Antidiabetic Evaluation of Medicinal Plants *Dillenia indica*, *Solanum indicum* and *Solanum torvum* from North East Region of India

A Thesis

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requirements for the Degree of

Doctor of Philosophy

By

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Under the supervision of

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STATEMENT

I do hereby declare that the content embodied in this thesis entitled as **"Antidiabetic Evaluation of Medicinal Plants *Dillenia indica*, *Solanum indicum* and *Solanum torvum* from North East Region of India"** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India under the joint supervision of Prof. Utpal Bora and Prof. Arun Goyal.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Antidiabetic Evaluation of Medicinal Plants *Dillenia indica*, *Solanum indicum* and *Solanum torvum* from North East Region of India"** by **Manoj Gadewar (Roll No.126106031)** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision at the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India and this work has not been submitted elsewhere for a degree.

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Dedicated to

To My

Father, Mother and Family

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"It's just a new beginning"

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SYNOPSIS

Introduction

Diabetes mellitus is a heterogeneous metabolic disorder of carbohydrate, fat and protein, affecting a large number of populations in the world (Pareek *et al*., 2009). The estimated burden of diabetes in India was 22 million in 1990, 28 million in 1995 and 33 million in 2000. Non insulin dependent diabetes mellitus (NIDDM) is the most common form of diabetes constituting nearly 90% of the diabetic population in any country with varying numbers in different geographical regions (Warjeet, 2011). According to World Health Organization projection, the population with diabetes is likely to increase to 300 million or more by the year 2025 (Meenakshi *et al*., 2010). The occurrence of consequences associated with diabetes are found to be high in countries like India (31.7%), China (20.8%) and United State of America (17.7%) (Balaraman *et al*., 2010). It is predicted that by 2030, India, China and the United States will have the largest number of people with diabetes (Frode and Medeiros, 2008). Diabetes mellitus is not a single disorder but a group of metabolic disorders characterized by chronic hyperglycemia, resulting from defects in insulin secretion, insulin action, or both. Increased thirst, increased urinary output, ketonemia and ketonuria are the common symptoms of diabetes mellitus, which occur due to the abnormalities in carbohydrate, fat, and protein metabolism. Appearance of ketone bodies in blood or urine indicates ketoacidosis and requires immediate medical attention to prevent diabetic complications (Craig *et al*., 2009).

Diabetes mellitus has caused significant morbidity and mortality due to microvascular and macrovascular complications (Thevenod, 2008). Diabetes is mainly attributed to the rapid rise in unhealthy life style, urbanization and aging. Hyperglycaemia which is the main symptom of diabetes mellitus generates reactive oxygen species (ROS) which cause lipid

peroxidation and membrane damage. ROS plays an important role in the development of secondary complications in diabetes mellitus such as cataract, neuropathy and nephropathy. Diabetic complications are mainly mediated through oxidative stress such as increased production of ROS or impaired antioxidant defense systems. Enhancement of lipid peroxidation, alteration in antioxidant enzymes and impaired glutathione metabolism are the main factors involved in the development of diabetes (Dewanjee *et al.*, 2009).

Type I occurs mainly due to destruction of β-cells which most either be immune mediated or idiopathic, whereas Type II diabetes occurs mainly due to insulin resistance or with relative insulin deficiency. Diabetes is also associated with lifestyle factors and genetics (Craig *et al*., 2009). Other factors which are involved in the development of diabetes are the genetic material such as chromosomal and mitochondrial DNA mutation. In some cases congenital rubella and cytomegalovirus infection also lead to diabetes mellitus. Sometimes, drugs and other chemicals such as pentamidine, nicotinic acid, glucocorticoids, thyroid hormone, α adrenergic agonists, thiazides, β-interferon and so on an cause diabetes mellitus.

Abnormalities in the pancreas such as pancreatitis, pancreatectomy, neoplasia, cystic fibrosis and fibrocalculous pancreatopathy can also contribute in the development of diabetes. There are other factors related to the immune system such as 'Stiff-man' syndrome and anti-insulin receptor antibodies that are involved in the development of diabetes. Diseases associated with the pancreas such as aromegaly, Cushing's syndrome, glucagonoma, phaeochromocytoma, hyperthyroidism and aldosteronoma can also mediate diabete mellitus. There are some other genetic syndromes such as Down syndrome, Klinefelter syndrome, Huntington's chorea, Laurence-Moon-Biedl syndrome, Myotonic dystrophy and Prader-Willi syndrome which were also involved in the development of diabetes in some cases (Craig *et al*., 2009).

Currently available drugs for the treatment of diabetes mellitus are insulin, sulfonylureas, biguanides, α-glucosidase inhibitors, and glinides, which can be used alone or in combination with other drugs to achieve better effect. Many of these oral antidiabetic drugs possess a number of serious adverse effects thus; the management of diabetes with drugs without any side effects is still a challenge (Pareek *et al*., 2009).

Herbal medicines have been used for the treatment of various diseases; a huge number of the population in the world is entirely dependent on traditional system of medicines (Meenakshi *et al*., 2010; Feshani *et al*., 2011). A number of medicinal plants and their formulations are used for treating diabetes in traditional medicine as well as in ethnomedicinal practices (Pareek *et al*., 2009). From the ethnobotanical information; about 800 plants which may possess anti-diabetic potential have been found (Venkatesh *et al*., 2010; Warjeet , 2011; Patel *et al*., 2011). Herbal medicines have fewer side effects, are safe and easy availability (Balaraman *et al*., 2010; Dewanjee *et al.*, 2009) compared to the synthetic hypoglycemic drugs.

Northeast India is considered as an ecological hot spot for a wide variety of medicinal plants. Ethnic communities inhabit the area, each having their own traditional medical cures for different diseases such as malaria, skin aliments and diabetes. In the recent years considerable attention has been directed towards the antidiabetic potential of medicinal plants and their herbal formulation in the management of disease.

Considering the important role of medicinal plants in the effective treatment of diabetes without producing much more adverse effects, our research mainly focused on pharmacological screening as well as toxicity evaluation of indigenous medicinal plants mainly found in North east India in different animal models.

The present investigation was carried out on “Plant based traditional knowledge for the treatment of diabetes” with the following objectives:

1) Identification and acquisition of the selected plant/ fruit

2) Extraction of plant/fruit using soxhlet extraction

3) Preliminary phytochemical analysis and screening of antioxidant activity using *In vitro* methods.

4) To investigate the effect of plant extracts in experimental animal model with special reference to antidiabetic activity.

The present work entitled "Antidiabetic evaluation of medicinal plants *Dillenia indica, Solanum indicum and Solanum torvum* from North East Region of India" has been divided into 5 chapters. This study deals with the collection, identification, extraction, preliminary phytochemical analysis, *in vitro* evaluation of antioxidant activity and antidiabetic evaluation of these plants in streptozocin (STZ)-induced diabetic rats. The various parameters like serum glucose, lipid profile and markers of hepatic function such as ASAT and ALAT were estimated.

Chapter 1 is the General Introduction which embodies the brief review of literature dedicated to the importance of diabetes and its type, global prevalence, their aetiology, various targets for drug action and various classes of drugs used for treatment and management of diabetes. This chapter elaborately reviewed the experimental animal models for induction of diabetes and their use in screening the antidiabetic activity of unknown compounds. This chapter also emphasized on the commonly used medicinal plants for treatment of diabetes, here we reviewed around 60 medicinal plants describing their possible mechanism of antidiabetic action in various animal models. This chapter also elaborately reviewed the importance of antidiabetic minerals from herbs like zinc, iron, chromium, vanadium etc and their role in prevention of disease.

Chapter 2 describes antidiabetic activity of fruit extract of *Dillenia indica* in streptozocin (STZ) induced diabetic rats. Acute toxicity studies and *in vitro* cytotoxicity

studies in L929 cell line revealed the non-toxic nature of the fruit extract of *Dillenia indica*. There was no lethality or any toxic reactions found with the selected dose until the end of the study period.

The results of the study have shown that the fruit extract at a dose of 400 mg kg^{-1} body weight has a marked hypoglycemic activity (61.2%) by improvement of the glucose tolerance test in normoglycemic rats and by lowering the blood glucose levels in STZ-induced diabetic rats. The extract also showed lipid lowering effect by decreasing the elevated level of triglycerides and cholesterol by 12% and 21.1% respectively, and beneficial effect by increasing the level of high density cholesterol in the rats suffering from type 2 diabetes.

The high proportion of total phenolic and total flavonoid content present in the crude extract supported the key role in free radical scavenging and/or reducing. The free radical scavenging activity of the fruit extract was tested through DPPH method and depends on interaction between antioxidant and the generated free radicals. The methanolic fruit extract of *Dillenia indica* was able to scavenge the stable DPPH radicals thereby reducing the oxidative stress. The methanolic fruit extract of *Dillenia indica* improved the altered level of ASAT by 58 and ALAT by 52.8% at 400 mg kg^{-1} as compared to diabetic control rats.

The results presented in this study suggest that the presence of one or more antidiabetic phytoconstituents in the methanolic fruit extract of *Dillenia indica* was needed to improve the physiology of rats affected with type 2 diabetes. These bioactive constituents contributed to normalizing the blood glucose and also improved the serum lipid parameters and oxidative stress unlike synthetic hypoglycemic agent like glibenclamide. The results suggested that bioactive constituents responsible for improving the physiology of type 2 diabetic rats need to be isolated and well characterized to contribute better for the therapy of type 2 diabetes.

Chapter Three describes the antidiabetic activity of fruit extract of *Solanum indicum* in STZ-induced diabetic rats. The extract showed concentration-dependant DPPH free radical scavenging activity, thus, play a pivotal role in reducing oxidative stress associated with diabetes. Acute toxicity studies in rats and *in vitro* studies in mouse fibroblast cell line revealed the non toxic nature of the extract. There was no lethality or any toxic reactions found with the selected dose until the end of study. Thus, the No Observed Adverse Effect Level (NOAEL) of the extract was greater than 5000 mg kg^{-1} body weight. The extract controlled post prandial hyperglycaemia by producing dose dependant α- amylase inhibition. Insulin resistance and impaired insulin secretion are the characteristic features of diabetes in our study. The extract caused marked reduction in fasting blood glucose level and also improved loss of body weight associated with diabetes.

The results of the study have shown that the methanolic fruit extract of *Solanum indicum* at a dose of 200 mg kg^{-1} body weight has a marked hypoglycemic activity by improvement of the glucose tolerance test in normoglycemic rats and by lowering the blood glucose levels from the 10^{th} day up to the 30^{th} day of treatment in STZ-induced diabetic rats. Oral administration of the extract also improved the elevated level of serum triglycerides and cholesterol which are increased due to faulty lipid metabolism and mobilization of free fatty acids. The extract also corrected the elevated level of ALAT and ASAT, thereby, prevented the hepatic damage caused by STZ.

All these observations ascribe a pivotal role to *Solanum indicum* in developing an antidiabetic drug of natural origin. Further research is required for identifying the molecular target and isolation of bioactive molecules. Further pharmacological validations of this plant will help in the development of effective herbal antidiabetic medicines in the near future.

Chapter Four discusses about the antidiabetic and antioxidant effect of *Solanum torvum* leaf extract in STZ diabetic rats. Acute toxicity studies confirmed that the LD_{50} of

Solanum torvum leaf extract was above 5000 mg kg^{-1} body weight as there was no observed toxic effect and lethality amongst all animals.

Phytochemical analysis of the leaf extract revealed the presence of alkaloids, glycosides, phenolic and flavonoid compounds which could be responsible for scavenging the stable DPPH free radicals thereby, imparting its antioxidant role.

Treatment of diabetic rats with leaf extract of *Solanum torvum* for 30 days showed dose and time dependant glucose reducing effect. The more prominent effect was observed at 200 mg kg^{-1} body weight. The antidiabetic effect of methanolic extract of *Solanum torvum* was compared against standard hypoglycaemic drug, glibenclamide, and could be related to the stimulatory action/insulin mimetic action on the existing pancreatic β-cells and the capacity of plant extract to scavenge the free radicals to prevent further damage similar to that of the standard drug.

The extract corrected the elevated levels of serum triglycerides and cholesterol while increased the level of serum HDL-C hence, it has a potential to be used in treatment of hyperlipidemia associated with diabetes. The extract lowered the elevated level of hepatic biomarker enzymes (ASAT and ALAT), thereby prevented further hepatic damage caused by the action of STZ.

Hence, the leaf extract of *Solanum torvum* was found to have a high margin of safety and thus seems to have a promising value for the development of a potent phytomedicine for diabetes, though, further comprehensive pharmacological investigations are needed to elucidate the exact mechanism of action of the extract.

CONTENTS

CHAPTER 1

Introduction and review of literature

1. Diabetes

Diabetes mellitus is a heterogeneous metabolic disorder characterized by increased serum glucose level. The symptoms of diabetes are polyurea, polyphagia and polydipsia. The hyperglycemia associated with diabetes causes disturbance in carbohydrate, fat and protein metabolism leading to insulin deficiency or resistance or both (WHO, 1999). Hyperglycemia increases the risk of both macrovascular and microvascular complications like retinopathy, nephropathy, neuropathy and coronary artery diseases.

The global incidence of diabetes mellitus was 246 million people in 2007 which according to the International Diabetes Federation (IDF), is likely to touch 380 million by the year 2025 (WHO, 2009). The World Health Organization estimates that at present, close to 347 million people are diabetic and this number is likely to escalate to 438 million by the year 2030 (WHO, 2013), if proper control and management strategies are not put in place.

The prevalence of diabetes in all age groups worldwide was estimated to be 2.8% in 2000, and is expected to rise to 4.4% in 2030 (WHO, 2002; Wild *et al*., 2004).

Diabetes mellitus is classified into insulin dependent diabetes mellitus (IDDM) or Type I and non insulin dependent diabetes mellitus (NIDDM) or Type II. Type I diabetes encompasses cases due to autoimmune destruction of pancreatic β-cells for which neither its aetiology nor pathogenesis is known (idiopathic). Type II diabetes includes the common major form of diabetes which results from defect in insulin secretion associated with insulin resistance (WHO, 1999).

1.1 Pathogenesis of diabetes mellitus

Chronic hyperglycemia of diabetes component is associated with prolonged damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (The Expert Committee on the Diagnosis and classification of Diabetes, 1997). Several pathophysiological processes are involved in the development of diabetes mellitus. The report further suggests that these processes involve autoimmune destruction of the pancreatic β-cells with subsequent deficiency of insulin or its resistance. Deficiency and insufficient action of insulin on target tissues leads to abnormality in carbohydrate, fat and protein metabolism (The Expert Committee on the Diagnosis and classification of Diabetes, 1997).

Chronic hyperglycemia associated with diabetes impairs growth and makes the individual susceptible to various infections. Long term complications include retinopathy, chronic renal failure and peripheral neuropathy with increased risk of foot ulcers. Acute life threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis (Davis, 1996). The various symptoms of diabetes include polyuria, polydipsia, polyphagia, and blurring of vision (Davis, 1996).

Oxidative stress and involvement of free radicals play an important role in the pathogenesis of diabetes mellitus (Riturparna and Neeraj, 2007). Hyperglycemia increases the generation of tissue damaging reactive oxygen species (ROS) by glucose autoxidation and non-enzymatic protein glycosylation (Brownlee, 1995). One of the major sites at which oxidative complications to diabetes take place is the vascular endothelium. Hyperglycemia also increases the generation of ROS such as superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) which reduce nitrogen oxide (NO) bioavailability in cultured endothelial cells (Riturparna and Neeraj, 2007) and in vascular tissue (Wild *et al*., 2004). Endothelial dysfunction is a well-documented characteristic phenomenon in diabetes mellitus (Wild *et al*., 2004) and is attributed to decreased vasorelaxant and increased contractile responses to physiological and pharmacological stimuli (Riturparna and Neeraj, 2007).

1.2 Classification of diabetes mellitus

World Health Organization (WHO) classifies diabetes mellitus into insulin dependent diabetes mellitus (IDDM) or type I, and non insulin dependent diabetes mellitus (NIDDM) or type II. Type I diabetes mellitus, has multiple genetic predispositions and is related to environmental factors that are still poorly defined. The rate of β-cell destruction appears to be age dependent being rapid mainly in infants and children and slow in adults (The Expert Committee on the Diagnosis and classification of Diabetes, 1997). The primary manifestation of the disease is generally ketoacidosis, while some may have modest fasting blood glucose level which can rapidly change to severe hyperglycemia in presence of infectious diseases and stress (Davis, 1996).

Insulin dependent diabetes mellitus or type I diabetes, also referred to as juvenile onset diabetes, is usually first diagnosed in children, teenagers and young adults. In these patients, the β-cells of the pancreas stop producing insulin due to destruction of the immune system. The other category is type II diabetes whose onset starts usually after 40 years of age

(Kaplan, 1989), and accounts for approximately 90-95% of diabetes mellitus cases worldwide. It is also called adult's onset diabetes which affects individuals who have insulin resistance generally associated with insulin deficiency (Abel *et al*., 2001). Insulin resistance is defined as an inadequate response to circulating insulin by insulin target tissues like adipose, skeletal muscle and liver; and this usually precedes the characteristic hyperglycemia in type II diabetes (Schenk *et al*., 2008).

Most patients with type II diabetes are obese which causes some degree of insulin resistance (Abel *et al*., 2001). This form of diabetes frequently goes undiagnosed for many years because hyperglycemia develops gradually and earlier stages are often not severe enough to get noticed as the classic symptoms of diabetes (The Expert Committee on the Diagnosis and classification of Diabetes, 1997). Although patients with this form of diabetes may have insulin levels that appear normal or elevated, higher blood glucose levels would be expected to result in even higher insulin values had their β-cells function been normal (Abel *et al*., 2001). Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. The risk of developing this form of diabetes increases with age, obesity and lack of physical activity.

Other forms of diabetes mellitus include; gestational diabetes and brittle diabetes (WHO, 1985). These forms are associated with monogenic defects in β-cell function inherited in an autosomal dominant pattern and are frequently characterized by the onset of hyperglycemia at an early age, generally before the age of 22 years. They are referred to as maturity onset diabetes of the young (MODY) and are characterized by impaired insulin secretion with minimal or no defects in insulin action (WHO, 1985).

1.3 Major organs affected by diabetes mellitus

The physiologic effects of insulin in the body are far reaching. These also directly correlate to the effects seen in the body of either too much or too little insulin in circulation.

The net effect of the hormone involves the storage and utilization of carbohydrates, proteins and fats. Most of the effects of insulin are seen in the adipose tissue, skeletal, cardiac and smooth muscles, liver and the pancreas. Adipose tissue is involved in the maintenance of normal glucose levels in the body. Its primary role is the storage of energy as triglycerides with glucose disposal being the primary role for the skeletal muscles (Huang and Czech., 2007). Adipose tissue has a number of glucose transporters such as GLUT 4, GLUT 8 and GLUT 12, that are responsible for shuttling glucose into the cells. GLUT 4 is the main hexose transporter and is highly expressed in the adipose tissue (Huang and Czech, 2007).

Visceral fat depots found in adipose tissue have very high lipolytic rates resulting in the release of large amounts of fatty acids into the system. Insulin normally suppresses the lipase and adipocytes triglyceride lipase enzyme that hydrolyses intracellular triglyceride but in the insulin resistant state, the activity of this enzyme is enhanced resulting in a free fatty acid flux (Duncan *et al*., 2007). Adipose tissue releases large amounts of a protein known as tumor necrosis factor (TNF-α), which plays a major role in the repression of many genes in the body which are responsible for the uptake and storage of glucose as well as fatty acids. TNF-α also mediates the inflammatory process which is associated with obesity and type II diabetes (Ruan and Lodish, 2002).

Skeletal muscles store glucose in the form of glycogen which is oxidized when needed to generate energy (Huang and Czech, 2007). It accounts for about 75% of the whole body insulin-stimulated glucose uptake (Perriot *et al*., 2001).

Glucose is transported into the cells through a specialized transmembrane sugar transporter known as GLUT 4, which catalyzes transport of glucose through the plasma membrane. This transporter works in tandem with others like GLUT 1, 5 and 12 to enhance glucose transport via facilitated diffusion. When insulin binds to its receptors tyrosine, phosphorylation of protein substrates occurs which activates the P13 kinase pathway.

Subsequent signaling pathways are activated with GLUT 4, eventually moving from its intracellular stores to the plasma membrane (Perriot *et al*., 2001). In type II diabetic patients there is as much as a 90% reduction in the levels of GLUT 4 that are responsive to insulin due to translocation of transporters and the resulting signaling pathways.

Insulin influences liver activity by either promoting anabolism or inhibiting catabolism. The liver helps the body to maintain normal blood glucose concentrations in fasting and postprandial states. Low insulin level increases glycogenolysis and increases hepatic glucose production (Lewis *et al*., 2002).

The liver has the maximum storage capacity for glycogen where insulin promotes synthesis of glycogen and also inhibits the breakdown of glycogen into glucose. These effects are mediated by changes in the activity of enzymes in the glycogen synthesis pathway. Insulin inhibits the expression of key gluconeogenic enzymes such as G-6-phospatase leading to elevated levels of glucose production in the liver (Luca and Olefsky, 2007). Insulin increases protein and triglyceride synthesis and very low density lipoproteins (VLDL) by the liver (Greenspan and Gardner, 2001). Individuals with type II diabetes have a higher incidence of liver function transferases (LFTs) abnormalities. The most common abnormality is elevated alanine aminotransferase (ALT).

1.4 Contributing factors for diabetes mellitus

The major contributing risk factors for increasing the prelevance of diabetes are ageing, obesity, sedentary lifestyle, smoking, psychological stress and low birth weight (Riturparna and Neeraj, 2007). In obesity associated type II diabetes mellitus, there is an increased accumulation of visceral fat which contains pro-inflammatory molecules such as tumour necrosis factor α (TNF-α), which are involved in the regulation of insulin sensitivity (Qatanani and Lazar, 2008). Other molecules such as adiponectin whose levels are low in

obese patients increase insulin sensitivity and reduce glucose output and fatty acid oxidation in the liver (Qatanani and Lazar, 2008).

Poor dietary choice is a major contributing factor to obesity and associated disorders like type II diabetes mellitus. Epidemiological evidences have demonstrated that saturated fatty acid intake is associated with increased risk of insulin resistance, diabetes and impaired glucose tolerance (Lichtenstein and Schwab, 2000). Foods rich in *trans*-fatty acids and high ratio of saturated to unsaturated fats result in weight gain and predisposition to diabetes. Foods such as red meat, refined grains, sweets and high fat dairy products have been linked to risks of type II diabetes. In contrast, weight loss is characterized by reduction in fat cell mass especially visceral fat which contains inflammatory markers associated with insulin resistance and decreased insulin sensitivity. Reduced visceral fat due to weight loss is accompanied by decreased adipose TNF-α release leading to improved insulin sensitivity (Mlinar *et al*., 2006).

1.5 Diagnosis of diabetes mellitus

According to WHO the diagnosis criterion for diabetes mellitus based on detection of plasma glucose level is FBG 100–125 mg dL^{-1} (5.6–6.9 mM). The new diagnostic criteria for diabetes mellitus have been greatly simplified. The OGTT which was previously recommended in 1979 by the National Diabetes Data Group (National Diabetes Data Group, 1979), has been replaced with the recommendation that diagnosis be based on two fasting plasma glucose levels of 250 mg dL^{-1} (13.8 mM) or higher (WHO, 2002; WHO, 2009). Measurement of the fasting plasma glucose level is the preferred diagnostic test, but any combination of two abnormal test results can be used. Fasting plasma glucose is considered as the primary diagnostic test because it predicts adverse outcomes like retinopathy (WHO, 1985; Gutteridge, 1999).

1.5.1 Conventional management of diabetes mellitus

The mainstay of non pharmacological treatment of diabetes is diet and physical activity (WHO, 2002). Other methods of treatment include; acupuncture, hydrotherapy, mineral supplementation and conventional hypoglycemic drugs like exogenous insulin and oral hypoglycemic agents (WHO, 2002). Presently available drugs for treatment and management of diabetes include sulfonylurea, biguanide, α-glucosidase inhibitor, thiazolidinedione and meglitinide.

1.5.1.1 Insulin

Insulin is a peptide hormone secreted by the β cells of pancreas. It is a diapeptide hormone composed of peptide chain A and B linked by disulphide bridges, and constituted by 51 amino acids having total molecular weight of 5802 daltons (Fig. 1). Its iso-electric point is pH 5.5. The A chain comprises 21 amino acids and the B chain 30 amino acids. The A chain has an N-terminal helix linked to an anti-parallel C-terminal helix; the B chain has a central helical segment. The two chains are joined by 2 disulphide bonds, which join the N- and C-terminal helices of the A chain to the central helix of the B chain. In pro-insulin, a connecting peptide links the N-terminus of the A chain to the C-terminus of the B chain (Dodson and Steiner, 1998).

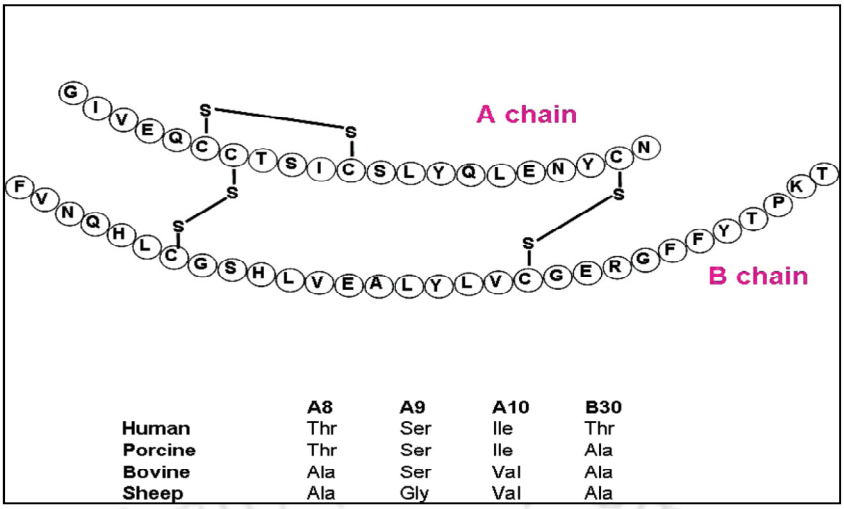


Fig. 1 Amino acid sequences of human, porcine, bovine, and sheep insulin (Reproduced with permission, David, 2004)

Insulin plays an important role in maintaining normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects.

1.5.1.1.1 Various actions of insulin

Insulin's action at the cellular level encompasses carbohydrate, lipid and amino acid metabolism and mRNA transcription and translation.

1.5.1.1.2 Carbohydrate Metabolism

Insulin acts at multiple steps in carbohydrate metabolism; it increases glycogen synthesis and decreases its breakdown by dephosphorylation of glycogen synthase and glycogen phosphorylase kinase respectively. Glycolysis is stimulated and gluconeogenesis inhibited by dephosphorylation of pyruvate kinase (PK) and 2, 6 biphosphate kinase. Insulin enhances the irreversible conversion of pyruvate to Acetyl CoA by activation of the intra-mitochondrial enzyme complex, pyruvate dehydrogenase (Denton and Tavare, 1997).

1.5.1.1.3 Lipid Metabolism

Insulin stimulates fatty acid synthesis in adipose tissue, liver and lactating mammary glands along with formation and storage of triglycerides in adipose tissue and liver. Fatty acid

synthesis is increased by activation and increased phosphorylation of acetyl-CoA carboxylase, while fat oxidation is suppressed by inhibition of carnitine acyltransferase. Triglyceride synthesis is stimulated by esterification of glycerol phosphate, while triglyceride breakdown is suppressed by dephosphorylation of hormone sensitive lipase. Cholesterol synthesis is increased by activation and dephosphorylation of HMG CoA reductase while cholesterol ester breakdown appears to be inhibited by dephosphorylation of cholesterol esterase. The metabolism of phospholipids is also influenced by insulin (Liu and Barrett, 2002).

1.5.1.1.4 Protein Synthesis

Insulin promotes protein synthesis. It affects transcription of specific mRNA as well as translation of mRNA into proteins in the ribosomes. Insulin action decreases mRNA for liver enzymes such as carbamoyl phosphate synthetase, a key enzyme in the urea cycle. Effects on translation are widespread and influenced by both insulin itself and by various growth factors like IGF-1(Hunter and Garvey, 1998).

1.5.1.2 Various forms/types of insulin

The various types of insulin preparations were depicted in (Table 1) as below,

Table 1.Various types of insulin preparations (Hunter and Garvey, 1998)

Drug (brand)	Onset time[a]	Peak time[a]	Duration[a]	Comments
Rapid-acting				
Insulin aspart (NovoLog)	10-20 min	1-3 hr	3-5 hr	Administer within 15 min before or immediately after meals
Insulin glulisine (Apidra)	25 min	45-48 min	4-5 hr	
Insulin lispro (Humalog)	15-30 min	0.5-2.5 hr	3-6.5 hr	
Short-acting				
Insulin regular (Novolin R, Humulin R)	30-60 min	1-5 hr	6-10 hr	Administer 30 min before meals
Intermediate-acting				
Insulin NPH (Novolin N, Humulin N)	1-2 hr	6-14 hr	16-24+ hr	Cloudy appearance
Long-acting				
Insulin detemir (Levemir)	1.1-2 hr	3.2-9.3 hr	5.7-24 hr (dose-dependent)	Do not mix with other insulins
Insulin glargine (Lantus)	1.1 hr	None	24 hr	
Premixed				
70% Insulin aspart protamine/30% insulin aspart (NovoLog Mix 70/30)	10-20 min	1-4 hr	24 hr	Cloudy appearance Administer within 15 min before meals
75% Insulin lispro protamine/25% insulin lispro protamine (Humalog Mix 75/25)	15-30 min	2 hr	22 hr	
50% Insulin lispro protamine/50% insulin lispro protamine (Humalog Mix 50/50)	15-30 min	2 hr	22 hr	
70% Insulin NPH/30% insulin regular (Humulin 70/30, Novolin 70/30)	30 min	1.5-12 hr	24 hr	Cloudy appearance Administer within 30 min before meals
50% Insulin NPH/50% insulin regular (Humulin 50/50)	30-60 min	1.5-4.5 hr	7.5-24 hr	

1.5.1.3 Sulfonylurea: Eg. Tolbutamide, glyburide, glibenclamide

Sulphonyl urea derivatives are widely used either alone or in combination with other oral hypoglycemic agents for treatment of type II diabetes mellitus. It contains sulphonic acid urea nucleus. Various analogues were prepared by substituting different functional groups on sulphonyl urea nucleus at different positions. The potency and efficacy of new analogues may differ significantly from each other depending on the nature of substituted functional group; however, they possess desired therapeutic action (Malender, 2004).

These drugs act by inhibiting K_{ATP} channels in the plasma membranes of pancreatic β cells. This inhibition results in stimulation pancreatic β cells triggering the release of insulin by the process of exocytosis (Foye *et al*., 1995). These drugs may be used as substitutes for biguanides and first line agents for treatment of type II diabetes (Foye *et al*., 1995). These drugs are not prescribed for type I diabetes as they require functioning pancreatic β-cells for the release of insulin to produce the desired therapeutic effect on serum/plasma (Greenspan and Gardner, 2001).

1.5.1.4 Biguanides: Eg**.** Metformin, phenformine

These drugs act by increasing peripheral utilization of glucose by sekeletal muscles, liver and red blood cells. This drug act in the presence of endogenous insulin produced by functioning pancreatic β- cells (Zhou *et al*., 2001).

Metformin is a widely used biguanide in the treatment of obese patients with type II diabetes and those with high blood glucose who are not responsive to other therapies. It activates adenosine monophosphate protein kinase (AMPK) in liver cells; therby, increasing the oxidation of free fatty acids and glucose uptake by cells. An overall reduction in the rate of lipogenesis and hepatic glucose production is normally observed (Rahimi *et al*., 2005).

The main side effect associated with metformin therapy is increased risk of lactic acidosis which is common in patients suffering from peripheral vascular diseases, renal

insufficiency, hepatic, pulmonary and cardiovascular disease (Greenspan and Gardner, 2001). Other adverse effects associated with biguanides are fatigue, weakness, dizziness, nausea, nephrotoxicity and shortness of breath (Greenspan and Gardner, 2001).

1.5.1.5 Thiazolidinediones: Eg. Pioglitazone, Rosiglitazone

Pioglitazone and rosiglitazone act by increasing the sensitivity of peripheral tissues to insulin by affecting the expression of specific genes. These drugs act by binding and activating γ peroxisome proliferator activated receptor (PPAR-γ), a nuclear receptor (Qatanani and Lazar, 2007). Some of the effects of this gene expression include, increase in the expression of glucose transporters, decreased glucose and increased differentiation of preadipocytes into adipocytes (Greenspan and Gardner, 2001). The high affinity of these drugs to PPAR-γ is important in the management of insulin resistance since large numbers of adipocytes differentiate from smaller ones to produce TNF-α which increases insulin resistance. Thiazolidinediones therefore, suppress the expression of these adipokines involved in insulin resistance (Sharma and Staels, 2007).

1.5.1.6 α- Glucosidase inhibitors: Acarbose, Voglibose

Polysaccharides are hydrolyzed to oligosaccharides and disaccharides in the presence of enzyme α- amylase, which is further hydrolyzed by the action of another enzyme α-glycosidase to monosaccharide. This is absorbed through the gut wall and enters the systemic circulation, which in turn increases postprandial glucose levels (Ranilla, 2010; El-Kaissi S, 2011). These drugs delay the breakdown of complex sugars to simple sugars which inhibit absorption of glucose across the intestinal wall. These drugs may have beneficial effect on insulin resistance and glycemic control in patients suffering with diabetes (Barrett, 2011; Nair, 2013). Most common adverse effects associated with these drugs are flatulence, abdominal pain and diarrhea.

1.6 Role of medicinal plants in the treatment and management of diabetes

Currently available drugs for treatment of diabetes mellitus can be used alone or in combination with other drugs to achieve a better effect. Since most of these oral antidiabetic agents have serious adverse effects, management of diabetes without any side effects is still a challenge (Pareek *et al*., 2009).

Although large numbers of herbal medicines are being used for the treatment of various diseases, still majority of the population in the world is entirely dependent on allopathic medicine (Meenakshi *et al*., 2010; Feshani *et al*., 2011). A number of medicinal plants and their formulations are used for treating diabetes in traditional medicine as well as in ethnomedicinal practices (Pareek *et al*., 2009). Ethnobotanical information suggests that as many as 800 plants may possess anti-diabetic potential (Venkatesh *et al*., 2010, Warjeet, 2011; Patel *et al*., 2011). Popularity of herbal treatments is due to their fewer side effects, safety, effectiveness, low cost and easy availability (Balaraman *et al*., 2010; Dewanjee et *al*, 2009) as compared to the synthetic hypoglycemic drugs. Many of the traditional plants and the bioactive compounds derived from them have been used for the treatment and management of diabetes (Fig. 2).

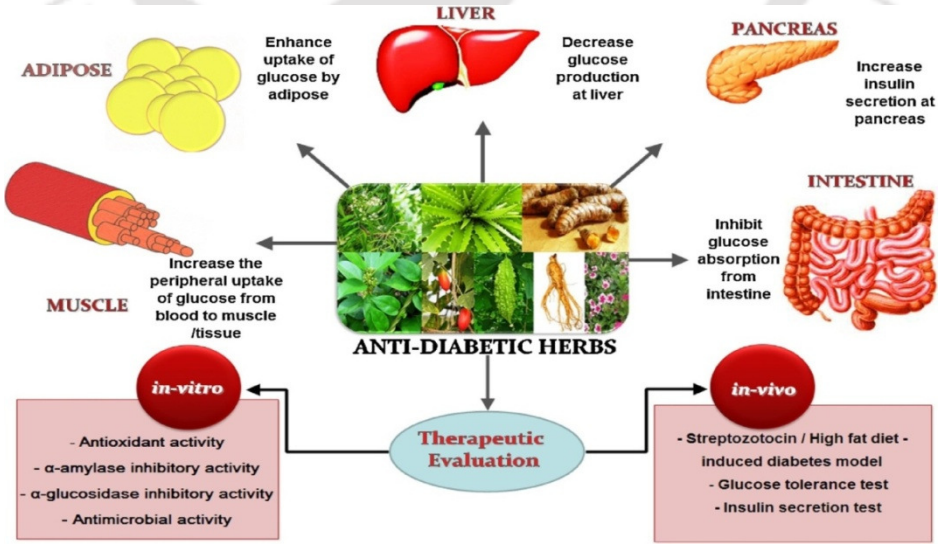


Fig. 2 Antidiabetic herbs, various mechanisms of actions and persisting models of their therapeutic evaluation (Feshani et al., 2011).

Systematic study of herbal medicines involving scientific validation of the bioactive principles, standardization, qualitative and quantitative phytochemical analysis, mechanism of action, preclinical and clinical applications and potential herbal drug interaction have validated their effectivity in modern era of medicine (Rai and Carpinella, 2006).

Several studies reported that bioactive principles/molecules derived from a variety of plants had therapeutic efficacy comparable to chemotherapeutic agents with the added advantage of not having any side effects. Developments in the field of molecular biology helped in understanding the molecular mechanism of many herbal drugs which differ in many aspects from that of synthetic drugs/agents (Rai and Carpinella, 2006).

The hypoglycaemic activity of some medicinal plants has been identified and experimentally demonstrated both in *In vivo* and *In vitro* diabetic models and documented in several studies depicted in Table 2.

These plants include *Azadirachta indica* (Prabbakar *et al*., 2013), *Cassia occidentalis* linn (Laxmi *et al*., 2010), *Colocynthis citrullus* (Abdel-Hassan *et al*., 2000), *Ocimum gratissimum*, *Momordica charantia* (Joseph and Jini, 2013) and *Zingeber officinale* (Asha *et al*., 2011) amongst many others. The potency and chemical composition of bioactive principle derived from the plants mainly depend on the age of the plant, collection, processing and method employed for extraction (Kunle *et al*., 2012).

Although herbal medicines are usually perceived as being natural, affordable, safe and free from adverse/toxic effects (Wheelwright, 1994). The presence of associated phytoconstituents makes it necessary to carry out toxicity studies on them.

1.7 Commonly used medicinal plants in the treatment of diabetes mellitus

Table 2. Medicinal plants used in the treatment of diabetes

S. No	Plant name	Part(s) extract	Mechanism of action	Experiment model For induction of diabetes	Reference
1	*Boldoa purpurascens*	Leaves	Ethanolic and aqueous extracts of leaves effectively reduced the blood glucose due to presence of D-pinitol and other flavonoids.	Alloxan	Gonzalez Mosquera *et al*.,2013
2	*Boerhaavia diffusa*	Leaves/roots	Treatment with the leaf extract resulted in significant reduction in serum glucose and cholesterol, free fatty acids, phospholipids and triglycerides levels.	STZ	Pari *et al*.,2004
3	*Boswellia serrata*	Gum and resin	Extracts prevent pancreatic islet destruction and consequent hyperglycemia in a diabetic animal model.	STZ	Shehata *et al*.,2011
4	*Gynura procumbens*	Leaves	The extract has exerted its hypoglycemic effect by promoting glucose uptake by the muscles.	STZ	Hassan *et al*.,2010
5	*Hiptage benghalensis*	Leaves	The methanolic extract effectively regenerated the beta cells of the pancreas.	Alloxan	Maheshwari *et al*.,2013; Algariri *et al*.,2013
6	*Hibiscus sabdariffa*	Whole	The aqueous extract of H. effectively inhibited α-amylase and α-glucosidase responsible for antidiabetic effect.	STZ	Peng *et al*.,2011
7	*Hyptis suaveolens*	Leaves	It possesses significant antihyperglycemic activity which might be due to the stimulating effects on glucose utilization and antioxidant enzyme.	STZ	Mishra *et al*.,2011; Ademiluyi *et al*.,2013
8	*Nigella sativa*	Whole plant/seed	*N. sativa* and its bioactive compound thymoquinone (TQ) protected and preserved beta cell integrity by decreasing oxidative stress.	STZ	Al Wafai *et al*.,2010 Abdelmeguid *et al*., .,2013
9	*Olea europea*	Leaves	Polyphenolic components of olive leaves on redox homeostasis that may have a role in the maintenance of β-cell physiology.	STZ	Cumaoglu *et al*.,2011 Cumaoglu *et al*.,2011

10	*Opuntia joconostle*	Whole plant	The frequent consumption of *O. joconostle* by humans in the diet may contribute to prevent and control the complications associated with type 2 diabetes mellitus.	STZ	Paiz *et al*.,2010 Ali *et al*.,2012
11	*Artemisia afra*	Leaves	Leaf extract promoted the regeneration of pancreatic β-cells, and enhanced glucose utilization in the diabetic rats.	STZ	Afolayan *et al*.,2011; Sunmonu *et al*.,2013
12	*Albizia odoratissima*	Aerial parts	Methanolic extract showed significant reduction in blood glucose and lipids level.	Alloxan	Rajan *et al*.,2010 ; Kumar *et al*.,2011
13	*Acacia nilotica*	Leaves	Leaf extract showed hypoglycaemic and anti-platelet aggregation activity in diabetic rats.	STZ	Asad *et al*.,2011
14	*Achyranthes rubrofusca*	Leaves	The extract showed significant increase in the antioxidant enzymes levels like SOD, CAT and glutathione expression.	Alloxan	Geetha *et al*.,2011; Omara *et al*.,2012
15	*Alangium salvifolium*	Barks	The extract normalizes the blood serum parameters pertaining to liver function test and also reduces blood glucose.	Alloxan	Sharma *et al*.,2011
16	*Coscinium fenestratum*	Stems	Alcoholic extract significantly altered the glycolytic enzymes, gluconeogenic enzyme and various toxicological parameters in treated diabetic rats.	STZ	Malarvili *et al*., 2011; Punitha *et al*.,2005
17	*Cistus laurifolius*	Leaves	Ethanolic extract reduced the blood glucose level and inhibited the activity of enzymes α-amylase and α-glucosidase.	STZ	Orhan *et al*.,2013
18	*Combretum lanceolatum*	Flowers	Crude extract of flowers showed antihyperglycemic activity.	STZ	Dechandt *et al*.,2013; Enomoto *et al*.,2004
19	*Cinnamomum zeylanicum*	Whole plant	Treatment with Cinnamon polyphenols showed hypoglycemic and hypolipidemic effects through the repairing of pancreatic β-cells in diabetic mice.	STZ	Li *et al*.,2013
20	*Emblica*	Fruits	It improved the elevated level of blood	STZ	Akhtar *et al*.,2011; Nain

	officinalis		glucose, lowered low density lipoprotein and cholesterol levels in human patients.		*et al*.,2012; Puppala *et al*.,2013
21	*Ficus amplissima*	Barks	The extract showed beneficial effects on lowering the blood glucose level and other toxicological parameters.	STZ	Arunachalam *et al*.,2013
22	*Ficus deltoidea*	Fruits	Reduced the blood glucose levels. Showed dose dependant inhibitory activity on α-glucosidase and α-amylase.	Yeast α-glucosidase, rat intestinal α -glucosidase & α-amylase inhibition assay	Misbah *et al*.,2013; Adam *et al*.,2012
23	*Gmelina arborea*	Barks	It increases the plasma insulin level and showed significant reduction in serum glucose concentration.	STZ	Pattanayak *et al*.,2011
24	*Ricinus communis*	Leaves	The extract has a significant antidiabetic effect and this effect may be due to the presence of saponins, flavonoids and other constituents present in the leaves.	STZ	Mann *et al*.,2013
25	*Salvia miltiorrhiza*	Roots and rhizome	The total poly phenolic acids fraction could ameliorate hyperglycemia, hyperlipemia and improved insulin resistance in diabetic rats.	STZ	Huang *et al*.,2012 Yang *et al*.,2011
26	*Sphaeranthus indicus*	Flowers	Decreases the blood glucose level and improves the glucose metabolism in type 2 diabetes due to changes of liver glycogen and insulin level.	STZ	Prabhu *et al*.,2008 Kharkar *et al*.,2013
27	*Selaginella tamariscina*	Whole plant	It showed hypoglycemic effect due to increase in the level of insulin.	STZ	Zheng *et al*.,2011 Zheng *et al*.,2013
28	*Bougainvillea spectabilis*	Roots and barks	Aqueous extracts showed significant increase in glucose-6-phosphate dehydrogenase activity and hepatic, skeletal muscle glycogen.	STZ and alloxan	Bhat *et al*.,2011; Geethan *et al*.,2008

29	*Brassica juncea*	Seeds	Ethyl acetate fraction significantly reduced the thiobarbituric acid reactive substance levels of serum and hepatic and renal mitochondria. It is beneficial in attenuating the oxidative damage involved in diabetes.	STZ	Yokozawa *et al*.,2002; Yokozawa *et al*.,2003
30	*Bauhinia Variegata*	Whole plant/leaves	Ethanolic extract and its major active constituent roseoside have enhanced insulin release activity through *in vitro* studies. The presence of insulin like protein in chloroplasts of plant may indicate its involvement in carbohydrate metabolism during diabetic condition.	STZ and alloxan	Azevedo *et al*.,2006 ; Frankish *et al*.,2006
31	*Byrsonima crassifolia*	Fruits and seeds	Extracts from fruits and seeds of *B. crassifolia* increased the levels of endogenous antioxidants like SOD, GSH and CAT. Hepatic glycogen content, glucose-6-phosphatase (G6Pase) plasma insulin and reduced the blood glucose concentration.	STZ	Perez-Gutierrez *et al*.,2010
32	*Terminalia chebula*	Fruits	Improves the insulin secretion from the pancreatic islets as well as glucose metabolism and reduces the blood glucose.	STZ	Kumar *et al*.,2006
33	*Teucrium orientale*	Stems,leaves and flowers	The effect of extracts on antioxidant enzyme activity is due to the high contents of flavonoids and polyphenol components which were involved in the healing process of free radical-mediated diseases including diabetes and its complications.	STZ	Dehghan *et al*.,2013
34	*Zizyphus spina-christi*	Leaves	Improves glucose utilization in diabetic rats by increasing insulin secretion.	STZ	Michel *et al*.,2011
35	*Psidium guajava*	Leaves	Inhibits the activity of α-glucosidase and stimulated glucose metabolic enzymes in	Alloxan	Divya *et al*.,2012 Huang *et al*.,2011

			liver tissue.		
36	*Pongamia pinnata*	Whole parts	Pongamol and Karanjin are the active compounds responsible for antidiabetic effect.	STZ	Tamrakar *et al*.,2011 Jaiswal *et al*.,2011 Goli *et al*.,2012
37	*Parquetina nigrescens*	Whole plants	Decreases the blood glucose level due to increased secretion of insulin and lowering lipogenesis.	Alloxan	Saba *et al*.,2010
38	*Potentilla discolor bunge*	Whole plants	Antidiabetic and hypolipidemic properties of extracts through strong antioxidant nature and a protective action on pancreatic beta cells.	STZ	Zhang *et al*.,2010
39	*Dioscorea spp*	Whole plant	Allantoin, an active compound may improve glucose utilization in skeletal muscle through β-endorphin dependent and independent pathways that decrease plasma glucose.	STZ	Niu *et al*.,2010
40	*Dodonaea viscosa*	Aerial parts	The extract showed antidiabetic and hypolipidaemic activities.	STZ	Veerapur *et al*.,2010; Veerapur *et al*.,2010
41	*Entada phaseoloides*	Seeds	Extract normalized hyperglycemia, reversed the state of dyslipidemia and tissue steatosis associated with diabetes.	HFD and low dose STZ	Zheng *et al*.,2012
42	*Gymnema montanum*	Leaves	It showed the antidiabetic effect by suppressing carbohydrate absorption from the intestine and thereby reducing blood glucose.	STZ	Ramkumar *et al*.,2010
43	*Mucuna pruriens*	Seeds	Seed extract showed significant reduction in blood glucose and body weight.	STZ	Majekodunmi *et al*.,2011 Bhaskar *et al*.,2008
44	*Merremia emarginata*	Whole plant	The methanolic extract shows antidiabetic action through pancreatic β-cells	STZ	Gandhi *et al*.,2012

			regeneration and increase level of insulin.		
45	*Sida cordifolia*	Aerial parts	Improvement in the blood glucose and insulin level.	STZ	Kaur *et al*.,2011
46	*Solanum surattense*	Leaves	Improve the plasma insulin level and reduce the blood glucose concentration.	STZ	Gupta *et al*.,2011 Sridevi *et al*.,2011
47	*Solanum xanthocarpum*	Leaves	The results indicate that extracts effectively reduce the blood glucose level and oxidative stress markers.	Alloxan	Poongothai *et al*.,2011
48	*Sansevieria roxburghiana*	Whole plant	Potential antidiabetic action is due to modulation of endogenous antioxidant status and increase the level of insulin.	STZ	Haldar *et al*.,2010
49	*Phoenix dactylifera*	Fruits	Crude extract improved the serum glucose level and liver functions along with lipid profiles.	Alloxan	Michael *et al*.,2013
50	*Kigelia pinnata*	Flower	Floral extract showed antidiabetic and antihyperlipidemic activity.	STZ	Kumar *et al*.,2012
51	*Lantana aculeata*	Mature roots	High concentration of oleanolic acid present in the roots is responsible for antidiabetic effect.	Alloxan	Khan *et al*.,2010 Kumar *et al*.,2012
52	*Malva parviflora*	Leaves	Hexane extract showed significant reduction in the blood sugar level.	STZ	Perez-Gutierrez.,2012
53	*Piper longum*	Roots	The plant extract is capable of managing hyperglycemia and complications of diabetes in STZ induced diabetic rats.	STZ	Kumar *et al*.,2013 Nabi *et al*.,2013
54	*Scoparia dulcis*	Whole plant	Scoparic acid and diterpenoid isolated from the extract stimulate the insulin secretion from isolated islets.	STZ	Md Zulfiker *et al*.,2010 Latha *et al*.,2009
55	*Syzygium cordatum*	Leaves	The isolated phytoconstituents oleanolic acid and ursolic acid were responsible for blood glucose lowering effect.	STZ	Deliwe and Amabeoku .,2013; Musabayane *et al*.,2010
56	*Terminalia*	Fruits	Phytoconstituents present in the crude	STZ	Latha and Daisy *et*

	bellerica		extract could be responsible for the modulation of c-AMP and intracellular calcium levels in the β-cells of the pancreas thereby causes release of insulin.		*al*.,2013
57	*Murraya koenigii*	Leaves	It showed hypoglycemic, lipid lowering and antioxidant effect.	STZ	Arulselvan *et al*.,2007
58	*Mirabilis jalapa*	Roots	Trigonelline is the major bioactive component of *M. jalapa* and showed better hypoglycaemic effect.	STZ	Zhou *et al*.,2011 Zhou *et al*.,2013 Sarkar *et al*.,2012
59	*Sapindus trifoliatus*	Fruits	The extract has insulin like activity and the antihyperglycemic effect of the extract due to an increase in peripheral glucose utilization.	Alloxan	Sahoo *et al*.,2010

1.8 Essential antidiabetic mineral elements from herbs

Medicinal plants are balanced sources of macro and micronutrient minerals that are useful in the management and treatment of diabetes. These micronutrients serve as essential co-factors and co-enzymes for metabolic reactions which regulate and support various cellular reactions like citric acid cycle, amino acid metabolism, glycolysis and lipid metabolism for energy production (Shils,1999). Micronutrients play an important role in the prevention and treatment of complications associated with both type I and type II diabetes (Mooradian, 1994). For instance, magnesium acts as a co-factor in various enzymes associated with glucose oxidation and transport and thereby, plays an important role in insulin release. Deficiency of magnesium has been associated particularly with diabetic retinopathy.

1.8.1 Manganese

It acts as a co-factor to various vital enzymes that regulate different biochemical reactions like antioxidant enzyme (manganese superoxide dismutase), involved in the protection of cell membranes preventing disruption of carbohydrate, protein and lipid metabolism (George, 2004).

1.8.2 Zinc

It is involved in the synthesis and transduction of insulin receptors (Ezaki, 1989). It plays an important role in the regulation and release of insulin secretion by pancreatic β-cells and glucose utilization by muscles and fat cells (Song, 1998). The ability to synthesize and secrete insulin is severely impaired in the zinc deficient state (Ezaki, 1989).

1.8.3 Chromium

It is an essential micronutrient which serves as a co-factor in insulin regulatory activity and plays an important role in normal glucose and lipid metabolism. It facilitates insulin binding and subsequent uptake of glucose into the cell and thereby, decreases fasting

blood glucose level (Mooradian, 1994; Baker, 1996). Oral supplementation of chromium corrects these problems in patients with type II diabetes.

1.8.4 Vanadium

It acts as a co-factor for enzymes essential in energy metabolism and plays an important role in the regulation of intracellular signaling. It reduces the rate of glycogenesis and gluconeogenesis in liver (Cohen, 1995). Oral supplementation of 20 μg/day; vanadyl sulfate at a dose of 100 mg/day improved insulin sensitivity associated with type II diabetes (Cohen, 1995).

1.8.5 Molybdate

It is the micronutrient of choice in the treatment of severe insulin resistance in diabetes. It stimulates pancreatic β- cells thereby, increasing insulin secretion. It is an effective anti hyperglycemic agent in diabetics with severe insulin resistance. It is associated with substantial reduction of hyperinsulinaemia and increase in pancreatic insulin secretion.

Medicinal plant toxicity could be attributed to the high levels of mineral elements. For instance, trivalent chromium sources are not toxic. However, hexavalent chromium toxicity from industrial exposure through inhalation has been associated with increased incidence of lung cancer. In experimental animals, ingestion of chromate resulted in liver and kidney damage (Fishbein, 1988). Epigastric pain, diarrhea and vomiting were caused by high zinc intake from food stored in galvanized containers. Supplement of, as little as, 25 mg of zinc resulted in reduced absorption of copper, presumably because of competition (Kaplan, 1989). Lead toxicity produced neurological, gastrointestinal, renal, immunological, endrocrinological and hematopoietic changes in humans (Kaplan, 1989). Supplementation with vanadyl compounds at oral doses of 50-125 mg/day caused cramps, loosened stools and green tongue in all patients and fatigue and lethargy in some individuals (Dimond, 1963).

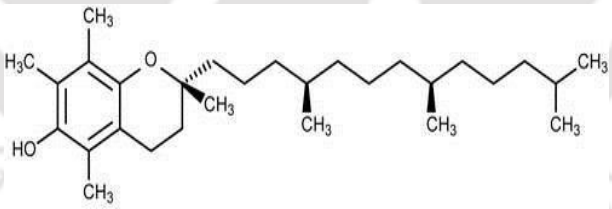
1.9 Essential vitamins with hypoglycemic activity from herbs

Safety and efficacy of medicinal plants in the management of diabetes mellitus is further potentiated by the presence of vitamins in appropriate amounts. These vitamins include; α-tocopherol, retinol, ascorbic acid and carotenoids such as lycopene, β-carotene and β-cryptoxanthin.

1.9.1 Retinol

Retinol or vitamin A is a subclass of retinoic acids. It helps in the regulation of immune functions which reduces the intensity of infectious diseases (Bates, 1995). Vitamin A is required for normal functioning of the visual system, maintenance of cell function for growth, epithelial integrity, production of red blood cells, immunity and reproduction (Bates, 1995). There are different forms of vitamin A such as β carotene, which is found in plants. It is an essential nutrient that cannot be synthesized in the body so it must be obtained through diet (Bates, 1995). Deficiency of vitamin A increases vulnerability to a range of illnesses including diarrhoea, measles, and respiratory infections (Rice *et al*., 2004).

1.9.2 α-Tocopherol



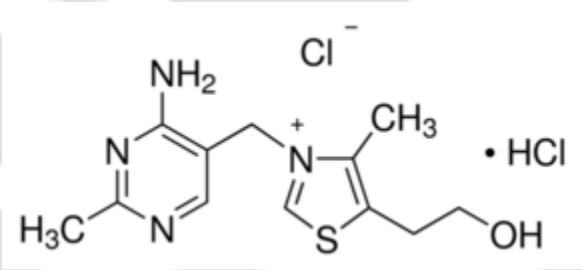
α- Tocopherol

α- Tocopherol also called vitamin E, is a collective name for a group of fat-soluble compounds with distinct antioxidant activities that includes tocopherols and tocotrienols (Traber, 2006). These vitamins are found naturally in some foods and available as a dietary supplement. Naturally occurring vitamin E exists in eight chemical forms (alpha, beta, gamma and delta-tocopherol and tocotrienols) that have varying levels of biological activity (Traber, 2006). The only form of tocopherol that is nutritionally important to humans is α-

tocopherol (Health, 2011). Serum concentration of α-tocopherol depends on the liver, which takes up the nutrient after the various forms are absorbed from the small intestine. The liver preferentially re-secretes only α-tocopherol *via* the hepatic α-tocopherol transfer protein (Traber, 2006).

Vitamin E is a fat-soluble antioxidant which stops the production of reactive oxygen species (ROS) formed during oxidation of fat. It also prevents or delays the chronic diseases associated with free radicals (Traber, 2006). In addition to its activities as an antioxidant, vitamin E is involved in cell signaling, regulation of gene expression and other metabolic processes (Traber, 2006). Usual dietary intake is estimated at 7–11 mg/day. The recommended daily/dietary allowance (RDA) for alpha tocopherol is 15 mg/day for people of 15 years of age and older. Natural vitamin E (d-alpha tocopherol) has approximately twice the bioactivity of synthetic forms of the vitamin (dl-alpha tocopherol) (Sarubin, 2000).

1.9.3 Thiamine



Thiamine

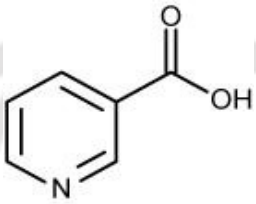
Thiamine regulates α-keto acid decarboxylation and transketolation. Decarboxylation reactions are an integral part of carbohydrate metabolism. Thiamine is involved in the alpha-keto acid decarboxylation of pyruvate, alpha-ketoglutarate, the branched-chain alpha-keto acids such as leucine, isoleucine and valine metabolites. Transketolation is involved in pentose phosphate pathways (Fattal *et al*., 2011).

Thiamine is converted to its active form, thiamine pyrophosphate. The thiamine-dependent enzymes are important for the biosynthesis of neurotransmitters and for the production of

reducing substances used in oxidative stress defenses as well as for the biosynthesis of pentoses used as nucleic acid precursors.

Deficiency of thiamine results in dry and wet beriberi, a peripheral neuropathy, cardiomyopathy with edema, lactic acidosis and Wernicke–Korsakoff syndrome. Various manifestations of thiamine deficiency are nystagmus, ophthalmoplegia, ataxia, confusion, retrograde amnesia, cognitive impairment and confabulation (Fattal *et al*., 2011).

1.9.4 Nicotinic acid and Nicotinamide

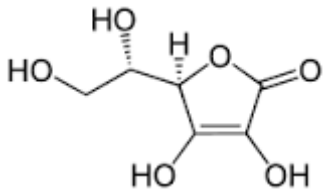


Nicotinic acid

Vitamin B_3 occurs in nicotinic acid and nicotinamide. The active coenzyme forms, nicotinamide adenine dinucleotide (NAD) and NAD phosphate, are essential for the function of enzymes and normal carbohydrate, lipid and protein metabolism (Frank and Bantle, 1999). As a vitamin, the two compounds function similarly, but in pharmacological doses they have distinct effects (Frank and Bantle, 1999). Nicotinic acid at a dose range of 1-3 g/day is effective in the treatment of dyslipidemia. However, its use in the treatment of diabetes has been limited due to negative effect on glycemic control (Sarubin, 2000).

Animal studies suggest that nicotinamide acts by protecting pancreatic β-cells from autoimmune destruction by maintaining intracellular NAD levels and inhibiting the enzyme, poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair. Nicotinamide also acts as a weak antioxidant of nitric oxide radicals (Kolb and Volker, 1999; Knip, 2000).

1.9.5 Ascorbic acid



Ascorbic acid

Vitamin C is an antioxidant which acts as an electron donor in eight various enzymes. Three of these participate in collagen hydroxylation and two in carnitine biosynthesis. Out of these three enzymes which participate in collagen hydroxylation, one is necessary for biosynthesis of catecholamine and norepinephrine, another is involved in amidation of peptide hormones, and the last one in tyrosine metabolism (Gulfraz *et al*., 2011).

Vitamin C protects low density lipoproteins *ex vivo* against oxidation and may function similarly in the blood. The antioxidant property of vitamin C stabilizes folate in food and plasma. Vitamin C promotes absorption of soluble non-haem iron by chelation or by maintaining the iron in reduced (ferrous, Fe^{2+}) form (Karau *et al*., 2012). However, the amount of dietary vitamin C required to increase iron absorption ranges from 25 mg upwards and depends on the amount of inhibitors, such as phytates and polyphenols, present in the meal (WHO, 2004).

1.9.6 Carotenoids from herbs

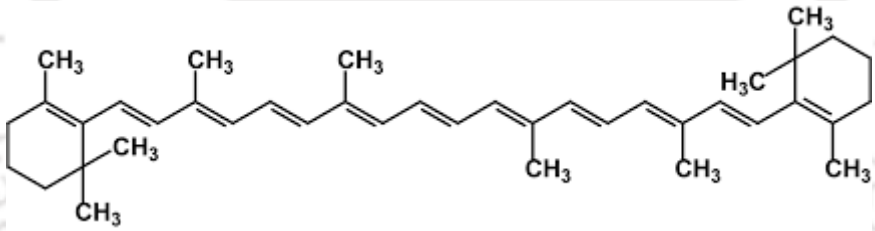
Among all the pigments present in living organisms, there is no doubt that the carotenoids are the most widely distributed in nature. They are found throughout the plant kingdom in both photosynthetic and non-photosynthetic organism including bacteria, fungi and algae. Animals are unable to synthesize carotenoids and therefore, they have to ingest them from dietary plants (Goodwin, 1980).

Carotenoids are natural pigments like lycopenes, which have two acyclic end groups, β-carotene which has two cyclohexene type end groups and oxygenated carotenoids called

xanthophylls. Examples of these compounds are zeaxanthin and lutein (hydroxy), spirilloxanthin (methoxy), echinenone (oxo) and antheraxanthin (epoxy) (Goodwin, 1980).

Many epidemiologic studies have associated high carotenoid intake with a decrease in the incidence of chronic disease. However, their biological mechanism remains elusive with multiple possibilities Carotenoids i) can be converted to retinoids to have provitamin A activity, ii) can modulate the enzymatic activities of lipoxygenases such as proinflammatory and immunomodulatory molecules, iii) can have antioxidants properties which are well above what is seen with vitamin A and iv) can activate the expression of genes which encode the message for production of a protein, connexin 43, which is an integral component of the gap junctions required for cell to cell communication. Such gene activation is not associated with antioxidant capacity and is independent of pro-vitamin A activity (Bendich, 1993).

1.9.7 β- carotene



β- Carotene

It is the main compound with provitamin A activity (Olson, 1993). When incorporated into the diet, it breaks down into two molecules of retinol (vitamin A) by the action of enzyme β-carotene-15 15'-dioxygenase in the intestine. However, β-carotene is not the only carotenoid with provitamin A activity. Any carotenoid with at least one unsubstituted β-ring can undergo similar cleavages and give rise to a vitamin A molecule. However, carotenoids such as α-carotene and β-cryptoxanthin can contribute substantially to the nutritional value of fruits and vegetables (Bendich and Olson, 1989).

Current attention is centered on the action of β-carotene as an antioxidant, as it may interfere with free radical oxidation such as the peroxidation of lipids, typical of many

degenerative diseases. Although it has been clearly demonstrated that β-carotene has a significant *in vitro* antioxidant effect, there is still no real proof of its *in vivo* function at the low concentrations in which it is found and under physiological conditions (Bendich, 1989).

There are 650 known naturally occurring carotenoids. Most of the carotenoids found naturally in fruits and vegetables have skeleton of 40 carbon atoms (C_{40}) and are biosynthesized from molecules of an intermediary C_{20} (geranylgeranyl diphosphate) giving rise to a phytoene, a generic precursor of a whole wide range of carotenoids present in the plant kingdom. The phytoene molecule undergoes a series of successive de-saturations (up to four), introducing new double bonds into the carbon chain. This results in the spreading of the double bond conjugation and thus, of the chromophore that is typical of these natural pigments and responsible for their chromatic properties (Britton, 1997).

1.9.8 Lycopene (ψ ψ-carotene)

It is an acyclic carotenoid containing 11 conjugated double bonds. It is naturally found in trans-form in raw tomatoes that imparts red color. Among the common dietary carotenoids, lycopene has the highest single oxygen quenching capacity *in vitro* and its antioxidant properties are probably related to risk reduction of certain types of cancers. It has been found that after air-drying at 80ºC, the number of hydroxyl phenol groups increases owing to the hydrolysis of flavonoid glycosides and / or the release of cell wall phenolics (Thadikamala, 2009). Processing promotes different side reactions that could affect the antioxidant activity of the plant products (Thadikamala, 2009).

1.10 Safety of herbal medicine

Systemic toxicity from herbal extracts depends on the route and site of exposure (Jothy *et al*., 2011). Direct tissue damage is usually the result of cellular destruction and this may have a biochemical or immunological basis. Many pathological lesions are of unknown mechanism, particularly the intermediate stages between the interaction of the toxin or its

metabolite with cellular constituents, and the start of the final degenerative changes that lead to cell death (Jothy *et al*., 2011).Toxic effects may be detected by gross pathological examination in the post mortem or histopathological examination after toxicity studies have been carried out. Some may also be detected using chemical analysis of body fluids (Timbrell, 1996). Efficacy and safety of medicinal plants have been studied using animal model such as BALB/c mice, albino mice, Wistar rats and rabbits.

1.11 Biochemical markers of medicinal plant extract toxicity

Biochemical markers are mainly applied to recognize, characterize and monitor treatment-related responses following an exposure to xenobiotics. They play three main roles in toxicology, i) to confirm exposure to a deleterious agent ii) to provide a system for monitoring individual susceptibility to a toxicant and iii) to quantitatively assess deleterious effects of a toxicant on an organism (Amacher, 2002). Because the liver is the general target for adverse effects of drugs and other chemicals, biomarkers of untoward hepatic response to xenobiotics are of particular interest to a pharmaceutical toxicologist (Abbort *et al*., 1997).

Liver function markers refer to peripheral indicators of hepatic synthetic and secretory activities, enterohepatic functions or perturbation of the hepatic uptake and clearance of circulating biomolecules (Amacher, 2002). Liver injury biomarkers include various peripheral proteins released in response to a cellular damage that are significantly altered within the liver. These include both circulating cytosolic, mitochondrial, or canalicular membrane markers and up-regulation or depletion of radical scavengers, modulators and stabilizers of intracellular damage (Amacher, 2002).

1.11.1 Blood urea nitrogen (BUN) and creatinine

These are nitrogenous end products of metabolism removed from the blood by the kidneys. BUN and creatinine are the most commonly used clinical serum biomarkers of renal damage. Urea is derived primarily from dietary protein, and protein turnover within the body

and creatinine is the product of muscle creatine breakdown (Hayes, 2008). BUN concentrations vary greatly with individual protein intake, protein metabolism, state of hydration and renal urea excretion. Creatinine concentration can vary with individual body muscle mass and measurement source (serum versus urine) and techniques. Elevation in BUN and creatinine can serve as clinical indicators of poor kidney function. However, creatinine is more sensitive than BUN (Hayes, 2008).

1.11.2 Alkaline phosphatase

Alkaline phosphatase (ALP), is a ubiquitous enzyme in the body needed in small amounts to catalyze specific chemical reactions. When present in serum in large amounts, it may signify bone or liver disease or a tumor in these organs (Bigoniya *et al*., 2009). In a healthy liver, fluid containing ALP and other substances is continually drained away through the bile duct.

In a diseased liver, the bile duct is often blocked retaining fluid in the liver. ALP accumulates and eventually escapes into the blood stream. The cells lining the small bile ducts in the liver produce ALP. Its origin differs from that of other diagnostic liver enzymes such as the aminotransferases (Bigoniya *et al*., 2009). If the liver disease is primarily of an obstructive nature (Cholestatic) involving the biliary drainage system, the ALP will be the first enzyme to be elevated. If on the other hand, the disease is primarily of the liver cells, the aminotransferases increase prominently.

1.11.3 Aspartate aminotransferase

Aspartate aminotransferase (AST) is a cytoplasmic and mitochondrial enzyme found in the liver, heart, skeletal muscles, kidney, pancreas, erythrocytes, lungs and brain tissue. When an injury affects these tissues, the cells are destroyed and AST is released into the blood stream. The amount of AST is directly related to the number of cells affected by the

injury, but the level of elevation depends on the length of time that the blood is tested after the injury.

Serum AST level increases eight hours after the cell injury, peaks at 24-36 hours, and returns to normal in three to seven days. If the cell injury is chronic (on-going), AST level remains elevated (Amacher, 2002). AST level is a valuable indicator of liver disease. It is used in combination with other enzymes like alanine aminotransferase (ALT) to monitor the cause of various liver disorders (Amacher, 2002).

1.11.4 Alanine aminotransferase

Alanine aminotransferase (ALT) is a cytoplasmic enzyme found in high concentration in the liver and to a lesser extent in skeletal muscles, kidney and heart. A rise in plasma ALT activity is an indicator of a damage to the cytoplasmic membrane. Liver cells contain more AST than ALT and in most conditions, damage to the cytoplasmic and mitochondrial membranes leads to a relatively greater increase in plasma AST activity than that of ALT. However, ALT is confined to the cytoplasm and mitochondria and its concentration is greater than that of AST (Zahra and Samaneh, 2012).

1.11.5 Creatinine kinase

Creatine kinase (CK) is the enzyme responsible for regeneration of ATP and in normal serum; at least 95% of the CK present is of skeletal muscle origin and is largely the result of leakage from skeletal muscles particularly during physical activity (Kaplan, 1989). Because of this, serum CK activity in healthy and active persons shows asymmetrical distribution skewed towards higher values.

Age, sex and race affect CK activity in serum. It has been established that values are lower in women than in men and also in morning than in evening. Values tend to be lower in hospitalized patients, possibly because bed rest reduces the amount of enzyme released from the muscles (Kaplan, 1989). The reference interval for total CK activity has been reported to

be 130 -253 µ/L in humans (Kaplan, 1989). In view of the wide variation in normal CK level in animals, a higher level of its activity is clinically significant (Kaneko, 1989).

1.11.6 Blood urea nitrogen

The major pathway of nitrogen excretion in humans is urea. Urea is synthesized in the liver from amino acids resulting from protein metabolism. The source of protein is from diet or tissues. Urea passes from the liver into the blood and filtered out by the kidneys. Urea production is increased by high protein diet or by catabolism caused by starvation, tissue damage or sepsis (Khajehdehi *et al*., 1998). Blood urea nitrogen (BUN) is used as first line of investigation of glomerular function. However, over 60% of glomeruli must be destroyed before blood urea nitrogen concentration significantly rises (McClellan *et al*., 1997).

Low blood urea nitrogen is observed in a conditions such as pregnancy (the most common cause in young women), over enthusiastic intravenous infusion and appropriate antidiuretic hormone (ADH) secretion. All these are caused by an increase in glomerular filtration rate (GFR). In children, it is due to decreased synthesis of proteins where amino acids are used for protein anabolism during growth, low protein intake, severe liver disease and inborn errors of urea cycle (Joan *et al*., 1988).

1.12 Experimental models for induction of diabetes

1.12.1 Chemical induced Diabetes

1.12.1.1Alloxan

Alloxan is a urea derivative, widely employed for induction of diabetes in experimental animals like rats, rabbits, mice and dogs. The severity of disease is concentration dependent of alloxan (Iranloye *et al*., 2011). It causes selective destruction by necrosis of pancreatic β- cells for induction of type II diabetes (Etuk, 2010).

Chemical Properties

Chemically, alloxan is 2,4,5,6 tetraoxypyrimidine; 2, 4, 5, 6- pyrimidinetetrone, which is an oxygenated pyrimidine derivative present as alloxan monohydrate in aqueous solution. It shows diabetogenic action on intraperitoneal, subcutaneous and intravenous administration. The dose of alloxan required for induction of diabetes depends on the animal species, route of administration and grade of disease severity (Federiuk *et al*., 2004). Alloxan is non toxic to human β-cells even at very high doses due to different glucose uptake mechanism as compared to animals (Eizirik *et al*., 1994 ; Tyrberg *et al*., 2001).

Mechanism of action

Alloxan treatment evokes a sudden rise in insulin secretion in presence or absence of glucose for a short duration followed by a complete suppression of the islet of langerhans response to glucose even at high concentrations (Szkudelski *et al*., 1998; Lachin and Reza, 2012). Alloxan action in pancreas is preceded by its rapid uptake by pancreatic beta cells. Moreover, in pancreatic beta cells, the reduction process occurs in the presence of reducing agents like reduced glutathione (GSH), cysteine, ascorbate and protein-bound sulfhydryl (-SH) groups (Lenzen *et al*.,1991; Zhang *et al*.,1992). Alloxan reacts with two -SH groups at the sugar binding site of glucokinase and results in the inactivation of enzyme. As a result, dialuric acid is formed which is then re-oxidized to alloxan establishing a redox cycle and generating reactive oxygen species (ROS) and superoxide radicals (Munday ,1998; Das *et al*.,2012).

The superoxide radicals liberate ferric ions from ferritin and reduce them to ferrous and ferric ions and undergo dismutation to yield hydrogen peroxide (H_2O_2). As a result, highly reactive hydroxyl radicals are formed in presence of ferrous and H_2O_2. Another mechanism that has been reported is the effect of ROS on the DNA of pancreatic islets. In the β-cells, alloxan causes DNA fragmentation and damage. Antioxidants like superoxide

dismutase, catalase and non enzymatic scavengers of hydroxyl radicals have been found to protect against alloxan toxicity (Ebelt *et al*., 2000). In addition, cytosolic free elevated Ca^{2+} has also been reported to constitute an important step in the diabetogenic action of alloxan. The calcium influx in to the pancreatic cells results from the ability of alloxan to open voltage dependent calcium channels. This causes supra physiological insulin release, that along with ROS, eventually causes damage of beta cells of pancreatic islets (Park *et al*., 1995).

1.12.1.2 Streptozotocin

Streptozotocin is a mono functional nitrosourea derivative isolated from *Streptomyces achromogenes* (Herr *et al*., 1967). It is widely used for induction of type I diabetes with renal complications in experimental animals. Low doses (60-90 mg kg^{-1}) of STZ were employed for induction of type- 2 diabetes. It has been used alone or in combination with other chemotherapeutic agents like vincristine, 5-fluorouracil, methyl-CCNU, procarbazine and 6-thioguanine, for the treatment of colorectal carcinomas, metastatic cancer of islets and other gastrointestinal cancers (Togni *et al*., 1982; Welt *et al.,* 1983; Clamon *et al*., 1987).

Mechanism of Action

Streptozotocin prevents DNA (Deoxyribonucleic acid) synthesis in mammalian and bacterial cells and renders special reaction with cytosine groups resulting in the degeneration and destruction of DNA. It enters the pancreatic cells *via* a glucose transporter-GLUT2 (Glucose transporter 2) and brings about alkylation of DNA. Further STZ induces activation of poly adenosine diphoshate ribosylation and nitric oxide release. As a result of STZ action, pancreatic cells are destroyed by necrosis which in turn induces insulin dependent diabetes mellitus (Mythili *et al*., 2004; Patel *et al*., 2006).

1.12.1.3 Dithizone

Dithizone is an organo sulphur compound widely used for induction of diabetes in experimental animals. In dithizonised diabetic animals, the levels of serum iron, potassium

and zinc were found to be higher than normal but magnesium and copper levels remained unchanged (Halim *et al*., 1977). It acts as a chelating agent and forms complexes with lead, zinc and mercury. It is also used to assess the purity of human pancreatic islet preparations used for transplantation in patients with type I diabetes (Bavelsky *et al*., 1992).

Mechanism of Action

It gets transported across cell membrane by active transport and forms a complex with zinc and releasing protons that enhance diabetogenic action of dithizone. The addition of these complexing agents at pH 6 to the lipid vesicles containing entrapped zinc ions, acidifies the contents of these vesicles. Such proton release occurs within the zinc-containing insulin storage granules of pancreatic β-cells, solubilisation of insulin would be induced which leads to osmotic stress causing the rupture of granules and finally inducing diabetes (Epand, 1985).

1.12.1.4 Gold thioglucose

It is a glucose derivative of gold and mainly used in experimental animals for the induction of non insulin dependent diabetes mellitus with severe obesity.

Mechanism of Action

It induces experimental diabetes by promoting obesity which develops diabetes in genetically normal mouse strains. Gold thioglucose-treated DBA/2 (Dilute Brown Non-Agouti), C57BLKs and BDF1 mice gained weight rapidly and significantly increased non fasting plasma glucose level within 8-12 weeks. These mice showed impaired insulin secretion, mainly in early phase after glucose load and reduced insulin content in pancreatic islets (Karasawa *et al*., 2011).

1.12.1.5 Monosodium glutamate

Monosodium glutamate is a naturally occurring non- essential amino acid, it induces Type -II diabetes in experimental animals without producing polyphagia.

1.12.2 Virus-Induced Diabetes

Viral infection and β-cells specific autoimmunity may lead to the development of juvenile onset diabetes (Craighead, 1978). Gamble and co-workers reported newly diagnosed juvenile onset diabetes mellitus due to viral infections in 1960s (Gamble *et al*.,1996).

1.12.2.1 D- Variant Encephalomyocarditis

EMC- D virus can infect and destroy pancreatic beta cells in certain inbred strains of mice and produce insulin dependent hyperglycaemia (Yoon *et al*., 1980). Pre-treatment with cyclosporine-A increased severity and incidence of diabetes in ICR Swiss mice (Gould *et al*., 1985). Intraperitoneal injection of viral strain, NDK25, developed non-insulin dependent diabetes mellitus (Utsugi et *al*., 1992).

1.12.2.2 Coxsackie Viruses

Coxsackie viruses may also be a possible cause of diabetes in mice. They infect and destroy pancreatic acinar cells while leaving the adjacent islets of Langerhans intact. Coxsackie B4 virus has been associated with the development of insulin dependent diabetes mellitus in humans. Diabetes induced by Coxsackie virus infection is a direct result of local infection leading to tissue damage, inflammation and release of sequestered islet antigen. These result in re-stimulation of resting auto reactive T cells, indicating that the islet antigen sensitization is an indirect consequence of the viral infection (Lansdown and Brown, 1976; Horwitz *et al*., 1998).

1.12.3 Hormone-Induced Diabetes

1.12.3.1 Growth hormone-induced diabetes

Repeated administration of growth hormone may lead to the development of type II diabetes with renal complications (Thirone *et al*., 2002). In experimental animals like cats and adult dogs repeated administration of growth hormone produces diabetes due to the

destruction and loss of pancreatic β- cells with severe symptoms of ketonemia and ketonuria (Campbell *et al*., 1954).

1.12.3.2 Corticosteroid-induced diabetes

Prolonged use of glucocorticoids, especially dexamathasone and prednisolone, for the treatment and management of inflammation can lead to diabetes. Glucocorticoids oppose insulin action and stimulate gluconeogenesis in the liver, resulting in a net increase in hepatic glucose output and causes hyperglycemia, insulin resistance and hyperlipidemia (Heather *et al*., 2012).

1.13 Plants under investigation in the study

1.13.1 *Dillenia indica*

1.13.1.1 Pharmacognostical characteristics of *Dillenia indica*

Fig. 3 Different parts of the plant, *Dillenia indica*

1.13.1.2 Scientific classification

Kingdom Plantae

Subdivision	Angiospermae
Division	Phanerogamae
Class	Dicotyledonae
Subclass	Polypetalae
Order	Dilleniales
Family	Dilleniaceae
Genus	Dillenia
Species	indica Linnaeus or speciosa Thunberg, pentagyna Roxburgh or hainanensis Merrill

1.13.1.3 Vernacular names

English	Elephant apple
Hindi	Chalta, girnar
Sanskrit	Bhavya, ruvya
Marathi	Karambal
Assamese	Chalita, Outenga
Telugu	Peddakalinga
Tamil	Akku, Ugakkay, uva, uvav, uvatteku
Kannada	Betta Kanigala, Kondukanagala

1.13.1.4 Distribution

The plant *D. indica* is widely distributed in many Asian countries like India, Malaysia, Nepal, Bhutan, Indonesia and Thailand. In India, it is found in Himalayan and sub Himalayan regions such as Bihar, North Bengal, Madhya Pradesh and Assam (Khanum and Khan, 2007; Khare,2007).

1.13.1.5 Description

It is a small to medium sized evergreen shrub and about 30 m tall. The leaves are arranged in a fascicle manner at the end of the branches. The petiole are narrowly winged, 2.5-5 cm long, channelled, sheathed; leaf blade oblong or obovate oblong, 15–40 × 7–14 cm, secondary veins (20–) 30–40(–70) on either side, parallel, margin serrate, apex is acute. The young branchlets are brown in colour and contain leaf scars with the veins close and running in to serrature. The bark is reddish brown in colour. The flowers are solitary, 12-20 cm in diameter and bud more than 5 cm in diameter. Sepals are 5 in number, near rounded, orbicular, concave, 4-6 cm in diameter, thick and fleshy. The fruit of *Dillenia indica* contains fleshy proteinacious aril which is used in jelly, pickle and curry preparations.

1.13.1.6 Chemicals constituents

Dillenia indica contains kaempferol glucoside, betunaldehyde, flavonoids like rhamnetin, 10% tannin, dihydro-isorhamnetin, naringenin, quercetin, dillenetin, betulinic acid, lupeol, and myricetin (Shah, 1978; Khanum and Khan, 2007; Khare, 2007). Ethanolic extract of the stem bark contains two flavonoids i) quercetin derivative and ii) kaempferol glucoside. It also contains triterpenoids (Srivastava and Pande, 1981). Four different compounds stigmasterol, betunaldehyde, lupeol and betulinic acid, were isolated from methanolic extract of the stem bark by using column chromatographic fractionation with n-hexane (Parvin *et al*., 2009).

Apart from these chemical constituents, leaves of different species of *Dillenia* contain various chemicals like β-sitosterol, betulinic acid, lupeol and betulin (Dan & Dan, 1980). They are rich source of triterpenoids, steroids, tannins and flavonoids. Four different compounds, n-hentriacontanol, betulin, cycloartenone and sitosterol were isolated from the petroleum leaf extract. Chloroform extract contains betulinic acid (Mukherjee, 1981). Fractionation of methanolic leaf extract with n- hexane yielded various compounds like betulinic acid, β-sitosterol, stigmasterol as well as dillenetin (Md Muhit *et al*., 2010).

Phytochemical investigation of acid hydrolyzed extract showed presence of kaempferol. Fresh leaves contained 7-glucosides of naringenin which get oxidized to ten corresponding flavonols (Bate-smith and Harborne, 1975) and dihydrokaempferide. Betulinic acid isolated from different fractions like n-butanol, methanol, ethyl acetate and water quantified by validated HPTLC analysis showed highest concentration of 97.99 mg/g of fraction in ethyl acetate fraction (Kumar *et al*., 2010).

Methanol fruit extract of *Dillenia indica* contained 34% of total phenolics, polysaccharides like arabinogalactan (Md. Abdille *et al*., 2005). It also contained saponins, colouring agents, sterols, glycosides, free amino acids, fixed oil, proteins, sugars and tannins (Uppalapati and Rao, 1980).

1.13.1.7 Traditional/ Ayurvedic uses

Different parts of the plant are used in traditional and folk medicine by tribal communities for the treatment and management of various ailments. Traditionally, decoction of the whole plant of *Dillenia indica* is used as universal antidote, for the relief of fever and as an aphrodisiac (Nadkarni *et al*., 1954; Shah, 1978; Khanum, 2007).

Stem bark of *Dillenia inidca* is used for prophylactic treatment of cholera, chronic progredient sores and sores caused by mercury poisoning. It is applied topically to remove the poison around the wound caused by spider bite (Nadkarni *et al*., 1954; Shah, 1978; Khanum *et al*., 2007; Janick and Paull, 2008).

Decoction of fruits is used to control dandruff and prolonged hair fall and as a tonic to combat weakness. Ripe fruits are used as galactogogue, supuration of boils and removing flatulence (Rastogi *et al*., 2000). The seeds and unsaponifiable matter possess antibacterial, antifungal and other antimicrobial properties. The fruits are widely used in indigenous ayurvedic medicine for nervousness and the mucilage is applied on wounds of burns (Janick and Paull, 2008).

1.13.1.8 Pharmacological applications

Pharmacologically, it has been reported that *Dillenia indica* shows anti-inflammatory, antiproliferative, hepatoprotective, antimicrobial, antidiarrheal, antileukemic, antioxidant and cytotoxic activities.

Fractionation of methanolic fruit extract of *Dillenia indica* with different solvents like ethyl acetate, n-butanol and water showed antileukemic activity against various human leukemic cell lines HL60, K562 and U937.

1.13.2 *Solanum torvum*

1.13.2.1 Pharmacognostical characteristics of *Solanum torvum*

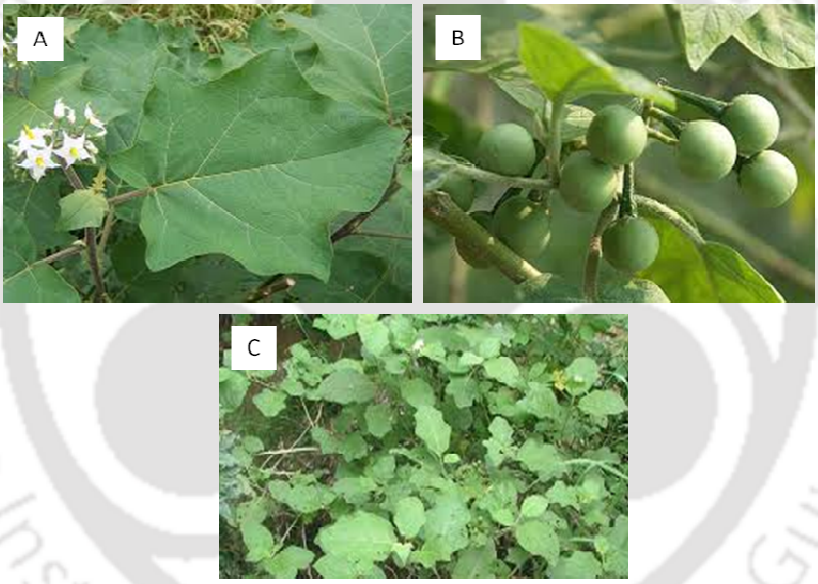


Fig. 4 Different parts of the plant *Solanum torvum*
A) Leafs and flowers of *Solanum torvum* B) Fruits of *Solanum torvum* C) Whole plant

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	Solanum L

Species	*Solanum torvum* sw

1.13.2.3 Vernacular names

English	West Indian Turkey Berry
Hindi	Bhurat, Bhankatiya
Sanskrit	Brihati
Marathi	Marang
Folk	Ran-Baingan, goth-begun

1.13.2.4 Description and distribution

Solanum torvum Sw. (Solanaceae), commonly known as Turkey berry, is native of and cultivated in West Indies and Africa (Adjanohoun *et al*., 1996). It is also distributed in different parts of India and mainly found in the moist farms at an elevation of 1000 m MSL. It is small a to medium erect spiny shrub that is usually 2 or 3 m in height and 2 cm in basal diameter, but may reach up to 5 m in height and 8 cm in basal diameter.

The fruits and leaves are widely used in Camerooninan traditional and folk medicine. The fruits are edible and utilized as vegetable in regular culinary preparations mainly in southern parts of India.

1.13.2.5 Chemical constituents

Preliminary phytochemical analysis of the fruit extract revealed the presence of various phytoconstituents like saponins, glycosides, alkaloids, tannins, flavonoids, vitamins B and C fixed oils and iron salts (Sivapriya and Srinivas, 2007). It also has a number of other chemical constituents like neochlorogenin 6-O-β-D-xylopyranosyl-(1→3)- β-D-quinovopyranoside (Arthan D.,2002), kaempferol (Lu *et al*., 2009) and quercetin (Gonzalez *et al*., 2004), solagenin 6-O-β-Dquinovopyranoside (Agrawal,1989), neochlorogenin 6-O-β-D-quinovo-pyranoside (Zhu, 2003), isoque-rcetin (Iida *et al*., 2005), solagenin 6-O-α-Lrhamnopyranosyl-(1→3)-β-Dquinovopyranoside (Yahara *et al*.,1996), rutin (Lu *et al*.,

2009) and neochlorogenin 6-O-α-L- rhamnopyranosyl-(1→3)-β-Dquinovopyranoside (Mahmood,1985)

1.13.2.6 Traditional/Ayurvedic uses

Decoction of the fruits is used in cases of liver and spleen enlargement and cough (Siemonsma and Piluek, 1994). Leaves are used as haemostatic, diuretic and sedative. Ripened fruits are used in the treatment and management of pain, as an antioxidant (Sivapriya and Srinivas, 2007) and haemopoietic agents (Kala, 2005). It is also used for the treatment of arterial hypertension, wounds, tooth decay, fever and reproductive problems (Ndebia *et al*., 2007).

1.13.2.7 Pharmacological applications

Solanum torvum showed various activities like antioxidant (Waghulde *et al*., 2011), neuroprotective (Mohan *et al*., 2010), antifungal (Karuppusamy *et al*., 2009, Satish *et al*., 1999), antibacterial (Lalitha *et al*., 2010), antihypertensive (Nguelefack *et al*., 2008), antiulcer (Telesphore *et al*., 2008), analgesic, anti-inflamatory and cardio protective activities (Atta and Alkofahi, 1997).

1.13.3 *Solanum indicum*

1.13.3.1 Pharmacognostical characteristics of *Solanum indicum*

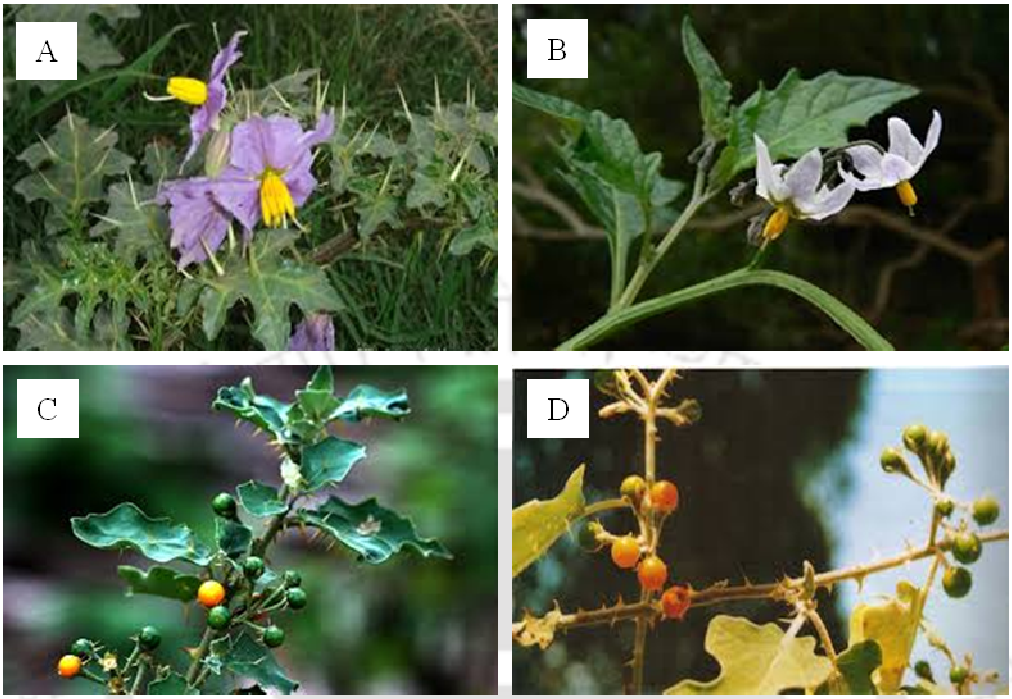


Fig. 5 Different parts of the plant *Solanum indicum* (A) Whole plant B) Flowers C) Unripened fruits D) Ripened fruits)

1.13.3.2 Scientific classification

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	Solanum L
Species	*Solanum indicum*

1.13.3.3 Vernacular names

Hindi	Badi Kateri, Vanabhanta
Telugu	Pedda Mulaka, Tella Mulaka
Bengali	Rambegun, Vyakud

Tamil	Anachundai, Papparamalli,
Marathi	Dorali

1.13.3.4 Description and distribution

Solanum indicum Linn (Synonym: Solanum anguivi) belongs to the family Solanaceae commonly known as Byakur, Guta begun, Kata begun, Brihati and Indian Night shade. It is a much branched perennial under shrub up to 1.8 m height. It is a stiff, prickly herb; prickles stout, recurved. The leaves are 7.5- 15 cm long, 2.5-10 cm broad, alternate, lobed, entire spines present on petiole and midrib. It is distributed throughout India and all over the tropical and subtropical regions of the world (Chopra *et al*., 1992; Kirtikar and Basu, 1975).

1.13.3.5 Chemical constituents

The fruits, leafs and roots of *Solanum indicum* contain chemical like alkaloids, solanine and solanidine, disogenin, solamargine, waxes, fatty acids, β-sitosterol, solasornine, lanosterol and so on (Chopra *et al*., 1992; Kirtikar and Basu, 1975 ; Bhakta, 2004 ; Bhattacherya , 1982).

1.13.3.6 Traditional/ Ayurvedic uses

Different parts of the plant like root, leaf, fruits and stem are used in traditional medicine for treatment of blood disorders, sore throat, rhinitis, cough, asthma, loss of appetite and anorexia, sexual disorders, worm infestation, hiccup, abdominal pain, fever, urinary complications, inflammation, insomnia and so on.

Solanum indicum has been used in folk medicine for the treatment of oedema and wound infections, ascites and inflammatory toothache (Huang *et al*., 2008). Different solvent extracts of *Solanum indicum* showed anti-inflammatory, wound-healing, anti-hypersensitive (Bahgat *et al*., 2008) and anticancer activities (Ma *et al*., 2006).

CHAPTER 2

In vivo and *In vitro* antidiabetic evaluation of *Dillenia indica* fruit extract in Streptozocin-induced diabetic rats

2.1 Introduction

Diabetes mellitus is the principal cause of morbidity and mortality in human populations (Steppan *et al*., 2001). It is a metabolic disease characterized by hyperglycemia, polydipsia and polyuria and causes complications to the eyes, kidneys and nerves. It is also associated with an increased incidence and complications of cardiovascular disease (Pickup and Williams, 1991). It currently affects an estimated 15.1 million people in North America, 18.5 million in Europe, 51.4 million in Asia and just under 1 million in Oceania (Kuhlmann, 1996). It is estimated that globally, the number of people will rise from 151 million in the year 2000 (Amos *et al*., 1997) to 300 million by 2025 (King *et al*., 1998).

The clinical diagnosis of diabetes includes the presence of hyperglycemia, glycosuria and sometimes, drowsiness or coma. The World Health Organization (WHO) criteria define diabetes by the fasting plasma glucose (FPG) level of 140 mg dL^{-1} (7 mM) or greater or

postprandial 2 h plasma glucose (PG) level of 200 mg dL^{-1} (11.1 mM) or greater during an oral glucose tolerance test (WHO, 1985).

National Institutes of Health recommends the following criteria for diagnosis of diabetes:

a. Fasting (overnight) venous plasma glucose concentration greater than or equal to 140 mg dL-1 on, at least, two separate occasions.

b. Venous plasma glucose concentration greater than or equal to 200 mg dL^{-1} at 2 h post ingestion of 75 g of glucose and at least, one other sample during the 2 h test.

2.1.2 Regulation of glucose metabolism by insulin and pathophysiology of diabetes

Plasma glucose concentration are effectively maintained within a fairly narrow range despite wide fluctuations in the body's supply (e.g. meals) and demand (e.g. exercise) for nutrients (Gerich, 1993). Changes in plasma blood glucose levels are moderated by the actions of the liver primarily under the control of insulin and glucagon (Unger and Orci, 1981). Insulin, secreted by the β-cells of the pancreas, lowers the concentration of glucose in the blood by inhibiting hepatic glucose production and stimulating the uptake and metabolism of glucose by the muscle and adipose tissue (Davis and Granner, 1996).

All forms of diabetes mellitus are due to a decrease in the circulating concentration of insulin (insulin deficiency) and a decrease in the response of peripheral tissue to insulin (insulin resistance). These abnormalities lead to alterations in the metabolism of carbohydrates, lipids, ketones, amino acids; the central feature of the syndrome is hyperglycemia. Insulin plays a key role in regulating both glycogenolysis and gluconeogenesis in liver (Cherrington *et al*., 1987). The absence or deficiency of insulin's effects not only engenders an increased hepatic net extraction of glucogenic amino acids, lactate, glycerol and their conversion to glucose, but also stimulates both the quantity and activity of gluconeogenesis enzymes, such as glucose-6-phosphatase (Glc-6-Pase), fructose-1,6-bisphosphatase and pyruvate carboxylase (Weber, 1964; Taunton *et al*.,1974). The

enzyme, Glc-6-Pase, catalyzes the terminal step in both gluconeogenic and glycogenolytic pathways, so it is a key determinant in the production of glucose by the liver. Both m-RNA levels and activity of Glc-6-Pase are low in the fed and reefed states, where insulin levels are elevated. Both mRNA levels and activity of Glc-6-Pase are elevated in diabetic rats and administration of insulin to diabetic rats results in the reduction in the level of mRNA as well as the activity of Glc-6-Pase enzyme (Argaud *et al*., 1996; Massillon *et al*., 1996).

Insulin has many actions within the central nervous system (CNS), including reducing food intake and body weight and interacting in predictable ways with other controllers of meal size (McGowan *et al*., 1990). On the other hand, its anabolic effects in peripheral tissue would promote weight gain. These two major actions of insulin tend to counter balance each other, as the peripheral anabolic effect of insulin would cause weight gain yet the appetite would be suppressed via insulin's central catabolic action (Schwartz *et al*., 1994). It is believed that insulin and leptin (Zhang *et al*., 1994), an adipose tissue hormone, modulate energy homeostasis, such as causing change in food intake and body weight at the brain level (Woods *et al*., 1998).

Hypoinsulinemia and low circulating leptin concentrations may contribute to hyperphagia via upregulation of hypothalamic neuropeptide Y (NPY) system in uncontrolled type I diabetes (Havel *et al*., 1998). However, in this kind of diabetes, the extreme hypoinsulinemia causes a wasting of peripheral tissue and consequent weight loss due to the lack of a peripheral insulin anabolic effect, even though there is also a concomitant enhanced appetite in this situation (The DCCT study group, 1988).

Insulin stimulates lipoprotein lipase activity and promotes fat and muscle storage of both exogenously derived triglycerides as well as that produced endogenously (Eckel and Yost, 1987). It also inhibits the hormone-sensitive lipase in adipose tissue and thus, inhibits the hydrolysis of triglycerides stored in the adipocytes. Elevations in plasma triglycerides and

cholesterol are evident in diabetic animals. This is related to decreases in activity of insulin-dependent lipoprotein lipase and in the apoprotein content of lipoproteins (Tavangar *et al*., 1992; Sparks *et al*., 1992) necessary for the recognition and efficient lipolysis of the triglyceride-rich particles at the sites of their uptake.

Steady state levels for insulin mRNA appears to be important for the regulation of insulin production. Insulin mRNA levels varied with the change in demand for insulin in several experimental conditions and correlated directly with rates of insulin biosynthesis when both were measured *in vivo* (Permutt *et al*., 1984; Giddings *et al*., 1985).

In a rat model for diabetes, maintenance of glucose homeostasis correlated with maintenance of pancreatic insulin mRNA content. When pre diabetic or mildly glucose intolerant rats were challenged with a diabetogenic agent, maintenance of normal glucose levels correlated with increases in insulin mRNA content. When this adaptive response failed, hyperglycemia worsened (Giddings *et al*., 1985). After administration of STZ and alloxan, a marked reduction in insulin mRNA level was observed (Mulder *et al*., 1995). The insulin gene is present as a single copy in most species. However, in rats, two nonallelic insulin genes (Insulin I gene and Insulin II gene) are expressed (Clark and Steiner, 1969; Lomedico *et al*., 1979). Their mRNAs are quite similar, being approximately 93% homologous in the coding regions with only 34 of 439 nucleotides different (Ullrich *et al*., 1977). Insulin I gene has been observed to be expressed in pancreas, but insulin II, the ancestral gene, is expressed not only by pancreas but also by extra pancreatic tissue, including the yolk sac and fetal liver (Giddings and Carnaghi, 1989). The two rat insulin genes may function independently. The conversion products, insulin I and II are usually stored in unequal amounts. The ratio of the cellular contents of insulin I over insulin II fluctuates between 1 and 2 in a basal fed or fasting state, but increases 2- to 4-fold during pregnancy or chronic hyperglycemia. Glucose is an important modulator of the rate of insulin biosynthesis, through changes in mRNA

levels (Kakita *et al*., 1982). Rat β-cells exhibit a differential regulation of biosynthesis of the two insulin isoforms at the level of both transcription and translation. This leads to an increase in the ratio of insulin I over insulin II in terms of both their respective mRNA content as well as their peptide content (Ling *et al*., 1998).

2.1.3 Free radicals and the complications of diabetes

The causes of death in the diabetic population changed drastically after the introduction of insulin therapy by (Banting and Best in 1922). While insulin and other medical treatments can control many aspects of diabetes, numerous complications are not uncommon. The microvascular, neuropathic and macrovascular complications are a major health problem for patients with either Insulin Dependent Diabetes Mellitus (IDDM) or Non Insulin Dependent Diabetes Mellitus (NIDDM) (Herman and Crofford, 1998). Oxidative damage appears to be involved in the pathogenesis of long term complications in diabetes, based on the increased concentration of lipid peroxidation products and the accumulation of advanced glycosylation end products and glycoxidation products in tissue proteins of diabetic patients with complications. Enzymatic and non enzymatic oxidation of lipids and carbohydrates yield reactive carbonyl compounds, including aldehydes derived from lipid peroxidation and dicarbonyl sugars derived from glucose, which are key intermediates in the chemical modification and cross-linking of proteins in diabetes (Baynes, 1995). Oxygen free radicals (OFRs), such as superoxide ($O2^{\bullet -}$), hydrogen peroxides (H_2O_2) and hydroxyl radicals (OH•) are implicated in the pathophysiology of ischemia/reperfusion injury and atherosclerosis. Oxidation of lipids in plasma lipoproteins and in cellular membranes is associated with the development of vascular disease in diabetes. Much of the experimental evidence suggests that diabetes and hyperlipidemia alone are not sufficient to provoke vascular disease but oxidative stress may be an important and independent risk factor in the development of vascular disease. Although antioxidant therapy has not been adequately

tested, it may provide an important defense against oxidative damage and the development of complications in diabetes.

Fig. 2.1 Different parts of *Dillenia indica.*

Different parts (fruits, leafs and whole plant) of the plant *Dillenia indica* were used in traditional and folk medicine by tribal communities for the treatment and management of various ailments. Traditionally, decoction of the whole plant of *Dillenia indica* is used as a universal antidote, for the relief of fever and as an aphrodisiac (Nadkarni *et al*., 1954; Shah, 1978; Khanum, 2007).

Pharmacologically, it has been reported that *Dillenia indica* has anti-inflammatory, anti-proliferative, hepatoprotective, antimicrobial, anti-diarrheal, anti-leukemic, antioxidant and cytotoxic activities. However, the antidiabetic activity of the fruit extract has not been reported. Hence the present study was focused on the investigation of the antidiabetic and antioxidant activities of methanolic fruit extract of *Dillenia indica* in STZ-induced diabetic rats.

2.2 Materials and methods

2.2.1 Chemicals, reagents and kits

Streptozocine (STZ), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), α-amylase, 3, 4, 5-Dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT), modified eagle media (MEM) and fetal bovine serum (FBS) were procured from Sigma-Aldrich, USA. Folin-Ciocalteu reagent for estimation of total phenolics was obtained from Himedia. Mouse fibroblast cell line (L929) was obtained from the National Centre for Cell Sciences, Pune, India. All other reagents and solvents used for the study were of analytical grades.

2.2.2 Collection and identification of plant material

Unripened fruits of *Dillenia indica* were bought in the month of August from the local market near IIT Guwahati, India. The plant was authenticated by a taxonomist at the Department of Botany, Guwahati University, India and a voucher specimen (17785) was deposited at the Herbarium for future reference.

2.2.3 Processing of plant materials

The fruits were thoroughly washed with distilled water to remove soil and dirt and then chopped and dried under shade at room temperature around 25^{o}C for about one month. The chopped pieces of dried fruits were grounded and sieved to obtain a fine powder. The powdered plant material was kept in closed dry plastic bags. Approximately, five hundred grams of coarse powder of *Dillenia indica* fruit was extracted with methanol by hot soxhlet extraction method for 24 h. The extract was concentrated using a rotary evaporator (Rotavapor, R-215). The concentrated extract was lyophilized to get a powder (yield 13.3% w/w) and used for further study.

2.2.4 Determination of total phenolic content in the plant extract

The concentration of phenolics in the plant extract was determined by spectrophotometric method (Singleton *et al*., 1995). Methanolic solution of the extract at concentration of 1 mg mL^{-1} was used in the analysis. The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of extract, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 mL methanol, 2.5 mL 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% of $NaHCO_3$. The samples were thereafter, incubated at room temperature at 25^{o}C for 30 min. The absorbance was measured using spectrophotometer (Carry 100 BIOUV–Vis, Varian, CA, USA) at λ_{max} of 765 nm. The samples were prepared in triplicate for each analysis and the same procedure was repeated for the standard solution of gallic acid (10-100 µg mL^{-1}) and the calibration curve was constructed. Based on the calibration curve of gallic acid, the concentration of total phenolics was measured (mg mL^{-1}); the content of phenolics in the extracts was expressed as gallic acid equivalent (mg of GA g^{-1} of fruit extract).

2.2.5 Determination of total flavonoids content in the plant extract

Total flavonoid content in the fruit extract was determined by spectrophotometric method (Quettier *et al*., 2000) with slight modification. 1 mg mL^{-1} solution of extract was made in methanol and to this, 1 mL of 2% $AlCl_3$ solution prepared in methanol was added. The sample was incubated for 60 min at 25^{o}C. The absorbance was measured using spectrophotometer at λ_{max} of 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration curve was constructed. Based on the measured absorbance, the concentration of flavonoids was read (mg mL^{-1}) on the calibration

curve; and the content of flavonoids in the extract was expressed in terms of quercetin equivalent (mg of quercetin g^{-1} of fruit extract).

2.2.6 Evaluation of antioxidant activity

The ability of the plant extract to scavenge DPPH free radicals was assessed by the standard method (Takeo *et al*., 1994) adopted with minor modifications (Kumarasamy *et al*., 2007). The stock solution of extract was prepared in methanol to achieve the concentration of 1 mg mL^{-1}, Dilutions were made to obtain different concentrations of 10, 20, 30, 40, 50 and 100 µg mL^{-1}. Diluted solutions of extract (1 mL each) was mixed with 0.1 mM DPPH solution and incubated in the dark at 25^{o}C for 30 min, the absorbance was recorded at 517 nm. The control sample contained all the reagents except the extract. Percentage inhibition was calculated using the following equation,

Percentage inhibition= {Absorbance of control-Absorbance of sample/absorbance of control} × 100

The IC_{50} value was determined from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data was presented as mean values ± standard deviation (n = 3).

2.2.7 Maintenance of cell line

The normal mouse fibroblast (L929) cell line and Human breast cancer cell line (MCF-7) were purchased from NCCS, Pune and maintained according to the standard protocol. The cells were maintained in Modified Eagle's Medium (DMEM) supplemented with 10% (V/V) foetal bovine serum (FBS) and 1% antibiotic, antimycoyic solution (1000 U mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin sulphate, 5 mg mL^{-1} gentamycin and 25 µg mL^{-1} amphotericin B). The cells were grown in humidified atmosphere in CO_2 in an incubator.

2.2.8 Treatment of cells

Mouse fibroblast cells (L929) and human breast cancer (MCF-7) cell lines were cultured and maintained according to supplier guidelines. Cytotoxicity of fruit extract was assessed by 3- 4, 5-dimethylthiazole-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) dye conversion assay. L929 and MCF-7 cells at a density of 1×10^4 per well were cultured in a 100 µL of cell culture medium (DMEM: Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum in a 96 well cell culture plate. After 24 h, cultured cells were treated with a series of different concentrations of fruit extract ranging from 10 to100 µg mL^{-1} in serum free media and incubated further for 24 h. This was followed by the removal of the media and treatment with MTT dye at a final concentration of (0.5 mg mL^{-1}) and further incubated for 4 h. Finally, 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the blue formazan precipitate and the absorbance was measured at 570 nm using a microplate reader (Tecan, Model 680). The cell viability was expressed as a percentage of the control by the following equation,

$$\% \text{ cell viability} = A_t / A_c \times 100$$

Where A_t and A_c represent absorbance of the plant extract treated and control cells respectively (A=3; where n is the no. of independent experiments).

2.2.9 α- Amylase inhibition assay

The α-Amylase inhibition assay was carried out according to the procedure reported by (Thirunavukkarasu, 2003). 500 µL of various concentrations (10, 20, 40, 60, 80, 100 µg mL^{-1}) of the fruit extract of *Dillenia indica* and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 µg mL^{-1}) were incubated for 10 min at 25°C. After pre-incubation, 500 µL of 1% starch solution dissolved in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. Finally, the reaction was stopped by adding 1 mL of 3, 5- dinitrosalicylic acid coloring reagent (Miller, 1959). The test tubes were then incubated in a boiling water

bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 mL of distilled water and the absorbance was measured at 540 nm (Nampoothiri *et al*., 2011). The percentage inhibition was calculated using the following formula,

$$\% \text{ inhibition} = A_b - A_t / A_b \times 100$$

Where A_b and A_t represent the absorbance of the blank and test respectively.

2.3 Animal experimentation

Healthy albino Wistar rats of either sex weighing 180-220 g were procured from the central animal house, Department of Pharmacology, College of Veterinary and Animal Sciences, Udgir, and used for the study. The animals were allowed to acclimatize for a period of two weeks in the animal house. The rats were housed in polypropylene cages, at an ambient temperature of 25±2ºC with 12 h light and dark cycle. The animals were fed with standard rat pellet diet (Hindustan Lever, Mumbai) and water *ad libitum*.

The experimental protocols and procedures used in the study were approved by the Institutional Animal Ethical Committee, College of Veterinary and Animal Sciences, Udgir, (Maharashtra) approval no. VCU/CPCSEA/IAEC/2/14 (II).

2.3.1 Acute toxicity study

The acute toxicity study was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines No. 423. The study was conducted in two phases using total number of thirty six rats. Overnight starved healthy adult Wistar rats were divided into six groups (n =6). In the first phase group 1, 2 and 3 animals (n=6) were fed with fruit extract of *Dillenia indica* in the increasing order of 5, 50 and 300 mg kg^{-1} body weight to establish the range of doses producing any toxic effect. In the second phase group 4, 5 and 6 animals (n=6) were administered with specific doses (1000, 2000 and 5000 mg kg^{-1} b.w) of the fruit extract to establish toxicity and determination of LD_{50} value.

The fruit extract was dissolved in 0.5% carboxy methyl cellulose (CMC) solution and given by oral route. All rats were monitored for 24 h for the signs of behavioural, neurological and autonomic profiles.

2.3.2 Oral glucose tolerance test (OGTT)

In order to determine the effect of the fruit extract of *Dillenia indica* on insulin activity, the (OGTT) was carried out on all four groups of rats (n=6) i.e., control, positive control and treated (in two different doses at 100 and 200 mg kg^{-1}). OGTT was performed by oral administration of glucose load of 2 g kg^{-1} body weight to overnight fasted rats. Blood samples were withdrawn from the tail vein at 30, 60, 90, 120 and 180 min after the oral glucose load and fruit extract administration and glucose levels were estimated using glucose estimation kit (Merck Ltd, Mumbai).

2.3.3 Induction of diabetes

Overnight starved experimental rats were injected with streptozotocin at a dose of 60 mg kg^{-1} body weight as described earlier (Annae *et al*., 1998) with slight modifications. STZ was injected intra-peritoneally after dissolving it in ice cold sodium citrate buffer (0.025 M, pH 4.0). The rats exhibiting blood glucose level ≥250 mg dL^{-1} were included in the study as stable hyperglycemic animals. Once the stable hyperglycemia was achieved, the rats were divided in to five different groups as follows,

The animals were divided into five groups of six animals each. Group I, untreated (control); Group II, STZ-treated diabetic rats; Group III, STZ-induced diabetic rats treated with glibenclamide (5 mg kg^{-1}); Group IV, STZ-induced diabetic rats treated with 200 mg kg^{-1} body weight of *Dillenia indica* fruit extract; Group V, STZ-induced diabetic rats treated with 400 mg kg^{-1} body weight of *Dillenia indica* fruit extract. After randomization into various groups, the treatment was continued for 30 days and serum biochemical parameters such as glucose, triglycerides and cholesterol were estimated at a regular time interval.

2.3.4 Collection of blood

The blood samples were collected by retro orbital sinus puncture under light ether anesthesia put into plastic test tubes, and was allowed to stand for 30 min to ensure complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 10 min and clear serum samples were aspirated off and stored at -20^{o}C until required for biochemical parameter analysis. The biochemical parameters estimated in the serum specimen were glucose, alanine aminotransferase (ASAT), aspartate aminotransferase (ALAT), serum lipid profile (Nicholas, 1956) by using auto-analyzer (Microlab100, Merck Ltd, Mumbai).

2.3.5 Estimation of serum glucose

Serum glucose was estimated by glucose oxidase (GOD) peroxidise (POD) method (Trinder, 1969). Glucose oxidase is an enzyme extracted from the growth medium of *Aspergillus niger*. Glucose is oxidized by glucose oxidase (GOD) to produce gluconate hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4 amino-antipyrene (4-AAP) and phenol in the presence of peroxidise (POD) to yield a red quinoeimine dye that is measured spectrophotometrically at 505 nm.

The absorbance of the color solution is directly proportional to the glucose concentration when measured at 505 nm and the glucose level in serum was expressed as mg dL-1.

2.3.6 Estimation of the activity of alanine aminotransferase

Principle: Alanine aminotransferase (ALT) catalyses the transfer of amino group from alanine to 2-oxoglutarate, resulting in the formation of pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340

nm, by means of lactate dehydrogenase coupled reaction (Gella, *et al*., 1985).The enzymatic reaction employed in the assay of serum glutamate-pyruvate transaminase is as follows.

2-oxoglutarate + L-aspartate ⇌ L-glutamate + Oxaloacetate

Oxaloacetate + NADH + H^+ ⇌ Malate + NAD^+

Reagent Preparation

Reagent A: Tris-150 mM (pH 7.3), L- Alanine-750 mM, lactate dehydrogenase-1350 U/L.

Reagent B: NADH-1.3 mM, 2-oxoglutarate-75 mM, sodium hydroxide-148 mM, sodium azide-9.5 g/L.

Working reagent: Reagent A (4 parts) was mixed with 1 part of Reagent B. The combined reagent was stable for 2 months at 2-8°C. The mixed reagent was stored in a dark place and protected from light.

Procedure: Rat serum was used as the sample. 10 µL of serum was reacted with 200 µL of working reagent and incubated at 37^0C for 10 min and the absorbance was measured at 340 nm. The activity was calculated and expressed in U/L.

2.3.7 Estimation of the activity of serum aspartate aminotransferase

Principle: Aspartate aminotransferase catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxaloacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm by means of malate dehydrogenase (MDH) coupled reaction (Gella, *et al*., 1985).

Reagent Preparation

Reagent A: Tris-121 mM (pH 7.8), L- aspartate-362 mM, malate dehydrogenase- 460 U/L, lactate dehydrogenase - 660 U/L, sodium hydroxide- 255 mM.

Reagent B: NADH-1.3 mM, 2-oxoglutarate-75 mM, sodium hydroxide-148 mM, sodium azide-9.5 g/L.

Working Reagent: Reagent A (4 parts) is mixed with 1 part of Reagent B. The combined reagent was stable for 2 months when stored at 2-8°C and protected from light.

Procedure: Rat serum was used as the sample. 10 µL of serum was mixed with 200 µL of working reagent and incubated at 37^0C for 10 min and absorbance was measured at 340 nm using a biochemical analyzer. The activity was calculated and expressed in U/L mixed reagent and estimation of ASAT was carried out using a biochemical analyzer

2-oxoglutarate + L-alanine ⇌ L-glutamate + pyruvate

Pyruvate + NADH + H^+ ⇌ Lactate + NAD^+

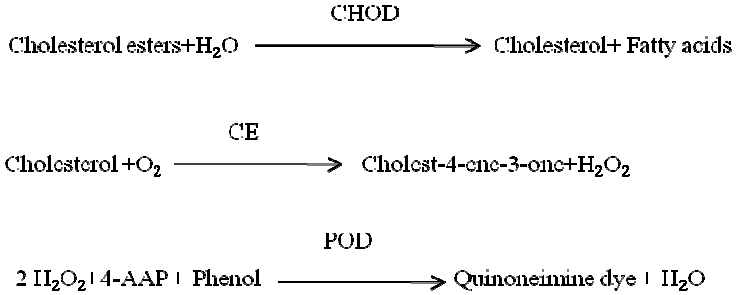
2.3.8 Estimation of serum cholesterol

Cholesterol in serum sample was estimated by cholesterol oxidase (CHOD) peroxidase/phenol/4-aminoantipyrine (PAP) method (Trinder, 1969). Cholesterol and its esters were released from lipoproteins by detergents. The series of reactions involved in this assay are as follows

1. Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase (CE) to cholesterol and free fatty acids.

2. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase (CHOD) to cholest-4-ene-3-one and H_2O_2.

3. In the presence of peroxidase (POD), the hydrogen peroxide formed combines with oxidative coupling of phenol and 4- amino antipyrine (4-AAP) to form a red color quinoneimine dye.



Reagent preparation

Reagent 1 (R_1) - Cholesterol standard

Reagent 2 (R_2)

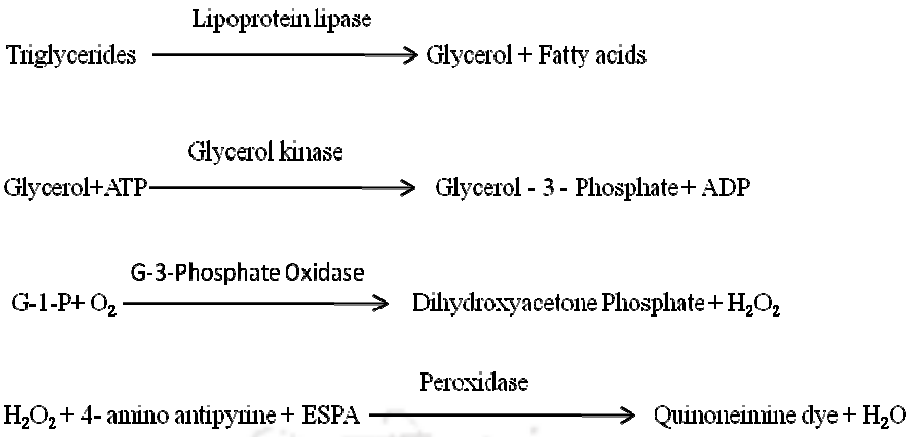
Pipes buffer	90 mM (pH 6.7)
Phenol	26 mM
Cholesterol oxidase	500 U/L
Cholesterol esterase	500 U/L
Peroxidase	1250 U/L
4- Amino antipyrine	0.4 mM

All reagents are ready for use and stable when stored at 2–8^{0}C.

Serum (10 µL) was reacted with 1 mL of reagent 2. The change in the absorbance was measured at 500-550 nm at 37^{o}C. The intensity of the color produced is directly proportional to cholesterol concentration in the serum. The concentration of cholesterol in serum was expressed in terms of mg dL-1 (Meiattini *et al*., 1978).

2.3.9 Estimation of serum triglycerides

Triglycerides are the esters of fatty acids and glycerol that do not circulate freely in plasma, but are bound to proteins and transported as macromolecular complexes called lipoproteins. Determination of triglyceride in the serum/plasma involves enzymatic or alkaline hydrolysis of triglycerides to glycerol and free fatty acids followed by either chemical or enzymatic measurement of the glycerol released (Trinder, 1969). The series of reactions involved in this assay are as follows,



Triglycerides are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol is then phosphorylated by adenosine-5'-triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine- 5'-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK).

G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase (POD) catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance which is maximum at 540 nm (Trinder,1969) The increase in absorbance at 540 nm is directly proportional to triglyceride concentration of the sample.

Reagent composition

Reagent 1 (R1) Triglyceride standard solution- 200 mg dL^{-1}

Reagent 2 (R2)

Pipes buffer	50 mM (pH 7.0)
p- chlorophenol	5.3 mM
Potassium ferrocyanate	10 mM
Magnesium salt	17 mM
4- Amino antipyrine	0.9 mM

ATP	3.15 mM
Lipoprotein lipase	1800 U/L
Glycerol kinase	450 U/L
Glycerol-3- phosphate oxidase	3500 U/L
Peroxidase	450 U/L

The reagent was stable for 18 months when stored at 2-8° C.

Procedure

Animal serum was used as the sample. 10 µL of serum was mixed with 1 mL of reagent 2, incubated for 5 min at 37°C and absorbance was measured at 540 nm using a biochemical analyzer (Merck, Microlab 100).

2.4 Statistical analysis

All the values of the experimental results were expressed as mean ± standard mean error (SEM) for evaluation of oral glucose tolerance test. One way analysis of variance (ANOVA) followed by Bonferroni post test was performed.

For the antidiabetic study (diabetic rats treated with the glibenclamide and fruit extracts at doses of 200 and 400 mg kg-1 body weight) and other parameters like aspartae aminotransferase, alanine aminotransferase, triglycerides, total cholesterol and body weight the data was analyzed by applying one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using graph pad prism (version 5.5) computer software. The results were considered statistically significant if $P < 0.05$.

2.5 Results and Discussion

2.5.1 Preliminary phytochemical analysis

Preliminary phytochemical analysis of the fruit extract of *Dillenia indica* revealed the presence of phenolic as well as flavonoid like compounds. Quantitative estimation of the methanolic fruit extract showed the presence of higher concentration of phenolic compounds. Total phenolic content of the fruit extract was found to be 98.5 µg gallic acid equivalent/gram of extract calculated from the gallic acid calibration curve depicted in (Fig. 2.2). Total flavonoid in the extract was found to be 104 µg of quercetin equivalent/gram of extract calculated from the calibration curve of quercetin (Fig. 2.3).

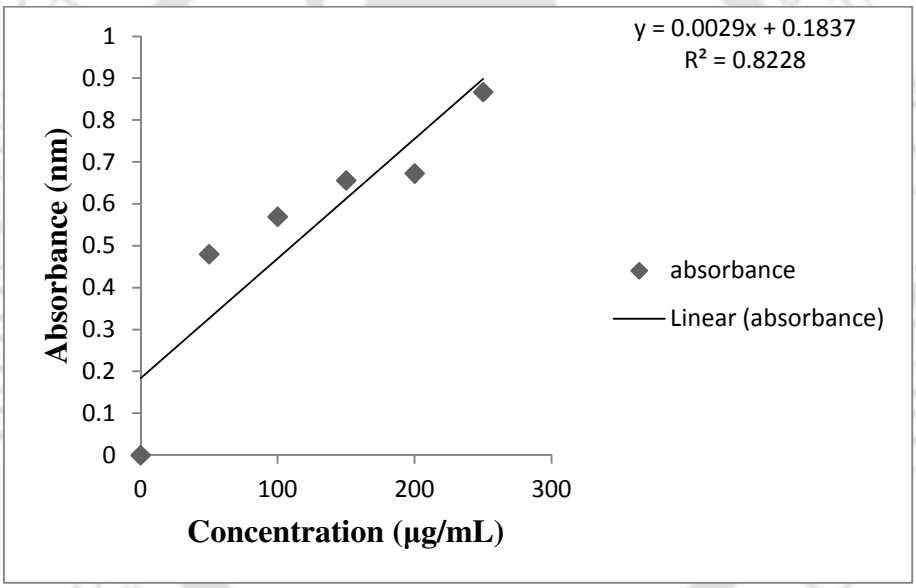


Fig. 2.2 Calibration curve for gallic acid.

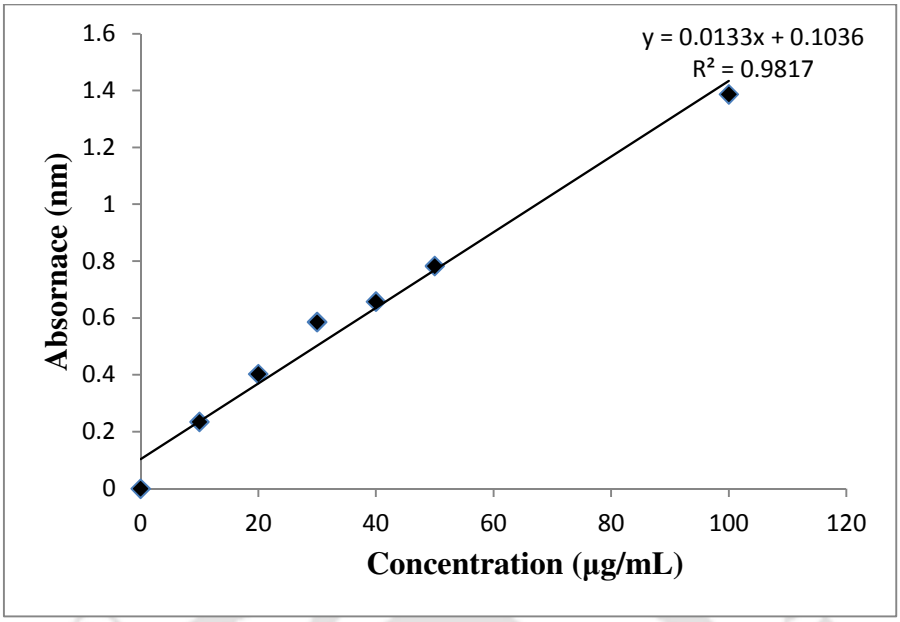


Fig. 2.3 Calibration curve for quercetin.

2.5.2 Antioxidant activity of fruit extract of *Dillenia indica*

The antioxidant activity of the *Dillenia indica* fruit extracts was measured on the basis of the scavenging ability of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical. The DPPH radical scavenging assay for varying concentrations of *Dillenia indica* extract (10-100 µg mL^{-1}) was shown in Fig. 2.4 and showed concentration dependant scavenging ability with maximum effect of 66.33% at 100 µg mL^{-1}. The EC_{50} of the extract was found to be 93.3 µg mL^{-1}.

It was observed that the radical scavenging effect of the extracts was positively correlated with their total amount of phenolic compounds. The radical scavenging effect of the fruit extract of *Dillenia indica* , which had a greater quantity of total phenolic compounds, was also determined to be stronger. Many studies in the literature present positive correlations between the quantity of phenolic compounds and the DPPH free radical scavenging effect (Sagar and Singh, 2011; Liu *et al*., 2009).

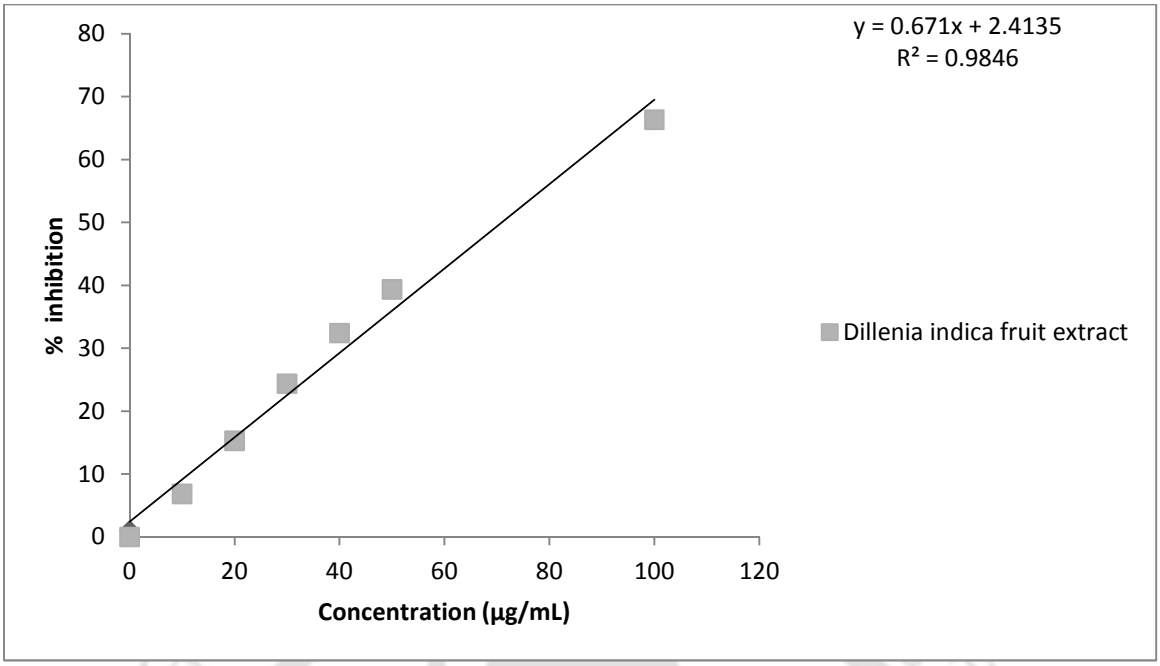


Fig. 2.4 DPPH radical scavenging activity of *Dillenia indica* fruit extract.

2.5.3 Cytotoxicity evaluation of the fruit extract of *Dillenia indica*

MTT dye conversion cytotoxicity assay triggered the activity of mitochondrial dehydrogenase of live mouse fibroblast cells (L929) and human breast cancer (MCF-7) cells that cleaved the tetrazolium ring in the tetrazolium salts. Only active mitochondria contain these enzymes and therefore, the reaction occurred in living cells. The L929 and MCF-7 cells treated with different concentrations of *Dillenia indica* fruit extract for 24 h did not cause any significant loss in the cell viability as compared with the untreated cells (Fig. 2.5 and Fig 2.6). The extract treated L929 and MCF-7 cells showed 67.7% and 59.7% viability, at a concentration of 100 µg mL^{-1}, respectively after 24 h. This confirmed that the *Dillenia indica* fruit extract is non toxic to normal cell (L929) and can be used for further study.

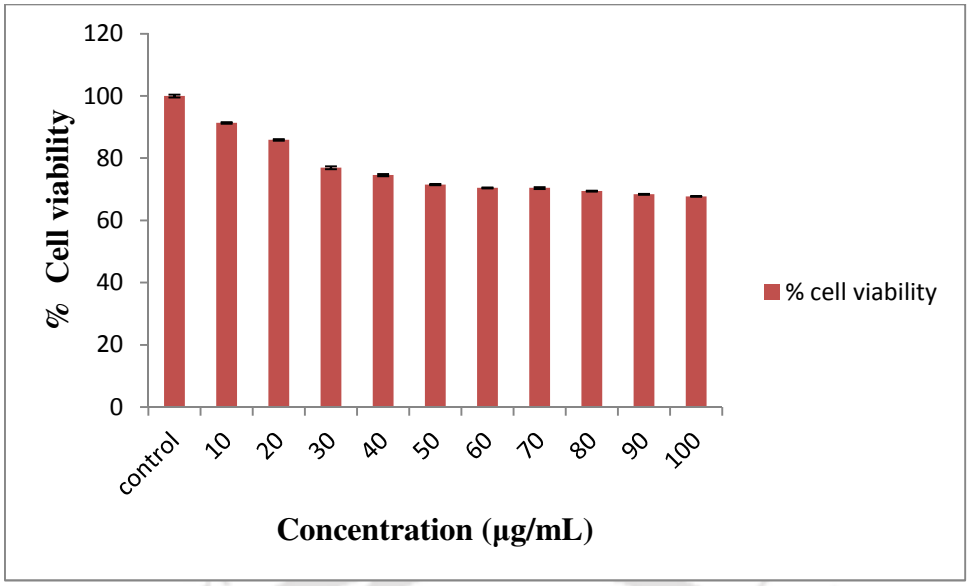


Fig. 2.5 MTT Cytotoxicity assay: cell viability of L929 cells exposed to different concentrations of *Dillenia indica* fruit extract.

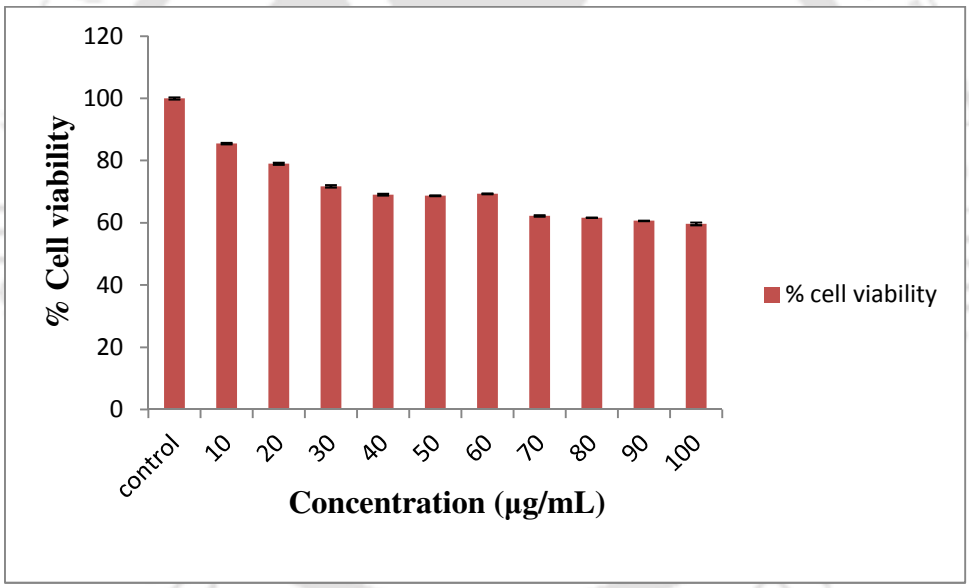


Fig. 2.6 MTT Cytotoxicity assay: cell viability of MCF7 cells exposed to different concentrations of *Dillenia indica* fruit extract.

2.5.4 Acute toxicity study

In phase I acute toxicity study, there was no sign of toxic effects like behavioral, motor and autonomic manifestation at doses ranging from 5 to 300 mg kg^{-1} body weight. Further to evaluate lethal dose (LD_{50}), in phase II higher doses of the extract were administered (1000 and 2000 mg kg^{-1} body weight) to the animals and it was found that there

was no any sign of toxic effects. Fifty percent of the animals administered with 5000 mg kg^{-1} body weight of extract showed sedation grooming effects and convulsion after 48 h of oral administration indicating that the LD_{50} of *Dillenia indica* fruit extract is 5000 mg kg^{-1}. Acute toxicity study revealed safe and non toxic nature of the extract. So further study was carried at lower doses and fixed at 200 and 400 mg kg^{-1}.

2.5.5 α-Amylase assay

Table 2.1 α-Amylase inhibitory effect of *Dillenia indica* fruit extract.

Treatment	Concentration (µg/mL)	% inhibition	IC_{50} (µg/mL)
Dillenia indica	10	28.1±0.4	43.3±0.5
	20	36.8±0.3	
	40	46.7±0.6	
	60	53.9±0.5	
	80	58.7±0.1	
	100	71.3±0.1	
Standard (Acarbose)	10	55.9±0.3	2.6 ±0.03
	20	65.7±0.6	
	40	73.8±0.7	
	60	82.5±0.8	
	80	82.9±0.5	
	100	93.6±0.6	

The inhibitory effect of the fruit extract of *Dillenia indica* against porcine pancreatic amylase is depicted in Table 2.1. The fruit extract of *Dillenia indica* showed concentration (10, 20, 40, 60, 80 and 100 µg mL^{-1}) dependant α-amylase inhibitory activity under *in vitro* conditions. At 100 µg mL^{-1} of *Dillenia indica* extract, the inhibitory activity was 71.3%, whereas, 10, 20, 40, 60 and 80 µg mL^{-1} of *Dillenia indica* extract, exhibited 28.1, 36.8, 46.7, 53.9 and 58.7% inhibitory activity. IC_{50} values of *Dillenia indica* extract and acarbose were found to be 43.3 µg mL^{-1} and 2.6 µg mL^{-1}, respectively.

α-Amylase is a digestive enzyme attached to the membrane of the brush border of the small intestine. It has a key role in the catalytic conversion of starches into monosaccharides

that can be absorbed and causes post prandial hyperglycemia. Previous study has established a relationship between hyperglycemia and increased α-amylase activity (Murakami and Ikeda, 1998); thus, hyperglycemia may be responsible for the increased α-amylase and α-glucosidase activities in the diabetic rats. However, the fruit extract of *Dillenia indica* showed dose dependant α- amylase inhibition which is similar to that of acarbose (a known α-amylase inhibitor). This finding is consistent with a recent study by Ademiluyi *et al*., (2014), where reduction in the activity of α-amylase was observed in diabetic rats fed with fermented soybeans.

The high proportion of phenolic compounds present in the crude extract of *Dillenia indica* could be responsible for bringing this inhibitory effect and may serve as an alternative to synthetic inhibitors, as polyphenolic compounds are strong inhibitors of α-amylase. Reports have suggested that they could serve as a potent alternative to synthetic inhibitors with little or no side effect (Ademiluyi *et al*., 2013).

2.5.6 The OGTT in normal Wistar rats

In oral the glucose tolerance test, it was observed that glibenclamide (5 mg kg^{-1}) caused significant attenuation in blood glucose level from 140 to 100 mg dL^{-1} ($P<0.001$), from 140 to 85 mg dL-1 ($P<0.01$) and from 140 to 77 mg dL^{-1} ($P<0.01$) at 60, 120 and 180 min respectively when compared with the vehicle treated group. The fruit extract of *Dillenia indica* showed significant ($P<0.001$) hypoglycaemic action by reducing the glucose level from 123 to 96 mg dL^{-1} at a dose of 200 mg kg^{-1} after 60 min of glucose load (Fig. 2.7). Oral administration of extract at 400 mg kg^{-1} showed marked reduction ($P<0.001$) in the glucose level from 140 mg dL^{-1} at 60 min to 84 mg dL^{-1} at 120 min of glucose load.

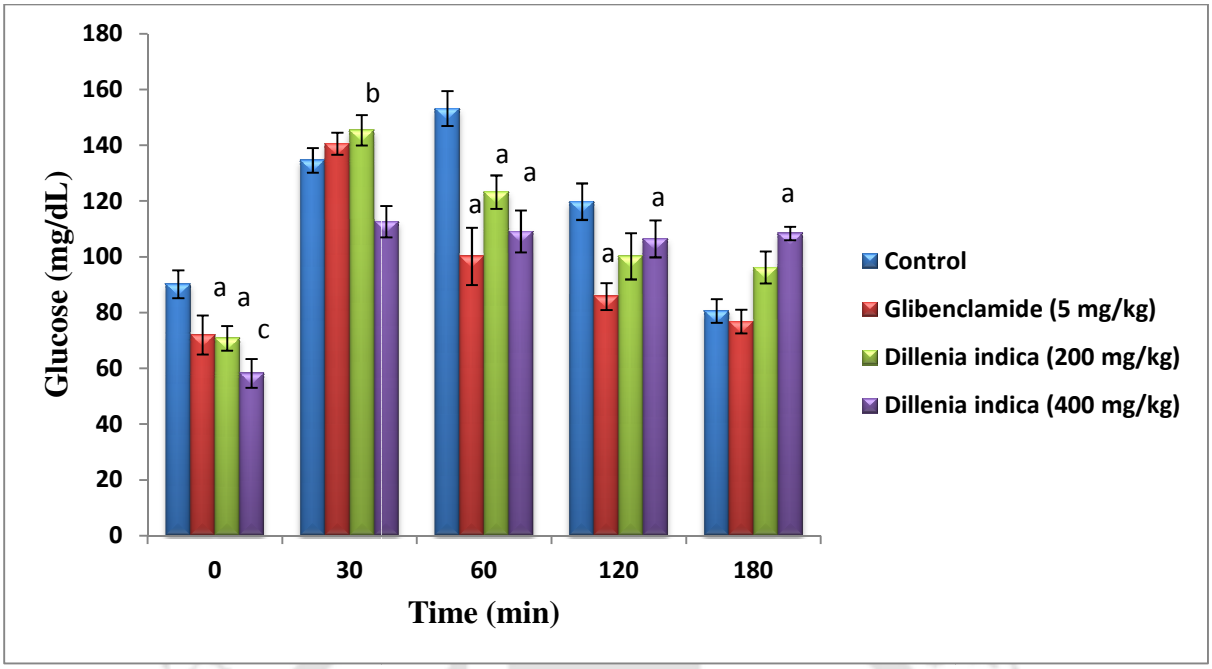


Fig. 2.7 Effect of *Dillenia indica* fruit extract on oral glucose tolerance test.

Each value represents mean ± S.E., n =6.

[a](P<0.001), [b](P<0.05) and [c](P<0.01) represent statistical significance vs control group.

The excessive amount of glucose load in animals stimulated the pancreatic β-cells for insulin release, and the secreted insulin may stimulate the peripheral utilization of glucose in diabetic rats. In all diabetic treated rats with fruit extract, the blood glucose concentration was lower than that of diabetic untreated rats at each time interval.

2.5.7 Repeated administration of fruit extract of *Dillenia indica* in STZ diabetic rats

Intra peritoneal administration of STZ (60 mg kg^{-1}) caused significant (P<0.001) increase in blood glucose level from 111 to 277 mg dL^{-1} in experimental animals and produced sustained hyperglycemia when compared to normal control rats. Once a day administration of glibenclamide (5 mg kg^{-1}) for 30 days caused a significant reduction (P<0.001) in the blood glucose level in STZ diabetic rats as compared to vehicle treated rats.

Table 2.2 Effect of *Dillenia indica* fruit extract on blood glucose levels in streptozotocin induced diabetic rats.

Treatment	0 days	10 days	20 days	30 days
Control (0.3 % w/v CMC)	88.6 ± 1.99	83.3 ± 1.96	96.4 ± 3.23	91.3 ± 1.36
Diabetic control	277.1 ± 1.63[a]	288.3 ± 2.93[a]	302.6 ± 6.12[a]	312.3 ± 1.27[a]
Positive control (Glibenclamide 5 mg kg-1)	269.1 ± 11.9	238.5 ± 1.48	121.02 ± 1.11[c]	101.02 ± 3.12[c]
Dillenia indica (200 mg kg-1)	279.5 ± 19.5	221.8 ± 3.14	176.5 ± 1.78[b]	139.2 ± 3.93[b]
Dillenia indica (400 mg kg-1)	279.2 ± 7.16	228.1 ± 8.17[b]	168.2 ± 3.26[b]	121.6 ± 1.62[b]

Values are mean ± S.E., n=6, Level of blood glucose is expressed as mg dL^{-1}, [a]P<0.001 highly significant when compared vs control group, [b]P<0.05 significant and [c]P<0.01 most significant when compared vs diabetic control.

Daily administration of the fruit extract of *Dillenia indica* at a dose of 200 mg kg^{-1} body weight for 30 days in STZ diabetic rats showed significant (P<0.05, 55.4%) reduction in the serum glucose level (Table 2.2) from the 20^{th} day onward and the effect was sustained till the 30^{th} day. The more prominent (61.2%) glucose lowering effect was observed at 400 mg kg^{-1} body weight for the 10^{th} day onwards and sustained till the end of the study The extract showed a statistically significant difference in serum glucose level compared with the diabetic control group (Table 2.2, P<0.05).

Streptozotocin induced diabetes is a well documented model of experimental diabetes. Previous report in literature indicates that the type of diabetes and characteristics differ with the employed dose of STZ and animal and species used (Kolb and Kroneke, 1993; Chattopadhyay *et al*., 1997). It has been stated that STZ diabetic animals may exhibit most of the diabetic complications mediated through oxidative stress (Matkovics *et al*., 1997; Kavalali *et al*., 2003). Oral administration of *Dillenia indica* fruit extract for 30 days resulted in 61.2% reduction in serum glucose level with respect to diabetic rat at 400 mg kg^{-1} body weight, which clearly explains the antidiabetogenic action of the extract.

The glucose lowering effect of the fruit extract of *Dillenia indica* was compared with glibenclamide, which is often used as a standard drug to compare the antidiabetic potential of variety of compounds in STZ induced diabetes (Cetto *et al*., 2000). The present investigation provides evidence that, the plant extract has lowered the high blood glucose level in diabetic rats to the normal level from day 10 onwards and it could be due to stimulation of existing pancreatic β-cells which is in accordance with the previous report (Cetto *et al*., 2000). The effect of the drug was retained in the body till the last days of the post treatment, indicating its longer tolerance to bio recycling in the body with no toxic effects.

2.5.8 Effect on body weight

Intra peritoneal administration of STZ (60 mg kg^{-1}) caused marked reduction in the body weight of diabetic rats from 208 to 158 g as compared with the normal rats at the end of the study period of 30 days. Oral administration of glibenclamide for 30 days showed significant ($P<0.001$) improvement in the body weight from 158 to 189 g compared with diabetic control rats. Treatment of diabetic rats with fruit extract of *Dillenia indica* at two different doses of 200 and 400 mg kg^{-1} body weight showed improvement in the loss of body weight i.e 17.9 and 19.3% respectively. Oral administration of fruit extract of *Dillenia indica* at 400 mg kg^{-1} showed more pronounced effect in the improvement of body weight as compared to glibenclamide, however, it remained lesser than the normal control rats (Table 2.3).

The characteristic loss of body weight in STZ diabetic rats was mainly due to the unavailability of carbohydrate for utilization as an energy source and increased protein catabolism (Musabayane *et al*., 2005). Improvement in the loss of body weight in the extract treated groups may be due to improved glycemic control and decreased protein breakdown.

Table 2.3 Effect of *Dillenia indica* fruit extract on body weight in streptozotocin induced diabetic rats.

Treatment	Initial	Final
Control (0.3 % w/v CMC)	198.1 ± 1.9	208.83 ± 1.56
Diabetic control	208.2 ± 2.1	158.83 ± 1.62
Glibenclamide (5 mg kg-1)	212.8 ± 2.2	189.00 ± 3.96[a]
Dillenia indica (200 mg kg-1)	212.3± 2.07	187.22 ± 4.3[c]
Dillenia indica (400 mg kg-1)	216.9 ± 1.83	189.47 ± 5.2[c]

Values are mean ± S.E., the body weight is expressed as g, n=6.
[a]Represent statistical significance (P<0.001) and [c] Represent statistical significance (P<0.01) when compared vs diabetic control rats.

2.5.9 Effects of *Dillenia indica* Extract (200 and 400 mg kg^{-1}) and glibenclamide (5 mg kg^{-1}), on lipids in STZ diabetic rats

STZ caused marked increase in the serum triglycerides, cholesterol and decrease in high density cholesterol level in diabetic rats (Table 2.4). Oral administration of *Dillenia indica* at a dose of 400 mg kg^{-1} body weight caused significant reduction (P<0.01) in serum triglycerides from 182 to 160 mg dL^{-1} and increase in HDL-C from 25 to 32 mg dL^{-1} (Table 2.4) as compared to the diabetic control rats. However, treatment of diabetic rats with extract at a dose of 200 mg kg^{-1} did not cause significant (P>0.01) change in the level of HDL-C and LDL-C.

Oral administration of *Dillenia indica* fruit extract at a dose of 400 mg kg^{-1} showed a significant (P<0.01) decrease in serum cholesterol and LDL-C levels, However, diabetic rats treated with extract at 200 mg kg^{-1} did not cause significant (P>0.01) change in serum cholesterol level as compared to diabetic rats. The daily administration of glibenclamide to STZ diabetic rats caused a significant decrease in the serum TG level from 182 to 135 mg dL^{-}

[1] (P<0.01) when compared to the diabetic control rats. Glibenclamide, however, did not decrease serum cholesterol and LDL-C concentrations (Table 2.4).

An abnormal increase in serum triglycerides and cholesterol levels in type II diabetes (Khan *et al*., 1995; Mitra *et al*., 1995) is associated with insulin resistance, excessive release of insulin in the blood stream, insulin sensitivity and hypercoagulability (Gingsberg, 1994). In the present study, treatment of diabetic rats with the fruit extract of *Dillenia indica* showed marked decrease in triglycerides, total cholesterol and LDL cholesterol, while increase in HDL cholesterol has been observed which may be due to improved insulin resistance and sensitivity or indirectly due to the influence on various lipid regulation systems. With respect to cholesterol lowering property of *Dillenia indica* fruit extract, it could be suggested that the constituents of the plant extract, may act as inhibitors of enzymes such as hydroxyl-methyl-glutaryl-CoA reductase, which participates in *de novo* cholesterol biosynthesis which is in line with previous reports (Reshma and Sushma, 2002).

Table 2.4 Effect of fruit extract of *Dillenia indica* on serum lipid profile in STZ induced diabetic rats.

Treatment	TC	TG	HDL-C	LDL-C
Control	166.7±5.1	140±2.6	43±1.3	95.69±1.3
Diabetic control	213.8±3.6[a]	182±3.8[a]	25±2.8[a]	151.6±3.0[a]
Glibenclamide (5 mg kg-1)	153.6±4.3[c]	135±4.2	40±3.1	86±2.8
Dillenia indica (200 mg kg-1)	186.8±3.9	175±2.8b	26±4.3	145±1.7
Dillenia indica (400 mg kg-1)	168.2±5.3[c]	160±4.1[c]	32±2.9[c]	104±3.6[c]

All the values were expressed in mean ± S.E., n=6, Level of TG, TC, HDL-C and LDL-C are expressed as mg dL^{-1}, [a]Represent statistical significance when compared vs control (P<0.001), [c]Represent statistical significance when compared vs diabetic control group (P<0.01).

2.5.10 Effect of extract on liver function

STZ caused significant (P<0.01) increase in the level of ASAT from 12 to 29.3 U/L and ALAT from 29 to 56.5 U/L as compared to normal control animals. Oral administration of *Dillenia indica* fruit extract at two different doses of 200 and 400 mg kg^{-1} body weight in

STZ-induced diabetic rats caused significant (P<0.01) decrease in the ALAT level from 56.5 to 26.7 U/L and ASAT level from 29.3 to 12.3. A more prominent effect was observed at 400 mg kg^{-1} as compared with that at 200 mg kg^{-1}.

Liver is the vital organ of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites. ASAT, ALAT are reliable markers of liver function (Ohaeri, 2001). STZ diabetic rat showed elevated level of ASAT and ALAT which might be due to the leakage of these enzymes from the liver cytosol into the blood stream which gives an indication of the hepatotoxic effect of STZ (Ramesh Babu *et al*., 2010). Treatment of the diabetic rats with fruit extract of *Dillenia indica* caused a decrease in the activity of ASAT and ALAT suggesting a potential role of the extract in preventing the liver damage caused by the action of STZ.

Table 2.5 Effects of methanolic fruit extract of *Dillenia indica* on liver function of streptozotocin induced diabetic rats.

Treatment	ASAT (U/L)	ALAT (U/L)
Control (0.3 % w/v CMC)	12.0 ±1.68	29.06 ± 1.56
Diabetic control	29.28 ± 3.17[c]	56.53 ± 5.62[c]
Glibenclamide (5 mg kg-1)	18.67 ± 2.33	39.48 ± 3.96
Dillenia indica (200 mg kg-1)	14.56± 2.07[b]	34.89 ± 1.87[c]
Dillenia indica (400 mg kg-1)	12.31 ± 2.16[b]	26.73± 2.16[b]

All values are expressed as mean± S.E., n=6.
[b](P<0.05) represent statistical significance compared vs diabetic control rats and [c](P<0.01) represent statistical significance compared vs normal control.

2.6 Conclusion

In conclusion, this study reports for the first time the antidiabetic activity of the fruit extract of *Dillenia indica* in STZ-induced diabetic rats. Acute toxicity studies and *in vitro* cytotoxicity studies in L929 cell line revealed the non-toxic nature of the fruit extract of *Dillenia indica*. There was no lethality or any toxic reactions found with the selected dose until the end of the study period.

The results of the study have shown that the fruit extract at a dose of 400 mg kg^{-1} body weight has a marked hypoglycemic activity (61.2%) by improvement of the glucose tolerance test in normoglycemic rats and by lowering the blood glucose levels in STZ induced diabetic rats. The extract also showed lipid lowering effect by decreasing the elevated level of triglycerides and cholesterol by 12% and 21.1% respectively, and beneficial effect by increasing the level of high density cholesterol in the rats suffering from type II diabetes.

The high proportion of total phenolic and total flavonoid content present in the crude extract supported the key role in free radical scavenging and/or reducing. The free radical scavenging activity of the fruit extract was tested with the DPPH method and depends on the interaction between antioxidant and the generated free radicals. The methanolic fruit extract of *Dillenia indica* was able to scavenge the stable DPPH radicals thereby, reducing the oxidative stress. The fruit extract of *Dillenia indica* improved the altered level of ASAT by 58% and ALAT by 52.8% at 400 mg kg^{-1} as compared to diabetic control rats.

The results presented in this study suggest that the presence of one or more antidiabetic phytoconstituent in the methanolic fruit extract of *Dillenia indica* improves the physiology of rats affected with type II diabetes. These bioactive constituents attributed to normalizing the blood glucose, also improving the serum lipid parameters and oxidative stress unlike synthetic hypoglycemic agent like glibenclamide. The results suggested that

bioactive constituents responsible for improving the physiology of type 2 diabetic rats need to be isolated and well characterized to contribute better for the therapy of type 2 diabetes.

CHAPTER 3

Evaluation of antidiabetic and antioxidant activity of *Solanum indicum* fruit extract in Streptozocin-induced diabetic rats

3.1 Introduction

Diabetes mellitus is a heterogonous metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism caused by defects in insulin secretion, insulin action or both (WHO, 1999). In 2000, an estimated 171 million people in the world had diabetes which is projected to increase to 366 million by 2030 (WHO, 2006). Hyperglycaemia associated with diabetes increases the risk of microvascular diseases like retinopathy, nephropathy and neuropathy and macrovascular (ischaemic heart disease, stroke and peripheral vascular disease) complications and thereby, induces the damage.

Diabetes mellitus is classified into insulin dependent diabetes mellitus (IDDM) or Type I and non-insulin dependent diabetes mellitus (NIDDM) or Type II. Type I diabetes encompasses cases due to pancreatic β-cell destruction and is prone to ketoacidosis and includes conditions attributed to an autoimmune process and β-cell destruction. Insulin

dependent diabetes is the common major form of diabetes which results from defects in insulin secretion with a major contribution from insulin resistance (WHO, 1999).

Diagnosis of diabetes mellitus is based on the measurement of fasting (7 mM or greater on two separate occasions) or random blood glucose (11 mM or greater) and glycated hemoglobin (HbA1C) level in serum/plasma.

Currently, available drugs for treatment of diabetes includes insulin secretagogues (sulfonylureas, meglitinides), insulin sensitizers (biguanides, thiazolidine-diones) and α-glucosidase inhibitors (miglitol, acarbose), which can be used alone or in combination with other drugs to achieve better effects (Ahmad, 2006).

The peptide analogues such as exenatide, liraglutide and DPP-IV inhibitors increase GLP-1 serum concentration and slow down the gastric emptying. Management of diabetes with insulin is associated with after effects like insulin resistance, anorexia nervosa, brain atrophy and fatty liver. In addition, insulin dependent diabetes mellitus is managed using drugs that control hyperglycemia such as amylin analogues. Sulphonylureas. A group of oral antidiabetic drugs acts by closure of ATP dependent channel. Metformin, a biguanide oral antidiabetic acts by limiting intestinal glucose absorption. Besides the side effects associated with the use of insulin, the side effects of most oral glucose-lowering drugs may include severe hypoglycemia at high doses, lactic acidosis, idiosyncratic liver cell injury, permanent neurological deficit, digestive discomfort, headache, dizziness and even death. Since the management of diabetes without any side effect is still a challenge, there is need to develop effective and safe drug therapy (Ahmad, 2006).

Herbs and herbal products have been used for several decades in traditional and folk medicine by different communities for management and treatment of various ailments and disorders. Herbal medicines are cost effective, safe, easily available and non toxic as

compared to synthetic drugs. The bioactive phytoconstutents derived from the plant extract have been found to have therapeutic activity comparable to the purified medication.

Sporadic reports on severe toxicity resulting from the use of herbal preperations are not well authenticated. Natural products obtained from plants specially contain various phytoconstituents, macro and micronutrients along with traces of various metals such as iron, zinc, chromium, vanadium and molybdenum. These micronutrients as well as some of the phytoconstituents like alkaloids, saponins and glycosides may produce serious adverse effect like allergic reaction, adverse drug interaction and potential herbs drug interaction and can interfere with laboratory tests (Ahmad, 2006). Hence, scientific validation regarding safety and efficacy of herbs and herbal products is necessary.

Effective and affordable drug therapy could be possible through the use of medicinal plants which are in vogue since time immemorial in traditional and folk medicine for treatment of various diseases including diabetes (Surendran *et al*., 2011).

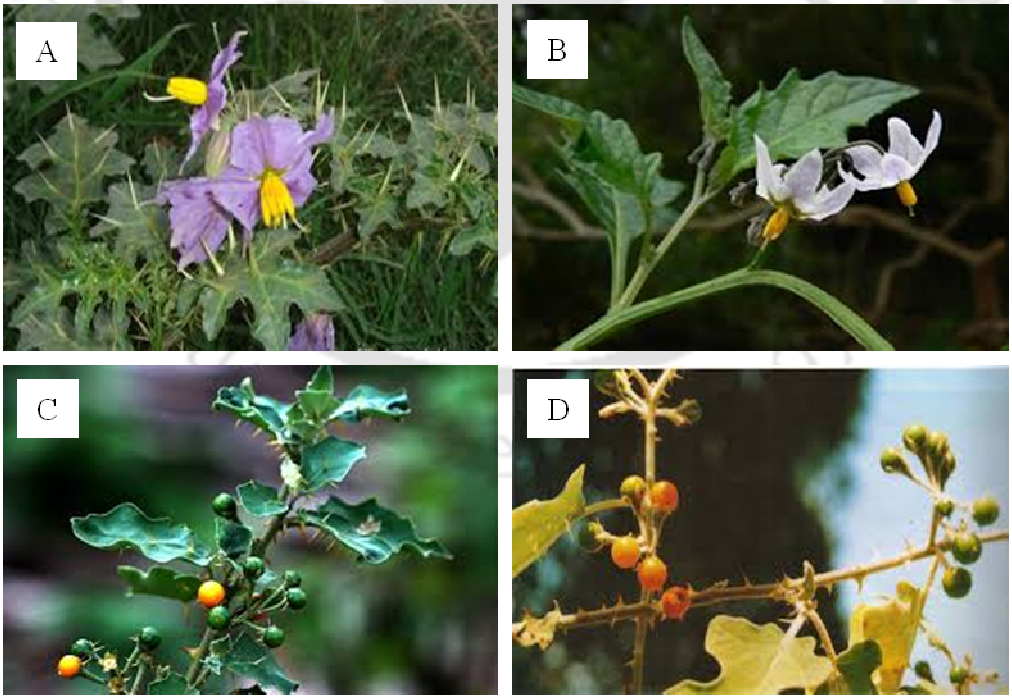


Fig. 3.1 Diffrent parts of the plant *Solanum indicum.* (A) Whole plant B) Flowers C) Unripened fruits D) Ripened fruits)

Solanum indicum Linn (Solanaceae) is distributed throughout India and all over the tropical and subtropical regions of the world (Kirtikar *et al*., 1975). Different parts of the plant are used by traditional practitioners in the treatment of loss of appetite and anorexia, blood disorders, rhinitis, cough, asthma, sore throat and hiccup, sexual disorders, abdominal pain, worm infestation, pain, fever, inflammation, insomnia, urinary complications, cardiac weakness and so on. The fruits and roots of the plant species contain wax, fatty acids, alkaloid solanine and solanidine, disogenin, lanosterol, β-sitosterol, solasornine, solamargine and solasidine (Chopra *et al*., 1992). Since the antidiabetic properties of *Solanum indicum* have not been reported earlier, an attempt was made in the present study to investigate its potential in the treatment of diabetes.

3.2 Materials and methods

3.2.1 Chemicals, reagents and kits

3, 4, 5-Dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT), 2, 2 -diphenyl-1- picrylhydrazyl (DPPH), ethidium bromide , acridine orange, Streptozocine and α-amylase were procured from Sigma-Aldrich Pvt. Ltd. USA. Folin–Ciocalteu reagent for estimation of total phenolics from Himedia and cell lines from the National Centre for Cell Sciences, Pune. All other reagents and solvents used for the study were of analytical grade.

3.2.2 Collection of plant material

Unripened fruits of *Solanum indicum* were collected in the month of December from the vicinity of Indian Institute of Technology Guwahati, Guwahati, India. The plant was authenticated by a taxonomist at the Department of Botany, Guwahati University, India and a voucher specimen (17783) was deposited at the Herbarium for future reference.

3.2.3 Processing of plant materials

The fruits were repeatedly washed with distilled water to remove soil and dirt and dried in the shade at 25^0C to the remove moisture. The dried fruits were grounded by using a

mixer grinder. The powdered plant material was kept at room temperature away from direct sunlight in air tight polythene bags. Methanolic extract was prepared from 500 g of the powder by hot soxhlet extraction method for 24 h. The extract was concentrated to about 50 mL using a rotary evaporator (Rotavapor, R-215). The concentrated extract was lyophilized to get a dried powder (yield 13.8%, w/w) which was stored for further studies.

3.2.4 Preliminary phytochemical analysis for detection of various components

3.2.4.1 Detection of alkoloids

Alkaloids were determined according to the method of Edeoga *et al*. (2005) with slight modification. 2.5 g of the dried fruits were extracted in 100 mL of 20% acetic acid in ethanol for 4 h. After 4 h, the extract was filtered using Whatman filter paper and the filtrate was concentrated to 25 mL using a rotary vaccum evaporator (Rotavapour, R-215). To the 25 mL of concentrated filtrate, ammonium hydroxide (10-20%) was added and the precipitate formed was collected and washed with dilute ammonium hydroxide solution and finally filtered using membrane filter (pore size 0.45 µm). The filtrate was discarded and the pellet obtained was dried at 25^0C and weighed (Edeoga *et al*., 2005).

3.2.4.2 Detection of saponins

Saponins were estimated as per the method given by Edeoga *et al*. (2005) with slight modification. 10 g of the dried fruits of *Solanum indicum* were mixed with 100 mL of 20% aqueous ethanol. The mixture was kept at 55°C in a water bath shaker for 4 h and then filtered. The obtained filtrate around 80 mL was again extracted in the same manner. The combined extract (the filtrate obtained in two steps) was concentrated to 40 mL in a water bath at 90°C. The concentrate obtained was transferred into a separating funnel and 10 mL of diethyl ether was added. After vigorous shaking, an aqueous layer was recovered by carefully discarding the ether layer. The process was repeated and 10 mL of n-butanol was added to 40 mL of the aqueous layer .The whole mixture containing 10 mL of n-butanol and 40 mL of

aqueous layer was washed in a separating funnel twice with 10 mL with 5% of NaCl. The upper portion was retained and heated in a water bath until evaporation to obtain a solid extract (Edeoga *et al*., 2005).

The extract obtained was subjected to various chemical tests to detect the presence of saponins as follows,

a) Foam test: 1g of the extract was diluted in 20 mL of distilled water and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins (Harborne, 1973).

b) 1 mg of the extract was treated with 1% lead acetate solution. Formation of white precipitates indicates the presence of saponins.

3.2.4.3 Detection of tannins

Qualitative estimation of tannins was carried out as per the method suggested by (Gurib-Fakim, 2006). 2 g of the fruits powder was extracted thrice in 70% acetone, centrifuged at 3000 rpm for 10 min at 15^{o}C and the supernatant was removed. Pellets from the three steps were mixed and the final volume adjusted to 3 mL by adding distilled water. The solution was mixed by vortexing and to this, 1 mL of 0.016M $K_3Fe(CN)_6$ was added followed by the addition of 1 mL 0.02M $FeCl_3$ in 0.10 M HCl. The solution was mixed by vortexing and the tubes were incubated at 25^{0} C for 15 min. The stabilizer was prepared by mixing water, dilute H_3PO_4 and 1% gum Arabic (3:1:1). To this mixture 5 mL of stabilizer was added and the absorbance was measured at 700 nm against blank. Standard curve was plotted using various concentrations of 0.001 M gallic acid (Gurib-Fakim, 2006).

3.2.4.4 Determination of total phenolics

Total phenolic content of the fruit extract of *Solanum indicum* was estimated according to the Folin–Ciocalteau method (Singleton and Rossi, 1999). Different concentrations (50, 100, 150, 200 and 250 µg mL^{-1}) of gallic acid in methanol were used for

preparing the standard curve. To 1 mL portion of each standard solution of gallic acid, 1.25 mL 10% Folin-Ciocalteu's reagent was added followed by the addition of 2.5 mL of 20% sodium carbonate after 5 min. The mixture was incubated at 25^0C for 30 min and the absorbance at 765 nm was measured. For the test solution, 1 mL of the fruit extract of *Solanum indicum* (1 g in 10 mL ethanol) was added to 2.5 mL of Folin-Ciocalteu's reagent. 2.5 mL of 20% sodium carbonate was added after 5 min and the reaction mixture incubated at 25^0C for 30 min and the absorbance was measured at a wavelength of 765 nm by a UV-VIS Spectrophotomer (Shimadzu, UV 2600). The results were compared to a gallic acid calibration curve and the total phenolic content of the fruit extract of *Solanum indicum* was expressed as milligram (mg) of gallic acid equivalent per gram (g^{-1}) of the extract.

3.2.4.5 Free radical scavenging activity by DPPH assay

Radical scavenging activity was determined by following the procedure described by Williams *et al*. (1995). 1 mg mL^{-1} stock solution of the fruit extract was prepared in methanol and used for preparing concentrations ranging from 10-100 µg mL^{-1}. From each dilution, 20 µL was mixed with 180 µL of 0.1 mM methanolic solution of DPPH and incubated in darkness at 25^0C for 30 min. The absorbance was measured at 517 nm. The percentage radical scavenging activity of the extract was calculated by using the following formula,

$$\text{Percentage radical scavenging activity} = [(Ab - Aa)/Ab] \times 100$$

Where Ab: absorbance of blank (methanolic DPPH), Aa: absorbance of test sample. The blank contained all reagents except the plant extract. Ascorbic acid at a concentration of 1 mg mL^{-1} was used as reference. The EC_{50} value of the extract was calculated using the Log dose inhibition curve (Koleva and Beek, 2002).

3.2.4.6 Cytotoxicity evaluation of crude extract

Measurement of cell viability and cell proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium

salts is now widely accepted as a reliable method to examine cell proliferation. MTT is reduced by metabolically active cells, by the action of dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometer. The MTT assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis and the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type, the linear relationship between cell number and signal produced is established, thus, allowing an accurate quantification of changes in the rate of cell proliferation (Mosmann, 1983).

The cytotoxicity of the extracts was tested against L929 mouse fibroblast cell. The cells were cultured in DMEM medium, supplemented with 11% fetal bovine serum at 37^0C with 5% CO_2. For the experiment, L929 cells were seeded in 96 well plates (10^5cells/well) in 100 µL of the medium. After 24 h, the extract at different concentrations was added to each well and incubated for 24 h. Growth of the cells were quantified by the ability of living cells to reduce the yellow dye, MTT, to a blue formazan product. At the end of 24 h of incubation, the media in each well was removed and replaced by fresh medium (100 µL) containing 0.5 mg mL^{-1} of MTT. Four hours (4h) later, the formazan product of MTT was dissolved in 100 µL of dimethyl sulphoxide (DMSO) and the absorbance was measured at 570 nm by using a microplate reader. The effect of the crude extract was quantified as the percentage of absorbance of reduced dye as control at 570 nm.

The L929 cells at a density of 1×10^5 cells were seeded in 96-well plates in 100 µL of medium and incubated for 24 h. After 24 h, the media was discarded and the cells were treated with the highest (500 µg mL^{-1}) and lowest (5 µg mL^{-1}) concentrations of the extract in serum free media and further incubated for 24 h. After that the media was discarded.

For enumeration of dead cells, acridine orange (AO) and ethidium bromide (EB) staining were applied to both treated and untreated live and dead cells (L929) and the analysis of cell the viability was carried out under an inverted microscope (Nikon, Eclipse TS100) equipped with fluorescence unit and digital camera (Nikon, Coolpix 5400).

3.2.4.7 α-Amylase inhibition assay

The α-amylase inhibition assay was carried out according to the procedure reported by Thirunavukkarasu, (2003). 500 µL of each of the concentrations ranging from 10-100 µg mL^{-1} of the fruit extract of *Solanum indicum* added with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.006 M NaCl and α-amylase solution (0.5 mg mL^{-1}) were incubated at 25°C for 10 min. After incubation, 500 µL of 1% (w/v) starch solution in 0.02M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were incubated at 25°C for 10 min. The reaction was stopped by adding 1 mL of 3, 5-dinitrosalicylic acid color reagent (Miller, 1959). Then, the test tubes were kept in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was diluted by adding 10 mL of distilled water and the absorbance measured at 540 nm. The % inhibition was calculated using the formula,

$$\% \text{ inhibition} = A_b - A_t / A_b \times 100$$

Where A_b is the absorbance of the blank, A_t is the absorbance of the sample (test)

3.3 Experimental animals

Healthy adult albino Wistar rats of either sex weighing 180–220 g were used for the study. The animals were allowed to acclimatize for a period of two weeks in the animal house at the Department of Pharmacology, College of Veterinary and Animal Sciences, Udgir, Maharastra, India. The rats were housed in polypropylene cages, maintained under standard laboratory conditions of 12 h Light/ dark cycle, ambient temperature of 25±2ºC and humidity of 35-60%. They were fed with standard rat pellet diet (Hindustan lever, Mumbai) and water

ad libitum. The experimental protocols and procedures used in this study were those approved by the Institutional Animal Ethical Committee, Department of Pharmacology, COVA Sciences, Udgir, (Maharastra), approval no.VCU/CPCSEA/IAEC/2/14 (II).

3.3.1 Acute toxicity study

Acute toxicity study was conducted in accordance with OECD guidelines No. 423. Healthy adult Wistar albino rats of either sex, starved overnight were divided into five groups (n =6) and fed orally with the fruit extract of *Solanum indicum* in increasing dose levels of 100, 500, 1000, 3000 and 5000 mg kg^{-1} body weight (Ghosh,1984). The rats were observed continuously for 2 h for behavioural, neurological and autonomic changes and after 24 and 72 h for lethality (Turner, 1965).

3.3.2 Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed according to the procedure described by Joy and Kuttan (1999) with slight modification. Healthy Wistar rats weighing 180-220 g were divided into four groups of six animals each: Group I which served as control received only vehicle (distilled water), Groups II, III and IV received only glibenclamide (5 mg kg^{-1}), fruit extract of *Solanum indicum* 100 and 200 mg kg^{-1} body weight, respectively. Glucose (2 g kg^{-1}) body weight was fed to the animals 60 min after dosing (Annie *et al*., 2006). Blood samples were withdrawn from the rat's tail veins after 0, 30, 60, 120 and 180 min of extract administration and glucose levels were estimated by glucose oxidase-peroxidase method.

3.3.3 Induction of non insulin dependent diabetes mellitus (NIDDM)

Prior to induction of diabetes, the rats weighing 180-220 g were subjected to fasting overnight (Szkudelski, 2001) but allowed free access to water until the end of the experiment. Hyperglycemia was induced by single intra peritoneal administration of streptozocin (60 mg kg^{-1} body weight dissolved in 0.1 M ice cold citrate buffer, pH 4.5) and the blood glucose

levels were monitored on day 0 and 72 h after STZ injection. The rats with blood glucose levels >250 mg dL^{-1} were considered diabetic and used for the study.

3.3.4 Experimental design

The experimental animals that received STZ were divided into five groups of six animals each. Control rats of group I were orally administered with 0.5% (w/v) carboxy methyl cellulose (CMC); Group II rats served as diabetic control; Group III rats were orally administered with glibenclamide, 5 mg kg^{-1} body weight in 0.5% (w/v) CMC; Group IV rats were orally administered with 100 mg kg^{-1} body weight of *Solanum indicum* fruit extract in 0.5% (w/v) CMC and Group V rats were orally administered with 200 mg kg^{-1} body weight of *Solanum indicum* fruit extract in 0.5% (w/v) CMC.

3.3.5 Collection of blood

The blood was collected in plastic test tubes and allowed to stand for 3 h to ensure complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 10 min and clear serum samples were aspirated off and stored frozen at -20^{o}C until required for analysis of biochemical parameters. The biochemical parameters determined in the serum specimen using the microlab 100 auto-analyzer were alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), triglycerides (TG), total cholesterol (TC) and high density lipoprotein cholesterol (HDL-C).

3.3.6 Blood sampling and blood glucose determination

Blood samples were drawn by sterilizing the tail of the animals with 10% alcohol and then nipping the tail at the start of the experiment and repeated after 0, 1, 2 and 3 h. Blood glucose levels were estimated with a glucometer. In experimentally induced diabetic rats, blood glucose level was estimated at interval of 10, 20 and 30 days.

3.3.7 Estimation of serum biochemical parameters

Various serum biochemical parameters like glucose, ALAT, ASAT, total cholesterol, and triglycerides were estimated using commercially available diagnostic kits (Ecoline, Merck) and the procedure was followed as described in chapter 2, sections 2.3.5, 2.3.6, 2.3.7, 2.3.8 and 2.3.9.

3.4 Statistical analysis

All the values of the experimental results were expressed as mean ± standard mean error (SEM). For evaluation of oral glucose tolerance test, two ways ANOVA followed by Bonferroni post test was performed.

For anti-diabetic studies and other parameters like aspartae aminotransferase, alanine aminotransferase, triglycerides, total cholesterol and body weight, the data were analyzed statistically by applying one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using graph pad prism (version 5.5) computer software. The results were considered statistically significant if $P<0.05$.

3.5 Results and Discussion

3.5.1 Preliminary phytochemical analysis

The preliminary phytochemical analysis of fruit the extract of *Solanum indicum* revealed the presence of alkaloids, glycosides, tannins and saponins.

3.5.2 Total phenolic content

Total phenolic content of the fruit extract of *Solanum indicum* was found to be 98.5 mg gallic acid equivalent/gram of extract calculated from the gallic acid calibration curve depicted in Fig. 3.2.

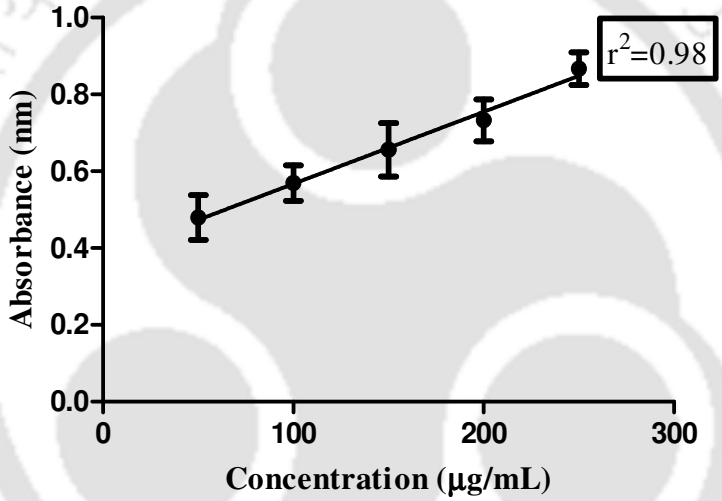


Fig. 3.2 Calibration curve for gallic acid.

Absorbance increased linearly with increase in the concentration of gallic acid. The phenolic content of the fruit extract was estimated by using this calibration curve.

3.5.3 Antioxidant activity evaluation of *Solanum indicum* fruit extract

The scavenging ability of *Solanum indicum* extract for DPPH radical is shown in Table 3.1. The results indicated that along with the increased concentration of extract, the DPPH scavenging ability was increased and showed maximum scavenging activity of 85.89% at 100 µg mL^{-1} of the extract (Table 3.1). The EC_{50} value of *Solanum indicum* extract was found to be 22.32 µg mL^{-1}. The extract showed excellent DPPH scavenging ability as the

EC_{50} value of the extract was lower than some reported values of crude extract of *Solanum* species (Kar et al., 2006).

DPPH scavenging assay is based on the reduction of DPPH in the presence of a proton donating substance (He *et al*., 2014). This assay has been used to evaluate the scavenging activity of natural compounds. The role of antioxidants depends upon their interaction with oxidative free radicals. The summary of DPPH method is that the antioxidants react with the stable free radical i.e., α, α- diphenyl-β-picrylhydrazyl (deep violet colour) and convert it to α, α-diphenyl-β-picrylhydrazine with discolouration. The discolouration indicates the scavenging potentials of the samples (extract) antioxidant such as phenolic compounds (Hossain and Muhammad, 2015). In the present study the fruit extract of *Solanum indicum* was able to discolour DPPH which confirmed the free radical scavenging potentials of the extract.

In the present study, it was found that the extract of *Soalnum indicum* contains substantial amount of phenolic compounds. It is the extent of the amount of phenolics present in this extract which is responsible for its marked antioxidant activity. Several reports have shown a close relationship between total phenolic contents and antioxidative activity of the fruits, plants and vegetables (Abdille *et al*., 2005, Deighton *et al*., 2000 and Vinson *et al*., 1998). Another possible mechanism for scavenging potential of the extract could be due to hydrogen donating capabilities to act as an antioxidant as it has been found that hydrocarbon, cysteine, glutathione, ascorbic acid, tocopherol and so on. reduce and decolourise α, α-diphenyl-β-picrylhydrazyl by their hydrogen free radical scavenging ability (Blois, 1958).

Table 3.1 DPPH scavenging assay of *Solanum indicum* extract.

Concentration (µg mL^{-1})	DPPH scavenging activity (%)
10	21.3±1.5
20	46.1±1.2
30	72.9±1.9
40	79.9±2.2
50	82.0±3.3
100	85.9±2.3

3.5.4 Cytotoxicity activity of *Solanum indicum* extract

MTT dye conversion assay triggered the activity of mitochondrial dehydrogenase of live mouse fibroblast cells (L929) that cleaved the tetrazolium ring of tetrazolium salts. Only active mitochondria contain these enzymes and therefore, the reaction occurred in living cells. Treated L929 cells with different concentrations of *Solanum indicum* extract (SIE) for 24 h, showed that the extract did not cause significant loss in cell viability compared to the control or untreated cells (Fig. 3.3). The extract-treated L929 cells showed 73% viability at a concentration of 500 µg mL^{-1} after 24 h of treatment. This confirmed that the fruit extract of *Solanum indicum* is non toxic and can be used for further study.

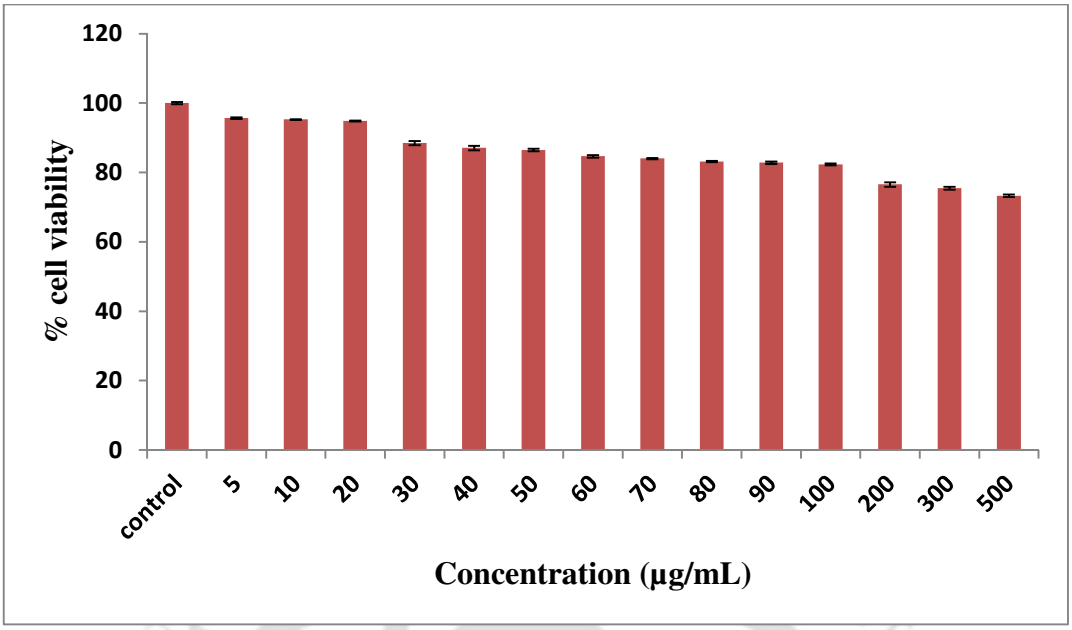


Fig. 3.3 MTT assay of *Solanum indicum* fruit extract.
L929 cells at a density of 1×10^5 were seeded in 96 well plates and treated with different concentrations of the extract for 24 h. The extract-treated L929 cells showed 73% viability at a concentration of 500 µg mL^{-1}.

3.5.5 Live and dead cell staining using acridine orange and ethidium bromide

Viability staining proved the membrane integrity of L929 cells based on the uptake or exclusion of a dye from the cells. Acridine orange (AO) is a membrane permeable dye that stained both live and dead cells, while ethidium bromide (EB) passed through the membrane of dead cells in untreated as well as treated cells. AO-stained cells emitted green color and EB staining turned the cells red. The untreated L929 cells gave intense green fluorescence and weak signals for red fluorescence which was expected for the control experiment (Fig. 3.4). Cells treated with the highest and lowest concentrations of the extract retained their viability confirmed by AO permeability causing a strong green fluorescence but a weak fluorescence for EB.

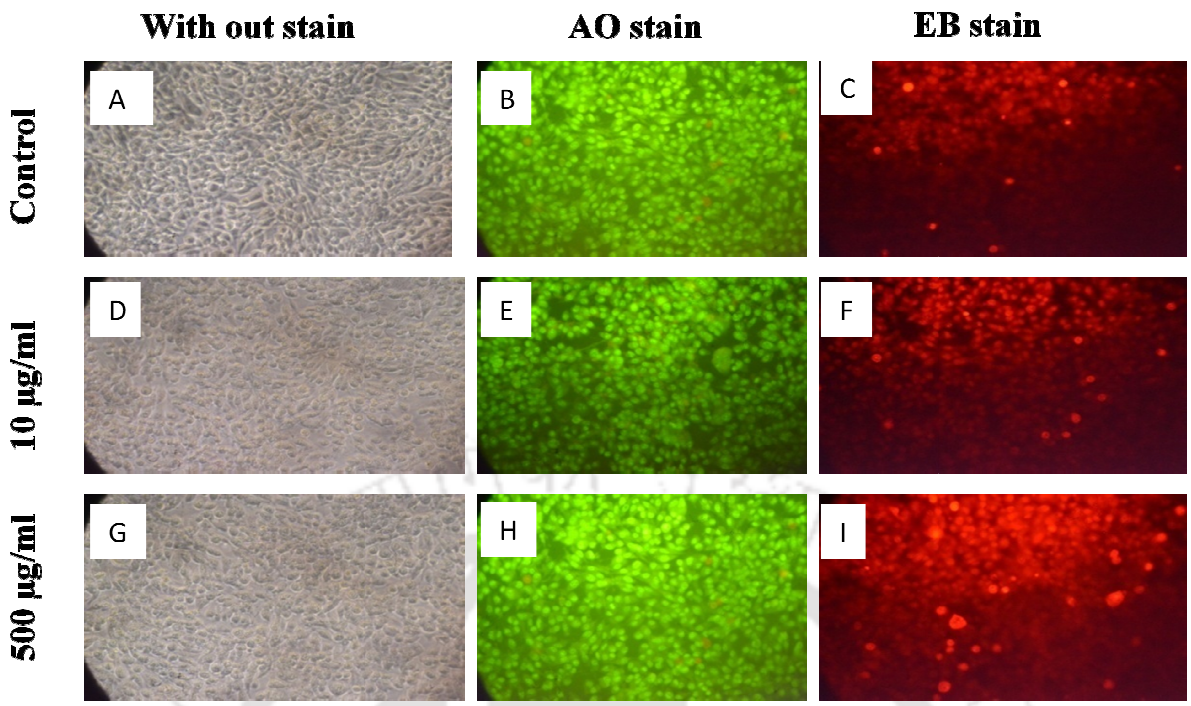


Fig. 3.4 Morphological study of untreated and cells treated with methanolic extract of *Solanum indicum* (A, D and G bright field images of L929 cell treated with extract; B, E and H cells treated with extract and stained with acridine orange; C, F and I cells treated with extract and stained with ethidium bromide)

Without stain there was no significant alteration in the cell morphology of untreated and the cells treated with 10 and 500 µg mL^{-1} *Solanum indicum* extract respectively (Fig.3.4. A, D and G); Untreated L929 cells emitted intense green fluorescence and weak signals for red fluorescence (Fig.3.4 B and C). The L929 cells treated with 10 µg mL^{-1} of extract showed similar pattern as compared to untreated cells (Fig.3.4. E and F). The cells on treatment with 500 µg mL^{-1} *Solanum indicum* extract (Fig.3.4.H and I) emitted intense green fluorescence but the intensity of red signals was more as compared to the cells treated with lower concentration of the extract.

3.5.6 α-Amylase inhibition

The inhibitory effect of methanolic extract of *Solanum indicum* against porcine pancreatic amylase is shown in Table 3.2. Under *in vitro* conditions the fruit extract of *Solanum indicum* showed concentration (10, 20, 40, 60, 80 and 100 µg mL^{-1})-dependant α-amylase inhibitory activity. At 100 µg mL^{-1} of *Solanum indicum* extract, the inhibition was

83.6%, whereas 10, 20, 40, 60 and 80 µg mL^{-1} of *Solanum indicum* extract, exhibited 38.1, 53.8, 59.7, 66.6 and 72.1% inhibitory activity respectively. IC_{50} values of *Solanum indicum* extract and acarbose were found to be 17.22 µg mL^{-1} and 2.65 µg mL^{-1}, respectively.

Table 3.2 α-Amylase inhibition assay of methanolic extract of *Solanum indicum*.

Treatment	Concentration (µg/mL)	% inhibition	IC_{50} (µg/mL)
Solanum indicum	10	38.1±0.4	17.2
	20	53.8±0.3	
	40	59.7±0.6	
	60	66.6±0.5	
	80	72.1±0.1	
	100	83.6±0.1	
Acarbose	10	55.9±0.3	2.6
	20	65.7±0.6	
	40	73.8±0.7	
	60	82.5±0.8	
	80	82.8±0.5	
	100	93.6±0.6	

Pancreatic α-amylase is a key enzyme involved in the digestion and hydrolysis of starches to simple sugars which are then absorbed from the intestine to enter in to the systemic circulation. This results in increase in the post-prandial glucose level (PPG), therefore, control of which becomes an important aspect in the treatment and management of type II diabetes (Eichler *et al*., 1984). The methanolic fruit extract of *Solanum indicum* showed concentration-dependant α amylase inhibitory activity. Few reports have suggested the potential role of natural polyphenols in carbohydrate hydrolyzing enzyme inhibitions like α-amylase, α-glucosidase (Lordan *et al*., 2013). In this study preliminary phytochemical analysis revealed the presence of phenolic and flavonoid compounds which could be responsible for the exhibition of the α-amylase inhibitory effect. This may also be considered as one of the mechanisms for the inhibition of α-amylase, thereby, regulating post prandial glucose level and furthermore, it can be used as a potential anti nutritional supplement.

3.5.7 Oral glucose tolerance test

In oral glucose tolerance test, the fruit extract of *Solanum indicum* showed significant (P<0.001) hypoglycaemic action at a dose of 100 mg/kg body weight after 60 min of glucose load (Fig. 3.5) and which was extended up to 120 min. Oral administration of the extract at a dose of 200 mg/kg body weight showed significant reduction (P<0.001) in blood glucose level from 60 min of glucose load up to 180 min.

The extract showed maximum glucose lowering effect at 3 h of oral administration, which indicated that it takes about 3 h or more to reach the target tissue through systemic circulation. Although, compared to the oral hypoglycemic agent, glibenclamide, the onset of action of extract was slow but it persisted for a longer duration of time.

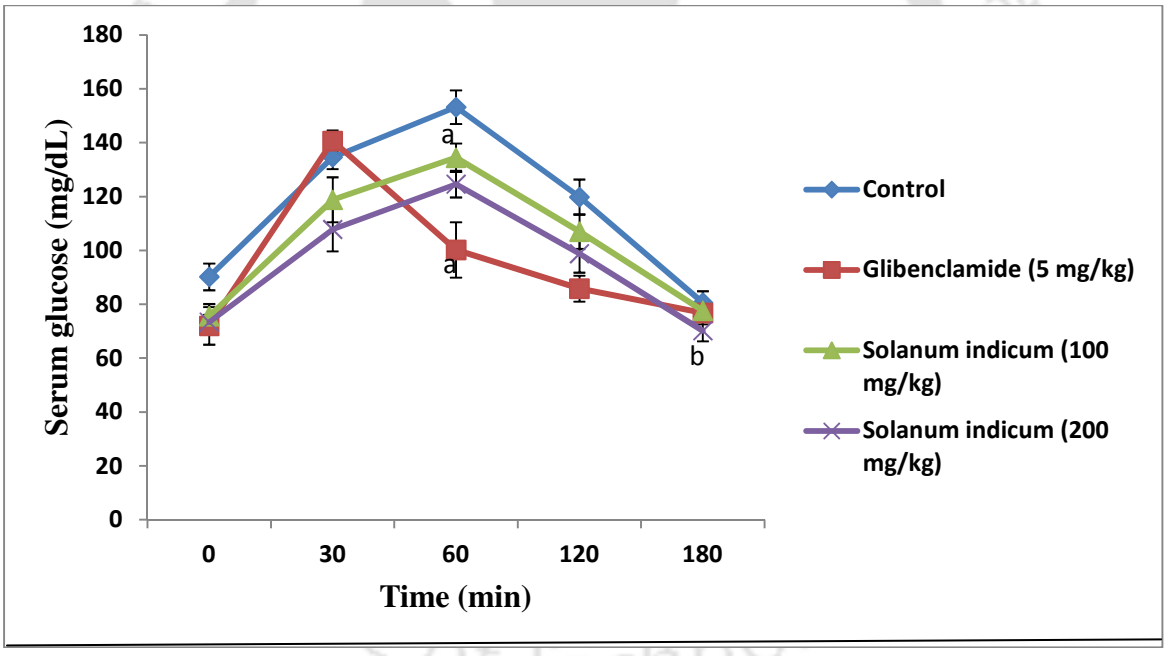


Fig. 3.5 Oral glucose tolerance test of *Solanum indicum* extract.

Each value represent mean ± S.E., n=6, Where, [a] (P<0.001) and [b] (P<0.01) represent statistical significance vs control animals.

3.5.8 Acute toxicity study

In acute toxicity study, oral administration of different doses (100, 500, 1000, 3000 and 5000 mg kg^{-1} body weight) of fruit the extract of *Solanum indicum* did not produce any toxic effects on motor function, general appearance as well as on behaviour during experimentation. This confirmed the non toxic nature of the extract. The body weight and food consumption of all animals was normal as compared to the vehicle treated groups. Animals in all groups did not exhibit any signs of adverse effect and thus, the No Observed Adverse Effect Level (NOAEL) of the extract was greater than 5000 mg/kg. For further studies, the doses were fixed as 100 and 200 mg/kg.

3.5.9 Effect of *Solanum indicum* fruit extract on fasting blood glucose

The fruit extract of *Solanum indicum* significantly reduced fasting blood glucose (FBG) level by 26 and 36.9% at a dose of 100 and 200 mg/kg body weight, respectively (Table 3.3). Abnormally high fasting blood glucose level is an indicator of insulin resistance and impaired insulin secretion which is severely disrupted in type II diabetes (Herrera *et al*., 2015). Hence, decrease in the FBG level could be due to improved insulin resistance and stimulation of existing pancreatic β cells by the action of the extract.

Table 3.3 Effect of *Solanum indicum* extract on the fasting blood glucose (mg dL^{-1}) level.

Group	Treatment	0 h	1 h	2 h	3 h	Reduction (%)
I	Control	88.6±9.8	97.5±6.2	101.5 ± 13.4	104 ± 11.8	-
II	Diabetic control	387.2±5.1	396.5±4.3	398.8±3.8	394.6±3.8	-
III	Glibenclamide (5 mg kg^{-1})	344.3±1.0	174.7±1.2	115.9±2.2	106.6±2.7	72.5
IV	*Solanum indicum* (100 mg kg^{-1})	389.7±4.8	347.6±3.6	316.5±3.9	286.4±6.2	26.0
V	*Solanum indicum* (200 mg kg^{-1})	355.2±4.7	323.6±3.4	279.1±5.6	244.4±4.3	36.9

3.5.10 Effect of *Solanum indicum* extract on serum glucose in STZ-induced diabetic rats

Intra peritoneal administration of STZ (60 mg kg^{-1} b.w.) caused significant increase in serum glucose level from 96 to 287 mg dL^{-1} as compared with the normal control rats confirming the induction of diabetes (Fig. 3.6). Daily administration of the fruit extract of *Solanum indicum* at all doses (100 and 200 mg kg^{-1}) resulted in significant ($P<0.05$) decrease in blood glucose from the 10^{th} day onwards and continued till the end of the study. The percentage reduction was 50.9, 52 and 55% after 10, 20 and 30 days, respectively at a dose of 100 mg kg^{-1}body weight while the percentage reduction of 52, 54.5 and 56% at 10, 20 and 30 days of treatment respectively was observed at a dose of 200 mg kg^{-1} body weight.

Previously reported literature indicates that the type of diabetes and characteristics differ with the employed dose of STZ, and animal and species used (Kolb and Kroneke, 1993; Chattopadhyay *et al*., 1997). It has been stated that STZ diabetic animals may exhibit most of the diabetic complications mediated through oxidative stress (Matkovics *et al*., 1997; Kavalali *et al*., 2003). Studies have also suggested the involvement of free radicals in pancreatic cell destruction (Lenzen, 2008). Glibenclamide is often used as an insulin stimulant in many studies and also used as a standard oral antidiabetic drug in STZ-induced

moderate diabetes to compare with the antidiabetic properties of a variety of hypoglycemic compounds (Andrade Cetto*et al.,* 2000).

After 30 days of oral administration of the fruit extract of *Solanum indicum*, there was a significant diminution of blood glucose level with respect to diabetic rat, which clearly explain the antidiabetogenic action of this extract. The possible mechanism by which *Solanum indicum* mediated its antidiabetic effect could be by potentiation of pancreatic secretion of insulin from existing β-cells of islets.

The hypoglycemic activity of extract was further validated and compared with standard drug, glibenclamide. The acute hypoglycemic effect of glibenclamide was evident from the stimulation of insulin release from the residual β-cells and inhibition of glucagon secretion (Fuhlendorff *et al*., 1998). The extract might possess insulin like effect on peripheral tissues either by promoting glucose uptake and metabolism or inhibiting hepatic gluconeogenesis. From the results of the present study, it may be suggested that the mechanism of action of plant extract may be similar to that of glibenclamide action.

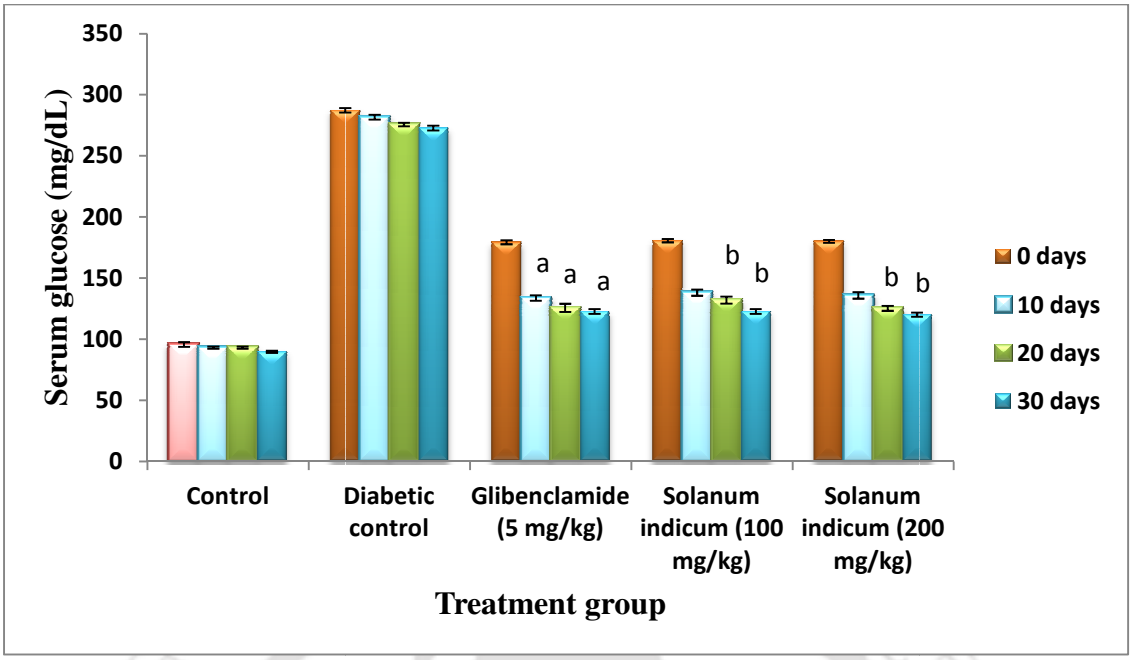


Fig. 3.6 Effect of *Solanum indicum* fruit extract on serum glucose in STZ-induced diabetic rats.

All the values were expressed as mean ±S.E., n=6.

[a]Represents statistical significance vs control (P<0.0001), [b] Represents statistical significance vs diabetic control group (P<0.05).

Fig. 3.7 represents the changes in body weight in normal and experimental diabetic rats. STZ produced significant loss in body weight from 200 to 141 g as compared to that of normal rats and continued to lose the weight until the end of the study. Oral administration of the fruit extract of *Solanum indicum* for 30 days showed significant (P<0.001) improvement in body weight from 141 to 175 and 178 g at a dose of 100 and 200 mg kg^{-1} body weight respectively as compared to that of diabetic control rats.

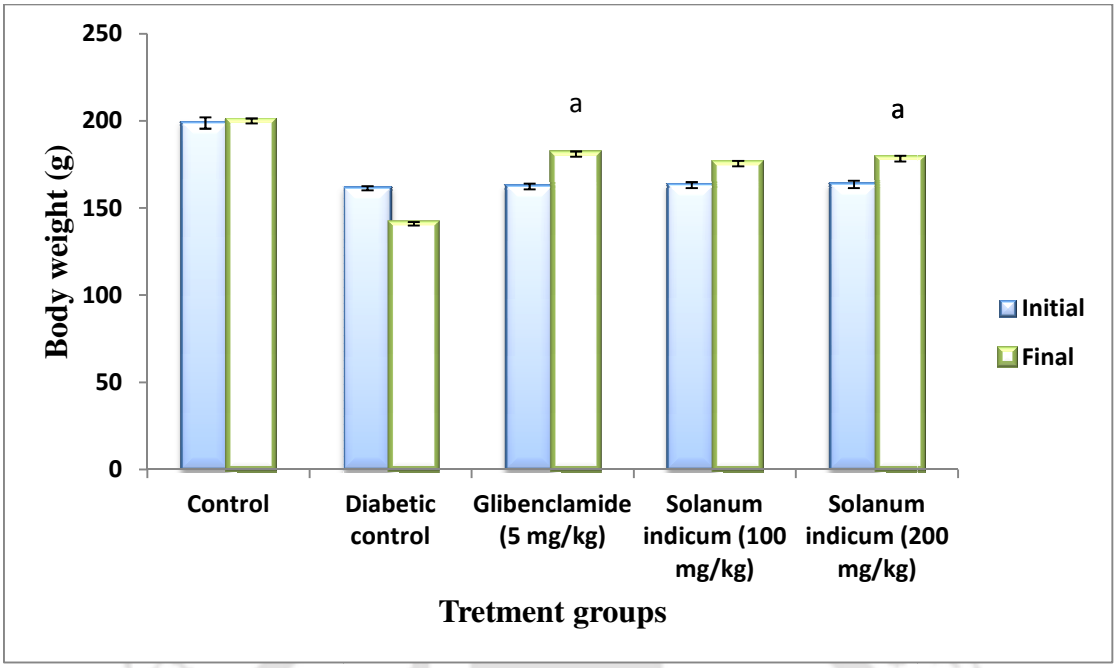


Fig. 3.7 Effect of *Solanum indicum* on body weight in STZ-induced diabetic rats.

All the values were expressed as mean ±S.E., n=6.
[a]Represents statistical significance (P<0.001) vs control group.

STZ-induced diabetes is characterized by severe loss in body weight (Al-Shamaony *et al.*, 1994) and this was also observed in the present study. The characteristic loss of body weight is mainly due to increased muscle wasting in diabetes (Swanston-Flatt *et al.*, 1990). *Solanum indicum* extract and glibenclamide administration controlled this loss in body weight. However, it did not normalize the body weight completely as it remained lesser than that of normal control rats. The decrease in body weight observed in diabetic rats might be the result of protein wasting due to unavailability of carbohydrate for utilization as an energy source (Musabayane *et al.*, 2005).

3.5.11 Effect of *Solanum indicum* fruit extract on ALAT and ASAT

STZ caused marked increase in the level of ASAT from 36 to 66 U/L and ALAT from 55 to 91 U/L as compared to that of normal rats. Oral administration of the fruit extract to diabetic rats significantly (P<0.01) suppressed elevated level ASAT from 66 to 48 and 46 at doses of 100 and 200 mg kg^{-1}, respectively (Fig. 3.8) at the end of 30^{th} day. The extract also

exhibited its effect on ALAT by suppressing the level from 91 to 72 and 61 at doses of 100 and 200 mg kg^{-1} respectively at the end of study.

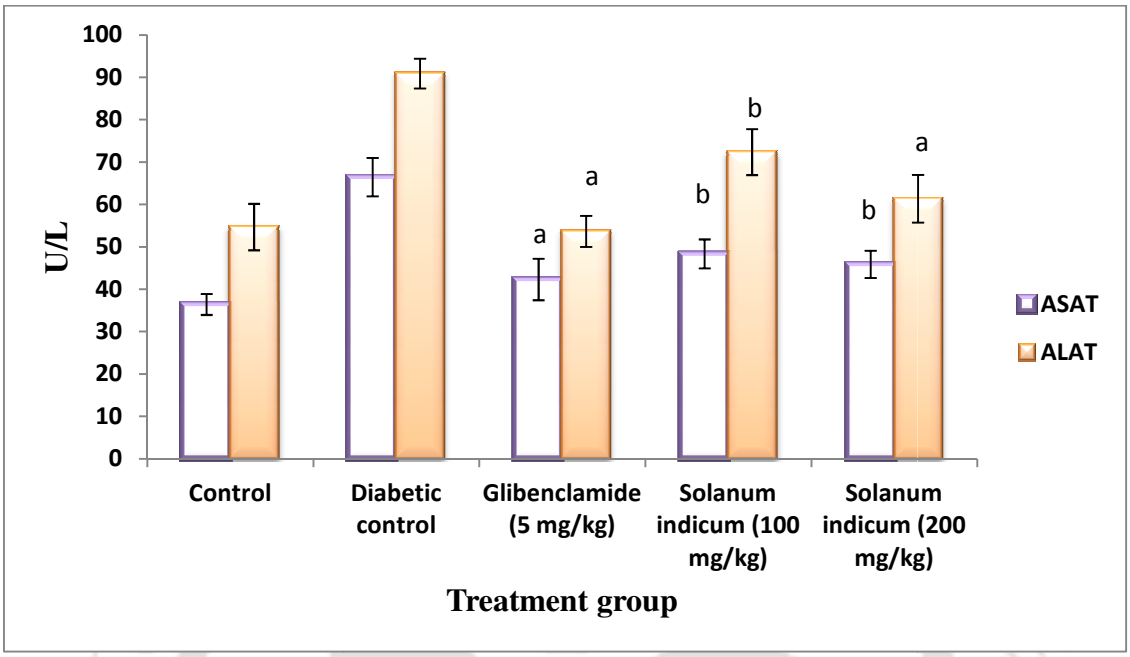


Fig. 3.8 Effect of *Solanum indicum* on ALAT and ASAT level in STZ-induced diabetic rats.

All the values were expressed as mean ±S.E., n=6.
[a]Represents statistical significance (P<0.001) vs control,
[b]Represents statistical significance vs diabetic control group(P<0.01).

Serum transaminases (ALAT and ASAT) are the enzymatic markers of hepatic function. In this study, increase in the activity of serum transaminase indicates the hepatic damage caused by the action of reactive oxygen species and chronic hyperglycaemia produced by STZ. Hyperglycaemia associated with diabetes causes reduced level of antioxidant due to over production of free radicals (Aragno *et al*., 2004). The later damage the membrane through lipid peroxidation of unsaturated fatty acids and alter its function (Memisogullari and Bakan, 2004; Ravi *et al*., 2004). Treatment of the diabetic rats with the extract reduced the activity of these enzymes as compared to that in the diabetic untreated group and consequently alleviated liver damage caused by STZ.

3.5.12 Effect of *Solanum indicum* fruit extract on serum lipid profile

In STZ diabetic rats increased levels of serum cholesterol were observed. Treatment of diabetic rats for 30 days with the fruit extract of *Solanum indicum* at doses of 100 and 200 mg kg^{-1} showed significant ($P<0.01$) reduction in serum cholesterol level from 20 days onward (Fig. 3.9) and continued till the end of the experiment. Oral administration of the extract to diabetic rats of a dose of 100 mg kg^{-1} showed 15.16, 17.7 and 20.14% reduction while administration of a dose of 200 mg kg^{-1} showed 17, 22 and 28.26% reduction in 10, 20 and 30 days of the treatment, respectively.

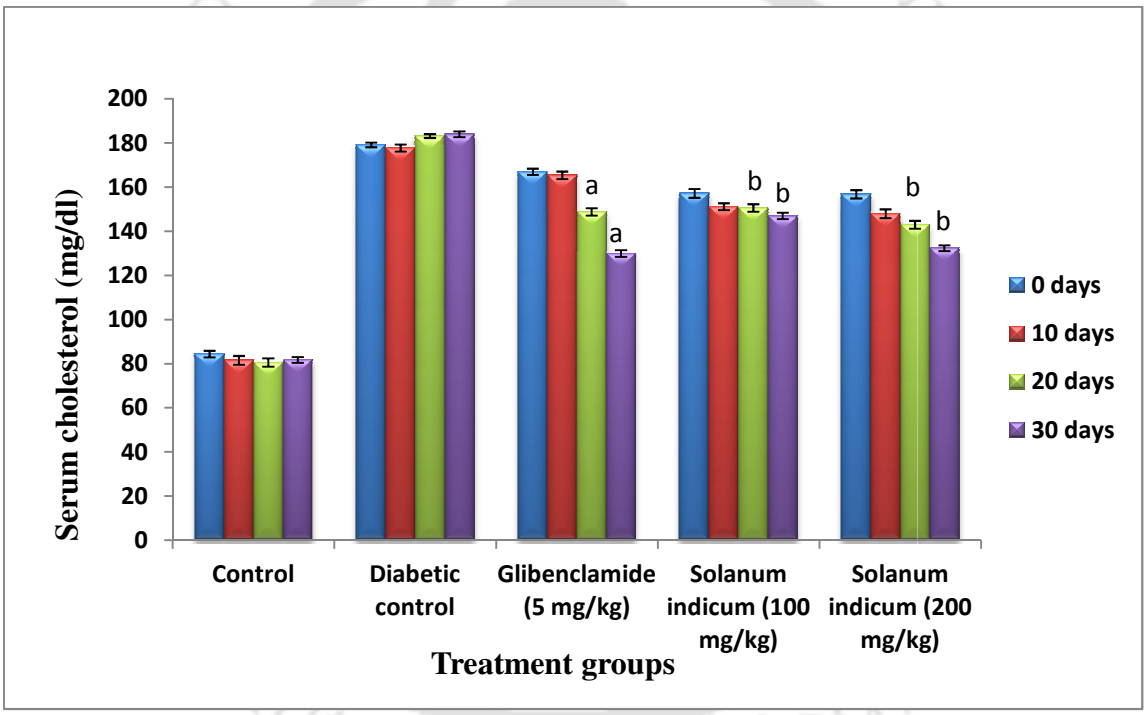


Fig. 3.9 Effect of *Solanum indicum* fruit extract on total cholesterol in STZ-induced diabetic rats.

All the values were expressed as mean ±S.E., n=6, [a] Represents statistical significance vs diabetic control (P<0.0001) and [b] Represents statistical significance vs diabetic control (P<0.01).

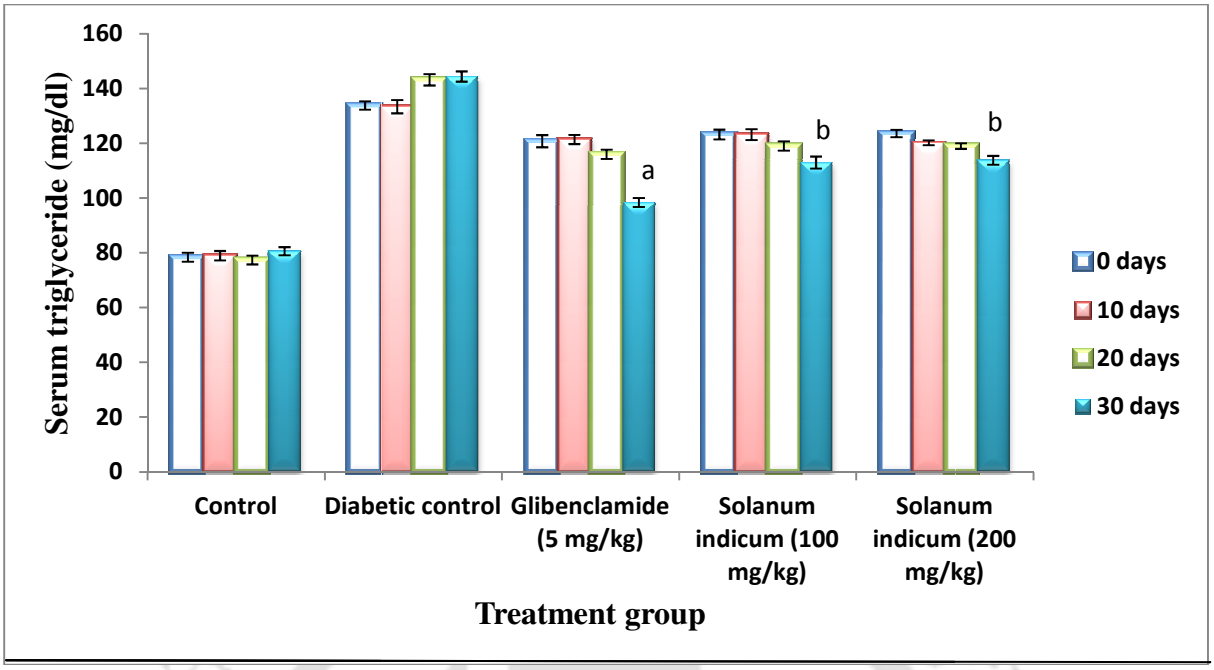


Fig. 3.10 Effect of *Solanum indicum* fruit extract on triglycerides in STZ-induced diabetic rats.

All the values were expressed as Mean ± S.E., n=6.

[a] Represents statistical significance vs diabetic control (P<0.0001) and [b] Represents statistical significance vs diabetic control (P<0.001).

Treatment of diabetic rats with the fruit extract of *Solanum indicum* showed a dose- and time-dependant significant (P<0.001) reduction in triglycerides level (9, 16.78 and 21.52% at a dose of 100 mg kg^{-1} while 9.77, 19.44 and 24.17% at a dose of 200 mg kg^{-1} in 10, 20 and 30 days of treatment respectively) compared to pre treatment levels (Fig. 3.10). The fruit extract of *Solanum indicum* at a dose of 200 mg kg^{-1} was more effective than 100 mg kg^{-1}in reducing the triglyceride levels.

Hypercholesterolaemia and hypertriglyceridemia are primary factors involved in the development of atherosclerosis and coronary heart disease which are the secondary complications of diabetes (Ananthan *et al*., 2003). Fruit extract of *Solanum indicum* significantly reduced serum triglycerides and total cholesterol in STZ diabetic rats. Thus, it is reasonable to conclude that the extract could modulate blood lipid abnormalities

3.6 Conclusion

The present study discusses the antidiabetic effect of the fruit extract of *Solanum indicum* in STZ induced diabetic rats. The extract showed concentration-dependant DPPH free radical scavenging activity, thus, plays a pivotal role in reducing oxidative stress associated with diabetes. Acute toxicity studies in rats and in vitro studies in mouse fibroblast cell line revealed the non toxic nature of the extract. There was no lethality or any toxic reactions found with the selected doses until the end of study. Thus, the No Observed Adverse Effect Level (NOAEL) of the extract was greater than 5000 mg kg^{-1} body weight.

The extract controlled post prandial hyperglycaemia by producing dose-dependant α-amylase inhibition. Insulin resistance and impaired insulin secretion are the characteristic features of diabetes, in our study, the extract caused marked reduction in fasting blood glucose and also improved loss of body weight associated with diabetes.

The results of the study have shown that the methanolic fruit extract of *Solanum indicum* at a dose of 200 mg kg^{-1} body weight has a marked hypoglycemic activity by improvement of the glucose tolerance test in normoglycemic rats and by lowering the blood glucose levels from the 10^{th} day up to the 30^{th} day of treatment in STZ-induced diabetic rats.

Oral administration of the extract also improved the elevated level of serum triglycerides and cholesterol which are increased due to faulty lipid metabolism and mobilization of free fatty acids. The extract also corrected the elevated level of ALAT and ASAT thereby, prevented the hepatic damage caused by STZ.

All these observations ascribe a pivotal role to *Solanum indicum* in developing an antidiabetic drug of natural origin. Further research is required for identifying the molecular target and isolation of bioactive phytoconstituents to find out the exact mechanism of its antidiabetic action.

CHAPTER 4

Evaluation of antidiabetic activity of *Solanum torvum* leaf extract in streptozocin-induced diabetic rats

4.1 Introduction

Diabetes mellitus (DM) is collective form of various metabolic disorders that affect more than 100 million population worldwide (6% of the population) and is expected to increase five times by the year 2025 (Grover *et al*., 2002; Kim *et al*., 2006). Glucose metabolism gets severely hindered due to improper insulin production from pancreatic β-cells (Jarald *et al*., 2008). Glycogen catabolism in liver increases due to low insulin level resulting in low hepatic glycogen content in diabetes. It is classified as insulin dependent diabetes mellitus (type I) and non insulin dependent diabetes mellitus (type II) and diagnosis of diabetes mellitus is based on measurement of fasting (7 mM or greater on two separate occasions) or random blood glucose level (11 mM or greater) and glycated hemoglobin HbA1C in the serum (Patel and Macerollo, 2010).

Present therapy for diabetes includes drugs like insulin secretagogues (sulfonylurea, meglitinides), insulin sensitizers (biguanides, metformin, thiazolidinediones) and α-glucosidase inhibitors (miglitol, acarbose). These oral hypoglycemics can be used alone or in combination with other drugs to achieve better effects. Many of these oral hypoglycemic agents possess a number of serious adverse effects, thus, the management of diabetes without any side effects is still a challenge (Pareek *et al*., 2009). Hence, there is a need to develop safe, effective and affordable drug therapy for the management and treatment of diabetes which could be obtained through the use of the medicinal plants as they may be safe and non toxic (Surendran *et al*., 2011).

Solanum torvum Sw. (*Solanaceae*), commonly known as Turkey berry is cultivated in Africa and West Indies (Adjanohoun *et al*., 1996). It also occurs commonly in the moist farms of India. The leaves of *Solanum torvum* are edible and are available in markets. They are utilized as a vegetable and regarded as an essential ingredient of the food in South India.

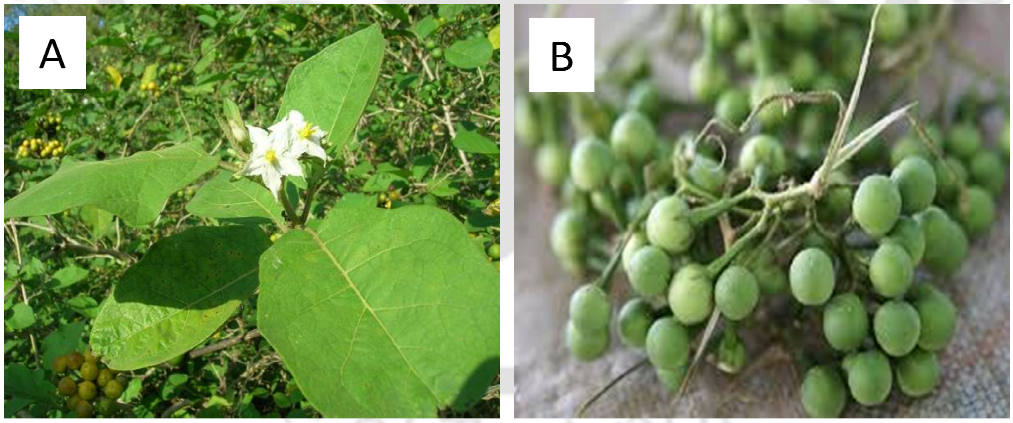


Fig. 1 A) Whole plant of *Solanum torvum* B) Fruits of *Solanum torvum.* (Adapted from http://luirig.altervista.org/flora/taxa/index2.php?scientific- name=solanum+torvum)

Solanum torvum is an erect spiny shrub that is usually 2 to 3 m in height and 2 cm in basal diameter, but may reach 5 m in height and 8 cm in basal diameter. It grows on all types of moist, fertile soil at elevations from near sea level to almost 1,000 m. It grows best in full sunlight and does well in light shade or shade for part of the day.

Decoction of leafs is given for cough ailments and is also considered useful in cases of liver and spleen enlargement (Siemonsma and Piluek, 1994). The plant is a sedative and diuretic and is used as a haemostatic. The ripened leaves are used in the preparation of tonic and haemopoietic agents and also for the treatment of pain (Kala, 2005). It has antioxidant properties (Sivapriya and Srinivas, 2007). It is intensively used worldwide in traditional medicine as a poison antidote and for the treatment of fever, wounds, tooth decay, reproductive problems and arterial hypertension (Ndebia *et al*., 2007).

Pharmacologically it has been reported that *Solanum torvum* has various activities like antifungal (Karuppusamy *et al*., 2009; Satish *et al*., 1999), antioxidant (Waghulde *et al*., 2011), antibacterial (Lalitha *et al*., 2010), antiulcer (Telesphore *et al*., 2008), antihypertensive and metabolic correction (Nguelefack *et al*., 2008), cardio protective, neuroprotective (Mohan *et al*., 2010) , analgesis and antiinflamatory activities (Atta and Alkofahi, 1997).

For many decades, plant-derived products have been used for the treatment and management of various ailments (Ridtitid *et al*., 2008).The medicinal plant contains various phytoconstituents like alkaloids, glycosides, tannins, flavonoids and phenolic compounds which are mainly responsible for producing various therapeutic effects. Before the screening of naturally derived products for various pharmacological activities, its evaluation for toxicity is foremost and essential in the initial step. Regardless of the antidiabetic activity of leaf extract of *Solanum torvum* reported by traditional practitioner's detailed knowledge about its antidiabetic effect has not been reported in any literature. Hence, the current study was carried out to evaluate the antioxidant and antidiabetic activity of metahnolic extract of *Solanum torvum* in experimentally-induced diabetic rats.

4.2 Materials and methods

4.2.1 Chemicals, reagents and kits

2, 2-diphenyl-1-picrylhydrazyl (DPPH), α-amylase, Streptozocine (STZ), 3, 4; 5-Dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT), modified eagle media (MEM) and fetal bovine serum (FBS) were procured from Sigma-Aldrich Pvt. Ltd. USA. Folin–Ciocalteu reagent for estimation of total phenolics was obtained from Himedia Pvt Ltd, Mumbai. Mouse fibroblastic cells (L929) cell lines were obtained from the National Centre for Cell Sciences, Pune, India. Glucose, triglycerides, total cholesterol and HDL-C kits were procured from Merck, Mumbai. All other reagents and solvents used for the study were of analytical grades.

4.2.2 Collection and identification of plant material

Leaves of *Solanum torvum* were collected during the month of August and September from the nearby area of IIT Guwahati campus, Guwahati, India. The plant was authenticated by a taxonomist at the Department of Botany, Guwahati University, India and a voucher specimen (17786) was deposited at the Herbarium for future reference.

4.2.3 Processing of plant material

The leaves were repeatedly washed with distilled water to remove soil and dirt; the leaves were allowed to dry under shade at 25^0C for about one month. The dried leaves were ground to obtain fine powder. The powdered dry leaf material was kept at 25^0C protected from direct sunlight. 300 g of leaf powder was subjected to repeated extraction by using hot soxhlet extraction method for 24 h. The extract was concentrated using a rotary evaporator (Rotavapor, R-215). The concentrated extract was lyophilized to get a powder (yield 16.9 % w/w) and used for subsequent experiment.

4.2.4 Determination of total phenolic content in the plant extract

The concentration of phenolics in the plant extract was determined by spectrophotometric method (Singleton *et al*., 1995) and as described in chapter 2 (section 2.2.4).

4.2.5 Determination of total flavonoids content in the plant extract

Total flavonoid content in the plant extract was determined by spectrophotometric method (Quettier *et al*., 2000) with slight modification and as described in chapter 2 (section 2.2.5).

4.2.6 Evaluation of antioxidant activity

The ability of the plant extract to scavenge DPPH free radicals was assessed by the standard method of Tekao *et al*, (1994), adopted with some modifications (Kumarasamy *et al*., 2007). The procedure was described in chapter 2 (section 2.2.6).

4.2.7 Cytotoxicity evaluation of the crude extract using MTT assay

Mouse fibroblastic (L929) cell line was cultured and maintained according to standard protocol. Cell cytotoxicity of the plant extract was carried out by the MTT (3-[4, 5-dimethylthiazole-2-yl] -2, 5-diphenyl tetrazolium bromide) dye conversion assay. L929 cells at a density of 1×10^4 per well were cultured in a 100 µL of culture medium (DMEM: Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum in a 96 well cell culture plate. After 24 h, cultured cells were treated with different concentrations of plant extract ranging from10 to100 µg mL^{-1} in serum free media and incubated further for 24 h. This was followed by the removal of the media and treatment with MTT dye at a final concentration of 0.5 mg mL^{-1} and further incubated for 4 h. Finally, 100 µL of dimethylsulfoxide (DMSO) was added to each well to dissolve the blue formazan precipitate and the absorbance was measured at 570 nm using a microplate reader (Tecan, Model 680). The cell viability was expressed as a percentage of the control by the following equation:

$$\text{Viability (\%)} = At/Ac \times 100$$

Where, At and Ac represents absorbance of the plant extract treated and control cells respectively.

4.2.8 α-Amylase inhibition assay

The α-amylase inhibition assay was carried out according to the procedure reported by Thirunavukkarasu, (2003) and as described in chapter 2 (section 2.2.9).

4.2.9 Experimental animals

The study was conducted on albino Wistar rats of either sex weighing 180–220g which were procured from the central animal house, Department of Pharmacology, College of Veterinary and Animal Sciences, Udgir, Maharastra. The animals were allowed to acclimatize for a period of two weeks in the animal house. The rats were housed in polypropylene cages, at an ambient temperature 25 ± 2ºC with 12 h light and dark cycle. The animals were fed with standard rat pellet diet (Hindustan lever, Mumbai) and water *ad libitum.*

The experimental protocols and procedures used in the study were approved by the Institutional Animal Ethical Committee, COVA Sciences, Udgir, (Maharashtra), approval No. VCU/CPCSEA/IAEC/2/14 (II).

4.2.10 Acute toxicity study

The acute toxicity study was conducted in accordance with the OECD guidelines No. 423. The study was conducted in two phases using a total number of 36 rats. Healthy adult Wistar rats of either sex weighing 150-200 g, starved overnight were divided in to six groups (n =6). In the first phase group 1, 2 and 3, animals were administered a single oral dose of *Solanum torvum* leaf extract 5, 50 and 300 mg kg^{-1} body weight (b .w.) respectively, to establish the range of doses producing any toxic effect.

In the second phase, further specific doses 1000, 2000 and 5000 mg kg^{-1} of *Solanum torvum* leaf extract was administered to groups 4, 5 and 6 rats for further determination of the LD_{50} value. The extract was dissolved in 0.5% vehicle i.e. carboxy methyl cellulose (CMC) solution and given by oral route. All animals were monitored for 24 h for signs of behavioural, neurological and autonomic changes and for acute toxicity including death.

4.2.11 Oral glucose tolerance test

In order to determine the effect of the leaf extract of *Solanum torvum* extract on insulin activity, the OGTT was carried out on all three groups of rats i.e., Group I control animals (administered vehicle only), Group II positive control (administered with glibenclamide 5 mg kg^{-1}), Group III and IV (administered with *Solanum torvum* extract, 100 and 200 mg kg^{-1} body weight respectively). OGTT was performed by oral administration of glucose load of 2 g kg^{-1} body weight to the overnight fasted animals. Blood samples were collected from the tail vein at 30, 60, 90, 120 and 180 min after the oral glucose load extract administration and the serum glucose levels were estimated.

4.2.12 Induction of experimental diabetes

Overnight starved experimental rats were injected with streptozotocin at a dose of 60 mg kg^{-1} body weight as described earlier (Sanae *et al*., 1998) with slight modifications. STZ was injected intra peritoneally (i.p) after dissolving in 0.025 M ice cold sodium citrate buffer at pH 4.0. The rats exhibiting blood glucose level ≥250 mg dL^{-1} were selected for the study. Once the stable hyperglycemia was achieved, the rats were divided into five different groups as follows,

Group I: untreated (control); Group II: STZ-treated diabetic rats; Group III: STZ-induced diabetic rats treated with glibenclamide (5 mg kg^{-1}); Group IV: STZ-induced diabetic rats treated with 100 mg kg^{-1} body weight of *Solanum torvum* extract; Group V: STZ-induced diabetic rats treated with 200 mg kg^{-1} body weight of *Solanum torvum* extract. After

randomization into various groups, the treatment was continued for 30 days and the serum biochemical parameters like glucose, triglycerides and cholesterol were estimated at a regular time interval of 10,20 and 30th day of study.

4.2.13 Collection of blood

The rats were anesthetized using light ether anaesthesia and blood was collected by retro orbital sinus puncture. The blood was collected in plastic test tubes and allowed to stand for 30 min to ensure complete clotting. The clotted blood samples were centrifuged at 3000 rpm at 4^0 C for 10 min and clear serum samples were aspirated and stored at -20^{o}C. The biochemical parameters determined in the serum specimen were glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC) and high density cholesterol (HDL-C) by using microlab100 auto analyzer.

4.2.14 Serum biochemical parameters

The various biochemical parameters like serum 1) glucose, 2) triglycerides, 3) cholesterol, 4) AST and 5) ALT were estimated as described in chapter 2 section (2.3.5, 2.3.6, 2.3.7, 2.3.8 and 2.3.9, respectively).

4.3 Statistical analysis

All the values of the experimental results were expressed as mean ± standard error Mean (SEM). For evaluation of oral glucose tolerance test, two ways ANOVA followed by Bonferroni post test was performed.

For the antidiabetic study (diabetic rats treated with the glibenclamide and leaf extracts at doses of 100 and 200 mg kg^{-1} body weight) and other parameters like aspartae aminotransferase, alanine aminotransferase, triglycerides, total cholesterol and body weight the data was analyzed statistically by applying one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using graph pad prism (version 5.5) computer software. The results were considered statistically significant if $P<0.05$.

4.4 Results and Discussion

4.4.1 Preliminary phytochemical analysis

Preliminary phytochemical analysis of methanolic leaf extract of *Solanum torvum* showed presence of alkaloids, glycosides and saponins.

Total phenolic content of the leaf extract was found to be 123.5 µg gallic acid equivalent/gram of the extract calculated from the gallic acid calibration curve depicted in Fig. 4.1. Total flavonoid in the extract was found to be 95 µg of quercetin equivalent/gram of the extract calculated from the calibration curve of quercetin.

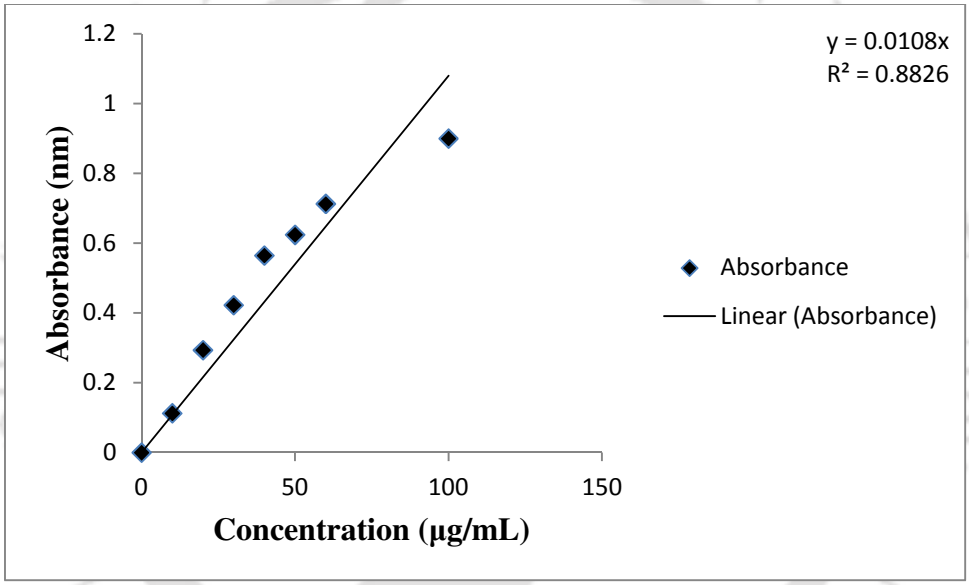


Fig. 4.1 Calibration curve for gallic acid.

4.4.2 Antioxidant activity evaluation of *Solanum torvum* extract

Preliminary phytochemical analysis revealed the presence of alkaloids, saponins and glycosides as well as the presence of high content of phenolics and flavonoids in the leaf extract of *Solanum torvum* and this prompted us to screen for the radical scavenging activity.

The leaf extract of *Solanum torvum* showed the highest DPPH scavenging activity of 66.3% at 100 µg mL^{-1} of the extract. The EC_{50} value was found to be 93.3 µg mL^{-1} and the DPPH radical scavenging activity was recorded in terms of % Inhibition (Fig. 4.2). The antioxidant compounds present in the plants may act by preventing the production of free

radicals or by neutralizing/scavenging free radicals produced in the body or reducing/chelating the transition metal composition (Amic *et al*., 2003). The results presented showed that the leaf extract of *Solanum torvum* had the lowest EC_{50} as compared to other *Solanum* species (Poongothai *et al*., 2011). In DPPH assay, the radical scavenging ability of a plant extract is based on its ability to decolourize the stable DPPH free radicals (deep purple colour) measured from changes in absorbance. This implies that the leaf extract of *Solanum torvum* decolourized DPPH so possessed the best ability to scavenge the DPPH radicals (Wettasinghe and Shahidi, 2000).

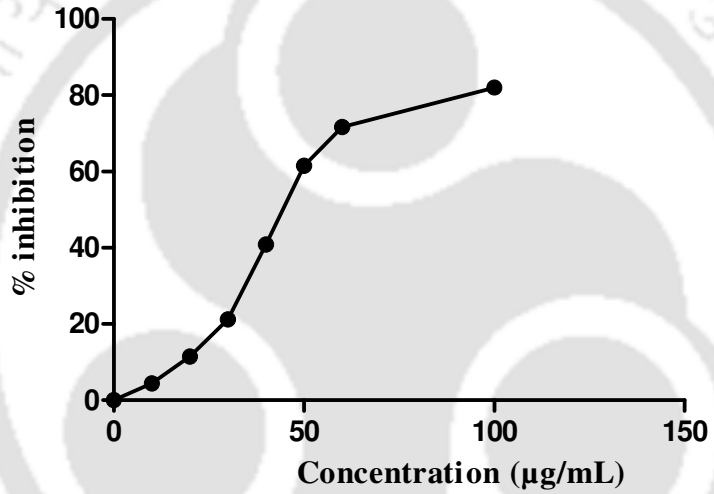


Fig 4.2 Antioxidant activity of *Solanum torvum* leaf extract.

4.4.3 Cytotoxicity evaluation of *Solanum torvum* leaf extract

MTT dye conversion assay triggered the activity of mitochondrial dehydrogenase of live mouse fibroblast cells (L929) that cleaved the tetrazolium ring in the tetrazolium salts. Only active mitochondria contain these enzymes and therefore, the reaction occurred in living cells. Treatment of L929 cells with different concentrations of *Solanum torvum* extract for 24 hours did not show any significant loss in cell viability compared to the untreated cells (Fig. 4.3). The extract treated L929 cells at the highest concentration (100 µg mL^{-1}) showed cell

viability of 60.26% after 24 h of treatment, confirming that the leaf extract was biocompatible and non toxic to the cells.

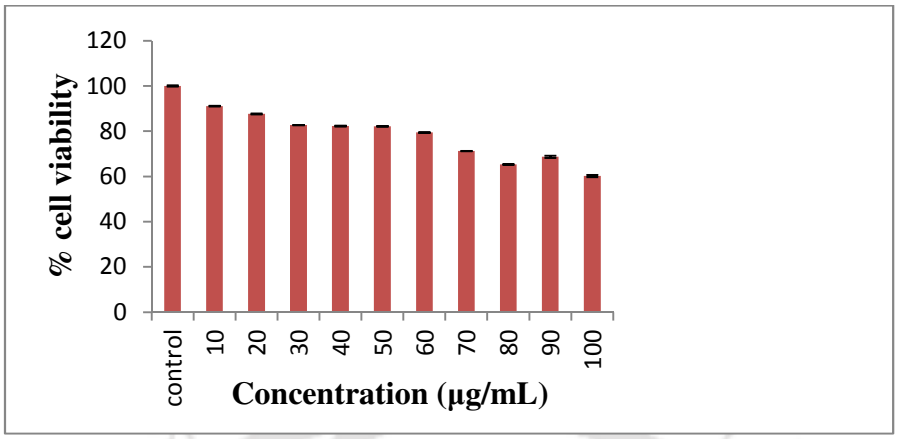


Fig. 4.3 MTT Cytotoxicity assay: cell viability of L929 cells exposed to different concentrations of *Solanum torvum* extract.

4.4.4 α-Amylase assay

The inhibitory effect of leaf extract of *Solanum torvum* against porcine pancreatic amylase is depicted in (Table 4.1). Under *in vitro* conditions, the leaf extract of *Solanum torvum* showed concentration (10, 20, 40, 60, 80 and 100 µg mL^{-1})-dependant α-amylase inhibitory activity. It showed maximum inhibition of 78.5% at100 µg mL^{-1}; whereas, 10, 20, 40, 60 and 80 µg $mL^{-1}$of *Solanum torvum* extract, showed 36.1, 46.8, 56.7, 63.9 and 68.6% inhibition, respectively. IC_{50} value of *Solanum torvum* extract was found to be 32.1 µg mL^{-1}.

Table 4.1 α-Amylase inhibitory effect of *Solanum torvum* leaf extract.

Concentration (µg mL-1)	% inhibition	IC_{50} (µg mL^{-1})
10	36.1±1.4	
20	46.8±0.8	32.1
40	56.7±0.6	
60	63.9±0.9	
80	68.6±0.2	
100	78.5±0.4	

α-Amylase orchestrates a catalytic conversion of complex carbohydrates into monosaccharides that can be absorbed. Reduction of postprandial hyperglycemia is the mainstay in the treatment paradigm of diabetes mellitus. Blood glucose level reduction can be confronted by extending the glucose absorption through the down regulation of carbohydrate hydrolyzing enzymes in the digestive tract. α-Amylase inhibitors play a pivotal role in blocking the release of glucose from dietary source of carbohydrates and extend glucose absorption leading to decreased postprandial plasma glucose levels and further minify postprandial hyperglycemia (Lebovitz, 1997). In our study, *Solanum torvum* leaf extract effectively inhibited the α-amylase activity *in vitro* which is in line with a previous report (Adisakwattana and Chanathong, 2011).

Preliminary phytochemical analysis of leaf extract of *Solanum torvum* revealed presence of phenolic and flavonoid compounds. Some flavonoids, such as rutin, myricetin, kaempferol and quercetin, have been previously reported to inhibit the activity of carbohydrate hydrolyzing enzyme, α-amylase (Kamalakkannan and Prince, 2006; Wang *et al*., 2010). The presence of flavonoid compounds in the crude extract of *Solanum torvum* could be responsible for the inhibitory effect and this may be one of the mechanisms for the regulation of post prandial hyperglycemia associated with diabetes.

4.4.5 Acute toxicity study

In the present study, the acute toxicity was carried out in two different phases, in phase one, the different doses of methanolic extract of *Solanum torvum* (50, 100, 300, 500 and 1000 mg kg^{-1} body weight) did not produce any sign of toxicity like grooming, defecation, neurological and behavioural changes and there was no lethality until the end of study period revealing the non toxic nature of the extract. In second phase, the extract showed toxic effect (more than 50% mortality in experimental animals) at the highest dose level (2000 and 5000 mg kg^{-1}b.w.). Since no toxic effects were found during the acute toxicity

study at lower doses, further evaluation was conducted on the antidiabetic and antioxidant potential of the leaf extract of *Solanum torvum* on experimental animal model using lower doses.

4.4.6 The OGTT in normal Wistar rats

In oral glucose tolerance test, oral administration of glibenclamide (5 mg kg^{-1}) caused significant (P<0.005) reduction in blood glucose level from 30 min upto180 min as compared to vehicle treated rats (Fig. 4.4). Leaf extract of *Solanum torvum* showed significant (P<0.05) reduction in blood glucose at a dose of 100 mg kg^{-1} from 60 min and sustained up to 180 min (P<0.005). Rats treated with the extract at a dose of 200 mg kg^{-1} showed significant reduction (P<0.05) in blood glucose level from 60 min and the effect was observed till 180 min after oral administration of glucose (P<0.005).

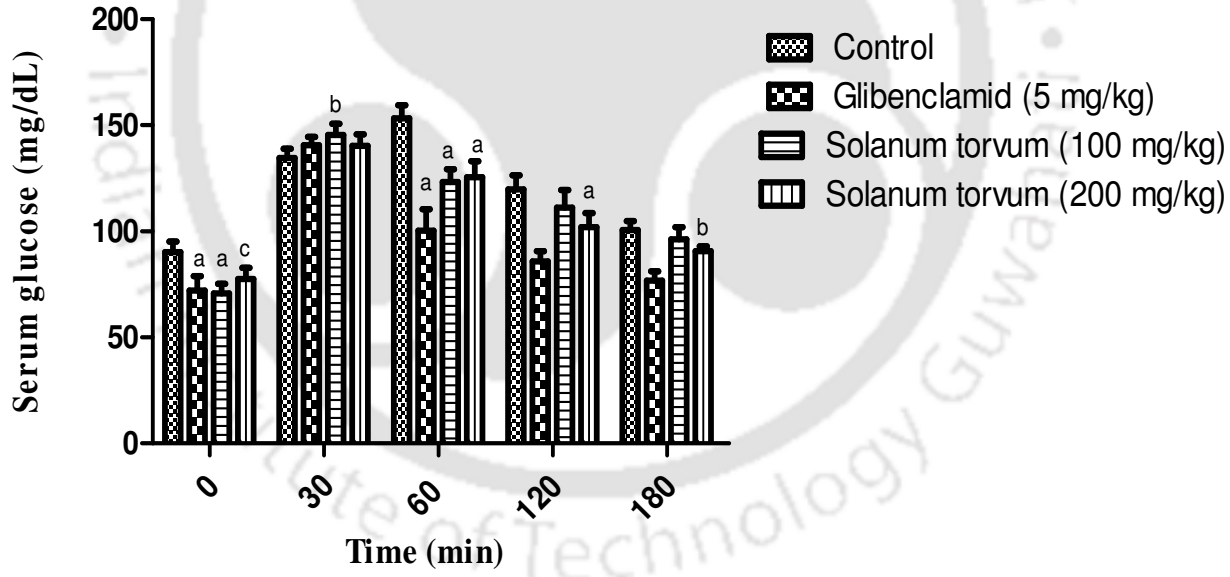


Fig. 4.4 Effect of *Solanum torvum* leaf extract on oral glucose tolerance test.
Each value represents mean ± S.E., n=6.[a] (P<0.001), [b] (P<0.05) and [c] (P<0.01) represent statistical significance when compared vs control group.

4.4.7 Repeated administration of *Solanum torvum* leaf extract in diabetic rats

As shown in Figure 4.5, intra peritoneal injection of STZ (60 mg kg^{-1} resulted in a significant (P<0.001) increase in the serum glucose level from 85 to 267 mg dL^{-1} as compared to those of control rats, confirming the stable hyperglycemia. Daily administration

of *Solanum torvum* leaf extract at all doses resulted in significant ($P<0.05$) decrease of blood glucose levels. The reduction was 13.58 and 14.98% respectively at the doses of 100 and 200 mg kg^{-1} on the 10^{th} day of treatment compared to the initial value. After 30 days of treatment with plant extract, the decrease in blood glucose level was 58.69% and 60.24% at doses of 100 and 200 mg kg^{-1} respectively. Animals treated with glibenclamide (5 mg kg^{-1}) showed a decrease in serum glucose level from 22.29% to 64.90% at the 10^{th} and 30^{th} day respectively compared to the diabetic control rats.

STZ has been extensively used to induce diabetes mellitus in experimental rat models. The intra peritoneal administration of STZ (60 mg kg^{-1}) selectively destroyed some population of pancreatic beta cells resulting in insulin deficiency and causing type 2 diabetes. In the present study, diabetic rats treated with the leaf extract of *Solanum torvum* showed significant decrease in serum glucose level and the hypoglycaemic effect of extract was compared with glibenclamide so it may be suggested that the mechanism of action of *Solanum torvum* extract may be similar to that of glibenclamide. This could be due to the potentiation, by the extract, of the pancreatic secretion of insulin from regenerated β-cells, or its action to release bound insulin from regenerated beta cells by inhibiting ATP sensitive K^+ channels like glibenclamide. Previous studies showed that phenolic compounds acted on ATP sensitive K^+ channels and regulated blood glucose (Pandey and Rizvi, 2009). The high proportion of phenolic compounds present in the leaf extract could be responsible for the antidiabetic effect.

Solanum species are a rich source of saponins and phenolics, particularly flavonoids, tannins and proanthocyanidins (Romussi *et al*., 1994; Sohretoglu and Sakar, 2004; Sakar *et al*., 2005) and possess antioxidant, anti-inflammatory and antidiabetic properties (Sohretoglu and Sakar, 2004; Sohretoglu *et al*., 2007). In the present study, preliminary phytochemical analysis of *Solanum trovum* leaf extract revealed the presence of alkaloids, glycosides,

tannins, phenolic and flavonoid compounds which may be responsible for producing glucose lowering effect.

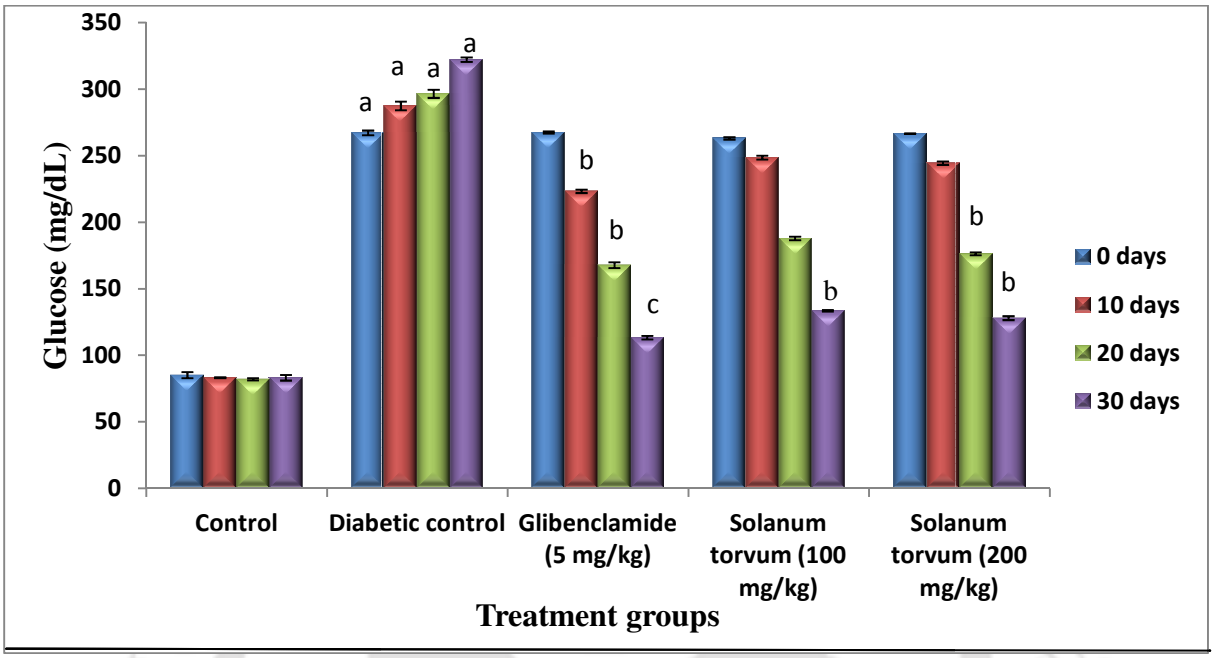


Fig. 4.5 Effect of *Solanum torvum* leaf extract on blood glucose.

Values are expressed as mean ± S.E., n=6.

[a]P<0.001 represent statistical significance when compared vs control group, [b]P<0.05 and [c]P<0.005 represent statistical significance when compared vs diabetic control group.

4.4.8 Effect of *Solanum torvum* leaf extract on the body weight

The initial and final body weights in the control, diabetic control and treated groups are summarized in (Fig. 4.6). At the end of experiment, diabetic control rats showed a considerable reduction in body weight by 3.59% (from 167 to 161 g) as compared to the normal control rats. Oral administration of *Solanum torvum* leaf extract during 30 days of treatment at doses of 100 mg kg^{-1} and 200 mg kg^{-1} showed significant (P<0.01) reduction in body weight as compared to diabetic control rats.

STZ-induced diabetes is associated with weight loss which is due to excessive protein catabolism, unavailability for providing structural proteins like amino acids for gluconeogenesis and poor glycemic control during insulin deficiency (Kasetti *et al*., 2010). In the present study, STZ treated rats showed prolonged loss in body weight as compared to normal control animals. Treatment with the leaf extract produced improvement in the body

weight in diabetic treated rats, the observed effect of the extract could be due to improved glycemic control.

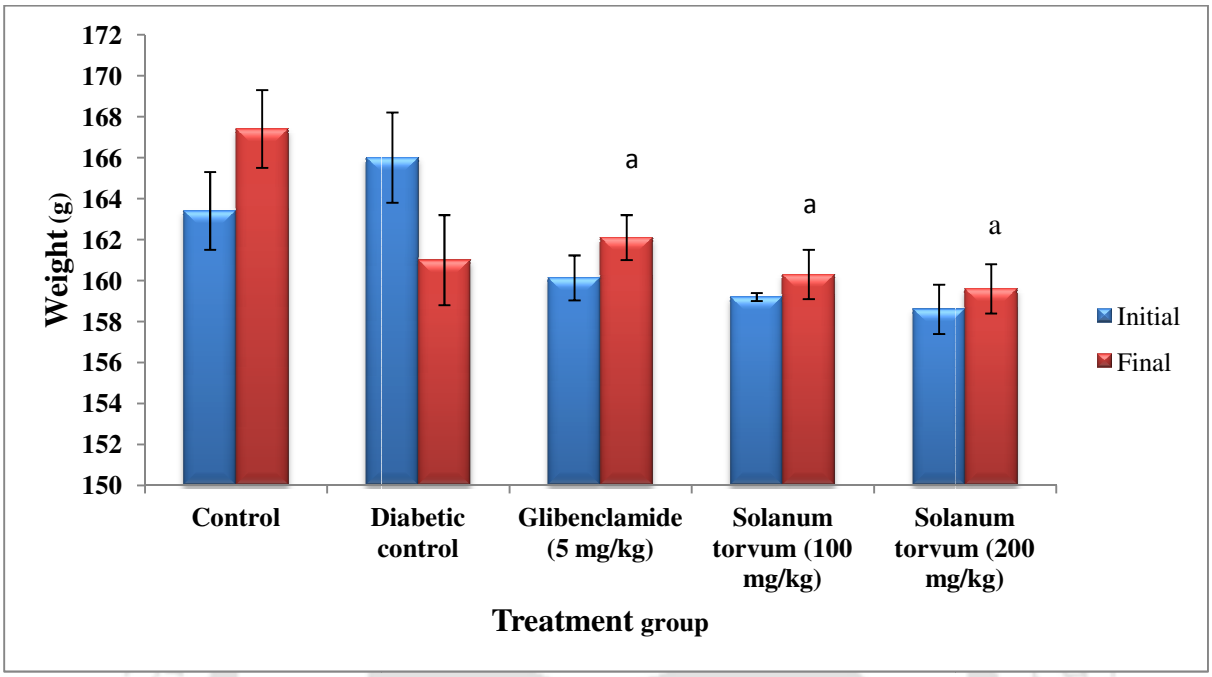


Fig. 4.6 Effect of *Solanum torvum* leaf extract on the body weights.

Values are the mean ± S.E., n=6,

[a]P<0.01 represent statistical significance when compared vs control group.

4.4.9 Effects of *Solanum torvum* leaf extract on serum lipid level in STZ diabetic rats

Figure 4.7 shows serum lipid profile in diabetic rats with chronic hyperglycaemia, as compared to the normal control rats, diabetic control rats have shown a significant increase in triglycerides (P<0.001; 35.97%), total cholesterol (P<0.001; 49.50%), LDL-cholesterol (P<0.001; 77.51%) and with a significant decrease in HDL-cholesterol (P<0.001; 14.28%). Daily administrations of the leaf extract of *Solanum torvum* for 30 days at the dose of 200 mg kg^{-1} significantly (P<0.05) decreased triglycerides (20.75%), total cholesterol (43.13%), LDL-cholesterol (53.48%) and increased HDL-cholesterol by 2.04% as compared to the diabetic control rats. However treatment of diabetic rats with the extract at a dose of 100 mg kg^{-1} did not producing significant lipid lowering effect.

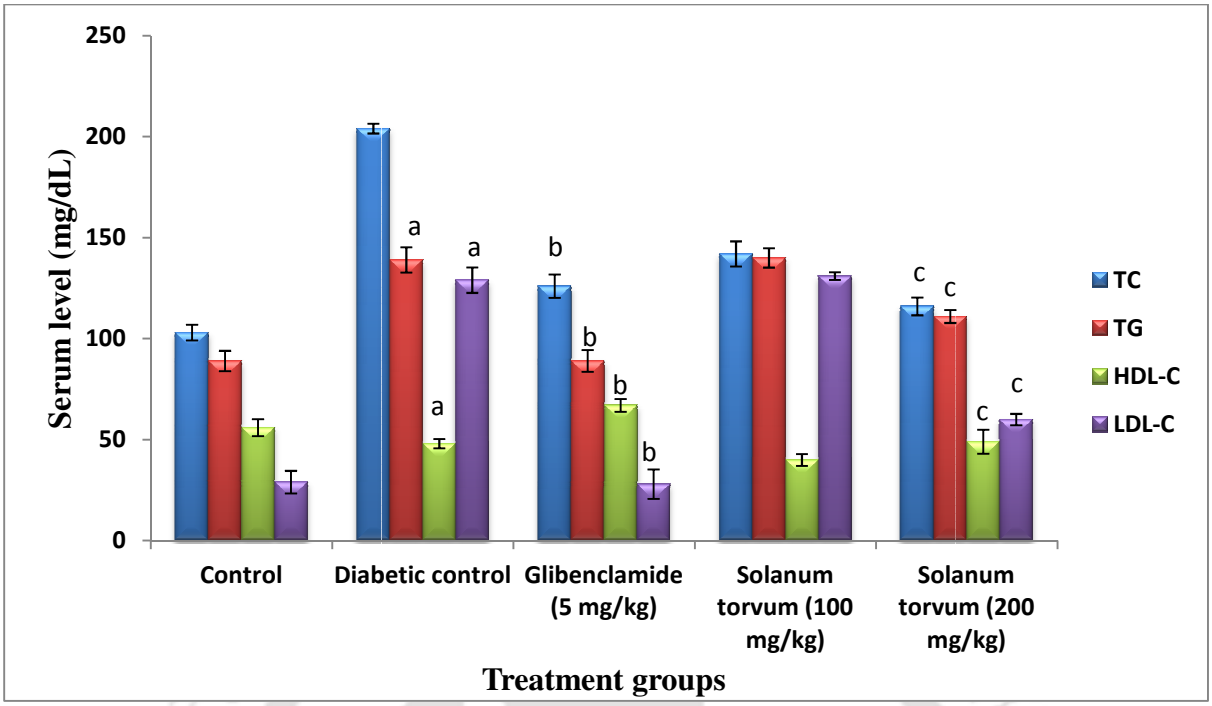


Fig. 4.7 Effect of *Solanum torvum* on serum lipid profile in STZ induced diabetic rats.

All the values were expressed in mean ± S.E., n=6.

[a]P<0.001 compared to normal control animals, [b]P<0.05 vs diabetic control, [c]P<0.005 vs diabetic control.

High blood glucose content is accompanied by elevation of serum lipid levels in STZ-induced diabetes (Kim *et al*., 2006 ; Sridevi *et al*., 2011). Remarkable hyperlipidemia in this group might be associated with uninhibited activities of lipolytic hormones on the fat depots (Al-Shamaony *et al*., 1994). In the present study, elevated level of cholesterol, triglycerides and LDL-C while decrease in HDL-cholesterol level was observed in the STZ-induced diabetic rats. In the present study, administration of the *Solanum torvum* leaf extract to the STZ diabetic rats significantly improved these parameters towards normal level. The observed hypolipidemic effect may be due to decreased cholesterogenesis and fatty acid synthesis and this may also be attributed to the enhanced glucose utilization. Thus, *Solanum torvum* leaf extract could have a potential to reduce long term cardiovascular complications in diabetic conditions and these effects of the extract have been demonstrated for the first time in the present study.

4.4.10 Effect of extract on liver function

STZ caused increase in the levels of ASAT and ALAT in experimental animals from 46 to 93 U/L and 17 to 37 U/L, respectively. Treatment of diabetic rats with the leaf extract of *Solanum torvum* at a dose of 100 mg kg^{-1} for 30 days resulted in significant ($P<0.001$) reduction in the elevated level of these enzymes (Fig. 4.8). Oral administration of extract at a dose of 200 mg kg^{-1} showed more significant reduction ($P<0.0001$) as compared to diabetic control rats.

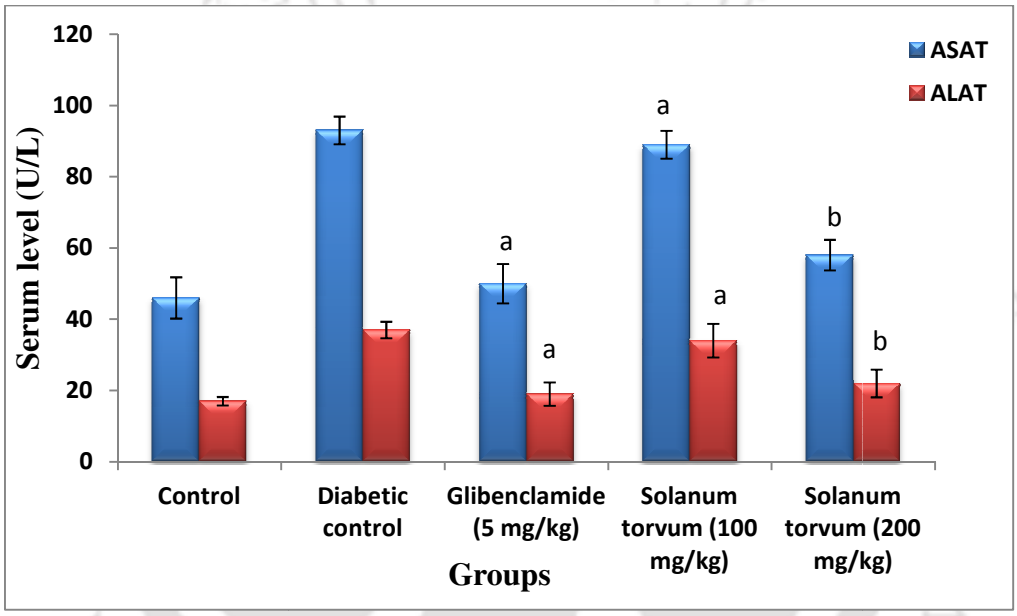


Fig. 4.8 Effect of *Solanum torvum* extract on liver function of streptozotocin induced diabetic rats.

Each value represents mean ± S.E., n=6.

[a] P<0.001 and [b] P<0.0001 represent statistical significance when compared vs diabetic control rats.

Serum enzyme activities can be used as biomarkers for monitoring the cytotoxicity of xenobiotics including STZ. In the present study, significant increases of ASAT and ALAT activities were estimated and compared with the diabetic control group. Treatment of diabetic rats with extract significantly decreased the activities of AST and ALT. Presently the reason for such effect of the extract was not clear. But, it is known that these enzymes have been considered as biomarkers of hepatic dysfunction and damage. In addition, the increase in the levels of AST and ALT in the serum of diabetic rats is mainly due to the leakage of these

enzymes from the liver cytosol into the blood stream (Navarro *et al*., 1993). Similar to our results, some earlier studies noted increases in ASAT and ALAT (Florence *et al*., 2014) levels in STZ-induced diabetic rats (Ozkol *et al*., 2013).

4.5 Conclusion

Recently, a number of studies have reported the potential role of medicinal plants in the treatment and management of diabetes. In this study, the antidiabetic activity of *Solanum torvum* leaf extract was analyzed. The parameters such as blood glucose, lipid profile and the liver marker enzymes like ASAT and ALAT in STZ-induced diabetic rats were monitered. Acute toxicity studies confirmed that the LD_{50} of *Solanum torvum* leaf extract was above 1000 mg kg^{-1} body weight as there was no observed toxic effect and lethality amongst all animals.

Phytochemical analysis of the leaf extract revealed the presence of alkaloids, glycosides, phenolic and flavonoid compounds which could be responsible for scavenging the stable DPPH free radicals thereby, exerting the antioxidant role.

Treatment of diabetic rats with the leaf extract of *Solanum torvum* for 30 days showed dose-and-time dependant glucose reducing effect. The more prominent effect was observed at 200 mg kg^{-1} body weight. The antidiabetic effect of the methanolic extract of *Solanum torvum* was compared against standard hypoglycaemic drug, glibenclamide and could be related to the stimulatory action/insulin mimetic action on the existing pancreatic β-cells and the capacity of the plant extract to scavenge the free radicals to prevent further damage similar to that of standard drug.

The extract corrected the elevated levels of serum triglycerides and cholesterol while increased the level of serum HDL-C hence, it has a potential to be used in treatment of hyperlipidemia associated with diabetes. The extract lowered the elevated levels of hepatic biomarker enzymes (ASAT and ALAT) thereby, prevented further hepatic damage caused by the action of STZ.

Hence, the leaf extract of *Solanum torvum* was found to have a high margin of safety and thus, *Solanum torvum* seems to have a promising value for the development of a potent

phytomedicine for diabetes, although, further comprehensive pharmacological investigations are needed to elucidate the exact mechanism of action of the extract.

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Summary and future prospects

Summary

It is being reported for the first time that the crude extracts of *Solanum indicum*, *Dillenia indica* and *Solanum torvum* have antidiabetic activity and has potential to be developed into a phytomedicine for treatment of diabetes. The results suggested that the presence of one or more antidiabetic phytoconstituents in the crude extract to improve the physiology of rats affected with non insulin dependent diabetes mellitus (NIDDM). These bioactive constituents attributed to normalise the blood glucose, also improved level of hepatic marker enzymes level of ASAT and ALAT and improved the physiology of diabetic rats.

Future prospects

- Identification, isolation and characterization of bioactive phytoconstituents is necessary to assess whether the individual isolated compounds retain or lose their activity or are more active than their parent crude extracts.
- Comprehensive screening and structural elucidation of polar compounds should be carried out to compare it with synthetic hypoglycaemic agents.
- The molecular mechanism underlying the antidiabetic and antioxidant activity of these plant species should be investigated.
- Polyherbal formulation of these plant extract with different doses should be tested to evaluate the combined effect in treatment and management of diabetes mellitus.

- Long term toxicity studies like cardiotoxicity, mutagenicity, reproductive toxicity and metabolic toxicity studies should be carried out for the plant extract which showed maximum glucose lowering effect.

Conference proceedings/ Papers

1) Gadewar M, Goyal A, Bora U. Novel herbal drug delivery system for diabetes. 4th Biennial International Conference on New Developments in Drug Discovery from Natural Products and Traditional Medicines, NIPER, Chandigarh (Poster presentation).

2) Gadewar M, Goyal A, Bora U. Lipid lowering agents. Pharma times 2015; 47(9): 11-12

Manuscripts under preparation

1) Antidiabetic and antioxidant evaluation of methanolic fruit extract of *Dillenia indica* in streptozocin-induced diabetic rats.

2) *In vivo* and *in vitro* evaluation of antidiabetic activity of *Solanum indicum* and *Solanum torvum* in streptozocin-induced diabetic rats.

Manuscripts from collaborative work

1) Sett A, **Gadewar M**, Sharma P, Deka M, Bora U. Green synthesis of gold nanoparticles using aqueous extract of *Dillenia indica*. Advances in Natural Sciences: Nanoscience and Nanotechnology 7 (2): 025005

2) Prashanth GK, Prashanth PA , **Manoj G**, Utpal B, Nagabhushana BM, Ananda S, Krishnaiah GM, Sathyananda HM. In vitro antibacterial and cytotoxicity studies of ZnO nanopowders prepared by combustion assisted facile green synthesis. Karbala International Journal of Modern Science 2015; 1(2): 67-77

3) PG Krishna, PP Ananthaswamy, **Manoj G**, U Bora, NB Mutta. In vitro antibacterial and anticancer studies of ZnO nanoparticles prepared by sugar fuelled combustion synthesis. Advanced Materials Letters 2017; 8(1): 24-29

VITAE

The author was born on July 1, 1981 in the city of Udgir, (Maharastra). He passed the Secondary Examination conducted by Maharastra State Board of Secondary and Higher Education (10th Class), Maharastra in 1996 and Higher Secondary Examination conducted by Maharastra State Board of Secondary and Higher Secondary Education (12th Class), Maharastra in 1998. He completed B. Pharmacy from College of Pharmacy, Nasik affiliated to Pune University, Pune (Maharastra) in April, 2003. He completed M. Pharmacy (Pharmacology) from JSS College of Pharmacy, Ooty, affiliated to Tamilnadu Dr. MGR Medical University, Chennai (Tamil Nadu) in March, 2007. Mr. Manoj Gadewar joined as a faculty (Asst Prof.) in Dept. of Pharmacology, Sri Kakatiya Institute of Pharmaceutical Sciences, Warangal (Telangana) and worked from Feb 2007- July 2012.

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