Screening of Leaf Extracts of *Amaranthus tricolor* Linn. for Antidiabetic Activities

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By

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(2013)

CERTIFICATE

*This is to certify that the thesis entitled "**Screening of Leaf Extracts of Amaranthus tricolor Linn. For Antidiabetic Activities**" is a bonafide research work carried out by Miss Aruna Patricia Juliet Colaco under my guidance and supervision and that no part thereof has been presented for the award of any other degree.*

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DECLARATION

*In accordance with the university ordinance, I hereby declare that the thesis entitled "**Screening of Leaf Extracts of Amaranthus tricolor Linn. For Antidiabetic Activities**" is my own work and that it has not previously been submitted in any form for assessment to another University or institution for any other qualification.*

Goa University ***Aruna Patricia Juliet Colaco***

25th February, 2013 ***Research Scholar***

DEDICATION

Courage doesn't always roar. Sometimes courage is the quiet voice at the end of the day saying, 'I will try again tomorrow'.

– Mary Anne Radmacher

This thesis is dedicated to my ***Dad****, who has spent the* ***last 23 years*** *of his life* ***battling Diabetes.*** *My* ***Mum****, who put aside her own dreams to* ***support*** *him round the clock. Who stood* ***tall and strong*** *for us through* ***nights and days.*** ***Lynn****, whose* ***unconditional love*** *and* ***constant support*** *has kept us together and made it all possible.*

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Aruna

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LIST OF ABBREVIATIONS

LDL	Low Density Lipoprotein
FRAP	Ferric Reducing Antioxidant Power
DPPH	1,1-diphenyl-2-picrylhydrazyl
CVD	Cardiovascular Diseases
WHO	World Health Organization
FDA	U.S. Food and Drug Administration
TP C	Total Phenol Content
ROS	Reactive Oxygen Species
ATP	Adenosine Triphosphate
GC-GR	Glucocorticoids-Glucocorticoid Receptor
PEPCK	Phosphoenolpyruvate Carboxykinase
G-6-Pase	Glucose-6-Phosphatase
GC	Glucocorticoid
ACTH	Adrenocorticotropic Hormone
GH	Growth Hormone
IR	Insulin Receptor

GLUT	Glucose transporter
KATP	ATP-sensitive potassium channel
DM	Diabetes Mellitus
ADA	American Diabetes Association
PCOD	Polycystic ovarian disease
GDM	Gestational diabetes mellitus
TG	Triglyceride
HDL	High Density Lipoprotein
IGT	Impaired glucose tolerance
IFG	Impaired fasting glycaemia
DKA	Diabetic ketoacidosis
IEC	International Expert Committee
NDDG	National Diabetes Data Group
NIH	National Institute of Health
Hb	Hemoglobin
DPP- 4	Dipeptidyl peptidase-4
CKD	chronic kidney disease
IDDM	Insulin dependent diabetes mellitus

STZ	Streptozotocin
PPP	Pentose phosphate pathways
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced form
G6PD	Glucose 6 phosphate dehydrogenase
LDH	Lactate dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide
AST	Aspartate transaminase
ALT	Alanine transaminase
CCl_4	Carbon tetrachloride
AE	Aqueous Extract – Fresh sample
AD	Aqueous Extract – Dry sample
ME	Methanolic Extract – Normal
MS	Methanolic Extract –Soxhlate
PE	Petroleum Ether Extract
R.T.	Room temperature (25ºC)
DW	Distilled Water

BHT	Butylated Hydroxy Toluene
AAE	Ascorbic Acid Equivalent
TAE	Tannic Acid Equivalent
PPA	Porcine Pancreatic Amylase
FBG	Fasting Blood Glucose
OECD	Organisation for Economic Co-operation and Development
AOT425	Acute Oral Toxicity - 425
BW	Body Weight
VLDL	Very Low Density Lipoproteins
TS	Tyrode's Salts
GOD	Glucose Oxidase
POD	Peroxidase
TCA	Tricholoro-acetic acid
ELISA	Enzyme-linked immunosorbent assay
TMB	3, 3', 5, 5'-tetramethylbenzidine
WBC	White Blood Corpuscle
RBC	Red Blood Corpuscle

PCV	Packed Cell Volume
TLC	Thin Layer Chromatography
FTIR	Fourier transform infrared spectroscopy
NMR	Nuclear Magnetic Resonance

PREFACE

This thesis is submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology and comprises of research work carried out by the author under the guidance of Prof. P. V. Desai, Department of Zoology, Goa University from June 2007 to January 2013.

Diabetes Mellitus, be it Type 1 (insulin dependent), Type 2 (insulin independent) or gestational, is initially characterised by an abnormal rise in blood glucose level or "hyperglycemia". After an initial diagnosis people often assume DM to be a non-serious condition. In reality it is a chronic crippling killer disease which emotionally, psychologically, physically and financially affects not only the patient but the family as well. Also known to accelerate aging, DM reduces the patient's life expectancy on average by 20 years.

The development of hyperglycemia may be due to several reasons; failure to produce the hormone insulin, cellular insulin resistance or stress. Uncontrolled hyperglycemia puts the patient at risk for a host of complications that can affect nearly every organ in the body by causing damage to the blood vessels, nerves, or both. This ultimately leads to complications such as blindness, kidney failure, cardiovascular diseases and nerve damage where patients lose sensation in their feet, putting them at risk of amputation because of delayed wound healing.

Maintenance of normoglycemia in diabetic patients is the most important and most basic step in the treatment of DM which slows down the accelerated aging process, buying time and reducing infirmity due to diabetic complications. For centuries people across the globe have depended on plant resources for their medicinal properties. *Amaranthus tricolor* is a commonly consumed leafy vegetable which is popularly grown in India. This thesis focuses on the antidiabetic and antioxidant properties of the plant and its role in decreasing diabetic morbidity by normalizing blood sugar levels through various mechanisms.

The present investigation comprising of in vivo and in vitro studies, biochemical assays, spectral and chromatographic studies, is an attempt to elucidate the protective and antidiabetic effects of *Amaranthus tricolor* leaf extracts in alloxan diabetic rats. Through this thesis an effort is being made to add more information to the current knowledge on plant therapies for Diabetes Mellitus, especially *Amaranthus tricolor* as it is a commonly consumed and easily available plant which is abundantly cultivated during the non monsoon seasons.

Chapter 1

Introduction & Review of Literature

1.1 Blood Glucose and its importance in the body

The glucose molecule is a simple, energy-rich monosaccharide, which represents an essential primary biological energy source, enabling the generation of ATP (adenosine triphosphate) following glycolysis. ATP powers the millions of biochemical reactions occurring in the human body (Cooper, 2000). The amount of ATP required continually fluctuates depending on the sudden physiological demands.

Without a constant supply of glucose none of the metabolic reactions would proceed. Hence the body regulates the availability of glucose in order to maximise its energy making potential (Wang et. al., 2006).

The blood sugar concentration or blood glucose level is the amount of glucose present in the blood of the individual. The body tightly regulates blood glucose levels as a part of metabolic homeostasis. These interactions within the body facilitate compensatory changes, to maintain balance or return systems to functioning within a normal range, which in turn are supportive of physical and psychological functioning. This process is essential to the survival of an individual and to a species.

An inability to maintain glucose homeostasis leads to a glucose imbalance which may lead to a hypoglycaemic or hyperglycaemic state. This hyperglycaemic state is called diabetes which may lead to morbidity. Medical interventions and a holistic approach can help restore glucose homeostasis and possibly prevent permanent damage to the organs.

1.2 Blood glucose homeostasis

Blood glucose homeostasis is the balanced maintenance of hepatic glucose release, transport and peripheral glucose disposal, which is achieved through a finely-tuned, coordinated network of metabolic, signalling and regulatory pathways. Blood glucose is maintained within a narrow physiologic range through a complex balance of dietary intake, de novo synthesis, glycogen storage and release, and insulin-dependent and insulin independent glucose uptake by tissues (Baum et. al., 2006). Glucose entry into the circulation is influenced by the rates of digestion and absorption of carbohydrates as well as the activity of the hepatic gluconeogenic and glycogenolytic pathways (Klover and Mooney, 2004; Postic et al., 2004). A critical factor in the clearance of glucose from the blood is insulin, a hormone produced by the pancreatic beta cells (Linder, 1991).

The liver regulates the rate of glucose appearance into the blood by balancing absorption of exogenous dietary glucose with endogenous production of glucose from gluconeogenesis and glycogen breakdown (Jahoor et. al., 1990; Pascual et. al., 1997; Balasubramanyam et. al., 1999; Christiansen et. al., 2000). The use of blood glucose by peripheral tissues occurs through insulin-dependent and insulin-independent transporters (Furtado et. al., 2002; Wood and Trayhurn, 2003).

1.3 Need for glucose homeostasis

Maintenance of blood glucose homeostasis is of utmost importance to human survival. Human plasma glucose concentrations are maintained within a relatively

narrow range throughout the day (usually between 55 and 165 mg/dl) despite wide fluctuations in the delivery (e.g., meals) and removal (e.g., exercise) of glucose from the circulation (Alsahli and Gerich, 2010). This is accomplished by a tightly linked balance between glucose production and glucose utilization regulated by complex homeostatic mechanisms. The brain requires 25% of the glucose circulating in the blood. If for no other reason the needs of the brain for this oxidizable glucose require the human body to closely regulate the level of glucose in the blood. An elevation or reduction, in blood glucose level triggers hormonal responses that re-establish glucose homeostasis.

Low blood glucose triggers release of glucagon from pancreatic alpha cells thereby stimulating the release of glucose into circulation. High blood glucose triggers release of insulin from pancreatic beta cells, prompting the uptake of glucose from circulation by the hepatic and extra hepatic tissues for the purpose of storage, in the form of glycogen. The liver is the main site for glucose storage in the form of glycogen and glucose synthesis via gluconeogenesis. The liver provides this energy metabolite to maintain blood glucose level in times of need and to provide glucose to extrahepatic tissues such as the brain, which uses approximately 25% of total body glucose.

In a state of energy deprivation, Hepatic GC-GR (Glucocorticoids-Glucocorticoid Receptor) signalling plays a critical role in maintaining blood glucose level, by directly controlling rate limiting enzymes of gluconeogenesis such as Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-Phosphatase (G6-Pase)

(Hanson and Reshef, 1997; Van Schaftingen and Gerin, 2002; Opherk et al., 2004). In turn, GC (Glucocorticoid) signaling impair glucose uptake in peripheral tissues, such as skeletal muscle and adipose tissues. Thus, glucocorticoids oppose insulin action, which limits glucose production through the inhibition of hepatic glycogenolysis and gluconeogenesis, and stimulates glucose uptake, storage, and utilization by other tissues (Andrews and Walker, 1999). In response to hepatic glucocorticoid receptor deficiency and the concomitant impairment of the liver to counteract low energy levels by gluconeogenesis, a compensatory increase in glucagon (Opherk et al., 2004) and a parallel decrease in insulin were observed (Opherk et al., 2004; Mueller et al., 2011). Elevated hepatic gluconeogenesis is a major contributor to hyperglycemia in type 2 diabetes. Growth hormone has both chronic and acute effects on glucose metabolism. The acute effects are designated as temporary insulin-like effects, and their physiological significance is not clear. The chronic effects of growth hormone (GH) oppose insulin action on glucose metabolism (Davidson, 1987).

Adrenocorticotropic hormone (ACTH) and growth hormone (GH) released from the pituitary, causes an increase in blood glucose level by inhibiting glucose uptake by extrahepatic tissues (Mueller et. al., 2012). Glucocorticoids also act to increase blood glucose levels by inhibiting glucose uptake. Cortisol is secreted by the adrenal cortex in response to increased ACTH levels (Ehrhart-Bornstein et. al., 1996). Stress and low level of blood glucocorticoids results in ACTH release. The primary functions of cortisol are to elevate blood glucose through hepatic gluconeogenesis, suppress the immune system, and aid in fat, protein and carbohydrate metabolism (Hoehn and Marieb, 2010). The adrenal medullary hormone, epinephrine, stimulates the

production of glucose by activation of glycogenolysis in response to stress. Cortisol plays an important role in glycogenolysis, the breaking down of glycogen to glucose-1-phosphate and glucose in liver and muscle tissue. Glycogenolysis is stimulated by epinephrine and/or norepinephrine, however cortisol facilitates the activation of glycogen phosphorylase, which is essential for the effects of epinephrine on glycogenolysis (Coderre et. al., 1991; Martin and Crump, 2003).

Certain tissues like the nervous system, red blood corpuscles (RBC), kidney and retina are very sensitive to changes in glucose levels. Use of blood glucose as a fuel is relatively constant for tissues including brain. These tissues account for obligatory glucose usage (Cahill, 1970). An excess or deficit of blood glucose for more than a few hours can result in the loss of consciousness and brain damage (Cryer, 2008).

Both hypoglycemia and hyperglycemia, are to be avoided in order to protect the brain, prevent cognitive dysfunction (Rosenthal et. al., 2001; Cox et. al., 2005) and functional brain failure (Cryer 2007). Because of limited availability of ketone bodies and amino acids, and the limited transport of free fatty acids across the blood–brain barrier, glucose is the exclusive source of energy for the brain, except under conditions of prolonged fasting (Owen et. al., 1967). Under the prolonged fasting state, ketone bodies increase several fold so that these may be used as an alternative fuel.

All activities in the brain rely heavily on glucose for energy (Siesjo, 1978; Weiss, 1986; Laughlin, 2004). Each brain region can carry out its given function through the metabolization of glucose from the bloodstream (Reivich and Alavi, 1983; McNay, et. al., 2001). It is generally thought that the brain cannot store or produce glucose and therefore requires a continuous supply of glucose from the circulation. Recent studies in animals, however, suggest that the brain contains negligible quantities of glycogen, localized almost exclusively to astrocytes (Phelps, 1972; Koizumi, 1974; Pfeiffer-Guglielmi et. al., 2003).

At physiological plasma glucose levels, phosphorylation of glucose is rate limiting for its utilization. However, because of the kinetics of glucose transfer across the blood–brain barrier, uptake becomes rate limiting as plasma glucose concentrations decrease below the normal range. Other tissues, most notably skeletal muscle, utilize a mixture of fuels (carbohydrates, lipids, and protein) determined by physiological conditions and substrate availability. Glucose use by skeletal muscle is increased during high intensity exercise (Holloszy and Booth, 1976). Consumption of dietary glucose in excess of immediate energy needs results in its storage as glycogen in liver or skeletal muscle or its conversion into fat via endogenous fatty acid synthesis. Consequently, maintenance of the plasma glucose concentration above some critical level is essential to the survival of the brain (Clutter et. al., 1990) and thus the organism (Wei et. al., 2000).

1.4 Digestion and absorption of carbohydrates

The enzyme α-amylase hydrolyses dietary polysaccharides to produce oligosaccharides and disaccharides in the gastrointestinal tract. Further the enzyme α-glucosidase hydrolyses the resulting disaccharides to produce glucose and other monosaccharides as shown in Fig. 1.

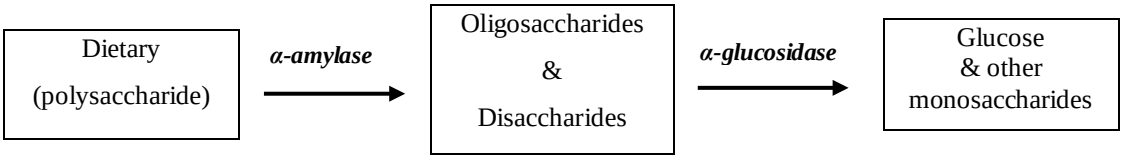


Fig 1: Breakdown of polysaccharides by α-amylase and α-glucosidase enzymes

Glucose and other monosaccharides (fructose and galactose) resulting from digestion of carbohydrates are absorbed through the small intestine into the hepatic portal vein. This results in elevation of the postprandial blood glucose level.

1.5 The role of pancreas in glucose metabolism

The pancreas plays a primary role in the metabolism of glucose by secreting the hormones, insulin and glucagon. Elevated postprandial blood glucose level stimulates pancreatic beta cells to secrete insulin which then facilitates the entry of glucose into the muscle and adipose tissues, thereby clearing excess glucose from the circulation (Sonksen and Sonksen, 2000). Insulin also stimulates the processes of glycolysis (catabolism of glucose) and glycogenesis (synthesis of glycogen from glucose) and

inhibits both hepatic gluconeogenesis and glycogenolysis thereby reducing the hepatic glucose output (Ahmed, 2004). The actions of insulin are opposed by glucagon, a hormone produced by the pancreatic alpha cells when the blood glucose level tends to be low. Glucagon inhibits glycogenesis and stimulates both gluconeogenesis and glycogenolysis which releases blood glucose into the blood circulation thereby raising the blood glucose level.

1.6 Insulin and its metabolic actions

The insulin molecule consists of two polypeptide chains connected by two disulfide bridges, with a third disulfide bridge linking parts of one chain. This two-chain structure has evidently been present throughout evolution, but major variations in the amino acid sequences are observed between species. Various mammalian insulins and non mammalian insulins usually have similar potencies in all mammals, including humans (Turner and Johnson, 1973)

Insulin apparently exerts its glucose-lowering effects by stimulating glucose uptake in tissues; skeletal muscle, suppressing fatty acid release from fat (adipose) tissue, and inhibiting production of glucose by the liver (Flakoll et. al., 2004). Muscle, liver, and fat, therefore, are widely viewed as the principal insulin-sensitive tissues in the body. Insulin is transported across the blood-brain barrier and is effective in suppressing food intake when given directly into the brain, and that insulin receptors are concentrated in brain areas involved in energy homeostasis.

The hypothalamus and brain stem are important centres involved in the regulation of feeding and monitoring of glucose status (Pénicaud et. al., 2002). Making the analogy with the beta cell of the islet of Langerhans, it has been proposed that glucose sensing could be assured in some cells of the brain by proteins such as glucose transporter 2, glucokinase and the ATP-dependent potassium channel. Furthermore, some pathological conditions such as diabetes and obesity have been shown to alter this glucose sensing system (Pénicaud et. al., 2002).

Metabolic actions of insulin results from its interaction with the insulin receptor (IR) found in all insulin responsive target cells (liver, muscle and adipose tissue) (Goldfine, 1987; James et. al., 1988; White and Kahn, 1994). Increased glycogen synthesis, glucose transport, and lipogenesis, and decreased gluconeogenesis, glycogenolysis, and lipolysis is brought about by normal insulin action (Klover and Mooner, 2003; Postic et. al., 2004).

1.7 Physiology of glucoregulation

As glucose enters the blood from the intestines, the blood glucose level begins to rise, triggering the release of insulin from the ß-cells of the Islets of Langerhans in the pancreas, into the blood circulation and is transported to all of the tissues of the body. In these tissues, insulin stimulates the cellular uptake of glucose present in the blood. As the body tissues take up glucose, the level of glucose in the blood will decrease. When this occurs, the release of insulin by the pancreas is halted.

Glucose is added to the blood by:

- Absorption from the intestine
- Breakdown of liver glycogen to glucose
- Gluconeogenesis

Glucose is removed from the blood by:

- Synthesis of fat (triglycerides)
- Conversion to liver glycogen
- Conversion to muscle glycogen

A balance of these processes keeps the blood sugar level in normal limits (70-100 mg/dl) throughout the day (American Diabetes Association, 2000).

Liver plays an important role in the;

- Uptake of glucose from blood
- Conversion of glucose to glycogen
- Release of glucose from glycogen
- Conversion of pyruvate to glucose

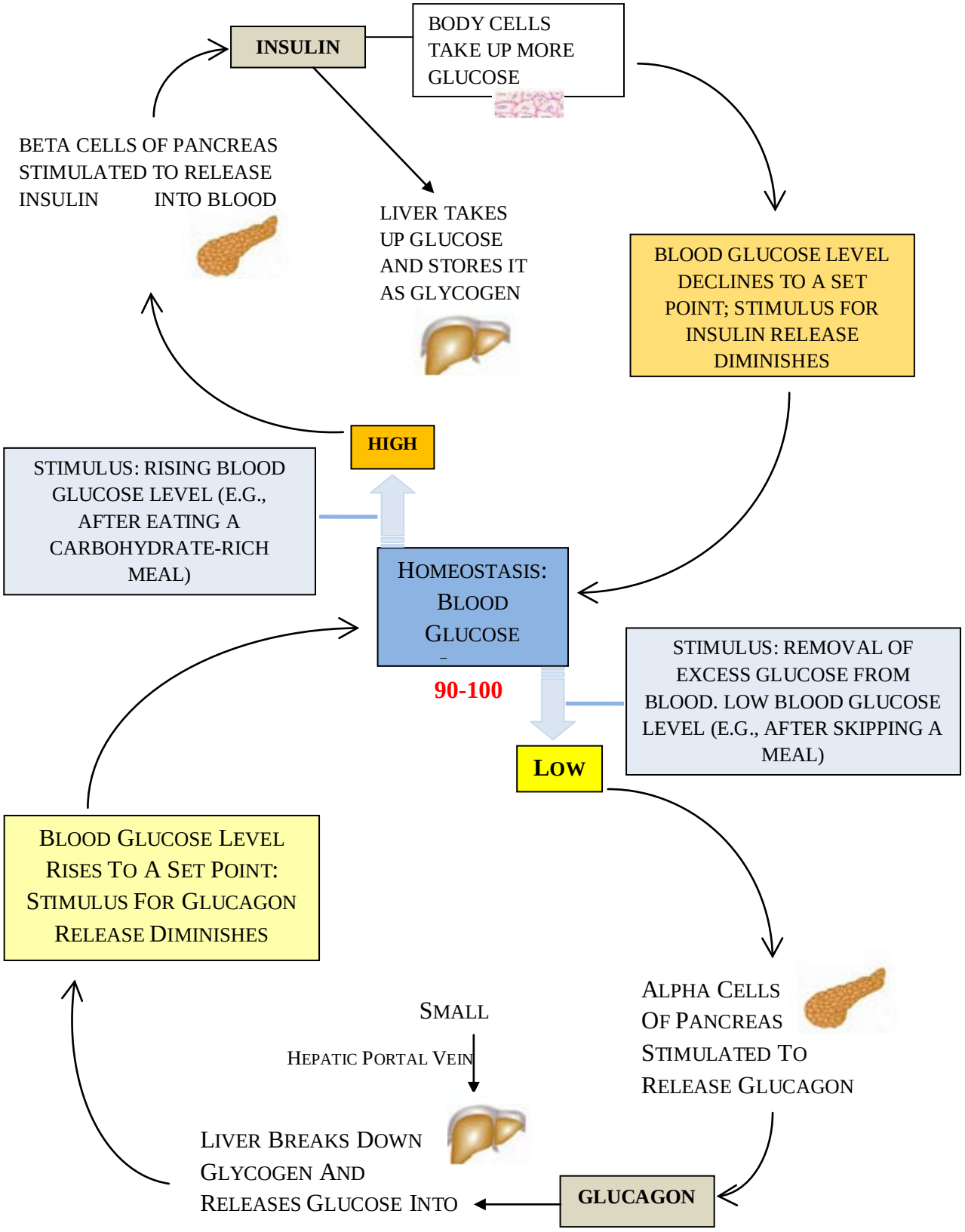
Insulin is a primary regulatory signal in animals, which facilitates the uptake of glucose from the circulation, by the cells. Some cells store glucose internally in the form of glycogen, while others take in and hold in lipids. In many cases insulin also

controls cellular electrolyte balances and amino acid uptake. Its absence turns off glucose uptake into cells, reverses electrolyte adjustments, begins glycogen breakdown and glucose release into the circulation by some cells, begins lipid release from lipid storage cells, etc (Guyton and Hall, 2003).

The blood glucose level in circulation is the most important signal to the insulin-producing cells. The hormone glucagon has an effect opposite to that of insulin, forcing the conversion of glycogen in liver cells to glucose, which is then released into the blood. Muscle cells lack the ability to export glucose into the blood. The release of glucagon is precipitated by low levels of blood glucose. Other hormones, notably growth hormone, cortisol, and certain catecholamines (such as epinepherine) have glucoregulatory actions similar to glucagon (Gerich et. al., 1979; Hoelzer et. al.,1986).

Insulin, a hormone that normally coordinates the disposal of glucose after a meal, becomes less effective in obesity that results in high blood-glucose concentration which is the hallmark of diabetes. The liver has a central role in glucose homeostasis because it extracts glucose from the bloodstream in times of plenty, and synthesizes glucose in times of need, thus buffering the body from extremes in glucose concentration (Nussey and Whitehead, 2001). The liver identifies these different energy states through changes in the blood insulin concentration. Insulin directly inhibits glucose production by binding to its receptor in the liver (Layden et. al., 2010).

Fig.2: The role of the pancreas in glucose homeostasis **(Guyton and Hall, 2003)**



Glucose is used as an efficient source of energy under normal conditions. Under fasting conditions triglycerides are converted to circulating fatty acids, and are further broken down in a process known as fatty-acid oxidation. In the fasting state, the liver maintains normal circulating levels of glucose (which is essential for brain function) by synthesizing glucose anew, in a process known as gluconeogenesis.

The capacity of the liver to synthesize glucose and burn fat is governed by changes in circulating levels of the hormones insulin and glucagon; that allow the liver cell to change between feeding and fasting metabolism (David and Wasserman, 2008). In insulin-resistant states, insulin is prevented from regulating glucose and fat metabolism due to the damaged phosphorylation of certain proteins that respond to insulin (Saltiel and Kahn, 2001). As a consequence, insulin-resistant individuals exhibit hyperglycaemia (high blood glucose), partly because of elevated gluconeogenesis in the liver.

Despite this inability to inhibit glucose production in the diabetic state, insulin still seems to be capable of shutting off the switch that normally promotes fat burning (fatty-acid oxidation) during fasting. This phenomenon, known as mixed insulin resistance, implies that the insulin signal is transmitted preferably to the fatty-acid-oxidation switch rather than the glucose switch. The unfortunate consequence being that insulin-resistant individuals not only turn hyperglycaemic but also tend to accumulate triglycerides in the liver, rather than breaking them down (White, 1998; Spiegelman and Heinrich, 2004).

1.8 Diabetes Mellitus (DM)

1.8.1 Definition

Diabetes mellitus is a group of diverse metabolic disorders characterized by a loss of glucose homeostasis resulting in hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (W.H.O., 1999; Pietropolo, 2001; DeFronzo, 2004) and distinctive complications that include premature atherosclerotic cardiovascular disease and small vessel disease manifested as retinopathy with potential loss of vision; nephropathy leading to renal failure; and peripheral neuropathy with a high risk of foot ulcers and amputations (National Diabetes Data Group, 1979).

Without enough insulin, body tissues, in particular, the liver, muscle and adipose tissues fail to take and utilize glucose from the blood circulation resulting in an elevated blood glucose level known as hyperglycemia. If the hyperglycaemic condition persists over a long period of time, this results in long-term damage of vital organs such as the kidneys, eyes, nerves, heart and blood vessels. Complications in some of these organs can even lead to death (Hirch, 1995; Cade, 2008; Huang et. al., 2011).

Insulin is a hormone secreted by the β-cells of the islets of Langerhans, which helps regulate the circulating blood glucose level in the body. It initiates the uptake and storage of glucose by most of the tissues in the body especially the liver, musculature and fat tissues (Roussel, 1998). Under the euglycemic condition, insulin markedly

stimulates glucose uptake in muscle but not liver (Sonksen and Sonksen, 2000). In contrast, hyperglycemia combined with hyperinsulinemia substantially increases glucose uptake in both tissues (Basu et. al., 2000).

1.8.2 Factors Causing Diabetes

Diabetes mellitus is considered to be a multi-factorial disease. Hereditary is known to play a major role in the development of diabetes by either increasing the susceptibility of the beta cells to viruses, or by favouring the development of auto immune antibodies against the beta cells, thus leading to their destruction (Achenbach et. al., 2005), or a simple hereditary tendency for beta cell degeneration.

Obesity also plays a role in the development of clinical diabetes. Under the influence of obesity the β-cells of the islets of Langerhans become less responsive to stimulation by increased blood glucose; therefore the blood insulin levels do not increase when needed. Another reason is that obesity decreases the number of insulin receptors in the insulin target cells throughout the body, thus making the amount of insulin that is available even less effective in promoting its usual metabolic effects (Kahn and Flier, 2000).

Humans are said to be biologically adapted to their ancestral food habits and environment, where in expenditure of energy was required to obtain food (Bellisari, 2008). The modern developed world has a surplus of easily accessible, ready to eat,

inexpensive food which is rich in carbohydrates and saturated fats. Indians are at a high risk of developing type 2 diabetes, due to a strong genetic predisposition. (Radha and Mohan, 2007) Urbanization is causing more and more people to incorporate unhealthy fast food diets into their stressful lives. A lack of regular exercise due to time constraints also contributes to the rising rates of obesity, type 2 diabetes and the resultant complications such as cardiovascular diseases (Diamond, 2011).

Recent clinical studies have shown that oxidative stress mechanisms in diabetes are triggered by acute glucose swings in addition to chronic hyperglycemia (Robertson et. al., 2004), demonstrating the importance for therapeutic interventions during acute and sustained hyperglycemic episodes (Maiese et. al., 2008, Ramachandran et. al., 2010). Numerous experimental and clinical observations have indicated that hyperglycemia may directly or indirectly contribute to excess formation of free radicals (Ceriello and Testa, 2009).

1.8.3 Classification of Diabetes Mellitus

Diabetes mellitus is a complex and heterogeneous disorder with diverse etiologic mechanisms; therefore, any given classification is arbitrary (cannot capture the disease in its entirety) but nevertheless useful. There are two major classes of diabetes mellitus: type 1, which accounts for 5–10% of cases, and type 2, which accounts for 90–95%. Gestational diabetes and other forms related to pancreatic disease, endocrine disorders, drugs, and genetic mutations make up a small percentage of all the cases (Chamany and Tabaei, 2010).

1.8.3.1 Type 1 diabetes

Type 1 diabetes (previously known as insulin-dependent, juvenile or childhood-onset) is characterized by complete insulin deficiency and requires daily administration of insulin for the survival of the individual.

The exact cause of type 1 diabetes is not known and the secret to its prevention is still not found despite decades of research devoted to the same topic. Race, age, family history, environment, and genetics - all play a role in its development. Environmental factors include early and prolonged exposure to cow's milk, Coxsackie B virus, human cytomegalovirus, and measles have been considered to increase the risk of T1DM (Ekoé et. al., 2008 Tom et. al., 2011). Association of HLA class antigens, such as the DR and DQ types, with increased risk of type 1 diabetes has been demonstrated in a variety of populations around the world (Dorman et. al., 1995).

It results from a progressive cellular-mediated autoimmune destruction of the pancreatic β-cells that leads to complete insulin deficiency (Atkinson and McLaren, 1994). The rate of β-cell destruction is rapid in the majority, particularly in infants and children, but may be a slower process in adults (American Diabetes Association 2010). Sudden β-cell failure causes ketoacidosis, which is often the first manifestation of type 1 diabetes. But more commonly a sluggish onset, with severe hyperglycemia and/or ketoacidosis is found, only in the presence of stress conditions or severe infections (Atkinson and McLaren, 1994).

The slow rate of β-cell destruction in adults may mask the presentation making it difficult to distinguish type 1 diabetes from type 2 diabetes (Wilkin, 2001; Rosenbloom, 2003). A C-peptide assay, which measures endogenous insulin production and a positive pancreatic autoantibody helps establish the diagnosis and distinguishes type 1 from type 2 diabetes (Pietropaolo et. al., 2000). This type of diabetes is known as Latent Autoimmune Diabetes in Adults (LADA), which is more common in the Caucasian population. The presence of autoantibodies indicates the need for early insulin treatment. (Zimmet et. al., 1994; Zimmet et. al., 1999)

1.8.3.2 Type 2 diabetes

T2DM (formerly called non-insulin-dependent or adult-onset), a complex metabolic disease with a multifactorial etiology under the influence of genetic and environmental factors, accounts for 90% of all diabetic cases. The most prominent of these environmental factors are excessive calorie intake and a sedentary lifestyle, leading to obesity which is a potent risk factor for Type 2 Diabetes (Swinburn et. al., 2004). Race, age, family history and co-morbid conditions such as polycystic ovarian disease (PCOD) or Gestational diabetes mellitus (GDM) are important risk factors for developing insulin resistance and type 2 diabetes (Alberti et. al., 2004). It is often associated with central or visceral obesity, cardiovascular risk factors such as hypertension, and abnormalities of lipoprotein metabolism with the characteristic dyslipidemia of elevated triglycerides and low high-density lipoprotein cholesterol (Henry, 2001). The symptoms may be similar to those of Type 1 diabetes, but are often less severe. As a result, the disease may be diagnosed once complications arise,

which may be several years after onset of the disease. Until recently type 2 diabetes was seen only in adults, but it is also now seen to occur in children.

Type 2 diabetes is characterized by complex metabolic derangements, with two main metabolic defects: insulin resistance, a decreased response of peripheral tissues to insulin and β-cell dysfunction that is manifested as inadequate insulin secretion or relative insulin deficiency in the face of insulin resistance and hyperglycemia (D'Adamo and Caprio, 2011). Thus, insulin secretion is defective in these individuals and insufficient to compensate for insulin resistance. Peripheral (muscle and fat) and hepatic insulin resistance precedes the development of hyperglycemia (Takahashi et. al., 1993; Vuguin et. al., 2004).

Inorder to compensate the insulin-resistant state, β-cells initially increase basal and postprandial insulin secretion (DeFronzo, 1997). In due course, the β-cells capability to compensate diminishes and do not respond appropriately to the impairment in glucose disposal. Insulin resistance and loss of β-cell function, together eventually lead to the deterioration of glucose homeostasis and to the development of hyperglycemia (Fernández et. al., 2001). At a later stage fasting hyperglycemia develops, secondary to the excessive hepatic glucose production. The insulin secreting capacity in these patients is often enough to prevent ketosis and ketoacidosis, but becomes evident during periods of severe stress or acute medical illness. This type is beginning to become more common in both developed and developing countries, afflicting younger individuals, and, particularly, ethnic minorities.

1.8.3.3 Gestational Diabetes Mellitus (GDM)

This form of diabetes complicates ~4% of all pregnancies, but the true prevalence may range from 1 to 14% depending on the population studied. The American Diabetes Association (2009) has stated that GDM now represents nearly 90% of all diabetes during pregnancy. Due to its high incidence; the previous recommendation of screening only high-risk patients has now been changed to the early screening of all pregnant women.

GDM is often diagnosed through prenatal screening, rather than reported symptoms. The symptoms of GDM are very similar to T2DM. Evaluation for GDM should be done early in pregnancy, particularly in women at high risk (marked obesity, personal history of GDM, glycosuria, or a strong family history of diabetes). Early screening and diagnosis of GDM is essential as timely monitoring and initiation of therapy will reduce perinatal morbidity and mortality (Magee et. al., 1993; Langer et. al., 1994). Preliminary results have shown a direct correlation between high blood sugar and poor outcome for both mother and baby without a glycemic threshold. Mothers diagnosed with GDM during pregnancy have to be assessed regularly post delivery, as they are at a risk of developing diabetes later in life. (Perkins et. al., 2007). Even with a negative postpartum test these patients need further monitoring as they remain at an increased risk of developing type 2 diabetes and cardiovascular disease (Magee et. al., 1993; Langer et. al., 1994).

1.8.3.4 Pre-Diabetes Mellitus

Impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG)

Impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG) are intermediate conditions in the transition from normality and diabetes. An individual whose glucose level does not meet the criteria for diabetes but is higher than normal is termed as a pre-diabetic. These kind of individuals normally have impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) and are at a high risk of progressing to the type 2 diabetic state, particularly when therapeutic interventions such as lifestyle changes or medications are not provided upon diagnosis, (Unwin et. al., 2002) although this is not inevitable.

1.9 Pathologic features of Diabetes Mellitus

The pathologic features of diabetes mellitus can be attributed to the following reasons:

1) A decreased utilization of glucose by the body cells, with a resultant increase in blood glucose concentration to as high as 300 to 1200 mg/dl.

2) An increased fat metabolism from the fat storage areas, causing abnormal fat metabolism as well as deposition of cholesterol in arterial walls, leading to atherosclerosis.

3) A depletion of protein in the tissues of the body.

4) Loss of glucose through the urine in the diabetic condition.

5) A dehydrating effect of elevated blood glucose levels in the diabetic condition.

All diabetic symptoms are due to the pathological lack of the hormone insulin. *Polyuria* is the excessive elimination of urine, due to the osmotic diuretic effect of glucose in the kidney tubules. *Polydipsia* is excessive drinking of water due to uncontrolled thirst, which is due to dehydration resulting from polyuria. Inspite of a tendency toward *polyphagia* or excessive eating, considerable amount of *weight loss* is seen due to the failure of glucose (and protein) utilization by the body. *Asthenia* which is a general lack of energy caused mainly due to loss of body protein (Patton and Fuchs, 1989).

The symptoms of type 2 diabetes which tend to develop gradually over time due to complications include:

- Blurred vision
- Numbness or tingling in the hands or feet
- Slow healing of wounds and sores

In the early stages of the disease, symptoms may be non-existent, which is very unfortunate because organ damage sets in when hyperglycemia begins. Hence it is very important for people who may be at high risk of diabetes to get their blood sugar levels checked regularly where an early diagnosis can be accomplished through inexpensive blood testing.

1.10 Complications of Diabetes Mellitus

The complications of diabetes mellitus can involve multiple systems throughout the body that are susceptible to the detrimental effects of oxidative stress and apoptotic cell injury (Maiese et al., 2007). This is mainly due to the increased glucose concentration in the circulating blood. Severe long term abnormalities can result such as eye complications, heart disease, kidney and foot problems if blood sugar levels are poorly controlled. These complications are of two types microvascular complications that include retinopathy, nephropathy, neuropathy and peripheral vascular disorders and macrovascular complications that include cardiovascular and cerebrovascular disorders (American Diabetes Association, 2010).

Diabetes increases the risk of heart disease and stroke. 50% of the diabetic population die of CVD (primarily heart disease and stroke). Neuropathological conditions in the extremities, combined with reduced blood circulation, increases the chances of diabetic ulcers and ultimately gangrene and amputation. Diabetic retinopathy is the main cause of blindness, and occurs due to long-term accumulated damage of the small retinal blood vessels. in the retina. After 15 years of diabetes, approximately 2% of people become blind, and about 10% develop severe visual impairment (W.H.O., 2012).

Diabetes is among the leading causes of kidney failure. Diabetic neuropathy affects upto 50% of people with diabetes. Although many different problems can occur as a result of diabetic neuropathy, common symptoms are tingling, pain, numbness, or

weakness in the feet and hands (Boulton, 2005). The overall risk of dying, amongst diabetic people is at least double the risk of their peers without diabetes. Over time, diabetes unquestionably causes permanent damage the vital organs of the body such as the heart, blood vessels, eyes, kidneys, and nerves.

Table 1 : Neuropathic symptoms and signs in diabetes mellitus

(Windebank and Feldman, 2001)

Sensory	▪ Negative symptoms: numbness, deadness, "cotton wool feeling," "thick," "less sensitive," loss of dexterity, painless injuries, ulcers. ▪ Positive symptoms: burning, prickling, pain, hypersensitivity to light touch, stabbing, electric shock-like, tearing, tight, band-like.
Motor	▪ Proximal weakness: difficulty rising from a seated position, difficulty climbing stairs, falls secondary to knees "giving out," difficulty raising arms above the shoulders (as in combing or shampooing hair). ▪Distal weakness: difficulty turning keys or opening jars, impaired fine hand coordination, toe scuffing, tripping, foot slapping.

1.10.2 Acidosis and Coma

Diabetic ketoacidosis (DKA) is a state of metabolic decompensation in which insulin deficiency (relative or absolute) causes both hyperglycemia and excess production of ketoacids, resulting in metabolic acidosis (Kitabchi et. al., 2009). The osmotic diuresis of hyperglycemia causes dehydration, which aggravates the metabolic acidosis. The

osmotic diuresis of hyperglycemia causes dehydration, which aggravates the metabolic acidosis. Hyperlipidemia, hypophosphatemia, brain and pulmonary edema, pancreatitis are complications of DKA. High mortality in adult patients with DKA is due to multiple organ failure (cardiac, renal, hepatic, and pulmonary). Elevated pancreatic enzymes, such as amylase and lipase, are correlated with the degree of hyperglycemia, acidemia, and dehydration (Rizvi, 2003).

1.11 Complications of Diabetes Therapy

Insulin therapy may increase patient's risk of acquiring viral hepatitis because of the exposure to needles. Adhering to good infection-control practices should significantly reduce this risk. There is a rare association between the use of oral hypoglycemics and hepatic injury, but Bloodworth and Hamwi (1961) reported that sulfonylureas can cause chronic hepatitis with necro-inflammatory changes, the mechanism of which is not known. Chlorpropamide appears to be the most hepatotoxic of these drugs. Cholestatic hepatitis occurs in 0.5% of people on the drug. Jaundice gradually develops over 2–5 weeks and resolves in virtually all patients when the drug is stopped (Levinthal and Tavill, 1999). Although very uncommon, acetohexamide and glyburide can cause acute hepatocellular necrosis, and fatalities have been reported.

At least two cases of granulomatous hepatitis thought secondary to glyburide have been reported in the literature (Saw et., al., 1996).

The biguanides, such as metformin hydrochloride, have not been associated with liver injury, though lactic acidosis can be associated with its use in the treatment of

diabetes, but is reported to occur occasionally and usually in patients with major contraindications to the drug (Silvestre et. al., 2007). Patients with a predisposition toward "Chronic liver disease" tend to develop lactic acidosis, possibly due to a reduced ability of the liver to clear lactate. It is therefore listed as a contraindication (Sulkin et. al., 1997). Troglitazone (Rezulin), an oral antihyperglycemic agent acts primarily by decreasing insulin resistance. The drug package insert carries a warning that severe idiosyncratic hepatocellular injury, usually reversible but possibly leading to death or liver transplantation, has been reported in patients using the medication, usually during the early months of therapy (Jaeschke, 2007).

In patients with T1DM or T2DM, hypoglycemia is a frequent complication, due to the use of exogenous insulin. Because of its possible detrimental effects on the central nervous system, hypoglycemia is considered to be the main limiting factor for achieving near-normal glycemic control (Cryer, 2002). This condition is to be avoided inorder to protect the brain and prevent cognitive dysfunction. Some risk factors for severe hypoglycemia due to diabetes complications include renal insufficiency, gastroparesis which causes unpredictable and delayed food absorption, poor vision, and (rarely) insulin antibodies. The symptoms of hypoglycemia include; sweating, trembling, dizziness, hunger, confusion, seizures and loss of consciousness (if hypoglycemia is not recognized and corrected) (Lin et. al., 2010).

1.12 Diagnostic Criteria

The presence of characteristic symptoms such as thirst, polyuria, unexplained weight

loss, blurring of vision, recurrent infections and, in more severe cases, pre-coma compels an individual to visit the doctor for a diagnosis. These symptoms are normally seen in symptomatic individuals, making the diagnosis of the disease simpler. But often, the person may be totally asymptomatic and mild hyperglycemia may persist for years with gradual tissue damage developing (American Diabetes Association, 2008).

The International Expert Committee (IEC) appointed by the American Diabetes Association has defined normal fasting plasma glucose level as being no higher than 100 mg/dl. The IEC has recently introduced the concept of impaired fasting glucose levels with plasma glucose levels between 100mg/dl and 125 mg/dl. This represents a scale, where the lower the glucose level the better. The National Diabetes Data Group (NDDG) from the National Institute of Health (NIH), USA has proposed the use of glycosylated hemoglobin (HbA1c) levels for diagnostic purposes, because of the discrepancies between fasting plasma glucose and postprandial values, and also as HbA1c gives an average estimation of the blood glucose level of the individual over a period of 3 to 4 months (Sikaris, 2009).

Diagnosis of diabetes at an earlier stage is important in preventing diabetes related complications. The pathology tests commonly requested by the consulting doctors to diagnose diabetes are fasting blood glucose, postprandial blood glucose, oral glucose tolerance testing and HbA1c (Godkar and Godkar, 2000). The diagnosis of diabetes is established by noting elevation of blood glucose by any one of three criteria given

below. In the symptomatic individual this is easier but in asymptomatic people (or in the absence of unequivocal hyperglycemia) once an abnormal test has been found it must be confirmed by a further test involving any one of the three methods.

1.12.1 Random Blood Glucose

A random or casual blood glucose concentration greater than 200 mg/dl, with classical signs and symptoms of diabetes, may suggest the presence of hyperglycemia.

1.12.2 Fasting Blood Glucose

The fasting blood sugar level in the early morning is normally 80 to 90 mg/dl, and 110 mg/dl is generally considered to be the upper limit of normal. The fasting blood glucose concentration of a diabetic individual is almost always above 100 mg/dl and often above 140 mg/dl. A fasting blood level above this value often indicates diabetes mellitus or, less commonly, either pituitary diabetes or adrenal diabetes.

1.12.3 Oral Glucose Tolerance Test

The glucose tolerance test is a standard test used to determine whether or not the body produces sufficient insulin to promote the uptake of glucose from the blood after a high glucose load. This test is administered after an overnight fast which ensures that the level of blood glucose is low enough, to avoid the release of more than trivial amounts of insulin.

When a normal fasting person ingests 1 gm glucose/kg body weight, the blood glucose level rises from approximately 90 mg/dl to 120 to 140 mg/dl and falls back to below normal within about 2 hours. On ingestion of glucose a pre-diabetic or a diabetic individual, a much greater than normal rise in blood glucose level occurs and a gradual reduction to the control value is seen only after 4 to 6 hours; furthermore it fails to fall below the control level, depending on the degree of the diabetic condition. This slow fall of the curve and its failure to fall below the control level illustrates that the normal increase in insulin secretion following glucose ingestion does not occur in the diabetic person, and a diagnosis of diabetes mellitus can usually be definitely established on the basis of such a curve.

1.12.4 Glycosylated Haemoglobin (Hb1Ac)

Glycosylated hemoglobin (HbA1c) is the best measure of long-term glycemic control, since it represents the average blood glucose levels over several months (Thomas and Elliott, 2009). Glycemic control is defined as excellent if the measured HbA1c is < 6.5%,very good if HbA1c is 6.5 to 7.0% , good if HbA1c is 7.1 to 7.5%, acceptable if HbA1c is 7.6 to 8.0% and poor if HbA1c is > 8.0% (Al-Shoumer et. al., 2008).

1.12.5 Acetone Breath

Small quantities of acetoacetic acid, which increase greatly in severe diabetes, can be converted to acetone, which is volatile and is vaporized into the expired air. One can frequently make a diagnosis of diabetes mellitus simply by smelling acetone on the

breath of a patient. The severity of the diabetic state can be assessed through the quantitative chemical detection of ketoacids in the urine.

1.13 Treatment of Diabetes Mellitus

The first line of any treatment of diabetes mellitus involves the lowering and maintenance of blood glucose, and other known risk factors, which damage blood vessels, to near normal levels. Interventions that are both cost saving and feasible in developing countries include moderate blood glucose control, blood pressure control and proper foot care. People with T1DM require insulin while; people with T2DM are treated with oral medication, but may also require insulin depending on the severity of their condition. Early screening and treatment for retinopathy (which causes blindness), blood lipid control (to regulate cholesterol levels) and screening for early signs of diabetes-related kidney disease are some of the other cost saving interventions. These measures should be supported by a healthy diet, regular physical activity, a normal body weight and cessation of tobacco and alcohol use. Pharmacologic treatment of diabetes improves glycemic control, controls hypertension and reduces blood lipid concentrations, which in turn reduces the occurrence and progression of diabetic complications (Wolever, et. al., 2008). Phenotyping of an individual and targeted drug therapy can minimize risks and maximize efficacy (Ko, et. al., 2009). For e.g. with a better understanding of the molecular mechanisms of diabetes, patients with genetic defects encoding the β-cell pathways were found to be more responsive to sulphonylurea therapy than metformin treatment.

1.13.1 Oral antidiabetic drugs

α-Amylase inhibitors

α-Amylase inhibitors, also known as starch blockers prevent dietary starches from being absorbed by the body. Starches are complex carbohydrates that cannot be absorbed unless they are first broken down by the digestive enzyme amylase and other secondary enzymes (Marshall and Lauda, 1975; Choudhury et.al., 1996). Research has demonstrated that α-Amylase inhibitors, when administered with a starchy meal, reduce the usual rise in blood sugar level of both healthy people and diabetes. (Layer et. al., 1986; Boivin et. al., 1987; Boivin et. al., 1988; Lankisch et. al., 1998). The side effects of amylase inhibitors consumed in high quantities may lead to diarrhea, due to the effects of undigested starch in the colon (Boivin et. al., 1987; Boivin et. al., 1988).

α-Glucosidase inhibitors

α - glucosidase inhibitors (AGIs) like Voglibose are known to inhibit hydrolysis of disaccharides to monosaccharides in the intestinal mucosa. Thus inhibiting carbohydrate absorption, and lowering the blood glucose level in T2DM patients. Treatment with Voglibose was found to prevent an increase in body weight (Negishi et. al., 2008). Alpha- glucosidase inhibitors (acarbose, miglitol, voglibose) are widely used in the treatment of patients with T2DM and have a lowering effect on postprandial blood glucose and insulin levels (Van de Laar et. al., 2005).

Biguanides

Metformin, a biguanide, is one of the most common drug used as first line antihyperglycemic agents in the treatment of T2DM, which acts primarily by lowering hepatic glucose production and may also improve insulin resistance (Charbonnel et. al., 2006). Metformin has been approved for use in the treatment of T2DM for nearly three decades in many countries. It is the only antidiabetic agent that has been shown to reduce mortality in patients newly diagnosed with T2DM and the only antidiabetic agent not associated with increased morbidity and mortality in patients with CVD, including heart failure (Eurich et. al., 2009).

Third generation sulphonylurea drug

Sulfonylureas act by stimulating insulin release from pancreatic β cells. They have been a cornerstone of type 2 diabetes pharmacotherapy for over 50 years. Although sulfonylureas are effective antihyperglycemic agents, individual variability exists in drug response namely pharmacodynamics, disposition namely pharmacokinetics, adverse effects (Aquilante, 2010) and are ineffective in the treatment of T1DM. The third generation of sulphonylurea, glimepiride stimulates nitric oxide production and thereby inhibits cytokine-induced nuclear factor (NF)-κB activation in endothelial cells and confers protective effects on vascular endothelial cells. They are preferable sulphonylurea agents in the treatment of type 2 diabetes and vascular diseases (Kojima et. al., 2009).

Thiazolidinediones

Thiazolidinediones, also known as glitazones, are used in the treatment of T2DM. Pioglitazone is a widely used member of this drug family. It interacts with PPAR-γ receptors that are located predominantly in adipose, hepatic and skeletal muscle cells. Modulation of these receptors adjusts the regulation of genes involved in metabolic control and also reduces insulin resistance (Smith et. al., 2005). PPAR-γ receptor activation increases glucose and lipid uptake, increases glucose oxidation, decreases free fatty acid concentration and decreases insulin resistance. PPAR-γ receptor activation also stimulates adipocyte differentiation resulting in more and smaller fat cells. Hepatic fat is significantly decreased with improvements in glycemic control and correction of dyslipidemia. Insulin action is improved by various mechanisms: increasing expression, synthesis and release of adiponectin from fat cells; increasing expression of genes that increase glucose oxidation and lowering plasma free-fatty acid levels (Lebovitz and Banerji, 2001).

Dipeptidyl peptidase-4 (DPP- 4) inhibitors

DPP-4 inhibitors offer a new therapeutic approach for the management of patients with type 2 diabetes (Charbonnel et. al., 2006). Sitagliptin is a once daily, orally active, competitive and fully reversible inhibitor of dipeptidyl peptidase-4, the enzyme that is responsible for the rapid degradation of the incretin hormone glucagon-like peptide-1 (GLP-1). It is the first in this new class of antihyperglycemic agents to gain regulatory approval for the treatment of type 2 diabetes. It is normally recommended in combination with other drugs, after the treatment based on a

combination of diet and metformin fails (Gadsby, 2009). Sitagliptin improves glycemic control by reducing both fasting and postprandial glucose concentrations, leading to clinically meaningful reductions in glycosylated hemoglobin levels (Deacon, 2007).

1.13.2 Insulin

Insulin is the primary treatment for all patients with type 1 diabetes and for type 2 diabetic patients who cannot adequately control their blood sugar by diet and exercise or by oral hypoglycemic agents (Nathan et. al., 2006). Novel long and short acting insulin analogues, the so-called 'designer insulins', developed through genetic engineering in the 1990s, paved the way for more physiological insulin therapy. Newer analogues exist as monomers and are absorbed much faster (insulin aspart or lispro) or absorbed very slowly (insulin glargine or detemir). The newer analogues have increased the stability, less variability and selective action which will help in developing individualized treatment suitable to specific patient characteristics and will improve glycemic control (Kaur and Badyal, 2008). Short-acting analogues have rapid onset and shorter duration of action. The peak of onset corresponds more closely with the postprandial glucose peak and is administered immediately before meals. This avoids postprandial hypoglycemia that occurs due to long duration of action of soluble insulin. The agents are insulin lispro, insulin aspart and insulin glulisine (Mannucci et. al., 2009). Long-acting analogues have a longer duration of action, and provide 24 hour control, with minimum variation in absorption and are given once a day. Two long acting insulin analogues insulin glargine and insulin detemir have

made significant improvements in the management of type 1 diabetes both in terms of improvement in glycemic control and in reducing hypoglycemia rates (Philips and Scheen, 2006). Albulin is the newest insulin analogue and displays characteristics of a potent long acting insulin analogue that can be evaluated for use as a novel insulin therapy for patients with insulin-dependent diabetes (Duttaroy et. al., 2005). Inhaled insulin drugs have faster onset of action, even faster than intravenous route and large surface area of lungs causes more systemic absorption. If long-term safety and efficacy are confirmed, inhalation will become the first non-subcutaneous route of insulin administration for widespread clinical use. Exubera, an insulin product for pulmonary delivery in powder form is the first inhalational drug to be approved by food and drug administration (Mandal, 2005).

1.13.3 Alternative Medicines

Many patients with DM combine alternative and traditional medicine. Alternative medicine has long been part of most cultures throughout the world. The most common forms of alternative medicine are herbs, chiropractic care, yoga, relaxation, acupuncture, ayurveda, biofeedback, chelation, energy healing, Reiki therapy, hypnosis, massage, naturopathy, and homeopathy. It is estimated that at least a third of patients with diabetes use some dietary supplements (Birdee and Yeh, 2010). Information on the effect of alternative medicine on diabetes care has started to emerge. Recent decades have witnessed the resurgence of alternative therapies mainly due to its efficacy and no side effects. The use of plant by man for the treatment of diseases is an age long practice (Prohp et. al., 2008). Currently diabetes mellitus is

managed by a combination of diet, exercise, oral hypoglycemic drugs and sometimes insulin injections (Bastaki, 2000). However the synthetic hypoglycemic drugs, which are currently the main form of treatment, have been shown to have undesirable side effects and high secondary failure rates (Bailey, 2000; Erasto et. al., 2005). In addition, these drugs cannot be afforded by the majority of people living in rural communities of developing countries because of their high cost (Bailey, 2000). These limitations, of currently available antidiabetic pharmacological agents have motivated researchers all over the world to seek out alternative antidiabetic remedies. In particular, consideration is given to plants and herbs used by traditional healers and herbalists as antidiabetic remedies with the hope of discovering new natural products that can be used or developed into safe, inexpensive and effective antidiabetic remedies.

In the treatment of diabetes mellitus, non-pharmacologic measures (diet, exercise and weight loss therapies) remain a critical component of therapy. Herbal preparations alone or in combination with oral hypoglycaemic agents sometimes produce a good therapeutic response in some resistant cases where modern medicines alone fail (Ghosh, et. al., 2004). Dietary management includes the use of traditional medicines that are mainly derived from plants (Swanston, et. al., 1991) Even now approximately 80% of the third world population is almost entirely dependent on traditional medicines (Srinivasan, 2005).

Under the given circumstances a number of medicinal plants and herbs have been studied and validated for their hypoglycaemic potential using experimental animal

models of diabetes (Kesari, et. al., 2005; Ruzaidi, et. al., 2005; Karato, 2006) and clinical studies involving diabetic patients. (Jaouhari et. al., 1999; Herrera-Arellano et. al., 2004; Jayawardena et. al., 2005). In addition, bioactive compounds of most of these plants have been isolated and identified (Grover, et. al., 2002; Jayawardena, et. al., 2005). However, mechanisms of action whereby most of these plants and/or their products exert their blood glucose lowering effects on tissue or organs remain unknown.

1.14 Global Burden of Diabetes Mellitus

The prevalence and incidence of diabetes is increasing by leaps and bounds worldwide every year. In 2000, the estimated prevalence of diabetes among adults was 2.8% or 171 million people. The prevalence of diabetes is expected to increase to 4.4%, or 366 million people, by 2030. In absolute numbers, India is and will continue to be the country with the most individuals living with diabetes, projected in 2030 to have nearly 80 million people with diabetes. Individuals with diabetes have twice the all-cause mortality rate and 2–4 times the cardiovascular disease mortality rate of individuals without diabetes (National Diabetes Statistics, 2008). The majority of deaths in individuals with type 1 diabetes occur in middle and late adulthood. In the early years after diagnosis, acute coma is the leading cause of death. Diabetes is the leading cause of chronic kidney disease (CKD) and is associated with excessive cardiovascular morbidity and mortality (USRDS, 2007; Herzog, et. al., 2008). Anemia is common among patients diagnosed with diabetes and CKD and greatly contributes to patient outcomes (Toto, 2005; Vlagopoulos, et. al., 2005). Observational studies

indicate that low Hb levels in such patients may increase risk for progression of kidney disease and cardiovascular morbidity and mortality (New, et. al., 2008). The leading causes of death in individuals with type 2 diabetes are diseases of the heart (55%), diabetes (13%), malignant neoplasms (13%), and cerebrovascular disease (10%) (Geiss et. al., 1995). Life expectancy of persons with diabetes aged 55–74 years is on average 4–8 years less than that of persons without diabetes (Gu et. al., 1998). With the growing number of individuals who will develop diabetes, the costs that society will bear in direct medical costs, decreased quality of life, and years of life lost will be immense, disproportionately affecting developing countries and racial and ethnic groups most at risk for diabetes (Poretsky, 2010). While managing diabetes and preventing its complications are critical to improving the life of a diabetic patient, prevention of diabetes and its complications through individual and community interventions will support those at risk for obesity and diabetes. Poverty influences not only the development of type 2 DM but also complications of DM (Poretsky, 2010).

Phase one results of the Indian Council of Medical Research – India Diabetes (ICMR-INDIAB) Study suggests that currently around 62.4 million people live with diabetes in India, and another 77.2 million people are on the threshold, with pre-diabetes. These results have been published in an article authored by Anjana et. al., (2011) published in the current issue of *Diabetologia*. Phase one results of the Indian Council of Medical Research – India Diabetes Study (ICMR-INDIAB) suggests that India currently has 62.4 million diabetic people India, and another 77.2 million people are pre-diabetics.

For the first time, the *World health statistics 2012* has reported that the global average prevalence of people with raised blood glucose levels is around 10% and up to one third of populations in some Pacific Island countries have this condition. Left untreated, this can lead to diabetes which in turn can lead to cardiovascular disease, blindness and kidney failure. In August 2011, approximately 346 million people worldwide were known to be diabetic and in 2004, an estimated 3.4 million people died from consequences of high blood sugar (W.H.O., 2012). These were the detected and reported cases. More than 80% of diabetes deaths occur in low and middle income countries. Fig 3 projects the global diabetic population by the year 2030.

Fig 3: Projections of the diabetic epidemic for the year 2030

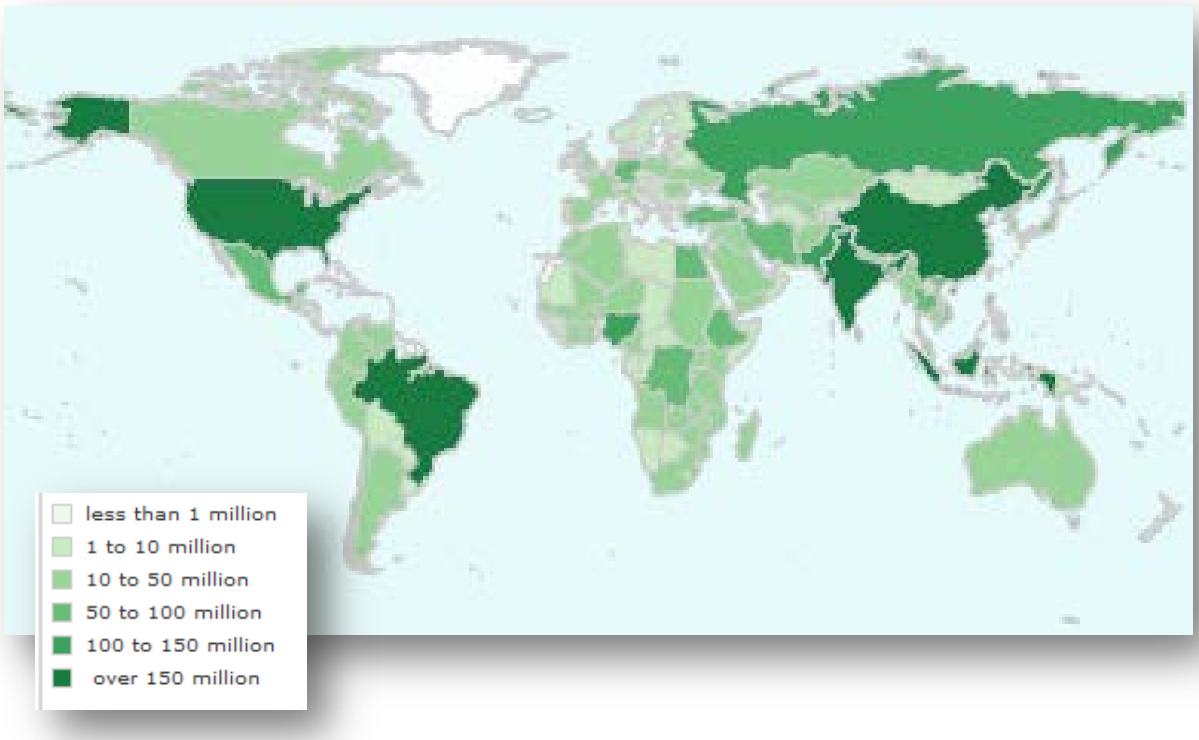


Table 2: List of countries with highest numbers of estimated cases of diabetes for 2000 and 2030

	2000		2030	
Ranking	**Country**	**Diabetics (millions)**	**Country**	**Diabetics (millions)**
1	**India**	**31.7**	**India**	**79.4**
2	China	20.8	China	42.3
3	United States	17.7	United States	30.3
4	Indonesia	8.4	Indonesia	21.3
5	Japan	6.8	Pakistan	13.9
6	Pakistan	5.2	Brazil	11.3
7	Russian Federation	4.6	Bangladesh	11.1
8	Brazil	4.6	Japan	8.9
9	Italy	4.3	Philippines	7.8
10	Bangladesh	3.2	Egypt	6.7

Source: Data from Wild et. al., (2011)

Review of Literature

1.15 History of diabetes and its treatment

The history of diabetes and its treatment, spans over a vast period of 3,500 years and has advanced a long way from magic, religious rituals, traditional herbal remedies, insulin and in recent decades, chemical therapy. The first documented reference of diabetes mellitus - the Egyptian *Ebers Papyrus* written in approximately 1550 B.C. describes the diabetic condition as a wasting disease in which the sufferer produced sweet-tasting urine, and also provides the earliest documentation about the use of plants in the treatment of conditions associated with diabetes (Bailey and Day, 1989). The Greek physician Aretaeus used the word diabetes (Greek- siphon) 1600 years later and noted the “excessive amount of urine which passed through the kidneys” (Sanders 2002).

In India, the early Ayurvedic texts such as the *Sushruta Samhita* and the *Charaka Samhita* (4th to 5th century B.C.) describe the use of about 760 and 500 species of medicinal plants, respectively, including those prescribed for diabetes associated conditions like glycosuria, polyphagia, and polyuria (Soumyanath 2005). *Ben Jing*, written in about 104 B.C., in China contains a detailed description of 252 plant species used to treat diabetes. In medieval Persia, Avicenna (980–1037) provided a detailed account on diabetes mellitus in "The Canon of Medicine", describing the abnormal appetite, collapse of sexual functions, the sweet taste of diabetic urine and recognized a primary and secondary form of diabetes. He also described diabetic gangrene, and prescribed a treatment for diabetes - a mixture of lupine, trigonella (fenugreek), and zedoary seed, which considerably reduced excretion of sugar, and is still prescribed in modern times (Simmonds and Howes, 2006). It was Thomas Willis’s observations of diabetes in 1674 (Ahmed, 2002) and Matthew

Dobson's experimentations in 1776 that convincingly established the diagnosis of diabetes as the presence of abnormally large quantities of sugar in the urine and blood. In the first half of the 19th century Claude Bernard (1857), discovered glycogen (Young, 1957) and established the role of the liver in glycogenesis, and the concept that diabetes is caused due to excess glucose production.

In 1869, Paul Langerhans submitted the famous histological finding, the pancreatic islets in his doctoral dissertation at the University of Berlin, without knowing their function. Although diabetes had been recognized and treatments have been known since ancient times, the pathogenesis of diabetes has only been understood experimentally since about 1900. Merring and Minkowski (1889) discovered the endocrine function of the pancreas, when they observed that total pancreatectomy in a dog resulted in the production of fatal diabetes. In 1910, Sir Edward Albert Sharpey-Schafer suggested that people with diabetes were deficient in a single chemical produced by the pancreas—he proposed calling this substance "insulin", from the Latin "insula", meaning island, in reference to the insulin-producing islets of Langerhans in the pancreas (Patlak, 2002). In the early 1900s, Georg Zuelzer, a German scientist, found that injecting pancreatic extract into pancreactectomised dogs and patients could help control diabetes (Rosenfeld, 2002).

The discovery and therapeutic application of the insulin hormone in the 1920s by Frederick Banting and Charles Best (1922); and its purification and isolation by John Jacob Abel (1926) was a miraculous development in the treatment of diabetes that enabled affected individuals to live an almost-normal life (Rosenfeld, 2002). However it soon became apparent, that insulin did not cure diabetes. Elliot Joslin (1934) noted

that following the introduction of insulin, mortality from diabetic coma had fallen significantly from 60% to 5%, yet, deaths from diabetic gangrene (of the foot and leg) and other complications had risen significantly. He developed a team approach to diabetes care, which included patient education, medical nutrition therapy, exercise, prompt treatment of foot infections, and, when necessary, specialized surgical care (Sanders, 2002).

In 1935, Roger Hinsworth differentiated between two types of diabetes: "insulin sensitive" (type I) and "insulin insensitive" (type II), which helped to open up new avenues of treatment. In 1946, Auguste Loubatières reported the hypoglycaemic effect of a sulphonamide (2254 RP), a drug initially used to treat typhoid. In this study, which added to the belief of the existence of the dual pathogenesis of diabetes mellitus, he observed that the hypoglycaemic effect of 2254 RP in normal and partially pancreatectomized dogs (not in totally pancreatectomized dogs), depended on the concentration of sulphonamide in the plasma (Loubatières-Mariani, 2007). Humulin - the first biosynthetic human insulin; identical in chemical structure to human insulin, was mass produced in 1982, using recombinant DNA technology, by the pharmaceutical firm Eli Lilly (Chance and Frank, 1993). Dr Gerald Reaven's (1988) identified, the constellation of symptoms associated with diabetes mellitus, now called metabolic syndrome (Alberti et. al., 2005; Reaven, 2009).

Along with the increase in knowledge and understanding of the pathogenicity of this disease in the 1900's, came the advent of new and more potent drugs, which promised abolishment of defective glucose homeostasis, but did not reduce morbidity or the mortality of the affected patients. Over a span of the next 20 years, researchers,

physicians and patients realised that the newer synthetic oral hypoglycaemic drugs had undesirable and serious ramifications and high secondary failure rates (Davis and Alonso, 2004; Shaw, 2006).

Scientific evidence demonstrates that much of the morbidity and mortality of diabetes can be reduced by aggressive treatment with diet, exercise, and improved blood glucose control. Despite all the advancements in diabetic therapy, recent research suggests that the commonly prescribed drugs for type 2 diabetes and insulin therapy for type 1 diabetes cannot cure and may delay the onset of; but not prevent the morbidity and death from end stage diabetic complications such as kidney failure, paralysis, microvascular and macrovascular complications resulting in gangrene and retinal derangements, and cardiovascular diseases, such as heart attacks and stroke (American Diabetes Association, 2005).

1.16 Plant therapy for diabetes mellitus

Alongside the conventional synthetic therapies, a huge chunk of the worlds' diabetic population is entirely dependent on traditional plant medicines or incorporate plants having antidiabetic activity into their daily diet. The plant-based, traditional medicine system continues to play a crucial role in health care. About 80% of the world's inhabitants rely on traditional medicines for their primary health care (Owolabi et al., 2007). The historic role of plants in healing declined early in the twentieth century with the ascension of synthetic drugs, even though a number of basic medicines, such as opium, strychnine, and cocaine, were of botanical origin (Morton, 1968).

Norman Farnsworth in 1966 published a 44 page phytopharmalogical survey on the biological and phytochemical screening of plants and their activities. Morton (1968) attributed the resurgence in phytomedicine to three discoveries – i) penicillin and other antibiotics from natural moulds, ii) the realization that the ancient tranquilizer of India derived from Rauwolfia was superior to what chemists could devise, and iii) the vast reduction in costs of production of medication. Marles and Farnsworth (1995) published a review on more than 1200 species of plants reported to be used to treat diabetes; investigated for - antidiabetic activity; the diversity of plant constituents with hypoglycaemic activity, their mechanisms of action; methods for bioassay of hypoglycaemic agents and their potential toxicity problems.

India has about 45,000 plant species, of which several thousand claim to possess medicinal properties. Many of these medicinal plants and herbs are part of our Indian diet as spices (Kim et. al., 2006; Mathur et. al., 2011; Kuroda et. al., 2012), vegetables (Platel and Srinivasan, 1997, Tiwari, 2007) and fruits (Teixeira et. al., 2004; Rai et. al., 2009; Dewanjee et. al., 2011). Research on plants mentioned in ancient literature or used traditionally in the treatment of diabetes, conducted in the last few decades, , show potential anti-diabetic property of varying potency (Grover et. al., 2002). W.H.O has recommended the use of indigenous plants as alternative medicine in the management of diabetes mellitus, particularly in developing countries where safe modern drugs, health centres and resources are limited or lacking (W.H.O, 2002). Research suggests that using an antidiabetic plant in whole form or as complex extracts may offer many benefits due to the presence of multiple active components.

The fact that approximately 600,000 plant species existing on the earth, only some 5% have been specifically investigated chemically or pharmacologically is a challenge to chemists specializing in natural substances and to pharmacologists.

According to W.H.O (1977) “a medicinal plant” is that which contains substances of therapeutic purposes or which are precursors for the synthesis of useful drugs. This definition distinguishes those plants whose therapeutic properties and constituents have been established scientifically and plants that are regarded as medicinal but which have not yet been subjected to thorough investigation. Herbal medications have been used for the treatment of variety of ailments; a huge number of populations in the world are entirely dependent on traditional medicines (Meenakshi et. al., 2010, Feshani et. al., 2011). A number of medicinal plants and their formulations are used for treating diabetes in Ayurvedic medicine system as well as in ethnomedicinal practices. From the ethnobotanical information, about 800 plants which may possess anti-diabetic potential have been found (Patil et. al., 2011).

Several plants have been used in treating diseases without any knowledge on their proper functions and constituents and as dietary adjuvants. This practice may be due to its fewer side effects compared to the synthetic hypoglycemic agents and because of their safety, effectiveness, and availability (Modak et. al., 2007). There are roughly about 200 pure compounds from botanical sources reported to possess blood glucose lowering effect. The compounds may be alkaloids, carbohydrates, glycosides, flavonoids, steroids, terpenoids, peptides and amino acids, lipids, phenolics, glycopeptides and iridoids (Marles and Farnsworth, 1994). Many antidiabetic products of herbal origin are now available in the market. Plants have been the

primary source of drugs and many of the currently available drugs have been directly or indirectly derived from plants. For example, the popular hypoglycemic drug glucophage (metformin) is derived from *Galega officinalis* (Grover et. al., 2002). About 800 plant species have been reported to possess antidiabetic properties (Alarcon-Aguilara et. al., 1998). A wide array of plant derived principles belonging to compounds mainly alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides, and terpenoids have demonstrated bioactivity against hyperglycemia (Ivorra et. al., 1988; Marles and Farnsworth, 1995). Mentreddy et. al., (2005) stated that several plant species have been used for prevention or management of diabetes by the Native Americans (Johnston, 1987), Chinese (Foster, 1993; Vuksan, 2000), South Americans (Garcia, et. al., 2001), and Asian Indians (Subbalakshmi and Naik, 2001; Grover et. al., 2002).

The ethnobotanical information reports a huge number of plants species (approximately 1200 species) have been screened for antidiabetic potential on the basis of ethnomedicinal use (Singh, 2011). Herbal treatments for diabetes have been used in patients with insulin dependent and noninsulin dependent diabetes, and their complications.

1.17 Screening medicinal plants for antidiabetic activity

The screening phase of medicinal plants is commonly used to assess the desired activity, in this case the antidiabetic or hypoglycemic activity of medicinal plants and can generally be grouped into three main categories – In vivo bioassays, In vitro bioassays and enzyme inhibition based assays.

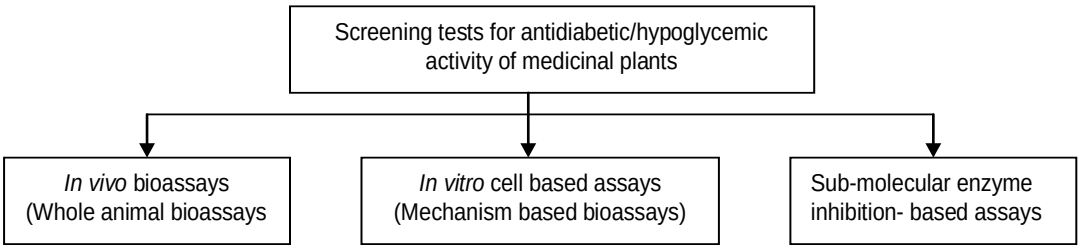


Fig 4: Assessment of the antidiabetic/hypoglycemic potential of medicinal plants

1.18 In vivo bioassays

In vivo bioassays for screening plants for antidiabetic activity and other antidiabetic remedies are usually carried out in normal or diabetic animals (mostly rats and mice) in which experimental diabetes has been induced via chemical, dietary, surgical or genetic manipulations (Rees and Alcolado, 2005; Masiello, 2006; Day and Bailey, 2006; Srinivasan and Ramarao, 2007; Fröde and Medeiros, 2008). By far the most commonly used animal models for screening plants for antidiabetic activity are the chemically (alloxan and streptozotocin) induced diabetic animal models (Fröde and Medeiros, 2008). Ethical and practical considerations make it impossible to screen large numbers of samples using animal models of diabetes, even though they appear to be more useful in screening plants for their antidiabetic activity than in vitro bioassay screening techniques. (Day and Bailey, 2006; Fröde and Medeiros, 2008). It is necessary to reemphasize that natural products display several effects besides lowering blood glucose in these experimental models. In view of the lack of parallel studies of their toxicity, these models of diabetes induced by either alloxan or STZ are considered a screening step in the search for drugs for the treatment of diabetes (Ye et. al., 2002; Srinivasan and Ramarao, 2007)

1.19 Animal Models of Diabetes Mellitus

Animal models of diabetes have had a major role in shaping our current understanding of diabetes. Two major purposes of animal models are to improve the understanding of the physiology of diabetes and to promote the development of new therapeutic compounds. Important contributions have come from both classical laboratory animal models and new ones made using advanced methods of gene manipulation. The majority of studies published in the field of ethno-pharmacology between 1996 and 2012 employed chemical induced model (Kumar et. al., 2012).

The ideal animal model should be simple to describe, easy to produce, maintain, and study while accurately reproducing the human phenotype. The choice of animal model depends on the experimental question under investigation. Without a doubt, rodent models have been instrumental in describing pathways that have led to pancreatic β-cell destruction, evaluating potential causes of type 1 diabetes and providing proof-of-principle for the potential of interventions (Roep et. al., 2004).

1.19.1 Species consideration

A survey of the ethnobotanical literature on antidiabetic activity reveals that, rats or mice are the animals of choice for diabetes research. Rats being 10 fold larger than mice are more preferred for physiology experiments, mainly because their physiology is more similar to humans and their larger size makes repeated blood sampling and other techniques (surgical and non surgical) easier (European Commission, 2010). The use of larger animals such as dogs, rabbits and primates allows very sophisticated physiology experiments, such as those requiring frequent blood sampling, longitudinal tissue sampling, and use of multiple chronic indwelling catheters.

However, experiments using these animals, calls for specialised expertise and facilities, and are costlier to maintain.

1.19.2 Sex Genetics and age

Sex related effects must always be taken into account when deciding the choice of animal model. Choeiri et. al., (2005) found the diabetic phenotype to be more severe and progressive in males. These effects are thought to be mediated by the sex steroid hormones. However, not all models have more severe diabetes in males. The background genetic effects are very significant and must be considered in every animal experiment. Ideally, background genotype should be controlled for by using littermates, by using genetically identical animals, and/or by using animals in which the phenotype or genes have been backcrossed onto particular background for at least 10 generations (Haluzik and Reitman, 2002).

Diabetes mellitus is a systemic disease affecting many tissues. Some features of diabetes pathophysiology can be studied using cultured cells (Geraldes et. al., 2009). However, other aspects of the disease cannot yet be studied except in whole animal models. Classically animal models have been used for identification of regulatory and metabolic pathways, for genetic studies of candidate genes for obesity, diabetes, and insulin resistance and for discovery of new drugs and subsequent testing of their efficacy and safety. Using animal models for the study of diabetes is safe, relatively inexpensive and practical. Of course, known differences between the physiology of the model animal and human must be taken into account when interpreting the results. Each and every drug that goes into the market is not passed until and unless it is studied on the laboratory animals, which forms the preclinical study phase of a

clinical trial (Pugsley et. al., 2008). The characteristic features of disease and pathological changes during disease in small animals (rats or mice) are near about similar to that of human beings. Diabetes mellitus is generally induced in laboratory animals for experimental purpose by several methods: chemical, surgical and genetic (immunological) manipulations.

1.19.3 Experimental Models of Diabetes Mellitus

The animal models employed for screening anti-diabetic agents can be classified into three main categories, enlisted below (Vogel and Vogel 2002):

A. Induced Experimental Diabetes Mellitus

B. Genetically Diabetic Animals

C. Miscellaneous Models

A. Induced experimental Diabetes Mellitus

This category can be further sub-classified as under:

a) Pancreatectomy

von Merring and Minkowski (1889) discovered that complete surgical pancreatectomy in a dog resulted in severe type 1 diabetes mellitus (Young 1961). This technique has since been extensively used by scientists in various animals (Cobb and Merrell, 1984; Stump et. al., 1988; Hardikar et. al., 1999), exhibiting classical diabetic symptoms of polyuria, polydipsia, polyphagia, and severe glucosuria.

b) Chemically induced diabetes

Several drugs, such as theophylline, aspirin, isoniazid and nalidixic acid can cause transient hyperglycaemia when overdosed, but only streptozotocin, alloxan and the

rodenticide Vacor are known to cause permanent diabetes (Ferner, 1992). Symptoms of diabetes and obesity induction have been found by compounds such as dithizone (Hansen et. al., 1989), goldthioglucose (Heydrick et. al., 1995) or monosodium glutamate (Sartin, 1985). Goldberg and Eshchenko, (1991) used various chelators, such as 8-(p-toluene-sulfonylamino)-quinoline (8-TSQ) and 8-(benzenesulfanylamino)-quinoline for induction of diabetes in experimental animals. The diabetogenic action of pure anterior pituitary growth hormone in adult cats and dogs, on repeated administration, induces intensive diabetes with all symptoms including severe ketonuria and ketonemia, while rats do not show any sign of diabetes on a similar treatment, but grow faster (Young, 1941; Cotes et. al., 1949). Cortisone can induce hyperglycemia and glucosuria in treated rats (Ingle, 1941). Guinea pig anti-insulin serum can also induce a transient reversible diabetic syndrome (Wright, 1968), due to neutralization by insulin antibodies of endogenous insulin secreted by the injected animal. Viral infections and beta-cell specific autoimmunity may induce juvenile-onset (type I) diabetes mellitus (Craighead, 1978). Infection and destruction of pancreatic beta-cell by D-variant of encephalomyocarditis virus (EMC-D) is known to induce diabetes (Vialettes et. al., 1983).

Alloxan Diabetes

Alloxan has been reported to act by selectively destroying the β-cells of the pancreas without affecting other islet cells, thereby, reducing insulin secretion (Weaver et. al., 1978), and inducing diabetes mellitus in the alloxan treated animals (Dunn et. al., 1943; Dunn & McLetchie, 1943). Weaver, et. al., (1978) and Malaisse, et. al., (1982) have suggested that this cytotoxic effect involves both a rapid uptake of alloxan and a

drug-induced generation of oxygen-containing radicals and peroxide (Heikkila, et. al., 1976; Grankvist, et. al., 1979; Fischer & Hamburger, 1980).

Surveys on chemically induced diabetes in animals were given by Frerichs and Creutzfeldt (1971). Hyperglycemia and glucosuria have been described in several species upon alloxan administration by several researchers, in dogs (Tasaka, et. al., 1988), rabbits (Baily and Baily, 1943), rats (Goldner and Gomori, 1944) and in other species (Frerichs and Creutzfeldt, 1968). Alloxan resistance has been found in Guinea pig (Maske and Weinges, 1957). The dosage and treatment regimen for alloxan diabetes has been elaborated for the most frequently used species. The most frequently administered dose of alloxan ranges between 40 and 200 mg/kg body weight, depending on the route of administration. In most species a triphasic time course is observed: an initial rise of glucose is followed by a decrease, probably due to depletion of islets from insulin, again followed by a sustained increase of blood glucose. Alloxan generally produces greater cytotoxicity owing to its conversion to anionic radicals. Alloxan induction of diabetes results in occurrence of hyperglycemia and uricosuria in 70% of the animals. The rest of the animals either die or are only temporarily hyperglycaemic (Bänder, 1969).

B. Genetically diabetic animals can be further sub-classified as under:

Rodents have been described to exhibit spontaneous diabetes mellitus on a hereditary basis. Since the discovery of leptin (Zhang et. al., 1994) and its downstream signal transduction cascade (Friedman and Halaas, 1998), new insight of the genetics of diabetic and obese animal disease models were derived.

a) Spontaneously diabetic rats

Spontaneously diabetic animals of diabetes may be obtained from the animals with one or several genetic mutations transmitted from generation to generation (e.g., ob/ob, db/db mice) or by selected from non-diabetic outbred animals by repeated breeding over several generation. These animals generally inherited diabetes either as single or multigene defects (Srinivasan and Ramarao, 2007).

b) Transgenic Animals

Transgenic animals are generally helpful in giving insights into gene regulation and development, pathogenesis and finding new targets and the treatment of disease. In general, transgenic animals particularly mice are usually created by transferring and altering the site or level of expression of functional gene (transgene) or by deleting specific endogenous genes (knockout) or placing them under the control of alternate promoter regions.

C. Miscellaneous Models

Invertebrate animal models

The silk worm *Bombyx mori* when fed a high-glucose diet (10% glucose containing diet) for 3 days, exhibits a 4 fold increase in hemolymph sugar level, body size and weight when compared with silk worms fed a normal diet. *Caenorhabditis elegans* and Drosophila are also being used to study diabetes mellitus. Kuznetsova et. al., (2007) presented the streptozotocin (STZ) model of insulin-dependent diabetes in the mollusc *Anodonta cygnea*.

Diet Induced Metabolic Dysregulation

Higgins et. al., (2010) reported that the non human primate Baboon (*Papio hamadryas* sp.) when fed a high sugar, high fat diet for a duration of 8 weeks, showed an increase in body fat and triglyceride concentration, along with a change in percentage glycosylated hemoglobin (HbA1c) and adipokines. Diabetes related complications develop in rats when fed a diet rich in sucrose or other refined sugars, but not when fed a starch or stock diet (Velasquez et. al., 1972; Panchal et. al., 2011).

1.20 In vitro assays

Cell based assays commonly used to screen or evaluate the antidiabetic activity of medicinal plants belongs to a class of in vitro bioassays known as "mechanism based assay" (Soumyanath and Srijayanta, 2006; Frode and Medeiros, 2008). A mechanism-based bioassay differ from an ordinary cell culture bioassay in that it can provide a possible mechanism of action at the same time that the plant material is screened for biological activity (Benjamin, et al., 1994). Two different types of mechaism based in vitro bioassays are commonly used to assess the antidiabetic/hypoglycemic of medicinal plants and/or products: the insulin secretion stimulation (Gray and Flatt, 1997, 1999) and the glucose uptake (utilization) biosasays (Soumyanath and Srijayanta, 2006; van de Venter et. al., 2008).

1.21 Glucose uptake (utilization) bioassay

Gemmill and Hamman (1941) showed that the invitro presence of insulin improves the uptake of glucose by isolated rat diaphragm. The action of insulin on glucose transport is the classical example of metabolism being controlled by the permeability of membranes. This bioassay assesses the ability of plant materials to enhance glucose

uptake by insulin target tissues such as diaphragm, skeletal muscle or liver (Soumyanath and Srijayanta, 2006; van de Venter et al., 2008).

Best et. al., (1926) from his experiments in spinal eviscerated preparations demonstrated that glucose which decreases in concentration from the media under the action of insulin is equal to the sum of the glycogen deposited in the muscles and the glucose equivalent of the oxygen absorbed. This was followed by several studies on the distribution of nonmetabolized sugars in eviscerated dogs by Levine et. al., (1949), rabbits (Wick and Drury, 1953), and nephrectomised rats (Helmreich and Cori, 1957). More recently the hindquarter (Mahler et. al., 1968; Ruderman et. al., 1971) and the hemicorpus of the rat (Jefferson et. al., 1972) have been shown to provide sensitive tools for the evaluation of insulin effects in major intact and composite structures of skeletal muscle.

The effect of insulin on glucose consumption in human peripheral skeletal muscle has also been assessed by measurements of the arterio-venous concentration difference in the forearm (Andres et. al., 1961). This method has yielded clear cut evidence that glucose uptake is a function of the insulin concentration in the physiological range (Christensen and Ørskov, 1968), but since it is complicated, it has not been used in many laboratories. The mere size of this major target for the action of insulin, the skeletal muscle; indicates its obvious role in blood glucose homeostasis (Sinacore and Gulve, 1993). Numerous preparations have been proposed over the years for the study of how insulin controls glucose transport in skeletal muscle cells.

Various studies hence have used different skeletal muscles, but not without limitations. Pain and Manchester (1970) and Rogus and Zierler (1973) used the extensor digitorum longus from baby rats, while Chaudry and Gould (1969) and Kohn and Clausen (1971) reported the use of the soleus muscle, which can be easily prepared with intact fibres and are quite convenient for most invitro studies of glucose transport and metabolism as well as the measurement of ionic fluxes (Clausen et. al., 1973). However, diffusion through the interstitial space represents a rate limiting factor for the exchange of solutes in these preparations, and that spontaneous contractures may occur if the muscles are not thoroughly oxygenated.

Isolated muscle preparations have been developed in order to obtain more versatile tools for the detailed study of the mechanisms of glucose transport and insulin action. Since the work of Gemmill (1940), the isolated rat diaphragm muscle (originally proposed by Meyerhof and Himwich in 1924) has become the classical choice for such investigations, being easy to prepare and sufficiently thin to allow rapid equilibration of oxygen and substrates between the cells and the incubation medium. Groen et. al., (1952) and Liebecq, (1954) demonstrated that quarter diaphragms had a smaller response to insulin than hemidiaphragms. In the isolated rat hemidiaphragm, a stimulating effect of insulin can be detected down to concentrations below 10µU/ml (Groen et al., 1952), which is close to the level measured in human plasma with an immunoassay (Bronner, 1975). This preparation has been the most widely used for the bioassay of insulin like activity in the plasma (Vallance-Owen and Wright, 1960), in drugs (Borrebæk and Walaas, 1963; Adnitt and Frayn, 1972) and the study of insulin action.

A glucose uptake bioassay is generally performed by incubating the target tissue in a sterile buffer containing glucose or/and insulin in the presence and absence of the candidate plant extract (Benjamin et al., 1994). Glucose in aliquots of the incubation media is then measured (Frost and Lane, 1985). A major disadvantage of these techniques is that only "acute" or immediate effects are measured, whilst effects that may only be apparent after chronic exposure to the antidiabetic compound are overlooked (Soumyanath and Srijayanta, 2006).

1.22 Sub-molecular enzyme inhibition-based assays

Some antidiabetic agents are known to exert their blood glucose lowering effects through inhibition of specific carbohydrate metabolizing enzymes. Several researchers (Hara and Honda, 1990; Kim et. al., 2005; Ali et. al., 2006; Bhandari et. al., 2008) have investigated the ability of plant extracts to inhibit the activities of enzymes such as α-amylase, α-glucosidase, hexokinase (glucokinase) and glucose 6-phosphatase by means of in vitro sub-molecular enzyme inhibition assays. An antidiabetic agent may exert its blood glucose lowering effect by stimulating insulin secretion from pancreatic beta-cells, enhancing glucose uptake by fat and muscle cells, altering the activity of some enzymes that are involved in glucose metabolism or slowing down the absorption of sugars from the gut (Tanira, 1994; Cheng and Fantus, 2005).

Consumption of plant foods is associated with lowered risk of major chronic diseases including diabetes, cardiovascular diseases (Hu, 2003; Liu, 2003) and cancer.

Plant compounds may influence glucose metabolism by several mechanisms;

i) inhibition of carbohydrate digestion and glucose absorption in the intestine

ii) stimulation of insulin secretion from the pancreatic β-cells

iii) modulation of glucose release from liver

iv) activation of insulin receptors and glucose uptake in insulin-sensitive tissues

v) modulation of hepatic glucose output.

The liver plays a major role in the regulation of blood glucose levels in tight cooperation with peripheral tissues. As estimated, the liver is responsible of taking up one third of the postprandial glucose (Cherrington, 1997), and stores effectively glucose as glycogen *via* glycogenesis.

Over the years researchers (Koti et. al., 1996; Zulet et. al., 1999; Khan et. al., 2007) have explored the differences in the activity of carbohydrate-metabolizing enzymes under dietary and drug influences. Some antidiabetic remedies, such as Metformin exerts its blood glucose effects by inhibiting endogenous glucose production by the liver through the process of gluconeogenesis and glycogenolysis (Bastaki, 2005; Agius, 2007).

Several researchers (Grover et. al., 2000; Rathi et. al., 2002) have investigated the restorative effects of various plants and their extracts on the activities of the gluconeogenic enzyme, glucose-6-phosphatase and the glycogenolytic enzyme, hexokinase, glucose-6-phosphate dehydrogenase, aspartate transaminase, alanine

transaminase, lactate dehydrogenase, in insulin dependent (skeletal muscle and liver), insulin-independent tissues (kidneys and brain), in the normal and diabetic conditions.

Hexokinase converts glucose to glucose-6-phosphate, while Glucose-6-Phosphatase, hydrolyzes glucose-6-phosphate to glucose in the glycolytic pathway and is the enzyme that ultimately controls the release of glucose by the liver (Matschinsky, 2006). Enzyme activities of gluconeogenesis have been shown to increase during the course of diabetes, with a simultaneous increase in the glycogenolytic and lipolytic pathways. Diabetes also has been accompanied with a decrease in the enzyme activities of the glycolytic and pentose phosphate pathways (PPP) (Punitha et. al., 2005). Three major metabolic anomalies that contribute to a hyperglycaemic state in diabetes mellitus are defective glucose-induced insulin secretion, increased hepatic glucose output and the incapability of insulin to stimulate glucose uptake in the peripheral target tissues (Garvey, 1992). Pancreatic β-cells appropriately alter their rates of insulin secretion in response to fluctuations in the levels of these calorigenic molecules, with glucose playing the dominant role in the regulation of insulin secretion. Eventually, glycolysis and gluconeogenesis are two reciprocally regulated pathways that prevent wasteful operation of both pathways at the same time. The rate of glycolysis is determined by the concentration of glucose and the rate of gluconeogenesis by the concentrations of lactate and other precursors of glucose.

Glucose-6-phosphate dehydrogenase catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate, with a simultaneous oxidation of $NADP^+$ to NADPH. This maintains adequate levels of glutathione in its reduced form and helps to overcome oxidative stress (Oni et. al., 2005).

Mering and Minkowski (1946) and Hédon (1946) found that the glycogen content of the liver and the skeletal muscles, during experimental pancreatic injury induced-diabetes, is much decreased (Weber, 1946). Tuerkischer and Wertheimer, (1946) suggested that a decrease in the glycogen content of the diabetic liver is often considered to be the direct result of the diabetic disturbance in metabolism, caused either by an increased glycogenolysis or by a decreased ability to synthesize glycogen.

1.22.1 Glucose-6-phosphatase

Glucose-6-phosphatase (G6Pase) is an important regulatory enzyme in the gluconeogenic pathway that hydrolyzes glucose-6-phosphate resulting in the creation of a phosphate group and free glucose. Glucose is then exported from the cell via glucose transporter membrane protein (Ghosh et. al., 2002). This catalysis completes the final step in gluconeogenesis and glycogenolysis and therefore plays a key role in the homeostatic regulation of blood glucose levels (Nordie et. al., 1985), which gets altered during the course of uncontrolled diabetes.

Along with the conversion of glucose-6-phosphate to glucose, it simultaneously, provides hydrogen, which binds with $NADP^+$ in the form of NADPH and enhances the synthesis of fats from carbohydrates, i.e. lipogenesis (Koti et. al., 2011). The activities of G6Pase is found to be increased in the diabetic state as compared with the normal rats.

1.22.2 Hexokinase

Hexokinase is an important unidirectional enzyme that catalyzes the first reaction of glycolysis, through the phosphorylation of glucose, forming glucose-6-phosphate. Thus via this process hexokinases maintain the downhill concentration gradient that favors the facilitated transport of glucose into cells. This reaction also initiates all physiologically relevant pathways of glucose utilization, including glycolysis and the pentose phosphate pathway (Robby and Hay, 2006).

1.22.3 Glucose 6 phosphate dehydrogenase

G6PD is a regulatory enzyme which converts glucose-6-phosphate into 6-phosphoglucono-δ-lactone and is the rate-limiting enzyme of the pentose phosphate pathway (PPP), and one of a number of glycolytic enzymes. It is part of a metabolic pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). NADPH, the principal antioxidant, or reducing agent, in turn maintains the level of glutathione in these cells that helps protect against oxidative damage.

Diabetes is associated with increased levels of oxidants leading to complications of the heart, eye, kidney and blood vessels. Thus, maintaining healthy cells requires appropriate regulation of the pentose phosphate pathway (PPP). Xu et. al., (2005) has reported that the diabetic condition causes a major decrease in the activity of G6PD. Decreased G6PD activity leads to decreased NADPH, a significant cause of the increased oxidative stress seen in diabetes that causes kidney disease, vascular disease and other complications (Zhang et. al., 2000). NADPH is also required for the production of nitric oxide, which acts as a vasodilator leading to a lowering of blood

pressure. Hence a lack of NADPH would play an important role in the development of hypertension in patients with diabetes (Paravinci and Touyz, 2008). NADPH is also required by a number of other cellular reactions including the WBC enzyme NADPH oxidase, which is required for proper bacterial killing. Thus lack of NADPH leads to impaired antioxidant function, making cells susceptible to damage; decreased nitric oxide, leading to hypertension; and decreased white blood cell function, increasing susceptibility to infections (Stanton, 2012).

1.22.4 Lactate dehydrogenase (LDH)

Vizir (1977) studied the activity of LDH in blood serum of 120 patients suffering from DM and reported a significant elevation of total LDH activity. This was confirmed by Nair et. al.,(1985). Coulson and Anderson, (1967) reported a decrease in LDH levels in the kidney of the alloxan-diabetic rat, while Beitner and Lahat, (1973) reported a decrease in the liver tissue levels of LDH. Elük and Yeúün, (2002), reported elevation in plasma LDH in alloxan induced diabetes in rabbits.

Lactate dehydrogenase catalyzes reduction of the keto group in pyruvate to a hydroxyl, yielding lactate, as NADH is oxidized to NAD^+. This forms the last step in the glycolytic pathway (Alcazar et. al., 2002). When the exertion is brief and intense, the skeletal muscles ferment glucose to lactate. Lactate released to the blood may be taken up by other tissues, or by skeletal muscle after exercise, and converted via lactate dehydrogenase back to pyruvate, which may be oxidized in Krebs cycle or (in liver) converted back to glucose via gluconeogenesis (Brooks et. al., 1999). Lactate serves as a fuel source for cardiac muscle as well as brain neurons.

1.22.5 Aspartate transaminase

AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells, and is commonly measured clinically as a marker for liver health. Aspartate transaminase catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. Elevated levels of this enzyme is indicative of the health status of the liver (Reichling and Kaplan, 1988).

1.22.6 Alanine transaminase

When muscles produce lactate during times of decreased oxygen, alanine is also produced. This alanine is shuttled to the liver where it is used to make glucose. AST is a part of the gluconeogenic cycle. Alanine plays a key role in glucose–alanine cycle between tissues and liver. In muscle and other tissues that degrade amino acids for fuel, amino groups are collected in the form of glutamate by transamination. Glutamate can then transfer its amino group through the action of alanine aminotransferase to pyruvate, a product of muscle glycolysis, forming alanine and α-ketoglutarate. The alanine formed is passed into the blood and transported to the liver. A reverse of the alanine aminotransferase reaction takes place in liver. Pyruvate regenerated forms glucose through gluconeogenesis, which returns to muscle through the circulation system. The glucose–alanine cycle enables pyruvate and glutamate to be removed from the muscle and find their way to the liver. Glucose is regenerated from pyruvate and then returned to muscle: the energetic burden of gluconeogenesis is thus imposed on the liver instead of the muscle (Nelson et al., 2005).

Acid Phosphatase and Alkaline Phosphatase

Diabetes mellitus is a metabolic disorder which is associated with altered activity of various enzymes (Beltiore et. al., 1973). The phosphatases (acid and alkaline) usually play a role in the production and transport of inorganic phosphate, which is necessary for a large number of metabolic reactions (Osagie, 1992). Inorganic phosphate plays a vital functional role in energy transfer and metabolic regulation and is also an important structural constituent of many biomolecules. An increase in tissue phosphatase activities are reported in serum as well as in the liver of alloxan mediated diabetic rats (Drabkin and Marsh, 1947; De et. al., 1956; Hanna et. al., 1997). The enhanced activity of these enzymes has been tentatively interpreted as a manifestation in serum of the increased phosphatase activity that may occur in tissues in the diabetic state.

1.23 Effect on insulin secretion

In most published studies, investigation of the effect of medicinal plant extract on insulin secretion in vivo has involved the use of streptozotocin or alloxan induced animal models of diabetes. Both alloxan and streptozotocin causes destruction of pancreatic beta cells resulting in reduced insulin secretion (Szuldelski, 2001; Fröde and Medeiros, 2008). In streptozotocin and alloxan induced animal models of diabetes, insulin is markedly depleted but not absent (Pushparaj et. al., 2001; Walde et. al., 2002; Fröde and Medeiros, 2008). For this reasons these animal models have been widely used to study the effect of antidiabetic remedies on insulin secretion *in vivo.* Normal insulin action leads to increased glycogen synthesis, glucose transport, and lipogenesis, and decreased gluconeogenesis, glycogenolysis, and lipolysis (Kahn and Flier, 2000; Newsholme and Dimitriadis, 2001; Saltiel, 2001).

1.24 Inhibition of Carbohydrate digesting enzymes

One method of keeping blood sugar level in check is the consumption of foods possessing a low glycemic index, which are known to reduce the risk of diabetes and cardiovascular diseases as well as their complications. Contributing to the problem of chronic post-prandial glucose surges is the age-related impairment of the body's ability to utilize calories efficiently, particularly carbohydrate calories. Even if a reduced carbohydrate intake is maintained, the aged body struggles to process any excess carbohydrates. This implies that every carbohydrate-laden meal poses a threat to aging humans' healthy life span because of unavoidable age-related metabolic inefficiencies in carbohydrate metabolism. An alternative to the low glycemic index diet, are products that slow down the absorption of carbohydrates through the inhibition of enzymes responsible for their digestion. There is a rapidly growing body of research on these products which include the alpha-amylase and glucosidase inhibitors (Barrett and Udani, 2011**)**.

1.24.1 Assay of α-amylase inhibitory activity

The enzyme α-amylase plays an important role in the digestion of starch and glycogen. Thus the inhibition of α-amylase is considered a vital strategy in the treatment of carbohydrate uptake disorders (Kandra, 2003). Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources (de Sales et. al., 2012). The potential role of the medicinal plants as inhibitors of α-amylase has been reviewed by several authors. A variety of plants have been reported to show α-amylase inhibitory activity (Boivin et. al., 1988; Le Berre-Anton et. al., 1997; Prashanth et. al., 2001; Kobayashi

et. al., 2003; Conforti et. al., 2005; Karthic et. al., 2008), especially against the porcine pancreatic amylases (McCue and Shetty, 2004)

Elliott and Leopold (1953) postulated and proved the activation and inhibition of alpha-amylase during the germination *Avena sativa*. Verbeek and Dumitru (1964) also reported the same in barley. Black and Altschul (1965) reported the inhibition of a-amylase by aflatoxin from Aspergillus species. McGeachin and Reynolds, (1961); McGeachin et. al., (1961; 1966; 1968) in an attempt to study relationships between amylases of crude tissue extracts in a variety of animal species, have concluded that the amylase enzyme, is immunologically and structurally distinct depending on its source (hepatic, pancreatic, salivary or serum amylases).

Marshall, (1975) compiled a review on the detection, assay, specificities, properties, and physiological effects of plant derived proteinaceous inhibitors of the digestive enzyme, α-amylase. Granum,(1979) studied alpha amylase inhibitors found in daily consumed food and their activity after heat processing, which was seen to drastically decrease the activity as compared to the non treated food. Layer et. al., (1986), reported a crude bean-derived amylase inhibitor ("starch blocker") that contained minimal anti-amylase activity without any modification in carbohydrate assimilation in normal subjects and in patients with non-insulin-dependent diabetes mellitus.

α-amylase is an important digestive enzyme secreted by the salivary glands and the pancreas that converts starches into glucose in the small intestine. Currently, the method to determine the levels of α-amylase inhibitor is based on the measurement of α-amylase activity resulting by the different iodine staining power in the presence or

absence of an inhibitor during the action of the enzyme on soluble starch or by using an alkaline reactive whose brown reduction products are determined photometrically as reported by Bernfeld, (1955). Crude plant extracts are generally tested for inhibition of α-amylase activity, using an enzyme-starch system (Ou et. al., 2001).

1.25 Amaranthaceae

A survey of literature with reference to species of Amaranthaceae used traditionally for antidiabetic actions is given in summarised form in the following Table 2.

Table 2: Species of Amaranthaceae Reported in Literature to Be Used Traditionally to Treat Diabetes

Species	Distribution	Part Used	Comments about Activity
Achyranthes aspera L.	Africa, India, Australia	All	Lowers blood glucose levels in alloxan-treated rabbits (Akhtar and Iqbal, 1991) may act by providing certain necessary elements like Ca, Zn, Mg, Mn, and Cu to the β-cells.
Aerva sanguinolenta Blume	S.E. Asia	All	Reported to have antidiabetic activity (Marles and Farnsworth, 1995)
Atriplex halimus L.	Middle East	Leaf	Extract is reported to have an insulin-potentiating effect (Shani et al., 1972)

Beta vulgaris L.	International	Root, leaf	Betavulgarosides II and IV and oleanane triterpenoid saponins have hypoglycemic activity (Murakami et. al.,1999) Extracts reduce serum lipids, sialic, and uric acid, glucose, and lipid peroxidation in rats. The extracts protect the liver (Ozsoy–Sacan et. al., 2004)
Chenopodium ambrosioides L.	India	Leaf	Used to treat diabetes (Neame and Pillay, 1964). Hypoglycemic activity in high-fat fed STZ diabetic Mice (Song et. al., 2011)
Hammada salicornica **R.Br**.	Arabia	Aerial parts	Reduces blood glucose in alloxan treated mice (Ajabnoor et. al., 1984) and dogs (Hand et. al., 1989)
Spinacia oleracea **L.**	Africa, Europe, India	Leaf	Extracts possess antidiabetic activity (Marles and Farnsworth, 1995). Reduces hyperglycemia in alloxan

			diabetic rats (Kumar and Longathan, 2010) (Gomathi et. al., 2010)
Amaranthus spinosus	India	Leaf	Reduces elevated blood glucose in STZ treated rats (Sangameswaran and Jayakar, 2008)
Celosia argentia	India	Seeds	Reduces the increase of blood glucose in alloxan-induced diabetic rats (Vetrichelvan et. al., 2002), (Shan et. al., 2005). Reduces elevated blood glucose in STZ treated rats (Ghule et. al., 2010)
Amaranthus caudatus	India	Leaf	Reduces elevated blood glucose, body weight changes, and improves lipid profile of STZ induced rats. (Girija et. al., 2010)

var. Oscar blanco		Seed	Inhibition against alpha amylase by Methanolic, Ethyl acetate, and Hexane extracts (Conforti et. al., 2005).
var. Victor red		Seed	Inhibition against alpha amylase by Methanolic, Ethyl acetate, and Hexane extracts (Conforti et. al., 2005).
Amaranthus viridis	India	Leaf	Reduces elevated blood glucose, body weight changes, and improves lipid profile of STZ induced rats. (Krishnamurthy et. al., 2011)
Amaranthus esculantus		Whole Plant	Reduces hyperglycemia and improves insulin secretion (Kim et. al., 2006)

1.26 *Amaranthus tricolor*

1.26.1Scientific Nomenclature

Order:	*Caryophyllales*
Family:	*Amaranthaceae*
Subfamily:	*Amaranthoideae*
Genus:	*Amaranthus*
Common Name:	Lal Saag, Tambdi Bhaji, Amaranth, Chinese spinach, Pigweed, Tampala, Joseph's coat.

1.26.2 Vernacular Names:

Assamese:	Bishalya karani, Bishalya
Bengali:	Dengua
Hindi:	Lal sag, Chaulai
Kannada:	Dantina soppu, Chikka harive soppu
Marathi:	Chavalaayi, Ranmaath- Tambada maath
Oriya:	Bajjisag
Sanskrit:	Alpamarisa, Marsha, Meghanada, Panyasaka, Raktatandula
Tamil:	Thandukkeerai, Cherikkirai
Telugu:	Thotakoora, Bhadara Dantu koora
Malayalam:	Cheera, Chenjeera

Fig 5: *Amaranthus tricolor* – Representative plant.

a. b.

Fig 6: Leaves of the *Amaranthus tricolor* plant – a. Lancet shape; b. Ovoid shape.

1.26.3 Geographical Distribution and Habitat

The genus Amaranthus has a worldwide distribution and is most abundant in the tropics. It is not plentiful in countries with a cold climate. The main vegetable type of amaranth, *Amaranthus tricolor* is said to have originated from South or South East Asia (Grubben and Sloten, 1981) and from there has been carried and spread through the tropics and the temperate zone (Martin and Telek, 1979) by emigrants.

It is the most widespread species, cultivated frequently in lndia, Indonesia, and the Pacific islands to Papua-New Guinea, in the North to Japan, Northen China, seldom in West Africa. It has been introduced more often by people from Asia to other regions of the world, e.g. to the USA: It might have also escaped as weed. Many species of amaranth are grown as vegetables throughout the tropics and Eastern Asia (Feine et. al., 1979). Though only *A. tricolor* has been extensively cultivated, primarily in Southern China (Martin and Ruberte, 1979).

1.26.4 Description

Amaranthus tricolor is an erect, tropical, annual herb with long-stemmed leaves and small black seeds, which can attain a maximum height of 5 feet. The leaves are elliptical to lance-shaped or broad-ovate, dark green, light green or red and can attain a length of upto 5 inches. The leaves are notched or rounded at the tips. Clusters of flowers are axillary. often spherical or slightly spherical, and with a reduced terminal spike, but occasionally the terminal spike is well developed. There are three sepals. The fruit is dehiscent with a dehiscing lid. The seeds are black and relatively small with about 1200-2900 seeds/gram.

It is a quantitative short-day plant, which is an advantage in the sub-tropics where the generative stage is retarded during summer. Due to rapid growth, water consumption is

high. The crop normally uses about 6 mm/day and prefers fertile, well drained soils with a loose structure. The mineral uptake is very high.

1.26.5 Culinary Uses

Amaranthus tricolor is consumed on a daily basis as a stir fry, in soups, curries or salads and has a delicious, slightly sweet flavour. Goan delicacies prepared using this leafy vegetable include steaming the leaves plain, or with grated coconut, or with jackfruit seeds or dal and is also consumed as a soup. It is normally cooked in the same way spinach is cooked. The leaves and the softest portions of the shoots are usually boiled in several changes of water and then separated from the cooking liquid (Martin and Telek, l979), though they traditionally are steamed in Uganda (Stafford et. al., 1976). *A. tricolor* leaves are combined with condiments to prepare soup in Nigeria (Okiei and Adamson, 1979; Oke, 1983); used in salads, boiled and mixed with a groundnut sauce in Mozambique (Oliveira and de Carvalho, 1975); or pureed into a sauce and served over (farinaceous) vegetables in West Africa (Martin and Telek, 1979).

The flavor of raw and cooked vegetable amaranth was reported as equal to or better than that of spinach or other similar greens (Martin and Ruberte, 1979; Daloz, 1980; Abbott and Campbell, 1982). Chinese spinach (*A. tricolor*), is commonly heat processed (Keshinro and Ketiku, 1979; Wills et. al., l984); typical processes include cooking, steaming, blanching, stir-frying, and baking (Ajayi and Osibanjo, 1980; Oke, 1983; Saunders and Beciker, l984; Stafford et. al., 1976; Bassir and Fafunso, 1975).

Singh et. al., (2009) reported that products like biscuit, mathi, matar and sev supplemented with *Amaranthus tricolor* leaf powder had significantly higher protein, fat, ash and fibre

contents as compared to their control. Consumption of such value added products would contribute in improving the nutritional status of the population especially the vulnerable section.

1.26.6 Therapeutic Uses

Amaranthus tricolor possesses a variety of medicinal values. A survey of folk literature revealed the following claims; some of which are being practiced till date. Goan folklore suggests the plant to be highly nutritious, especially for convalescing and anaemic patients. Consumed in the capacity of a liver tonic, the plant is also said to have a cooling effect on the body and its consumption is recommended during the summer months due to its diaphoretic nature. It is highly recommended as a good food with medicinal properties for young children, lactating mothers and for patients with fever and anemia.

The leaves and stem are said to be purgative by nature, and are used in poultices (fresh or as dried powder) to treat inflammations, boils and abscesses, gonorrhoea, orchitis and haemorrhoids. lt is said to prevent calcium retention. It is also used as a diuretic and in treatment for bladder distress. The whole plant is astringent in nature (Chopra et. al., 1956) and a decoction of the root is said to be used with *Cucurbita moschata* to control haemorrhage follorving an abortion (Duke and Ayensu, 1985).

A decoction of very old plants is taken internally to improve vision and strengthen the liver. Its use as a *antihelmintic* has been mentioned in some places the decoction of the plant is highly recommended in severe menorrhagia, and has also been found beneficial in diarrhoea, dysentery and hemorrhage from the bowels. It has likewise been used as a local

application in ulceration of the mouth and throat, in leucorrhoea, and as a wash to foul, indolent ulcers (Chopra et. al., 1956). However it is scarcely used at the present day.

The fruits of *Amaranthus tricolor* are chewed by tribals for cure of cold and cough (Rai and Nath, 2003). The roots of *Amaranthus tricolor* are useful in dysentery. Due to it fibrous nature it aids in digestion and rectifies constipation. It is said to purify the blood after childbirth, strengthen hair roots, and increase blood volume. In some regions a puree of the leaves is applied to venomous animal bite wounds (Buragohain, 2011). The leaves are believed to have febrifugal properties. Tribals in the Chatthisgarh district claim that consumption of this plant on a daily basis aids in weight loss and regulates menstrual disorders and spotting.

It is well known for curing a variety of ailments such as throat infections, toothache, eczema, gonorrhoea, leucorrhoea and impotence. Ethnobotanically the herb is used as blood purifier, tonic in dropsy, as an ascaricide, earaches, sore throat, and bronchitis. The roots, leaves and stems are eaten in bilious disorders. Roots and seeds are used in impotence, against colic, gonorrhea, eczema and have galactagouge properties. A decoction of the *A. tricolor* roots with *Cucurbita pepo* Linn. is used to control hemorrhages following abortion. Roots are considered as demulcent and in the form of decoction used for piles and diarrhoea in children (Kirtikar and Basu, 2003).

In older texts *Amaranthus tricolor* is also referred to as *Amarantthus gangeticus* var. melancholicus, which refers to the history of the medical use of this plant in the treatment of choleric disorders. In Ayurveda the plant is said to pacify vitiated vata, pitta, constipation, urinary retention, edema, anemia, reduced eye sight, menorrhagia. leucorrhea, and as a stimulant and tonic for general weakness.

A survey of available literature reveals that *Amaranthus tricolor* possesses hepatoprotective in carbon tetrachloride induced liver cirrhosis (Al-Dosari, 2010). An antiviral protein that imparts high resistance to sunnhemp rosette virus has been purified from the dried leaves of *A. tricolor* (Roy et. al., 2006). Potent cyclooxygenase and human tumour cell growth inhibitory activities have also been identified from the leaves and stems of *A. tric*olor (Jayaprakasam et. al., 2004).

An 800mg/kg BW dose of an alcoholic extract of *A. gangeticas* leaves for a period of two weeks, before radiation exposure, significantly protected the normal functioning of the biochemical activities in the brain of inbred Swiss albino mice, thus proving to be beneficial for clinical use as a radioprotector (Verma et. al., 2002).

Various extracts of *Amaranthus tricolor* leaves at a dose of 200mg/kg BW, were seen to have very good antiulcers properties when tested on five different experimental models of gastric ulcers (Devaraj and Krishna, 201l). The plant as a whole is consumed as a pot-herb and is considered to be wholesome.

1.26.7 Ayurvedic properties of *Amaranthus tricolor*

Rasa : Kashava. Madhura

Guna :Guru, Snigdha

Virya : Seeta

1.26.8 Other Uses

In many areas *Amaranthus tricolor* is used as fodder or as ornamental plants. The red dye obtained from the mature leaves of *A. tricolor* leaves are used to colour alcoholic

beverages in Bolivia and northwestern Argentina, to colour maize dough in Mexico and the southwestern United States (Sauer, 1950), and to dye foods and beverages in Ecuador (Jain and Hauptli, 1980). This dye is sourced from red-violet pigments - the betacyanins amaranthin and isoamaranthin (Piatelli et. al., 1969). Dye use seems limited to cultures that do not grow amaranth as a grain crop (Sauer, 1950). Yellow and green dyes can also be obtained from the whole plant (Grae, 1974).

1.26.9 Phytochemical Constituents

Amaranthus tricolor is said to be a rich source of protein, which is highly essential to the growth and development of any living organism. The plant is also a rich source of β-carotene, zeaxanthin, lutein, violaxanthin-neoxanthin (Wills and Rangga. 1996). It is rich in minerals (calcium, iron, magnesium, phosphorus, potassium, zinc, copper and manganese) and vitamins (vitamin A, vitamin B6. vitamin C, riboflavin and foliate)(Shukla et. al., 2006, Rao et. al., 2010) Jerz et. al., (2007) isolated two chlorophyll breakdown products l32-hydrory-(132-S)-phaeophytin-a and chlorophyll-b methoxylactone by preparative high-speed countercurrent chromatography' from lipophilic extracts of the aerial parts of *A. tricolor*.

The major unsaturated fatty acids found to be present in *A. tricolor* are linoleic acid in seeds (49%), stems (46%) and linolenic acid in leaves (42%), while the major saturated fatty acid in seeds, stems, and leaves is palmitic acid at l8-25% of total fatty acids (Fernando and Bean-1984). Mature leaves of *A. tricolor* contain red-violet pigments, the betacyanins amaranthin and isoamaranthin (Piatelli et. al., 1969). Carbohydrates, glycosides, phenolic compounds, flavonoids, saponins, steroids, steroidal glycosides, tannins, polyphenols, sterols and lipids are present in the plant (Rao et. al., 2010; Clemente

and Desai, 2010). Vitamins C and A are present at nutritionally significant levels, averaging 420 ppm of vitamin C and 250 ppm of §-carotene (Wills et. al., 1984). Trace quantities of vitamin B-12 like activities were also found in *A. hypochondriacus* leaves, though the exact nature of this activity could not be concluded (Jathar et. al., 1974). Minerals such as potassium, iron, magnesium, and calcium (Table-6) exist also in significant concentrations, with average values of 287 ppm of iron and 2.1 % calcium (dry matter). The presence of large amounts of oxalate(s) ranging from 0.2 to 11.4% (dry weight), may limit availability of these nutrients. It also contains large amounts of protein and essential amino acids, such as lysine (De Macvean and PÖll, 2002).

1.27 The Challenge

The aforementioned composition, properties and historical, current and future applications of *Amaranthus tricolor* demonstrates the food potential of this underutilized and underestimated crop. Nevertheless, there are still lacunae present in the role of amaranth as an economically important plant with untapped medicinal value-mainly because of lack of sufficient experimental data.

1.28 Lacunae

The survey of literature and the literature on diabetes clearly indicate the increasing need to develop novel, safe, and efficacious botanical therapeutics for diabetes which is one of the major human diseases. Not only this, one needs to keep on exploring more and more plant materials to mitigate diabetes and its perils. Therefore *Amaranthus tricolor*, a traditionally used plant, known for its nutritional value was selected to study antidiabetic effects.

1.29 Aim, significance and objectives of the study

The main aim of the study was to screen the leaf extracts of *Amaranthus tricolor* for antidiabetic activity and to investigate the possible mechanism (s) of action whereby the aqueous and methanolic leaf extracts of *A. tricolor* exerts blood glucose lowering effects or properties. In a pilot study conducted a crude extract of *Amaranthus tricolor* showed promising antidiabetic activity in alloxan mediated diabetic mice. Such interesting claims warranted further study of the effect of the extracts on the major organs of the body, to check for any deleterious or beneficial side effects.

Hence the present study was undertaken to –

- ✓ Screen various extracts of *Amaranthus tricolor* leaf for antidiabetic activity and any beneficial or deleterious effects associated with its regular consumption.
- ✓ To explore the mechanism of antidiabetic action.

The objectives of the study to achieve the above mentioned goals were:

1. To induce diabetes through i.p injection of alloxan for the purpose of antidiabetic studies (Acute and Sub-acute).
2. To prepare extracts of *A. tricolor* using various solvents.
3. To administer extracts to various groups to evaluate glucose levels at specific intervals.
4. To analyse effects of vehicle and extracts.
5. To analyse extracts for phytochemical constituents beginning with separation of components through column fractionation.
6. To administer the fractionated components to various groups to evaluate glucose levels at specific intervals.

Chapter 2

Materials & Methods

The methods that were followed in order to achieve the objectives of this study are divided into two main sections in this chapter - Plant Studies and Animal Studies.

2.1 Plant Studies

2.1.1 Materials used in this study

2.1.1.1 Plant material

Fresh *Amaranthus tricolor* (Amaranthaceae) plants were purchased from various local markets in Goa, between January 2007 and April 2012. The samples were selected based on their freshness, and quality of leaves. The identity of the plant was confirmed by Dr. Janarthanam, Department of Botany, Goa University. A herbarium sheet was prepared bearing access number (GUBH-PVAC-0515).

2.1.1.2 Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade. Organic solvents used for extraction of the plant material included chloroform, ethyl acetate, methanol, petroleum ether and hexane (all purchased from S.D. Fine Chemicals). Enzyme α-amylase, and substrate potato starch were purchased from Sigma Chemical Co St. Louis M.O., (USA). TLC plates Merck. Spray reagents, and plant standards were from SD fine chemicals

2.1.1.3 Apparatus and Equipment

General laboratory glassware including volumetric flasks, measuring cylinders, beakers, pestle and mortar, filter papers, test tubes, micro pipette, weighing balances and water

bath, all available in the Department of Zoology, Goa University, were used in the study. Specific equipment used in the study included both preparative and analytical thin layer chromatography (TLC) plates, TLC Visualization UV-Vis chamber, Rotavapor, Nanodrop spectrophotometer, and Shimadzu UV-Vis spectrophotometer, NMR, FTIR etc. The NMR studies were carried out in Shraddha Analyticals, Mumbai and the FTIR and melting point studies were conducted in the Department of Chemistry, Goa University.

2.1.2 Preparation of Herbarium Sheet

Materials required

1. 2% solution of mercuric chloride in 95% ethanol: 2 gms of mercuric chloride was dissolved in 100 ml of freshly prepared 95% ethanol.
2. Acid free paper
3. Enamel tray
4. Blotting papers
5. Heavy weight press

The fresh *Amaranthus tricolor* plant was rinsed with distilled water to wash off any mud or debris. The stem and roots of the plant was slit intermittently lengthwise. The plant was then placed in an enamel tray containing mercuric chloride solution for two hours. The plant was then lifted up and placed onto a stack of acid free blotting paper and arranged properly as it should appear on the herbarium sheet. Once the plant was placed on the sheet, another stack of blotting paper was placed on top of the plant,

forming a sandwich. This paper sandwich stack was then placed under a constant heavy weight and left undisturbed for 48 to 72 hours to press and dry. The pressed plant was then mounted onto the herbarium sheet carefully and fixed into place with glue and if required plastic holders. An identification tag was pasted onto the sheet in order to log the details of the plant collected.

2.1.3 Processing of the plant material

The fresh plants were collected from the local farmers, during the harvesting period, which is roughly 20 to 30 days after seeding. The plants were brought back to the laboratory and washed free of mud with normal tap water thoroughly with a final rinse of distilled water.

2.1.4 Fresh plant material

The leaves were plucked and rinsed ten times of any debris or dirt, in distilled water. The leaves were then patted dry with blotting paper and used for the experiments.

2.1.5 Dried plant material

The rinsed leaves were dried overnight in an oven at 40°C (not exceeding 50°C) following the suggestion by Khamsah et. al., (2006). The dried leaves were milled into a fine powder using a dry grinder and sieved to yield particles of uniform size.

2.1.6 Preparation of Plant Extracts

2.1.6.1 Preparation of Aqueous Extract (AE)

The fresh leaves were blended with cold distilled water for 3 min having solid:liquid ratios of 1:3 (AE 200) and 1:1.5 (AE 400). In each the slurry was strained through a cheese cloth folded eight times to sieve out all fibres and yield the aqueous extract. The residue was re-blended and all filtered juice combined. One millilitre of the extract was dried in an oven at 40°C to a constant weight to determine the yield of extract present in 1 ml distilled water.

2.1.6.2 Preparation of Aqueous Extract from dried plant material (AD)

The aqueous extract from dried leaves (AD), was prepared by boiling ten grams of dry leaf powder with 100 ml of water for one hour and the resulting decoction was filtered as mentioned above.

2.1.6.3 Preparation of Methanolic Extract (ME)

50 gms of dried powder of plant leaves was weighed and transferred into a 1000 ml conical flask. 800 mL of solvent (absolute methanol) was added into the beaker and the plant-solvent mixture was left to macerate for 48 h at 27-28°C with intermittent shaking to aid the extraction process. Each extract was filtered using Whatman No.1 filter paper. The filtrate was collected and the residue was re-extracted twice. The two extracts were then pooled. The solvent (absolute methanol) in the extract was removed under reduced pressure at 40°C using rotary evaporator and evaporated separately at 40°C and the dried extracts were reconstituted (one mg/ml) with the

respective solvents before using for the assays. The extracts were stored in glass vials at 4℃ until further use.

2.1.6.4 Preparation of Methanolic Extract (Soxhlet) (MS)

For the methanolic soxhlet extract (MS), ten grams of dried leaves' powder was soxhletted with 100 ml of methanol at 70℃ for one hour and the resulting extract was filtered evaporated separately at 40℃ and the dried extracts were reconstituted (one mg/ml) with the respective solvents before using for the assays. The extracts were stored in glass vials at 4℃ until further use.

2.1.6.5 Preparation of Petroleum Ether Extract (PE)

Petroleum ether extract (PE) was obtained by mixing ten grams of dry leaves' powder with 100 ml of petroleum ether at 70℃ for four hours and the resulting extract was filtered and evaporated separately at 40℃ and the dried extracts were reconstituted (one mg/ml) with the respective solvents before using for the assays. The extracts were stored in glass vials at 4℃ until further use.

2.1.7 Phytochemical Work

2.1.7.1 Determination of extraction yield (% yield) (Ogunsina et. al., 2011)

The yield (%, w/w) from the dried extracts was calculated as:

$$\text{Yield } (\%) \quad = \frac{W1}{W2} \times 100$$

Where, W1 is the weight of dried extract after evaporation of solvent and W2 is the weight of plant powder.

2.1.7.2 Qualitative phytochemical work

The below mentioned qualitative chemical tests were performed for establishing the phytochemical profile of the leaves/aerial portion of *Amaranthus tricolor*. These qualitative determinations were carried out using standard analytical procedures as described by Raaman (2006).

2.1.7.2.1 Detection of alkaloids (Raaman, 2006)

Solvent free extract, 50 mg was stirred with a few ml of dilute HCl and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows:

A. Mayers test

To a few ml of filtrate a drop or two of Mayer's reagent was added by the side of the test tube. A white creamy precipitate indicated the test as positive. The Mayer's reagent was prepared as below:

Mayer's Reagent: Mercuric chloride (1.358 gms) dissolved in 60 ml of water and potassium iodide (5 gms) was dissolved in 10 ml of water. The two solutions were mixed and made up to 100 ml with water.

B. Wagner's test

To a few ml of filtrate, a few drops of Wagner's reagent were added by the side of the test tube. A reddish brown precipitate confirmed the test as positive. The Wagner's reagent was prepared as below.

Wagner's Reagent: Iodine (1.27 gms) and potassium iodide (2 gms) was dissolved in 5 ml of water and made up to 100 ml with distilled water.

C. Hager's test

To a few ml of filtrate, 1 or 2 ml of Hager's reagent (saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicated positivity of the test.

D. **Dragendorff's test**

To a few ml of filtrate 1 ml of Dragendorff's reagent was added. A prominent yellow precipitate indicated the test as positive. The Dragendorff's reagent was prepared as below.

Dragendorff's reagent: Stock solution: Bismuth carbonate (5.2 gms) and sodium iodide (4 gms) were boiled for a few minutes with 50 ml of glacial acetic acid.

After 12 hours the precipitated sodium acetate crystals were filtered off using a sintered glass funnel. Clear red brown filtrate 40 ml was mixed with 160 ml ethyl acetate and 1 ml water and stored in an amber coloured bottle.

Working solution: Ten ml of stock solution was mixed with 20 ml of acetic acid and made up to 100 ml with water.

2.1.7.2.2 Detection of Carbohydrates (Raaman, 2006)

The extract 100 mg was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests.

A. Fehling's test

One ml of filtrate was boiled on a water bath with 1 ml of each of Fehling's A and B. A red precipitate indicated the presence of sugar. The Fehling's A and B reagents were prepared as below.

Fehling's A: Copper sulphate (34.66 gms) was dissolved in distilled water and made up to 500 ml using distilled water.

Fehling's B: Potassium sodium tartarate (173 gms) and sodium hydroxide (50 gms) were dissolved in water and made up to 500 ml.

B. Barfoed's test

To a 1 ml of filtrate, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 min. Red precipitate indicated the presence of sugar. The Barfoed's reagent was prepared as below.

Barfoed's reagent: Copper acetate (30.5 gms) was dissolved in 1.8 ml of glacial acetic acid.

C. Benedict's test

To a 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minute. A characteristic coloured precipitate indicated the presence of sugar. The Benedict's reagent was prepared as mentioned below.

Benedict's reagent: Sodium citrate (173 gms) and sodium carbonate (100 gms) were dissolved in 800 ml of distilled water and boiled to make it clear. Copper sulphate (17.3 gms) dissolved in 100 ml distilled water was added to it.

2.1.7.2.3 Detection of glycosides (Raaman, 2006)

50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate was subjected to the following tests.

A. Borntrager's test (Evans, 1997)

To a 2 ml of filtered hydrolysate, 3 ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Development of pink colour indicated the presence of glycosides.

B. Legal's test

Fifty mg of the extract was dissolved in pyridine and sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycoside was indicated by development of a pink colour.

2.1.7.2.4 Detection of saponins (Raaman, 2006)

The extract (50 mg) was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A 2 cm layer of foam indicated the presence of saponins.

2.1.7.2.5 Detection of proteins and Amino acids (Raaman, 2006)

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

A. Millon's test

To a 2ml of filtrate, few drops of Millon's reagent were added. A white precipitate indicated the presence of proteins. The Millon's reagent was prepared as below.

Millon's reagent: Mercury (1gm) was dissolved in 9 ml of fuming nitric acid. When the reaction was completed, equal volume of distilled water was added.

B. Biuret test

An aliquot of 2 ml of filtrate was treated with one drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicated the presence of proteins.

C. Ninhydrin Test for amino acids

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) were added to two ml of aqueous filtrate. A characteristic purple colour indicated the presence of amino acids.

2.1.7.2.6 Detection of Phytosterols (Raaman, 2006)

Libermann-Burchard's test

The extract (50 mg) was dissolved in 2 ml acetic anhydride. To this one or two drops of concentrated sulphuric acid was added slowly along the sides of the test tube. An array of colour change showed the presence of phytosterols.

2.1.7.2.7 Detection of Fixed Oils and Fats (Raaman, 2006)

A. Spot test

A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil.

B. Saponification test

A few drops of 0.5 N alcoholic potassium hydroxide solution were added to a small quantity of extract along with a drop of phenolphthalein. The mixture

was heated on water bath for 2 hrs. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

2.1.7.2.8 Detection of Phenolic Compounds and Tannins (Raaman, 2006)

A. Ferric chloride test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

B. Gelatin test

The extract (50 mg) was dissolved in 5 ml of distilled water and 2 ml of 1% solution of gelatin containing 10% sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

C. Lead Acetate test

The extract (50 mg) was dissolved in distilled water and to this, 3 ml of 10%lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

D. Alkaline reagent test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

E. **Magnesium and hydrochloric acid reduction**

The extract (50 mg) was dissolved in 5 ml of alcohol and a few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) were added. When any pink to crimson colour developed, presence of flavonol glycosides was inferred.

2.1.8 Quantitative phytochemical work

2.1.8.1 Determination of phytosterols (Libermann-Buchard method)

Quantitative determination of phytosterols was carried out by the Libermann-Buchard method (Shahidi, 2001) using β-sitosterol (1mg/ml) as the standard.

Reagents

1) **Std. steroid solution**, β-sitosterol (Concentration 1.0 mg/ml)
2) **Preparation of Libermann-Burchard reagent**

   The following reagents were mixed in cold condition to give the final reagent.

   i) 60 ml acetic anhydride

   ii) 30 ml acetic acid

   iii) 10 ml conc. sulphuric acid
3) **Glacial acetic acid**

Sample preparation

1 g sample was accurately weighed and placed in a Soxhlet apparatus. The sample was extracted with 60 ml of chloroform. Extraction was carried out for 2 hours. The chloroform extract thus obtained was concentrated to 50 ml.

Protocol

1 ml of concentrated chloroform extract was transferred into a 10 ml volumetric flask followed by the addition of 0.5 ml of glacial acetic acid and 5 ml of Libermann-Burchard reagent. The volume was made up to 10 ml with chloroform. The optical

density (O.D) taken at 618 nm after 20 min. at R.T. Standard curve for sterols was obtained by using β-sitosterol as standard phytosterol. 10 mg of β-sitosterol was accurately weighed in 10 ml volumetric flask and dissolved in chloroform. The volume was made to 10 ml with chloroform.

From the above solution of standard phytosterol 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml were taken into six different 10 ml volumetric flasks. In each flask, 0.5 ml of glacial acetic acid was added followed by 5 ml Libermann-Burchard reagent and the volume was made up with chloroform. The O.D's was taken at 618 nm after 20 min. at R.T. The graph of concentration vs. absorbance was plotted.

2.1.8.2 Total Phenolic Content Assay (Tannic Acid Equivalence Method)

The total phenolic content of the plant extracts were measured using the Folin-Ciocalteu method as described by Amin et. al., (2004). All samples and readings were prepared and measured in triplicate.

Reagents

1) **Standard stock solution of tannic acid (1 mg/mL):** 10 mg of dry tannic acid was dissolved in 10 ml of extracting solvent and further diluted to 500 ml with DW. The stock solution was stored at 4°C.
2) **Working standards of tannic acid:** Working standards of tannic acid (0.01-0.05 mg/mL) were prepared by diluting the stock solution with DW.
3) **Folin-Ciocalteau reagent:** Folin-Ciocalteau reagent was diluted 10-fold with deionised water. To 1 ml of Folin-Ciocalteau reagent 9 ml of deionised water was added. The solution was prepared afresh everytime.

Sample preparation

The extracts were prepared at concentrations of 1 mg/ml.

Protocol

In a test tube, 100 µl of extract was added to 0.75 ml of Folin-Ciocalteu reagent and mixed. The mixture was allowed to stand at R.T for 5 min. Then, 0.75 ml of 6% (w/v) Na_2CO_3 was added to the mixture and mixed gently. After standing at R.T for 90 min, the absorbance was read at 725 nm using the Nanodrop UV-Vis spectrophotometer. The standard calibration curve of tannic acid (0.01–0.05 mg/ml) was plotted.

2.1.8.3 Assay of tannins (Raw hide method) (W.H.O., 1998)

To prepare the plant material extract, introduce the quantity specified in the test procedure for the plant material concerned, previously powdered to a known fineness and weighed accurately, into a conical flask. Add 150 ml of water and heat over a boiling water bath for 30 minutes. Cool, transfer the mixture to a 250 ml volumetric flask and dilute to volume with water. Allow the solid material to settle and filter the liquid through a filter paper, diameter 12 cm, discarding the first 50 ml of the filtrate.

To determine the total amount of material that is extractable into water, evaporate 50 ml of the plant material extract to dryness, dry the residue in an oven at 105°C for 4 hours and weigh (T_1). To determine the amount of plant material not bound to hide powder that is extractable into water, take 80 ml of the plant material extract, add 6

gm of hide powder R and shake well for 60 minutes. Filter and evaporate 50 ml of the clear filtrate to dryness. Dry the residue in an oven at 105°C and weigh (T_0).

The quantity of tannins was calculated as percentage by using the following formula:

$$\frac{[T1 - (T1 - T0)]}{\omega} \times 500$$

Where, ω = weight of the plant material in grams.

2.1.8.4 Determination of lipids (AOAC, 1996)

1 gm of dried leaf powder sample was accurately weighed and placed in a Soxhlet apparatus. The sample was extracted with 70 ml of pet-ether. Extraction was carried out for 4 h. The pet-ether extract thus obtained was evaporated. The dried residue was weighed and the lipid content was determined in terms of pet-ether extract.

2.1.9 In Vitro Assays

2.1.9.1 Antioxidant activities

Plants proven to have antioxidant activities are known to have potent free radical scavenging activities.

2.1.9.1.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay (Blois, 1958)

Reagents

1. **0.1% methanolic DPPH**

   100 mg DPPH was dissolved in 50 ml of methanol. After DPPH dissolved the solution was made up to a total volume of 100 ml. The solution was prepared fresh and stored in an amber colour bottle.

2. **Butylated Hydroxy Toluene (0.16% BHT)**

   160 mg BHT was dissolved in 100 ml of methanol. The solution was stored in an amber colour bottle.

Sample preparation

The evaporated extracts were dissolved in methanol in the concentration of 1mg/ml which was used to determine its antioxidant activity.

Protocol

Qualitative Assay:

To 50 µl of plant extract, 100 µl of 0.1% methanolic DPPH was added in a microplate well and incubated for 30 min at R.T. in the dark. The wells containing samples were observed for any signs of discoloration ranging from purple to yellow and to pale pink colour changes were considered as strong and weak positive reactions respectively. The antioxidant positive samples were then subjected to further quantitative analysis. Each assay was repeated in triplicate.

Quantitative assay:

2.7 ml of methanol was added to 100 µl of plant extract in a test tube, followed by the addition of 200 µl of 0.1 % Methanolic DPPH. The control tubes were prepared by adding 200 µl of 0.1% Methanolic extract to 2.8ml of Methanol. The suspension was incubated for 30 min at R.T. in the dark and the absorbance of the solutions was read at 517 nm. The antioxidant activity of the sample was compared with known standard 0.16 % BHT.

The DPPH radical scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition } = \frac{100(C - S)}{C}$$

Where, C and S are the absorbance of control and test sample respectively, at 517 nm.

2.1.9.1.2 FRAP (Ferric Reducing Ability Power) Assay (Benzie and Strain, 1996)

Reagents

3. **Working FRAP reagent**
    - 200 ml of 300 mM sodium acetate.$3H_2O$, pH 3.6
    - 20 ml of 10 mM TPTZ (freshly prepared)
    - 20 ml of 20 mM $FeCl_3$
    - 24 ml DW

    The above solutions were mixed well and brought to 37ºC before performing the assay.

4. **1 mM $FeSO_4.7H_2O$**

    0.278g $FeSO_4.7H_2O$ was dissolved in 1L of DW

Protocol

To 1.5 ml of freshly prepared working FRAP reagent, 50 µl of plant extract was added and vortexed for 30 seconds. The absorbance was then read at 593 nm at 0 min. Thereafter the samples were placed in a water bath at 37ºC. The absorbance was read after 4 min. The standard ($FeSO_4$) tubes were processed in the same way.

FRAP activity was calculated according to the following equation:

$$\text{FRAP value} = \frac{Sa \times FS}{St}$$

Where, Sa and St are the changes in absorbance of the sample and standard respectively, between 0 and 4th min, and FS is the FRAP value of the standard.

2.1.9.1.3 Phosphomolybdenum assay (Prieto et. al., 1999)

Reagents

- **0.6 M H_2SO_4 acid:** 1.7 ml H_2SO_4 was dissolved in 98.3 ml of DW.
- **Phosphomolybdenum reagent:** 40 mg $NaPO_4$ and 49 mg ammonium molybdate was dissolved in 10 ml of 0.6M H_2SO_4.
- **4% Oxalic acid:** 4 gm Oxalic acid was dissolved in 100 ml DW.
- **Stock Standard solution:** 50 mg of ascorbic acid was dissolved in 50 ml of 4% oxalic acid.
- **Working Standard solution:** 5 ml of ascorbic acid stock standard solution was dissolved in 45 ml of DW.

Protocol

0.1 ml of extract (100-1000µg/ml) was mixed with 0.9 ml of DW in a test tube. To this solution 1 ml of phosphomolybdenum reagent solution was added and mixed well. The test tubes were incubated at 95°C for 90 minutes. The solutions were cooled to R.T. and the absorbance read at 695 nm against a reagent blank.

The antioxidant capacity was expressed as Ascorbic Acid Equivalent (AAE).

Calculation:

$$\frac{OD\ of\ sample\ \times\ Conc\ of\ standard}{OD\ of\ standard}$$

2.1.9.2 Porcine pancreatic α-amylase inhibition

The aqueous and methanolic extracts were tested for inhibition against porcine pancreatic α-amylase following the method of McCue & Shetty (2005).

Reagents

1) **20 mM Sodium Phosphate Buffer, containing 6.7 mM NaCl, pH 6.9 at 20°C (**Buffer**):** 100 ml of the buffer was prepared in purified water by adding 0.24 gms Sodium Phosphate, Monobasic, Anhydrous, and 0.04 gms NaCl. The pH was adjusted to 6.9 at 20°C with 1 M NaOH.
2) **1.0% (w/v) Soluble Starch Solution (**Starch**):** 1 gm of potato starch was added to 25 ml of the above mentioned Sodium Phosphate buffer, containing 6.7 mM NaCl. Solubilization was facilitated by heating the starch solution in a glass beaker directly on a heating/stir plate with constant stirring. The solution was brought to a boil and maintained at the same temperature for 15 minutes. The starch solution was allowed to cool to room temperature with constant stirring. The starch solution was returned to its original volume (25 ml) by adding required amount of purified water. Aliquots for assay were dispensed for assay with constant stirring.
3) **2M NaOH:** 7.999 gms of NaOH was dissolved in 100 ml deionised water
4) **Na-K Tartrate Solution:** 12 gms of Na-K Tartrate, Tetrahydrate, was dissolved in 8 ml of heated (50-70°C) 2M NaOH, with constant stirring, till the solution cleared. Care was taken to see that the solution was not boiled.

5) **96 mM 3,5-Dinitrosalicylic Acid Solution:** 0.437 gms of 3,5-Dinitrosalicylic Acid was added to 20 ml of heated (50-70°C) purified water, with constant stirring, till the solution cleared. Care was taken to see that the solution was not boiled.

6) **Color Reagent Solution:** To a 12 ml of heated (50-70°C) purified water, the above mentioned **Na-K Tartrate Solution** was added, followed by **96 mM 3,5-Dinitrosalicylic Acid solution.** The beaker was swirled till reagents completely dissolved. The solution was stored at R.T in an amber bottle.

7) **0.2% (w/v) Maltose Standard (STD):** 0.2 gms of Maltose, Monohydrate was added to 10 ml purified water.

Protocol

In a series of test tubes, kept on ice, 5 mg of powdered PPA was added to 2.7 ml deionised water. For each plant extract, a volume equivalent to 40µg total phenolic content was added to the each of the test tubes except the blank. The pH of the mixture was adjusted to 6.9. The above mixtures were diluted to a total volume of 3 ml. The amylase-plant extract mixtures were incubated for 1h at R.T. with constant gentle shaking. For the control tubes 0.1 ml of deionised water or methanol was used in place of the extract. α-Amylase inhibitory activity was determined using starch as the substrate by the colorimetric reaction using 3,5-dinitrosalicylic acid (Bernfeld, 1955) The amylase inhibitory activity was calculated in terms of % inhibition defined herein as the ratio of the amylase activity of the control (enzyme alone) to that of the enzyme extract mixture multiplied by 100.

2.1.10 UV-Vis Spectral Fingerprint of Aqueous and Methanolic Extracts

The AF and ME extracts were subjected to UV-Vis spectral scan (190 nm-1100 nm) on a UV-Visible spectrophotometer (UV-2450, Shimadzu, Japan). This was performed on full strength and serially diluted samples of the extract.

2.1.11 Chromatographic studies

2.1.11.1 Thin Layer Chromatography (TLC)

- TLC Plates (Merck Silica Gel 60 F_{254})
- Chromatographic glass chamber, with a tightly fitting lid (Borosil)
- TLC sprayer (Borosil)
- UV chamber having short (254 nm) and long (366 nm) wavelengths
- Rack for drying the TLC plates
- 5 ml Culture vials with tightly fitting cap (Borosil)

Preparation for TLC

Glassware: The glass culture vials used for preparation of the TLC samples were previously washed with distilled water, oven dried and rinsed 3 times with chloroform, and then air dried. The TLC chamber was saturated with the respective solvent system, by lining the walls of the TLC chamber with filter paper. The desired mobile phase solvent system was then poured into the chamber, to form a level of about 5 mm deep. The chamber was closed and allowed to stand for one hour at room temperature (25ºC).

Plant Sample: 10 mg of crude methanolic extract was weighed into a 5 ml glass culture vial and dissolved in 1 ml of chloroform by gently shaking for 2 min.

Choice of Solvent Systems

10 µl from the prepared TLC sample was spotted onto Merck Silica Gel 60 F_{254} TLC plates (3.5 cms × 10 cms). The trial and error method was used to determine the best solvent system for running TLC. The sample was chromatographed using various solvents as well as a combination of different solvents in varying ratios, ranging from polar to non polar, to find the best solvent system which renders the best separation. This solvent system was then applied to the column chromatography in order to separate the various compounds.

Development & Visualization of TLC plates using spray reagents

Reagents used:

- **10% Alcoholic KOH:** 10 grams of Potassium Hydroxide (KOH) was dissolved in 100 ml of methanol.
- **1 % $FeCl_3$:** 1 gram of Iron(III) chloride hexahydrate (FeCl3) was dissolved in 100 ml DW.
- **Vanillin-HCl:** 1 gram of vanillin was dissolved in 100 ml of hydrochloric acid (HCl) (37%)

2.1.11.2 Column Chromatography

The crude methanolic extract was run on a silica gel column (Mesh Size- 60-120), column length of 20 cms having inner diameter of 1.8 cms. The column was run at

25ºC with the following solvent ratios: 200 ml Hexane-100%, 200 ml Ethyl Acetate-Hexane -1:2 , 200 ml Ethyl Acetate-Hexane – 2:1, 200 ml Ethyl Acetate-Chloroform – 2:1, 200 ml Ethyl Acetate-Chloroform – 1:2, 200 ml Chloroform-100%. The column was then washed with 200 ml Methanol-100% to wash out any residue or remaining compounds, if any. 30 ml fractions were collected and similar fractions were pooled together after running TLC in a Hexane:Ethyl Acetate:Chloroform (1:3:3) solvent system.

2.1.12 Spectral studies of isolated powder (AT-C)

AT-C samples were checked for solubility in various solvents of varying polarities.

2.1.12.1 NMR

The samples were subjected to NMR analysis at 295ºC on a Varian 400 machine, after dissolving the sample in NMR grade D_20 and pyrimidine solvents.

2.1.12.2 FTIR

The isolated crystal was stored under vacuum. A few mg (not more than 300 mg) of dry IR-grade KBr was ground in an agate mortar until no evidence of crystallinity under an IR lamp. A very small quantity of sample, (1-2 mg), was added and ground into the KBr, until it was uniformly distributed throughout the KBr. The ground mixture was transferred to the pellet making die to make the pellet, which was loaded into the instrument and scanned.

2.1.12.3 UV-Vis Spectral Scan of Fraction No. 1:

The sample was dissolved in water, and a spectral scan was run on a UV-Vis Spectrophotometer (UV-Vis 2450 Shimadzu, Japan), against a water blank. The sample solution was scanned along the entire UV-Visible spectra.

2.1.13 UV-Vis Spectral Scan of Fraction No.2:

The sample was dissolved in chloroform, and a spectral scan was run on a UV-Vis Spectrophotometer (UV-Vis 2450 Shimadzu, Japan), against a chloroform blank. The sample solution was scanned along the entire UV-Visible spectra.

The spectra obtained of Fraction No. 1 and Fraction No. 2 were compared with available spectra in online literature and a spectral software "PhotoChem Cad (V2.1)" developed by the Lindsey Group, North Carolina State University.

2.2Animal Studies

2.2.1Materials used in this study

2.2.1.1Study animals

Colony bred healthy albino male rats (*Rattus norvegicus*), 10 weeks of age, were obtained from Mohan Bhat Laboratory Suppliers, Mangalore and maintained at the animal house facility of Goa University, India. The animals were fed on standard laboratory pellet diet with water ad libitum and housed at room temperature (25°C - 27°C) in polypropelyne cages with a 12h light/12h dark cycle.

For the glucose uptake experiment colony bred, healthy albino rats of either sex weighing between 200-220 g were taken for the study. Before commencing any experiment, the animals to be included in the respective study were grouped together for a period of 7 days for the purpose of acclimatization. The study was approved by the institutional animal ethics committee.

2.2.1.2Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade and purchased from S.D. Fine Chem Ltd., Mumbai. Glucose 6-phosphatase and Glucose 6-phosphate was purchased from Sigma-Aldrich Chemical Co, St. Louis M.O., (USA). Hexokinase and Tyrode's Salt was purchased from HiMedia Laboratories, Mumbai. Insulin (Lupin Ltd.), Ultra Sensitive Rat Insulin ELISA Kit, was purchased from Crystal Chem Inc., USA. All the kits used for the biochemical assays were purchased from Crest Biosystems, a division of Coral Clinical Systems, Goa.

2.2.2 Standardizations followed for the induction of diabetes in rats

A variation in blood glucose level (animals become more prone to blood glucose fluctuations) is noticed to be primarily due to sudden changes in what the rats are exposed to on any one day. For example; a change in cage, incompetent handling, change in any habitual pattern such as traffic in the animal room or sudden changes in temperature. The following standardizations were carried out in order to ensure reduced variability in blood glucose estimation and allow accurate replication.

2.2.2.1 Handling of animals

When the rats were first procured, they were allowed to acclimatize to the surroundings for preferably 2 weeks, prior to any form of experimentation. This allowed the rats to recover from any handling or travelling stress, and adapt to their new surroundings which included changes in diet, bedding, and mode of water administration, housing density, and health status. The rats were periodically handled on a daily basis inorder to acquaint them with the handler in a bid to reduce stress.

2.2.2.2 Inbreeding of rats for alloxan sensitive animals

The rats were inbred and crossed for 10 generations within the same colony, in order to have a similar genetic background. Animals were grouped and acclimatized for 7 days. At the end of 7 days the animals were fasted overnight and injected with alloxan. The FBG was measured at 72 hours post alloxan injection and was subsequently monitored every 3 days, to check for reversal of diabetes. One male and two or three females were chosen from each litter from the cages, wherein a higher percentage of the population showed desired susceptibility to alloxan. These animals were then mated to produce the next generation of offspring. Only males were used for the antidiabetic studies.

2.2.2.3 Blood sampling

The sampling of blood was done within 10 seconds of grasping the rat without causing excessive commotion so as to avoid unnecessary fluctuations in blood glucose level during sampling due to stress. Repeated sampling in live animals was performed only from the tail vein, under topical local anesthesia, using a portable

glucometer (One touch Horizon, Johnson & Johnson).

2.3 In vivo bioassays

2.3.1 Acute Oral Toxicity Test

The acute oral toxicity test was carried out as per OECD guidelines using the AOT425 software. Single oral administrations of the extract (0.1 - 2 g/kg BW) were administered to different groups of rats, with each group containing 5 rats. The animals were observed continuously for the first 4 hours and intermittently for the next 48 and 72 hours, following administration of the plant extracts. The rats were observed for grooming, hyperactivity, sedation, respiratory rate, convulsion and reflexes.

2.3.2 Standardization of alloxan dose and Induction of alloxan diabetes

Reagents

1) **Ice cold 0.9% Mammalian Saline:** 0.9 gms NaCl was dissolved in DW and chilled.
2) **2% Alloxan Monohydrate solution:** Prepared fresh just prior to injection. The weighed quantity of alloxan was dissolved in 0.9%Mammalian saline to give a 5% solution.

Animals were divided into 5 groups of 6 animals each and fasted over night for 16 hours. The rats were injected intraperitoneally with freshly prepared 2% alloxan monohydrate solution at doses ranging from 40mg/kg BW to 200 mg/kg BW, i.e. 40mg/kg BW, 50 mg/kg BW, 100 mg/kg BW, 150 mg/kg BW, 200 mg/kg BW. After the alloxan injection, they were returned to their respective cages and allowed free

access to drinking water. Food was introduced 30 minutes after alloxan injection. The animals were allowed to rest for a period of 72 hours. The fasting blood glucose levels of the animals and their conditions were monitored daily, in order to determine the degree and duration of hyperglycemia caused by each individual dose. This was done inorder to choose the appropriate alloxan dose for the induction of diabetes in the concerned experiments.

2.3.3 Induction of alloxan diabetes

Ice cold mammalian saline was used as the half life of alloxan in a buffered solution at pH 7.4 and at a temperature of 37℃ is 1.5 mins (Lenzen and Munday, 1991). The animals were kept on a 16 hour overnight fast. The body weights of the animals were taken and the alloxan dose was calculated accordingly at 140 mg/kg BW. The alloxan powder was weighed out into marked 1.5 ml microcentrifuge tubes, which was dissolved in ice cold saline just prior to injection. The entire time limit starting from dissolving the alloxan in saline to injection into the intraperitoneal cavity of the animal did not exceed 10 seconds. Failing which, the activity of alloxan is considered to decrease, leading to haphazard results.

After 72 hours, rats with fasting blood glucose levels >110 mg/dl were included in the study, and maintained further for a period of 2 to 3 days for establishment of hyperglycemia. Animals with established diabetes were segregated into mildly diabetic (110-250 mg/dl) and highly diabetic (250 mg/dl-400 mg/dl).

2.3.4Grouping of Experimental Rats

The highly diabetic animals obtained were grouped into 5 groups of 6 animals each. One group was maintained as the non diabetic, non treated group (Normal Control). The normal and diabetic rats were divided among the following groups:

Group I - Normal Control (distilled water orally)

Group II - Diabetic Control (distilled water orally)

Group III - Diabetic + 200 mg/kg BW Aqueous Extract (AE 200)

Group IV - Diabetic + 400 mg/kg BW Aqueous Extract (AE 400)

Group V - Diabetic + 200 mg/kg BW Methanolic Extract (ME 200)

Group VI - Diabetic + 400 mg/kg BW Methanolic Extract (ME 400)

Treatments with the plant extracts started 72 hours after alloxan injection and establishment of hyperglycemia. Control (normal) groups were orally administered a volume of distilled water equivalent to the volume of the extract administered. All the extracts were administered orally by gavage.

2.3.5Screening for antidiabetic activity (Acute study)

The control and alloxan-diabetic rats were divided into their respective groups and fasted overnight. Whole blood glucose was estimated just prior to oral administration of the *A. tricolor* leaf extracts. This reading was considered as the 0 hour value. Whole blood glucose was then measured intermittently with a glucometer (One Touch Horizon, Johnson & Johnson) at 3 hours, 6 hours, 9 hours and 12 hours after

administration of a single dose of the vehicle or *A. tricolor* leaf extracts. After the administration of the *A. tricolor* leaf extracts the experimental animals were given free access to water and no food till the end of the experimental period. This study was performed in triplicate. And a graph was plotted based on the mean values obtained from the study.

2.3.6 Treatment of diabetic rats with *Amaranthus tricolor* extracts for 3 weeks

Body weights were taken on days 1 and day 21 of the experiment. After the last dose, the animals were fasted for 12 hours and sacrificed under anaesthesia.

2.3.6.1 Sample and Tissue preparation

Blood was collected by cardiac puncture into clean sterile vials containing EDTA for study of haematological parameters. The remaining blood was collected in clean sterile glass centrifuge tubes and allowed to clot in the refrigerator. The separated serum was aspirated out into sterile centrifuge tubes and centrifuged at 3500 rpm for 10 min to separate any remaining cellular components. Each serum sample was pipetted in duplicate into clean sterile micro centrifuge tubes and stored at -4° C until further analysis. The liver, kidney, skeletal muscle and pancreatic tissues were excised and rinsed free of blood, with ice cold mammalian saline. The tissue was trimmed free of any extra tissue/fat, blotted dry and weighed. The tissues used for the assays were homogenised in ice cold mammalian saline, except for the Glucose 6-phophatase assay, where the tissues were homogenized in ice cold 250 mM sucrose-EDTA solution (Punitha et. al., 2005). The homogenates were centrifuged (3000×g, 10 min, 4ºC) and the resulting supernatants were assayed immediately. For the amylase and

lipase assays the pancreatic tissue of each rat was homogenized in ice cold mammalian saline and centrifuged (5000×g, 20 min, 4°C) and the resulting supernatants were assayed immediately.

2.3.6.2 Biochemical assays

The biochemical assays for the above mentioned groups were carried out using the serum, kidney, liver and skeletal muscle tissue samples of the control and treated animals. Blood glucose levels, lipid profile, hematological parameters and liver function tests were estimated on day 21 of the experiment. All determinations were made in the pre-prandial state. Glucose, Total Cholesterol (TC), Triglyceride (TG), High density lipoprotein (HDL), Low density lipoprotein (LDL), Very low density lipoprotein (VLDL), Total protein (TP), bilirubin, Aspartate transaminase (AST), Alanine transaminase (ALT), Lactate dehydrogenase (LDH), Alkaline Phosphatase (AlP), and Acid Phosphatase (AcP) were assayed from the collected serum; and tissue homogenates using commercially available kits (Crest Biosystems, Goa). G6Pase, G6PD, and Hexokinase were assayed from the tissue homogenates. Protein was estimated by Lowry's method for tissue enzyme assays.

2.3.6.3 Biochemical Assay Protocols

2.3.6.3.1 Glucose (GLU012 – Crest Biosystems) (Trinder, 1969)

Glucose is oxidised to gluconic acid and hydrogen peroxide in the presence of glucose oxidase (GOD). Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine (4-AAP) by the catalytic action of peroxidase (POD) to form a red coloured

quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of glucose present in the sample.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase (GOD)}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + 4\text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase (POD)}} \text{Red Quinoneimine Dye} + H_2O$$

Reagents

1) **Glucose Reagent**: Phosphate Buffer 100 mM; pH 7.0; GOD ≥ 15 KU/L; POD ≥ 1000 U/L; 4-AAP 0.3mM; Phenol 5 mM; Non-Reactive Stabilizers and Preservatives.
2) **Glucose Standard** (100 mg/dl)

Protocol

Clean dry test tubes were labelled as Blank (B), Standard (S) and Test (T), and the following reagents were pipette into the tubes:

Addition Sequence	Blank (ml)	Standard (ml)	Test (ml)
Glucose Reagent	**1.0**	**1.0**	**1.0**
Distilled Water	**0.01**	**-**	**-**
Glucose Standard	**-**	**0.01**	**-**
Sample	**-**	**-**	**0.01**

The tubes were mixed by inverting gently and then incubated at 25°Cfor a period of 30 minutes. The absorbance of the standard (Abs. S) and test sample (Abs. T) was measured against the blank at 505 nm within 60 minutes.

Calculations:

$$\text{Total Glucose (mg/dl)} = \frac{Abs.T}{\text{Abs. S}} \times 100$$

2.3.6.3.2 Insulin Assay

The Ultra Sensitive Rat Insulin ELISA kit (90060, Crystal Chem Inc., USA) has been used for the quantitative determination of serum insulin of rats following exposure to plant extracts (Algandaby et. al., 2010) or test compounds (Hassan et. al., 2010).

Principles of the Assay

1. **First reaction:** Rat insulin in the sample was bound to the guinea pig anti-insulin antibody coated on the microplate well.
2. **Washing:** Unbound material was removed by washing.
3. **Second reaction:** Horse radish peroxidase (POD)-conjugated anti-insulin antibody was then bound to the guinea pig anti-insulin antibody/rat insulin complex immobilized to the microplate well.
4. **Washing:** Excess POD-conjugate was removed by washing.
5. **Enzyme reaction:** The bound POD conjugate in the microplate well was detected by the addition of the 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution.
6. **Measurement of absorbance**
7. **Evaluation of results:** The insulin concentration was determined via interpolation using the standard curve generated by plotting absorbance versus the corresponding concentration of rat insulin standard.

Preparation of Rat Serum

Blood was collected through cardiac puncture and allowed to clot. The serum was aspirated into a microcentrifuge tube and was centrifuged for 20 min at 2,000 x *g*.

Hemolysis was avoided during preparation. Samples requiring dilution were diluted using the Sample Diluent (marked "G").

Assay Materials

	Description
1	Antibody-coated Microplate (6x8 well modules)
2	Rat Insulin Standard, Lyophilized 2.56ng/vial for 100 µL
3	Anti-Insulin Enzyme Conjugate Stock Solution
4	Enzyme Conjugate Diluent
5	Enzyme Substrate (TMB) Solution
6	Enzyme Reaction Stop Solution (1 N Sulfuric Acid)
7	Sample Diluent
8	Wash Buffer Stock Solution (20X Concentrate)
9	Frame for affixing the microplate well module
10	Plastic microplate cover

Preparation of reagents

Prior to use, all reagents were brought to room temperature (25°C), and were stored at 2-8°C immediately after use. Before use, the reagents were mixed thoroughly by gentle agitation or swirling.

1. Antibody-coated microplate was removed from the sealed foil pouch after the pouch had been equilibrated to room temperature and was used the same day as the pouch was opened.

2. Rat insulin stock solution: "Rat Insulin Standard, Lyophilized" was reconstituted by careful addition of 100 µl of distilled or deionized water to the vial. The vial was

gently inverted until the contents were completely dissolved. This stock solution contained 25.6 ng/ml of rat insulin. The reconstituted rat insulin stock solution was stored for one week at 2-8°C as it is considered stable for that period, after which it was discarded and fresh standards were prepared.

3. Anti-insulin enzyme conjugate: The required volume of anti-insulin enzyme conjugate solution was prepared immediately before use for six modules, by mixing 3.6 ml of "Anti-Insulin Enzyme Conjugate Stock Solution" with 1.8 ml of "Enzyme Conjugate Diluent", and mixed completely to ensure a homogeneous and clear solution. Foaming was avoided during mixing.

4. Enzyme substrate solution was provided as a ready-to-use preparation. Once the bottle was opened, the enzyme substrate solution was kept in the dark for one week at 2-8°C, after which it was discarded.

5. Enzyme reaction stop solution (1N H_2SO_4) was provided as a ready-to-use preparation.

6. Sample diluent was provided as a ready-to-use preparation. Once the bottle was opened, the sample diluent was kept for one week at 2-8°C, after which it was discarded.

7. The **"Wash Buffer Stock Solution"** was brought to 1 L with distilled or deionized water in a volumetric flask. The solution was mixed well before use. The wash buffer was kept for one week at 2-8°C, after which it was discarded.

Preparation of working rat insulin standards

1. Fifty µl of sample diluent and 50 µl of rat insulin stock solution (25.6 ng/l) were pipetted into a polypropylene microtube labelled 12.8 ng/ml, and

mixed thoroughly.

2. Fifty µl of sample diluent was dispensed into seven polypropylene microtubes labeled 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/ml, respectively.

3. Fifty µl of the 12.8 ng/ml standard was dispensed into the 6.4 ng/mL microtube, and mixed thoroughly.

4. Fifty µl of the 6.4 ng/ml standard was dispensed into the 3.2 ng/ml microtube, and mixed thoroughly.

5. This dilution scheme was repeated using the remaining microtubes.

6. Fifty µl of sample diluent was dispensed into one polypropylene microtube labelled 0 ng/ml.

Note: The working insulin standards were prepared shortly before use in polypropylene microtubes and discarded after use.

Preparation of working rat insulin standards (wide range)

	Rat Insulin concentration (ng/ml)								
	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0
RISS*(µl)	50								
SD(µl)**	50	50	50	50	50	50	50	50	50
		50	50	50	50	50	50	50	
		↗	↗	↗	↗	↗	↗	↗	
Total (µl)	100	100	100	100	100	100	100	100	50

RISS*: Rat Insulin Stock Solution (25.6 ng/mL); SD: Sample Diluent**

Assay Procedure

First reaction:

1. The antibody-coated microplate modules were removed from the sealed foil pouch after the pouch had been equilibrated to room temperature and the microplates were affixed to the supporting frame.
2. Into each well, 95 µl of sample diluent was dispensed.
3. 5 µl samples (or 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 ng/ml worklng rat insulin standards) were pipetted into the wells.

   ***Note**: Each standard and sample was assayed in duplicate. A 10 µl or better precision pipette was used when dispensing small volumes (5 µl).*
4. The microplate was covered with the plastic microplate cover and incubated for 2 hours at 4°C.

Second reaction:

5. The well contents were aspirated and washed five times using 300 µl of wash buffer per well. After each wash, any remaining solution was removed by inverting and tapping the plate firmly on a clean paper towel.
6. 100 µl per well of anti-insulin enzyme conjugate was dispensed into each washed microwell.
7. The microplate was covered with the plastic microplate cover and incubated for 30 minutes at room temperature.

Third reaction:

8. The well contents were aspirated and washed seven times using 300 µl of

wash buffer per well. After each wash, any remaining solution was removed by inverting and tapping the plate firmly on a clean paper towel.

9. Immediately 100 µl per well of enzyme substrate solution was dispensed and allowed to react for 40 minutes at room temperature. During the enzyme reaction, care was taken to avoid exposing the microplate to light.
10. The enzyme reaction was stopped by adding 100 µl per well of enzyme reaction stop solution.
11. Absorbance was measured at 450 nm and 630 nm within 30 minutes using a plate reader. (A630 values were subtracted from A450 values).

Determining the insulin concentration

1. The mean absorbance for each set of duplicate standards or samples was determined.
2. Using linear graph paper, the insulin standard curve was constructed by plotting the mean absorbance value for each standard on the Y axis versus the corresponding standard rat insulin concentration on the X axis. ***Note****: A standard curve was plotted every time the assay was performed.*
3. Rat insulin concentrations in the samples were interpolated using the standard curve and mean absorbance values for each sample.

2.3.6.3.3 Protein Assay (Lowry et. al., 1951)

Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour formed is due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The

intensity of colour depends on the amount of aromatic amino acids present in the protein.

Reagents

1) **Alakaline sodium carbonate solution**: (20g/L Na2CO3 in 0.1 mol/L Na, K tartrate).
2) **Copper sulphate** (5g/L CuSO4.5H2O in 10g/L Na.K tartrate).
3) **Alkaline solution** was prepared on the day of use by mixing 50 ml of alkaline sodium carbonate and 1.0 ml of copper sulphate.
4) **Folin–Ciocalteau reagent** (The reagent was diluted with equal volume of DW prior to use.)
5) **Standard protein** (0.2 mg Bovine Serum Albumin per millilitre of DW).

Protocol:

Alkaline solution (5.0 ml) was added to sample solution (0.1 ml), mixed and allowed to stand at room temperature for 10 minutes. Subsequently Folin-Ciocalteau reagent was added while shaking. The above mixture was incubated for 30 minutes and extinction was read against the appropriate blank at 750 nm. The protein concentrations of samples were measured using a protein standard curve. For standard curve bovine serum albumin concentrations in the range of 0.02 - 0.14 mg/ml were used.

Calculations:

$$\frac{OD\ of\ sample\ \times\ Conc\ of\ standard}{OD\ of\ standard}$$

2.3.6.3.4 Glycogen Estimation (Carroll et. al., 1956)

Reagents

1) **Deproteinizing reagent (10% TCA):**10 grams of TCA was dissolved in 100 ml of distilled water. The TCA solution was stored at 4℃ until further use.
2) **95% Ethanol:** 5 ml of DW was added to 95 ml of Absolute Ethanol. The ethanol solution was stored at 4℃ until further use.
3) **Anthrone reagent:** 250 mg of anthrone powder was dissolved in concentrated H_2SO_4. The anthrone reagent was stored in an amber colored bottle at 4℃ until further use.

Protocol

100 mg of tissue was weighed and deproteinized by homogenizing tissue in 10% TCA in a pre-chilled mortar and pestle. The resulting homogenate was centrifuged at 3000 rpm for 10 min and the supernatant transferred into another test tube. 1 ml aliquots of the homogenate were taken in triplicate into fresh test tubes. To each test tube was added 5 ml of 95% ethanol and the solutions were mixed well. The tubes were kept overnight at 4ºC to precipitate. The tubes were then centrifuged at 3000 rpm for 10 min. The supernatant was discarded and tubes kept inverted on a blotting paper and were gently tapped to dry. After 5 min 1 ml DW was added and gently inverted to mix. To 1 ml of sample was added 4 ml of anthrone reagent, followed by Folin-Ciocalteau reagent. The tubes were kept in boiling water bath for 15 min. The absorbance was read against blank at 620 nm. The glycogen concentrations of samples were measured using a glycogen standard curve.

Calculations:

$$\frac{OD\ of\ sample\ \times\ Conc\ of\ standard}{OD\ of\ standard}$$

2.3.6.3.5 Total Protein (TPR010 - Crest Biosystems) (Biuret Assay)

Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

$$\text{Proteins} + \text{Cu}^{++} \longrightarrow \text{Blue Violet Coloured Complex}$$

Reagents

1) Biuret Reagent
2) Protein Standard (8 g/dl)

Protocol:

The following reagents were pipetted into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Addition Sequence	B (ml)	S (ml)	T (ml)
Biuret Reagent	**1.0**	**1.0**	**1.0**
Distilled Water	**0.02 ml**	**-**	**-**
Protein Standard (S)	**-**	**0.02**	**-**
Sample	**-**	**-**	**0.02**

The contents of the tubes were mixed well by inverting gently and incubated at R.T. for 30 minutes. The absorbance of the Standard (Abs. S) and Test Sample (Abs. T) were measured against the Blank, at 550 nm within 60 minutes.

Calculation:

$$\text{Total Proteins (g/dl)} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 8$$

2.3.6.3.6 Total Bilirubin Assay (Modified Jendrassik and Grof's Method)

Bilirubin reacts with diazotised sulphanilic acid to form a coloured azobilirubin compound. The unconjugated bilirubin couples with the sulphanilic acid in the presence of a caffein- benzoate accelerator. The intensity of the colour formed is directly proportional to the amount of bilirubin present in the sample.

$$\text{Proteins} + \text{Cu}^{++} \longrightarrow \text{Blue Violet Coloured Complex}$$

Reagents

1. Total Bilirubin Reagent
2. Total Nitrite Reagent
3. Artificial Standard (10 mg/dl)

Protocol

The above mentioned reagents were pipetted into clean dry test tubes labelled as Blank (B) and Test (T) as follows:

Addition Sequence	B (ml)	T (ml)
Total Bilirubin Reagent	**1.0**	**1.0**
Total Nitrite Reagent	**-**	**0.05**
Sample	**0.1**	**0.1**

The contents of the tubes were mixed well by inverting gently and incubated at RT for 10 min. The absorbance of the Test Samples (Abs.T) was immediately measured against their respective Blanks at 546 nm.

Calculation:

$$\text{Total bilirubin(mg /dl)} = \text{Abs. T} \times 13$$

Cholesterol (CHO012 - Crest Biosystems) (Allian et. al., 1974)

Cholesterol esterase (CHE) hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol Esterase (CHE)}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase (CHO)}} \text{Cholestenone} + H_2O$$

$$H_2O_2 + 4\text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase (POD)}} \text{Red Quinoneimine Dye} + H_2O$$

Reagents

1. Enzyme Reagent 1
2. Enzyme Reagent 2
3. Cholesterol Standard (200 mg/dl)

Composition

Goods buffer 50 mM; pH 7.0; CHE≥100 U/L; CHO≥100 U/L; POD≥1000 U/L; 4-AAP 0.3 mM; Phenol 4 mM; Non Reactive Stabilizers, Detergents and Preservatives.

Working reagent was prepared when desired by mixing together 0.8 ml of Enzyme Reagent 1 and 0.2 ml of Enzyme Reagent 2

Protocol

The following reagents were pipetted into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Addition Sequence	B (ml)	S (ml)	T (ml)
Working Reagent	**1.0**	**1.0**	**1.0**
Distilled Water	**0.01**	**-**	**-**
Cholesterol Standard (S)	**-**	**0.01**	**-**
Sample	**-**	**-**	**0.01**

The contents of the tubes were mixed well by inverting the tubes gently several times and incubated at R.T. for 15 minutes. The absorbance of the Standard (Abs. S) and Test Sample (Abs. T) was measured against the Blank at 505 nm within 60 minutes.

Calculation:

$$\text{Cholesterol (mg/dl)} = \frac{Abs.T}{Abs.S} \times 200$$

2.3.6.3.7 Triglycerides (TG 010 - Crest Biosystems) (Fossati and Prencipe, 1982)

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

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol} + \text{Free Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol 3 Phosphate} + \text{ADP}$$

$$\text{Glycerol 3 Phosphate} + \text{O}_2 \xrightarrow{Glycerol\ \ 3\ PO} Dihydroxyacetone\ phosphate + H_2O_2$$

$$H_2O_2 + 4\text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase (POD)}} \text{Red Quinoneimine Dye} + \text{H}_2\text{O}$$

Reagents

1) Enzyme Reagent 1
2) Enzyme Reagent 2
3) Triglycerides Standard (200 mg/dl)

Working reagent was prepared by mixing together 0.8 ml of Enzyme Reagent 1 and 0.2 ml of Enzyme Reagent 2 just prior to use in the assay and was stored at 2 to 8°C.

Protocol

The following reagents were pipetted into clean dry labeled test tubes:

Addition Sequence	B (ml)	S (ml)	T (ml)
Working Reagent	**1.0**	**1.0**	**1.0**
Distilled Water	**0.01**	**-**	**-**
Triglycerides Standard	**-**	**0.01**	**-**
Sample	**--**	**-**	**0.01**

The contents of the tubes were mixed well and incubated at R.T. for 15 min. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) was measured against Blank, at 505 nm within 60 minutes.

Calculation:

$$\text{Triglycerides(mg / dl)} = \frac{Abs.T}{Abs.S} \times 200$$

$$\text{VLDL} = (\text{TotalCholesterol}) - \left(\frac{\text{Triglycerides}}{5}\right) - (\text{HDL Cholesterol})$$

2.3.6.3.8 HDL Cholesterol kit (HDL010 - Crest Biosystems) (Grillo et. al., 1981)

When the serum is reacted with the Polyethylene Glycol contained in the precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the Cholesterol (CHOD/PAP) reagent.

Reagents

1) Enzyme Reagent 1
2) Enzyme Reagent 2
3) Precipitating Reagent
4) HDL Standard (25 mg/dl)

Working Reagent was prepared by mixing together 0.8 ml of Enzyme Reagent 1 and 0.2 ml of Enzyme Reagent 2 just prior to the assay.

Precipitation of VLDL & LDL:

The following reagents were pipetted into clean dry test tubes:

Precipitating Reagent	**0.1 ml**
Sample	**0.1 ml**

The contents of the tubes were mixed well by inverting the tubes gently several times and incubated at R.T. for 5 minutes and centrifuged at 2500-3000 rpm to obtain a clear supernatant to be used in the following cholesterol assay:

Cholesterol assay:

Protocol

The following reagents were pipetted into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Addition Sequence	B (ml)	S (ml)	T (ml)
Working Reagent	**1.0**	**1.0**	**1.0**
Distilled Water	**0.05**	**-**	**-**
HDL Standard (S)	**-**	**0.05**	**-**
Sample	**-**	**-**	**0.05**

The tubes were mixed well and incubated at 37°C for 15 min. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) was measured against Blank, at 505 nm within 60 min.

Calculation:

$$\text{HDL Cholesterol (mg/dl)} = \left(\frac{\text{Abs .T}}{\text{Abs .S}}\right) \times 25 \times 2$$

Where 2 is the dilution factor due to the deproteinization step

$$\text{LDL Cholesterol} = (\text{Total Cholesterol}) - \left(\frac{Triglycerides}{5}\right) - (\text{HDL Cholesterol})$$

2.3.6.3.9 Pancreatic Insulin Content Measurement (Mu et. al., 2006)

Reagents

1. **Acid-ethanol:** 1.5% of conc HCl was diluted with 70% ethanol.
2. **1M Tris, pH 7.5:** 12.11 gms Tris base was dissolved in 100 ml of DW

Protocol

The pancreatic tissue from the rats were isolated, and cut free of fat and connective tissues. The wet weight (mg) of each pancreas was taken. The pancreatic tissue was homogenized in 3 ml ice cold acid ethanol in a pestle and mortar, kept on ice. The homogenate was transferred to a polypropelene centrifuge tube. The mortar and pestle was rinsed with an additional 3 ml of acid ethanol, and added to the tube of tissue homogenate (total of 6 ml), which was incubated overnight at 4°C. After incubation the tube was centrifuged at 2400 rpm for 15 min at 4°C. The supernatant was transferred to a fresh 15 ml conical tube, and stored at -10°C. This was done with all the pancreatic tissue collected from the different groups of *A. tricolor* treated and untreated diabetic rats.

The acid ethanol pancreatic extract was neutralized with 1M Tris, pH 7.5 in a 1:1 ratio. The neutralized extract was then assayed for insulin content using the Elisa insulin assay kit (Crystal Chem, USA). Protein content of the neutralized extract was also determined by the biuret assay. Pancreatic insulin content was expressed in terms of ng/mg of protein/gm tissue.

2.3.6.3.10 Amylase EC 3.2.1.1 (AMY 010 - Crest Bioystems) (IFCC, 1999)

α-amylase catalyses the hydrolysis of a 2 Chloro-4 nitrophenol salt to chloronitrophenol (CNP). The enzyme present in serum consists of both pancreatic and salivary origin. The salivary amylase is inhibited by a monoclonal antibody mix and only the activity of the pancreatic function is measured as an increase in

absorbance due to the formation of chloronitrophenol which is proportional to the pancreatic amylase activity in the sample.

Reagents

1) Buffer Reagent
2) Substrate Reagent

Working Reagent was prepared by mixing 4 parts reagent 1 with 1 part reagent 2.

Protocol

Pipette into clean dry test tubes labeled as calibrator (C), and test (T):

Addition Sequence	**T (ml)**
Buffer Reagent	**0.8 ml**
Sample	**0.2 ml**
Incubate at the assay temperature for 5 minutes	
Substrate Reagent	**0.2 ml**

Mix well and read the initial absorbance A at 405 nm after 1 min and repeat the absorbance reading after every 1, 2 and 3 min.

Calculation:

$$Pancreatic\ Amylase\ Activity\ (U/L) = \Delta A/\text{min} \times 2290$$

2.3.6.3.11 Lipase E.C.3.1.1.3 (LIP010 - Crest Biosystems) (Lott et. al., 1986)

Pancreatic lipase catalyses the hydrolysis of Triolein, in the presence of colipase to form monoglycerides and fatty acids. The rate of decrease in turbidity measured at 340 nm is proportional to the lipase activity. The activities of other lipases in the serum are inhibited by the cholic acid salts in the reagent.

$$\text{Triolein} + 2H_2O \xrightarrow{\text{Lipase}} \text{Monoglyceride} + 2\ \text{Oleic Acid}$$

Reagents

1) Lipase Reagent
2) Calibrator (for 1 ml) – is reconstituted with 1 ml DW. It is allowed to stand for 10 mins with occasional mixing, and stored at -10ºC until use.

Protocol

Pipette into clean dry test tubes labeled as calibrator (C), and test (T):

Addition Sequence	C (ml)	T (ml)
Lipase Reagent (L1)	**1.0**	**1.0**
Calibrator (C)	**0.04**	**-**
Sample	**-**	**0.04**

The solution was mixed well and incubated at 37ºC for 4 minutes. The initial absorbance A, for the calibrator (C) and test (T) was read at 340 nm. Another absorbance A_2 of the calibrator and test was read after exactly 5 min. The change in absorbance A for both the calibrator and test was calculated.

Calculation: For calibrator $\Delta AC = A_2C - A_1C$

For test $\Delta AT = A_2T - A_1T$

$$\text{Lipase (U /L)} = \frac{\Delta AT}{\Delta AC} \times Concentration\ of\ Calibrator$$

2.3.6.3.12 Alkaline Phosphatase EC 3.1.3.1 (ALP 010 – Crest Biosystems) (Bowers and McCommb, 1972)

ALP at an alkaline pH hydrolyses p-Nitrophenylphosphate to form p-Nitrophenol and Phosphate. The rate of formation of p-Nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample.

$$\text{p} - \text{Nitrophenylphosphate} \xrightarrow{\text{Alkaline phosphatase}} \text{p} - \text{Nitrophenol} + \text{Phosphate}$$

Reagents

1) Buffer Reagent
2) Substrate Tablet

Preparation of Working Reagent: 1 Substrate Tablet was dissolved in 3.2 ml of Buffer Reagent and the solution stored in dark at 2-8°C for 15 days..

Protocol

Addition Sequence	**T (ml)**
Working Reagent	**1 ml**
Incubate at the assay temperature for 1 minute and add	
Sample	**0.02**

The tubes were mixed well and the initial absorbance A_o was read at 405 nm after 1 minute. The absorbance was read after every minute for 3 min.

Calculation:

$$\text{ALP activity (U/L)} = (\Delta A/\text{min}) \times 2754$$

2.3.6.3.13 Acid Phosphatase EC 3.1.3.2 (ACP 010 – Crest Biosystems) **(**Hillman, 1971**)**

AcP at an acidic pH hydrolyses di-Sodium Phenylphosphate to form phenol. The phenol formed reacts with 4-Aminoantipyrine in the presence of Potassium Ferricyanide, as an oxidising agent, to form a red coloured complex. The intensity of the colour formed is directly proportional to the activity of AcP present in the sample. Tartrate inhibits prostatic AcP and the testing in its presence is done to find the non prostatic AcP. The difference between the activities of the total and non prostatic AcP gives the activity of the prostatic AcP.

$$\alpha - \text{Naphthylphosphate} + H_2O \xrightarrow{\text{Acid phosphatase}} \alpha - \text{Naphthol} + \text{Phosphate}$$

$$\alpha - \text{Naphthol} + \text{Fast Red TR Salt} \longrightarrow \text{Diazo dye complex}$$

Reagents

1) Buffer Reagent
2) Substrate Reagent
3) Colour Reagent
4) Tartrate Reagent
5) Phenol Standard (10 mg/dl)

Protocol

Addition Sequence	B (ml)	S (ml)	C (ml)	T (ml)	TS (ml)
Distilled Water	**1.1**	**1.05**	**1.0**	**1.0**	**1.0**
Buffer Reagent (L1)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Substrate Reagent (L2)	**0.10**	**0.10**	**0.10**	**0.10**	**0.10**
Mix well and allow to stand at 37°C for 3 min and add					
Tartrate Reagent (L4)	**-**	**-**	**-**	**-**	**0.02**
Sample	**-**	**-**	**-**	**0.1**	**0.1**
Phenol Standard (S)	**-**	**0.05**	**-**	**-**	**-**
Mix well and allow to stand at 37°C for 60 mins and add					
	1.0	**1.0**	**1.0**	**1.0**	**1.0**
Sample	**-**	**-**	**0.1**	**-**	**-**

The tubes were mixed well after each addition. The absorbances of the Blank (Abs.B), Standard (Abs.S), Control (Abs.C), Test (Abs.T) and Tartrate Stable (Abs.TS) were measured at 505 nm against distilled water.

Calculation:

$$\text{Total AcP activity (K. A. Units)} = \frac{\text{Abs. T} - \text{Abs. C}}{\text{Abs. S} - \text{Abs. B}} \times 5.0$$

2.3.6.3.14 Alanine transaminase EC 2.6.1.2 (GPT 020 – Crest Biosystems) (Bergmeyer et. al., 1986)

Alanine transaminase (ALT) catalyzes the transfer of amino group between L-Alanine and α-Ketoglutarate to form Pyruvate and Glutamate. The pyruvate formed reacts with NADH in the presence of Lactate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the ALT activity in the sample.

$$\mathrm{L-Alanine + \alpha-Ketoglutarate \xrightarrow{Alanine\ \ transaminase} Pyruvate + L-Glutamate}$$

$$\mathrm{Pyruvate + NADH + H^{+} \xrightarrow{Lactate\ \ dehydrogenase} Lactate + NAD^{+}}$$

Reagents

1) Enzyme Reagent
2) Starter Reagent

Working reagent was prepared by mixing together 0.8 ml of Enzyme Reagent and 0.2 ml of Starter Reagent just prior to the assay.

Protocol

The reagents and samples were pipetted into labelled microcentifuge tubes in the following sequence.

Addition Sequence	**T (ml)**
Enzyme Reagent	**0.8 ml**
Sample	**0.2 ml**
Incubate at the assay temperature for 1 min and add	
Starter Reagent	**0.2 ml**

The tubes were gently inverted to mix the contents well and the initial absorbance A_o was read at 340 nm at R.T. The absorbance was read after every minute for 3 minutes.

Calculation:

$$ALAT\ activity\ (U/L) = (\Delta A/min) \times 952$$

2.3.6.3.15 Aspartate transaminase EC 2.6.1.1 (GOT 020 - Crest Biosytems) (IFCC, 1986)

Aspartate transaminase (AST) catalyzes the transfer of amino group between L-Aspartate and α-Ketoglutarate to form Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with NADH in the presence of Malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance,s proportional to the AST activity in the sample.

$$\mathrm{L-Aspartate + \alpha-Ketoglutarate} \xrightarrow{\text{Aspartate transaminase}} \mathrm{Oxaloacetate + L-Glutamate}$$

$$\mathrm{Oxaloacetate + NADH + H^+} \xrightarrow{\text{Mal ate dehydrogenase}} \mathrm{Malate + NAD^+}$$

Reagents

1) Enzyme Reagent
2) Starter Reagent

Working reagent was prepared by mixing together 0.8 ml of Enzyme Reagent and 0.2 ml of Starter Reagent just prior to the assay.

Protocol

Addition Sequence	T (ml)
Enzyme Reagent	**0.8 ml**
Sample	**0.2 ml**
Incubate at R.T. for 1 min and add	
Starter Reagent	**0.2 ml**

The solution was mixed well and the initial absorbance A was read at 340 nm. The Absorbance was read after every minute for 3 min.

Calculation:

$$ALAT\ activity\ (U/L) = (\Delta A/min) \times 952$$

2.3.6.3.16 Lactate Dehydrogenase EC 1.1.1.27 (LDH 010 - Crest Biosystems) (SFBC, 1982)

LDH catalyzes the reduction of pyruvate with NADH to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance, which is proportional to the LDH activity in the sample.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

Reagents

1. Buffer Reagent
2. Starter Reagent

Composition

Tris Buffer 100 mM; pH 6.8; Pyruvate 1.2 mM; NaCl 200 mM; NADH 0.18 mM; Non Reactive Stabilizers; Detergents and Preservatives.

Protocol

Pipette into a clean dry test tube labeled as Test (T):

Addition Sequence	**(T)**
Buffer Reagent	**0.8 ml**
Sample	**0.05 ml**
Incubate at R.T. for 1 min and add	
Starter Reagent	**0.2 ml**

The solution was mixed well and the initial absorbance A_0 was read at 340 nm. The absorbance was read after every minute for 3 min.

Calculation:

$$\text{LDH activity (U/L)} = (\Delta A/\text{min}) \times 3333$$

2.3.6.3.17 Glucose-6-Phosphate Dehydrogenase EC 1.1.1.49 (Worthington, 1988)

One unit reduces one micromole of pyridine nucleotide per minute at 30°C and pH 7.8 under the specified conditions.

Reagents

- 0.055 M Tris·HCl buffer pH 7.8 containing 0.0033 M $MgCl_2$
- 0.006 M Nicotinamide adenine dinucleotide phosphate, monosodium salt, (NADP), (TPN). Note: NADP may vary in salt form and degree of hydration. When using NAD, prepare 0.06 M solution.
- 0.1 M Glucose-6-phosphate
- 5 mM Glycine buffer, pH 8.0
- 5 mM Glycine buffer, pH 8.0 containing 0.1% bovine serum albumin

Protocol

The spectrophotometer was adjusted to 340 nm and the temperature of the spectrophotometer cuvette cell was set to 30°C. Into each cuvette was pipetted the reagents in the following sequence as mentioned in the table given below:

Reagent	ml
0.055 M Tris·HCl buffer, pH 7.8 with 0.0033 M $MgCl_2$	**2.7**
0.006 M NADP (or 0.06 M NAD)	**0.1**
0.1 M Glucose-6-phosphate	**0.1**

The cuvette was incubated in spectrophotometer cuvette chamber at 30°C for 7 to 8 min in order to achieve temperature equilibration and to establish a blank rate, if any. 0.1 ml of the diluted enzyme was added and the increase in A_{340}/min for 4 to 5

minutes was recorded. The A_{340}/minute was calculated from the initial linear portion of the curve.

Calculation:

$$\text{Units/mg protein} = \frac{\Delta A\ 340/\text{min}}{6.22 \times \text{mg protein/ml reaction mixture}}$$

The increase in absorbance was recorded as enzyme activity and was expressed as unit per gram per minute in tissue.

2.3.6.3.18 Hexokinase EC 2.7.1.1 (Worthington, 1988)

One unit of activity reduces one micromole of NAD^+ per minute at 30°C and pH 8.0 under the specified conditions.

Reagents:-

- 0.05 M Tris*HCl buffer, pH 8.0 with 13.3 mM $MgCl_2$
- 0.67 M Glucose in above Tris·$MgCl_2$ buffer
- 16.5 mM Adenosine 5'Triphosphate in above Tris·$MgCl_2$ buffer
- 6.8 mM NAD in above Tris·$MgCl_2$ buffer

Protocol

The spectrophotometer was adjusted to 340 nm and the temperature of the spectrophotometer's cuvette cell was set to 30°C.

Pipette into each cuvette as follows:

Reagent	ml
Tris·MgCl2 buffer	**2.28**
0.67 M Glucose	**0.50**
16.5 mM ATP	**0.10**
6.8 mM NAD	**0.10**
G-6-PDH	**0.01**

The cuvette was incubated in spectrophotometer cuvette chamber at 30°C for 6 to 8 min in order to achieve temperature equilibration and to establish a blank rate, if any. At zero time 0.1 ml of the enzyme solution was added and mixed thoroughly. The increase in A_{340}/min for 3 to 4 minutes was recorded. The A_{340}/minute was calculated from the initial linear portion of the curve. The enzyme activity was expressed as unit per gram per minute in tissue.

Calculation:

$$\text{Units/mg protein} = \frac{\Delta A\ 340/\text{min}}{6.22 \times \text{mg protein/ml reaction mixure}}$$

2.3.6.3.19Glucose-6-Phosphatase EC 3.1.3.9 (Baginsky et. al., 1974)

Glucose-6-phosphatase catalyzes the conversion of glucose-6-phosphate to glucose.

Protocol

Into each cuvette was pipetted the reagents in the following sequence:

Reagent	ml
Sucrose/EDTA buffer	**0.1**
Glucose-6-phosphate (100 mM)	**0.1**
Imidazole buffer (100 mM, pH 6.5)	**0.1**
Homogenate	**0.1**
Sucrose/EDTA buffer	**0.1**

The solution was thoroughly mixed by inverting the tube several times. The tubes were then incubated at 37°C for 15 min. The enzymatic activity was terminated by the addition of 2 ml of TCA/ascorbate (10%/2%, w/v), and the solution was centrifuged at 3000 r.p.m. for 10 min. To 1 ml of clear supernatant were added 0.5 ml of ammonium molybdate (1% w/v) and 1 ml of sodium citrate (2% w/v). The absorbance was measured at 700 nm. The enzyme activity was expressed as unit per gram per minute in tissue.

2.3.8 Hematological Parameters

Whole blood was collected into EDTA treated vials and inverted gently several times to facilitate mixing, so as to not allow the blood to coagulate. The uncoagulated blood was loaded into the automated hematology analyzer (Abott Diagnostics CELL-DYN 1800, Asia-Pacific) and the following parameters were analyzed: WBC Count, RBC Count, Hemoglobin and PCV%.

2.3.9 Effect of fractions from methanolic extract on BGL in diabetic rats (Acute Study)

The non diabetic and alloxan diabetic rats were grouped as follows:

Group I - Normal Control (distilled water orally)

Group II - Diabetic Control (distilled water orally)

Group III - Diabetic + 200 mg/kg BW Fraction 1 (AT-C)

Group IV - Diabetic + 200 mg/kg BW Fraction 2 (F- 2)

Group V - Diabetic + 200 mg/kg BW Fraction 3 (F- 3)

Group VI - Diabetic + 200 mg/kg BW Fraction 4 (F- 4)

Group VII - Diabetic + 200 mg/kg BW Fraction 5 (F- 5)

Group VIII - Diabetic + 200 mg/kg BW Fraction 6 (F- 6)

Group IX - Diabetic + 200 mg/kg BW Fraction 7 (F- 7)

The rats were then fasted overnight before commencement of the experiment, with free access to drinking water. Whole blood glucose was estimated just prior to oral administration of the fractions obtained from the methanolic *Amaranthus tricolor* leaf

extract. This reading was considered as the 0 hour value. Whole blood glucose was then measured intermittently with a glucometer (One Touch Horizon, Johnson & Johnson,USA) at 3 hours, 6 hours, 9 hours and 12 hours after administration of a single dose of the vehicle or 200 mg/kg BW of the respective fractions. After the administration of the fractions, the experimental animals were given free access to water and no food till the end of the experimental period. This study was performed in triplicate. And a graph was plotted based on the mean values obtained from the study.

2.3.10 In Vitro Bioassays

2.3.10.1 Glucose uptake by isolated rat diaphragm and skeletal muscles

Reagents

1) **0.9% Mammalian Saline**
2) **Tyrode's solution containing 2% glucose:** 9.56 grams of Tyrode salts (HiMedia) was dissolved in 1000 ml of distilled water (as mentioned on package leaflet). The solution was stirred well and 20 grams of glucose was added and stirred again. Sodium bicarbonate. The solution was stored at 4°C until further use.
3) **Insulin diluent:** Plain 0.9% mammalian saline is sufficient to dilute regular insulin. In case the insulin is diluted in a plastic vial, 300mg of albumin may be added to the diluent in order to reduce adsorption of insulin to plastic. The insulin diluent was stored at 4°C until further use.
4) **0.4U/ml regular insulin:** 4U of regular insulin is gently mixed with 10 ml of ice cold insulin diluent in a 30 ml sterilized glass culture tube kept on ice. This diluted insulin was prepared afresh, just prior to the

commencement of the experiment. Any remaining unused solution was discarded.

2.3.10.1.1 Construction of Apparatus

The in vivo glucose uptake assay was carried out according to Walaas and Walaas (1952), Chiasson et. al., (1981) and Chattopadhyay et al. (1992) with minor modifications. Isolated rat diaphragm and skeletal muscle were used to study the uptake of glucose and its conversion to glycogen by the exposed tissues in the presence of insulin or any test compound or extract. For this study a glucose uptake apparatus was constructed in the laboratory.

2.3.10.1.2 Setting up the experiment

All the reagents were brought to R.T. prior to the commencement of the experiment except the insulin solution. Six groups were made, with each group containing a set of six graduated polypropylene test tubes. The solutions were dispensed into the respective tubes (Table 2a), without adding the plant extracts. The plant extracts were added 5 minutes prior to the addition of tissue to the tubes. The tube rack was kept in a water bath at 37°C. The volumes of all the test tubes were made up to 4 ml with distilled water kept at 37°C to match the volume of the test tubes of Group IV.

2.3.10.1.3 Tissue collection and preparation

Albino rats of either sex were fasted overnight, with free access to water. The animals were anesthetized and the diaphragms and soleus muscle were dissected out quickly with minimal trauma to the tissues. Each diaphragm was divided into two equal halves (hemi-diaphragms) by cutting along the central tendon, rinsed quickly in pre-

warmed saline and placed into the pre-warmed tubes (37°C) containing Tyrode's solution with the respective additives. Two hemi-diaphragms and skeletal muscle from the same animal were not used for the same set of experiment. Six hemi-diaphragms and skeletal muscles were used for each group.

The tubes containing media were placed in a 37ºC water bath to equilibrate the temperature. After the temperature across all the tubes was brought to 37ºC the tissues were added. The apparatus was then assembled onto the tubes in the tube rack. A constant bubbling of sterile 98% O_2 (Ghosh et. al., 2004) was maintained for the entire duration of the experiment. The temperature was constantly monitored and maintained at 37°C for 30 minutes. The hemi-diaphragms and soleus muscle placed in the test tubes were incubated for 30 min at 37°C, with constant bubbling of sterile medical oxygen (98%) with constant but, gentle shaking.

Groups for the glucose uptake and conversion to glycogen assay

Group I	2 ml of TS + 2% glucose
Group II	2 ml TS + 2% glucose + 0.62 ml of 0.4 U/ml insulin
Group III	2 ml TS + 2% glucose + 1.38 ml AE 400
Group IV	2 ml TS + 2% glucose + 0.62 ml of 0.4 U/ml insulin + 1.38 ml AE 400
Group V	2 ml TS + 2% glucose + 1.38 ml ME 400
Group VI	2 ml TS + 2% glucose + 0.62 ml of 0.4 U/ml insulin+ 1.38 ml ME 400

At the end of the experiment, the tissues were removed from the respective tubes, patted dry and the weight of each tissue was recorded. The tissues were further processed to determine their glycogen content. The glucose content of the incubated medium was measured using a glucose oxidase kit. Glucose uptake by the hemi diaphragm and soleus muscle respectively was calculated as the difference between the glucose content in the control and experimental incubation medium.

2.4 Statistical analysis

The results obtained were expressed as mean ± SD. Comparisons were made between normal and alloxan-induced diabetic rats, and between treated and untreated alloxan-induced diabetic rats using Student's t-test (Microsoft Office Excel 2007). Differences were considered significant at $p < 0.05$; $p < 0.001$ was considered highly significant.

Chapter 3

Results

3.1 Plant Studies

In the present study the aqueous and methanolic extracts of leaves of *Amaranthus tricolor* have been evaluated, for their potential to lower elevated blood glucose levels in the diabetic rats and for improvement of the overall diabetic morbidity. The *Amaranthus tricolor* plants were identified, and validated by the Department of Botany, Goa University. Fresh leaves of *Amaranthus tricolor* were ground with distilled water and some of the leaves were dried overnight as mentioned in the methodology section. Fig. 7 a and b, are specimens of the *Amaranthus tricolor* plants used in this study. The entire plant is reddish in colour with the exception of the root which comprises approximately $1/5^{th}$ of the entire length of the plant. The leaves are found at the apical region, and comprise the edible region of the plant.

3.1.1 Determination of percent yield

The Table 4 gives the percent yield of leaf extracts of *Amaranthus tricolor* obtained by using different solvents/techniques. It is obvious from the table that ME gives the highest yield while PE gives the least. The aqueous extracts, AE and AD, obtained were dark reddish brown and semisolid in consistency, while the methanolic extracts (ME and MS) were dark green in colour and semisolid in consistency. The petroleum ether extracts (PE) were pale yellow in colour with semisolid consistency.

3.1.2 Qualitative phytochemical analysis

The Table 5 shows the different phytochemical tests carried out for screening of various chemical constituents of *Amaranthus tricolor* leaves. The phytochemical analyses indicate the presence of proteins, amino acids, tannins, sterols and polyphenols in the aqueous extracts. The methanolic extracts contain tannins, sterols and polyphenols.

a. b.

F**ig 7: Amaranthus tricolor – a. Specimen Plant; b. Herbarium Sheet**

Table 4: Percent yield of *Amaranthus tricolor* leaf extracts from 100 gms of leaf material.

Extract	Solvent Used	Material	Extract Yield (%)
AE (Grinding)	Water	Fresh leaf	**4.2**
AD (Maceration)	Water	Dried powdered leaf	**3.7**
MN (Maceration)	Methanol	Dried powdered leaf	**8.13**
MS (Soxhilation)	Methanol	Dried powdered leaf	**7.4**
PE (Maceration)	Petroleum Ether	Dried powdered leaf	**1.6**

The values calculated as average of n=25.

3.1.3 Quantitative phytochemical assays

The quantitative phytochemical assays as depicted in Table 6 indicate that the majority of phytoconstituents detected in 1.0 gram of dry sample of *Amaranthus tricolor*, were lipids (2.0%), polyphenols (1.561%), tannins (1.25%) and Phytosterols (0.178%). Proteins (1.5%), and 30% Fibre.

3.2 In Vitro Studies

3.2.1 Screening of *Amaranthus tricolor* for antioxidant activity

The aqueous (AD, AE), methanolic (ME, MS) and petroleum ether (PE) extracts were evaluated *in vitro* for protective antioxidant activities using DPPH, FRAP and phosphomolybdenum assays and they are expressed in Fig. 8, Table 7 and Table 8.

3.2.1.1 Quantitative DPPH and FRAP Assays

The free radical scavenging activities of the aqueous extracts (AE and AD) were comparable with the activity expressed by the commercial antioxidant butylated hydroxy toluene (BHT). FRAP analysis confirmed that the aqueous extracts have very high free radical scavenging activities as compared with the FRAP standard ($FeSO_4$). The free radical scavenging activity of the aqueous extracts of *Amaranthus tricolor* as revealed by the DPPH assay, is comparable to that of the standard butylated hydroxy toluene (BHT). The aqueous and petroleum ether extracts exhibited high DPPH inhibitory activity likethat of standard BHT. The antioxidant capacity of the aqueous extract as measured by the FRAP assay was much higher at 14.26 mM/L as compared with $FeSO_4$ standard at 2mM/L.

Table 5: Phytochemical Screening of *Amaranthus tricolor* Extracts

Compound Classes	AE	AD	ME	MS	PE
Alkaloids					
Mayer's test	-	-	-	-	-
Wagner's test	-	-	-	-	-
Hager's test	-	-	-	-	-
Dragendorff's test	-	-	-	-	-
Carbohydrates					
Fehling's test	-	-	-	-	-
Barfoed's test	-	-	-	-	-
Benedict's test	-	-	-	-	-
Glycosides					
Borntrager's test	-	-	-	-	-
Legal's test	-	-	-	-	-
Saponins					
Froth test	+	-	-	-	-

Notes: **(+) - indicates positive reaction to the chemical test**

(-) - indicates negative reaction to the chemical test

Table 5: Phytochemical Screening of *Amaranthus tricolor* Extracts

Compound Classes	AE	AD	ME	MS	PE
Proteins & Amino acids					
Millon's test	+	-	-	-	-
Biuret test	+	-	-	-	-
Ninhydrin test	+	-	-	-	-
Phytosterols					
Libermann-Burchard's	+	-	+	+	+
Fixed Oils & Fats					
Spot test	-	-	-	-	-
Saponification test	-	-	-	-	-
Phenolics					
Gelatin test	-	-	-	-	-
Lead Acetate test	+	-	+	-	-
Alkaline reagent test	+	+	+	+	-
Mg & HCl reduction	-	-	-	-	-
Tannins					
Ferric chloride test	+	+	+	+	-

Notes: **(+) - indicates positive reaction to the chemical test**

(-) - indicates negative reaction to the chemical test

3.2.1.2 Phosphomolybdenum Assay

Total antioxidant activity assay revealed that 1 g of dry leaf powder has antioxidant activity equivalent to 0.035g/ml of ascorbic acid. Therefore, it is considered that 1 gram dry leaf powder is equivalent to 0.035 grams ascorbic acid in millilitre.

The total phenolic contents (TPC) and total antioxidant capacity (TAC) of the various leaf extracts of *Amaranthus tricolor* are graphically presented in Fig. 9. The aqueous extracts (AE and AD) exhibited higher TPC and TAC than the other tested extracts. All the extracts showed TPC dependant change in TAC, with the exception of the methanolic (ME) extract, which showed moderate TAC activity despite having a low TPC contents. The correlation between the DPPH, FRAP, TAC activities and TPC is presented in Table 8. The highest correlation is observed between the TPC and FRAP assays, whereas a very weak but positive correlation is observed between the FRAP and TAC assays.

3.2.2 Porcine pancreatic α-amylase Inhibition Assay

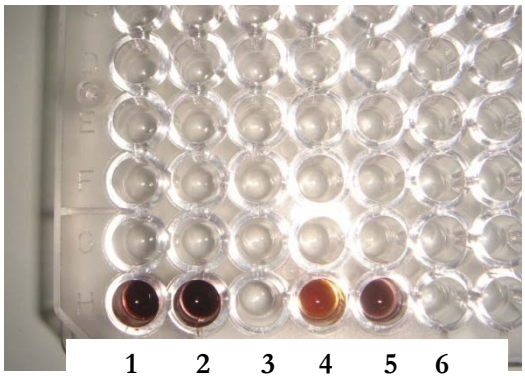
Amylase inhibition is presented in terms of percent inhibition of porcine pancreatic α-amylase (PPA) activity. The PPA inhibitions promoted by the aqueous and methanolic extracts at varying concentrations against porcine pancreatic α-amylase are presented in Fig 10 and Fig 11. The results are expressed in terms of percent inhibition against the phenolic content in the given concentrations of the extracts. Amongst all the prepared extracts in this study, the aqueous (AE) and the methanolic (ME) extracts promoted higher α-amylase inhibition than the other extracts. A dose dependant inhibition of porcine pancreatic a-amylase activity was noticed for each of the extracts, but the aqueous extracts induced more α-amylase inhibition than the methanolic extracts.

Table 6: Quantitative determination of major classes of phytoconstituents

Phytoconstituent	Content (% w/w)
Lipid	2.0
Phytosterols	0.178
Polyphenols	1.561
Tannins	1.25
Protein in 1 gm wet weight of leaf sample	1.5977

The values calculated as average of n=25.

Fig. 8 Photograph of qualitative DPPH Assay of all the extracts based on the colour change of the solution.



Photograph of DPPH Assay Plates showing colour change. Lane 1 – (ME) Methanol extract, Lane 2 – (MS) Methanol extract (Soxhlet), Lane 3 –Standard BHT (positive control), Lane 4 – (AE) Aqueous extract, Lane 5 – (PE) Petroleum Ether extract, Lane 6 – (AD) Aqueous extract

Table 7: 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay (Qualitative)

DPPH Antioxidant Colour Change	
Aqueous (AE)	Dark to light yellow
Aqueous (AD)	Dark to light yellow
Petroleum Ether(PE)	Dark to light yellow
Methanol Normal (ME)	No colour change
Methanol Soxhlet (MS)	No colour change
Butylated hydroxytoluene (BHT)	Colourless

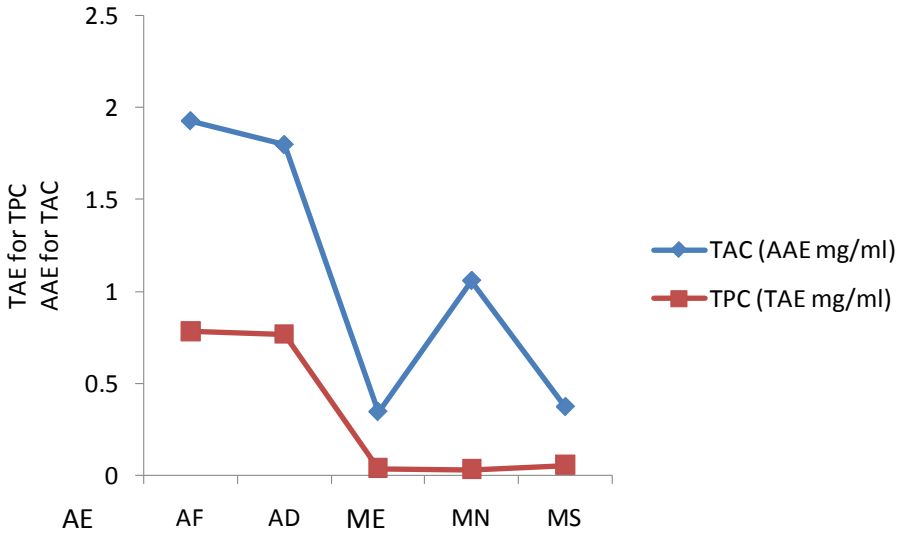
The values calculated as average of n=5.

Table 8: Antioxidant activities by DPPH and FRAP assays.

Extract	*DPPH (% inhibition)*	*FRAP (mM)*
Aqueous (AE)	65.72 ± 1.84	14.26
Aqueous (AD)	64.65 ± 0.39	14.26
Petroleum Ether(PE)	64.35 ± 0.84	1.46
Methanol Normal (MN)	2.68 ± 0.23	-6.1333
Methanol Soxhlet (MS)	5.46 ± 0.27	-5.8666
Butylated hydroxytoluene (BHT)	78.11 ± 0.04	-
$FeSO_4$	-	2

The values calculated as average of triplicate (n=3) ± SD.

Fig 9: Total Phenolic Contents and Total Antioxidant Capacity of various extracts of *Amaranthus tricolor*



The values calculated as average of triplicate (n=3).

Table 9: Correlation coefficients (R2) between antioxidant activities and Total Phenolic Content

	FRAP	TAC	TPC
DPPH	0.839069	0.233614	0.574214
FRAP	-	0.665343	0.926856
TAC	-	-	0.825426
TPC	-	-	-

DPPH *- 2,2-Diphenyl-1-Picrylhydrazyl,* ***FRAP*** *Ferric reducing Ability of Plasma,* ***TAC*** *Total Antioxidant Capacity,* ***TPC*** *Total Phenolic Content*

3.3 Spectral fingerprints of the aqueous and methanolic extracts

The spectral fingerprints of the aqueous and methanolic extracts are expressed in Fig 12 and Fig 13. The methanolic extract shows a higher absorption between 350 nm to 500 nm suggesting a higher percentage of carotenoid content as compared to the aqueous extracts. However the aqueous extracts show a higher absorption in the UV range (200 nm-350 nm) than the methanolic extracts, suggesting the possible presence of protein or amino acid content. Both the aqueous and methanolic extracts depict a peak between 650 nm to 700 nm, the intensity of which is stronger in the methanolic extract. This could possibly be part of the chlorophyll a or b twin peaks. The spectra were compared with spectra available online and from the Photochem Cad spectral library (Fig 14. and Fig 15).

3.4 Chromatographic studies

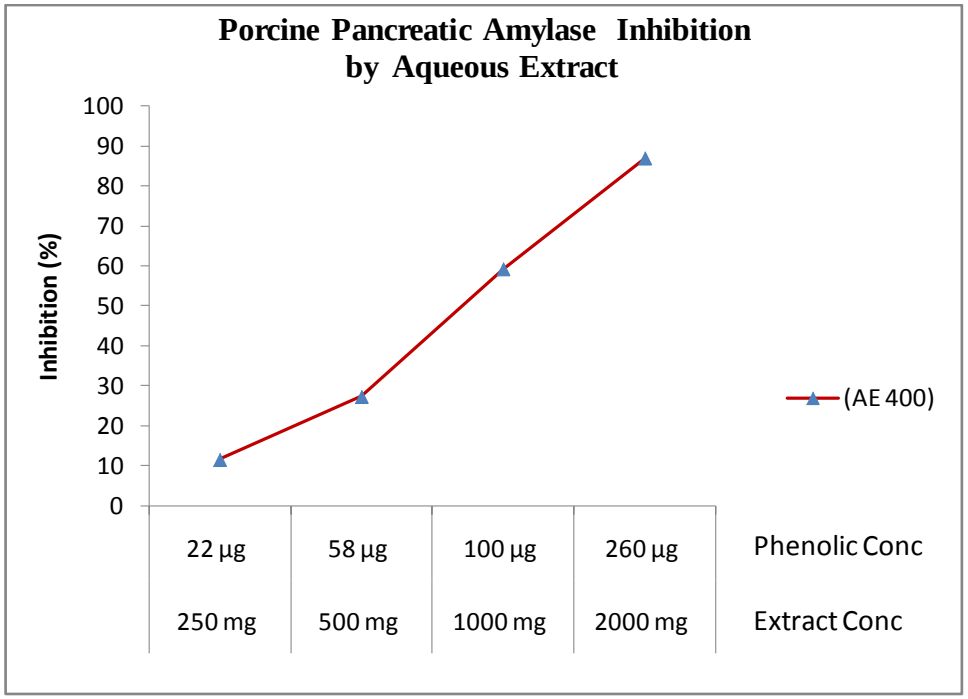
3.4.1 Thin Layer Chromatography

Thin layer chromatography of the methanolic extract in Hexane:Chloroform:Ethyl Acetate (1:3:3) solvent system yielded 11 visible bands. On exposure to iodine vapours a total of 13 bands could be visualized (Fig 16). The TLC plates were developed and visualized using 10% Alcoholic KOH, 1 % $FeCl_3$ and Vanillin-HCl. The TLC plates did not develop and show any visualizations with the KOH or Vanillin-HCl spray.

3.4.2 Column chromatography of Methanolic Extract

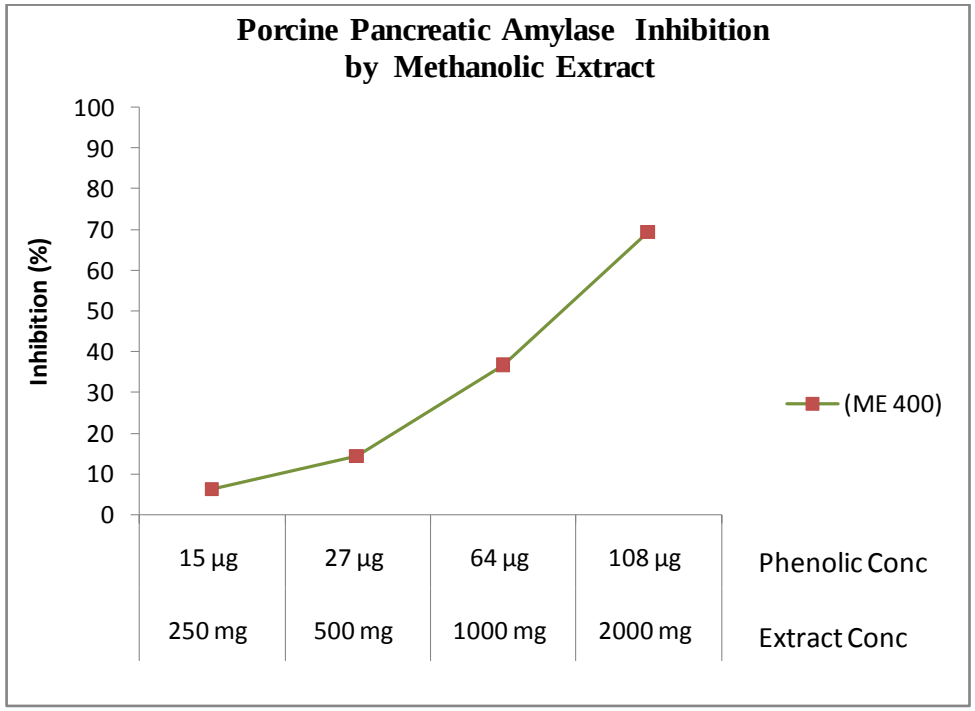
An attempt to separate the bands as fractions was done using column chromatography. After running the methanolic extract on a silica gel column with Hexane, Chloroform and Ethyl Acetate in varying ratios and a final methanolic wash, a total of 48 fractions of 30 ml each were collected. Each fraction was run on TLC along with the starting sample and similar fractions were pooled together to yield two pure fractions and five mixed fractions.

Fig 10: Inhibition of Porcine pancreatic α-amylase (%) by *Amaranthus tricolor* aqueous extract



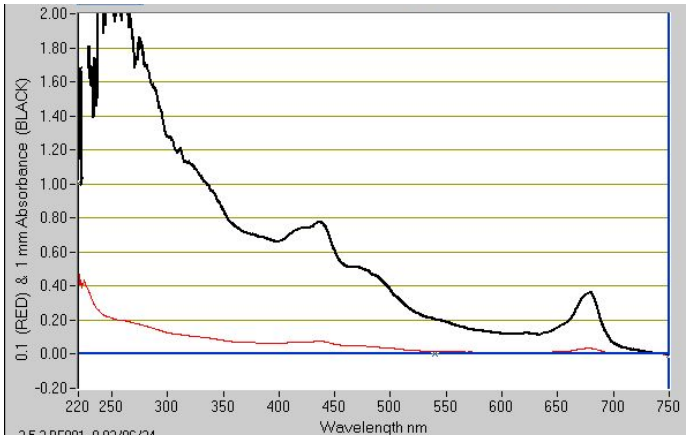
Inhibition of Pancreatic Procine Amylase by *Amaranthus tricolor* aqueous extract (AE) at various concentrations (10-1500 µg/ml). The α-amylase inhibition was analyzed colorimetrically using 3,5-dinitrosalicylic acid from PNPG at 405 nm after 30 minutes of incubation at 37°C. Results are expressed as mean of percent inhibition ± S.D against phenolic content in the given concentrations of the aqueous (AE) extract.

Fig 11: Inhibition of Porcine pancreatic α-amylase (%) by *Amaranthus tricolor* methanolic extract



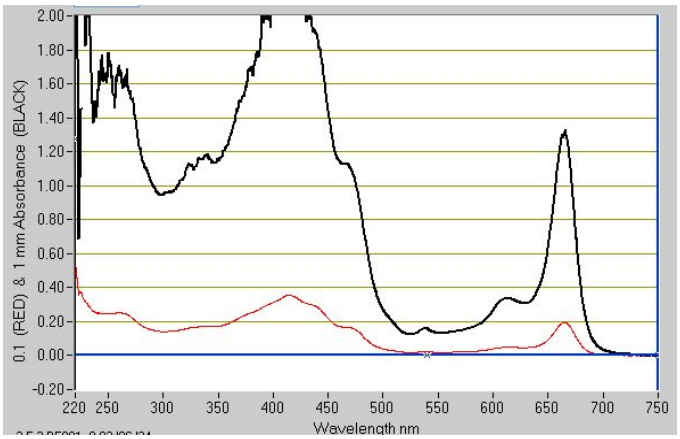
Inhibition of Pancreatic Procine Amylase by *Amaranthus tricolor* methanolic extract (ME) at various concentrations (10-1500 µg/ml). The α-amylase inhibition was analyzed colorimetrically using 3,5-dinitrosalicylic acid from PNPG at 405 nm after 30 minutes of incubation at 37°C. Results are expressed as mean of percent inhibition ± S.D against phenolic content in the given concentrations of methanolic (ME) extract.

Fig 12: Spectral finger print of Aqueous Extract (AE)



UV-Vis Spectral Scan was performed, with sample in water at 25ºC; at moderate scan speed from 1100 nm to 190 nm on UV-Vis 2450, Shimadzu, Japan.

Fig 13: Spectral finger print of the Methanolic Extract (ME)



UV-Vis Spectral Scan was performed, with sample in methanol at 25ºC; at moderate scan speed from 1100 nm to 190 nm on UV-Vis 2450, Shimadzu, Japan.

Fig 14: UV-Vis Spectra of Chlorophyll a from Photochem Cad Spectral library

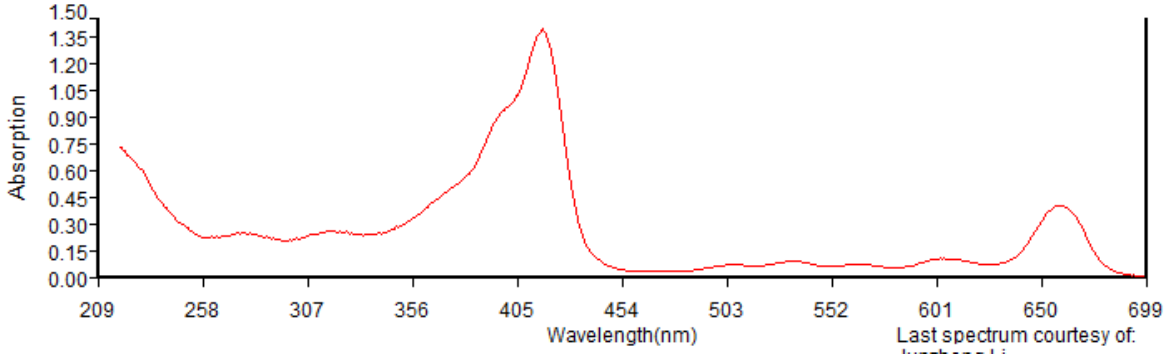
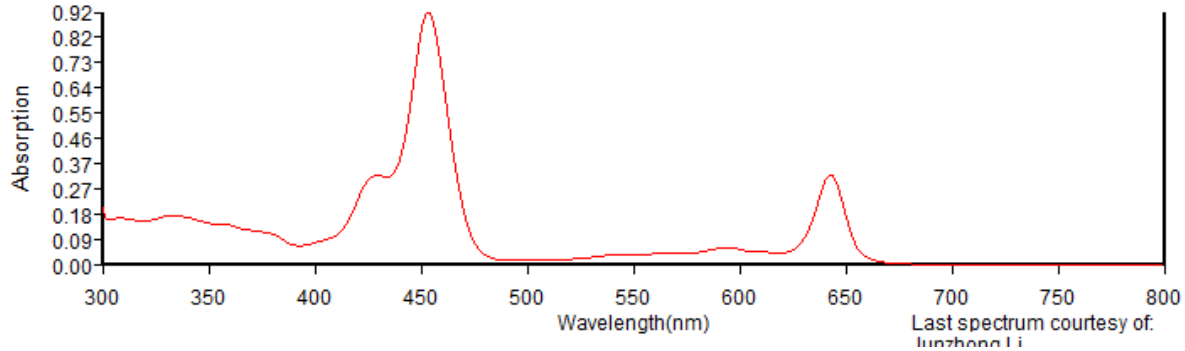


Fig 15: UV-Vis Spectra of Chlorophyll b from Photochem Cad Spectral library



3.5 Spectral studies of isolated powder

Upon evaporation of solvent, Fraction 1 yielded dark yellowish needle like powder, which on recrytallization yielded a pure white powder. The UV-Vis spectra of the uncrystallized and recrystallized powder in are depicted by Figs. 19 and 20. The FTIR spectrum (Fig. 18) revealed peaks in the 1200-1300cm^{-1} range, a peak at 2300 cm^{-1} and at 1700 cm^{-1} (Fig:). The melting point of the compound was found to be between 290-320°C. HNMR and CNMR analysis did not reveal any peaks besides the solvent peaks (Figs. 21, 22, 23, 24).

3.6 UV-Vis Spectra of Fraction 2

Upon evaporation of solvent, Fraction 2 yielded a dark reddish orange viscous liquid. The UV-Vis spectrum of Fraction 2 in methanol is depicted in Fig. 25. Comparison of this spectrum with online spectral libraries (Fig. 26) and available literature revealed that Fraction 2 was beta carotene.

Based on the results from the percent yield, the plant antioxidant assays and the amylase inhibition assay the aqueous (AE) extract and methanolic (ME) extract were chosen for further antidiabetic studies. Based on the results from the acute oral toxicity test, 1/10th of the highest dose administered and its double were chosen as the doses required to be evaluated for its therapeutic properties.

Hence for all the further physiological studies these two extracts were chosen in two concentrations each and were designated as AE 200, AE 400– Aqueous extract; and ME 200, ME 400 – Methanolic extract.

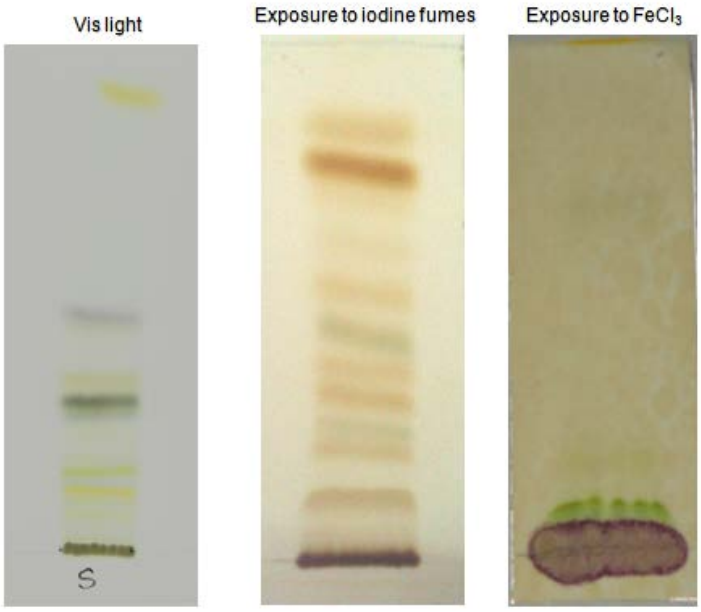


Fig 16: Thin Layer Chromatograms of *Amaranthus tricolor* Methanolic extract; developed on Merck Silica Gel 60 F254 plates in a Hexane:Chloroform:Ethyl Acetate (1:3:3) solvent system. Visualized in a. Visible Day Light; b. Exposure to iodine fumes; c. Exposure to $FeCl_3$

Table 10: Comparison of experimental Rf values of methanolic extract with available literature.

No	Band Colour Vis	Band Colour Iodine	Rf Vis	Rf Iodine	Literature Rf	Possible Component	Q/F, UV 256/ 365
1	Yellow-orange	Light brown	0.95	0.95	0.93-0.98	Carotene	-
2	-	Dark Brown	-	0.82	-	Polyphenol/ Flavonoid	Q, UV 256
3	-	Brown	-	0.75	-[b]	unknown	-
4	-	Brown	-	0.69	0.64[a]	Tannins?	-
5	Grey	Grey brown	0.56	0.59	0.55	pheophytin a	-
6	Grey *	Light grey brown	0.53	0.51	0.47-0.54	pheophytin b	-
7	Blue-green	Bluish Brown	0.46	0.48	0.46	chlorophyll a	-
8	Yellow-Green	Greenish Brown	0.44	0.42	0.42	chlorophyll b	-
9	Yellow	Brown	0.36	0.35	0.41	xanthophylls	-
10	Yellow	Brown	0.27	0.30	0.31	xanthophylls	-
11	-	Brown	-	0.25	-[b]	unknown	-
12	Yellow	Brown	0.17	0.20	0.17	xanthophylls	-
13	Yellow	Brown	0.11	0.10	0.15	xanthophyll	-
14	-	Faint Brown	-	0.05	0.06[a]	Tannins**	-
15	Purplish brown**	Blackish Brown	baseline	baseline	0.01[a]	Tannins**	-

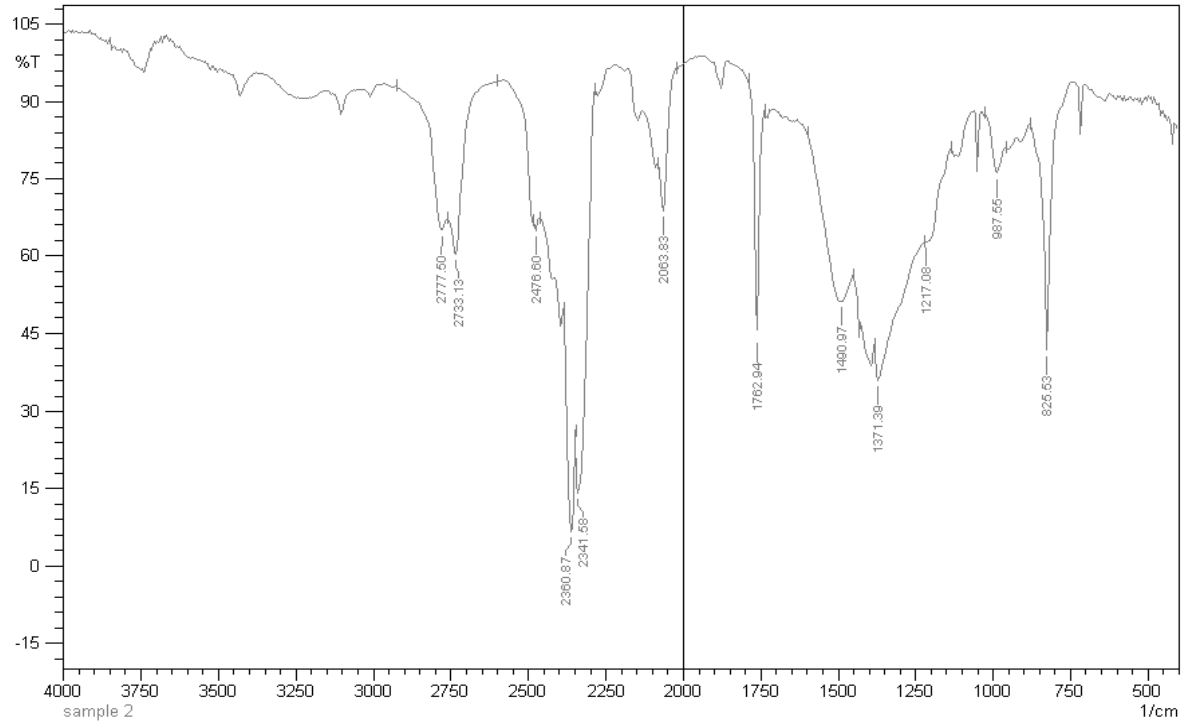
** Upon exposure to $FeCl_3$; Q –Quenching; F-Fluorescence; [a] (Yamuna Devi et. al., 2012); [b]Literature strongly suggests polyphenols or flavonoids, though this requires further investigation.

The above table is based on mean values derived from repeated TLC runs, (n=20).

Fig 17: Column Chromatograhy set up and collected fractions

Spectral properties of isolated compound

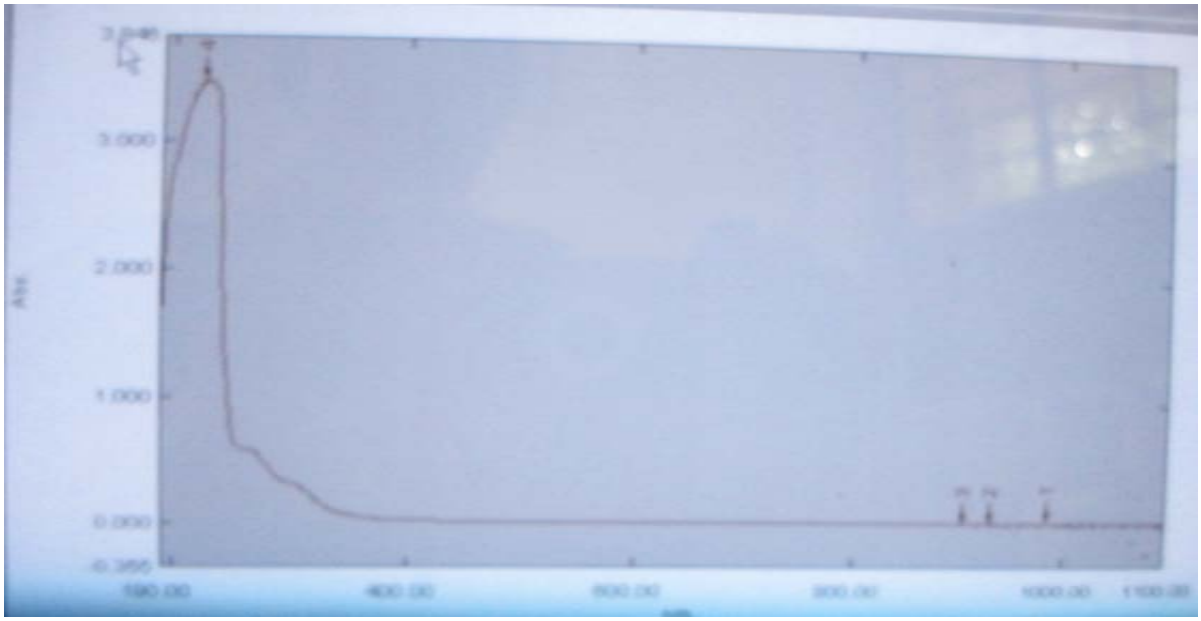
Fig 18: Fourier transform infrared spectroscopy (FTIR) of isolated compound (recrystallized) from Fraction 1



IR spectrum (KBr, v, cm-1):

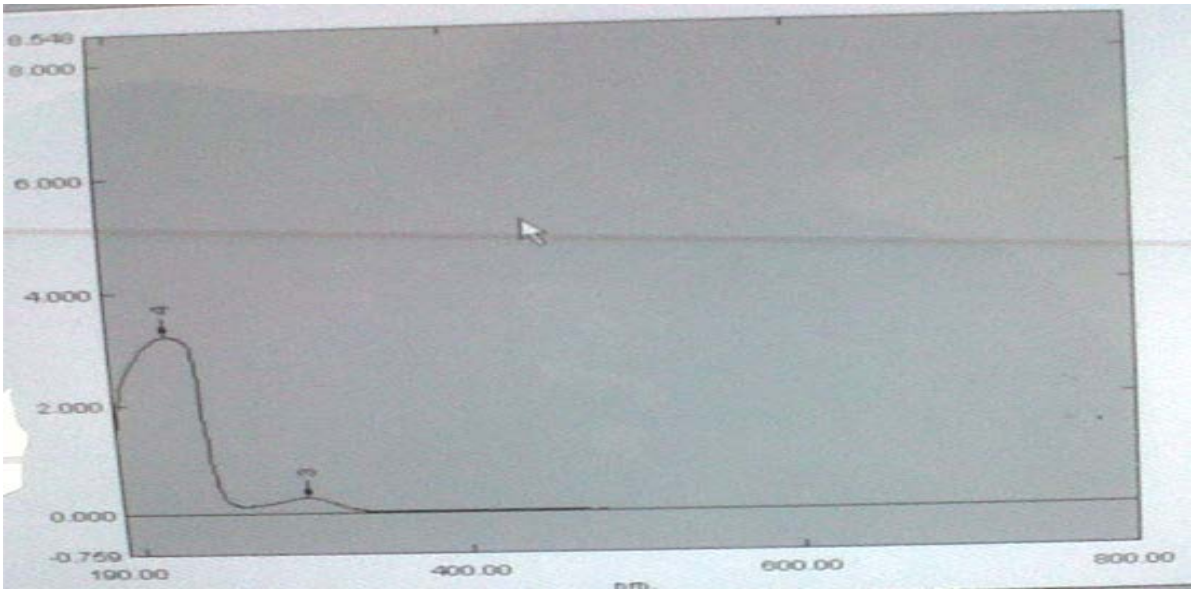
peak at 1200-1300	Aromatic phenyl group
peak at 2300	Cyano /triple bond
peak at 1700	Cyclic ester/amide

Fig 19: UV-Vis Spectra of isolated compound, unrecrystallized (Fraction 1)



UV spectrum (H20, λmax, nm): 220±4, 304±4

Fig20: UV-Vis Spectra of isolated compound, recrystallized in methanol (Fraction 1)



UV spectrum (H20, λmax, nm): 220±4, 304±4

Fig 21: HNMR Spectra of isolated compound (Fraction 1) in D_2O

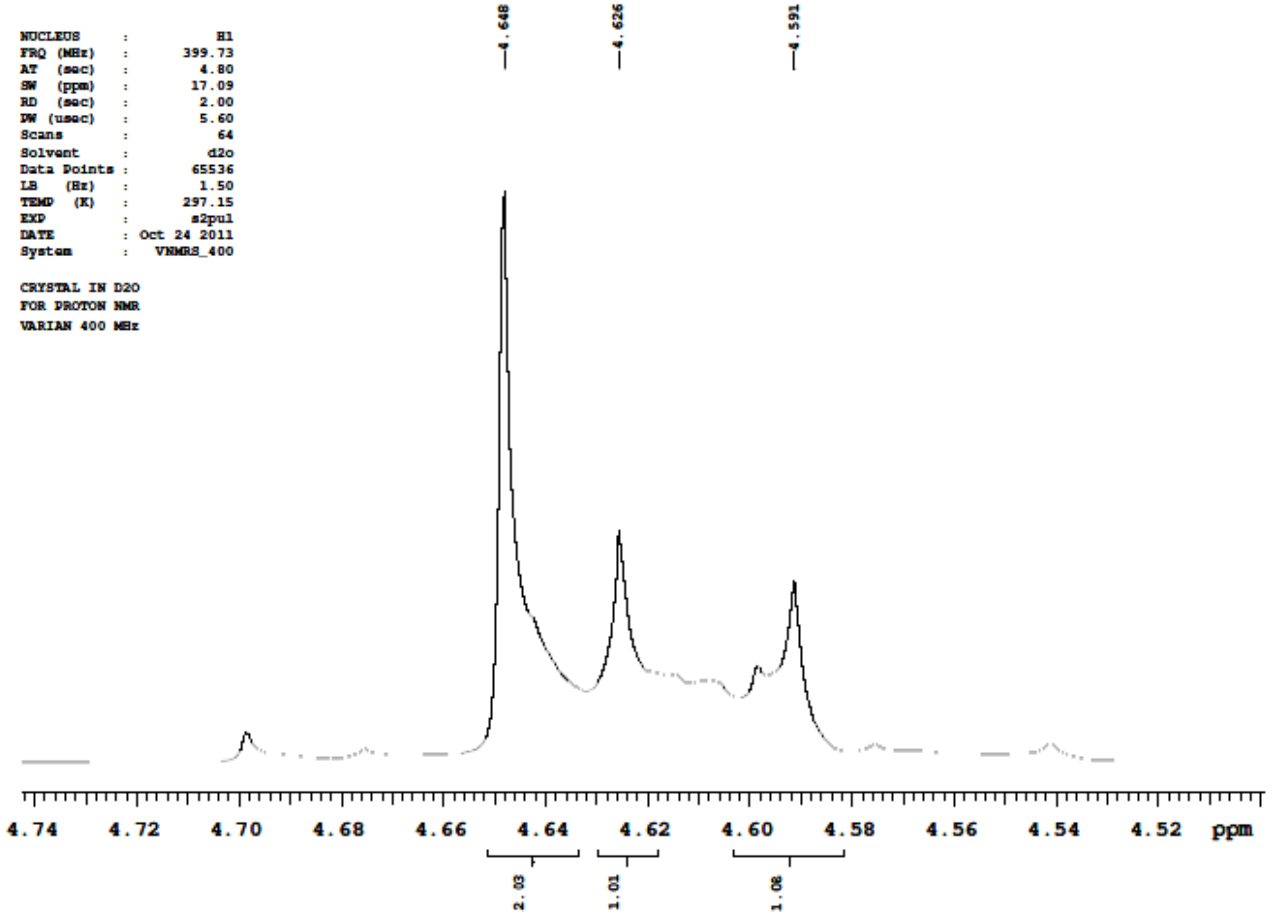


Fig 22: CNMR Spectra of isolated compound (Fraction 1) in D_2O

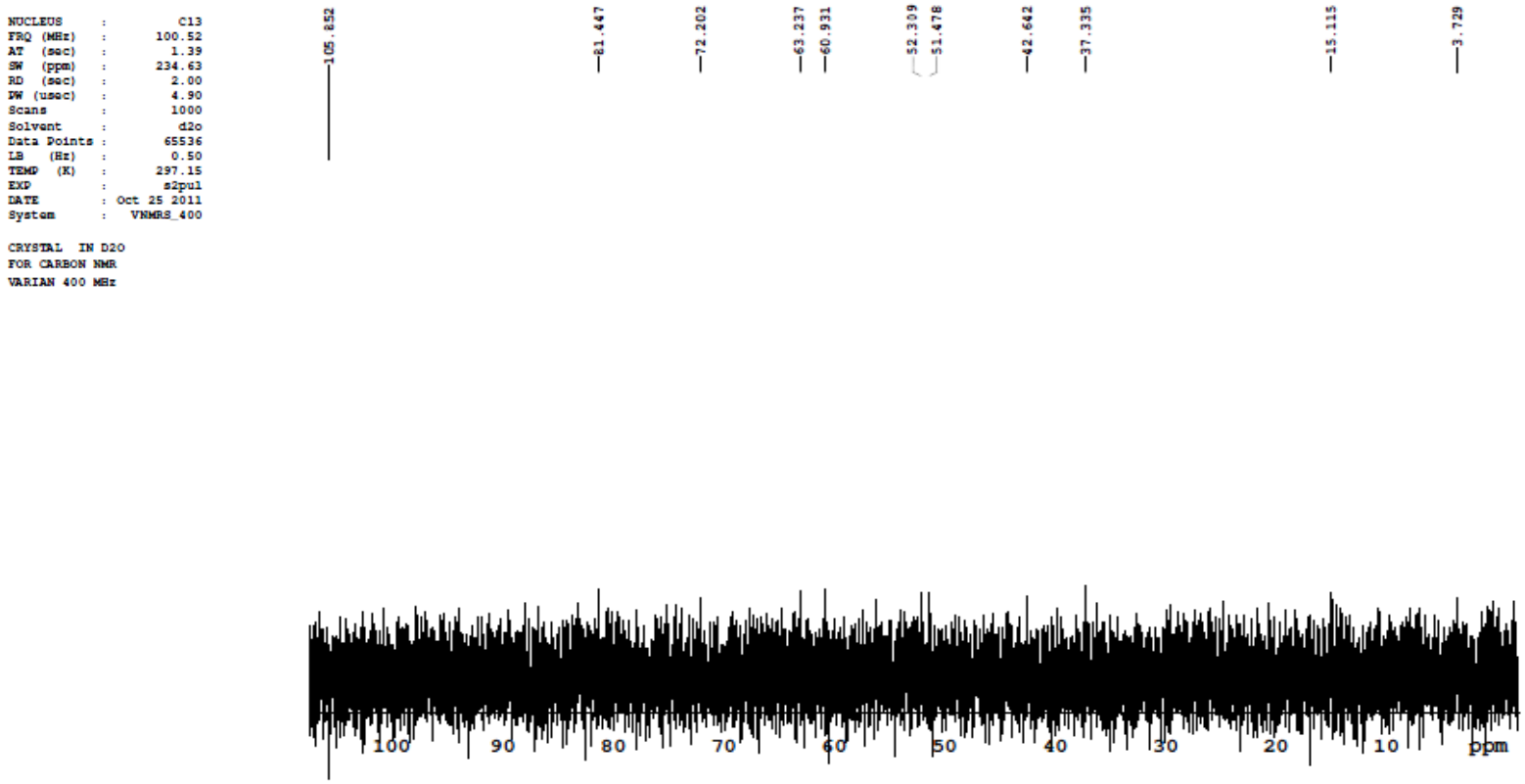


Fig 23: HNMR Spectra of isolated compound (Fraction 1) in DMSO

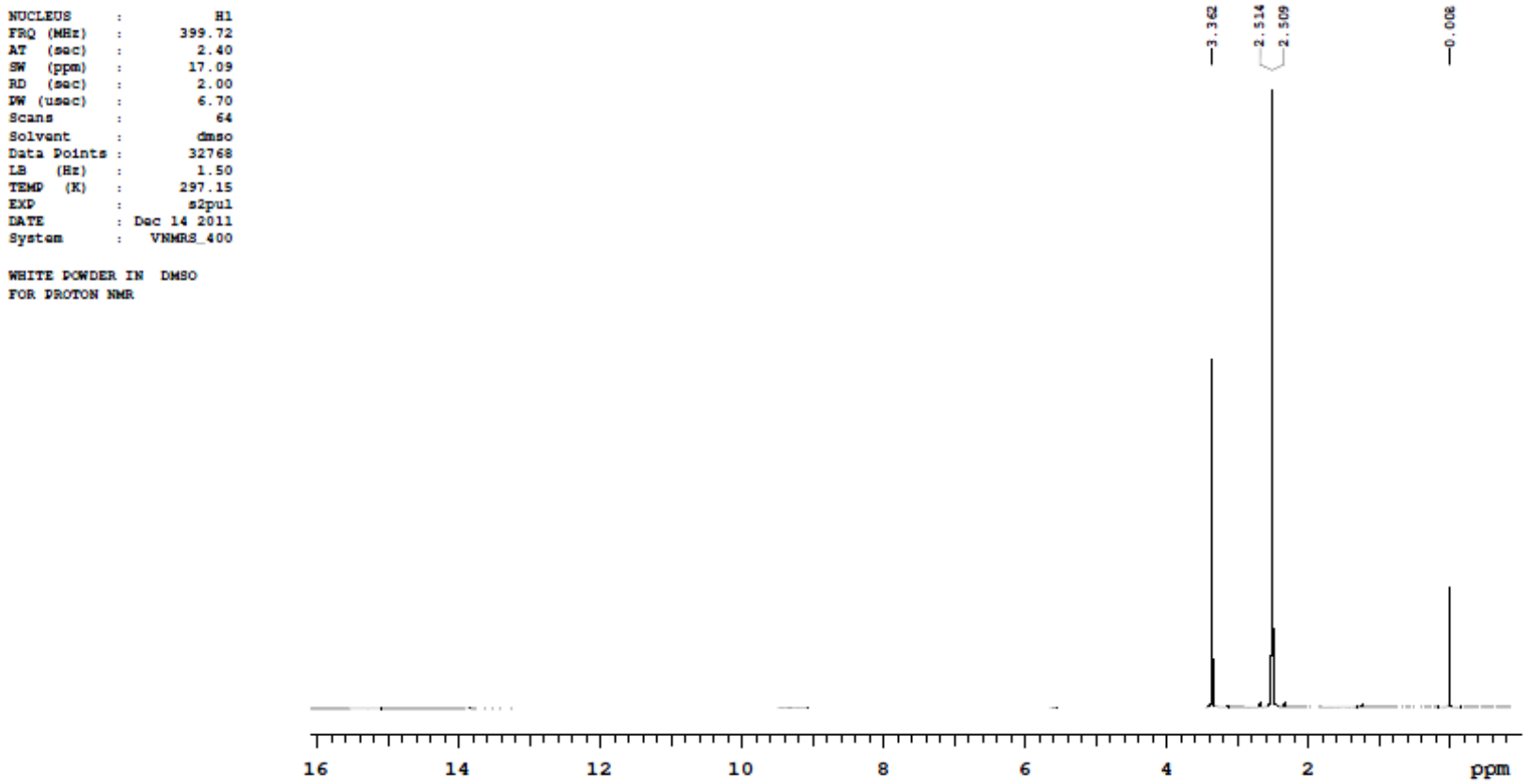


Fig 24: CNMR Spectra of isolated compound (Fraction 1) in DMSO

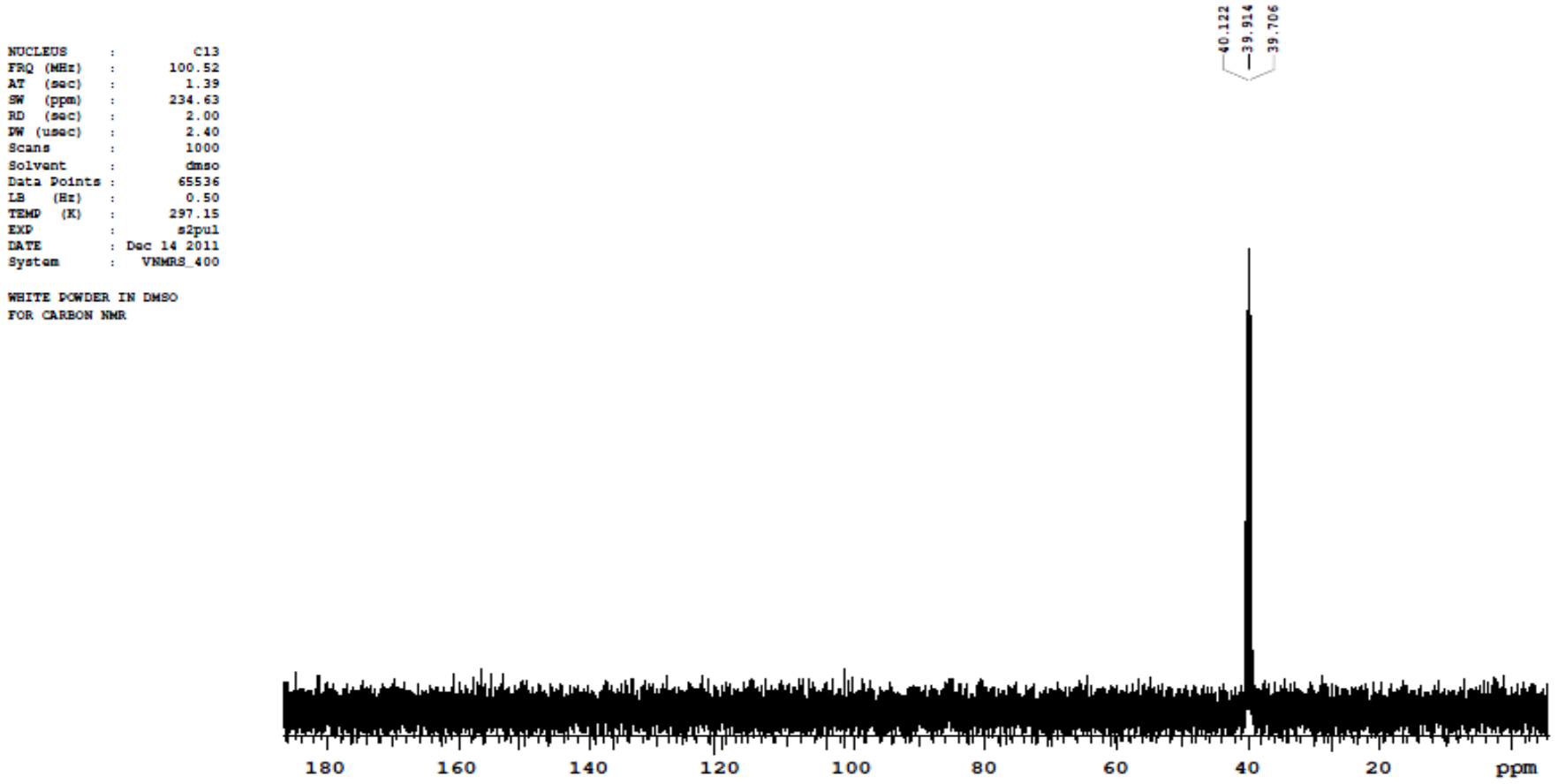


Fig 25: UV-Vis Spectra of isolated compound (Fraction 2)

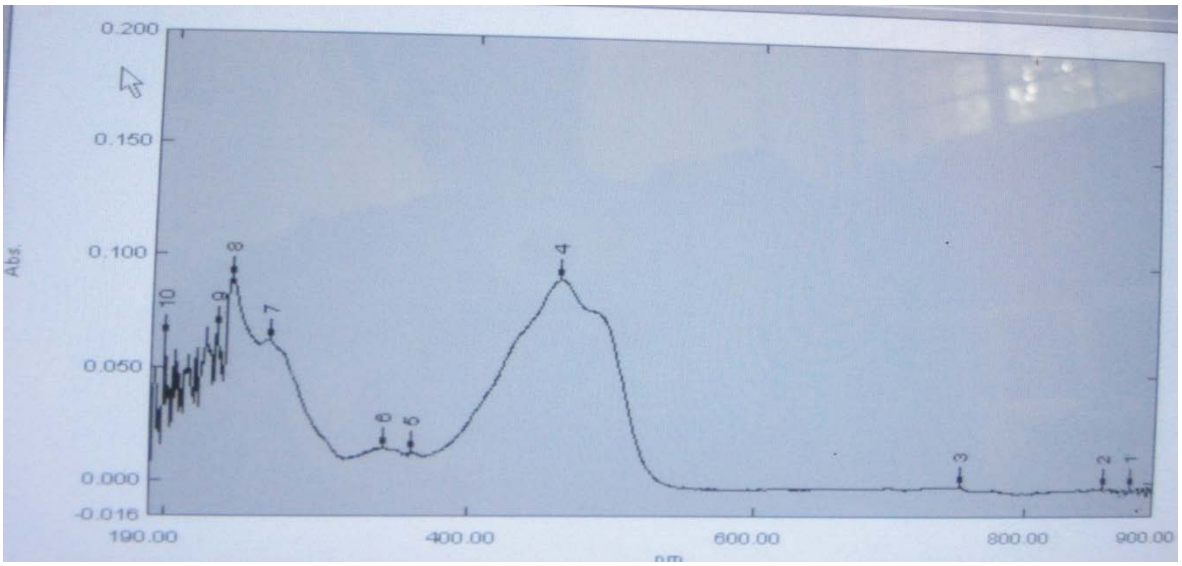
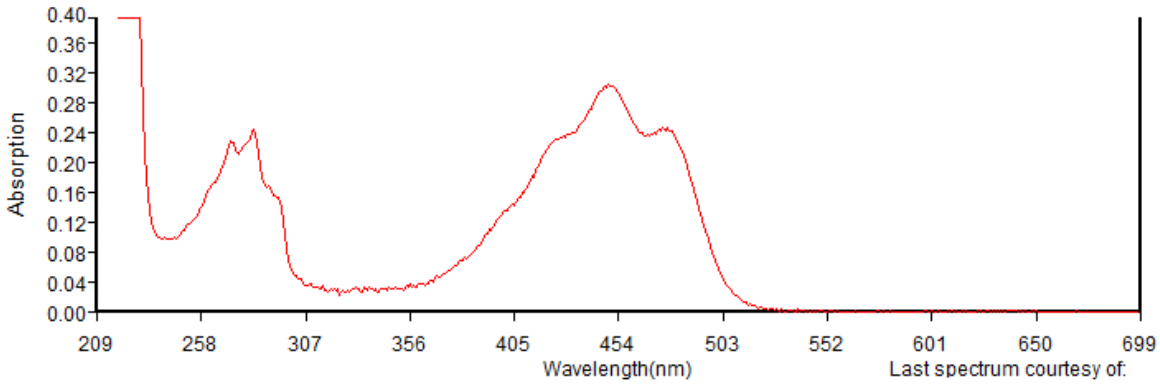


Fig 26: UV-Vis Spectra of beta carotene from Photochem Cad spectral library



3.7 Animal Studies

3.7.1 Standardizations followed for the induction of alloxan diabetes

3.7.1.1 Inbreeding of rats

The rats were inbred and crossed for 10 generations within the same colony, in order to have a similar genetic background.

3.7.1.2 Selection of alloxan doses

The dose of alloxan chosen for this research work was 145 mg/kg BW. This dose was seen to cause alloxan diabetes in 95% of the population with 1-2% mortality.

3.7.1.3 Observations on alloxan mediated diabetic rats.

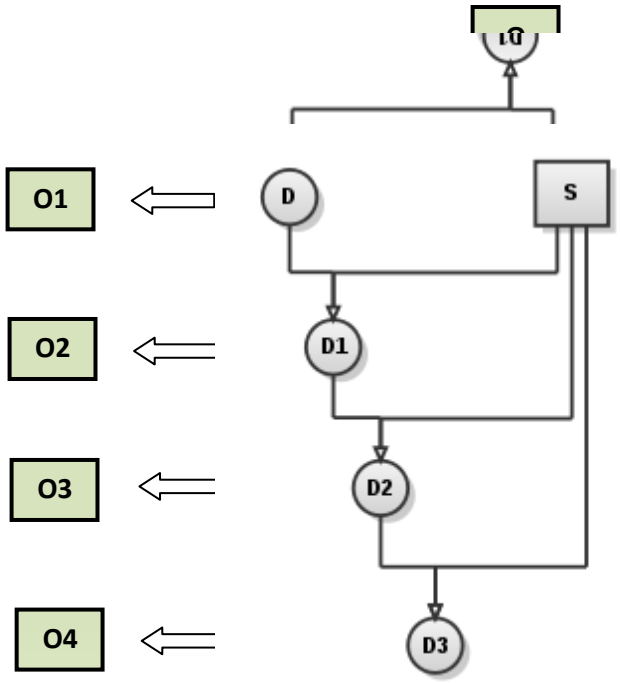
The alloxanic diabetic rats appeared to have:

- Fatigue, lethargy, subdued behavior, lack of interest in surroundings.
- Drastic loss of body weight and cadaver like appearance
- Loss of lustre of fur.
- Increased urination - bedding always wet.
- Increased thirst.
- Loss of body warmth (hypothermia), animal feels cold and clammy to the touch.
- Normal pinkish tinge of extremities replaced by bluish tinge after onset of diabetes.

Further, the rats exhibiting fasting blood glucose level $\leq$ 200 mg/dl, were injected again with the same dose of alloxan until diabetes developed and confirmed.

Some of the rats died due to uncontrolled and extremely high diabetes. Such rats emanated acetone like odour from the body indicating that the rats died due to ketoacidosis. These rats had blood glucose levels equal to ≥ 480 mg/dl. The percentage of animals responding in a similar fashion to the diabetogenic agent (alloxan) was higher (80%) in inbred animals as compared to the animals (45%) which were not part of the inbred colony.

Fig 27: Flow chart depicting the inbreeding of rats sensitive to alloxan.



S - (Son), D - (Daughter), O - (Offspring comprising males and females). S and D are from the same litter exhibiting the desired susceptibility to alloxan.

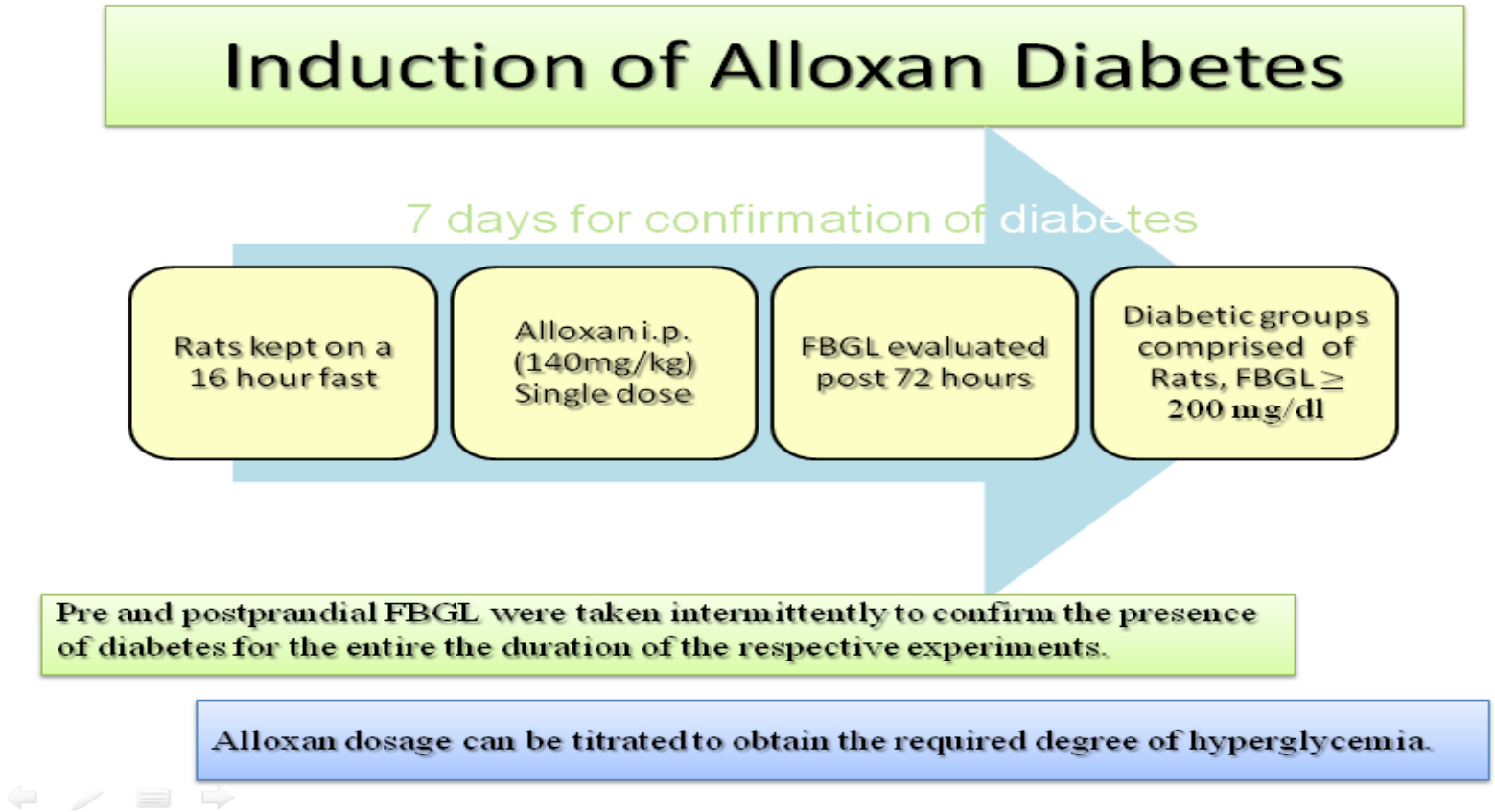


Fig 28: Flowchart depicting the induction of alloxan diabetes in rats.

After the induction of alloxan diabetes, the control as well as diabetic rats were administered the extracts in a bid to study its in vivo effect on the diabetic state.

3.7.2 In vivo bioassays

3.7.2.1 Acute Oral Toxicity Test

Oral administration of extracts of *Amaranthus tricolor* in mice did not show any visible symptoms of distress, morbidity or mortality during the observation period. Both the aqueous as well as the methanolic extracts were safe upto doses of 2 g/kg BW. Based on the results from the acute oral toxicity tests, 1/10th of the highest dose and its double was administered, i.e. 200 mg/kg BW and 400 mg/kg BW, were chosen as the doses to be evaluated for their therapeutic properties.

3.7.2.2 Screening for antidiabetic activity (Acute study)

Alloxan induced elevation of blood glucose level beyond that found in controls suggest a diabetic state. Such diabetic rats were subjected to a single dose of either AE200 (200 mg/kg BW) or AE400 (400 mg/kg BW). The Fig. 29 describes the dose dependant reduction of hyperglycemia by the leaf extracts of *Amaranthus tricolor*. It is clear from the figure that the AE200 and AE 400 treated diabetic rats exhibited significant reduction in blood glucose level by the 3rd hour. After administering AE200, the blood glucose level of diabetic rats lowered for 9-12 h. However, AE400 administration continued to decrease the blood glucose level beyond 9 h, suggesting a prolonged antidiabetic effect. The diabetic rats administered with ME200 and ME400 also exhibited a gradual dose dependant drop in blood glucose level, but not as effective as the AE200 and AE400 extracts.

3.7.2.3 Treatment of diabetic rats with *Amaranthus tricolor* extracts for 3 weeks

3.7.2.3.1 Body Weight

Diabetic controls exhibited a significant decrease ($p \leq 0.05$) in body weight as compared with normal controls. The diabetic animals given AE 400 showed a lesser percentage of weight loss or no weight loss when compared with the diabetic controls who exhibited a higher percentage of weight loss.

3.7.2.3.2 Glucose

The diabetic groups treated with aqueous extracts (AE200 and AE400), showed a highly significant ($p \leq 0.05$) decrease in blood glucose level by the end of 3 weeks of treatment. These results are given in Fig 30. It is obvious from the figure that AE400 was more effective than AE200, while ME200 and ME400 were relatively less effective.

3.7.2.3.3 Insulin

Serum insulin level of the diabetic rats was much less as compared to the normal rats (Fig. 31), which confirmed the destruction of pancreatic islets by the diabetogenic agent alloxan. This fall in serum insulin level was reflected in the insulin content of the pancreas (Fig. 32), there by confirming Type 1 diabetes in the diabetic groups. Treatment of the diabetic rats with the aqueous or the methanolic *Amaranthus tricolor* extracts did not restore or show any improvement in the serum insulin level nor did it have any sort of beneficial effect on the pancreatic insulin content.

3.7.2.3.4 Glycogen

The alterations in glycogen content in the liver and skeletal muscles are presented graphically in Fig 33 and Fig 34. The AE400 treated diabetic group showed the greatest improvement in glycogen contents of the liver. The glycogen level of skeletal muscles was maintained to a nearly normal level by AE200 and AE400. Administration of ME400 also seemed to improve the glycogen level of skeletal muscles.

3.7.2.3.5 Total Protein

Significant decrease in serum and liver total proteins is observed in untreated alloxan diabetic rats as compared to normal rats. Upon treatment with aqueous and methanolic extracts, the total protein levels of the aqueous treated rats show enhanced serum and liver total protein levels than the methanolic extracts, although the protein levels do not get restored to normal levels (Fig. 35 and Fig. 36).

3.7.2.3.6 Total Bilirubin

The diabetic rats seem to possess an increased level of serum bilirubin than the normal rats (Fig. 37). Treatment with AE400 reduces the elevated bilirubin content in the serum to near normal values. A dose dependent improvement in serum bilirubin is seen in both the aqueous and methanolic treated groups.

3.7.2.3.7 Hypolipidemic study

Serum cholesterol, triglyceride and LDL levels were significantly higher ($p \leq 0.05$) in diabetic controls, while HDL levels were significantly decreased ($p \leq 0.05$) in diabetic controls, as compared to normal controls. Following the treatment of diabetic rats with AE

400, a significant reduction ($p \leq 0.05$) in serum cholesterol, triglyceride and LDL and a significant increase ($p \leq 0.05$) in HDL were noticed (Fig. 38).

3.7.2.3.8 Amylase activity in serum and pancreas

The changes in serum amylase activity after administration of aqueous and methanolic extracts to alloxan diabetic rats are presented graphically in Fig. 39. The diabetic rats exhibit a significant (threefold) increase ($p \leq 0.05$) in serum amylase activity. The AE and ME extracts lowered the serum amylase activity of diabetic rats. However, only AE400 could restore the amylase activity to a relatively normal level.

The effects of aqueous and methanolic extracts on amylase activity in pancreatic tissue of alloxan diabetic rats are expressed in Fig 40. The amylase activity of pancreas significantly increased (threefold) in alloxan diabetic rats. It is obvious from the graph that only AE400 could restore pancreatic amylase level to a relatively normal state.

3.7.2.3.9 Lipase activity in serum and pancreas

The changes in lipase activity of serum of aqueous and methanolic extracts treated alloxan diabetic rats are expressed in Fig. 41. The diabetic rats exhibit a significant (twofold) increase ($p \leq 0.05$) in serum lipase activity. From the graph it is observed that the AE400 extract, could restore the lipase activity to a nearly normal level.

Fig. 42 depicts graphically the effects of aqueous and methanolic extracts on lipase activity in pancreas of alloxan diabetic rats. The lipase activity of pancreas increased more than two fold in alloxan diabetic rats. All the extracts could lower the pancreatic lipase activity.

3.7.2.3.10 Alkaline Phosphatase

The alterations in hepatic Alkaline Phosphatase (AlP) activities are graphically represented by Fig 43. The alloxan diabetic rats showed a decline in hepatic AlP activity. The hepatic AlP activity of diabetic rats exposed to different concentrations of aqueous and methanolic extracts improved significantly but remained below that of controls. AE400 induced better recovery of AlP activity. The alterations of Alkaline Phosphatase (AlP) activities in the serum of the various groups of rats are graphically presented in Fig 44. The diabetic rats exhibited an increase in serum AlP activity. None of the extracts could restore the serum AlP activity to a near normal level.

3.7.2.3.11 Acid Phosphatase

Fig. 45 graphically represents the AcP activities of hepatic tissue of normal, diabetic and extract treated diabetic rats. The diabetic animals show a significant decrease in hepatic AcP activity. None of the extracts could restore the hepatic AcP activity to a normal level. The variations in serum AcP activities of the control and experimental rats are graphically given in Fig 46. Alloxan diabetic rats exhibited rise in AcP activity of the serum, and none of the extracts could restore the serum AcP activity to a normal level.

3.7.2.3.12 Alanine transaminase

The alterations in ALT activities of the liver tissue are presented in Fig. 47. The ALT activity of the liver, after the administration of aqueous and methanolic extracts in the alloxan diabetic rats, elevated in comparison to that found in the diabetic rats. The diabetic rats had low hepatic ALT activity than controls. In general, the AE and ME improved the hepatic ALT activity. The alterations in the serum ALT activity after administration of

aqueous and methanolic *Amaranthus tricolor* extracts to alloxan diabetic rats are presented graphically in Fig. 48. The diabetic rats exhibit a significant increase ($p \leq 0.05$) in serum ALT activity. The diabetic rats exposed to AE400 exhibit restoration of serum ALT activity to a nearly normal level.

3.7.2.3.13 Aspartate transaminase

The alterations of hepatic AST activities are graphically represented in Fig. 49. Rats made diabetic with alloxan showed a decline in hepatic AST activity when compared with non-diabetic rats (controls). The AST activity showed an increase in the hepatic tissues of diabetic rats dosed with different concentrations of aqueous and methanolic extracts of *Amaranthus tricolor*. The highest increase in AST activity was seen in the diabetic group treated with the aqueous extract (AE400), at a dose of 400 mg/kg body weight. Though administration of methanolic extracts (ME 200 and ME 400) at 200 mg/kg body weight and 400 mg/kg body weight respectively caused an increase in AST activity, it did not show full recovery to normal values but the data suggests that the extracts elevated the decreased AST activity promoted by diabetes.

The alterations of AST activities in serum are presented in Fig. 50. Alloxan diabetic rats exhibit an increase in AST activity as compared to non-diabetic rats (controls). The AST activity showed a dose dependant decrease in the hepatic tissues of diabetic rats fed with, aqueous extracts of *Amaranthus tricolor*. The most significant decrease in AST activity was seen in the diabetic group treated with the aqueous extract (AE400). The ME extracts did not bring the serum AST activity to the normal (control) level.

3.7.2.3.14 Lactate dehydrogenase

The levels of LDH activities of the hepatic tissues of the normal, diabetic and treated rats are shown in Fig 51. Alloxan diabetes reduced drastically the LDH activity of the liver. Only AE400 extract could improve the liver LDH activity to a nearly normal state. Alterations in renal LDH activity in the *Amaranthus tricolor* extract fed diabetic rats are expressed graphically in Fig 55. Alloxan diabetes reduced LDH activity of the kidney significantly ($p \leq 0.05$) by 73%. It appears from the graph that the AE400 could induce a relatively better recovery of renal LDH activity.

3.7.2.3.15 Glucose 6 phosphate dehydrogenase

Fig 52 depicts graphically the G6PD activity of the liver of the normal and diabetic rats. Glucose 6 phosphate dehydrogenase activity decreased in the liver of diabetic rats. The administration of AE400 and ME400 to the diabetic rats evoked recovery of G6PD activity in the hepatic tissue, however it was not completely restored to a normal level. Fig. 56 graphically describes the effects of *Amaranthus tricolor* extracts on Glucose 6 phosphate dehydrogenase (G6PD) activities of the renal tissue of the diabetic rats. It appears that diabetes elevates the kidney G6PD activities and only ME200 could restore it to a nearly normal level.

3.7.2.3.16 Hexokinase

Fig 53 shows the changes in hexokinase activity of the hepatic tissue of normal, diabetic and extract treated diabetic groups. The diabetic rats show a excessive drop ($p \leq 0.05$) in the hexokinase, activity. Only AE400 treatment could elevate the hexokinase activity effectively but it could not be restored to a normal level.

The effects of AE and ME on renal hexokinase activities on diabetic rats are graphically presented in Fig. 57. The diabetic rats show a significant rise ($p \leq 0.05$) in the levels of hexokinase activity. It appears that all the extracts could significantly reduce the kidney hexokinase activity of the diabetic rats.

3.7.2.3.17 Glucose 6 phosphatase

Fig. 54 expresses alterations in the liver G6Pase activities of the control and experimental rats. The glucose-6-phosphatase activity in the liver of alloxan diabetic rats shows a significant (three-fold) increase ($p \leq 0.05$) as compared to that found in control (normal rats). Administration of aqueous (AF) and methanolic (MN) extracts to the diabetic rats cause a significant reduction ($p \leq 0.05$) in hepatic glucose-6-phosphatase activity. However, none of the treatment regimen could restore the hepatic G6Pase activity to a normal level.

In Fig. 58, the renal glucose-6-phosphatase activities of normal, alloxan diabetic and treated rats are expressed. It appears that alloxan diabetes increases kidney G6Pase activity beyond the normal level but none of the extracts could restore it to a normal state.

3.7.3 Hematological Parameters

The effect of AE400 on Hb%, PCV%, RBC and WBC counts are presented in Table 11. The diabetic rats showed a highly significant ($p \leq 0.001$) decrease in Hb%, PCV%, RBC and WBC counts as compared with the normal controls. From the data it is clear that AE 200 and AE 400 had a significant effect ($p \leq 0.05$) on the Hb level in the diabetic rats as compared to controls. The rise in WBC and RBC counts was negligible in the extract treated groups except in the AE400 fed group, as compared to the diabetic controls, while

% PCV increased significantly ($p \leq 0.05$). The AE 400 extract fed rats showed significant improvement ($p \leq 0.05$) in WBC, RBC count and a highly significant improvement ($p \leq 0.001$) in PCV and Hb levels as compared to the diabetic rats.

3.7.4 Effect of Fractions from methanolic extract on BGL in diabetic rats - (Acute Study)

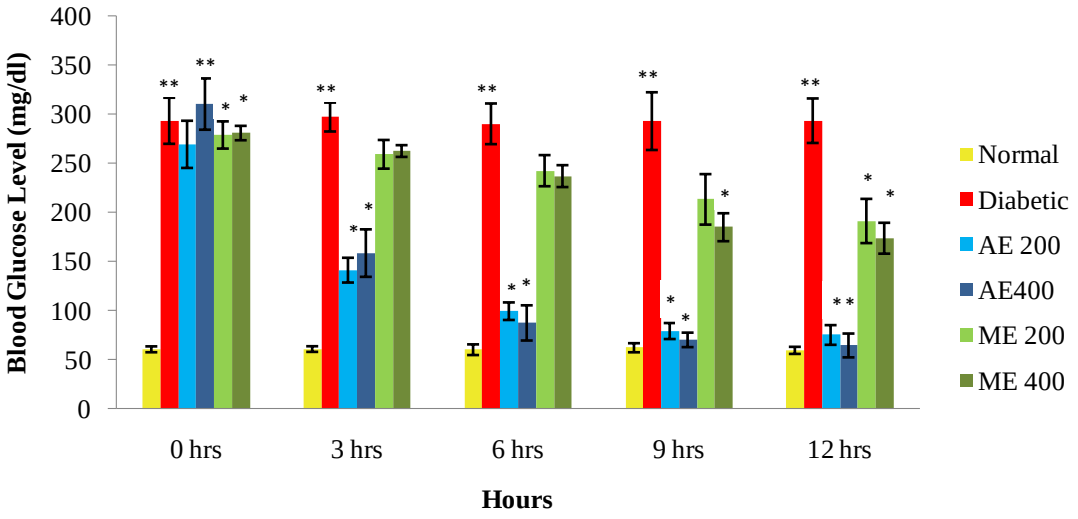
Alloxan induced diabetic rats were subjected to a single dose of 200 mg/kg BW of each fraction. The Fig. 59 illustrates the dose dependant reduction of hyperglycemia by the leaf extracts of *Amaranthus tricolor*, along with the effect of the respective fractions on the diabetic rats. It is clear from the figure that besides the AE and ME extracts, none of the fractions exhibit a significant reduction in fasting blood glucose level. However a minor nonsignificant reduction is seen in the diabetic rats administered Fraction 1, Fraction 2, Fraction 6 and Fraction 8.

3.8 In Vitro Assay

3.8.1Glucose Uptake Study

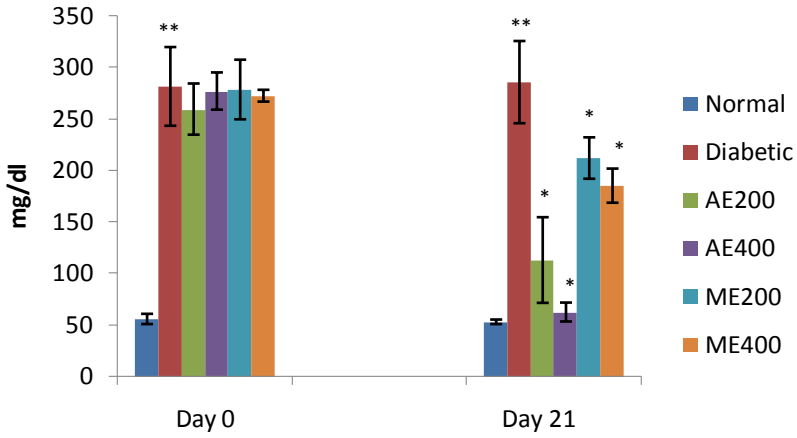
Fig. 60 and 61 are photographs of the Glucose uptake apparatus designed and assembled in the Physiology laboratory of Department of Zoology, Goa University. The glucose uptake by the isolated diaphragm and skeletal muscles are tabulated in Table 12 and 13. From the tables it is observed that the uptake of glucose and its conversion to glycogen is highest in the groups containing tissue, incubated in the presence of the extracts only. The groups where in the tissues were incubated with extract as well as insulin resulted in poorer absorption and conversion to glycogen.

Fig. 29: Dose dependant reduction of hyperglycemia by *Amaranthus tricolor* leaf extracts in alloxan-induced diabetic rats – Acute Study



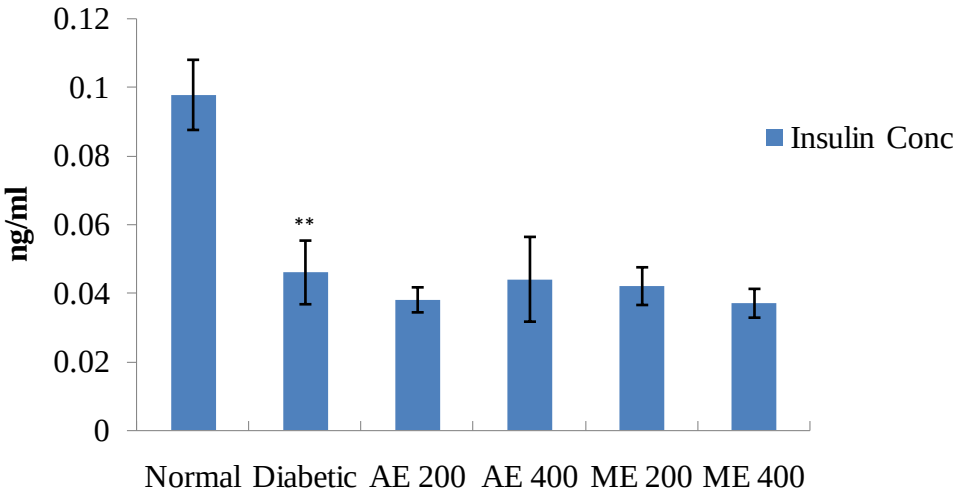
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤.05 vs. respective control rats.

Fig. 30: Effect of aqueous and methanolic *Amaranthus tricolor* extracts on Blood Glucose Level



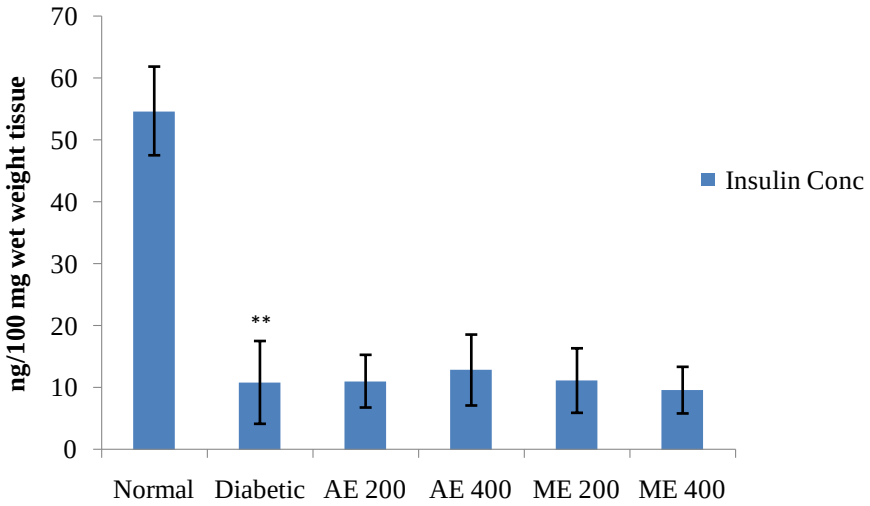
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p ≤.05 vs. respective control rats.

Fig. 31: Effect of *Amaranthus tricolor* leaf extracts on Serum Insulin



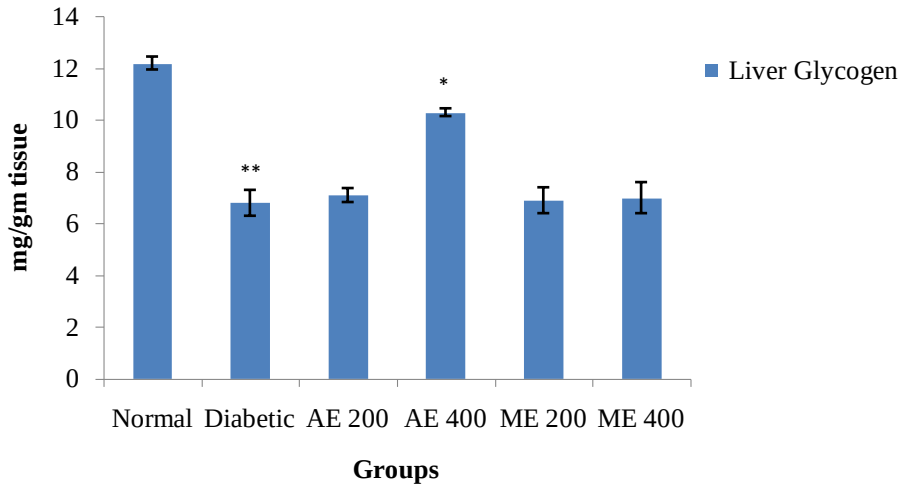
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p ≤.05 vs. control diabetic rats; double asterisk (**), p ≤0.05 vs. respective control rats.

Fig. 32: Effect of *Amaranthus tricolor* leaf extracts on Insulin Content in Pancreas



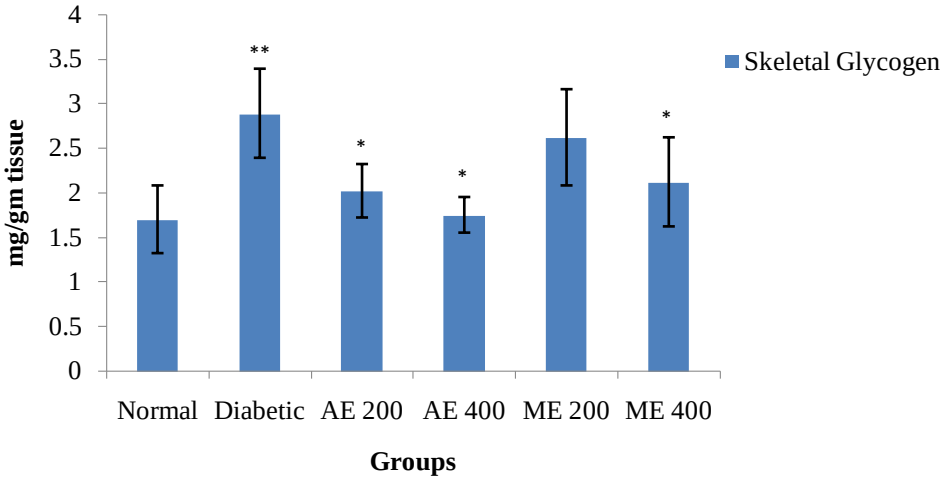
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats.

Fig. 33: Effect of *Amaranthus tricolor* leaf extracts on Glycogen content in Liver



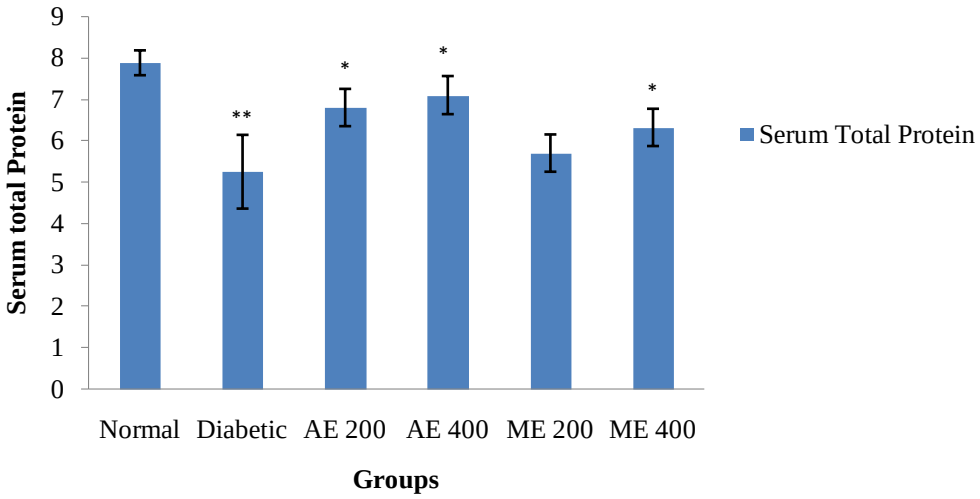
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats

Fig. 34: Effect of *Amaranthus tricolor* leaf extracts on Glycogen content in Skeletal Muscle



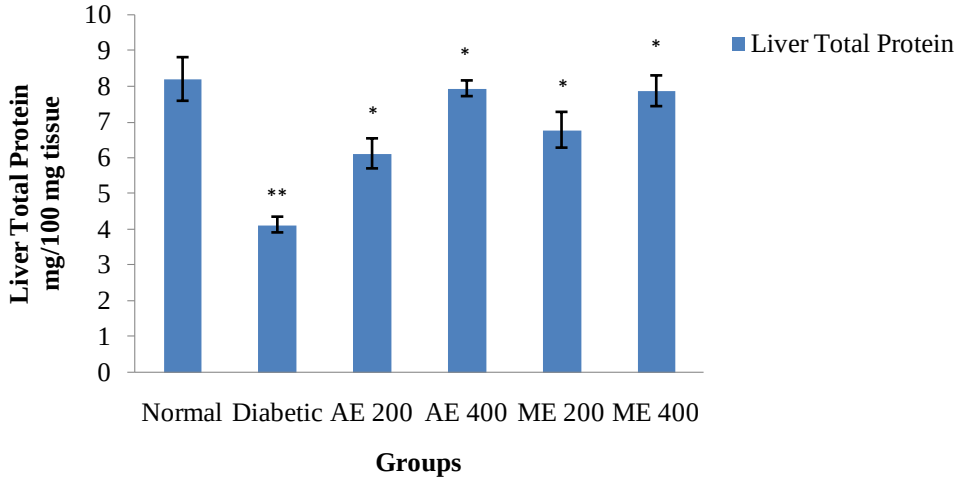
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats

Fig. 35: Effect of *Amaranthus tricolor* leaf extracts on Total Protein in Serum



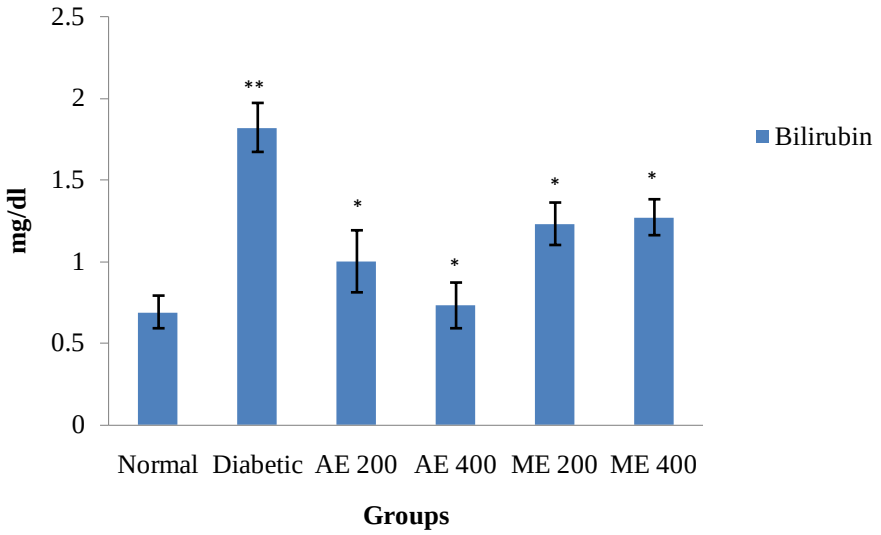
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats

Fig. 36: Effect of *Amaranthus tricolor* leaf extracts on Total Protein in Liver



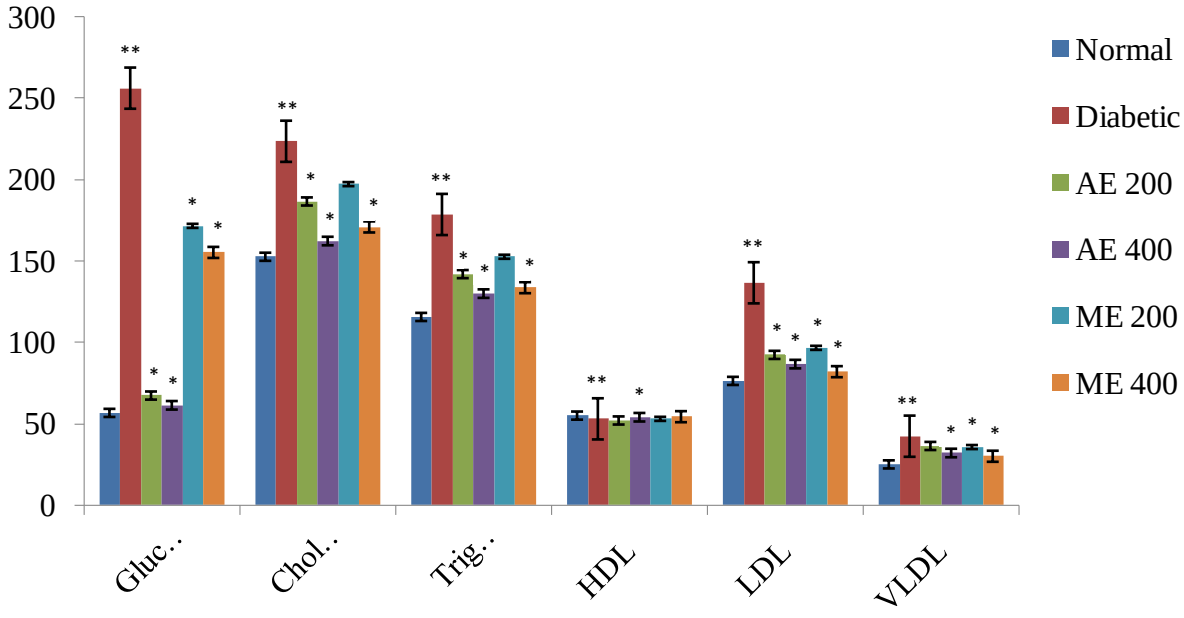
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats

Fig. 37: Effect of *Amaranthus tricolor* leaf extracts on Serum Bilirubin



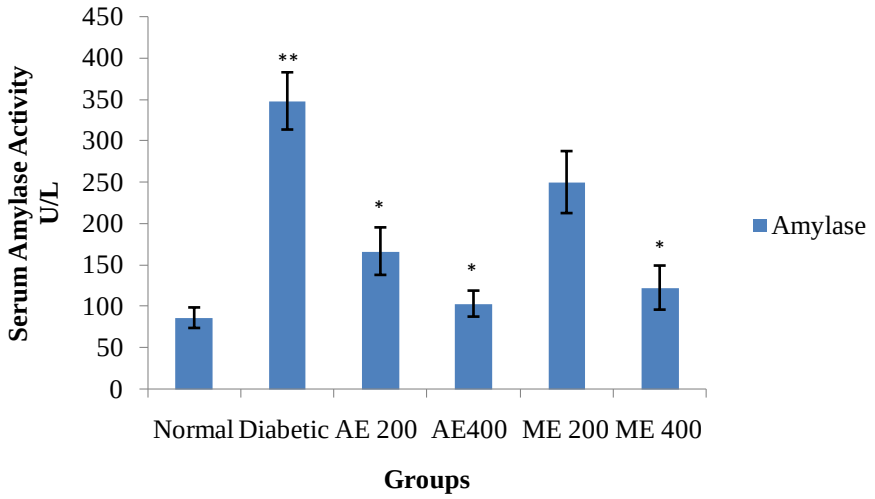
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats

Fig. 38: Effect of *Amaranthus tricolor* extracts on glucose and lipid profile



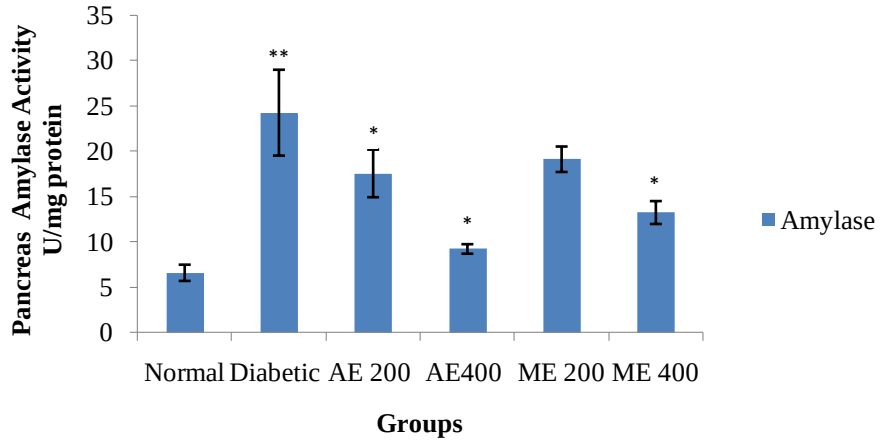
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats

Fig. 39: Effect of *Amaranthus tricolor* leaf extracts on Amylase activity in Serum



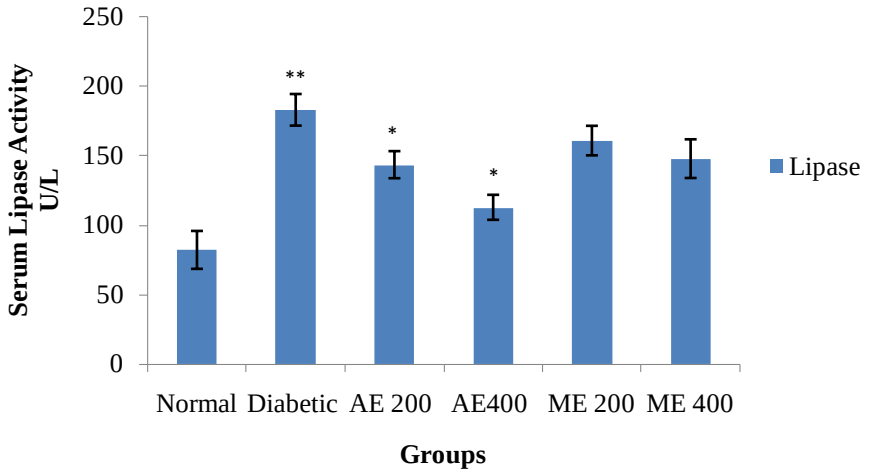
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats.

Fig. 40: Effect of *Amaranthus tricolor* leaf extracts on Amylase activity in Pancreas



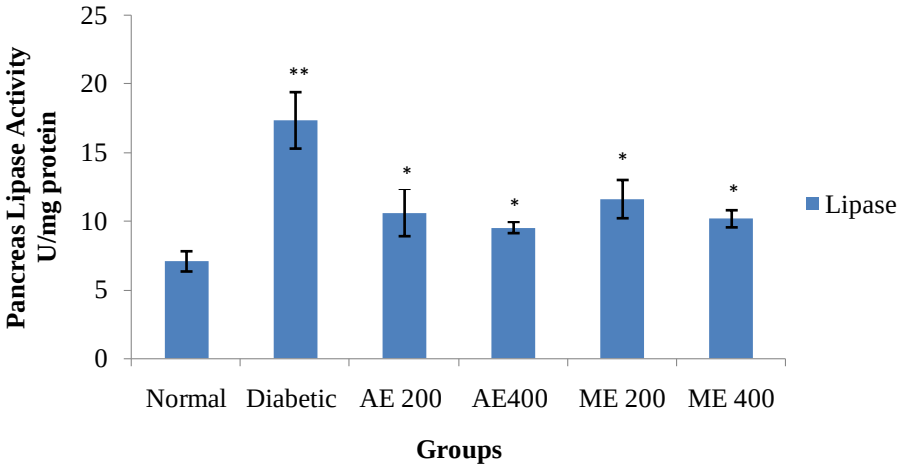
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats.

Fig. 41: Effect of *Amaranthus tricolor* leaf extracts on Lipase activity in Serum



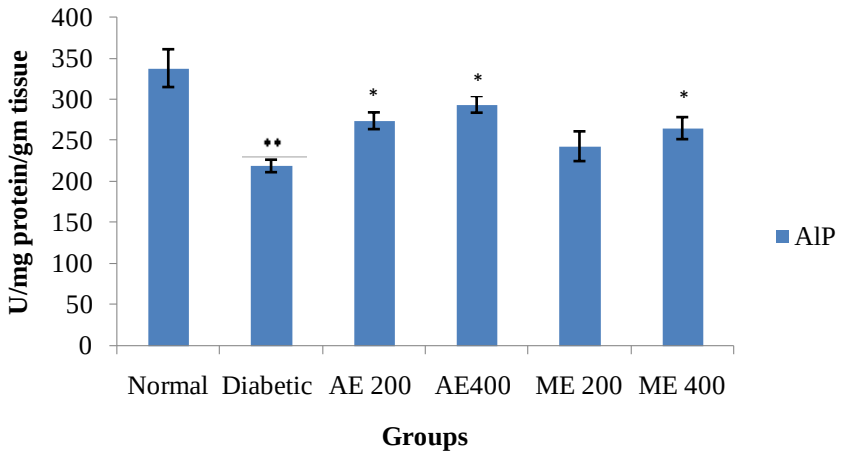
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs. control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats.

Fig. 42. Effect of *Amaranthus tricolor* leaf extracts on Lipase activity in Pancreas



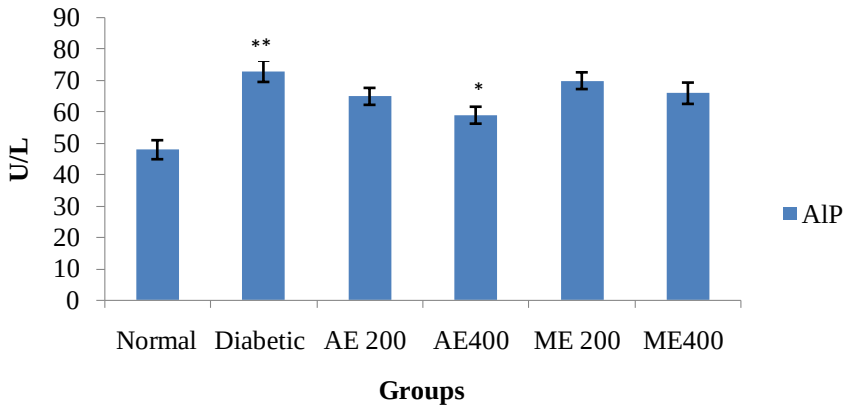
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: ^{p} p < 0.05, ^{q} p < 0.001 - normal vs.diabetic rats; ^{a} p < 0.05, ^{b} p < 0.001 - treated vs diabetic rats.

Fig. 43: Effect of *Amaranthus tricolor* leaf extracts on Alkaline phosphatase activity in Liver



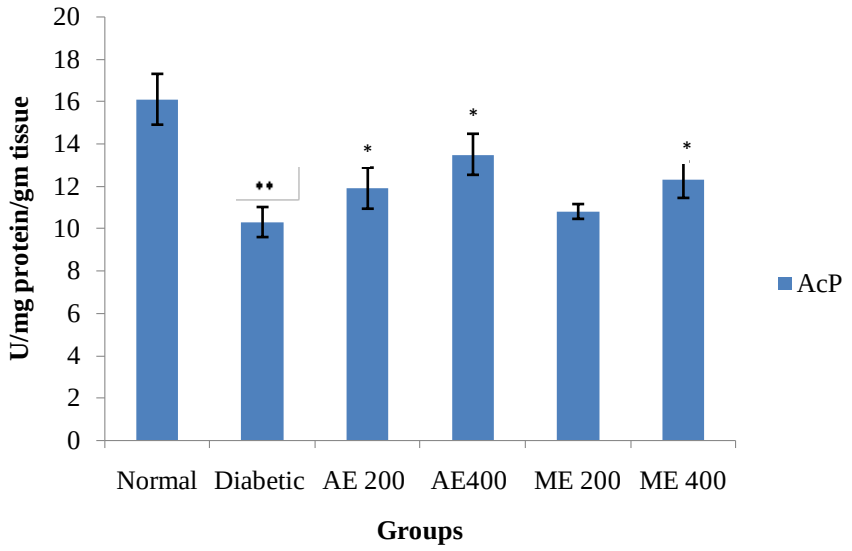
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p≤0.05 vs.control diabetic rats; double asterisk (**), p≤0.05 vs. respective control rats.

Fig. 44. Effect of *Amaranthus tricolor* leaf extracts on Alkaline phosphatase activity in Serum



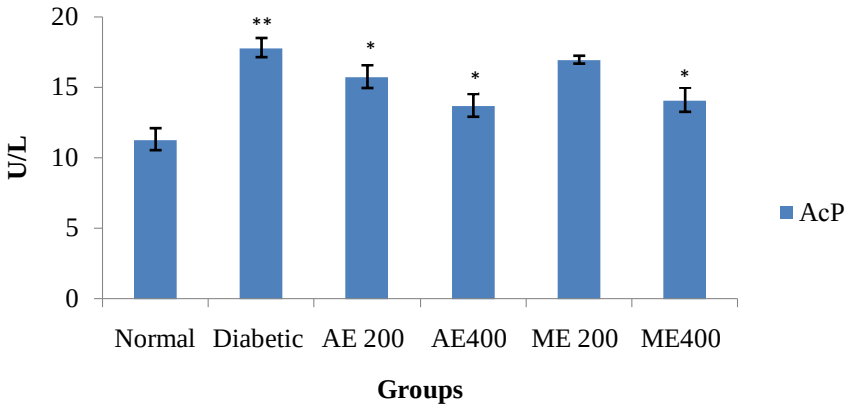
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 45: Effect of *Amaranthus tricolor* leaf extracts on Acid phosphatase activity in Liver



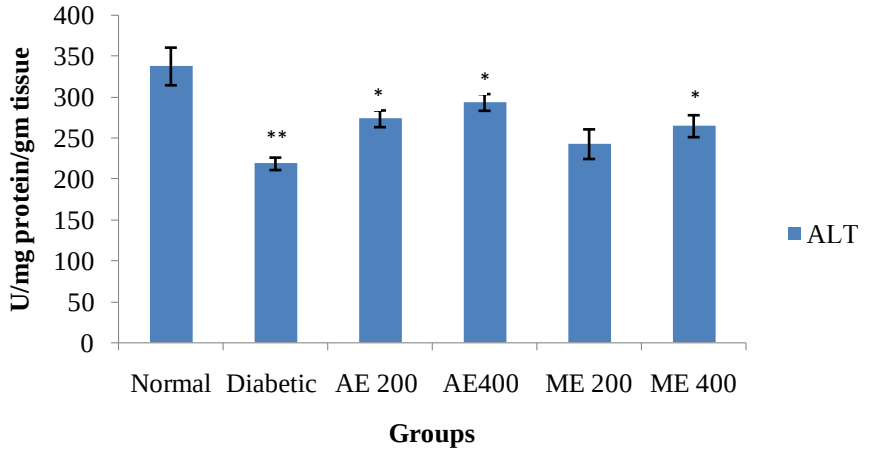
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 46: Effect of *Amaranthus tricolor* leaf extracts on Acid phosphatase activity in Serum



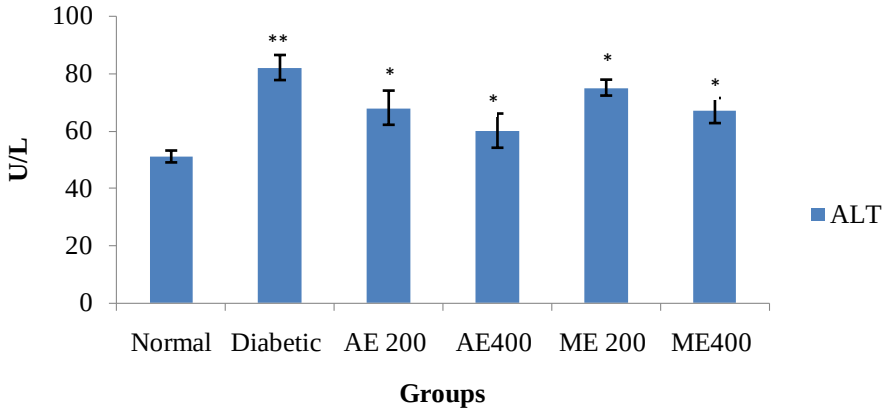
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 47: Effect of *Amaranthus tricolor* leaf extracts on Alanine transaminase activity in Liver



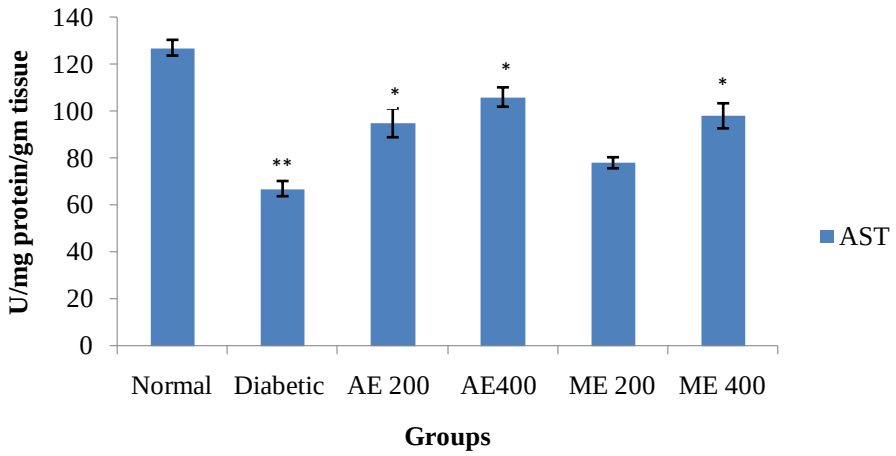
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 48: Effect of *Amaranthus tricolor* leaf extracts on Alanine transaminase activity in Serum



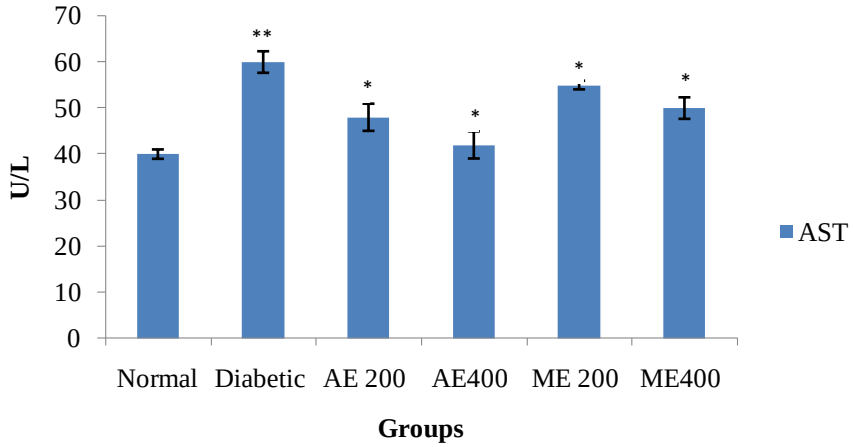
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 49: Effect of *Amaranthus tricolor* leaf extracts on Aspartate transaminase activity in Liver



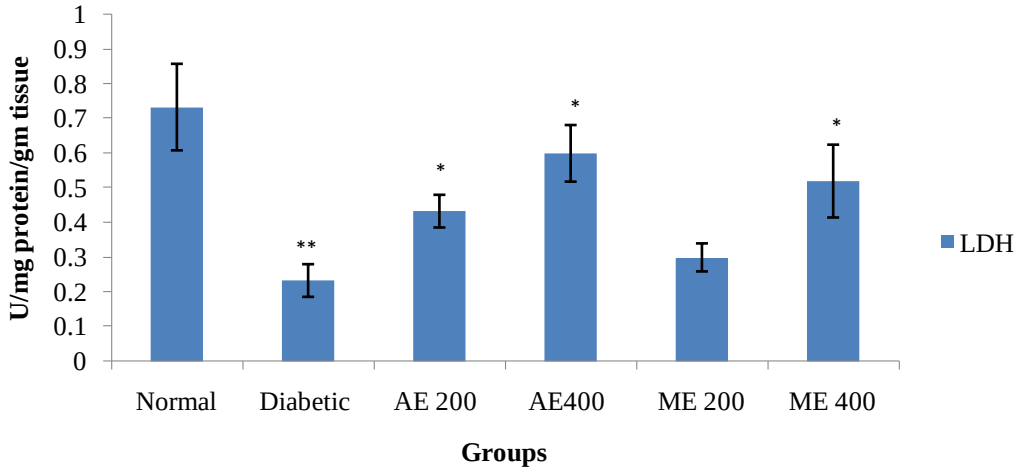
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 50: Effect of *Amaranthus tricolor* leaf extracts on Aspartate transaminase activity in Serum



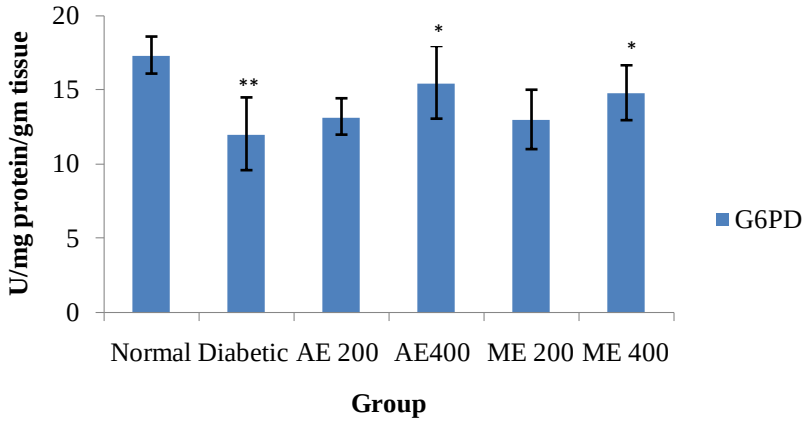
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 51: Effect of *Amaranthus tricolor* extracts on Lactate dehydrogenase activity in Liver



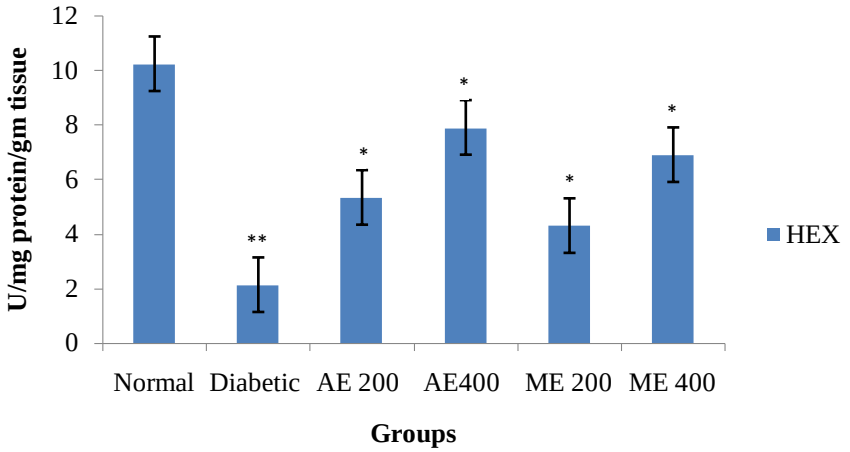
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 52: Effect of *Amaranthus tricolor* leaf extracts on Glucose 6-phosphate dehydrogenase activity in Liver



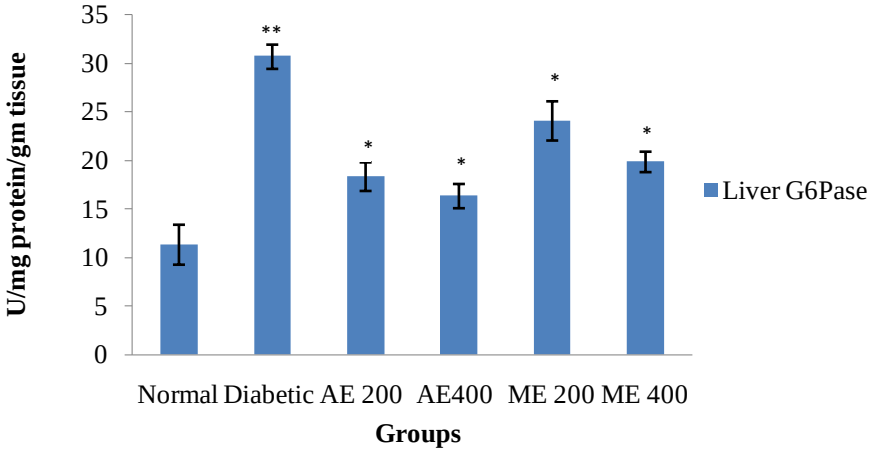
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 53: Effect of *Amaranthus tricolor* leaf extracts on Hexokinase activity in Liver



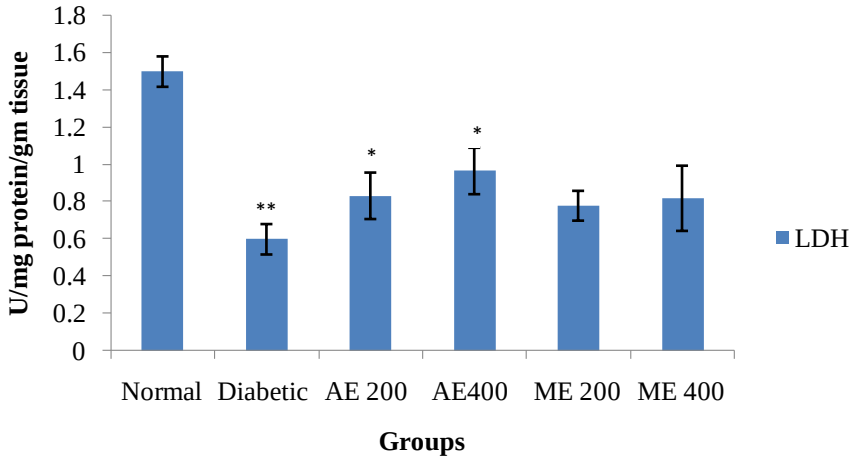
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 54: Effect of *Amaranthus tricolor* leaf extracts on Glucose 6-phosphatase activity in Liver



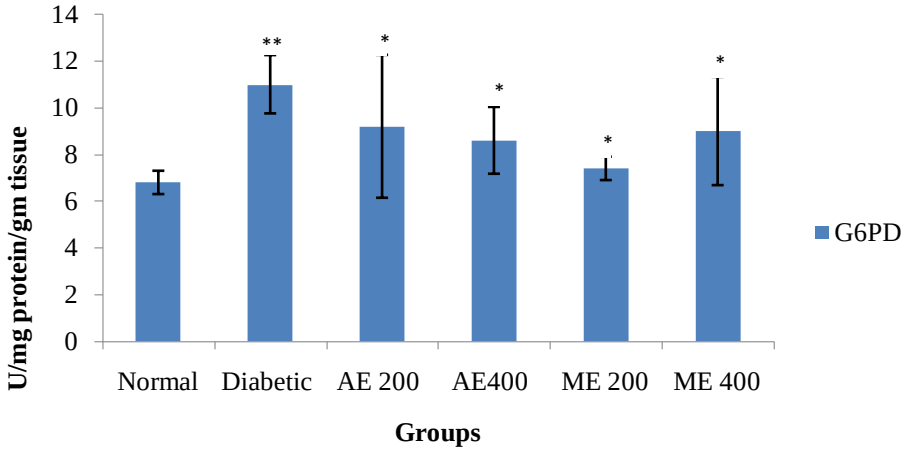
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 55: Effect of *Amaranthus tricolor* extracts on Lactate dehydrogenase activity in Kidney



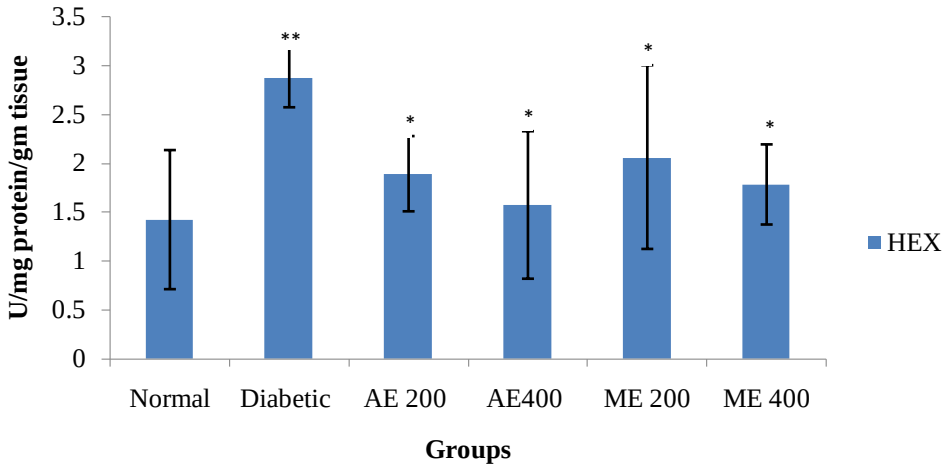
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 56: Effect of *Amaranthus tricolor* extracts on Glucose 6-phosphate dehydrogenase activity in Kidney



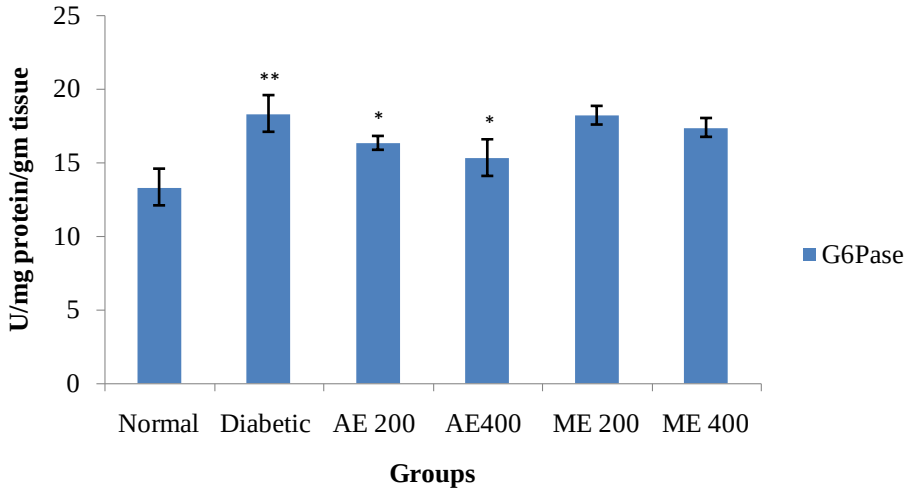
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 57: Effect of *Amaranthus tricolor* extracts on Hexokinase activity in Kidney



Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Fig. 58: Effect of *Amaranthus tricolor* extracts on Glucose 6-phosphatase activity in Kidney



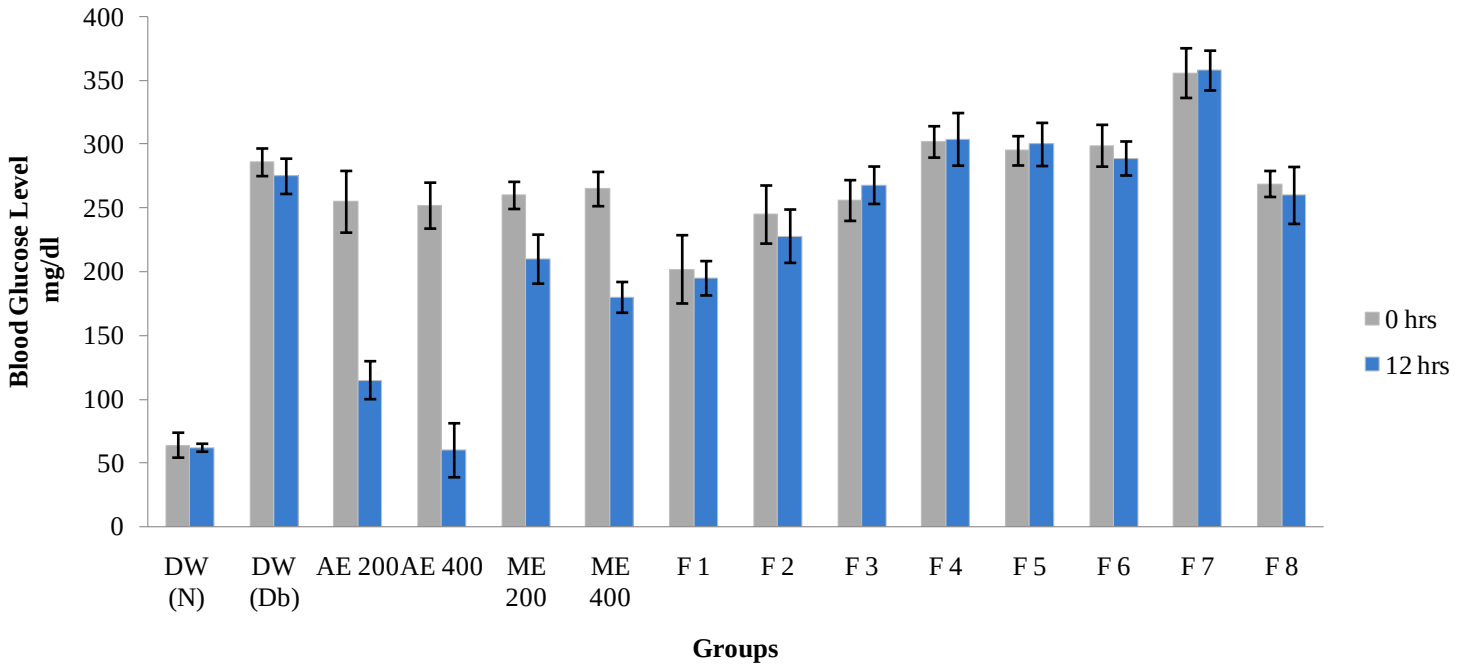
Data represents mean ± S.D (n = 6 for each group). The values are statistically significant and presented as follows: single asterisk (*), p < 0.05 vs.control diabetic rats; double asterisk (**), p < 0.05 vs. respective control rats.

Table 11: Effects of *Amaranthus tricolor* leaf extracts on the hematological parameters in alloxan induced diabetic rats

Parameters	Normal	Diabetic	AE 200	AE 400	ME200	ME 400
WBC ($x10^3$/ml)	5.38 ± 0.47	3.12 ± 0.16 [b]	3.19 ± 0.53	3.36±0.48[y]	3.09±0.48	3.15±0.52
RBC ($x10^6$/ml)	5.6 ± 0.34	4.28 ±0.49 [b]	4.38 ± 0.43	4.88±0.71[y]	4.3±0.37	4.55±0.5
PCV (%)	38.40 ± 2.33	33.85 ± 2.85 [a]	35.69 ± 3.2 [y]	37.58±1.78[z]	33.95±2.96	34±3.12
Hb (g/dl)	12 ± 0.71	9.54±0.97 [b]	10.00 ± 1.1	10.96±0.9[z]	9.67±0.83	9.58±0.93

Where - a = p≤0.05, b =≤0.001 when compared to normal groups; y = p≤0.05, z=≤0.001 when compared to diabetic groups.

Fig. 59: Effect of leaf extracts and fractions of *Amaranthus tricolor* on fasting blood glucose of alloxan-induced diabetic rats - Acute study



	Normal	Db	AE 200	AE 400	ME 200	ME 400	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8
% Change	-3.125	-3.846	-54.901	-76.19	-19.23	-32.075	-3.465	-6.938	4.687	0.662	1.694	-3.344	0.561	-3.345

In Vivo Bioassays

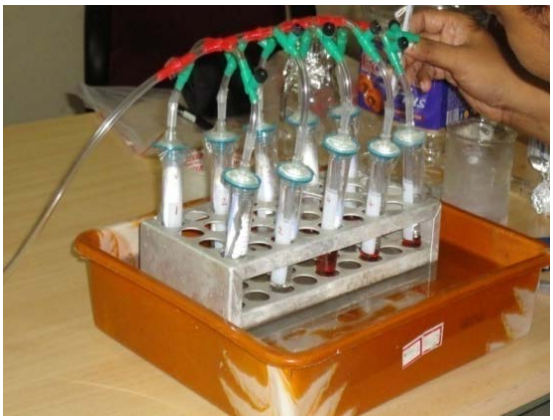


Fig 60: Glucose uptake apparatus

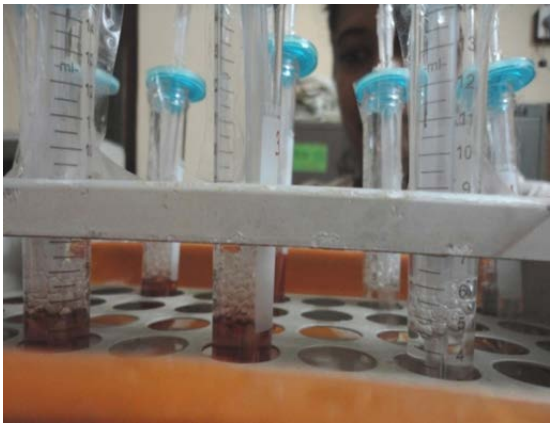


Fig 61: Enlarged view of glucose uptake apparatus tubes containing tissue and incubation media. Constantly gassed at 37°C with sterile 98% O_2.

Table 12: Effect of *Amaranthus tricolor* leaf extracts on glucose uptake in isolated Hemi-diaphragm of rat

Groups	Glucose in media	% Change of glucose in media	Glycogen /gm wet weight tissue
TS + Glucose + HD	**598.7**	**↓ 4.834**	**0.34**
TS + Glucose + Insulin + HD	**569.76**		**↑ 0.393**
TS + AE400 + Glucose	**615.13**	**↓ 20.05**	**0.507**
TS + AE400 + Glucose + HD	**491.86**		
TS + Glucose + Insulin + AE400	**616.27**	**↓ 20.188**	**0.391**
TS + Glucose + Insulin + AE400 + HD	**527.91**		
Groups	**Glucose in media**	**% Change of glucose in media**	**Glycogen /gm wet weight tissue**
TS + Glucose + HD	**496.72**	**↓ 5.615**	**0.33**
TS + Glucose + Insulin + HD	**468.83**		**↑ 0.38**
TS + ME400 + Glucose	**561.51**	**↓ 17.357**	**0.40**
TS + ME400 + Glucose + HD	**464.05**		
TS + Glucose + Insulin + ME400	**563.88**	**↓ 8.512**	**0.20**
TS + Glucose + Insulin + ME400 + HD	517.91		

TS – Tyrode's Salts; HD - Hemidiaphragm

Table 13: Effect of *Amaranthus tricolor* leaf extracts on glucose uptake in isolated Skeletal muscle of rat

Groups	**Glucose in media**	**% Change of glucose in media**	**Glycogen mg/gm wet weight tissue**
TS + Glucose + SM	578.92	↓ 5.517	0.38
TS + Glucose + Insulin + SM	546.98		↑ 0.39
TS + AE400 + Glucose	615.13	↓ 25.32	0.640
TS + AE400 + Glucose + SM	459.38		
TS + Glucose + Insulin + AE400	616.27	↓ 12.418	0.338
TS + Glucose + Insulin + AE400 + SM	539.74		
Groups	**Glucose in media**	**% Change of glucose in media**	**Glycogen /gm wet weight tissue**
TS + Glucose + SM	485.43	↓ 4.448	0.25
TS + Glucose + Insulin + SM	463.84		↑ 0.29
TS + ME400 + Glucose	561.51	↓ 18.561	0.30
TS + ME400 + Glucose + SM	457.29		
TS + Glucose + Insulin + ME400	569.88	↓ 8.898	0.22
TS + Glucose + Insulin + ME400 + SM	519.17		

TS – Tyrode's Salts; SM – Soleus Muscle

Chapter 4

Discussion

4.1 Plant Studies

Amaranthus tricolor is a commonly consumed, abundantly available leafy vegetable, which forms an integral part of the local staple diet. In a preliminary assessment Amaranths were reported to be important contributors to improvement of the nutritional content of rural and urban people, among other known leafy vegetables **(**Odhav et. al., 2007). Normally consumed in the capacity of a traditional hepatoprotective (Clemente and Desai, 2012) and haematinic agent (Clemente and Desai, 2011), the plant and its family are well known for their high protein content (Mlakar et. al., 2010).

4.1.1 Phytochemical screening

Traditional healers mainly use crude preparations of plants in the treatment of diseases. A majority of these preparations are aqueous based. Many scientific studies relevant to natural medicinal chemistry and related areas have used polar solvents, non-polar solvents or a combination of both to extract bioactive compounds from plants (Mendonça-Filho, 2006; Raaman, 2006; Sasidharan et. al., 2010).

Solvents of different polarities tend to extract compounds of identical polarity. For example, methanol and water being polar solvents are known to extract highly polar compounds such as glycosides, carbohydrates, amino acids and their derivatives (Bruneton, 1999; Scalbert, et. al., 2005). Whereas Petroleum ether or hexane are known to extract compounds of low polarity such as fatty acids, waxes, some alkaloid, and terpenoid aglycones (Ayaffor et. al., 1994; Cowan, 1999).

Previous studies indicate the presence of proteins and amino acids (National Research

Council, 2006), phytosterols (Fernando and Bean, 1984), and betalain pigments in *Amaranthus tricolor*. The plant is well known in many parts of India and across the globe as being highly nutritive. The present study shows that *Amaranthus tricolor* contains polyphenols, tannins, phytosterols, lipids, proteins, amino acids and flavonoids. Almost all of these classes of compounds have been scientifically proven to lower blood glucose levels in varying degrees, in a diabetic condition.

4.1.2 Screening of *Amaranthus tricolor* for antioxidant activity

In the last decade, interest in the antioxidant activity of plant extracts has grown considerably due to the knowledge that free radicals responsible for onset and aggravation of ailments can be quenched by antioxidants of plant origin. Antioxidants due to their radical scavenging activity are useful for the management of those ailments.

4.1.2 .1 DPPH Assay

Decrease in absorbance of DPPH solution (i.e. from purple to yellow) depends on intrinsic antioxidant activity of antioxidant as well as on speed of reaction between DPPH and antioxidant (Azlim et. al., 2010). The DPPH radical scavenging activity assay assessed the ability of the extract to donate hydrogen or to scavenge free radicals. DPPH radical is a stable free radical. When it reacts with an antioxidant compound which can donate hydrogen, it is reduced to diphenylpicrylhydrazine. The changes in colour (i.e.from deep-violet to light-yellow) can be measured spectrophotometrically. The potent DPPH scavenging activity indicates the presence of polyphenols (Gupta and Prakash, 2009; Kang et. al., 2010). In the present study *Amaranthus tricolor* extracts show high DPPH scavenging activity indicating the presence of polyphenols and the utility of extracts in

mitigating free radical effects to reduce diabetic morbidity upto a certain extent.

4.1.2 .2 FRAP

The FRAP assay is based on an electron-transfer reaction, wherein a ferric salt is used as an oxidant (Benzie and Strain, 1996) and provides a measure of the reducing ability of the plant extracts. Here a reduction of ferric to the ferrous form is observed in the presence of reductants (antioxidants) in the tested samples, which serves as an indicator of the potential antioxidant activity of the tested *Amaranthus tricolor* extracts. The FRAP activity exhibited by all the extracts in the decreasing order are - Aqueous (AD & AE) > Pet Ether (PE) > Methanolic (MN & MS).

4.1.2 .3 Phosphomolybdenum Assay

This assay has been routinely used to evaluate the total antioxidant capacity of extracts. The basic principle to assess the antioxidant capacity through phosphomolybdenum assay includes the reduction of Mo (VI) to Mo (V) by the plant extract possessing antioxidant compounds. The method is utilized for the spectrophotometric quantitation of total antioxidant capacity and employs cost-effective reagents (Prieto et. al., 1999). Therefore the present work clearly shows the antioxidant capacity of *Amaranthus tricolor* extracts through DPPH, FRAP and phosphomolybdenum assays.

4.1.2 .4 Total Phenolic Content

Folin-Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurs under alkaline

condition provided by sodium carbonate. The intensity of blue colour reflects the quantity of phenolic compounds, which can be measured using spectrophotometer (Conforti et. al., 2006). The FCR actually measures a given sample's reducing capacity. The total phenols assay by FCR is convenient, simple, and reproducible. As a result, a large body of data has been accumulated, and it has become a routine assay in studying phenolic antioxidants.

The AD extract exhibits higher total phenolic content than the other tested extracts, which contain significantly lower total phenolic content. When compared with other plants containing high phenolic content, *Amaranthus tricolor* contains a reasonable quantity of phenolic contents, indicating it as a potential source of naturally occurring polyphenols. According to Prior et. al., (2005), the Folin-Ciocalteau assay gives a crude estimate of the TPC present in an extract, whereas the free radical scavenging assay is not only specific to polyphenols. Besides, various phenolic compounds respond differently in DPPH assay, depending on the number of phenolic groups they have (Singleton and Rossi, 1965).

The results also suggest that extraction by methanol could give higher phenolic content as compared to other solvents. The findings are in agreement with Pérez et. al., (2007) who found that methanol was the most efficient solvent as compared to ethanol and water for extracting phenolic compounds from rosemary leaves. Yang et. al., (2007) reported that methanol extract of lotus rhizome had the highest yield and total phenolic recovery. Methanol is said to be the most suitable solvent in the extraction of phenolic compounds due to its ability to inhibit the reaction of polyphenol oxidase that causes the oxidation of phenolics and its ease of evaporation compared to water (Yao et. al., 2004).

4.1.2 .5 Correlation between TPC and primary antioxidant activity

Numerous publications have applied correlation to the total phenols assay by FCR and an ET-based antioxidant capacity assay (e.g., FRAP, TEAC,etc.) and often9 found excellent linear correlations between the “total phenolic profiles” and “the antioxidant activity”.

The total antioxidant capacity and total phenolic contents of the AD, PE and MS extracts correlate with each other. But the MN extract does not show any correlation at all with total phenolic contents. The MN extract shows moderate antioxidant activity as compared to that of aqueous extract (AD) but shows very low total phenolic contents. However it is also possible that other phytoconstituents besides the polyphenols may have antioxidant activity.

The present work corroborates the general belief that antioxidant activities go hand in hand with total phenolic contents, but there are reports that there need not be a perfect correlation between antioxidant activity and total phenolic contents of plants since terpenes, sterols when present show antioxidant activity (Sengul et. al., 2009; Sikder et. al., 2010). The current work reports that *A. tricolor* has lipids, polyphenols, tannins and phytosterols as well (Clemente and Desai, 2011).

Normally food is enriched by synthetic antioxidants, which unfortunately promote hepatotoxicity and carcinogenicity besides their normal antioxidant activity (Anagnostopoulou, 2006). Therefore there is an upsurge in demand of natural polyphenols as they retard oxidative degradation of lipids and improve quality and nutritional value of food (Laughton et. al., 1989; Bravo, 1998). Plant phenolics constitute one of the major

groups of compounds acting as primary antioxidants or free radical terminators, thus making it necessary to determine the total amount in plant material (Gursoy and Tepe, 2009). The significant antioxidant activity exhibited by most of the extracts of *A. tricolor* validates the traditional claim of medicinal value of this plant. The present work shows that, the aqueous and petroleum ether extracts of *Amaranthus tricolor* exhibit high free radical scavenging activity, as it contains compounds which act as natural scavengers, indicating its possible potential in arresting cellular damage.

Research suggests that people with diabetes have more free radicals than people who do not suffer from this disease. It is thought that free radicals play a role, both in causing diabetes and exacerbating its long term effects. Hence antioxidants are of utmost importance and are found in high proportions in a number of fruits and vegetables. Carotenoids such as β-carotene, along with the antioxidant vitamins E and C appear to be particularly important for diabetics. *Amaranthus tricolor* could be highly beneficial for treating and preventing diabetic complications such as heart disease and nerve damage, since it is an excellent source of β-carotene, vitamin E and vitamin C.

4.1.3 Porcine Pancreatic α-Amylase Inhibition

A major goal in the treatment of diabetes mellitus is to maintain near normal blood glucose levels in both the fasting and postprandial state (Alwan, 1994). One therapeutic approach to decrease postprandial hyperglycemia is to suppress the production and/or absorption of glucose from the gastrointestinal tract through inhibition of either α-amylase or α-glucosidase enzymes (Thulé, 2012). The pancreatic a-amylase hydrolyses dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to

supply the body with energy. Disaccharides produced by α-amylase are hydrolyzed further by α-glucosidases to produce glucose and other monosacharides, which are readily absorbed in the small intestines (Smith et. al., 2005).

Both the *A. tricolor* leaf extracts (aqueous and methanol) investigated in the present work exhibited inhibition of α-amylase activity even in in vitro experiments. This observation suggests that porcine pancreatic α-amylase is inhibited by mainly polar components of the *A. tricolor* extracts. It was observed that the aqueous extract exhibited greater inhibition of PPA activity, than the methanolic extract. In this regard other in vitro studies have attributed the α-amylase inhibitory activity of some plant material extracts to the presence of tannins (Bhandari et. al., 2008) and to the presence of flavonoids, polyphenols and their glycoside derivatives (Jung et. al., 2006). Therefore the ability of *A.tricolor* extracts to inhibit α-amylase activity indicates its potential to help decrease postprandial hyperglycemia.

4.1.4 Spectral Studies of Fraction1 and Fraction 2

The hygroscopic compound isolated from the methanolic extract of *A. tricolor* was subjected to spectral analysis. When compared with available literature, the spectral data suggests that the isolated compound (AT-C) may belong to the flavonoid class of phytochemicals. The UV-VIS spectrum of AT-C has absorption bands at 304 nm and 220 nm, which are characteristic of flavonoids and their derivatives.. The flavonoids spectra typically consist of two absorption maxima in the ranges 230-285 nm (band I) and 300-350 nm (band II). The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoids. Saxena. et. al., (2012) reported the presence of

a flavanoid from *Acorus calamus*, having similar absorption peaks in UV-Vis and the IR range. Fraction 2 when compared with literature available and the PhotoChem Cad spectral library was found to be beta carotene.

4.2 Animal Studies

4.2.1 Standardizations followed for the induction of alloxan diabetes

4.2.1.1 Inbreeding of rats

Systematic inbreeding and maintenance of inbred strains of laboratory mice and rats is of great importance for biomedical research. The inbreeding guarantees a consistent and uniform animal model for experimental purposes (Boo and Hendriksen, 2005). By using inbred animals (instead of out bred ones), with respect to the test of a new drug, a lower number of test animals are needed due to a lower error variance, as their response to the drug would be similar (Hendriksen, 1987). To maintain the uniformity, the use of full sib (brother × sister) mating is necessary and one has to ensure that the original parents are no more than five generations away, due to the possibility of self mutations (Christensen, 2003).

Experimental diabetes in animals has provided considerable insight into the physiological and biochemical derangements of the diabetic state. The diabetogenic agent alloxan induces diabetes mellitus by disrupting the balance between cellular antioxidant defences and free radical formation as well as by causing necrosis of the β-cells in the pancreas (Dunn et. al., 1943, Jorns et. al., 1997). Due to the destruction of the β-cells and subsequent decrease in circulating insulin level, there is a parallel increase in the blood glucose level. Therefore, the technique of using inbreeding rats and exposing them to

alloxan appears to be the appropriate approach for developing diabetic state. Diabetes-induced hyperlipidemia is attributable to excess mobilization of fat from adipose tissue due to the underutilization of glucose (Abbate and Brunzell, 1990). The basal blood glucose level of the normal rats was seen to range between 50 mg/dl and 72 mg/dl and corresponds with the findings of previous workers (Ahmed et. al., 2010).

4.2.2 InVivo Bioassays

4.2.2.1 Acute Oral Toxicity Test

The oral safety dosage of the extract using Acute Oral Toxicity Program (AOT 425) guidelines was greater than 2000 mg/kg body weight. Any compound with an oral LD50 > 1000 mg/kg can be considered a low toxicity and safe agent (Clarke and Clarke, 1977). The extract fed diabetic animals did not show any signs of distress or discomfort at doses exceeding 1g per kg body weight. Their overall behaviour did not suggest any toxic effects on them by the plant extracts. Hence it appears that *A. tricolor* extracts are safe for consumption since there are no symptoms of toxicity expressed by the test animals.

4.2.2.2 Screening for antidiabetic activity (Acute Study)

Hyperglycemia is an important factor in the development and progression of the complications of diabetes mellitus (Luzi, 1998). A reduction in hyperglycemia decreases morbidity of the disease. The extracts were screened for antidiabetic activity by dosing overnight fasted diabetic rats with the respective doses assigned for each group. Among all the extracts the methanolic and aqueous exhibited the most potent antidiabetic activity. The maximum reduction in blood sugar level was observed by the second hour. The reduction in blood glucose levels continued upto 12 hours. However the aqueous extract

was more effective in bringing down the blood glucose to near normal level than that affected by the methanolic extract.

4.2.2.3 Treatment of diabetic rats with *A. tricolor* extracts for 3 weeks

4.2.2.3.1 Body Weight

Diabetes is considered as a disease of starvation in the midst of plenty. Weight loss is a very serious issue in the management of diabetes owing to the degeneration of the adipocytes and muscle tissues to make up for energy loss from the body due to frequent urination and over-conversion of glycogen to glucose. Diabetic animals treated with *A. tricolor* extracts showed positive weight gain as compared to the diabetic controls. The weight gain in the extract fed diabetic groups suggest the utilization of proteins from the extract for repair and tissue building that causes weight gain. The analysis of aqueous extracts shows a reasonable amount of protein, which may be the contributing factor to weight gain seen by the animals as mentioned above.

4.2.2.3.2 Glucose and Insulin

Treatment of alloxan-diabetic rats with the aqueous and methanolic extracts reduced the fasting blood glucose level as compared with the diabetic control group. In the current study, serum insulin levels in alloxan induced diabetic rats were markedly reduced. The aqueous and methanolic leaf extracts of *A. tricolor* appear to lower blood glucose levels of alloxan induced diabetic rats without increasing the serum insulin levels. The aqueous and methanolic extracts appeared to have no effect on the insulin levels in the diabetic rats. This suggests that the plant extracts do not help to decrease high blood glucose level

through elevation of insulin synthesis, vis a viz level of stimulation of residual beta cells or regeneration of beta cells in the pancreas of the diabetic rats.

4.2.2.3.3 Glycogen

A decrease in the glycogen content of the diabetic liver is often considered to be the direct result of the diabetic disturbance in metabolism, caused either by an increased glycogenolysis or by a decreased ability to synthesize glycogen (Tuerkischer and Wertheimer, 1946).

The glycogen content in the liver and skeletal muscle tissues were found to be markedly decreased in the alloxan diabetic state. Similar findings were reported in liver (Babu et. al., 2002) and skeletal muscle (Daisy et. al., 2009) tissues of rats made diabetic with alloxan. This decrease in glycogen content of liver and skeletal muscle of diabetic rats is due to lack of insulin in the Type 1 diabetic state. The diabetic animals treated with *A. tricolor* for duration of 3 weeks, showed an improvement in the glycogen level in both the hepatic and skeletal muscle tissue. This increased glycogen content in the liver and muscle corresponds with the decrease in blood glucose level upon *A. tricolor* extract treatments. This suggests the mobilization of blood glucose into the liver glycogen reserve.

4.2.2.3.4 *Amaranthus tricolor* as a Hepatoprotective agent

Such interesting claims warrant further study of the effect of the extracts on the major organs of the body, to check for any deleterious or beneficial side effects. As the liver plays a major role in the metabolism of drugs it is necessary to ascertain the effects of the aqueous and methanolic extracts on the liver function of the alloxan induced diabetic rats.

Enzyme activities in tissues are used as 'markers' of early toxicity mediated by administered foreign compounds in experimental animals (Adesokan and Akanji, 2004). AlP is a membrane bound enzyme while ALT and AST are cytosolic and mitochondrial enzymes. These enzymes are highly concentrated in the liver and kidney, and found in minor quantities in other organs. They are only found in the serum in significant quantities when the cell membrane becomes leaky and even completely ruptured **(**Cotran et. al., 1989). A rise in serum level or decrease in tissue level of these intracellular enzymes is an index of tissue damage especially of liver and kidney cells (Moss and Rosalki, 1986). LDH activity in serum increases during tissue necrosis.

In alloxan-diabetic rats the activities of serum AST, ALT, LDH, AcP and AlP were significantly increased ($p \leq 0.05$) and serum total protein concentration decreased as compared to the non-diabetic control group. In contrast, the activities of AST, ALT, LDH, AcP and AlP decreased in the hepatic tissue of alloxan-diabetic rats. The increase in the activities of serum AST, ALT, LDH, AcP and AlP is generally seen in diabetes induced hepatic impairment, and may be mainly due to the leakage of these enzymes from the necrotic liver into the blood stream (Navarro et. al., 1993), indicating hepatotoxic effects of alloxan, visà-vis diabetes. However oral treatment of the diabetic rats with the plant extracts over a period of 3 weeks, showed a significant decrease ($p \leq 0.05$) in the serum transaminases (AST, ALT) and an increase in the liver transaminases (AST, ALT) as compared to the diabetic control group. Treatment of alloxan diabetic groups with *A. tricolor* extracts for 3 weeks shows restoration of the activities of the enzymes to near normal levels, indicating that these treatments recover the liver damage induced by alloxan.

In diabetic animals, the variations in the levels of AST, ALT, AlP, AcP and LDH are directly related to changes in metabolism where in these enzymes are involved (Bopanna et. al., 1997). The increased activities of transaminases in the blood of diabetics indicate increased gluconeogenesis and ketogenesis. Diabetes and hyperlipidaemia also cause cell damage by altering the cell membrane architecture, which results in enhanced activities of AlP in diabetic rats (Udayakumar et. al., 2009). It has been reported that diabetics exhibit elevated serum alkaline phosphates level (Grossi, 1998; Iwamoto et. al., 2001; Siddiqui et. al., 2005; Shaheen et. al., 2009). A change in serum AcP and AlP activities could be a direct reflection of its substrate, as both the enzymes are associated with the interconversion of lactate and pyruvate.

Therefore rise in serum AlP and AcP activities in the present work suggests that the alloxan induced diabetes caused liver damage. This may be attributed to sloughing off of damaged cells and the release of AlP and AcP into the blood. Rise in liver AlP and AcP activity is essential to improve the membrane transport of the intact tissue. The function of acid phosphatases in the production, transport, and recycling of phosphate is critical for the metabolic and energy transduction processes of the cell.

A decrease in serum AST, ALT, LDH, AcP and AlP activities in aqueous and methanolic extracts treated groups indicates the protective effect on liver function, particularly with reference to ketogenesis and gluconeogenesis. Alloxan induced diabetic rats showed a significant decrease in hepatic total proteins as compared to the control group, which could be attributed to hepatic injury. Besides serum total protein levels were also seen to be decreased. After *A. tricolor* leaf extract treatment an increase in serum and hepatic total

protein concentration was seen. Diabetes is well known to delay wound healing. Proteins are essential to wound healing because they build, maintain and repair body tissues. Increased protein intake speeds healing, where as inadequate protein intake is known to delay wound healing (Wilson and Wilson, 2006**)**. Amaranthus extracts reduce protein breakdown and provide proteins as *A. tricolor* is rich in protein as evidenced by the protein assay of *A. tricolor* leaves.

Alloxan diabetes increased the level of serum bilirubin as compared to control rats. This elevation indicates liver malfunction as confirmed by the changes in the activities of serum and liver enzymes. Administration of *A. tricolor* produced a significant decrease ($p \leq 0.05$) in serum bilirubin of alloxan-diabetic rats as compared to the diabetic control rats. This could be due to increased liver uptake of bilirubin and decreased production of bilirubin by liver. The increase in serum bilirubin (hyper-bilirubenimia) may be a result of the decreased liver uptake, conjugation or increased bilirubin production from hemolysis (Rana et. al., 1996). Treatment of the diabetic rats with repeated doses of aqueous extract of *A. tricolor* shows restoration of the normal enzyme activities and bilirubin levels in the diabetic rats. The methanolic extract also exhibits restorative behaviour though to a lesser extent.

4.2.2.3.5 Hypolipidemic Study

Hyperlipidemia is a metabolic complication of both clinical and experimental diabetes (Bierman, et. al., 1975). In animals, the administration of diabetogenic doses of alloxan induces hyperlipidemia (Velminsky et. al., 1970). Hypercholesteremia and

hypertriglyceridemia have been reported to occur in diabetic rats (Goodman and Gilman, 1985).

Administration of the aqueous extract of *A.tricolor* to diabetic rats significantly reduce cholesterol, triglyceride and LDL level, and increased HDL level. Tannic acid, a major component of tannins has the capacity to decrease blood glucose level by stimulating glucose transport while inhibiting adipogenesis. Various studies have shown that phytosterols and polyphenols have the potential to reduce hyperlipidemic conditions (Lee et. al., 2003) and blood glucose level (Li and Sinclair, 2002). Therefore, reduction in blood glucose and blood lipid levels in the diabetic rats can be attributed to the action of the phytosterols, polyphenols and tannins present in *A. tricolor*.

4.2.2.3.6 Hematological Parameters

The studies on a few haematological parameters clearly show that the diabetes leads to reduction in the RBC, WBC count along with reduction in PCV and Hb levels. The diabetic rats receiving leaf extracts of *A. tricolor* exhibit improvement in Hb and PCV values and partially of WBC and RBC counts. This suggests the hematinic properties of *A. tricolor*. The differential blood count showed decrease in WBCs and RBCs in the extract-fed normal rats. Tannin may be responsible for cell lyses of WBCs and RBCs as tannin is known to cause cell lysis (Toffazal et. al., 2002); however, there is a need to investigate further whether tannin selectively promotes lysis of WBCs and how majority of the RBCs escape tannin mediated toxicity. In diabetes mellitus, additional factors may contribute to anemia, such as increased hemolysis due to advanced glycosylation end-products (AGE) on the RBC membranes or iron and vitamin B12 deficiencies (Donnelly, 2003).

Recent studies have shown that anemia is a key indicator of early impairment of organ functions in diabetic patients and if left untreated, can cause significant renal and cardiac damage (O'Connell, 2003) resulting ultimately in death. AE II-treated rats showed significant increase in Hb concentration, which could be partly attributed to its high iron content (Rangarajan et. al., 1998), suggesting *A. tricolor*'s antianaemic properties. Since the administration of the aqueous extract of *A. tricolor* resulted in a significant reduction in blood glucose level and an increase in haemoglobin, it is felt that this plant is a potential natural source of antidiabetic, hypolipidemic and blood tonic medicine. Though, the plant has been used traditionally as a general tonic and good natural source of iron, it should be consumed with caution, as this study shows that it has a tendency to induce reduction of WBC count.

4.2.2.3.7 Amylase

The findings of the present study show that the administration of *A. tricolor* extracts to alloxan mediated diabetic rats significantly reduce pancreatic α-amylase activity, which plays a key role in the digestion of carbohydrates. This was indicative of lowered levels of absorbable glucose being formed from the digestion of carbohydrate and leading to reduced levels of blood glucose. The inhibition of pancreatic α-amylase activity in the human digestive tract represents one of the therapeutic approaches commonly used for the control and prevention of postprandial hyperglycemia in non-insulin-dependent diabetic patients through reducing the uptake of glucose released by those enzymes from starch (McDougall, 2005, Hamden et. al., 2011).

4.2.2.3.8 Lipase

Pancreatic lipases perform essential roles in the digestive transport and processing of dietary lipids into more simple forms that can be more easily absorbed and transported throughout the body. The present study also shows that *A. tricolor* extract administration to the alloxan diabetic rats reduce elevated pancreatic lipase activity, a decrease that is responsible for the hydrolysis of non-absorbable dietary triglycerides into absorbable monoglycerides and free fatty acids, which, in turn, leads to the decrease of plasma cholesterol and TG level (Carriere et. al., 2001, O'Donovan et. al., 2003; Prieto-Hontoria et. al., 2009). This represents one of the therapeutic approaches commonly used for the control and prevention of dyslipidemia.

Diabetes is known to reduce the activities of enzymes involved in the glycolytic and pentose phosphate pathways while increasing the activities of gluconeogenic, glycogenolytic and lipolytic pathways (Weber et. al., 1966, Storey and Bailey, 1978). In the diabetic state there are alterations in the specific activities of several glycolytic, NADPH generating and gluconeogenic enzymes in the liver (Belfiore et. al., 1973). The liver is the main site for glucose uptake, glucose phosphorylation and the entry of glucose-6-phosphate into the metabolic pathway (Cahill et. al., 1959). However the present work shows that *A. tricolor* extracts control the diabetes by influencing liver function independant of rise in insulin level. *A. tricolor* extracts do not promote elevation of insulin level. In the present study, the activities of G6PD, hexokinase and lactate dehydrogenase enzymes were significantly lowered in the livers of the diabetic rats as compared to the normal, while the activity of glucose 6 phosphatase was significantly increased in the diabetic state, while in kidneys it is observed that the activities of G6PD, glucose 6-phosphatase and hexokinase enzymes were significantly elevated in the diabetic rats as

compared to the controls. But during diabetes there was a significant decrease in the LDH activities during diabetes.

4.2.2.3.9 Glucose 6 phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) is the principal source of the intracellular reductant, NADPH, which is required by many enzymes, including enzymes of the antioxidant pathway (Zhang et. al., 2011) and is the rate limiting enzyme in the pentose-phosphate pathway. Previous studies have shown that high glucose i.e., diabetes, decrease G6PD activity in various cells of the body (endothelial, kidney, liver, and RBC) leading to oxidative damage, cellular dysfunction, and ultimately organ damage (Zhang, et. al., 2000; Xu et. al., 2005; Diaz-Flores, et. al., 2006). The treatment of extracts of *A. tricolor* leaves to the diabetic rats lead to improvement in the G6PD activities in liver and kidneys. G6PD is a regulatory enzyme that converts glucose-6-phosphate into 6 phosphogluconolactone for regulating glycolytic pathway. Also this enzyme plays an important role in maintaining level of NADPH, the principle antioxidant in turn maintaining the level of glutathione in cells to protect against oxidative damage (Shan et. al., 1990). However, it needs further investigation to confirm the rise in NADPH and glutathione levels under the influence of *A. tricolor* leaf extracts. Nevertheless the improvement of G6PD suggests the protective action of *A. tricolor* leaf extract against the diabetic morbidity vis a viz the oxidative damage and improvement in glycoytic pathway upto a certain extent. High glucose has been shown to increase ROS in many cell types in patients with diabetes due to a combination of increased production of ROS along with decreased antioxidant function (Baynes and Thorpe, 1999, Maritim et. al., 2003, Forbes et. al., 2008).

4.2.2.3.10 Hexokinase

Hexokinase plays a central role in the maintenance of glucose homeostasis, as it catalyzes the conversion of glucose to glucose-6-phosphate. It is an important regulator of glucose storage and disposal in the liver (Ghosh, 1984). Hexokinase begins the first step in the metabolic sequence as a phosphorylating enzyme to convert dietary and mobilized glucose in the presence of ATP to glucose-6-phosphate. Hence, the rate of glycolysis (glucose utilization) mainly depends upon the level of hexokinase activity in a cell (Muthuraman, 2009). The present work shows that the liver of diabetic rats has a low hexokinase activity while the kidney has high hexokinase activity. The low hexokinase activity prevents entry of glucose into the glycolytic pathway leading to lesser utilization of glucose, thereby further elevating the blood glucose level. The elevated kidney hexokinase activity in diabetes indicates higher utilization of glucose by the renal tissue. This could be attributed to increased metabolic demand of energy by the renal tubules under stress.

Alloxan is known to inhibit thiol dependant enzymes such as hexokinase (Jörns et. al., 1997). The treatment of diabetic rats with *A. tricolor* leaf extracts result in improvement of hexokinase activity indicating elevated utilization of glucose by the hepatic tissue. This could contribute to the reduction of elevated sugar levels of the rats. This indicates *A. tricolor* is partially controlling diabetes through improving hexokinase activity of the liver. The decline in hexokinase activity of the kidney of rats exposed to *A. tricolor* extracts suggests the reduction of metabolic stress of the diabetic kidneys.

4.2.2.3.11Glucose-6-phosphatase

From the present work it is clear that in a state of diabetes, the glucose 6-phosphatase

activities elevate in liver and kidney but upon *A. tricolor* leaf extract treatment this enzyme activity is significantly reduced though it does not come back to a normal non diabetic level. The elevation of glucose 6-phosphatase activity in the liver and kidney in a diabetic state indicates the conversion of glucose 6-phosphate to glucose thereby adding more glucose to elevate further the hyperglycaemic state. The significant reduction of glucose 6-phosphatase activity in diabetic rats treated with *A. tricolor* leaf extracts suggests that the reconversion of glucose 6 phosphate to glucose is significantly reduced thereby preventing the excessive rise in glucose level. Thus it appears that *A. tricolor* leaf extracts partly control the diabetes by preventing the excessive rise of glucose level in the blood mediated by glucose 6-phosphatase through their inhibitive action on glucose 6-phosphatase.

4.2.2.3.12 Effect of Fractions of *Amaranthus tricolor* on fasting blood glucose of alloxan induced diabetic rats – Acute Study

The fractionation of *A. tricolor* methanolic extract resulted in the isolation of two important compounds. AT-C exhibited very weak hypoglycemic activity as compared to the aqueous and methanolic extracts. However we believe that AT-C in combination with the carotenoid fraction may improve the diabetic condition greatly. There is ample evidence in literature to support this claim. Carotenoids have been shown to reverse cellular damage caused by chronic hyperglycemia in various organs (Bastaki, 2005) retina (Kowluru and Kennedy, 2001; Muriach et. al., 2006), and improve the overall diabetic morbidity.

4.2.2.3.13Carotenoids

Carotenoids are now known to play more important roles beyond their classical functions

with respect to nutrition and vision (Tee, 1988). With their highly conjugated double bonds, carotenoids may act as free radical traps or antioxidants, and therefore play an important role in cancer prevention (Peto, et. al., 1981; Olson, 1986; Temple and Basu, 1988).

Carotenoids are very important natural antioxidants that help preventing several human diseases. Although carotenoids are widely used as colorants, they also play an important role as precursors of vitamin A and powerful antioxidants (Heinonen et. al., 1989; Philip and Chen, 1988; Hulshof et. al., 1997; Bauernfeind, 1972). They can also be considered as preventive factors against several diseases, such as cardiovascular, carcinogenic (liver, medulla or prostate tumours) and vision failure (cataracts and blindness). Recent studies about other diseases, such as arteriosclerosis, rheumatism, Parkinson disease and infertility have revealed new functionalities of these molecules (Henriques et. al., 2005).

4.2.3 In Vitro Assays

4.2.3.1 Glucose uptake

The estimation of glucose content in rat hemi-diaphragm and skeletal muscles is a commonly employed and reliable method for in vitro study of peripheral uptake of glucose. The in vitro tests have indicated that *Amaranthus tricolor* has the ability to enhance glucose uptake significantly in the isolated rat hemidiaphragm and skeletal muscle tissues of rat, and may convert it into stored glycogen through glycogenesis. The *A. tricolor* extracts were found to be more effective than insulin alone. The groups containing both the extract and insulin exhibit a lower uptake of glucose and subsequently a lower conversion rate to glycogen than the groups containing the plant extract alone, which

exhibits the highest glucose uptake and conversion to glycogen. It appears that drug interaction between the extract and insulin, or competitive inhibition for the insulin receptor site could have occurred when given simultaneously

From the results obtained in this study we may deduce that several enzyme activities change in diabetes mellitus. The increase and decrease of these enzyme activities seem closely related to the diabetic metabolic alterations. Some however are indirectly related to the diabetic state, either by being expressions of acute tissue damage caused by episodes of severe decompensation, or complications that may develop during the chronic course of diabetic disease.

The present study shows that the feeding of *Amaranthus tricolor* leaf extracts to diabetic rats results in alterations in the metabolism of glucose with subsequent reduction of serum glucose concentration. The hypoglycaemic activity of *Amaranthus tricolor* involves increased glycogen synthesis in the liver and muscles and also enhanced uptake of glucose in the peripheral tissues. The findings indicate promising antioxidant capacity of *A. tricolor* and needs to be further investigated to ascertain its full potential in both modern and traditional system of medicines.

Fig 62: Primary Antidiabetic activity of *Amaranthus tricolor* in alloxan induced diabetic rats

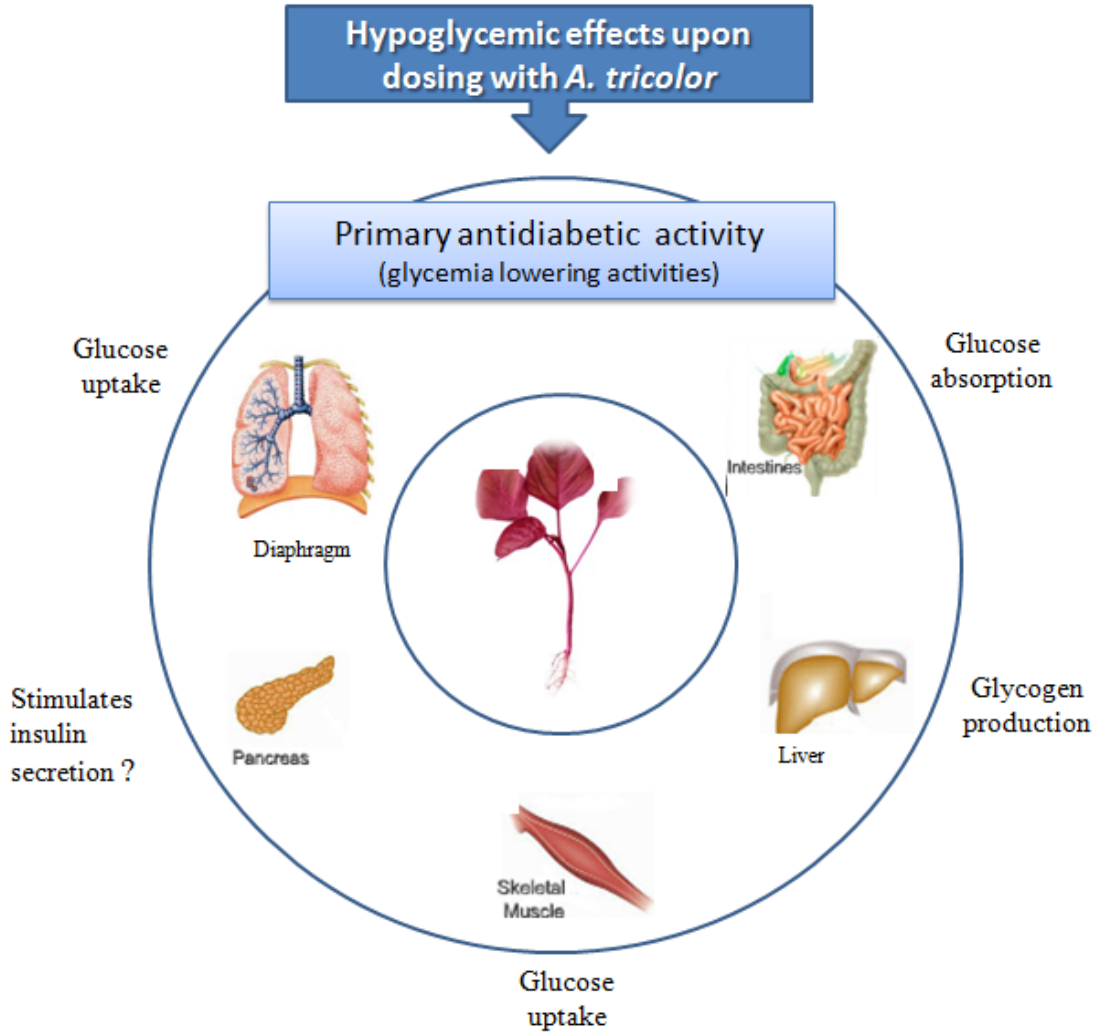
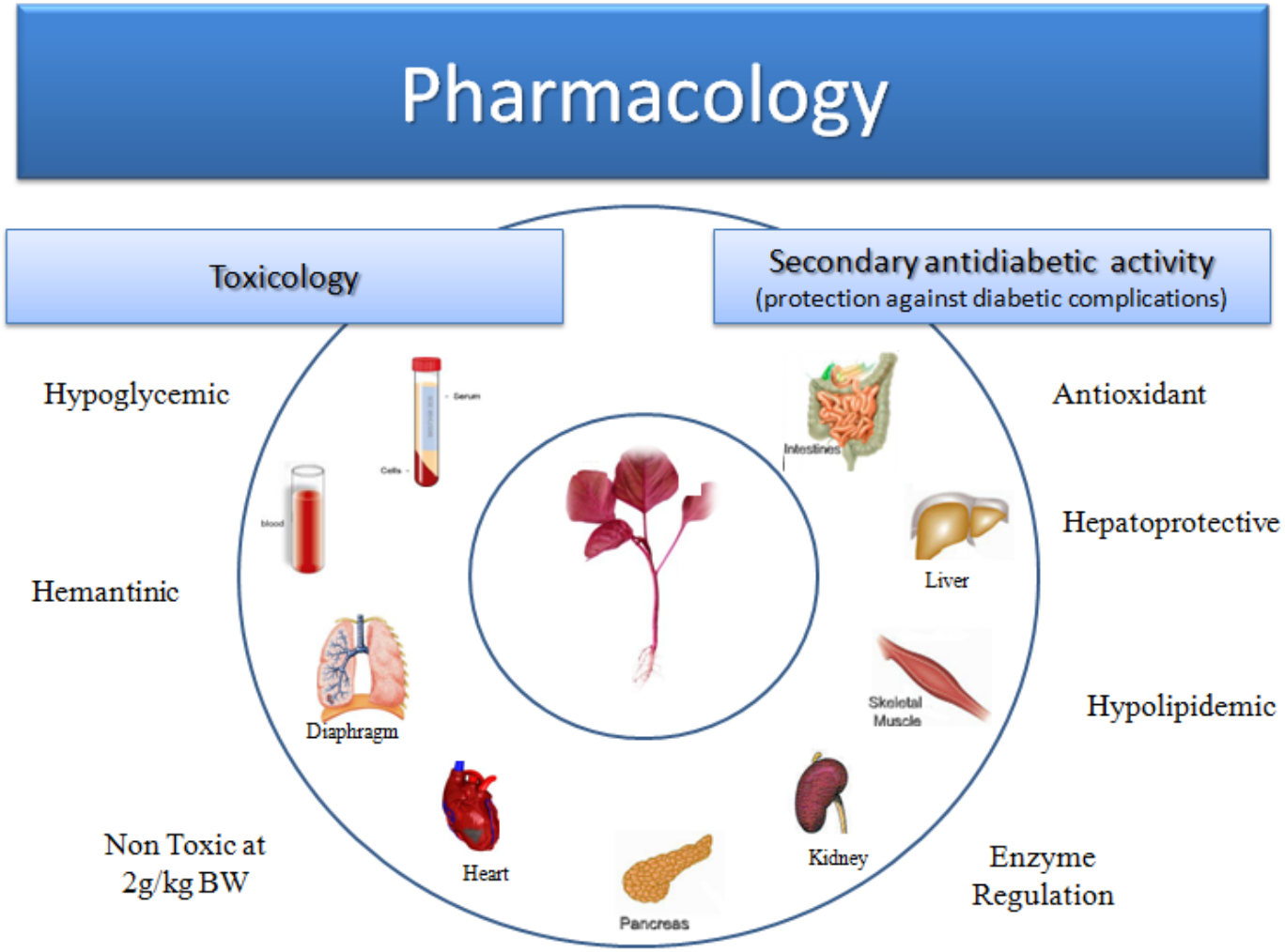


Fig63: Pharmacological activities of *Amaranthus tricolor* on the diabetic state in alloxan induced diabetic rat.



5 Conclusion

Therefore, it is concluded the *Amaranthus tricolor* leaf extracts alleviate hyperglycemia through improvement of glucose uptake, enhancement of activities of enzymes involved in glycolysis and glycogenesis and through the reduction of gluconeogenic enzyme activities as well as inhibition of pancreatic α-amylases. Further *Amaranthus tricolor* leaf extracts have no influence on the insulin levels vis a viz on insulin synthesis by pancreatic β cells. Also *Amaranthus tricolor* leaf extracts help alleviate the diabetic morbidities like loss of weight, hyperlipemia and hematic losses. Besides the present results have categorically and significantly thrown light on the antioxidant properties of the *Amaranthus tricolor* leaf extracts.

6 Future Work

In order to produce an authentic formulation of antidiabetic herbal medicine from *Amaranthus tricolor*, one has to isolate and purify, antioxidants, flavonoids, carotenoids, polyphenols, tannins and phytosterols and find out their appropriate effective proportions for mitigating diabetes. Further one has to identify the factor responsible for reduction of WBC'S in non-diabetic animals.

Summary

of the Thesis

Diabetes mellitus is a syndrome initially characterized by loss of glucose homeostasis (Wolff, 1993), resulting from nonproduction of or defects in insulin production, insulin action, or both. One of the main challenges in managing diabetes is maintaining blood glucose at near normal level with almost no episode of major fluctuation.

Complications in diabetic patients arise from the non-maintenance of normal or near normal blood glucose level (Nathan et. al., 2005). Studies in India indicate that more than 50% of people with diabetes have poor glycaemic control, uncontrolled hypertension and dyslipidaemia, and a large percentage have diabetic vascular complications (Raheja, 2001; Rema, 2005; Nagpal, 2006; Ramachandran, 2008).

The first line of drug treatment for diabetic patients in general, begins with the introduction of oral hypoglycemic agents, dietary restrictions coupled with exercise regimen and eventually administration of insulin in more severe cases and Type 1 diabetes. Synthetic hypoglycaemic agents can produce undesirable and serious side effects (Lamer, 1985), which have led to an increased demand for antihyperglycemic agents with fewer or no side effects. WHO has recommended the use of indigenous plants as alternative medicine, in the management of diabetes mellitus, particularly in developing countries where safe modern drugs, health centers and resources are limited or lacking (WHO, 2002).

Majority of the potential alternative therapies are often unverified, there is lack of quality assurance, no standardization of actual phytochemical content and lack of knowledge of their mechanisms of action as well as side effects. There is an increasing need to develop novel, safe, and efficacious botanical therapeutics for diabetes which is one of the major

human diseases.

Amaranthus tricolor as it is a commonly consumed and easily available plant cultivated for its economic value. which is abundantly cultivated during the non monsoon seasons. The phytochemical analysis of the plant revealed the presence of proteins, amino acids, tannins, sterols and polyphenols in the aqueous extracts. The methanolic extracts contain tannins, sterols and polyphenols. The aqueous (AD, AE), methanolic (MN, MS) and petroleum ether (PE) extracts were evaluated *in vitro* for protective antioxidant activities using DPPH, FRAP and phosphomolybdenum assays. The aqueous extracts were seen to possess the highest antioxidant property amongst all the other extracts.

Amongst all the prepared extracts in this study, the aqueous (AE) and the methanolic (MN) extracts promoted higher inhibition of porcine pancreatic a-amylase activity than the other extracts. The aqueous extracts induced more amylase inhibition than the methanolic extracts.

A UV-Vis spectral scan was carried out inorder to establish a spectral fingerprint for the administered extracts. Thin layer chromatography of the methanolic extract in Hexane:Chloroform:Ethyl Acetate (1:3:3) solvent system yielded 13 bands, comprising of beta-carotene, plant pigments and possibly polyphenols and tannins. An attempt to separate these bands as fractions using column chromatography resulted in two pure fractions and five mixed fractions. Fraction 1 resulted in a white powder which was subjected to UV-Vis, NMR and FTIR analysis. Fraction 2 resulted in a dark orange thick

liquid which was subjected to UV-Vis and online spectral analysis. Analysis revealed that Fraction 1 was possibly a flavonoid like compound, while Fraction 2 was pure beta carotene.

In the AOT test, both the aqueous as well as the methanolic extracts showed no visible signs of toxicity of doses upto 2g/kg BW. The alloxan induced diabetic rats were screened for antidiabetic activity by subjecting them to single doses of AE200, AE400, ME200 and ME400. A dose dependant reduction of hyperglycemia was noted upto 12 hours after the administration of the leaf extracts of *A. tricolor*, with the aqueous extract (AE) being more efficacious than the methanolic extract (MN).

The diabetic rats were subjected to *Amaranthus tricolor* leaf extract treatment over a period of 3 weeks, in order to study the effect of long term administration on the diabetic state. Upon administration of the extracts, an improvement in the overall physiology of the diabetic animals was noted. A tendency to approach normalcy is observed in the treated diabetic groups.

Administration of *A. tricolor* plant extracts for a period of 3 weeks lowered serum glucose in diabetic rats to near normal levels, but did not have any beneficial effect on serum insulin level or insulin concentration in the pancreas. The extract treated diabetic rats gained weight and the hepatic and skeletal muscle tissues showed an improved increase in glycogen content, over the duration of the treatment as compared to the untreated diabetic rats. Total protein in the serum and hepatic tissue of diabetic rats was found to be low as compared to the normal rats. Diabetic rats treated with *A. tricolor* aqueous and methanolic extracts showed a reversal of decreased total protein levels in both serum and liver tissue.

Extract administration resulted in an increase in hepatic AST, ALT, AcP and AlP activity and a decrease in serum AST, ALT, AcP, AlP activity along with a decrease in serum bilirubin, suggesting a hepatoprotective behavior. The levels of serum HDL increased while, that of triglycerides, cholesterol, LDL and VLDL decreased post *A. tricolor* treatment suggesting that the plant possesses hypolipidemic properties. The extracts restored the amylase and lipase activity in both serum and pancreatic tissue to a relatively normal state.

The effect of *Amaranthus tricolor* on key enzymes in carbohydrate metabolism such as Hex, G6P, G6PD and LDH was studied in an attempt to elucidate the blood glucose lowering mechanism of action of this plant. In the diabetic state there is an increase in G6Pase activity, while a decrease in Hex, G6PD and LDH activity in hepatic tissue is observed. In the renal tissue an increase in Hex, G6Pase and G6PD activity and a decrease in LDH activity are observed. Treatment of diabetic rats with *Amaranthus tricolor* reverses the enzyme activities in both tissues. Hepatic Hex, G6PD and LDH activities increase, while G6Pase activity decreases and Renal Hex, G6Pase, G6PD activities decrease while LDH activity increases.

Treatment of diabetic rats with *Amaranthus tricolor* fractions did not exhibit any relevant decrease in the fasting blood glucose. AT-C (Fraction 1) exhibited very weak hypoglycemic activity, where as Fraction 2 did not exhibit any hypoglycaemic activity when compared to the aqueous and methanolic extracts.

The uptake of glucose from the media and its conversion to glycogen in vitro, by the

hemidiaphragm and skeletal muscle tissues was found to be highest on exposure to the extracts alone. Exposure to insulin alone or insulin in combination with the plant extracts showed lower uptake and conversion of glucose to glycogen.

The outcome of this study suggests that *Amaranthus tricolor* is a potent antidiabetic plant rich in antioxidants and possesses hypolipidemic as well as hepatoprotective properties. It is seen to be beneficial in the treatment of disease associated anemic conditions by increasing Hb. The findings indicate promising antioxidant capacity of *A. tricolor* and needs to be further investigated to ascertain its full potential in both modern and traditional system of medicines.

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Contributions from this thesis

1. *Clemente, A.C., Desai, P.V. (2012). Hepatoprotective effects of Amaranthus tricolor Linn. extracts on the alloxan diabetic rat (Rattus norwegicus). Int J Bio Pharm App Sci, 1(4), 594-603.*

2. *Clemente, A.C., Desai, P.V. (2012). Evaluation of the total phenolic content and primary antioxidant activity of various extracts of Amaranthus tricolor Linn. J Pharm Res. 5(3), 1596-1599.*

3. *Clemente, A.C., Desai, P.V. (2012). Hematological studies and antioxidant activities of Amaranthus tricolor leaf extract on rat. National Seminar on Advanced Zoology & Life Processes. Department of Zoology, Goa University.*

4. *Clemente, A.C., Desai, P.V. (2011). Evaluation of the Hematological, Hypoglycemic, Hypolipidemic and Antioxidant Properties of Amaranthus tricolor Leaf Extract in Rat. Trop J Pharm Res, 10(5), 595-602.*

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Research Article

Evaluation of the Hematological, Hypoglycemic, Hypolipidemic and Antioxidant Properties of *Amaranthus Tricolor* Leaf Extract in Rat

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Abstract

Purpose: *To investigate the effect of Amaranthus tricolor leaf extract on some biochemical parameters in diabetic and normal rats*

Methods: *A. tricolor aqueous extract was assayed for antioxidant properties using ferric reducing ability of plasma (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and phosphomolybdenum assay. The effect of the leaf extract on serum glucose and triglyceride, total cholesterol, low density lipoprotein (LDL), very low density lipoprotein (VLDL), elevated high density lipoprotein (HDL), body weight and hematological parameters were assessed in diabetic and normal rats. The extract doses used were 200 and 400 mg/kg body weight. Acute toxicity studies were also carried out*

Results: *In the extract doses, 200 and 400 mg/kg, reduced blood glucose levels in a dose-dependant manner, from 168.0 ± 18.5 mg/dl at 0 h to 43.0 ± 9.3 mg/dl at the 12th hour and from 148.50 ± 22.1 mg/dl at 0 h to 37.250 ± 6.3 mg/dl at the 12th hour, respectively. Oral administration of 400 mg/kg of the extract for 21 days significantly reduced ($p < 0.001$) serum glucose, serum triglyceride, total cholesterol, low density lipoprotein, and very low density lipoprotein, but elevated ($p < 0.05$) high density lipoprotein in diabetic experimental rats, compared to diabetic control. The extract prevented a decrease in body weight in treated diabetic rats and promoted an improvement in haemoglobin levels. Total antioxidant activity assay revealed that 1 g of dry leaf powder was equivalent to 0.035g/ml of ascorbic acid. The extract showed no toxicity up to 2 g/kg body weight.*

Conclusions: *This study shows that the aqueous extract of Amaranthus tricolor possesses some beneficial antidiabetic properties that warrant further research.*

Keywords: *Amaranthus tricolor, Anti-hyperglycemia, Anti-hyperlipidemia; Amaranthus, Antioxidant activity, Phosphomolybdenum*

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Evaluation of the total phenolic content and primary antioxidant activity of various extracts of *Amaranthus tricolor* Linn.

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ABSTRACT

The total phenolic contents and antioxidant potentials of aqueous dried leaf (AD), methanolic (MN & MS) and petroleum ether (PE) extracts of *Amaranthus tricolor* were evaluated. The free radical scavenging activity, ferric reducing ability and total antioxidant capacity of the extracts were tested using three different in vitro antioxidant systems (2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and Phosphomolybdenum assays). The AD extracts exhibited the highest total phenolic contents, while PE extracts exhibited the least. AD and PE extracts exhibited significant antioxidant activities but MN and MS extracts showed lowest activities. However the total antioxidant capacity as depicted by phosphomolybdenum assay was higher in MN than in PE extracts. A strong correlation between the DPPH and FRAP values was observed (R^2=0.8390). The antioxidant assays also showed significant correlations with the total phenolic contents.

Key words: *Amaranthus tricolor*, Antioxidant, Phenolic, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP)

INTRODUCTION

Plants have been investigated for their medicinal properties throughout the world, mainly due to their potent pharmacological activities, low toxicity and economic viability.[1] It is well documented that indigenous antioxidants avert the harmful effects of oxidative stress, and therefore increasing the interest in the protective biochemical functions of such natural antioxidants of plant origin.[2, 3]

In the last decade the hunt for naturally occurring antioxidants has grown tremendously since free radicals are known to quench various ailments that affect human health. Consumption of herbal antioxidants improves health. This necessitates the search for natural plant products which could effectively intervene in the onset and morbidity of the disorders and diseases.[3] It is well documented that expression of many disorders or ailments/diseases is associated with the generation of reactive oxygen species or free radicals.[4, 5] Reactive oxygen species (ROS) are often produced as by-products of biological reactions or as a result of intake of exogenous elements which foster oxidative damage,[2] leading to a wide range of biological malfunctions prompting DNA damage, carcinoma, cardiovascular, metabolic and neuro-degenerative disorders, as well as acceleration of senescence.[5, 6] A potent broad spectrum ROS scavenger may serve as a possible preventive intervention for mitigating free radical mediated cellular damages and diseases.[6]

With increased awareness of ROS mediated diseases, worldwide people are turning to alternative herbal medicines rich in antioxidants, with almost no side effects in comparison to allopathic therapies. Phenolic compounds with high antioxidant properties are reported to heal free radical mediated diseases including diabetes mellitus.[7, 15] Amaranthus is one of such plants known traditionally for its healing effects, nutritional and medicinal values,[11, 12, 13, 14] while *Amaranthus tricolor* is known for its purple pigments, amaranthine and isoamaranthine,[13] as well as for its potent antidiabetic, antihyperlipidemic,[14] antiviral,[14] antitumor,[17] and hepatoprotective activities.[18]

The present study therefore seeks to investigate the antioxidant properties of various solvent extracts of *A. tricolor* and their correlations, if any with the phenolic contents.

MATERIALS & METHODS

Fresh *Amaranthus tricolor* plants were collected from a local source in Farrenda, Goa, India. The plant was identified by Department of Botany, Goa University and a voucher specimen (GUBH-PVAC-0515) was deposited for future reference. The leaves were plucked, rinsed in cold distilled water and dried at 50°C overnight in an oven to a constant weight. The dried leaves were ground and sieved.

Preparation of extracts:

The aqueous extract from dried leaves (AD), was prepared by boiling ten grams of dry leaf powder with 100 ml of water for one hour and the resulting decoction was filtered. Methanolic extracts (MN) was prepared by mixing and shaking of ten grams of dry leaf powder at 27-28°C with 100 ml of methanol for 24 hrs and resulting extract was filtered. For the methanolic soxhlet extract (MS), ten grams of dried leaf powder was soxhelated with 100 ml of methanol at 70°C for one hour and the resulting extract was filtered. Petroleum ether extract (PE) was obtained by mixing ten grams of dry leaf powder with 100 ml of petroleum ether at 70°C for four hours and the resulting extract was filtered. All filtered extracts were evaporated separately at 40°C and the dried extracts were reconstituted (one mg/ml) with the respective solvents before using for the assays.

Total Phenolic Content:

The total phenolic content of the aqueous (AD), methanolic (MN & MS) and petroleum ether (PE) extracts were assayed using the Folin-Ciocalteu method[19]. Both, samples and tannic acid standards were prepared in triplicate. Stock standard solution of tannic acid was prepared by dissolving one microgram of tannic acid in one millilitre of extracting solvent and diluting to 50 ml of distilled water. Working standards were prepared as required by

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HEPATOPROTECTIVE EFFECTS OF *AMARANTHUS TRICOLOR* LINN. EXTRACTS ON THE ALLOXAN DIABETIC RAT (*RATTUS NORWEGICUS*)

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ABSTRACT

The present study investigates the effect of *Amaranthus tricolor* on some hepatic enzyme activities in alloxan-induced diabetic rats. The serum levels of glucose, total protein, and bilirubin were significantly increased in alloxan diabetic rats as compared to control rats. A significant increase ($p \leq 0.05$) in the activities of transaminases, i.e. - aspartate aminotransferase (AST) and alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), in the serum of alloxan diabetic rats was noted, whereas the activities of the same enzymes decreased in the hepatic tissue as compared to the control rats. Daily oral administration of *A. tricolor* aqueous and methanolic extracts (400 mg/kg body weight) for a seven day period significantly restored the disturbed enzyme activity to their normal levels. The serum total protein was also normalized and an improvement in body weight was seen in the extract fed rats when compared with the diabetic control group. The present study shows that *A. tricolor* besides its potent antioxidant and antidiabetic activities, upon regular consumption, may consequently alleviate or mitigate liver damage caused by diabetes.

Keywords: *Amaranthus tricolor*, **aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alloxan diabetes**

INTRODUCTION

Diabetes mellitus arises from impaired glucose metabolism due to defective insulin secretion or action, and is initially characterized by hyperglycemia, glucosuria and increased thirst. In recent decades diabetes mellitus has assumed epidemic like proportions. Doubts about the efficacy and safety of the oral hypoglycemic agents have