Hypolipidemic Effect of Diallyl Disulphide in Alloxan Induced Diabetic Rats

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Certificate

This is to certify that this thesis entitled "*Hypolipidemic Effect of Diallyl Disulphide in Alloxan Induced Diabetic rats*" is a bonafide work of Mr. Naveen Kumar S and was carried out under our supervision and guidance in the Department of Biochemistry, Shri B.M.Patil Medical College, Hospital & Research Centre, Vijaypur, Karnataka, India.

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DECLARATION

I declare that the thesis entitled *"Hypolipidemic Effect of Diallyl Disulphide in Alloxan Induced Diabetic rats"* has been prepared by me under the guidance of Professor Dr J.G. Ambekar, Department of Biochemistry, BLDE University's Shri B.M.Patil Medical College, Hospital and Research Centre, Vijaypur, Karnataka, India. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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DEDICATION

I dedicate this thesis to

My Parents

Shri Manohar Kumar Sambu & Smt Shakuntala Sambu

&

My Teachers

ABSTRACT

Background- Diabetes Mellitus is a chronic metabolic disorder which may lead to various complications, the important being dyslipidemia leading to Coronary Heart Disorders (CHD), the major cause for morbidity and mortality in diabetic patients. Diabetes Mellitus could be treated by nutritional therapy/drug therapy and others. But the drug therapy would have its own limitations and side effects. To overcome from this an herbal extract is recommended, such as Diallyl Disulphide (DADS) a principle compound of Garlic oil.

Aim- To assess the hypolipidemic effect of Diallyl Disulphide (DADS) in alloxan induced diabetic rats.

Materials and Methods- Healthy adult Wistar strain male albino rats weighing around 200-250 grams were randomly selected from the animal house at BLDE University's Shri B.M.Patil Medical College, Hospital and Research Centre, Vijaypur, India. Diabetes was induced using alloxan and was treated with DADS. After a stipulated time the rats were anesthetised and sacrificed to collect the blood and liver tissue. Various Lipid parameters, glucose, lipoprotein lipase, TBARS, total thiols, AST, ALT, HMG CoA Reductase, fecal bile acids were estimated in the blood, feces and homogenised liver tissue using standard procedures. Liver histological section were prepared and observed under the microscope.

Statistics- One way ANOVA followed by post hoc't' test is done.

Result- There was significant decrease in the blood and liver tissue lipid parameters of DADS treated alloxan induced diabetic rats when compared to the alloxan induced diabetic rats. There was also a significant reversal of histological and biochemical changes in the liver of DADS treated alloxan induced diabetic rats when compared to the alloxan induced diabetic rats.

Conclusion- From this study it can be concluded that the DADS a principle compound of garlic, definitely has the hypolipidemic and hepato-protective effect in diabetic rats, which may reduce the morbidity in diabetic cases due to dyslipidemia without the adverse effects.

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ADP	Adenosine Diphosphate
AIDS	Acquired Immuno Deficiency Syndrome
ALT	Alanine Transaminase
AST	Asparatate Transaminase
ATP	Adenosine Triphosphate
CETP	Cholesterol Ester Transfer Protein
CHD	Coronary Heart Disease
CPCSEA	Committee for the Purpose of Control and Supervision of Experimental Animals
DADS	Diallyl Disulphide
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra acetic acid
EFA	Esterified Fatty acids
FFA	Free Fatty Acids
GDM	Gestational Diabetes Mellitus
GLUT	Glucose Transporter
GSH	Glutathione (Reduced)
H&E	Haematoxylin and Eosin
HDL	High Density Lipoprotein
HHS	Hyperglycemic Hyperosmolar State

HIV	Human Immuno deficiency Virus
HMG CoA	Hydroxy Methyl Glutaryl Coenzyme A
HMIT	H^+ Coupled Myo Inositol Transporter
HSSL	Hormone Sensitive Lipase
HTGL	Hepatic Triglyceride Lipase
IAEC	Institutional Animal Ethical Committee
IDDM	Insulin Dependent Diabetes Mellitus
IDL	Intermediate Density Lipoprotein
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IUPAC	International Union for Pure and Applied Chemistry
LCAT	Lecithin Cholesterol Acyl Transferase
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
MODY	Maturity Onset Diabetes of Young
NAC	N- Acetyl Cysteine
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NDDG	National Diabetes Data Group
NIDDM	Non Insulin Dependent Diabetes Mellitus
PEP	Phospho Enol Pyruvate
PFK	Phospho Fructo Kinase

PKC	Pyruvate Kinase C
PLTP	Phospho Lipid Transfer Protein
RNA	Ribo Nucleic Acid
ROS	Reactive Oxygen Species
sdLDL	Small Dense LDL
SDS-PAGE	Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
SGLT	Sodium Dependent Glucose Transporter
SOD	Super Oxide Dismutase
STZ	Streptozotocin
TBARS	Thio Barbituric Acid Reactive Substances
TCA	Tri Chloro Acetic acid
TFA	Total Fatty acids
TNF α	Tumor Necrotic Factor α
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

Chapter 1

Introduction

Diabetes mellitus is the most severe metabolic disorder of the 21st century, mainly affecting the biochemical activities in almost every cell of the human body. It is estimated that in the year 2000, 171 million people had diabetes and this is expected to get double by the year 2030. Insulin deficiency or insufficiency alters the carbohydrate and protein metabolisms, leading to elevation in blood glucose. This elevation when persists for a period of time, it leads to hyperglycemia which in turn presents as a syndrome known as diabetes mellitus. Diabetes mellitus and its complications pose an immense amount of social and economic burden on the health infrastructure and resources across the globe.

Several pathological processes either in single or in combination are known to cause diabetes such as: obesity resulting in insulin resistance and autoimmune destruction of β-cells leading to insulin deficiency. Inadequate secretion of insulin leads to deficiency in insulin action and/ or diminished tissue responses to insulin. The disease is thus primarily characterized by abnormalities in the carbohydrate, fat and protein metabolism.

Marked symptoms of hyperglycemia include polydipsia, weight loss, polyuria, polyphagia and blurred vision. Uncontrolled diabetes may contribute to the development of many complications with acute complications such as – hyperglycemia along with ketoacidosis or the non-ketotic hyperosmolar syndrome and chronic complications such as hyperlipidemia, retinopathy, nephropathy and neuropathy. Mounting evidence from experimental and clinical studies suggest that oxidative stress plays an important role in the pathogenesis of Diabetes mellitus. Free radicals will be generated in a disproportionate way during diabetes by glucose oxidation. Abnormally high levels of these free radicals and the decline in antioxidant defence mechanisms are known to cause the damage of cellular organelles and enzymes.

Membrane glucose transporters are known to play a key role in the transportation of glucose across the membrane. However altered or defective function of these transporters as in seen in diabetes mellitus is known to cause the hyperglycemia.

From the ancient past, many plants and their extracts were known to have beneficiary effects (hypolipidemic) for the management of diabetes mellitus. Studies conducted by the World Health Organisation (WHO) observed that 80% of world population relies on medicinal plants for their primary health care needs (Ngugi MP *et al*., 2012). Allium species are known to have hypoglycemic and hypolipidemic effects, which are employed to control diabetes mellitus.

In the present study, an attempt was made to assess the hypolipidemic and other biochemical effects of principle disulphide of garlic (Diallyl Disulphide) in alloxan induced diabetic rats.

Chapter 2

Review of Literature

Diabetes mellitus (DM) is well acknowledged as a chronic metabolic disease causing death and disability globally. The problem has reached pandemic proportions (Suja K *et al*., 2014). DM is a metabolic disorder characterized by chronic hyperglycemia and disturbances of carbohydrate, protein and fat metabolism associated with absolute or relative deficiency in insulin secretion and/ or insulin action (Ozougwu JC *et al*., 2013). DM is often referred to as "starvation in the midst of plenty" because the intracellular levels of glucose are low, although the extracellular levels may be extremely high (Burnstock G *et al*., 2013). Uncontrolled hyperglycemia leads to life threatening complications that may result from acute metabolic decomposition, while long standing metabolic derangements are frequently associated with permanent and irreversible functional and structural changes in the cellular level itself (Abbas EK *et al*., 2009).

Global scenario – The rapidly increasing prevalence of DM worldwide is one of the most serious and challenging health problems in the 21st century. The number of people with diabetes had grown faster than expected. In 2007, 246 million people (roughly 6%) were affected worldwide and it is estimated that this will increase to 380 million or 7.35% by 2025. Furthermore, it is estimated that there are even more people (308 million or 8.1%) with impaired glucose tolerance (IGT). These people have a significant risk of developing type II diabetes mellitus. (Inge AMV *et al*., 2010). Type II diabetes is the commonest form of diabetes constituting almost 90% of diabetic population. There will be 42% increase i.e., from 51 to 72 million, in the developing countries. Countries with the largest number of people with diabetes are India, China, and the USA. In developing countries, the majority of people with diabetes are in the age group of 45-64 years and in developed countries around 65 years (Kesavadev JD *et al*., 2003). DM is the seventh leading cause of death in the United States and is the major cause of heart disease and stroke, and leading cause of kidney failure,

non traumatic lower limb amputations, and new cases of blindness among adults. It affects 25.8 million people of all ages (8.3%) of the US population. DM is the leading cause of kidney failure.

Indian scenario – Currently, India ranks highest with its largest number of diabetic subjects in any given country. WHO has already declared India as 'global capital of diabetes' (Jali MV *et al*., 2006). In 1970's the prevalence of diabetes among urban Indian population was reported to be 2.1%, this has now risen to 12.1% (Apoorva SM *et al*., 2013). It has been estimated that presently, 19.4 million individuals are affected by DM and these numbers are expected to increase to 57.2 million by the year 2025 (Mohan V., 2004) according to WHO.

2.1. History of Diabetes mellitus:

The history of DM probably dates back to the beginning of human kind encompassing centuries, generations and civilizations. A historical review of the events surrounding the evolution of our current knowledge of DM must examine the oldest civilization including Babylonians, Assyrians, Egyptians, Chinese and Japanese as well as the centuries of the Greeks, Romans, Europeans and Americans.

Before Christ, Egyptians (1500 BC) had described an illness associated with the passage of much urine. Charaka and Sushruta (600-400 BC) recognised many aspects of this disorder and called it 'Madhu meha' (rain of honey) after noticing the sweet taste of urine. It was Greek physician: Aretacus of Cappadocia gave the name 'Diabetes'.

The earliest of the Chinese medicine texts is based on the works of Haug-ti of 2697 BC. Records were preserved on Lacquer on strips of bamboo or palm leaves. The Chinese and Japanese also recognized the symptoms of diabetes, but were even less restrained with their description and wrote "The urine of diabetes was very large in amount and it was so sweet that it attracted dogs".

Before Christ, Egyptians (1500 BC) had described an illness associated with the passage of much urine. Egyptians used strips of papyrus, reeds fastened together and shaped into rolls, on which they inscribed information. These strips subsequently became permanent records (Saima YQ *et al*., 2012). The most interesting of the papyri is papyrus ebers, written about 1500 BC, which records medical knowledge of ancient days. This contains a record of abnormal polyuria now believed to be related to diabetes. This probably represents the first recorded reference to the symptoms of diabetes.

The Hindu Medical writings of Charaka and Sushruta (600-400 BC) refer to diabetes as honey urine (madhu meha) (Ritu Lakhtiakia, 2013). It describes diabetes as "A disease of the rich and one that is brought about by gluttony or never indulgence in flour and sugar. This disease is ushered in by the appearance of the morbid secretions about the teeth, nose, ears and eyes. The hands and feet are very hot and burning. The surface of the skin is shiny as if oil had been applied to it, this accomplished by the thirst and the sweet taste in the mouth. The different varieties of this disease are distinguished from each other by the colour of the urine".

Although DM had always been present, it was Aretaeus who is credited for naming this medical illness. He made first complete clinical description of diabetes, describing it as "a melting down of flesh and limbs into urine" (Neils M *et al*., 2008). The term 'Diabetes Mellitus', is a Greek word meaning 'Siphon Sweet' (Dia-cross, biano-go, mellitus-sweet i.e., sweet is being siphoned). It is a wasting disease, because energy giving glucose is being filtered across the body as sweet urine.

In 1674, Thomas Willis, a physician, anatomist and a professor of natural philosophy at Oxford, discovered (by tasting) that the urine of diabetic patient was sweet. This was actually a rediscovery, for unbeknownst to him, an ancient Hindu document by Sushruta in

India in about 400 BC had described the diabetic syndrome as characterized by a "honeyed urine" (Madhu meha). Dabson in 1755 demonstrated the presence of sugar in urine. Matthew Dobson of Manchester of England demonstrated in 1776 that diabetics actually excrete sugar in urine. In 1778 Cawley reported (without particular comment) that he observed a shrivelled pancreas with stones in a diabetic patient at autopsy. This may have been the first published reference to the pancreas in relation to human diabetes. John Rollo noted that the amount of sugar excreted depends primarily on type of food ingested. Foods containing grains and fruits increased glycosuria whereas meat (protein and fat) resulted in comparatively lower excretion of sugar. In 1815, Chevreuil showed that blood sugar behaved as if it is grape sugar (i.e., dextrose/glucose). Later, specific methods of analysis were devised and used to measure glucose as the major "reducing substance" in the plasma and urine. Thus Rollo's predictions were confirmed that in diabetes a rise in blood sugar level causes the excretion of sugar and that the 'seat' of diabetes was outside of the kidneys.

Bouchardat in 1850 proved that sugar in diabetic urine was in fact glucose. The part of pancreas not involved in digestion was identified by Langerhans in 1869 and was named after him. In 1888 Cawley diagnosed diabetes for the first time by demonstrating the presence of sugar in urine. He observed that the disease may result from injury to pancreas as had already been observed in experimental animals in 1682 by Brunner. In 1889 Von Merin and Minkowiski accidentally discovered that pancreatectamised dogs became diabetic in addition to developing digestive disorders. In 1891, Minkowski demonstrated clearly that the pancreas was a gland of internal secretion and that a small portion of the gland, when implanted under the skin of freshly depancratised dog, prevented the appearance of hyperglycaemia until the implanted tissue was removed or had degenerated spontaneously. In 1909, de Meyer identified that substance from the islets of Langerhans that prevented diabetes and named it

'Insulin' from the Latin word insulae or islands. It was only in 1921 that Banting and Best were able to purify Insulin from pancreas and showed that it lowered the blood glucose levels in diabetic dogs.

Sanger worked out the complete amino acid sequence of Insulin and was awarded the Nobel Prize in 1960. Dorothy Hodgkin, another Nobel laureate worked on the crystal structure of Insulin.

The following chart gives chronological order in the understanding of the disease - diabetes mellitus (Leo PK *et al*., 1994).

Table – 1 Important milestones in diabetes mellitus

Date	Source	Observation
15th Century BC	Ebers papyrus (Egypt)	Clinical Description of Polyuric condition resembling diabetes.
2nd Century BC	Galen (Rome) Aretaeus (Cappadocia)	Clinical Description of polyuric condition resembling diabetes.
5th Century	Sushrutha and Charaka (India)	Clinical description including sugary urine complication including gangrene and obese thin patient distinguished.
10th Century	Avicenna (Arabic)	Clinical description including sugary urine complication including gangrene and impotence.
19th Century		
1810-20	William pront (England)	Diabetic coma described.
1850	Bouchardat	Presence of sugar in diabetic persons is Glucose.
1869	Paul Langerhans (Germany)	Pancreatic Islands identified.
1888	Cawley	Presence of sugar in the urine due to injury to pancreas.
20th Century		
1909	Demeter	Identified the substance from pancreas and called

		insulin.
1922	Fredrick G.Banting Charles H.Best James B.Collip	Isolation and first clinical use of Insulin.
1936	Paul Kimmeisteil and Cliford Wilson (USA)	Described nodular glomerular lesions in the diabetic patient with proteinuria and hypertension.
1955	Frank and Fuchs	Hyperglycemic drugs (sulphonyl - urea Introducted).
1957	Unger	Hypoglycemic drug Phenformin is introduced.
1969	Dorothy Hodgkin (England)	Determined the 3 – D structure of Insulin.
1971	Pierre Freychet (USA)	Identified Insulin receptors.
1993	Diabetes Control and complications trial (USA)	Strict glycemic control reduces the risk of diabetic microvascular complications in IDDM.

2.2. Classification of Diabetes mellitus:

National diabetes data group (NDDG) in 1974 proposed a systemic classification of diabetes mellitus. This was adopted by WHO in 1980 and later modified in 1985, further lot of research has taken place in the field and information has accumulated, this necessitated a revision of WHO classification.

International expert committee, working under the sponsorship of the American Diabetes Association was established in May 1995 to review the classification and diagnosis of diabetes based on etiology (American Diabetes Association, 2010).

According to American Diabetic Association (American Diabetes Association, 2010), DM is classified into four clinical classes.

- Type I Diabetes – Results from β-cell destruction, usually leading to absolute insulin deficiency.

- Type II Diabetes – Results from a progressive insulin secretory defect on the background of insulin resistance.
- Other specific types of Diabetes – Due to other causes like genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis) and drug or chemical induced diabetes (such as treatment of AIDS and or after organ transplantation).
- Gestational Diabetes – Diabetes diagnosed during pregnancy.

2.2.1. Type I diabetes (absolute insulin deficiency):

- Immune mediated – it accounts for only around 10% of those with diabetes. Previously known by the term insulin – dependent diabetes or juvenile – onset diabetes, results from a cell – mediated autoimmune destruction of the pancreatic β-cells, here auto antibodies to islet cells can be detected that about 85-90% among individuals with fasting hyperglycemia.
- Idiopathic – Only a minority of patients with type I diabetes fall into this category mostly of African or Asian origin. It is an inherited autoimmune β-cell destruction with no immunological evidence.

2.2.2. Type II diabetes (insulin resistance, relative insulin deficiency):

It accounts for ~90-95% of those with diabetes, previously referred to as non-insulin-dependent diabetes or adult – onset diabetes. It is a metabolic disorder that is characterized by insulin resistance, relative insulin deficiency and hyperglycemia. In Type II diabetes, pancreas usually produces enough insulin. However, the body does not use it effectively. The condition known as "Insulin resistance" occurs when the cells do not respond to insulin's attempt to enter with glucose. The pancreas responds by producing more and more insulin. When the cells do not respond, high levels of glucose build up in the blood, leading to type II

diabetes. In type II DM, cells are always present regardless of the duration and severity of the disease, but lack any signs of functional activity. It was primarily seen among adults over age of 40 and now increasingly seen in children and adolescent. Obesity is found in approximately 55% of patients diagnosed with type II DM. People with type II DM often need to take prescription drugs to lower blood sugar levels along with dietary and lifestyle changes to control the problem.

2.2.3. Other specific types of diabetes:

i. **Genetic defects of beta cell function –** This form of diabetes can be seen at early age (before 25 years) which is also called as maturity-onset diabetes of the young (MODY) leading to hyperglycemia with deficiency in secretion of insulin and no defects in insulin action.

ii. **Genetic defects in insulin action –** Any mutations to the insulin receptor can lead to hyperinsulinemia and hyperglycemia which in turn can lead to metabolic abnormalities.

iii. **Diseases of the exocrine pancreas –** Any injury to the pancreas can cause diabetes, the processes include pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma.

iv. **Endocrinopathies –** Several hormones (e.g., Growth hormone, cortisol, glucagon and epinephrine) in excess are known to antagonize insulin action and cause diabetes.

v. **Drug or chemical-induced diabetes –** Many drugs can impair insulin secretion and cause diabetes in individuals with insulin resistance.

vi. **Infections –** Certain viruses are also associated with the β-cell destruction.

vii. **Uncommon forms of immune-mediated diabetes –** Binding of anti-insulin receptor antibodies to the insulin receptor blocks the binding of insulin to its receptor in target tissues which in turn can lead to diabetes.

viii. **Other genetic syndromes associated with diabetes –** Several genetic syndromes are accompanied with the increased incidence of diabetes mainly the chromosomal abnormalities (American Diabetes Association, 2010).

2.2.4. Gestational diabetes mellitus (GDM):

GDM has been defined as any type of glucose intolerance diagnosed during pregnancy. In most cases, spontaneous resolving after delivery is common. GDM definition applied whether or not the condition persisted after pregnancy and did not exclude the possibility that unrecognized glucose intolerance may have begun concomitantly with the pregnancy. This definition supports the classical detection and classification of GDM. The number of pregnant women with undiagnosed type II diabetes has increased.

The following are the major changes proposed in the new classification:

- The term insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) are eliminated.
- The terms type-I and type-II diabetes retained with Arabic numerals being used rather than Roman numerals.
- The stage impaired glucose tolerance (IGT) has been retained. A new entity named impaired fasting glucose (IFG) has been introduced.
- Gestational diabetes mellitus (GDM) is retained as defined by World Health Organization (WHO) and National Diabetes Data Group (NDDG). But more selective rather than universal screening during pregnancy is now recommended.

2.3. Diagnostic criteria for diabetes mellitus:

The National Diabetes Data Group and World Health Organization have issued diagnostic criteria for diabetes mellitus as follows-

1. Symptoms of diabetes plus casual random plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/L). Casual refers to the plasma glucose levels at any time in a day. The classical symptoms of diabetes include polyuria, polydipsia and unexplained weight loss.
2. Fasting plasma glucose ≥ 126 mg/dl (7.0 mmol/L). Fasting is defined as no calorie intake for at least 8 hours.
3. Two hour plasma glucose ≥ 200 mg/dl (11.1 mmol/L) during an oral glucose tolerance test. The test is done by giving 75g of anhydrous glucose in water as per WHO.
4. In a patient with classic symptoms of hyperglycemia, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/L) (American Diabetes Association, 2010).

2.4. Diagnosis and monitoring of diabetes:

A large number of diabetics are as yet unidentified despite the global awareness about diabetes. Most of the diabetics are detected on the strength of their postprandial glycosuria. Some of the methods are used for detection and diagnosis of hyperglycemia is as follows -

1. **Urine analysis:** It is one among the diagnostic tests firstly performed in patients with suspected diabetes. Glycosuria is the first symptom associated with diabetes and which provides the first clue to the development of the disease. However, urinary glucose may also be found in non-diabetic subjects. Absence of glycosuria, on the other hand cannot be taken to indicate absence of diabetes.

The finding of ketonuria in the presence of high urinary glucose or ketonuria in the absence of glucose excretion and concomitant low blood glucose also indicates diabetes. Detection of proteinuria or microhematuria is suggestive of diabetic nephropathy.

2. **Blood analysis:** Fasting and post-prandial blood glucose levels are the second set of diagnostic tests for hyperglycemia. Where these tests are not conclusive, a glucose tolerance tests is also performed which clearly indicates the borderline range and pathological range. Other tests include the detection of insulin and insulin-antibodies in the diabetic's blood. In type II diabetes mellitus however, the insulin level would be normal or slightly elevated. Currently, C-peptide analysis is also carried out to find out whether insulin is being synthesized and released or not.
3. **Glycosylated haemoglobin:** HbA_{1C} forms normally 4 – 6 % of the total haemoglobin. In diabetes, this value increases to above 8%. This glycosylated haemoglobin provides an index to the average glucose level in diabetes.

2.5. Metabolic complications of diabetes:

Conventional treatment of diabetes with diet, insulin and oral hypoglycemic drugs have been highly successful in controlling hyperglycemia, thereby prolonging life, unfortunately it has been not able to prevent other complications associated with diabetes. These complications include acute and chronic complications described below:-

2.5.1. Acute complications include:

1. **Diabetic Ketoacidosis:**

Diabetic Ketoacidosis (DKA) was formerly considered as the hall mark of type I diabetes mellitus. Insulin deficiency leads to breakdown of triacylglycerols in the

adipose tissue thus elevating the free fatty acid (FFA) content of the plasma. Increased glycogen or increased glucagon/ insulin ratio (caused by a decrease in insulin) stimulates the degradation of FFA via β- oxidation. As are results of these increased ketone bodies leading to ketoacidosis and related complication of diabetes (Kasper DL *et al*., 2005).

2. Hyperglycemic hyperosmolar state (HHS):

This state is in elderly individuals with type 2 DM, with a week history of polyuria, weight loss and diminished oral intake that leads to mental confusion, lethargy or coma. The physical examination reflects profound dehydration and hyperosmolality along with hypotension, tachycardia and altered mental status. This may also lead to serious illness such as myocardial infarction or stroke (Kasper DL *et al*., 2005).

2.5.2. Chronic Complications include:

1. Heart diseases:

Heart diseases occur in diabetics and it is one of the major causes of mortality among them. The co-existence of hypertension and diabetes contributes to the development of heart diseases.

2. Eye diseases (Ophthalmologic complications):

Diabetes mellitus is the leading cause of blindness between the ages of 20 and 74 in the United States. The data from U.S. study has revealed that 10% of new blindness at all ages and 20% of new blindness between the ages 45 and 74 are because of diabetic retinopathy. These incidences may be even higher in India.

Diabetic retinopathy is classified into non-proliferative and proliferative types. The primary effect of diabetes on the retina for the development of diabetic retinopathy appears to be on its capillaries. The exact mechanisms leading to damage are still largely unknown. But there may be alterations in retinal blood flow and breakdown in the blood – retinal barrier

resulting in abnormal leakage from retinal blood vessels appear to be the major cause of events (Ismail GM., 2014).

3. Kidney diseases (Diabetic Nephropathy):

Diabetic nephropathy is also one of major complication associated with diabetes. During this condition, number of functional abnormalities is present in the kidney in early diabetic. At this stage there will be 20 – 30% increase in the glomerular filtration rate without a proportional rise in renal plasma flow. In long term diabetics the most striking alteration are found in the glomeruli and the blood vessels (Allah RSA *et al*., 2007).

4. Diseases of the nervous system (Diabetic Neuropathy):

It is also one of the chronic complications associated with diabetes. Till the middle of the 19^{th} century, diabetes was believed to be a disorder of the central nervous systems. Involvement of the peripheral nervous system by diabetics is referred to as diabetic neuropathy and the metabolic aspects of diabetes remains unknown (Jennifer AT., 2008).

In addition to these are a wide variety of other disorders associated with diabetes. However at the present time, a direct metabolic connection is not known in almost all of these disorders.

2.6. Insulin:

2.6.1. Chemistry of Insulin:

Insulin is solely synthesised in beta cells of islets of langerhans situated in pancreas with 200 units or 8 mg in adult human and adult rat with 10 micro grams of insulin. It is a polypeptide consisting of two chains, A and B, linked by two interchain disulfide bridges that connect A7 cysteine to B7 cysteine and A20 to B19. A third intrachain disulfide bridge connects residues 6 cysteine and 11 cysteine of the A chain. The location of these three disulfide bridges is invariant, and the A and B chains have 21 and 30 amino acids,

respectively, in most species/ the covalent structure of human insulin (molecular mass 5.734 KDa) and a comparison of the amino acid substitutions found in a variety of species. Substitutions occur at many positions within either chain without affecting bioactivity and are particularly common at positions 8, 9 and 10 of the A chain. Thus, this region is not crucial for bioactivity (Murray k *et al*., 2000).

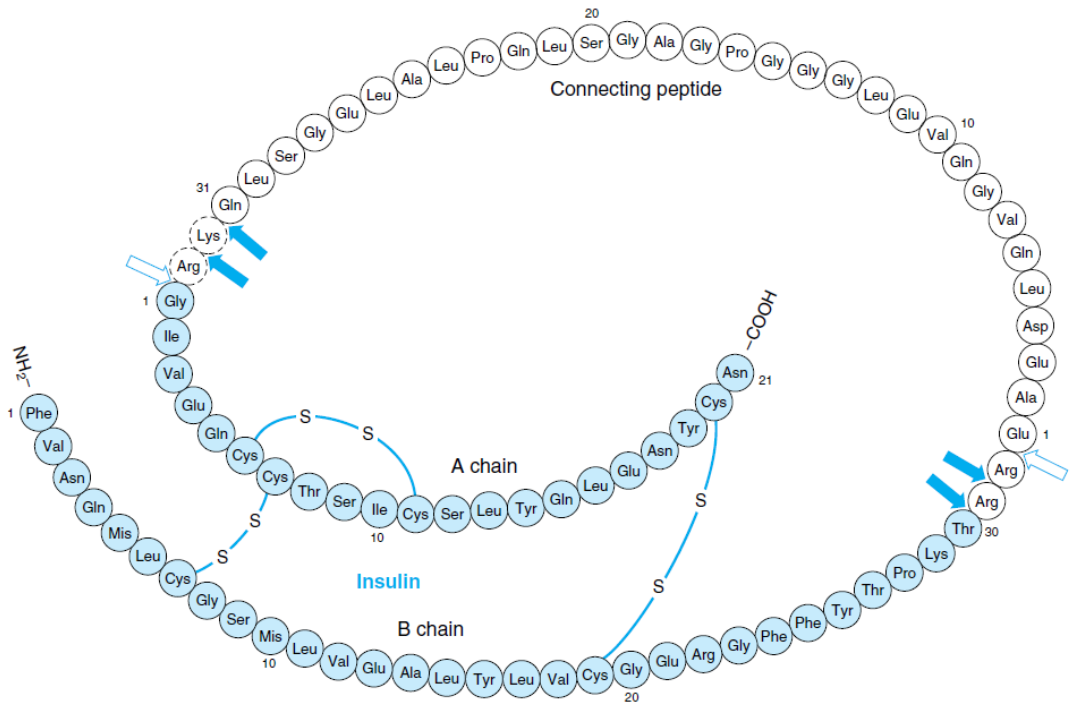


Figure – 1 Structure of human proinsulin.

Insulin and C – peptide molecules are connected at two sites by dipeptide links. An initial cleavage by a trypsin – like enzyme (open arrows) followed by several cleavages by a carboxypeptidase – like enzyme (solid arrows) results in the production of the heterodimeric (AB) insulin molecule (light blue) and the C – peptide.

Several positions and regions are highly conserved, including

1. The positions of the three disulfide bonds.
2. The hydrophobic residues in the carboxyl terminal region of the B chain, and
3. The amino and carboxyl terminal regions of the A chain.

Insulin is synthesised as a preprohormone of molecular weight ~15,000 which is later converted to proinsulin of molecular weight ~9000. The conversion of proinsulin involves cleavage of peptide bonds at specific sites that result in the formation of equimolar amounts of insulin and C – peptide occurs in the immature clathrin coated secretory granules (Murray k *et al*., 2000).

2.6.2. Regulation of Insulin secretion:

The human pancreas secretes 40-50 units of insulin daily, which represents 15-20% of the human stored insulin in the gland. In the fasting state, insulin concentration is 5-10 U/ml maintained by secretion of about 0.25-1.5 Units of insulin per hour into the portal vein. Insulin secretion is an energy requiring process that involves the microtubule – microfilament system in the β cells of the islets. The main character of beta cell is to function as 'fuel sensor' capable of adapting the rate of insulin secretion to the variations in plasma glucose levels and other energetic substrates (amino acids, ketone bodies, fatty acids). The threshold concentration for secretion is the fasting plasma glucose levels (80-100 mg/dl), and the maximal response is obtained at glucose levels between 300-500 mg/dl. It is accepted that an increase of the ATP/ADP ratio results in the inhibition of ATP – sensitive K^+ efflux channels. This causes depolarization of the β-cells and activation of voltage sensitive Ca^{2+} channels. The Ca^{2+} influx results in insulin secretion (Murray k *et al*.., 2000).

2.6.3. Metabolism of Insulin:

Insulin circulates in blood as free monomeric hormone. Insulin half life is about 5-6 min in humans. Pro-insulin half life is 17 min, C-peptide half life is 30 min, major organs involved in insulin metabolism are liver, kidneys and muscle (Pedro I *et al*., 2008). About 50% of the insulin that reaches the liver via portal vein never reaches the general circulation.

Proteolytic degradation of insulin in the liver occurs both at the cell surface and after receptor mediated internalization. Insulin with insulin receptors are internalized called endosomes the site of initiation of degradation. Some insulin is also delivered to lysosomes or lysosome related vesicle near the golgi for degradation. Mainly two enzyme systems are responsible for the metabolism of insulin. The first is Insulinase an endopeptidase, which acts at several sites preferentially $A_{13} - A_{14}$ and $B_9 - B_{10}$. Insulinase is inhibited by sulphydryl inhibitors and chelators such as EDTA and phenantrolene. A second insulin degrading enzyme is hepatic glutathione – insulin transhydrogenase (Arun V *et al*., 2014). This enzyme reduces the disulfide bonds and the individual A and B chains are rapidly degraded. In addition to insulin degradation, the signal created by insulin at the cellular level is reversed by phosphotyrosine phosphatase.

2.6.4. Insulin Receptor:

Insulin action begins after the binding of the hormones to a specific glycoprotein receptor on target cells. The insulin receptor is a heterodimer consisting of two subunits α and β in the configuration of α_2 β_2 linked by disulfide bonds. Both subunits are glycosylated.

The α subunit (135 KDa) is entirely extracellular, binds insulin via cysteine rich domain. The β subunit (95 KDa) is a transmembrane protein and a cytosolic domain. The insulin binding domain of the mature receptor is primarily in the α subunit. Proteolysis of β subunit does not influence insulin to α subunit appreciably (Murray k *et al*., 2000).

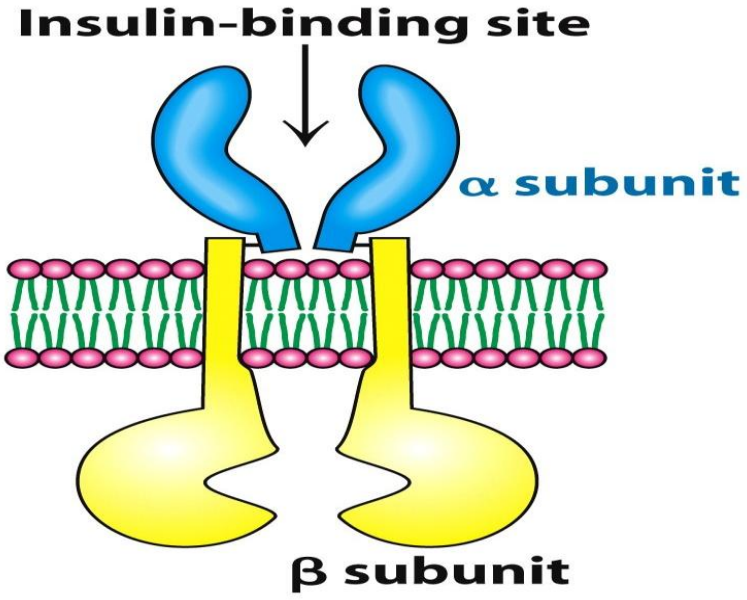


Figure – 2 Structure of insulin receptor

The α subunit contains 26 cysteine residues between residues 155 and 312. This cysteine rich region may be involved in forming disulphide bonds with the β subunit as well as with another α subunit. This region may also be important in insulin binding. The insulin receptor is constantly synthesized and degraded and its half-life is 7-12 hours. The precursor of the human insulin receptor has 1382 amino acids with a molecular weight 1,90,000. And the gene for insulin receptor is located on chromosome 19 (Murray k *et al*., 2000).

2.6.5. Mechanism of action of Insulin:

The first event that takes after the binding of insulin to its receptor is auto-phosphorylation of tyrosine residue of the β subunit. Insulin is known to share its receptor with other growth hormone receptors by the intrinsic tyrosine kinase activity (Antti V *et al*., 1999).

Insulin is known to regulate enzymes controlling intermediary metabolism within minutes of its binding to the receptor. Insulin is capable of altering the concentration of some critical proteins and thereby exerting profound effects on various cellular processes.

It affects mRNA translation and in general protein synthesis. First it affects the rate of protein synthesis in selected tissues like liver, adipose tissue, skeletal and cardiac muscle at the level of mRNA translation. Secondly, it has positive and negative effects on the expression of specific genes.

Table – 2 Insulin regulated enzymes/proteins (O'brien RM *et al*., 1991)

Enzymes/Protein	Effect	Enzymes/Protein	Effect
Intracellular Enzymes		**Intracellular Enzymes**	
Pyruvate Kinase	↑	ATP Citrate Lyase	↑
Serine Dehydratase	↓	Ornithine decarboxylase	↑
Fatty Acid Synthase	↑	Aspartate aminotransferase	↓
Glutamine Synthase	↑/↓	Fructose 1.6 bisphosphatase	↓
Try amino transferase	↑/↓	Carbamoyl phosphate synthetase	↓
Protein disulphide isomers	↓	Glucose 6 phoshate Dehydrogenase	↑
PEP Carboxykinase	↓	Glyceraldehyde 3 P Dehydrogenase	↑
Glycerol 3 Phosphodehydrogenase	↑	Aldolase B	↑
PFK/Fructose 2,6 bisphosphatase	↑	Malic Enzyme	↑
Integral Membrane Proteins		**Secreted Proteins/Hormones**	
Insulin Receptor	↑/↓	Prolactin	↑

Growth Hormone Receptor	↑	Glucagon	↓
GLUT 2	↑	Growth Hormone	↓
GLUT 4	↑	Apolipoprotein B	↓
		Lipoprotein Lipase	↑
		α – Amylase	↑

↑ Stimulatory ↓ Inhibitory

2.6.6. Physiological action of Insulin:

Insulin is the primary regulator of the blood glucose level. It also plays an important role in regulating fat and protein metabolism. Its effects on muscle and liver tissues primarily concerned with the regulation of glucose metabolism.

2.6.6.1. Effects of insulin on carbohydrate metabolism:

Insulin secreted by the β-cells of islets of langerhans of pancreas directly into the hepatic portal blood. In response of hypoglycaemia, hyperinsulinemia and in certain circumstances to increased catecholamines, plasma insulin levels fall. Basal amount of insulin present after an overnight fast has an inhibitory effect on glucose production. As the insulin concentration doubles it inhibits the glucose production by 80% in the liver. Insulin action on muscle and adipose tissue is slow. Insulin modifies glucose uptake by the α-cell and thus can decrease glucagon secretion. Basal amount of insulin exerts a restraining effect on gluconeogenesis. Insulin decreases the free fatty acid uptake by the liver, which increases the oxidation rate of glucose within the liver. Basal amount of insulin inhibits glycogenolysis by about 60%. Though insulin is very important regulator of glucose production it acts in concert with glucagon to control the blood glucose level. Glucagon does the action quickly by stimulating hepatic glycogenolysis and gluconeogenesis. Insulin increases glucose

utilisation by transport across cell membrane and converting the glucose to glycogen and also oxidation (Sylvain JLM *et al*., 2010).

2.6.6.2. Effects of insulin on fat metabolism:

Insulin plays a central role in the regulation of adipose tissue metabolism and in the storage, mobilization of adipose tissue triacylglycerols. The integrated physiologic effects of insulin on lipid metabolism are summarized as follows:

1. Inhibition of free fatty acid mobilization from adipose tissue.

2. Suppression of adipose tissue lipolysis.

3. Stimulation of intra adipocyte free fatty acid re-esterification (Meena A *et al*., 2010).

4. Inhibition of plasma free fatty acid uptake and oxidation and shunts fatty acid to triacylglycerols (McGarry JD *et al*., 1980).

5. Suppression of circulating ketone body concentration.

6. Reduction in supply of free fatty acid substrate in the liver for ketogenesis.

7. Inhibition of intrahepatic ketogenesis.

8. Acceleration of peripheral ketone body clearance and catabolism.

9. Activation of lipoprotein lipase.

10. Increased clearance of triacylglycerols rich lipoprotein by peripheral tissues.

11. Stimulation of lipogenesis.

2.6.6.3. Effects of insulin on protein metabolism:

Insulin has an anabolic effect on protein metabolism, it stimulates protein synthesis and retard protein degradation. Hence insulin decreases amino acid concentration in plasma. Insulin decreases proteolysis of skeletal and cardiac muscle (Pidaran M *et al*., 2007).

Basal insulin level in muscle decreases free cathepsin-D activity hence stops the degradation of polyribosomes. A decrease in insulin level in muscle enhances the muscle ribosomal activity and protein breakdown (Kettelhut IC *et al*., 1988). During prolonged insulin deficiency in IDDM, amino acid oxidation increases. Insulin decreases transaminase activity and therefore limits the conversion of leucine to its alpha ketoacid in skeletal muscle. But in adipose tissue it promotes leucine oxidation (Hutson SM *et al*., 1980).

2.6.7. Insulin resistance:

The down regulation of insulin receptor is one of the common causes of insulin resistance. This is a normal metabolic event associated with diabetes mellitus. Pathological desensitization of peripheral target tissues to the action of insulin is the major manifestation of NIDDM. Insulin resistance in most NIDDM patients is due to defects that lie distal to insulin binding in the insulin action pathways (Flier JS., 1983). Although the exact mechanism is not known, a variety of post-receptor binding abnormalities have been identified including impaired tyrosine kinase function, reduced activity of the glucose transport system and diminished enzyme activities involved in intracellular glucose metabolism (Marshall S *et al*., 1991). There appears to be a regulatory triangle between dietary intake of metabolic substrate, the release of insulin from the endocrine pancreas and regulation of insulin sensitivity and responsiveness. Specifically, circulating levels of glucose and amino acids would modulate cellular metabolism through two independent but integrated control systems. The first system is the classical one in which glucose and amino acids act as potent secretagogues for the release of insulin. The second control system would act in tandem with the first by enabling insulin target tissues to continuously monitor circulating levels of glucose and amino acids. However, despite vigorous research into abnormalities in elements of the signalling pathway in the insulin resistance in obesity and NIDDM, the

specific intrinsic defect remains un-clear, for example, the mechanism causing insulin resistance in skeletal muscle are not the result of a simple defect of insulin action.

2.7. Glucose transporters:

Glucose in diet is transferred from the lumen of the small intestine, both the dietary and synthesised glucose to be transported to the target cells, this occurs with the help of transport proteins called glucose transporters (GLUT). The mechanism of uptake of glucose has been shown in the 1930's, but the details about this were given in 1980's.

Glucose transport was categorized into three –

1. The Na^+ dependent glucose co-transporters (SGLT).

2. The facilitative Na^+ independent sugar transporters (GLUT family).

3. Hormone sensitive transporters.

Particularly GLUT has implications for the controlling the delivery of glucose to mammalian cells. Different GLUTs have different affinity which is as follows –

The Glucose Transporter

Table - 3 Glucose transporters

Isoform	Class	Main tissue location	Transport
GLUT 1	I	Erythrocyte, brain, ubiquitous	Glucose
GLUT 2	I	Liver, pancreas, kidney, intestine	Glucose (low affinity), fructose
GLUT 3	I	Brain	Glucose (high affinity).
GLUT 4	I	Heart, muscle, white adipose tissue, brown adipose tissue, brain.	Glucose (high affinity).
GLUT 5	II	Intestine, testes, kidney	Fructose, glucose (very low

			affinity)
GLUT 6	III	Brain, spleen, leukocytes	Glucose
GLUT 7	II	n.d	n.d
GLUT 8	III	Testes, brain and other tissues	Glucose
GLUT 9	II	Liver, kidney	n.d
GLUT 10	III	Liver, pancreas	Glucose
GLUT 11	II	Heart, muscle	Glucose (low affinity)
GLUT 12	III	Heart, prostate, muscle, small intestine, white adipose tissue	n.d
HMIT	III	Brain	H^+ -myo-inositol
SGLT 1		Small intestine	Glucose
SGLT 2		Kidney	Glucose

GLUT – Glucose transporters, HMIT – H^+ Coupled myo-inositol transporter, SGLT – sodium glucose transporter, n.d – not determined.

2.7.1. The SGLT transport glucose (and galactose) via secondary active transport mechanism provided by Na^+-K^+ ATPase pump against a concentration gradient. In small intestine and the proximal tubules, glucose is transported across the luminal cells by SGLT. SGLT 1 is limited to certain tissues such as apical membrane of small intestine absorptive cells and renal proximal tubules.

A second type called SGLT 2, is of low affinity and is expressed on the apical membrane of renal convoluted proximal tubules.

2.7.2. Facilitative glucose transporters (GLUT): Utilize the diffusion gradient of glucose across plasma membranes and have different substrate specificities, kinetic properties and

tissue expression. In facilitative glucose transporter there are thirteen members named GLUT 1 – 12 and HMIT (H^+ Coupled myo-inositol transporter).

The facilitative sugar transporters have twelve membranes spanning regions with intracellular located amino and carboxyl-termini. These consist of conserved glycine and tryptophan residues which are essential for general facilitative transporter function. Based on dendrogram there are 3 subclasses (I-III) in GLUT family.

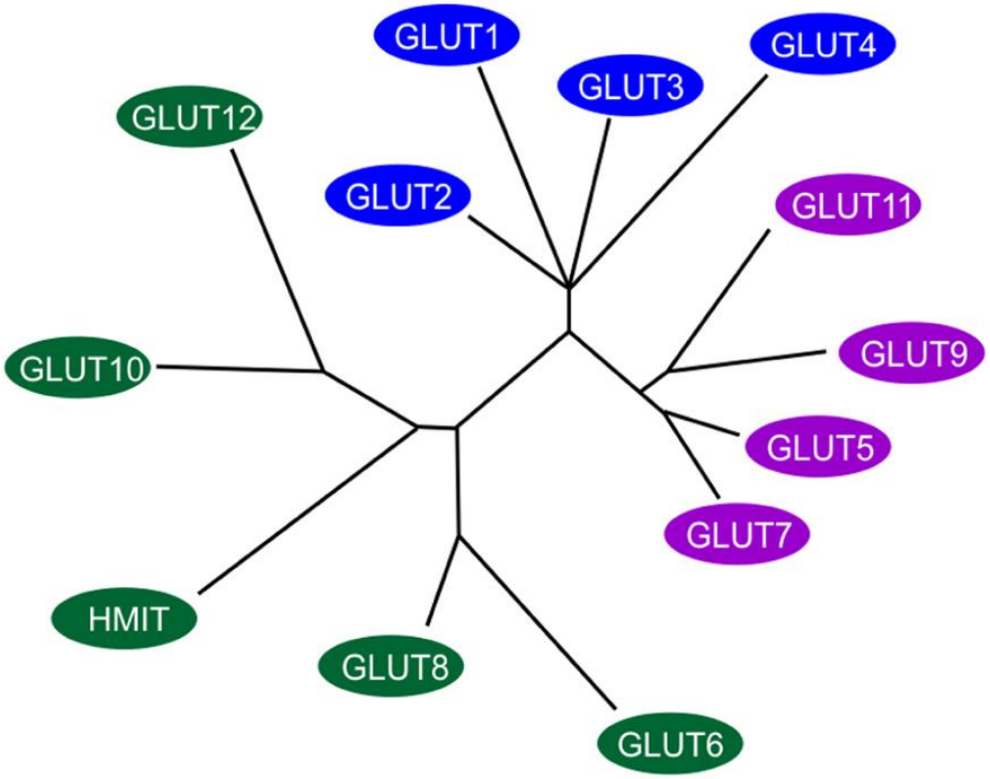


Figure – 3 Dendrogram of the glucose transporter (GLUT) family. The three classes of GLUT proteins are colour – blue, class I: red, class II: green, class III and HMIT, H^+ - coupled myo-inositol transporter.

2.7.2.1. Class I facilitative transporters:

This class contain GLUT 1-4, and these have been comprehensively characterized in terms of structure, function and tissue distribution.

GLUT – 1: The red cell transporter:

GLUT – 1 is expressed particularly in the brain (including blood – brain barrier) and erythrocytes. Moderate levels of expression observed in adipose tissue, muscle and the liver.

The band 4.5 glycoprotein on SDS-PAGE of erythrocyte membrane was identified as a glucose transporter (Baly DL *et al*., 1988). A similar glucose transporter from the rat brain expression library was found to have 98% homology with the red cell transporter in its amino acid sequence (Birnbaum MJ *et al*., 1986).

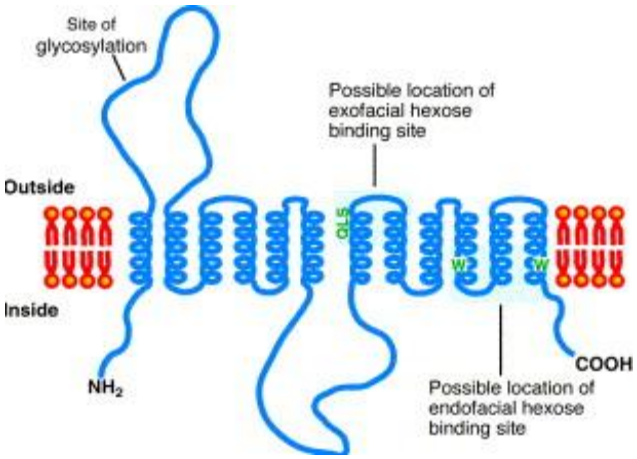


Figure – 4 Structure of GLUT – 1

It has 12 helical membrane spanning domains, with amino and carboxy termini on the cytosolic side of the membrane, a highly charged cytoplasmic domain consisting of 65 hydrophilic amino acids between the helices M6 and M7, and a 33 amino acid long extracellular domain between M1 and M2, with one asparagine linked oligosaccharide on Asn 45 (Meuckler M *et al*., 1985).

GLUT – 2: The liver glucose transporter:

GLUT – 2 is expressed primarily in pancreatic β-cells, the liver and the kidney. In the β-cells, GLUT – 2 is involved in the glucose sensing mechanism. While in the liver it is expressed on the sinusoidal membrane of hepatocytes and it allows for the bidirectional transport of glucose. GLUT – 2 is also found in enterocytes and proximal renal tubules,

involved in glucose and fructose transport across the cells. Is also had 55.5% homology with GLUT – 1.

GLUT – 3: The brain glucose transporter:

It has a high affinity for glucose, it is present where there is demand for glucose as a fuel particularly brain which disposes more than 50% of the glucose after a meal. GLUT – 3 has 64% homology with GLUT - 1 and 52% with GLUT – 2. The membrane topology was found to be similar to that of GLUT – 1. This is principally expressed in brain hence called brain glucose transporter.

GLUT – 4: The insulin responsive glucose transporter:

GLUT – 4 is found in the heart, skeletal muscle, brain and adipose tissue, it is responsible for the postprandial rise in plasma glucose levels. Insulin causes 20-30 fold increase in the rate of glucose transport across the plasma membrane of adipocytes. The effect of insulin on glucose transports are immediate occurring with a half time of 2-3 minutes. Various animals and human models of 'diabesity' exhibit reduced expression levels of GLUT 4 in adipose tissue, but not in muscle. The insulin stimulated glucose transport is due, at least in part to the translocation of an intracellular pool of glucose transporter to the plasma membrane (Suzuki K *et al*., 1980). Detailed studies indicated that the number of GLUT – 1 molecules on the plasma membrane increased approximately 3 fold. The rate of deoxyglucose transport increased 12 – 15 folds indicating that the translocation of GLUT – 1 alone was inadequate to explain the observed increase in the rate of glucose transport (Calderhead DM *et al*., 1988).

GLUT – 4 has 65%, 54% and 58% homology with GLUT – 1, 2 and 3 respectively. GLUT – 4 is stored in the intracellular storage vesicles. GLUT – 4 differs from other glucose

transporter in that about 90% of it is present in intracellular storage vesicles in the absence of insulin or other stimuli.

2.7.2.2. Class II facilitative transporter:

The class II facilitative transporter is headed by the fructose transporter GLUT – 5, GLUT – 7, GLUT – 9 and GLUT – 11.

GLUT – 5: Is expressed predominately in the small intestine, testes and kidney.

GLUT – 7: Least known member of the family, uncharacterized gene, the sites of expression are currently not known.

GLUT – 9: Expressed in the liver and the kidneys.

GLUT – 11: There are two splice variants – long and short forms consisting of 503 and 493 amino acid residues respectively. These two forms are expressed in a tissue specific manner. The short form has low affinity for glucose transport and expressed in heart or skeletal muscle. The long form of GLUT – 11 detected in liver, lung, trachea and brain, was shown to increase fructose transport (Stuart Wood I *et al*., 2003).

2.7.2.3 Class III facilitative transporter:

The class III facilitative transporters comprise five members – GLUT- 6, GLUT – 8, GLUT – 10, GLUT -12 and HMIT. One feature of this class is there is a characteristic glycosylation site on loop 9. The functionally important glycosylation site is found on loop 1 in other two classes. A second feature is the presence of targeting motifs.

GLUT – 6: Is expressed in the spleen, leukocytes and the brain.

GLUT – 8: Is present in the testis, brain, adipose tissue and skeletal muscle.

GLUT – 10: Is reported it's expressed in the insulin – sensitive tissues of skeletal muscle, heart, liver and pancreas.

GLUT – 12: Expressed in heart, small intestine, prostate, skeletal muscle, adipocytes and insulin – sensitive tissues.

HMIT: H^+ - coupled myo-inositol transporter, expressed predominantly in the brain (Stuart Wood I *et al*., 2003).

2.7.3. Glucose transporter and diabetes:

In IDDM, auto immune mediated destruction of beta cells occurs gradually over a variable period of time with an average of about 3 years of duration. However clinical manifestation of the abnormality is generally not observed until about 80% of the beta cells have been destroyed. Immunoglobulin from patients with new onset of IDDM interfered with the high K_m glucose transport of rat islet cells and with glucose stimulated insulin secretion (Bell G., 1991), suggesting an involvement of GLUT – 2 or a factor that influences the function of GLUT – 2.

Unlike the IDDM where auto-immune mechanisms were involved, in NIDDM the beta cell number is not affected (Leighton B *et al*., 1990). However, there appears to be a defect in the high K_m glucose transporter. In the rat model of NIDDM, using glucose intolerant Zucker fatty rats (Clark JB *et al*., 1983), GLUT – 2 was normal in prediabetic Zucker rats, but became virtually undetectable in animals with late severe diabetes (Johnson JH *et al*., 1990). However, even where GLUT – 2 was not completely absent, it was able to severely affect glucose stimulated insulin secretion suggesting the role for another glucose specific point in the glucose response pathway. Glucokinase was found to be the ideal partner for the GLUT – 2 dependent glucose response pathways (Iynedjian PB *et al*., 1989). Insulin independent glucose transport regulates insulin sensitivity. Insulin resistance is dependent on whether glucose is entering through GLUT – 2 or GLUT – 4 (Ebeling-pertti *et al*., 1998).

To generate signals for insulin secretion stimulatory concentrations of D – glucose (>7.5mM) is to be metabolised by aerobic glycolysis (Holz GG *et al*., 1992), where glucose is converted to glucose-6-phosphate, this is referred to as the glucose sensing mechanism.

Although the nature of the signals that mediate insulin release remain controversial, alterations in the cellular phosphate potential, cytosolic redox state, generation of phospholipid metabolites and others can induce insulin secretion. In fact all these may be required for a normal response (Holz GG *et al*., 1992). The latest signal identified was L – Arginine derived nitric oxide (Schmidt HH *et al.,* 1992).

2.8. Lipids:

Lipids, a heterogeneous group of compounds act as energy rich fuels of our diet and a major stored fuel of our body also helps the internal organs of our body by acting as a coating substance around the organs.

The tissue and plasma lipids in humans, generally comprises of triacylglycerols, phospholipids, cholesterol, cholesteryl esters and free fatty acids, the important physiological functions of these lipids are as follows –

- Triacylglycerols are a major energy store of the body.
- Phospholipids form the structural constituent of cell membrane (Subhankar Chowdhury, 2002).
- Cholesterol a structural constituent of cell membranes, precursor of steroid hormones and bile acids.

Fats that are absorbed from the diet (exogenous source) and the lipids synthesized from the liver (endogenous source), adipose tissues for utilization and also storage. These lipids being water insoluble carried in plasma as lipoproteins. Lipoproteins are complexes of macromolecular with hydrophobic lipids like cholesterol esters and triacylglycerols in plasma

with a central core of non-polar lipids – cholesteryl esters and triacylglycerols with a surface layer of polar lipids – phospholipids, apolipoprotein (Apo) and free cholesterol.

Based on density plasma lipoproteins are of four classes, with different compositional and functional properties (Refer table – 4)

1. Chylomicrons – (Lowest in density, largest in size, contain most percent of lipid, less percent of protein) derived from intestinal absorption of triacylglycerols.

2. Very low density lipoproteins (VLDL or pre β – lipoproteins) – derived from the liver for triacylglycerols export.

3. Low density lipoproteins (LDL or β – lipoproteins) – represent the final stage in the VLDL catabolism.

4. High density lipoproteins (HDL or α – lipoproteins) – involved in chylomicron and VLDL metabolism and in transport of cholesterol (Murray k *et al*., 2000).

Table – 4 Composition of lipoproteins

Sl.No	Lipoprotein	Triglyceride	Protein	Phospholipids	Cholesterol and Cholestryl esters
1	Chylomicron	90%	2%	3%	5%
2	VLDL	60%	5%	15%	20%
3	LDL	8%	20%	22%	50%
4	HDL	5%	40%	30%	25%

Apolipoprotein associated with lipoprotein particle have a number of diverse functions

- Serving as structural components of the particles.
- Providing recognition sites for cell surface receptors and
- Serving as activators or coenzymes for enzymes involved in lipoprotein metabolism.

These apolipoproteins are divided on the basis of structure and function into classes A to H, with most classes having sub classes. (refer table – 5) (Murray k *et al*., 2000).

Table – 5 Apolipoprotein of lipoproteins

Apolipoprotein	**Lipoprotein**	**Functions**
Apo A-I	HDL, Chylomicrons	Activates LCAT
Apo A-II	HDL, Chylomicrons	Inhibits LCAT
Apo A-IV	Secreted with chylomicrons but transfers to HDL	Involved in the formation of triacylglycerols rich lipoprotein, synthesized by intestine.
Apo B-100	LDL,VLDL,IDL	VLDL secretion from liver. Ligand for LDL receptor.
Apo B-48	Chylomicrons, Chylomicron ruminants.	Chylomicron secretion from intestine.
Apo C-I	VLDL, HDL, Chylomicrons	Activator of LCAT
Apo C-II	VLDL, HDL, Chylomicrons	Activator of lipoprotein lipase
Apo C-III	VLDL, HDL, Chylomicrons	Inhibits lipoprotein lipase also Apo C – II
Apo D	Sub fraction of HDL	May act as lipid transfer protein
Apo E	VLDL, IDL, HDL, Chylomicron remnants.	Triggers clearance of VLDL and chylomicron remnants.

2.8.1. Lipoprotein metabolism:

Chylomicrons are formed in the enterocytes, and they contain mainly the newly absorbed fatty acids as triacylglycerols with smaller amounts of cholesterol esters. The major protein component is apo B-48, these acquire apolipoproteins C and E from HDL. These particles are transported via the lymph into the blood, where they bind to lipoprotein lipase on the surface of capillary endothelial cells, thus leading to rapid hydrolysis of most of the triacylglycerols. Some phospholipids and the apolipoproteins A and C are transferred to HDL resulting in a residual particle called the chylomicron remnant. These remnants are cleared from the blood to the liver by several mechanisms. Thus, virtually all cholesterol absorbed from the intestine is delivered to the liver. The cholesterol in hepatocytes can enter metabolic pathways leading to formation of bile acids, can be incorporated into nascent lipoproteins or be stored within the cell.

VLDL is mainly formed in hepatocytes, and provides a pathway for the export of excess triacylglycerols from the liver cells. Triacylglycerols can be derived from hepatic de novo production, from plasma free fatty acids taken up by liver or from chylomicron remnants. The VLDL particle consists of a large amount of triacylglycerols and smaller amounts of cholesterol and phospholipids. The major protein component of the nascent VLDL is Apo B-100, and it also contains C and E apolipoproteins. In the blood, the triacylglycerols of VLDL are hydrolyzed in extra hepatic tissues by lipoprotein lipase leading to smaller, remnant particles including particles IDL (intermediate density lipoprotein). The surface components of the remnant particle, including phospholipids, free cholesterol and soluble apolipoproteins, are transported to HDL facilitated by plasma phospholipids transfer proteins (PLTP). VLDL remnants can then interact with LDL Apo B-receptors on hepatocytes via Apo E. The remnant particles, which contain several molecules of Apo E,

bind effectively to the LDL Apo B – receptors on hepatocytes via Apo E. The remnant particles, which contain several molecules of Apo E, bind effectively to the LDL Apo B-receptors and are rapidly taken up from the blood to the hepatocytes for catabolism. Particles with smaller amounts of Apo E remain longer in the blood. These are transformed to IDL and with further processing by hepatic lipase and the loss of the rest of Apo C and E they can form LDL. In most mammals, the majority of VLDL remnants are rapidly taken up by the liver, and a smaller amount is converted via IDL to LDL. In humans a much greater fraction of the remnants, perhaps even 50%, is converted to LDL.

LDL is mainly produced as an end product of the metabolism of VLDL, and it contains predominantly cholesterol esters added in small amounts of triacylglycerols, phospholipids and free cholesterol. LDL cholesterol is the main carrier of cholesterol in blood since LDL cholesterol normally accounts for about two-thirds of plasma total cholesterol. The exclusive apolipoprotein of LDL is Apo B-100, one LDL particle containing one Apo B molecule. LDL can be taken up from the circulation into hepatocytes by LDL Apo B – receptors on hepatocytes or extra hepatic cells. The binding to the receptors is mediated via recognition of Apo B-100. Due to the relatively low affinity of LDL for the hepatic LDL Apo B – receptors, as compared to the respective affinity of VLDL remnants, LDL circulates in the blood for about three days. Therefore, an appreciable fraction of blood LDL is taken up by many extra hepatic tissues via their LDL Apo B-receptors. Thus, LDL is the major particle responsible for transporting cholesterol to peripheral tissues.

Nascent HDL particles are either secreted by the liver or the intestine, or are assembled in the plasma from products of the catabolism of triglyceride rich lipoprotein (TRL). During the lipolysis of TRL in peripheral tissues, their surface components, phospholipids, cholesterol and apolipoproteins, are transferred to HDL. This is facilitated by

PLTP. These (Babu PS *et al*., 1997) components give rise to new HDL, or may be incorporated into pre-existing HDL particles. The major apolipoproteins of HDL are Apo A-I and Apo-II. In addition to being transferred from VLDL and chylomicrons, apolipoproteins may be secreted as free apolipoproteins, which then acquire lipids via an interaction with the cellular ATP binding cassette transporter (ABC). In both the mechanisms, discoidal, prebeta – HDL particles are formed. The plasma cholesterol – esterifying enzyme lecithin:cholesterol acyl-transferase (LCAT) circulates bound to these nascent and discoidal HDLs, and generates cholesterol esters from free cholesterol. These cholesteryl esters form the core of the spherical, now mature HDL particle. HDL cholesteryl esters may be transferred to Apo-B containing lipoproteins by cholesteryl ester transfer protein (CETP) in exchange for triacylglycerols. The triacylglycerols of HDL are hydrolyzed by hepatic lipase. The transfer of triacylglycerols and other surface components form the Apo-B containing lipoproteins and the elevation in the core cholesteryl ester amount due to the function of LCAT both increase the size of the HDL particle. Conversely the transfer of cholesteryl esters out of HDL by CETP and hydrolysis of HDL triacylglycerols and phospholipids by hepatic lipase will reduce the HDL size. Large HDL particles are often called HDL 2 and the smaller HDL particles are called HDL 3.

HDL is an important mediator of the reverse cholesterol transport, in which cholesterol from peripheral tissues is delivered to the liver, pre-beta HDL particles are specially adapted for mediating free cholesterol efflux from peripheral cells. Cholesterol is then esterified, generating larger cholesteryl ester rich-HDL particles. Next, the cholesteryl esters can be removed from the circulation to the liver with Apo-B containing lipoproteins, through selective uptake of special scavenger receptor BI (SR_B1), or as a part of an HDL particle uptake mechanism. The action of the different enzyme affecting and remodelling the

HDL composition contributes to the conversion of the mature HDL back to the pre-beta HDL, which is then capable of re-entering the HDL metabolism circle; thus the removal of cholesterol from the extra hepatic cells and the flow of the cholesterol to the liver are maintained.

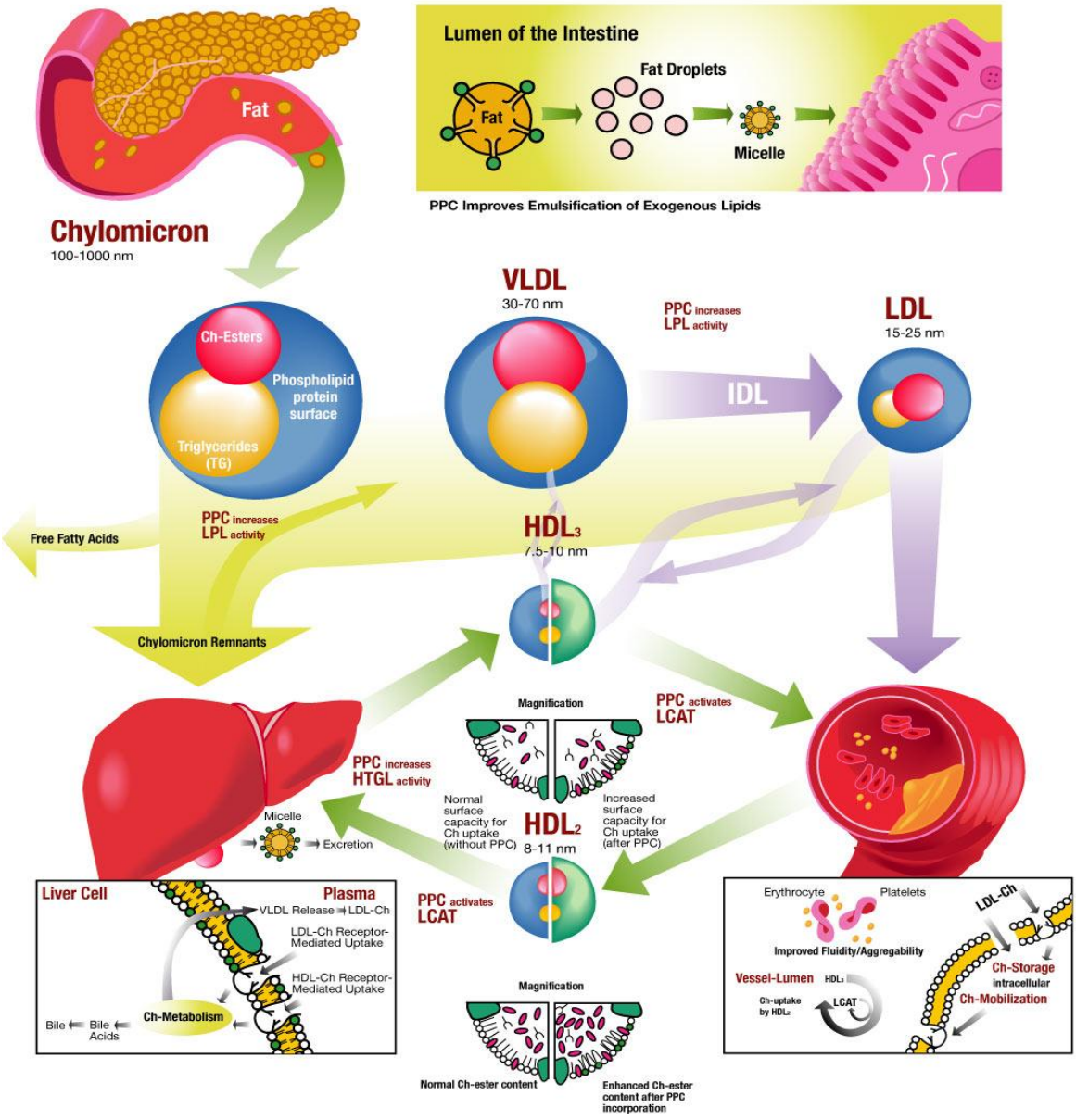


Figure – 5 Lipoprotein metabolism

2.8.2. Lipid disorders in diabetes:

The abnormalities associated with diabetes termed as dyslipoproteinemia or dyslipidemia. Where there may be changes in both the quality and quantity of the lipoproteins and these changes depends on the type of diabetes and the degree of glycemic control. Dyslipidemia is more frequent in type 2 diabetes and it also contributes to the high risk of coronary heart diseases (CHD) (Suzuki K *et al*., 1980).

Quantitative changes: The frequent quantitative change associated with type 2 diabetes is increased triglyceride and low HDL cholesterol concentration. Along with these there may also be elevation in the level of plasma total cholesterol in diabetes. The Multiple Risk Factor Intervention Trial has shown that the incidence of coronary mortality increases with increasing concentrations of plasma cholesterol in both those with or without diabetes.

Qualitative changes: Changes associated in diabetic subjects been found to have greater glycation of LDL particle which are more susceptible to oxidation, thus leads to increased oxidized LDL in diabetic subjects (Subhankar Chowdhury, 2002).

2.8.2.1. DYSLIPIDEMIA AND DIABETES MELLITUS:

Type I or Insulin-Dependent DM (T I DM):

The levels of lipids are generally higher in patients with type I diabetes mellitus with poor control over plasma glucose can be seen, because of the accumulation of chylomicrons and very low density lipoprotein (VLDL) which indicates the influence of elevated blood glucose level resulting in the alteration in the lipid metabolism. Abnormality in the lipoprotein composition can be evidenced by the elevated plasma triglyceride, elevated cholesteryl ester in VLDL was found in diabetic patients. But low density lipoprotein (LDL) may remain normal but the triglyceride levels in LDL or small dense LDL (sd LDL) are known to increase frequently, whereas the high density lipoprotein(HDL) cholesterol or the

ratio between cholesterol and triglyceride in HDL reduces in type I diabetes mellitus. But the compositional changes in lipoprotein can be brought back to normal by the use of hypoglycemic agents (Goldberg JI., 2001).

Type II or Non-Insulin-Dependent DM (T II DM):

The elevations of triacylglycerols in type II DM with suboptimal glucose control are not evident as that in type I DM. The levels of total and LDL-cholesterol in type II DM patients are often increased or subnormal. Elevated levels of small dense LDL have frequently been detected in type II DM patients. A decreased level of HDL-cholesterol is often detected in patients with type II DM. Those are associated with elevated levels of apolipoprotein B (Apo B) and decreased levels of Apo A1. Hypoglycemic therapy alone usually does not normalize the dyslipidemia in type II diabetic patients (Goldberg JI., 2001).

2.8.3. Lipoprotein metabolism in diabetes:

Diabetes mellitus may lead to many complications, one of the significant complication is dyslipidemia – alteration of lipid metabolism in uncontrolled glycemic levels. Dyslipidemia is a major risk factor for the development of macro vascular complications in type II diabetes patients. The features of dyslipidemia are the elevated levels of triacylglycerols, reduced levels of HDL cholesterol and increased levels of LDL-cholesterol (Howard BV., 1987). These altered lipid profile can be seen only primarily with insulin resistance (Krishnaswami V., 2010).

Many factors are involved in the development of dyslipidemia during diabetes including insulin resistance, disturbed fatty acid metabolism and hyperglycemia. The composition and amount of the lipoproteins are altered. Impaired action of insulin in adipocytes known to suppress intracellular hydrolysis of triacylglycerols with the release of free fatty acids into the circulation. This increased flux of free fatty acid into the liver leads to

the synthesis of triacylglycerols and its assembly and secretion of large VLDL, thus leads to hypertriglyceridemia.

There will be increased production of triglyceride rich – VLDL with decreased lipoprotein lipase activity and decreased catabolism of VLDL. There is an increased lipid exchange between triglyceride – rich VLDL and both HDL and LDL, possibly due to increased activity of CETP (cholesterylester transfer protein) and the excess VLDL pool (Ginsberg HN., 1987). This leads to the decrease of HDL cholesterol and the formation of triglyceride – rich HDL and LDL particles. In addition, the catabolism of HDL is increased because of the over activity of hepatic lipase. The finding has lead to the inhibition of CETP may increase the HDL – cholesterol levels (refer figure – 6).

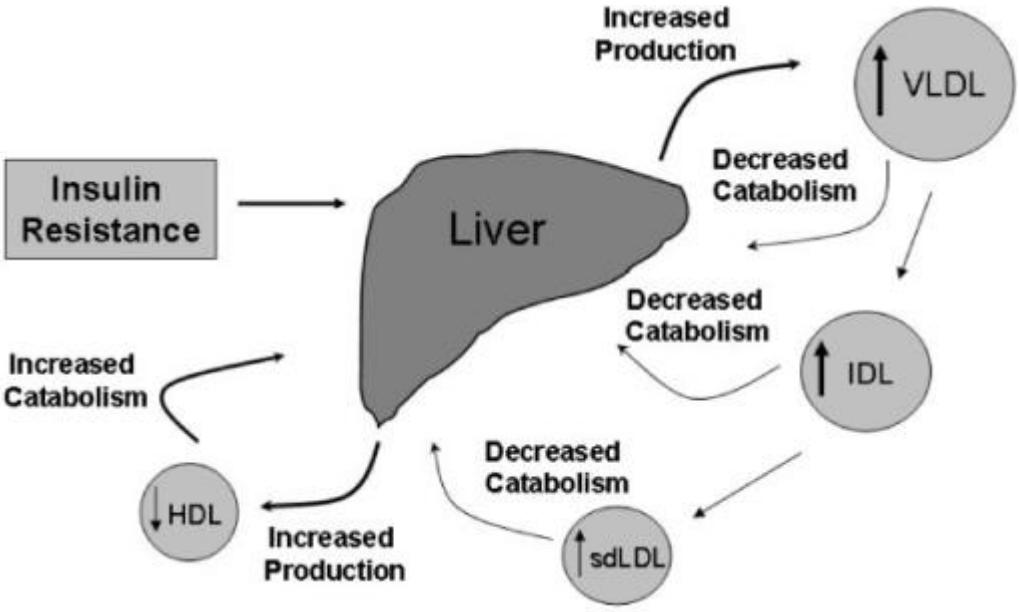


Figure – 6 Atherogenic dyslipidemia and changes in lipoprotein metabolism associated with type II diabetes mellitus (Krishnaswamy V., 2010).

Insulin resistance generally observed in type 2 diabetes mellitus is associated

i) With increased production of very low density lipoprotein (VLDL).

ii) With a reduction in the of intermediate – density lipoprotein (IDL) and small dense low density lipoprotein (sd LDL) catabolism.

iii) With increased production of high density lipoprotein (HDL) outweighed by increased catabolism.

One of the hallmarks of type II diabetes mellitus dyslipidemia is over production of very low density lipoproteins (Goldberg JI., 2001).

Defects in insulin action and hyperglycemia known to lead a change in plasma lipoprotein in diabetes with abnormalities in lipids. In uncontrolled diabetes hypertriglyceridemia and reduced HDL commonly occurs but these can be reversed by insulin therapy. These lipid abnormalities are more common in type 2 diabetes along with low density lipoprotein (LDL) converted to smaller low density lipoprotein.

Lipoprotein particles are also known to be modified by glycosylation in the presence of hyperglycemia (American Diabetes Association, 1993). The clearance of glycated LDL particles is prolonged and is susceptible to oxidation (Subhankar Chowdhury, 2002).

Insulin has a very important role in maintaining production of lipoprotein but the deficiency known to enhance the production of apolipoprotein B (apo B) major component of VLDL and LDL. Lipid known to regulate apo B production, apo B being a protein get degraded from translation but the interaction with lipid prevents. During diabetes lipolysis increases in adipocytes, releases more amount of free fatty acids because of poor insulinization. Thus increases the influx of free fatty acids to the liver a common abnormality associated with insulin resistant diabetes, this way cause an increase in VLDL secretion (Goldberg JI., 2001).

Insulin has a direct effect on production of apo B from liver and other proteins involved in the degradation of circulating lipoproteins and apo B. But the deficiency of

insulin/resistance may lead to increased production of apo B and other proteins such as apo C III – a small apoprotein, may increase VLDL by preventing the actions of LPL and inhibits uptake of lipoprotein via LDL receptor related protein. Hepatic lipase – an enzyme synthesized by hepatocytes hydrolyzes phospholipids and triacylglycerols on HDL and remnant lipoproteins. But the deficiency of insulin known to reduce the activity of this enzyme, thus affects the clearance of remnant lipoproteins.

Lipoprotein lipase (LPL) – major enzyme responsible for conversion of lipoprotein triglyceride into free fatty acids, synthesized by adipocytes, interacts with circulating triglyceride – rich lipoproteins such as VLDL and chylomicrons. But the activity of LPL reduced in diabetes, can be stimulated by insulin therapy (John D *et al*., 1994). But the enzyme hormone sensitive lipase (HSSL) play a role in the release of fatty acids from adipocytes, HSSL is inhibited by insulin.

LDL usually not always increases in diabetes, there is a chance that imbalance may occur, the production of LDL depends on the hydrolysis of the VLDL by LPL. Deficiency of LPL and poor glycemic control may lead to increased LDL that can be further reduced in diabetes after treatment.

Insulin normally inhibits hormone sensitive lipase in adipose tissue, but resistance of insulin is involved in evoking the changes in lipoprotein metabolism. The anti-lipolytic effect of insulin is reduced in adipose tissue leading to increased release of fatty acids. Because of which liver is exposed to a large amount of free fatty acid load, which could induce hepatic insulin resistance (Subhankar Chowdhury, 2002) and free fatty acids acts as substrates for increased production of VLDL. As a matter of fact, abnormal VLDL production and a deranged activity of lipoprotein lipase have been linked to insulin resistance. In addition to

these small dense LDL particles have been shown to be closely related to hypertriglyceridemia in insulin resistance (Carey DG *et al*., 1996).

The combination of hypertriglyceridemia, and increased numbers of LDL particles are associated with increased levels of HDL cholesterol, is now-a-days called hypertriglyceridemic hyperapoB.

Cholesterol which is found in lipoprotein also plays important role, which is substrate for the synthesis of steroid hormones and bile acids. Increased synthesis of cholesterol can be seen in liver during diabetes mellitus, this is mainly because of the increased activity of the lipogenic enzyme HMG CoA reductase, a rate limiting enzyme in cholesterol biosynthesis with the increased availability of NADPH, starting with the acetyl CoA.

There are four important enzymes that play a major role in controlling lipoprotein metabolism – Lipoprotein Lipase (LPL), Hepatic triglyceride lipase (HTGL), Lecithin cholesterol acyltransferase (LCAT) and cholesterylester transfer protein (CETP) defects in any of these mentioned enzymes leads to disturbances in the plasma lipids (Subhankar Chowdhury, 2002).

Lipoprotein abnormalities associated with diabetes can be summarised as below:

- Slow chylomicron clearance from the blood after diet, takes several steps after the entry of chylomicrons from blood stream via thoracic duct, apo C II, activator of LPL is transferred from HDL.
- Deficiency of lipases, triglyceride enriched lipoproteins converted to small denser forms.
- Development of hypertriglyceridemia because of the lack of LPL activity.
- Increased VLDL production.
- Reduced HDL in diabetes occurs because of the defective lipolysis.

- In poorly controlled diabetes, decrease in the LDL receptors can be seen.
- Very poor glycemic control may lead to increased LDL with the lack of LDL receptors for clearance.

2.9. TREATMENT OF DYSLIPIDEMIA:

Dyslipidemia is one of the complications associated with diabetes mellitus with uncontrolled blood glucose level. It is associated with elevated levels of total cholesterol, triacylglycerols, LDL cholesterol and decreased HDL cholesterol. If the lipid levels are not maintained may lead to coronary heart diseases, so it becomes essential to lower the lipid levels during diabetes mellitus. This condition can be treated by lifestyle change, glycemic control and by lipid modifying drugs. In order to achieve decreased levels of lipids some of the drugs have been given are called as lipid modifying drugs.

2.9.1. Lipid modifying drugs:

The major classes of drugs used to modify lipids in diabetic dyslipidemia are as follows

1. Statins (HMG CoA Reductase inhibitors)
2. Fibrates (Fibric acid derivatives)
3. Nicotinic acid
4. Bile acid sequestrants
5. Miscellaneous

Of these the first three are considered the first line therapy against hypercholesterolemia, with fibrates having effective action in lowering the triacylglycerols. Among these the most commonly used groups are the statins and fibrates.

Mechanism of action:

1. Statins – These drugs are structurally similar to hydroxyl methyl glutaryl coenzyme A (HMG CoA), a precursor of cholesterol and also competitive inhibitors of the rate limiting

enzyme in cholesterol biosynthesis, namely HMG CoA reductase and are best administered in the evening. Statins are the most effective drugs against lowering the LDL and triglyceride levels and can be utilized for both primary prevention (prevention in subjects have not yet suffered from the myocardial infarction) and secondary prevention (prevention of progression of CHD in those who have already sustained a myocardial infarction). Thus by the administration of statins coronary artery diseases can be reduced by about 30%.

2. Fibrates – These resemble short chain fatty acids and increase the β- oxidation of fatty acids, with diminished triglyceride synthesis and VLDL secretion from the liver, also increase the LPL activity in muscle and adipose tissue. Fibrates are thus effective in lowering triacylglycerols by 25-60%, with the beneficial increase in HDL cholesterol concentration along with LDL cholesterol lowering action. It also reduces hepatic VLDL production and increase hepatic LDL uptake.

3. Nicotinic acid – Very effective against dyslipidemia, it inhibits hepatic triglyceride production and VLDL secretion. Thus, decreases LDL cholesterol, triglyceride and increases HDL cholesterol to a significant extent.

4. Bile acid sequestrants – Are used as adjuncts to statins to further lower the cholesterol. These sequestrants results in decreased absorption of exogenous cholesterol and increased metabolism of endogenous cholesterol into bile acids. Which leads to increased expression of LDL receptors on liver cells results in increased removal of LDL from the blood, thus a reduced concentration of LDL cholesterol in plasma occurs.

5**. Miscellaneous** –

Orlistat – A pancreatic lipase inhibitor, known to cause significant reduction in total cholesterol, LDL cholesterol, triacylglycerols and apolipoprotein B in obese type II diabetes.

Acipimox – a nicotinic acid congener, causes significant decrease in triacylglycerols, total cholesterol and apolipoprotein B, but does not have an effect in elevating HDL cholesterol.

Fish oils – contain eicosapentaenoic acid and docosahexaenoic acid (n-3 polyunsaturated fatty acids) known to decrease triacylglycerols.

Above stated drugs can individually help in lowering the lipid levels in a dyslipidemic condition. Sometimes there need to treat dyslipidemia with a combination of different drugs depending on the severity of the lipid profile and also to overcome the adverse effects of some drugs alone (Subhankar Chowdhury, 2002.). These are summarised in table – 6.

Table – 6 Lipid modifying drugs

Type	Mechanism	Effect of lipid profile	Dose
HMG CoA reductase inhibitor (statin)	↓ Cholesterol synthesis ↑ LDL receptor	↓ LDL cholesterol 25-40% ↓ Triglyceride 10-30%	Lovastatin 10-80 mg/dl Simvastatin 5-80 mg/dl Provastatin 10-40 mg/dl Atorvastatin 5-80 mg/dl Fluvastatin 10-40 mg/dl
Bile acid sequestrant	↓ Reabsorption of bile acids in intestine → ↑ Synthesis of new bile acids and ↑ LDL receptor	↓ LDL cholesterol 20-30% ↑ HDL cholesterol ↑ Triglyceride	Cholestyramine 8-12 g BD or TD Colestipol 10-15 g BD or TD
Nicotinic acid	↓ Hepatic triglyceride synthesis ↑ ↓ Secretion of apoB 100 containing lipoprotein: ↓ VLDL → LDL conversion	↓ LDL cholesterol 15-25% ↓ VLDL cholesterol 25-35% ↓ Lp (a) 30% ↑ HDL cholesterol 25% ↓ Triglyceride 25-85%	50-100 mg TD; gradually increased to 1- 2.5g TD
Fibrate	↑ Fatty acid oxidation ↓ Hepatic triglyceride	↓ Triglyceride 25-40% ↑ HDL cholesterol	Gemfibrozil 600mg BD Bezafibrate 200mg TD

	synthesis ↑ LPL→ ↑ Triglyceride hydrolysis	↑ or↓ LDL cholesterol	(400 mg OD) Fenofibrate 100mg TD (200 mg OD) Ciprofibrate 100mg OD

2.10. Hyperglycemia Induced Oxidative Stress and Diabetic Complications:

Diabetic complications are not just because of hyperglycemia instead it is the chronic hyperglycemic glucose toxicity mediated through oxidative stress leads to diabetic complications. Hyperglycemia causes various pathological changes in arteries, peripheral nerves and small vessels. Vascular endothelial cells become primary vulnerable targets for hyperglycemic damage as glucose continuously flows through them leading to the production of ROS inside the aortic endothelial cells. The possible biochemical mechanisms to explain diabetes induced organ/tissue damage includes -hyperglycemia induced activation of protein kinase – C (PKC) isoforms, elevated formation of glucose derived advanced glycation end products and increased glucose flux mediated through aldolase reductase pathway (Brownless M., 1995).

The raised reactive oxygen species (ROS) increases the generation of tumour necrotic factor - α (TNF – α) and aggravates oxidative stress. Increased liberation of cytokines like TNF – α and interleukins has been implicated in the pathogenesis of insulin resistance. TNF – α is putative inhibitor of tyrosine phosphorylation of insulin receptor and post receptor signalling intermediates (Hotamisligil GS *et al*., 1994). TNF – α is a pleiotropic cytokine involved in many metabolic responses in both normal and pathophysiological states (Bonet MB *et al*., 1999). It has central role in obesity, modulating energy expenditure, fat deposition and insulin resistance. TNF – α may produce insulin resistance by a decrease in autophosphorylation of insulin receptor tyrosine substrate-1 into an inhibitor of insulin

receptor tyrosine kinase activity, decrease in circulating fatty acids, altering β – cell function and also increase in triacylglycerols and decrease in high density lipoprotein. TNF – α injection to healthy individuals reduces insulin sensitivity by inducing hyperglycemia without lowering plasma insulin levels. Adipocytes exposed to TNF – α become insulin – resistant, since insulin is not able to stimulate hexose transport. This appears to be a consequence of deregulation in expression of GLUT – 4, the insulin stimulable glucose transporter (Tiwari AK *et al*., 2002).

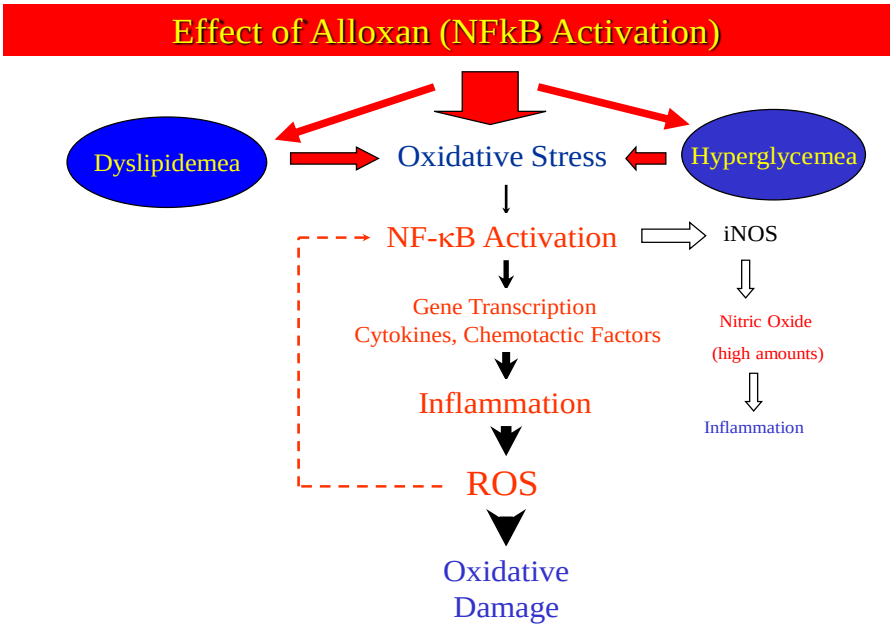


Figure – 7 Alloxan Induced Oxidative Stress

2.11. Medicinal Plants and their use in diabetes mellitus:

Over the centuries human beings are depending on plants for basic needs such as food, clothing and shelter, all obtained and manufactured from plant matrices (leaves, woods, fibers) and storage parts (fruits, tubers). Plants have also been utilized for additional purposes, namely as an arrow and dart poisons for hunting, poisons for murder, hallucinogens used for ritualistic purpose, stimulates for endurance, and hunger suppression, as well as inebriants and medicines. The plant chemicals used for these latter purposes are largely the

secondary metabolites (e.g., carbohydrates, amino acids and lipids) and are not directly involved in the growth, development or reproduction of plants. These secondary metabolites can be classified into several groups according to their chemical classes, such as alkaloids, terepenoids and phenolics. Secondary metabolites can be directly used as drug in their original form, these can also be used as drug precursors, templates for synthetic modification (Ramawat KG *et al*., 2008).

And the compounds derived from plants have been used as drugs, either in the original form or in semi-synthetic form which are mainly the secondary metabolites. These plant derived drugs called as plant extracts or 'phytomedicines' are been employed in the clinical trials for treatment of various diseases (Ramawat KG *et al*., 2008). Utilization of plants for medicinal purposes in India has been documented long back in ancient literature because they are essential for human survival (Manju paghal *et al*., 2010). Now a day's, herbal renaissance is happening all over the globe, because these herbal products symbolize safety in contrast to the synthetics, that are regarded as unsafe to human and environment. Herbs are known for their medicinal, flavouring and aromatic qualities (PP Joy *et al*., 1998). Herbal traditional system of medicine has been practiced in many countries worldwide because of the beneficial effects. A World Health Organization (WHO) study shows that 80% of world population solely relies on medicinal plants for their primary health care needs (Ngugi MP *et al*., 2012).

There are many traditional systems of medicines in the world to treat the diseases, such as Ayurvedic, Siddha, Unani and Chinese traditional systems which are used in many areas of the world. Among these Ayurveda is the most widely practiced of the Indian traditional medicine systems, but there are also other systems such as Siddha and Unani which are also used in the Indian subcontinent (Maury Umashanker *et al*., 2011).

Ayurveda, Siddha, Unani and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda is most developed and widely practiced in India. Ayurveda dating back to 1500-800 BC has been an integral part of Indian culture. The term comes from the Sanskrit root *Au* (life) and *Veda* (knowledge). Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and or reduced toxicity. The small fraction of flowering plant that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants (PP Joy *et al*., 1998).

There are more than 2,50,000 plant species on earth of which more than 80,000 are medicinal. India is one of the world's 12 biodiversity centres with the presence of over 45,000 different plant species. India's diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes (habitats of specific species). Of these, about 15,000-20,000 plants have good medicinal value. However, only 7,000-7,500 species are used for their medicinal values by traditional communities. In India, drugs of herbal origin have been used in traditional systems of medicines such as Unani and Ayurveda since ancient times. The Ayurveda system of medicine uses about 700 species, Unani 700, Siddha 600, Folk 600 and modern medicine around 30 species (PP Joy *et al*., 1998).

Hypoglycemic and hypolipidemic effects of plants:

Many plants and their extracts are used as therapeutic agents. Most commonly these are used extensively as hypoglycemic and hypolipidemic agent to treat diabetes induced hyperglycemia and hyperlipidemia in normal and experimental animals. Some of the commonly studied plants are Allium cepa, Aliu staivum, Momordica charantia etc., Active principles present in these are extracted and employed.

Table – 7 Plants having hypoglycemic and hypolipidemic activity (Kashikar VS *et al*., 2011)

Sl. No	Name of Plant	Part used	Model used	Reported mechanism of action
1	Acacia Arabica(Lam.) Muhl. Ex Willd. Common Name: Indian Gum Arabic tree [Family: Leguminosae]	Seed, Powdered seed (2,3 and 4 mg/kg)	Normal rats, alloxan rats, rabbits	It helps in the release of insulin from β-cells of pancreas
2	Allium cepa L. Common name: Onion [Family: Liliaceae]	Ether soluble fraction of onion (0.25 mg/kg p.o)	STZ rats	Reduces blood glucose levels
3	Allium sativum L. Common name: Garlic [Family: Alliaceae]	Ethanol, petroleum ether and ethyl acetate extract (0.25 mg/kg)	Alloxan rabbits	Acts as antioxidant by reacting with the proteins thiol group which is responsible for hypoglycemic property
4	Aloe vera (L.) Burm.f. Common name: Aloe [Family: Aloaceae]	Leaf pulp extracts	STZ rats	Helps in maintaining homeostasis of glucose by controlling the enzymes of

				carbohydrate metabolism also stimulates the release of insulin from pancreatic β-cells
5	Artemisis pallens wall Common name: Davana [Family: Compositae]	Aerial parts (100 mg/kg orally)	Alloxan rats	Increase the utilization of peripheral glucose
6	Azadirachta indica A. Juss. Common name: Neem [Family: Meliaceae]	Hydro alcoholic plant extract, crude ethanolic extract of the plant	STZ rats, alloxan albino rats	Inhibits epinephrine action on glucose, promotes the peripheral glucose utilization
7	Amaranthus esculents [Family: Amaranthaceae]	Whole plant, oil fraction	STZ rats	Insulin, Glucose
8	Biophytum sensitivum (L.) Common name: Life Plant [Family: Oxalidaceae]	Plant leaf extract	Alloxan rabbits	Helps in the insulin release

9	Caesalpinia bonducella (L.) Roxb. Common name: Chinese Cinnamon	Aqueous and 50% ethanolic seed extracts	STZ rats	Increases the release of insulin from pancreatic cells
10	Coccinia indica wight &Arn. Common name: Ivy gourd [Family: Cucurbitaceae]	Alcoholic leaf extract	Guinea pig, Alloxan dogs	Suppresses insulin, reduces enzymes of gluconeogenesis
11	Caesarea esculent Roxb. Common name: Camilla Fruit [Family; Flacourtiaceae]	Root extracts (300 mg/kg p.o)	STZ rats	Reduces blood glucose, hypoglycemic
12	Camellia sinensis Kuntze Common name: Greentea [Family: Theaceae]	Hot water extract of green tea	STZ rats	Increase insulin activity
13	Eugenia jambolana Lam. Common name: Indian black berry [Family: Myrtaceae]	Pulp extract of the fruits, alcoholic extract (100mg/kg	STZ rats, alloxan rats	Releases insulin, exhibits normoglycemia, peripheral glucose utilization

		p.o)		
14	Egyptian Morus alba [Family: Moraceae]	Alcoholic extract	STZ rats	Lipid peroxidation, insulin, glucose
15	Ficus bengalensis L. Common name: Banyan tree [Family: Moraceae]	Bark extract	STZ rats, alloxan rats	Enhances insulin secretion, inhibits insulinase enzyme
16	Hibiscus rosa sinensis L. Common name: China Rose [Family: Malvaceae]	Ethanol extract of the plant, alcoholic leaf extract	STZ rats,	Insulin secretion stimulation, glucose uptake and utilization
17	Helicteres isora L. Common name: Screw tree [Family: Sterculiaceae]	Ethanolic root extract (300 mg/kg after 9 days of administration)	Mice	Acts through insulin-sensitizing activty
18	Mangifera indica L. Common name: Mango [Family: Anacardiacea]	Aqueous leaf extracts (1 g/kg p.o)	STZ rats	Reduces the reabsorption of glucose from intestine
19	Momordica charnitia [Family: Cucurbitaceae]	Methanolic extract, isolated compounds/	STZ rats, STZ mice,	Glucose

		gourd aqueous extract/ leaves	alloxan rats	
20	Mucuna pruriens (L.) Common name: Velvet bean [Family: Leguminosae]	Powdered seeds (0.5, 1, & 2 g/kg), Plant extract, alcohol(200 mg/kg) extract of the plant (200 mg/kg)	Mice	Increases uptake of glucose
21	Murraya koenigii (L.) Spreng. Common name: Curry leaf tree [Family: Rutaceae]	Leaf powder	Normal rats	Decreases gluconeogenesis and glycogenolysis
22	Psidium guajava Linn. [Family: Myrtaceae]	Aqueous extract/whole plant	STZ rat	Glucose, lipids, insulin
23	Punica granatum L. Common name: pomegranate [Family: Punicaceae]	Ethanolic flower extract, plant extract (200 mg/kg for 30 days)	STZ rat	Anti-hyperglycemic
24	Raphamus stivus	Aqueous	STZ rat	Glucose, lipids,

	(Brassicaceae)	extract/whole plant		insulin
25	Salacia Oblonga Wal. [Family: Celastaceae]	Aqueous methanolic extract, aqueous methanolic extract	Zucker rat (OZR)	Inhibit the enzyme alpha-glucosidase activity
26	Viscum album L. [Family: Llorenthaceae]	Aqueous extract, ethnolic extract/whole plant	STZ rat	Lipid peroxidation, glucose
27	Sisyphus spinachristi [Family: Rhamnaceae]	n-butanol fraction, isolated compounds/ leaves	STZ rat	Insulin, Glucose

The plants belonging to the genus Allium group have been extensively studied for their hypoglycemic and hypolipidemic effect. Studies into the physiological and therapeutic effects of garlic and onion, their products and essential oils have been conducted since the early part of this century and very probably even before that. But these were necessarily limited by the lack of knowledge about the nature and the interrelationship of the chemical components in

the fresh tissue processed product or essential oil. Allium group of plants, specifically Allium sativum (garlic) been used for their hypoglycemic and hypolipidemic actions.

2.11.1. Chemistry of Garlic and its products:

Isolation and identification of active principles of garlic have been the long occupied interests of chemists and pharmacists. Subjecting the crushed cloves of garlic to steam distillation, Wertheim and Semmler obtained strong smelling oil with 0.1% and 0.2% yield which principally consisted of diallyl disulphide (DADS) together with smaller quantities of diallyl trisulphides and diallyl polysulphides and little of diethyl disulphide.

Cavallito *et al*., (1944) reported the occurrence of the active principle of garlic as Allicin. It was Stoll and Seebeck who reported for the first time the formation of Allicin by the degeneration of a precursor substance called Allin by an enzyme called Allinase.

Freshly peeled garlic has the following composition:

Table – 7a Chemical Contents of Garlic

Chemical Contents	**(g/100g wet weight)**
Moisture (%)	61.3 – 86.3
Proteins	2.2 – 6.20
Fat	0.2 – 0.3
Ash	0.6 – 1.5
Energy (cal)	30 – 140

Table – 7b Bulk elements of garlic

Bulk element	**(mg/100g wet weight)**
Calcium	50 – 90
Phosphorus	390 – 460
Potassium	100 – 120
Sodium	10 – 22
Magnesium	43 – 77
Aluminium	0.5 – 1
Barium	0.2 – 1
Iron	2.8 – 3.9

Table – 7c Trace elements of garlic

Trace element	**(mg/100g wet weight)**
Strontium	0.1 – 0.7
Barium	0.3 – 0.6
Copper	0.02 – 0.03
Zinc	1.8 – 3.1
Manganese	0.2 – 0.6
Chromium	0.3 – 0.5
Sulphur	65
Chloride	43

Table – 7d Vitamins of garlic

Vitamins	**(mg/100g wet weight)**
Thiamine	0.25
Riboflavin	0.08
Nicotinic acid	0.5
Ascorbic acid	5
Retinol	5 µg
Other B complex	Traces

Table – 7e In volatile Sulphur compounds of garlic

S – methyl cysteine sulfoxide	+
S – propyl cysteine sulfoxide	+
S – allyl cysteine sulfoxide	+++

Table – 7f Volatiles sulphur compounds of Garlic

Allyl propyl disulphide	6%
Diallyl disulphide	60%
Diallyl trisulphide	29%
Diallyl tetrasulphide	10.5%

Allicin:

Alliin (S – cysteine sulphoxide) constitute about 0.4% of the raw garlic. Allicinase – an enzyme involved in the conversion of alliin, which gives characteristic odour to garlic only after crushing. It can also be called as ‘Alliin lyase’ or Alliin alkyl sulphonate lyase’

(E.C: 4.4.4.4). This enzyme catalyse the conversion of allyl cysteine sulphoxide (alliin) to allyl sulphinic acid later on spontaneously changes to give diallyl thiosulphinate (allicin) finally get converted to diallyl disulphide (DADS) on warming/heating.

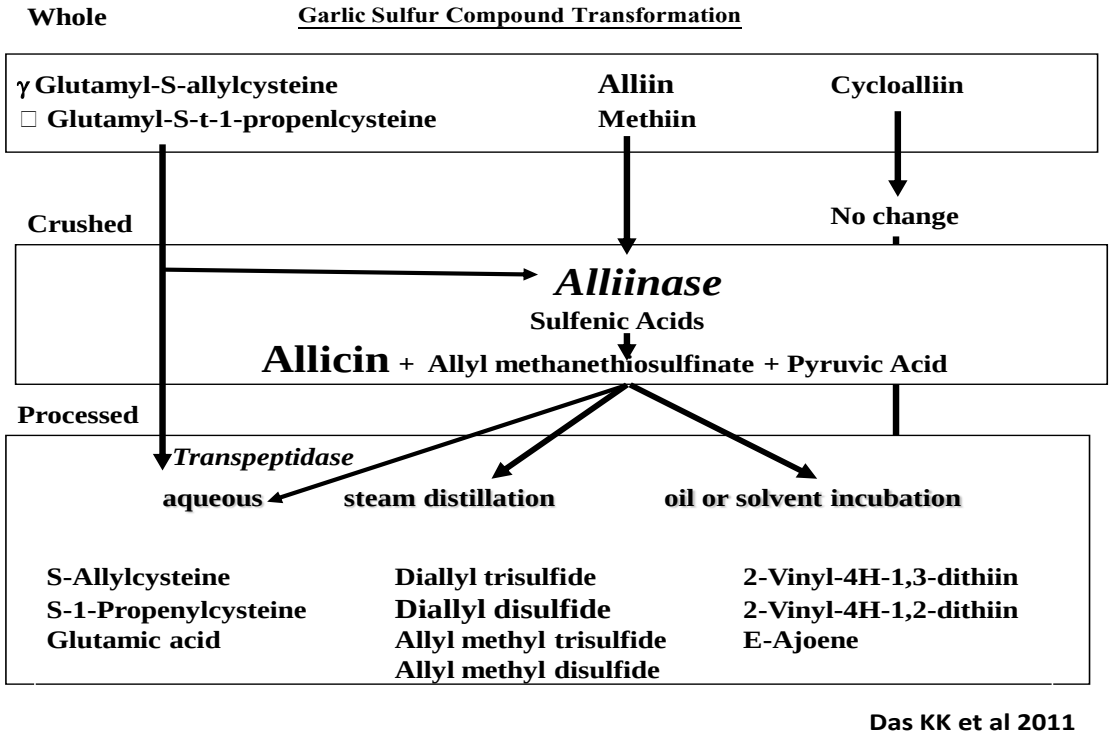


Figure – 8 Garlic Sulphur compound transformation

Later Mazelis and Crews (1968) showed purification of garlic allinase from that of the homogenate. Allicin on heating or upon steam get converted to disulphide i.e., Diallyl disulphide by losing oxygen. It is involved in inhibiting the sulphydryl enzymes and also non sulphydryl enzymes, which are associated. Some of the enzymes inhibited by garlic and are listed in table-8.

Table – 8 Enzymes inhibiting activity of garlic and its fractions:

Sl.No	Enzymes inhibited	Garlic fractions
1	Alcohol dehydrogenase	Allicin
2	Alkaline phosphatise	Allicin

3	Choline esterase	Allicin
4	Fatty acid oxygenase	Garlic
5	Glyoxalase	Allicin
6	Hexokinase	Allicin
7	LDH	Allicin
8	Protease	Garlic extract
9	SDH	Allicin
10	Triose phosphate isomerase	Allicin
11	Tyrosinase	Allicin
12	Urease	Allicin
13	Xanthine oxidase	Allicin

2.11.2. Biochemical functions of garlic and its extracts:

The natural disulphides and sulphoxides of garlic are known to possess hypoglycemic, hypolipidemic and fibrinolytic properties (Brahmachari HD *et al*., 1962).

The active principle of garlic oil is Diallyl disulphide (DADS)

$$CH_2 = CH - CH_2 - S - S - CH_2 - CH = CH_2$$

Seigers and Pentz (1990) showed that after an oral administration of alliin (60mg/kg body weight), only little unchanged alliin could be detected in the plasma and the bio-availability amounted to 16.5% within four hours. It has been concluded by the same authors that the removal or excretion of alliin as measured by the removal of thiol compounds is 15% in bile juice and about 50% in the urine in five hours and twenty four hours respectively.

The known nutritional and pharmacological properties of garlic can be attributed to their allyl compounds and to their broad variety of derivatives formed when these vegetables

cooked or processed. The biochemical and cellular functions of DADS, a natural component of garlic oil, can be summarized as follows:

1. Garlic products are hypoglycemic and insulinogenic agents and controls the side effects of diabetes like loss of weight, glycosuria, derangements in enzymes and metabolism of lipids and proteins (Augusti KT., 1996).

2. Lowering blood and tissue cholesterol & triacylglycerols levels, through its inhibitory effects on key cholesterol and fatty acids synthesizing enzymes and on the functions of thiol containing coenzymes (Augusti KT., 1977).

3. Reducing the platelets ability to aggregate the tendency of blood to clot and arteries ability to contract (Aptiz-Castro R *et al*., 1986).

4. Modulating the metabolic conversions of arachidonic acid to icosanoids through which garlic may have broad impacts on the immune functions, cell divisions/ multiplication, growth of normal and cancerous tissues, inflammatory responses, neurohormonal functions and on the behaviour of platelets and arteries.

5. To be an anti-cancer agent through its inhibitions of nitrosamine formation and its modulations of the metabolism of polyene carcinogens and perhaps through its effects on glutathione regulating enzymes and the enzymes involved in DNA replication, messenger RNA and protein synthesis.

6. Attenuating the toxic effects and subsequent liver damages caused by non-biological compounds (xenobiotics such as chemical pollutants and synthetic drugs).

7. To be an intestinal worm killer and a germicide/ pesticide when germs are brought into contact with raw garlic, onion or certain garlic allyl compounds (Moore GS *et al*., 1977).

8. One of garlic constituent azoene when administered in combination with conventional anti-HIV drugs may be a promising approach for treatment of AIDS (Aptiz-Castro R *et al*., 1986).

9. Serving as anti-oxidant, garlic thio-allyl compounds or related thiols have a solubility range between water soluble vitamins C and oil soluble vitamin D and have molecular configuration and active sites different from the vitamins. Thus they are complimentary to these two anti-oxidants (Block E *et al*., 1988).

10. Blood pressure reduction is observed (Augusti KT., 1996). This action of garlic is due to prostaglandin like effects.

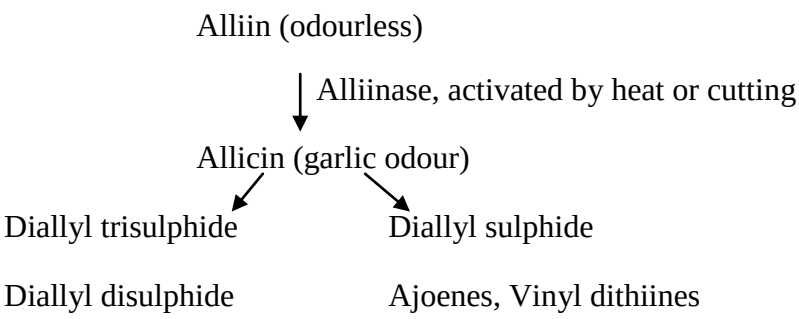
2.11.3. Garlic and its phytosulphur compounds – hypolipidemic effect:

From ancient period many plants and their extracts which are known to have hypoglycemic and hypolipidemic effects have been employed to control diabetes mellitus. There are many herbal products which are proved to have the beneficial effect in significantly lowering the blood glucose and lipid levels in diabetes mellitus. Among those plants garlic is one which is known to have a beneficial action against diabetes.

Garlic a member of the Liliaceae family, is one of the popular herbs used worldwide to treat various diseases. It is known to contain a variety of effective compounds that exhibit anticoagulant, antioxidant, antibiotic, hypercholesterolaemic and hypoglycemic as well as hypotensive activities (Kathi JK., 2000).

It is known to contain many sulphur compounds – at least 33 sulphur compounds, several enzymes, 17 amino acids and minerals such as selenium12. It contains a higher concentration of sulphur compounds than any other *Allium* species. The sulphur compounds are responsible both for garlic's pungent odour and many of its medicinal effects. One of the most biologically active compounds, allicin (diallyl thiosulfinate or diallyl disulfide) does not exist in garlic until it is crushed or cut; injury to the garlic bulb activates the enzyme allinase, which metabolizes alliin to allicin (Kathi JK., 2000).

Alliin metabolism



The observed hypolipidemic actions of garlic disulphide, DADS, is attributed to its

i) NADPH lowering effects (Farva D *et al*., 1989) as well as to its

ii) Sulphydryl exchange reactions that it undergoes with enzymes of lipid metabolism (Gilbert HF *et al*., 1981).

DADS + NADPH ⟶ Allyl thiol + NADP

Enzyme-SH + DADS ⟶ Enzyme-S-S-Enzyme + Allyl thiol

Alteration in carbohydrate metabolism during diabetes is also accompanied by disordered fat and protein metabolism. The characteristic features of diabetic dyslipidemia are a high plasma triacylglycerol concentration, low HDL – cholesterol concentration. Faulty glucose utilization causes hyperglycemia and mobilization of fatty acids from adipose tissue occurs for energy purpose. The lipid changes associated with diabetes are attributed to increased flux of free fatty acids into the liver secondary to insulin deficiency/ resistance. This results in excess fatty acid accumulation in the liver, which is converted to triacylglycerols (Nidhi Sharma *et al*., 2010). It has been shown that garlic can decrease plasma lipids, especially total cholesterol and low density lipoprotein cholesterol in patients with coronary artery disease (Kishu Tripathi, 2009). Yun – Yan Yeh *et al*., (1994) reported that garlic reduces plasma lipids by lowering hepatic cholesterol and triacylglycerols

synthesis by inhibiting the lipogenic enzymes. Hence exhibit hypolipidemic effect by depressing the key enzymes of the cholesterol biosynthesis, HMG CoA reductase and reduce the LDL cholesterol concentration is mainly by active compound of garlic, DADS.

It has been reported by Chun-chen *et al*., (2004) that the water soluble organosulphur compounds present in garlic and other Allium group plants. These are cysteine containing compounds such as N-acetyl cysteine (NAC), S-allyl cysteine, S-ethyl cysteine, S-methyl cysteine and S-propyl cysteine. Several studies have indicated that these above mentioned compounds have the inhibitory action on triacylglycerols and cholesterol biosynthesis along with the decreased activity of lipogenic enzymes in cultured rat hepatocytes. Apart from these some polyherbal ayurvedic formulation do possess antihyperglycemic, antihyperlipidemic and antioxidant effects in diabetic rats (Snehal SP *et al*., 2009).

It has been shown by many workers that feeding these phytochemicals for their hypolipidemic actions could result in certain untoward or harmful effects because of misuse or overuse, along with the beneficial effects. Garlic oil is reported to cause biochemical toxicity like increases in plasma transaminases, increases in urea as well as 100 mg garlic oil fed to a fasted rat could be fatal and the biopsy reports show that it is due to massive pulmonary oedema (Joseph PK *et al*., 1989).

The liver plays a central role in the metabolism of glucose and lipid. The liver tissue is mainly involved in the lipid metabolism through uptake, oxidation and metabolic conversion of free fatty acids, synthesis of cholesterol and phospholipids and secretion of lipoproteins. A profound alteration in the structure and composition of lipid profile occurs during diabetes (Umesh CSY *et al*., 2004). The accumulation of triacylglycerols leads to insulin to a reduction in insulin-mediated metabolic activity. There is a decrease in the lipogenic enzyme activity in liver and overall rates of hepatic lipogenesis. It has been shown

by Umesh CSY *et al*., (2004), sodium – orthovanadate and *Trigonella foenum – graecum* commonly called fenugreek seeds showed decreased activity of the hepatic lipogenic enzymes during diabetes as compared to control.

During diabetes altered lipoprotein metabolism occurs with elevations in plasma and tissue lipids. Along with this some alteration in enzyme activity do occur during diabetes, one such is decreased lipoprotein lipase activity associated with increased accumulation of lipids, especially triacylglycerols (Sarah Varghese *et al*., 1985). It is observed that serpasil (antihypertensive or hypotensive) treatment increased the lipolytic activity of lipase enzyme, also decreased the cholesterol in plasma and tissues of alloxan diabetic rats (Sarah Varghese *et al*., 1985). With all this, the agent also showed the hypocholesterolaemic effect may be due to its inhibitory effect on the enzyme HMG CoA reductase – regulatory enzyme in cholesterol biosynthesis. It also showed beneficial effect in decreasing the plasma and tissue phospholipids.

In diabetic condition altered lipid metabolism can be seen, indicated by the increased mobilization of free fatty acids from fat depots which occurs in the absence of insulin leads to hypercholesterolemia and hypertriglyceridemia. The antihperlipidemic effect of Cassia Auriculata flowers may be due to the down regulation of NADPH, a cofactor in the fat metabolism (Pari L *et al*., 2002).

2.12. Different models to induce experimental diabetes mellitus:

The various animal models for inducing diabetes are:

1) Chemical induced diabetes

a) Streptozotocin

b) Alloxan

c) Dithizone

d) Gold thioglucose

2) Surgical model of diabetes

3) Hormone induced diabetes

4) Genetic model of diabetes

a) Spontaneously developed diabetic rats

b) Genetically engineered diabetic rats

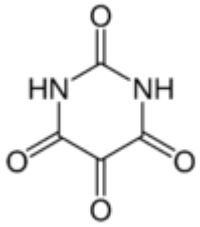
2.12.1. Chemical induced diabetes:

a) Streptozotocin:

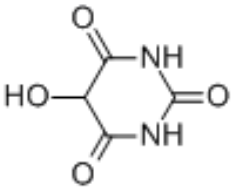
Streptozotocin is a synthetic nitrosoureido glucopyranose derivative isolated from fermentations of streptomyces achromogenes that is classically an anti-tumor, antibiotic and chemically is related to other nitrosureas used in cancer chemotherapy. Streptozotocin sterile powder are provided and prepared as a chemotherapy agent. Diabetes develops gradually and may be assessed after a few days, usually four days for mice and seven days for rats. Usually, a plasma glucose level of about 180-500 mg/dl indicates the induction of diabetes mellitus. Sometimes diabetic animals are maintained on insulin if the experiments are not to commence immediately to prevent the animal deaths (Williamson EM *et al*., 1996). Although streptozotocin is the most commonly used drug for induction of diabetes in rats (Balamurugan AN *et al*., 2003), there are some disadvantages to its use in chronic experiments, especially spontaneous recovery from high blood glucose levels by the development of functioning insulinoma and high incidence of kidney and liver tumours. These problems are due to strongly oncogenic action of streptozotocin (Camila AMO *et al*., 2004). Non insulin dependent diabetes mellitus (NIDDM) was induced by a single intraperitoneal injection of streptozotocin (60 mg/kg) and nicotinamide (120 mg/kg) to rats (Pellegrino *et al*., 1998). Nishiyama Y *et al*., (2013) reported that, injection of a single dose

of streptozotocin (200 mg/kg) induced rapid and permanent hyperglycemia in mice. Female mice are less susceptible to streptozotocin at both doses.

b) Alloxan:



Alloxan



Dialuric acid

Alloxan (2,4,5,6 – tertraoxypyrimidine; 2,4,5,6 – pyrimidinetetrone; 5,6 – dioxyuracil) is an oxygenated pyrimidine derivative. It is present as alloxan hydrate in aqueous solution. The IUPAC name is 1,3 – Diazinane – 2,4,5,6 – tetrone.

It was first originally isolated in 1818 by Brugnatelli and in 1838 by Wholer and Liebig synthesized a pyrimidine derivative, which they later called alloxan.

The compound was discovered by Justus Von Liebig and Friedrich wohler following the discovery of urea in 1928 and is one of the oldest named organic compounds that exist.

The alloxan model of diabetes was first described in rabbits by Dunn, Sheehan and McLetchie in 1943. Since then, alloxan diabetes has been commonly utilized as an animal model of insulin dependent diabetes mellitus (IDDM).

Alloxan is strongly acidic, hydrophilic and unstable substance with half life of 1.5 min at neutral pH and at 37^{o}C (Szkudelski T., 2001). It exerts its diabetogenic action within 24-48 hours of administration by selectively destroying the beta cells of islets of Langerhans in many species of experimental animals, when administered parenterally intravenously, intraperitoneally or subcutaneously. The dose of alloxan required for induction of diabetes varies with species, route of administration and nutritional status. Too small dose is not diabetogenic and too large dose may cause serious kidney damage with uremia. Larger

animals are administered with I.V route. Subcutaneous route is preferred in small animals. Alloxan is effective when given intraperitoneally in rats but the degree of liver damage tends to be higher. So cutaneous or I.V route of administration is preferred. Human islets are considerably more resistant to alloxan than those of the rat and mouse (Eizirik DL., 1994).

Diabetogenic substances are hydrophilic and non-diabetogenic substances are liphophilic (Jorns A *et al*., 1997). Hydrophilicity of alloxan was identified as a factor essential for diabetogenicity. However, selective uptake of alloxan by GLUT – 2 of beta cell is not a prerequisite for the diabetogenicity of alloxan (Ashok DC *et al*., 2007). The diabetes is a result of degeneration and resorption of the beta cells of the pancreatic islets, the alpha cells and acinar tissue being unaffected. Alloxan acts directly, promptly and specifically on the beta cells (Weaver DC *et al*., 1978).

Mechanism of action:

Alloxan acts by binding to insulin receptor causes autophosphorylation of tyrosine residue of the β subunit (Rosen OM., 1987), it has two distinct pathological effects; it selectively inhibits glucose – induced insulin secretion through specific inhibition of glucokinase, the glucose sensor of the β-cell, and it causes a state of insulin-dependent diabetes through its ability to induce ROS formation resulting in the selective necrosis of the beta cells. These two effects can be assigned to the specific chemical properties of alloxan, the common denomination being selective cellular uptake and accumulation of alloxan by the β-cells.

It is a very unstable chemical compound with a molecular shape resembling glucose. Both alloxan and glucose are hydrophilic and do not penetrate the lipid bilayer of the plasma membrane. The alloxan molecule is structurally so similar to glucose (Boquist L *et al*., 1983) that the GLUT-2 glucose transporter in the β-cell plasma membrane accepts this

glucomimetic and transports it into the cytosol. Alloxan does not inhibit the function of the transporter, and can therefore selectively enter beta cells in an unrestricted manner (Hammartrom L *et al*., 1967).

Selective inhibition of glucose-induced insulin secretion is the major pathophysiological effect of the thiol group reactivity of alloxan. Alloxan has a central 5-carbonyl group that reacts with thiol groups. Glucose is the most sensitive thiol enzyme in the beta cell, with a half maximal inhibitory concentration in the 1-10 μmol^{-1} range. Inhibition of glucokinase reduces glucose oxidation and ATP generation, thereby suppressing the ATP signal that triggers secretion. Inhibition of glucokinase is achieved within 1 min of exposure to alloxan (Konrad RJ *et al*., 2002).

The inhibition of glucose-induced insulin secretion is preceded by a very transient (1-2 min) stimulation of insulin secretion immediately after exposure to alloxan. This effect can be explained by an initial reduction of ATP consumption resulting from the blockade of glucose phoshorylation by glucokinase, which produces a transient increase in ATP in the β-cell and triggers a transient release of insulin (Weaver DC *et al*., 1978).

The inhibition of insulin secretion after exposure to alloxan is restricted to that induced by glucose, which induce insulin secretion through interaction with glucokinase. Glucose protects against alloxan-induced inhibition of glucose-induced insulin secretion because its binding to the sugar-binding site of glucokinase prevents the oxidation of the functionally essential thiol groups. The non-metabolisable seven carbon sugar mannoheptulose protects glucokinase through the same mechanis, but this alone is not sufficient to prevent alloxan-induced inhibition of insulin secretion. The glucose analogue 3-O-methylglucose, which is not a substrate of glucokinase, does however prevent inhibition. It

does this through competitive blockade of alloxan uptake into the beta cell via the GLUT-2 glucose transporter.

Alloxan can generate reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid, chemical redox cycling reactions between alloxan and dialuric acid and protective actions of cytoprotective enzymes. In the beta cells the toxic action of alloxan is initiated by free radicals formed in this redox reaction. Autoxidation of dialuric acid generates superoxide radicals and hydrogen peroxide, and in the Fenton reaction (Sakurai K *et al*., 1995), in the presence of a suitable catalyst (typically iron), hydroxyl radicals. The autoxidation of dialuric acid involves the intermediate formation of the alloxan radical (Malaisse WJ 1982; Winterbourne CC *et al*., 1989).

The reduction of alloxan to dialuric acid in the cell requires the presence of a suitable thiol, typically the tripeptide glutathione (GSH) to generate the redox cycling partner, dialuric acid, and oxidised glutathione. The triketone structure of alloxan is vitally important for this two-step reaction with glutathione, which generates the alloxan radical as an intermediate product. Alloxan can also generate ROS by reacting with thiol groups on proteins such as enzymes and albumin. During each typical redox cycle a small amount of 'Compound 305', an alloxan – GSH adducts is formed. The intracellular concentration of compound 305 increases in a time – dependent manner, which gradually decreases the amount of reduced GSH available in the cell for redox cycling. Thus producing a lower pro-oxidative ration between alloxan and GSH, rather than a higher antioxidative ratio (Sakurai K *et al*., 1991; Winterbourne CC *et al*., 1989).

The antioxidative enzyme SOD has a cytoprotective effect against both alloxan and dialuric acid in the presence of GSH by virtue of its ability to scavenge between dialuric acid and alloxan. The resultant suppression of dialuric acid autooxidation prevents the generation

of further ROS, although increasing the concentrations of the toxins can reinstate the toxic effects of both compounds (Munday R., 1988; Winterbourne CC., 1989).

Apparently, the superoxide radical is not the species responsible for the cytotoxicity of alloxan and dialuric acid. Several lines of evidence point to hydroxyl radicals as the principal cause. Hydroxyl radical (Heikkila RE *et al*., 1976) is the ultimate toxic ROS species, and its formation is prevented by the destruction of hydrogen peroxide by catalase. Optimum protection against the cytotoxic action of alloxan and dialuric acid is provided only by a combination of SOD plus catalase, which completely prevents redox cycling between alloxan and dialuric acid, and thus generation of all ROS species in this reaction pathway. Glucose also provides complete protection against all toxic effects of alloxan both in vivo and in vitro. This universal protection is achieved through the prevention of glucokinase inhibition and the preservation of the antioxidative defence mechanisms of the β-cell. The non-metabolisable glucose analogue 3-O-methyglucose also provides protection, but does this exclusively through the prevention of alloxan uptake into the beta cell via the GLUT-2 glucose transporter. Thus, it can be concluded that the pancreatic beta cell toxicity and the resultant diabetogenicity of alloxan are due to redox cycling and the generation of toxic ROS.

Apart from diabetogenic action alloxan is also known to cause a serious disturbance in intracellular calcium homeostasis by which alloxan elevates cytosolic free Ca^{2+} concentration in pancreatic β-cells (Kim HK *et al*., 2006; Park BH *et al*., 1995). And this effect is a result of many events such as – alloxan induced calcium influx from extracellular fluid, exaggerated calcium mobilization from intracellular stores and its limited elimination from the cytoplasm. This calcium influx is the result of the ability of the alloxan to depolarize the pancreatic β-cells (Takasu N *et al*., 1991) and depolarization of the cell membrane opens the voltage dependent calcium channels. Alloxan is also having a stimulatory effect on

mitochondrial Ca^{2+} efflux with inhibitory action on Ca^{2+} uptake by mitochondria (Lenzen S., 1992). Lastly alloxan is also involved in the inhibition of the liver plasma membrane Ca^{2+} - ATPase (Kim HR *et al*., 1994; Seckin S., 1993).

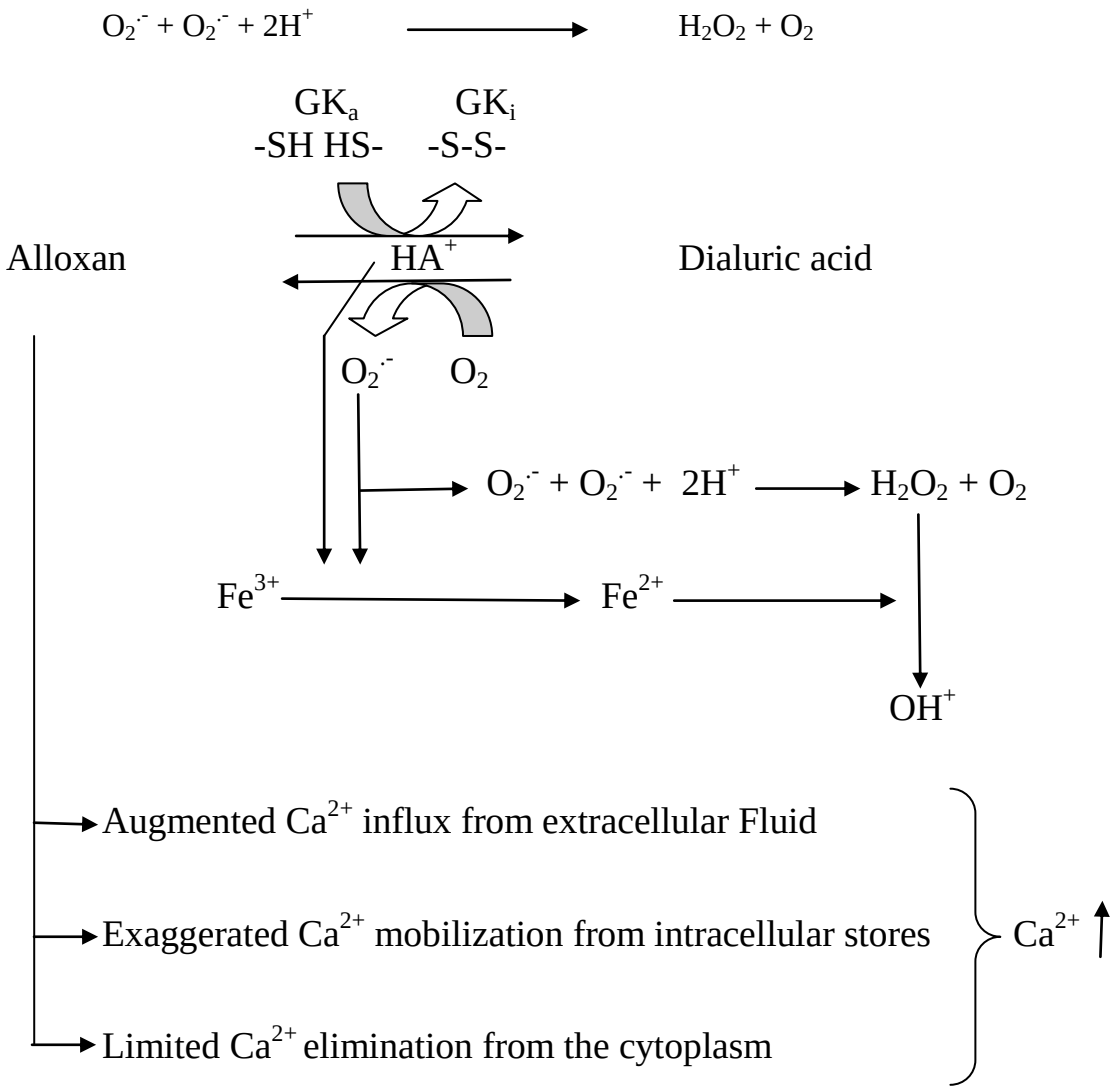


Figure – 9 Alloxan action

It is seen that alloxan administration leads to sudden release of insulin from β-cells (Kliber A *et al*., 1996) is due the alloxan induced augmentation in cytosolic Ca^{2+} concentration and the exaggerated concentration of this ion leads to supraphysiological insulin release, reactive oxygen species causes damage to pancreatic β-cells. It has been

demonstrated that the calcium channel antagonists can suppress hyperglycemia and the onset of alloxan diabetes in rats (Katsumata K *et al*., 1992).

Many investigators suggested that the diabetogenic dose of alloxan decrease –SH groups with a rise in glutathione peroxidase activity in the rat liver two minutes after administration (Szudelski T *et al*., 1998) with the elevation in the blood insulin level. It was also observed that alloxan also intensifies basal and epinephrine – induced biolysis in isolated rat adipocytes and insulin failed to restrict this effect (Kandulska k *et al*., 1999).

So, to induce diabetes by alloxan, animals should be examined at time intervals to minimize the adverse effects of alloxan action. The diabetogenic dose of alloxan is narrow, a slight overdose may lead to loss of animals. The loss of animals because of alloxan is likely due to the kidney tubular cell necrotic toxicity in the case of high dose of alloxan administration.

c) Dithizone:

Zinc chelating agent such as dithizone causes diabetes in laboratory animals. Dithizone has abilities to permeate membranes and to complex zinc inside liposomes with the release of protons, that can enhance diabetogenicity (Vineeta T *et al*., 2014).

d) Gold thioglucose:

Gold thioglucose developed obesity, induces diabetes in normal wistar strain rats. Gold thioglucose treated mice gained weight rapidly and significantly increase non fasting plasma glucose level within 8-12 weeks (Vineeta T *et al*., 2014).

2.12.2. Surgical model of diabetes:

Another technique used to induce diabetes is complete removal of the pancreas (pancreatectomy). Few researchers have employed this model in the previous years to explore effects of natural products with animal species such as rats, pigs, dogs and primates (choi SB

et al., 2004). Limitation to this technique include high level of technical expertise and adequate surgical environment, major surgery and high risk of animal infection, adequate post operative analgesia and antibiotic administration, supplementation with pancreatic enzymes to prevent malabsorption and loss of pancreatic counter regulatory response to hypoglycemia. More recently, partial pancreatectomy has been employed, but large resection (more than 80% in rats) is required to obtain mild to moderate hyperglycemia. In this case, small additional resection can result in significant hypoinsulinemia (Masiello, 2006).

2.12.3. Hormone induced diabetes:

Growth hormone has long distinguished history in diabetes, with possible participation in the development of renal complications (Vineeta T *et al*., 2014). Repeated administration of growth hormone in cats and adult dogs induces diabetes with all symptoms of diabetes including severe ketonemia and ketonuria. More prolonged administration of growth hormone produced permanent diabetes, there was loss of pancreatic islets tissue and of beta cells and only traces of insulin could be extracted from pancreas (Vineeta T *et al*., 2014).

2.12.4. Genetic model of diabetes:

a) Spontaneously developed diabetic rats:

These models permit the evaluation of the effect of a natural product in an animal without the interference of the side effects induced by chemical agents like streptozotocin, alloxan reported above. Several recent publications summarized the major advances in this field (Masiello, 2006). Example is the spontaneously diabetic Goto-Kakizaki rat which is a genetic lean model of type II diabetes originating from selective breeding over many generations of glucose-intolerant non diabetic wistar rats (Chen D *et al*., 2005). Mutant strains, obese diabetic mice are available such as the C57B/Ksj-db/db. With this model it is

possible to test for effects of plant extracts on blood sugar, body weight, insulin production and insulin resistant.

b) Genetically engineered diabetic mice:

In this case, rodents may be produced to over (transgenic) or under (knockout) express proteins, thought to play a key part in glucose metabolism (Masiello, 2006). Although significant advances in this field have arisen in recent years, especially with the advent of transgenic mice, there have been no studies carried out involving natural products on these models.

Chemical induction appears to be the most popularly used procedure in inducing diabetes mellitus in experimental animals. The best known drug induced diabetic model is the alloxan diabetes. It is capable of inducing both type I and type II diabetes mellitus with proper dosage selection. But the most commonly used drug is streptozotocin for reasons that are not well specified. The surgical, hormonal and genetic models require highly technical skills, may be associated with a high percentage of animal death and thus are rarely use. Alloxan induced diabetes model appears to be the most reliable and easily reproducible method of inducing diabetes mellitus in experimental animals.

Chapter 3

Aims & Objectives

In alloxan induced diabetic Wistar strain male rats:

1. To establish optimum effective dosage of Diallyl disulphide.
2. To determine the effect of Diallyl disulphide on:
    i. Plasma and Liver tissue lipids.
    ii. Liver tissue Cholesterol turnover.
    iii. Plasma Glucose and Lipoprotein lipase activity.
    iv. Oxidative stress and Antioxidant levels.
    v. Transaminases in plasma and liver tissue.
3. To evaluate the effect of Diallyl disulphide on liver histopathology.

Chapter 4

Methodology

4.1.1. Study design: Experimental.

4.1.2. Sample size: A total of 42 Wistar strain male albino rats weighing between 200-250 grams were used. Of these, 18 rats were divided into three groups to determine the optimum effective dosage and remaining 24 rats were divided into four groups to determine the effect of DADS and histopathological studies. Each group consisted of six rats.

4.1.3. Study duration: The total study duration was for four years (March 2011- February 2015), Of which, the first 6 months (March 2011- August 2011) was for a pilot study to standardize the laboratory procedures, the next 3 years (September 2011- August 2014) was dedicated for animal experimentation, laboratory investigations and review of literature. The final six months (September 2014 – February 2015) was for data compiling, analysis, review of literature and thesis writing.

4.1.4. Experimental animals: Healthy Wistar strain male albino rats weighing between 200-250 grams were randomly selected from the animal house facility at Dr. B.M.Patil Medical College, Hospital and Research Centre, BLDE University, Vijaypur, India.

4.1.5. Ethics: Experiments were conducted in accordance with Committee for the purpose of Control and Supervision of Experimental Animals (CPCSEA), New Delhi and Institutional Animal Ethical Committee (IAEC) of Dr. B.M.Patil Medical College, Hospital and Research Centre, Vijaypur, India.

4.1.6. Rearing & Maintenance: Animals were housed in polypropylene cages of 30 x 22 x 14cm with paddy husk as the bedding material. Bedding material was changed every 2 days and the cages were maintained in a controlled normal environment (25 ± 5^0C) with (12:12hr) light – dark cycles in an experimental room simulating natural conditions. Balanced rat pellet diet and water were provided *ad libitum*.

4.1.7. Induction of diabetes: Diabetes was induced in rats by intraperitoneal injection of freshly prepared aqueous alloxan monohydrate (150mg per kg body weight) (Ashok DC *et al*., 2007) in sterile water to overnight fasted rats. Later diet and water were provided *ad libitum*. Rats were labelled diabetic when urine sugar was positive and blood glucose level was >250mg/dl, for 7 consecutive days, after alloxan injection. Treatment was started on 8^{th} day after alloxan injection and was considered as first day of treatment.

4.1.8. Chemicals: Chemicals used in the present study for separation and estimation procedures were of analytical grade. Amino acids and ketoacids used for transaminases were of chromatographic purity. Special chemicals like Diallyl Disulphide (DADS) and Alloxan were procured from Sigma-Aldrich Chemical company, U.S.A.

4.2. Objective 1: To establish the optimum effective dosage of DADS.

4.2.1. Table – 9 Experimental design:

Group	Dosage of DADS	Treatment for 30 days	Diet	Sample
Group A (n=6)	50 mg/kg body weight	3 ml of suspension per kg body weight (50,100,150 mg/kg body weight of DADS respectively in group A,B,C) through gastric intubation	Lab diet and water was provided *ad libitum*	Blood & Liver tissue
Group B (n=6)	100 mg/kg body weight			
Group C (n=6)	150mg/kg body weight			

4.2.2. Method of sample collection: Rats were ether anaesthetised and sacrificed after the stipulated period. Blood was collected from internal jugular vein in heparinised blood collection tubes. Liver tissue was dissected out, smoothly blotted to dry, weighed and kept in clean dry beakers covered in aluminium foil and stored in deep freezer.

The blood samples were centrifuged for 6 min at 3000 rpm and clear plasma was employed for estimation of lipids – total lipids (Choudary K., 1989), total cholesterol (Harold V *et al*., 1991), triacylglycerols (Richard JH *et al*., 1974) and phospholipids (Nath RL., 1990).

Liver tissue was further homogenised using electric blender with chloroform-methanol solvent and centrifuged at 3000 rpm for 10min. The supernatant of chloroform-methanol (1:1 v/v) homogenate was used for estimation of lipids – total lipids (Choudary K., 1989), total cholesterol (Harold V *et al*., 1991), triacylglycerols (Richard JH *et al*., 1974) and phospholipids (Nath RL., 1990).

4.2.3. Statistical analysis: Data obtained was expressed in terms of mean $\pm$ SD. Statistical significance was established at $P<0.05$. Student't' test was performed to assess the significance of mean difference between the groups.

4.3. Objective 2: To determine the effect of DADS.

4.3.1 Table – 10 Experimental design:

Group	Name	Treatment for 30 days	Diet	Sample
Group I (n=6)	Normal Control	3 ml of normal saline per kg body weight through gastric intubation	Lab diet and water was provided *ad libitum*	Fecal material Blood & Liver tissue
Group II (n=6)	Diabetic Control			
Group III (n=6)	DADS treated Normal rats	3 ml of suspension per kg body weight (100 mg/kg body weight of DADS) through gastric intubation		
Group IV (n=6)	DADS treated Diabetic rats			

4.3.2. Gravimetric analysis: Body weights of all the animals from each group were recorded on the day one of initiating the treatment and on the day of sacrification. Liver weight was determined after dissecting out and blotting it dry on a single pan balance to evaluate the hepato - somatic index. Hepato - somatic index was calculated by obtaining the ratio of liver weight after dissection to the body weight at the time of sacrifice (Shaheenkousar HH *et al*., 2013).

4.3.3. Method of sample collection: One day prior to the animal scarification, each animal was kept in a separate cage and the fecal material of each animal was collected. Bile acids were extracted from this fecal material (Jan S *et al*., 1988) and bile acids content were estimated to assess cholesterol conversion to bile acids.

On completion of the stipulated period, rats were anaesthetised and sacrificed. Blood was collected from internal jugular vein in heparinised blood collection tubes. Liver tissue was dissected out, smoothly blotted to dry, weighed and kept in clean dry beakers covered in aluminium foil and stored in deep freezer.

Biochemical analysis - *Blood & Liver tissue*: Blood samples were used for estimation of various biochemical parameters such as - blood glucose (Richard J *et al*., 1974), total lipids (Choudary K., 1989), triacylglycerols (Richard JH *et al*., 1974), phospholipids (Nath RL., 1990), total fatty acids, free fatty acids (Nath RL., 1976), esterified fatty acids (Nath RL., 1976), total cholesterol (Harold V *et al*., 1991), LDL cholesterol, VLDL cholesterol (calculated by Frieldwald formula), HDL cholesterol (Harold V *et al*., 1991) and enzymes lipoprotein lipase (Edward DK., 1962), AST & ALT (Harold Varley, 1969).

Liver tissues from different groups were homogenised using electric blender with different solvents and centrifuged at 3000 rpm for 10min. This supernatant of these homogenates were used for biochemical analysis.

The supernatant of chloroform-methanol (1:1 v/v) homogenate was used for estimation of lipids – total lipids (Choudary K., 1989), total cholesterol (Harold V *et al*., 1991), triacylglycerols (Richard JH *et al*., 1974) and phospholipids (Nath RL., 1990). The supernatant of sodium arsenate (1% aq) homogenate was employed for HMG CoA reductase assay (Venugopal RA *et al*., 1975), where as the supernatant of phosphate buffer (pH-7.4) homogenate was employed to estimate total thiols (Colowich and Kaplan, 1957), enzymes-AST & ALT (Harold Varley, 1969). The TCA (5% aq) supernatant of the homogenate was used for TBARS estimation (Nadiger HA *et al*., 1986).

4.3.4. Statistical analysis: Data obtained was expressed in terms of mean $\pm$1 SD. Statistical significance was established at $P<0.05$. One way analysis of variance (ANOVA) was used to determine the significant difference between the various groups. One way ANOVA, if significant is followed by post hoc't' test to determine the level of significance between the groups.

4.4. Objective 3: To evaluate the effect of DADS on liver histopathology.

4.4.1. Experimental design: Same as explained for objective 2.

4.4.2. Tissue Processing: A part of liver tissue was kept in 10% formalin. Paraffin blocks were made as described below:

- Fixation : 10% formalin
- Dehydration : **a.** 70% alcohol – two hours.

b. 90% alcohol – two hours.

c. Three changes in absolute alcohol for two hours.

- Clearing : Xylene for two hours.
- Embedding : **a.** Four changes of paraffin wax for ½ hour each.

  b. Embedded in fresh filtered paraffin wax.

Five micron sections were cut using a rotary microtome. Sections were selected and mounted serially on air dried gelatinised slides.

4.4.3. Tissue staining:

4.4.3.1. Deparaffinization: Two changes of xylene were used each lasting two minutes to dissolve the paraffin.

4.4.3.2. Hydration: The sections were hydrated by passing them for two minutes each in descending grades of alcohol (100%, 90%, 70% & 50%) and finally distilled water.

4.4.3.3. Haematoxylin and Eosin staining procedure:

- Hydrated sections were stained with haematoxylin solution for three minutes.
- Washed in running tap water for five minutes.
- Differentiated in 1% acid alcohol for 30 seconds.
- Washed under running tap water for one minute.
- Bluing in 0.2% ammonia water for 30 seconds.
- Stained in 1% aqueous eosin for two minutes.

- Washed under running tap water for three minutes.
- Rinsed in 90% alcohol for 10-15 seconds.
- Dehydrated in two changes of absolute alcohol for 15-30 seconds.
- Cleared in two changes of xylene, two minutes each or until sections were clear.
- Mounted with DPX.
- The slides were cleaned and examined under light microscope and photomicrographs were taken with connected camera.

4.5. Method of evaluation:

A) Estimation of Glucose:

Glucose levels in plasma were determined using the modified method of Hultman as described by Richard J Henry *et al* (1974).

Principle:

Glucose reacts with O – toludine in glacial acetic acid when heated to yield a blue green N – glucosylamine derivative. The absorbance of this coloured complex was measured at 625 nm.

Reagents:

1. Ortho-toluidine reagent: 5.0g thiourea (reagent grade) and 90ml O-toluidine were mixed and was diluted to 1000ml with glacial acetic acid. For storage the reagent was refrigerated in amber bottles. As the reagent aged it yielded less colour for a given amount of glucose but proportionality between standard and unknown was maintained.

2. Glucose standard 100mg/100ml: 1.0g pure anhydrous glucose was dissolved in a litre of distilled water containing 1.5g benzoic acid. This solution was stable for use up to 2 years, when stored in a refrigerator.

Procedure:

1) 5.0ml Ortho-toluidine reagent was taken in three 10ml capacity test tubes, labelled 'blank', 'standard' and 'test'.

2) 0.1ml glucose standard and 0.1ml plasma were added to the standard and test respectively.

3) The tubes both standard and test along with blank (containing 5.0ml reagent and 0.1ml water) were incubated for 10mins in boiling water bath.

4) The incubated tubes were cooled under tap water to room temperature.

5) The optical density of standard and test was read against the blank at 625nm.

Calculations:

$$\text{Plasma glucose concentration (mg/dl)} = \frac{\text{OD of Test}}{\text{OD of Standard}} \times \frac{0.1}{0.1} \times 100$$

B) Estimation of Total lipids:

Total lipids were estimated in plasma or tissue homogenates by sulpho-vanillin reaction of Chabrol and Charronat, as proposed by Choudary (Choudary K., 1989).

Principle:

Lipids on heating with concentrated sulphuric acid are oxidized into ketones. The latter forms a pink colour with phosphoric acid and vanillin.

Reagents:

1) Vanillin 0.6%: 600mg of vanillin was weighed and dissolved in 100ml of 1% benzoic acid solution. This reagent remains stable for several years at room temperature.

2) Phosphoric acid: Analytical grade.

3) Concentrated sulphuric acid: Analytical Grade.

4) Standard lipid solution: 1g of pure triolein was weighed and dissolved in absolute ethanol and this was made up to 100ml with the same.

Procedure for plasma:

1) 0.1 ml of plasma was mixed with 1 ml of concentrated sulphuric acid in a test tube and incubated in boiling water bath for 10 minutes, later removed and cooled under tap water.
2) For the tube labelled as standard, 0.1 ml standard lipid solution and for tube labelled blank 0.1 ml distilled water was added in place of plasma in the procedure.
3) To all labelled tubes as standard, blank and test, 1 ml of vanillin (0.6%) was mixed with 4 ml phosphoric acid in another set of test tubes and to each tube 0.1 sulphated plasma from step 1 was added.
4) All the tubes were well shaken and incubated for 15 minutes at 37^0C. Later tubes were removed and cooled to room temperature.
5) The optical density of all the tubes was measured against blank at 540 nm.

Procedure for Tissue Homogenates:

0.1 ml chloroform-methanol (1:1 v/v) extract was taken in a clean and dry test tube and subjected to evaporation in a water bath maintained at 70^0C. To this, 1 ml of concentrated sulphuric acid was added and was proceeded as above from step 2.

Calculations:

$$1)\ \text{mg total lipids/100 ml plasma} = \frac{\text{O D of Test}}{\text{O D of Standard}} \times 1000$$

$$2)\ \text{mg total lipids/g tissue} = \frac{\text{O D of Test}}{\text{O D of Standard}} \times \frac{1}{0.005}$$

C) Estimation of Phospholipids:

Phospholipids were estimated in plasma or tissue homogenates by the modified method of Youngberg and Youngberg as described in "Practice of Biochemistry in Clinical Medicine" by Nath RL (1990).

Principle:

Phospholipids were extracted using ether-alcohol (1:3v/v) mixture. An aliquot of the ether-alcohol was dried and subjected to digestion using sulphuric acid and nitric acid to liberate phosphoric acid. The liberated phosphoric acid was estimated.

Reagents:

1) Ether-alcohol mixture: 1:3 v/v

2) Sulphuric acid 10N: This was prepared by diluting 10 ml of concentrated sulphuric acid to 36 ml with distilled water.

3) Concentrated Nitric Acid: Analytical grade reagent.

4) Ascorbic acid solutions 1%: This was prepared by dissolving 1 gm of ascorbic acid in distilled water and this was made up to 100ml with distilled water.

5) Ammonium Molybdate solution 1% in 0.5N Sulphuric acid: 1 g of pure ammonium molybdate was weighed and dissolved in 50 ml of glass distilled water with the aid of heats if necessary. Later this was transferred to 100 ml volumetric flask quantitatively and 10 ml of 0.5 N sulphuric acid was added and diluted up to the mark with distilled water.

6) Standard Phosphate Solution:

a) Stock Standard (10 mg phosphorus per 100 ml): 0.439 g of properly dried mono potassium dihydrogen phosphate (KH_2PO_4) analytical grade was weighed and dissolved in glass distilled water and made up to 1000 ml with the glass distilled water.

b) Working standard (0.2 mg phosphorus per 1000ml): From the stock solution 2 ml stock phosphate standard was taken in a 100ml volumetric flask, 2.5ml of 10N sulphuric acid was added and diluted up to the mark with glass distilled water. This contained 0.01 mg P/5ml.

Procedure for Plasma:

1) 0.2ml of plasma was taken in a glass stopper test tube to this 15ml ether-alcohol (1:3 v/v) mixture was added, mixed well for 3 minutes on vortex mixer.

2) 10ml supernatant was collected in another test tube and subjected to evaporation at 70^0C maintained in water bath.

3) The resultant residue was heated with 0.5ml 10N sulphuric acid slowly on a low flame, by keeping the test tubes in a slanting position.

4) When white fumes start coming from the dark digest, then cool and add a drop of concentrated nitric acid and again heated till it becomes clear and colourless (if any brown tinge persisted, heating was repeated with another drop of nitric acid). At last the digest was made up to 10ml with glass distilled water.

5) From this, 5ml was transferred into another test tube and to this 0.5ml of ascorbic acid solution and 0.5ml ammonium molybdate solutions were added with the start of the stop watch and mixed well.

6) The optical density was read against water blank at 5^{th}, 10^{th} and 15^{th} minutes at 640nm. The readings were plotted against time and the zero minute absorbance was found out by extrapolation. This optical density of unknown was denoted as 'U'.

7) In another similar test tube, 5ml working phosphate standard solution was taken and by following through the steps 5 and 6. The zero minute optical density obtained by extrapolation was standard 'S'.

Procedure for Tissue Homogenates:

1) 1ml chloroform-methanol (1:1 v/v) extract was taken in a clean and dry glass stopper test tube and subjected to evaporation at 70^0C maintained in water bath.

2) The residue was extracted with 15ml of ether-alcohol (1:3 V/V) mixture and mixed well in a glass stopper test tube.

3) From this, 10ml of ether-alcohol extract was taken in another test tube and followed as described for plasma.

Calculations:

$$\text{1) mg lipid phosphorus/100 ml plasma} = \frac{U}{S} \times 15$$

$$\text{2) mg lipid phosphorus/g tissue} = \frac{U}{S} \times \frac{1}{0.033}$$

The values obtained were converted to phospholipids and reported in micromoles/L plasma or micromole/Kg tissue (1mg/dl = 0.323 mmol/L).

D) Estimation of Triacylglycerols:

Triacylglycerols in plasma or in tissue homogenate were estimated by the modified method of Van Handle and Zilversmit as cited in "Clinical Chemistry – Principles and Practice" by Henry *et al.,* using Florisil to remove phospholipids (Richard JH *et al*., 1974).

Principle:

Triacylglycerols were extracted from plasma or tissue homogenates with chloroform. The phospholipids were removed by adsorption on florisil. Triacylglycerols were then saponified to give glycerol which was oxidized by periodic acid to formaldehyde. Excess hydroiodic acid was removed with sodium arsenate. Formaldehyde was estimated photo-metrically by reaction with chromo-tropic acid.

Reagents:

1) Chloroform: Reagent grade

2) Florisil: Suitable for chromatographic analysis

3) 0.2N Sulphuric acid: 2.8ml concentrated sulphuric acid, analytical grade was diluted to 500ml with glass distilled water.

4) 24N Sulphuric acid: 300ml glass distilled water was taken in a beaker and was kept in ice cold water bath. To this, 600ml concentrated sulphuric acid was added slowly with constant stirring and shaking. It was stored in amber coloured bottle in refrigerator.

5) Alcoholic KOH: 1.2ml of 33% KOH solution was mixed with 98ml rectified spirit.

6) Sodium metaperiodate: 1.07g of sodium metaperiodate was dissolved in glass distilled water and made up to 100ml.

7) Sodium arsenite: 5.0g arsenite oxide (analytical grade) was mixed with 2.25g sodium hydroxide pellets in a long test tube. To this, little glass distilled water was added and mixed thoroughly. This was made up to 10ml with glass distilled water.

8) Chromotropic acid reagent: 200mg chromotropic acid sodium salt (1,8 dihydroxy naphthalene and 3,6 disulphuric acid) was dissolved in 20 ml glass distilled water. To this, 90ml ice cold 24N sulphuric acid was added. This was prepared fresh, each time.

9) Standard triacylglycerol solution (stock): 100mg olive oil was dissolved in 100ml of chloroform and refrigerated.

10) Working standard solution: 1ml stock standard was diluted to 10ml with chloroform for ready use within a week after preparation.

Procedure for Plasma :

1) 0.2ml plasma was mixed with 200mg florisil and 4.8ml chloroform in a clean dry test tube. The tube was covered with aluminium foil. This was thoroughly vortexed for about 4 minutes.

2) 1ml each of chloroform extract were pipetted out into clean and dry tubes marked as 'T' and 'TC'. 0.5ml, each of working triacylglycerols were pipetted into clean and dry test tubes marked 'S' and 'SC'. 1ml chloroform alone was taken in tube marked 'B'.

3) These tubes were kept in a water bath maintained at 70^0C and the contents were evaporated to dryness.

4) 0.5ml alcoholic KOH was added to tubes marked 'S' and 'T'. These tubes were incubated at 70^0C for 20 minutes.

5) To all the tubes (B, S, SC, T & TC), 0.5ml of 0.2N sulphuric acid solution was added. To tubes marked 'B', 'SC' and 'TC' 0.5ml alcoholic KOH solution was also added. All the tubes were heated in a boiling water bath for 5 minutes to remove alcohol.

6) The tubes were removed from the boiling water bath cooled and 0.1ml sodium metaperiodate solution was added to each tube. The tubes were incubated at room temperature for 15minutes.

7) 0.1ml sodium arsenite solution was added to all these tubes, mixed thoroughly and were kept at room temperature for 5 minutes.

8) 5ml freshly prepared chromotropic acid solution was added to all these tubes and were mixed by lateral shaking. The tubes were heated on a boiling water bath for 30minutes.

9) The tubes were removed and cooled in running water. The absorbance was measured at 540nm.

Procedure for Tissue Homogenates:

0.1ml chloroform-methanol (1:1 v/v) extract was taken in a clean dry tube. To this, 200mg florisil and 4.9ml chloroform were added. The tubes were covered with aluminium foil and mixed well on a vortex mixer for about 4minutes.

1ml supernatant was used for estimation of triacylglycerols as described above.

Calculations:

$$1)\ \text{mg Triacylglycerol/100 ml plasma} = \frac{\text{O.D of T} - \text{O.D of TC}}{\text{O.D of S} - \text{O.D of SC}} \times 100$$

$$2)\ \text{mg Triacylglycerol/g tissue} = \frac{\text{O.D of T} - \text{O.D of TC}}{\text{O.D of S} - \text{O.D of SC}} \times 50$$

E) Estimation of Total Cholesterol:

Cholesterol in plasma and tissue was estimated using ferric chloride by Zak's method (Harold Varley *et al*., 1991).

Principle:

Zlatkis, Zak and Boyle used the red colour, which cholesterol in acetic acid solution gives with ferric chloride and sulphuric acid. This reaction was employed by a number of researchers previously.

Reagents:

1. Acetic acid: Analytical grade acetic acid was satisfactory.

2. Ferric chloride: 0.05 % Solution of $FeCl_3.6H_2O$ in the purified acetic acid.

3. Sulphuric acid: Analytical Grade.

4. Stock cholesterol standard: 100mg in 100ml of purified acetic acid.

5. Cholesterol standard for use: Dilute the stock standard 1 to 25 with the ferric chloride acetic acid reagent.

Procedure for Plasma:

1. To 0.1 ml of plasma, 10ml of the ferric chloride-acetic acid reagent was added in a glass stoppered centrifuge tube.

2. The above mixture was mixed well and allowed to stand for 10-15 minutes.

3. Later the mixture was centrifuged and 5 ml of the clear supernatant was transferred to glass stoppered centrifuged tube.

4. For the standard, 0.1 ml of normal saline and 10 ml of cholesterol standard were taken. From this, 5 ml was transferred to a second stoppered centrifuged tube.

5. For blank, 5 ml of the ferric chloride-acetic acid reagent was taken in third tube.

6. 3 ml of sulphuric acid was added to all the tubes from a burette, stoppered tightly and mixed well by repeated inversion.

7. All the tubes were allowed to stand for 20-30 min at room temperature.

8. Optical density of all the tubes was read against blank using a yellow filter at 560 nm.

Procedure for Tissue Homogenate:

0.1 ml Chloroform-methanol (1:1 v/v) extract was taken in a dry test tube. This was evaporated to dryness using a water bath maintained at 70^0C. To the above tube, 10 ml of ferric chloride-acetic acid reagent was added and proceeded through the second step as described above for plasma.

Calculations:

$$\text{mg of cholesterol per 100 ml plasma} = \frac{\text{O.D of test}}{\text{O.D of Standard}} \times \frac{0.2}{0.05} \times 100$$

$$= \frac{\text{O.D of test}}{\text{O.D of Standard}} \times 400$$

F) Estimation of HDL – Cholesterol:

HDL is the fraction of plasma lipoprotein with hydrated density of 1.063 – 1.21 g/ml. This is made up of equal proportions of proteins and lipids. The lipid portion constitutes: 28% phospholipids, 18% cholesterol and 4% triacylglycerols. Since measurement of cholesterol is simple, HDL is measured by determining its cholesterol content. HDL – cholesterol quantification is based on isolation and separation of HDL and determination of cholesterol in isolated HDL (Harold Varley, *et al*., 1991).

Principle:

LDL, VLDL and Chylomicrons were precipitated using phosphotungstate – magnesium as described in Varley (Alan H *et al*., 1988) and the cholesterol content of supernatant was estimated.

Reagents:

1. Phosphotungstic acid reagent: 22.5g phosphotungstic acid (analytical grade) was dissolved in 200ml glass distilled water, 80ml 1M sodium hydroxide solution was added and made up to 500ml with glass distilled water.

2. Magnesium chloride solution 2 mol/L: 101.7g $MgCl_26H_2O$ was dissolved in glass distilled water and made up to 250ml with same.

Procedure:

1. To 1ml plasma in a centrifuge tube, 0.1ml phosphotungstic acid reagent was added. The contents were vortexed for 10 seconds.

2. To this, 50µl magnesium chloride solution was added and again mixed for 10 seconds on a vortex mixer. Then it was centrifuged at ambient temperature for 30 minutes at 1500 rpm, ensuring the temperature did not rise appreciably.

3. The supernatant was carefully removed avoiding any surface deposit and put into a clean dry test tube.

4. Cholesterol was determined in this supernatant (Refer Cholesterol procedure).

5. The result was multiplied by 1.125 (dilution factor).

G) Estimation of Free Fatty Acids (FFA):

The determination of plasma Free Fatty Acids (FFA) (also known as Non – esterified or Un-esterified fatty acids – NEFA or UEFA) is a measure of that portion of the fatty acid pool that circulated in immediate readiness for metabolic needs. This is usually composed of C_{16} and C_{18} fatty acids. The FFA is estimated in a fresh plasma sample by the method of Dole and Meinertz as described by Nath RL (1976).

Principle:

FFA's are extracted from a sample of plasma by a mixture of heptanes and isopropanol. This was then washed with dilute sulphuric acid to remove non – FFA contaminants. The extracted FFA was titrated immediately against dilute thymol blue which acted as an indicator.

Reagents:

1. n – Heptane: Redistilled.

2. Extraction mixture: Prepared by mixing together 30ml isopropanol, 30 ml heptanes and 3ml 1N sulphuric acid.

3. Indicator: 0.1 % solution of thymol blue solution was prepared in water and diluted 10 times with redistilled ethanol.

4. Standard solution of sodium hydroxide: 0.02N NaOH was prepared by diluting carbonate free saturated NaOH solution (1.2 ml to 1000ml in CO_2 free glass distilled water). This was standardized and the normality was adjusted to 0.02 by adequate dilution.

5. Standard solution of palmitic acid: 0.0512g of (0.2 meq/L) recrystallised palmitic acid was weighed and dissolved in n – heptanes and made up to 1000 ml with the n-heptane.

Procedure:

Test: 0.1 ml fresh plasma was mixed with 10ml extraction mixture in a glass stoppered tube. The contents were vigorously shaken and allowed to stand at room temperature for 5-7 minutes. Then 4 ml of glass distilled water and 6 ml heptanes were added and shaken again. 3 ml aliquot of the upper phase in duplicate was taken in 15ml centrifuge tube. 1 ml indicator was added and titrated against 0.02 N NaOH taken in a micro burette till a green-yellow end point was obtained. The mean of the two titre readings were taken.

Blank: The titrations in duplicates were carried out with 2ml glass distilled water instead of plasma. The mean of the two titre readings were taken.

Standard: The titrations in duplicate were carried out using 2 ml glass distilled water instead of plasma and 6 ml standard solution of palmitic acid instead of 6 ml heptanes. The mean of the titre readings were taken.

Calculations:

$$\text{mEq of FFA /L} = \frac{T - B}{S - B} \times 0.6$$

Where T=mean titre reading of test

S=mean titre reading of standard

B=mean titre reading of blank

H) Estimation of Esterified Fatty Acids (EFA):

Esterified Fatty Acids were estimated in plasma by the method of Stern and Shapiro as described by Nath RL (1976).

Principle:

Hydroxylamine in alkaline solution reacts with esters of fatty acids to give hydroxamic acids, which produce a red violet colour with ferric chloride. The optical density of this colour was measured at 540nm.

Reagents:

1. Ether – alcohol mixture - 1:3 v/v

2. Hydroxylamine hydrochloride 14%: 14% of hydroxylamine hydrochloride was freshly prepared by weighing 14 g of hydroxylamine and dissolving in 10ml hydrochloride made up to 100ml with distilled water.

3. Ferric chloride 10%: 10g of ferric chloride ($FeCl_3 6H_2O$) was prepared by dissolving in 0.1N Hydrochloric acid and made to 100ml with the same.

4. Sodium Hydroxide 14%: Prepared by dissolving 14g of sodium hydroxide pellets in glass distilled water and made up to 100 ml with the same.

5. Hydrochloric acid 33%: Prepared by diluting 1 volume of concentrated hydrochloric acid (SG 1.18) and diluted with 2 volumes of glass distilled water.

6. Standard solution of triolein:

a) Stock standard: 1.18g of trolein was weighed and dissolved in ether alcohol (1:3v/v) mixture and made up to 100ml with the same.

b) Working standard: 5ml of the stock standard solution was diluted to 100ml with ether alcohol (1:3 v/v) mixture. 1ml of working solution contained 0.54 mg of fatty acids.

Procedure:

0.3ml of plasma was mixed with 6ml ether alcohol mixture in test tube, mixed and warmed on a hot water baht. It was cooled and filtered without pouring the precipitate through a fat free dry filter paper into a 15ml test tube. The extraction was repeated in the same way using 5ml and 4ml of the solvent successively. Volume of the combined filtrate was made up to 15ml and was mixed. Three stoppered cylinders were arranged as follows:

Reagent	Blank	Standard	Test
Ether – alcohol mixture (ml)	5.0	4.0	-
Working standard (ml)	-	1.0	-
Filtrate of test (ml)	-	-	5.0
Hydroxylamine hydrochloride (ml)	0.5	0.5	0.5
Shake and add			
Sodium hydroxide (ml)	0.5	0.5	0.5
Shaken, stoppered and kept at 25^0C for 20minutes, then add			
Hydrochloric acid (ml)	0.6	0.6	0.6
Ferric chloride (ml)	0.5	0.5	0.5

The contents of each tube mixed and the absorbance of standard(S) and unknown (U) were taken against blank (B) at 540nm.

Calculation:

$$\text{mg of Esterified fatty acids/100ml plasma} = \frac{U}{S} \times 540$$

The values were converted into meq/L and reported.

I) Estimation of Lipoprotein Lipase

Lipoprotein Lipase in the plasma has been estimated by method explained Edward D Korn (1962).

Principle:

Glycerol produced by the action of enzyme on triacylglycerols emulsion was oxidized by periodate to formaldehyde. Formaldehyde produced was then determined colorimetrically by the chromotropic acid reaction.

Reagents:

1. 0.5M ammonium sulphate: 33g of ammonium sulphate was dissolved in 500ml glass distilled water.

2. 20% Albumin: 2g of bovine albumin was dissolved in little glass distilled water. pH was adjusted to 8.5 with sodium hydroxide and made up to 10ml with glass distilled water.

3. Substrate: Activated triacylglycerols emulsion at a concentration of 2% with respect to triacylglycerols was used. Activated triacylglycerols were prepared by incubating a commercial triacylglycerols emulsion (2%) with an equal volume of plasma at 37^0C for 30minutes used as such.

4. 0.05M Sodium periodate: 11.4g of $HIO_4 2H_2O$ was dissolved in 900ml of glass distilled water, neutralized with 1N sodium hydroxide and was diluted to 1000ml with distilled water.

5. 0.5M Sodium arsenite: 22.5g of sodium hydroxide and 5g of arsenite oxide AS_2O_3 were dissolved in glass distilled water and made to 1000ml with the same.

6. 1N Sulphuric acid

7. Chromotropic acid reagent: 1g of chromotropic acid was dissolved in 100ml glass distilled water. Then 400ml ice cold 24N sulphuric acid (2 parts of concentrated sulphuric acid + 1 part of water) was added slowly.

Procedure:

1. Incubation: 0.4ml albumin was taken in a clean dry tube. 0.1ml ammonium sulphate solution, 0.1ml substrate, 0.1ml plasma and 0.3ml of glass distilled water were added. The

contents were mixed and the tube was incubated at 37^0C for 60minutes. An aliquot of 0.1ml was taken out at zero minute and transferred into another tube marked 'Control' containing 0.1ml of 1N sulphuric acid. At the end of the incubation period, another 0.1ml aliquot transferred into a tube marked 'Test' and containing 0.1ml of 1N sulphuric acid. The amount of glycerol in the 'Control' and 'Test' were determined.

2. Glycerol determination: The amount of glycerol content of the zero minute aliquot (control) and a sixty minute aliquot was determined by the procedure given earlier, starting from the 6^{th} step as described for plasma (Refer triacylglycerol estimation procedure).

Calculations:

Amount of glycerol Produced in 60minutes

= [glycerol present in the test] – [glycerol present in control] X 10 by 1ml plasma at 37^0C.

Enzyme activity:

Activity is expressed in terms of µmole glycerol produced / ml plasma.

J) Estimation of Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances were estimated in liver tissue TCA extract by the procedure explained by Nadiger HA *et al*., (1986).

Principle:

TBARS in trichloroacetic acid homogenates was determined by reacting them with thiobarbituric acid. The colour developed is measured at 535nm against distilled water.

Reagents:

1. Trichloroacetic acid 40%: 400g trichloroacetic acid dissolved in glass distilled water and diluted to 1000ml.

2. Trichloroacetic acid 5%: 40g trichloroacetic acid dissolved in glass distilled water and diluted to 1000ml. This was preserved in refrigerator.

3. Thiobarbituric acid 0.67%: 6.7g thiobarbituric acid was dissolved in glass distilled water and made up to 1000ml.

Procedure:

1. 1g tissue was homogenized with 9ml 5% cold trichloroacetic acid for 10minutes using potter elvehjem homogenizer fitted with Teflon piston.

2. 1ml homogenate was taken in a long test tube and to this, 1ml 40% trichloroacetic acid was added followed by 2ml of thiobarbituric acid.

3. The tube was incubated in boiling water bath for 10minutes.

4. The tubes were removed, cooled to room temperature centrifuged and the optical density of the supernatant was measured at 535nm using glass distilled water as blank.

Calculations:

Using the extinction coefficient of 1mole MDA which is equal to 1.53×10^{-5}

$$\mu\text{mole of MDA} = \text{O.D of Test} \times \frac{40}{0.156}$$

K) Estimation of Total Sulphydryl Groups

The total sulphydryl group in the renal tissue was estimated in liver tissue phosphate extract by nitroprusside reaction method (Colowick and Kaplan, 1957).

Principle:

Total sulphydryl groups of tissues were estimated by the nitroprusside reaction as explained by colowick and Kaplan (1957) using cysteine as standard sulphydryl compound.

Reagents:

1. Hydrochloric acid: 0.033N

2. Sodium hydroxide: 0.033N

3. Sodium chloride: Saturated

4. Sodium carbonate: 1.5M

5. Sodium nitroprusside: 0.067M

6. Sodium Cyanide: 0.067M

7. Standard sulphydryl reagent: 10mg cysteine mono hydrochloride in 100ml glass distilled water. This contains 0.57µmol SH group per ml.

Procedure:

1. 1g of tissue was homogenized with 9 parts if phosphate buffer solution pH 7.4. From this 0.2ml homogenate was taken in a test tube and to this, 0.1ml 0.033N hydrochloric acid, 1.2ml saturated sodium chloride solution and 0.2g of solid chloride were added. The tube was allowed to stand at room temperature for 10minutes. Then 0.1ml 0.033N sodium hydroxide was added. The above mixture was subjected to centrifugation to remove flocculated proteins.

2. After centrifugation, 0.8ml of the clear fluid was taken in another test tube. To this 0.1ml 0.067M sodium nitroprusside solution and 0.1ml each of 1.5M sodium carbonate and 0.067M sodium cyanide were added.

3. The contents of the tubes were mixed well and the optical density was read exactly after 30seconds at 520nm.

4. 0.2ml water for blank and 0.2ml water containing 0.05ml standard cysteine solution for standard were employed instead of tissue homogenate.

Calculations:

$$\text{µmol of SH groups / 0.02g} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 0.056$$

Hence

$$\text{µmol of SH groups / g} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 2.8$$

L) Estimation of Transaminases:

Glutamate oxaloacetate transaminase (GOT) or Aspartate amino transferase (AST) (EC:2.6.1.1) and glutamic pyruvic transaminase (GPT) or Alanine amino transferase (ALT) (EC: 2.6.1.2) levels in plasma were estimated by the colorimetric method of Reitman and Frankel (Harold Varley *et al*.,1969).

Principle:

AST: L – aspartic acid and α – ketoglutaric acid were incubated with the sample and the ratio of formation of oxaloacetic acid was determined directly as the dinitro phenyl hydrazine. The absorbance of the dinitro phenyl hydrazine derivatives were measured using a green filter, after adding alkali.

ALT: L – alanine and α – ketoglutaric acid were incubated with the sample and the rate of formation of pyruvate was determined directly as the dinitro phenyl hydrazine. Absorbance of the dinitro phenyl hydrazine derivatives were measured using a green filter, after adding alkali.

Reagents:

1. Phosphate buffer 0.1M pH 7.4: 23.86g of disodium hydrogen phosphate (Na_2HPO_4) and 4.36g of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in glass distilled water and made up to 2000ml with the same.

2. Pyruvate 2.0 mole/L: 0.110g of sodium pyruvate was dissolved in 500ml phosphate buffer. This was used for standard curve and prepared fresh as needed.

3. AST Substrate: 0.146g of α-ketoglutaric acid and 13.3g DL aspartic acid were taken in a beaker. Small quantities of 1N NaOH solution were added with stirring until the solution was complete. The pH of this solution was adjusted to 7.4 with 1N NaOH. This was made up to 500ml with phosphate buffer and refrigerated.

4. ALT Substrate: 0.146g of α-ketoglutarate and 8.9g of DL alanine were taken in a beaker. Small quantities of 0.1N NaOH solution were added with stirring until the solution was complete. pH of this was adjusted to 7.4 with 1N NaOH. This was made up to 500ml with phosphate buffer and refrigerated.

5. 2,4-dinitro phenyl hydrazine: 0.198g of 2,4-dinitro phenyl hydrazine was dissolved in a litre of 1N HCl and stored in dark bottle in refrigerator.

6. Sodium hydroxide: 0.4N

Procedure:

1. 1ml of AST/ALT substrate was taken into a clean dry test tube and incubated for 10minutes in a water bath maintained at 37^0C.

2. 0.2ml plasma / phosphate buffer extract of tissue was taken in both AST and ALT tubes and mixed.

3. The tube was incubated at 37^0C for 60minutes for AST (30minutes for ALT)

4. At the end of the incubation period (60minutes for AST and 30minutes for ALT), the tubes were removed from the water bath, cooled and 1ml 2,4-dinitro phenyl hydrazine reagent was added. The contents of the tubes were mixed well.

5. The tubes incubated for 20minutes at room temperature.

6. 10ml of 0.4N NaOH solution was added to each tube and mixed well by inversion. The tubes were allowed to stand at room temperature for 10minutes.

7. The optical density was measured against water as blank using green filter.

Procedure for Tissue Homogenate:

1:10 homogenate of tissue was prepared in phosphate buffer solution pH 7.4, centrifuged and the 0.2ml of supernatant was employed.

Calculation:

$$\text{AST units/ mg} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 30.5$$

$$\text{ALT units/mg} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 28.5$$

M) Estimation of Fecal Total Bile Acids:

Bile acids in faeces were estimated by the modified method of Petten Kopfer as given by Jan Siovall (1945).

Principle:

An aliquot of the purified fecal bile extract is evaporated and the bile acids present in the residue were made to react with furfuraldehyde in the presence of sulphuric acid. The intensity of the purplish colour formed was measured at 620nm.

Reagents:

1. Sulphuric acid 16N: This was prepared by mixing 445ml concentrated sulphuric acid analytical grade to 500ml glass distilled water kept in cold water bath. The acid was added in small portions. The resulting solution was made up to 1000ml with glass distilled water.

2. Furfuraldehyde AR grade 0.5%: The commercial sample of furfuraldehyde was redistilled before use. 0.5ml of this freshly distilled furfuraldehyde solution was diluted to 100ml with glass distilled water.

3. Ethanol: Purified by redistillation.

4. Sulphuric acid 2N: Prepared by diluting 16N sulphuric acid in this 1:8 proportion with glass distilled water.

5. Glacial acetic acid: Analytical grade.

6. Bile acid standard: Prepared by diluting 100mg cholic acid in 100ml glass distilled water.

Procedure:

1. Extraction of Bile acids: 24hours fecal matter was collected from rats, semi dried, weighed and pulverised with ethanol and refluxed for 120minutes. The ethanol portion was decanted in a conical flask, this step was repeated for two more times with ethanol and combined together. Later the combined extract was acidified with 1ml of 2N sulphuric acid and diluted with 20-30ml of glass distilled water.

2. This diluted ethanol extracts was again extracted with diethyl ether 2-3 times. The combined ether extract was made up to 30ml.

3. 2ml Ether extract was taken in a pyrex test tube and was evaporated to dryness at 70^0C.

4. 6ml of 16N sulphuric acid followed by 2ml furfuraldehyde solution were added.

5. 2ml ether and 2ml standard solution served as blank and standard respectively. They were processed through the above steps.

6. The tubes were heated for 13minutes at 65^0C. The tubes were removed and cooled.

7. 5ml glacial acetic acid was added to each tube and the contents of each tube was thoroughly mixed.

8. The optical density of test and standard were taken at 620nm against the blank set at zero.

Calculation:

$$\text{mg Bile acid / 24hours faeces} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times \frac{0.2}{2} \times 30$$

$$= \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 3$$

N) Estimation of HMG CoA reductase acitivity in liver tissue by indirect method:

An indirect method of assessing variation in 3-hydroxy 3-methyl glutaryl coenzyme A reductase (HMG CoA) activity in liver tissue. 3-hydroxy 3-methyl glutaryl CoA and Mevalonate concentrations in the tissue homogenate are estimated in terms of absorbance's and the ratio between the two is taken as an index of activity of the enzyme, which catalyzes the conversion of 3-hydroxy 3-methyl glutaryl CoA to Mevalonate. The ratio is known to increase under conditions in which activity of this enzyme in liver reportedly decreases (e.g., fasting, cholesterol feeding) and vice versa. The estimation of ratio 3-hydroxy 3-methyl glutaryl CoA/ Mevalonate in liver tissue is simple, reproducible and does not require any unusual chemicals (Venugopal Rao A *et al*., 1975).

Principle:

HMG CoA reductase (EC 1.1.1.34) is the sole rate limiting reaction of hepatic cholesterol synthesis in animals. Methods available for the assay of this enzyme in tissues are complex, here is an indirect method for assessing HMG CoA reductase activity. It is easily used in laboratories where more complicated measurement of the activity of this enzyme cannot be undertaken. HMG CoA and Mevalonate concentration in the tissue samples (homogenates) are estimated in terms of absorbance, calculated per gram of wet tissue. The ratio (absorbance of HMG CoA/absorbance of mevalonate) is taken as an index of the activity of HMG CoA reductase required to convert HMG CoA to Mevalonate in the presence of NADPH. If cholesterol biosynthesis is decreased in a clinical condition, the ratio by this method will increase, and vice versa.

Reagents:

1. Saline arsenate solution: 1g of sodium arsenate per litre of physiological saline.

2. Diluted perchloric acid (50m/L): Dilute 50ml perchloric acid to 1000ml with glass distilled water.

3. Hydroxylamine hydrochloride reagent (2 mol/L): 13.9g of hydroxylamine hydrochloride in 100ml glass distilled water.

4. Hydroxylamine hydrochloride reagent for mevalonate: Equal volumes of hydroxylamine hydrochloride reagent and water were freshly mixed before use.

5. Hydroxylamine reagent for HMG CoA: Equal volumes of hydroxylamine hydrochloride reagent and sodium hydroxide solution (4.5 mol/L) were freshly mixed before use.

6. Ferric chloride reagent: 5.2g of trichloroacetic acid and 10g of ferric chloride were dissolved in 50ml of 0.65 mol/L hydrochloric acid and dilute to 100ml with the latter.

Procedure:

HMG CoA/mevalonate ratio was measured in liver tissue of male albino rats weighing 150-200g range. Rats were divided into 3 different groups of 6 rats each. All the animals were kept on a standard diet. A part of the fresh liver tissue was homogenized with saline/arsenate solution to give a 10g/dl homogenate.

HMG CoA was determined by reaction with hydroxylamine at pH 5.5 and subsequent colorimetric measurement of the resulting hydroxamic acid by formation of complexes with ferric salts. Because mevalonate interferes in this estimation of acid or neutral pH, alkaline hydroxylamine was used to estimate specifically HMG CoA only. Possible interference by coenzyme A is also minimal when readings are taken at 540nm. Mevalonate was estimated by reaction with the same reagent, but at pH 2.1. At this pH the lactone form of mevalonate readily reacts with hydroxylamine to form the hydroxamate.

Analytical Procedure: Mixed equal volumes of the fresh 10% tissue homogenate and dilute perchloric acid were allowed to stand for 5 min and centrifuged (2000rpm, 10min). Treat

1.0ml of filtrate with 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG CoA), mix and after 5 min add 1.5ml of ferric chloride reagent to the same tube and shaken well. Readings were taken after 10min at 540nm against a similarly treated saline or arsenate blank.

Chapter 5

Results

5.1. To show the optimum dosage of DADS in alloxan diabetic rats:

It is evident from the table-11, that DADS at 100 mg/kg body weight significantly ($p<0.001$) lowers plasma as well as liver tissue total lipids, total cholesterol and triacylglycerols as compared to other concentrations (50 mg/kg body weight and 150 mg/kg body weight) of DADS employed on alloxan induced diabetic rats. Thus, from the present study it is observed that 100 mg/kg body weight of DADS had hypolipidemic effect in both plasma and tissue lipids.

Table – 11 Showing plasma and liver tissue total lipid, total cholesterol and triacylglycerols in diabetic rats treated with 50mg, 100mg and 150mg/kg body of DADS.

Concentration of DADS	Total Lipids		Total Cholesterol		Triacylglycerols	
	Plasma mg/dl	Tissue mg/g	Plasma mg/dl	Tissue mg/g	Plasma mg/dl	Tissue mg/g
50 mg/kg body weight Group A (6)	257± 18.0[a]	49.8±8.8 [a]	107±9.17 [a]	5.81±0.57[a]	89±6.49 [a]	43±6.28 [a]
100 mg/kg body weight Group B (6) Vijay V *et al*., 2013	203±23.6[b]	29.8±4.16 [b]	77±5.41[b]	4.03±0.32[b]	64±7.4[b]	32±8.2 [b]
150 mg/kg body weight Group C (6)	254.6±6.8[a]	50.5±9.79[a]	96.8±7.83[a]	5.36±0.50[a]	89±5.7[a]	49±6.43[a]

Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a, b) are significantly different from each other ($p<0.05$).

5.2. Gravimetric analysis:

Body weight of all the rats was recorded on 1st day of treatment and just before the scarification. Liver weight was taken after dissecting it out. Hepato somatic index is the ratio of liver weight after dissecting it out to body weight at the time of scarification. The percent

body weight change of the rats at the end of the experiment were significantly different among the four rat groups (p = <0.001). Result showed a significant loss in percentage body weight (p<0.001) in group II diabetic rats when compared to group I normal rats. A significant reduction in percent body weight loss (p<0.001) was observed in group IV rats as compared to group II rats.

The hepato somatic index was significantly different among the four rat groups (p = <0.001). Results in the table-12 narrate a significant raise in hepato somatic index (p<0.001) in group II rats as compared to group I rats. A significant improvement in hepato somatic index (p<0.001) was observed in group IV rats when compared to group II rats. No significant difference was observed either in percent body weight change or hepato somatic index in between group III and group II rats.

Table – 12 Showing changes in body weight and Hepato - Somatic index in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	**Initial Body weight (g)**	260±9 [a]	257±16.8 [a]	251.5±6.9 [a]	253.5±9.3 [a]	0.7444	0.5388
2	**Final Body weight (g)**	276.6±11 [a]	238±18.1 [b]	266.6±8.1 [a]	242±7 [b]	15.38	<0.001
3	**% Body weight Change**	6±1.34 [a]	-8.23±2 [b]	5.6±0.9 [a]	-4.7±1.8 [c]	120.3	<0.001
4	**Hepato-Somatic Index (g/Kg)**	29±0.49 [a]	36± 1.2 [b]	30.5±1.8 [a]	33.2±0.7 [c]	33.27	<0.001

Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a, b, c) are significantly different from each other (p<0.05).

Figure – 10

Graph Showing the % Body weight Gain in Normal (G-I), Alloxan Diabetic (G-II),DADS Treated Normal (G-III) and DADS Treated Alloxan Diabetic Rats (G-IV)

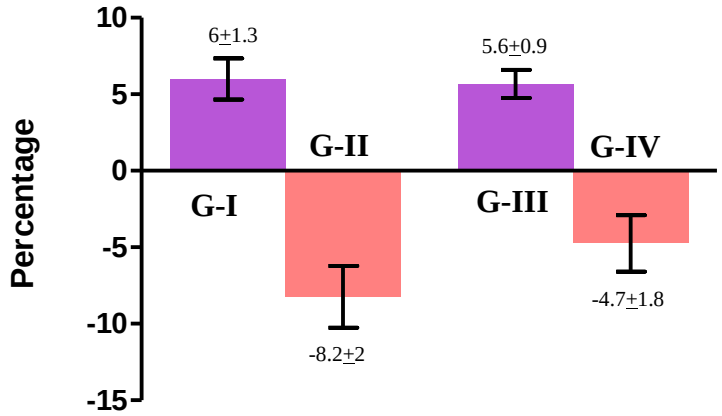
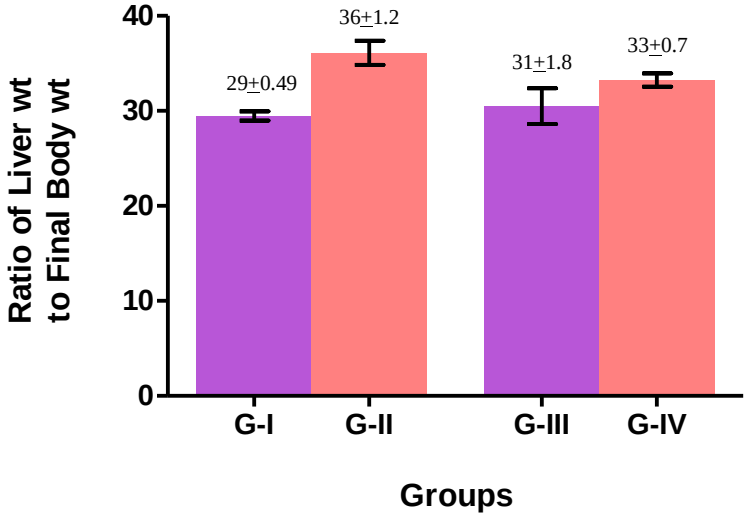


Figure – 11

Graph Showing the Hepato Somatic Index in Normal (G-I), Alloxan Diabetic (G-II),DADS Treated Normal (G-III) and DADS Treated Alloxan Diabetic Rats (G-IV)



5.3. Effect of DADS in alloxan induced diabetic rats:

i) Plasma and liver tissue lipids – Results showed a significant elevation in plasma total lipids, total cholesterol, triglycerides, phospholipids, free fatty acids, esterified fatty acids, total fatty acids, LDL cholesterol, VLDL cholesterol and decrease in HDL cholesterol levels in group II rats as compared to group I rats. No significant difference was observed in group III when compared to group I rats. A significant reduction in plasma lipids was observed in group IV rats as compared to the group II rats, except for esterified fatty acids, total fatty acids and HDL cholesterol in which no significant difference was observed.

Table – 13 Showing changes in plasma lipid profile in normal and alloxan induced diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	Total Lipids(mg/dl)	164± 18.8[a]	265±46.8 [b]	163.5±21.08 [a]	203.6±23.67[c]	14.83	<0.001
2	Triacylglycerols(mg/dl)	60±4.0 [a]	90±9.1 [b]	49.8±6.3 [c]	64±7.4 [d]	33.09	<0.001
3	Total Cholesterol(mg/dl)	58±4.5 [a]	100±6.1 [b]	53±4.8 [a]	77±5.41 [c]	89.33	<0.001
4	Phospholipids(mg/dl)	31±4.1 [a]	66.4±3.8 [b]	30.5±3.2 [a]	42.6±5.3 [c]	83.24	<0.001
5	Free Fatty acids(mg/dl)	4.66±0.64 [a]	6.96±0.61 [b]	4.4±0.3 [a]	5.8±0.39 [c]	26.35	<0.001
6	Esterified Fatty acids(mg/dl)	65±9.74 [a]	90.2±9.39 [b]	68±16.1 [a]	82±12.1 [b]	5.149	0.0090
7	Total Fatty acids(mg/dl)	69.75±9.2 [a]	97.16±9.26 [b]	72.4±16.1 [a]	87.8±11.82 [b]	6.335	0.0037
8	HDL Cholesterol(mg/dl)	35±5.08 [a]	19±2.3 [b]	27.5±4.96 [c]	24±7.64 [b]	8.470	0.0009
9	LDL Cholesterol(mg/dl)	10.3±7.2 [a]	63±5.9 [b]	15.5±2.2 [a]	40.4±5.38 [c]	105.7	<0.001
10	VLDL Cholesterol(mg/dl)	12.0±0.8 [a]	18.0±1.8 [b]	9.9±1.2 [c]	12.8±1.4 [d]	33.09	<0.001

Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$).

Results showed a significant raise in liver tissue total lipids, total cholesterol, triglycerides and phospholipids in diabetic rats as compared to normal rats. No significant difference was observed in group III and group I rats. A significant improvement was observed in group IV rats when compared to group II rats.

Table – 14 Showing changes in liver tissue lipids in normal and alloxan induced diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	Total Lipids(mg/g)	20±2.3 [a]	51±6.9 [b]	19.6±6.7 [a]	30±4.16 [c]	42.17	<0.001
2	Triacylglycerols(mg/g)	14±1.7 [a]	44±2.9 [b]	12.8±3.4 [a]	32±8.2 [c]	53.33	<0.001
3	Total Cholesterol(mg/g)	4.3±0.3 [a]	6.1±0.46 [b]	3.8±0.52 [a]	4.0±0.32 [c]	34.67	<0.001
4	Phospholipids(mg/g)	0.96±0.21 [a]	2.48±0.23 [b]	1.0±0.2 [a]	1.55±0.36 [c]	32.90	<0.001

Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$).

ii) Cholesterol turnover – In the present study, it was observed that alloxan significantly elevated plasma and liver tissue total cholesterol, in group II rats as compared to the group I rats. Treatment with DADS had decreased the plasma and liver tissue total cholesterol significantly in group IV rats when compared to group II rats.

The liver tissue HMG CoA/Mevalonate ratio were significantly different among the four rat groups ($p <0.001$). A non significant reduction in liver tissue HMG CoA/Mevalonate ratio was observed in group II rats as compared to group I rats. Supplementation with DADS had improved the liver tissue HMG CoA/Mevalonate ratio ($p<0.001$) in group IV rats as compared to group II rats.

The fecal bile acids were significantly different among the four groups ($p < 0.001$). A significant decrease in fecal bile acids ($p=0.003$) was observed in group II rats as compared to group I rats. Treatment with DADS had raised the fecal bile acids ($p<0.001$) in group IV rats as compared to group II rats. No significant difference was observed in either HMG CoA/Mevalonate ratio and fecal bile acids in between group III and group I rats.

Figure – 12

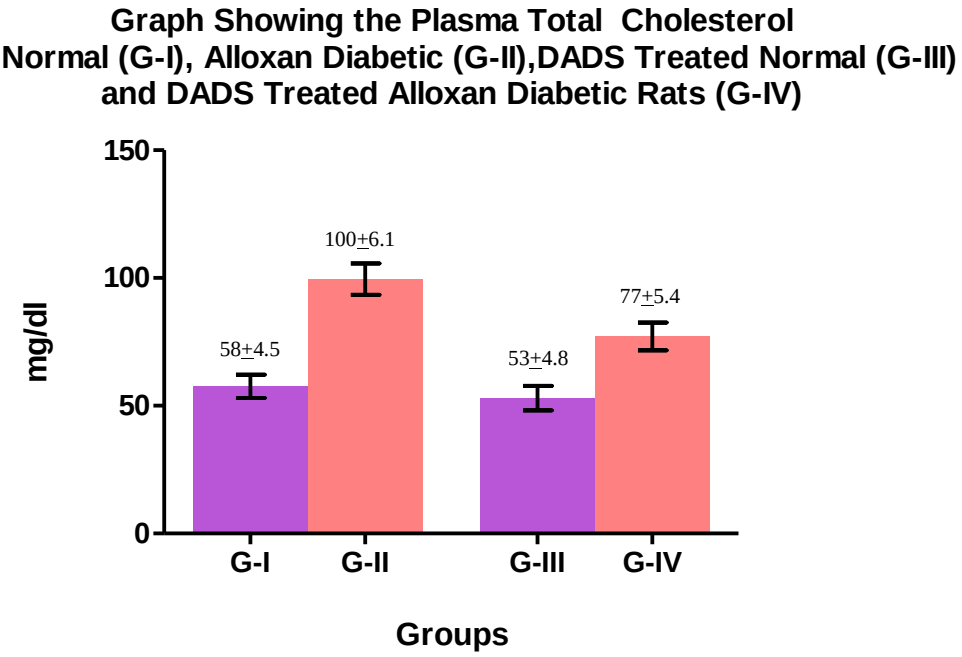


Figure – 13

Graph Showing the Liver Tissue Cholesterol in Normal (G-I), Alloxan Diabetic (G-II),DADS Treated Normal (G-III) and DADS Treated Alloxan Diabetic Rats (G-IV)

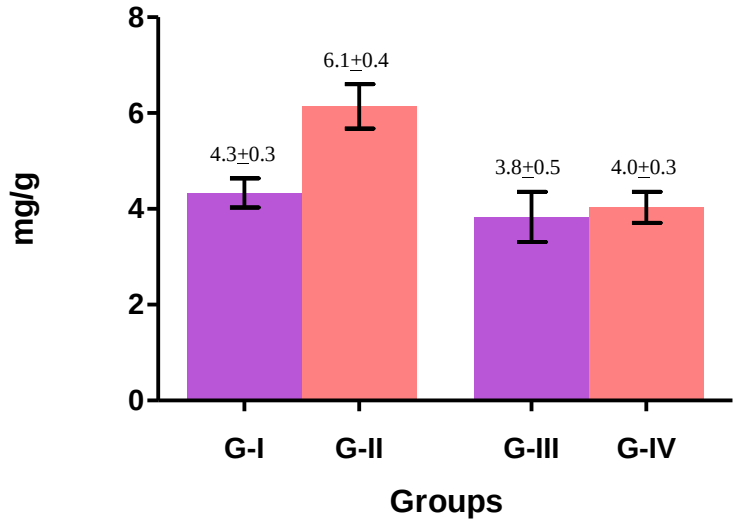


Figure – 14

Graph Showing the Liver Tissue HMG CoA / Mevalonate Ratio in Normal (G-I), Alloxan Diabetic (G-II),DADS Treated Normal (G-III) and DADS Treated Alloxan Diabetic Rats (G-IV)

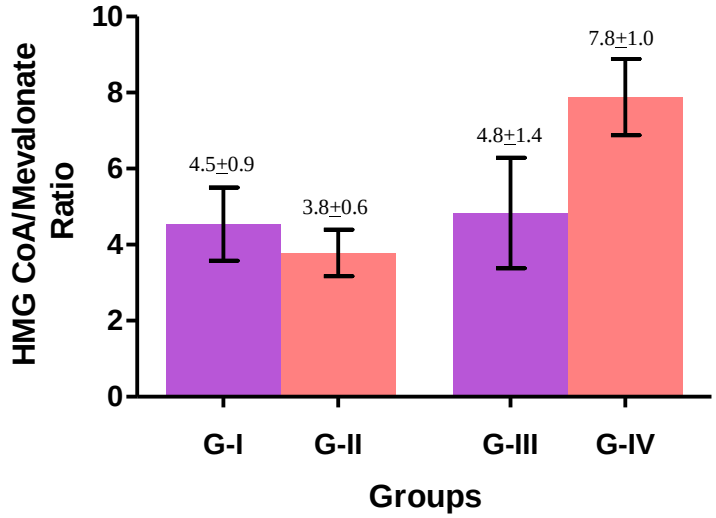
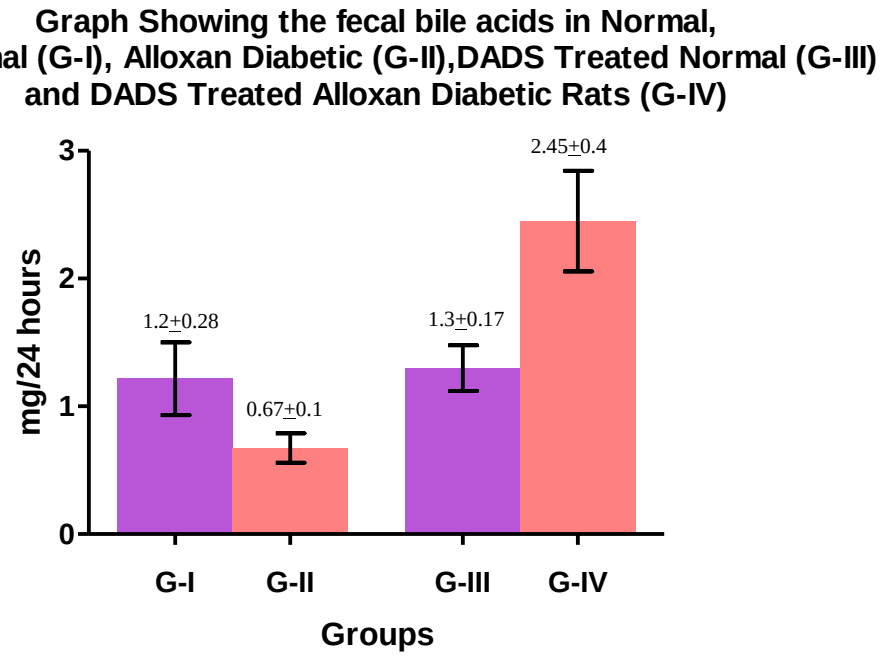


Figure – 15



iii) Blood glucose and Lipoprotein lipase activity – Blood glucose of the rats at the end of the experiment were significantly different among the four groups ($p<0.001$). A significant raise in blood glucose ($p<0.001$) was observed in group II rats in comparison with group I rats. Supplementation of DADS in group IV rats a significant improvement in blood glucose ($p<0.001$) was observed as compared to group II rats.

The lipoprotein lipase activity of the rats at the end of the experiment were significantly different among the four groups ($p<0.001$). A significant decrease in lipoprotein lipase activity was observed in group II rats as compared to group I rats. Treatment with DADS had significantly increased the lipoprotein lipase activity in group IV rats ($p=0.008$) as compared to group II rats. There was no significant difference in either blood glucose level or lipoprotein lipase activity in between group III and group I rats.

Figure – 16

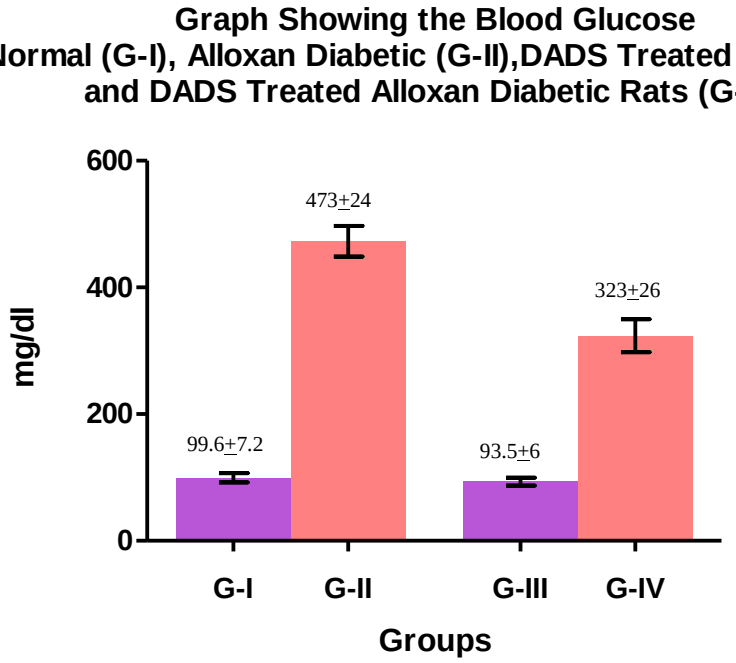
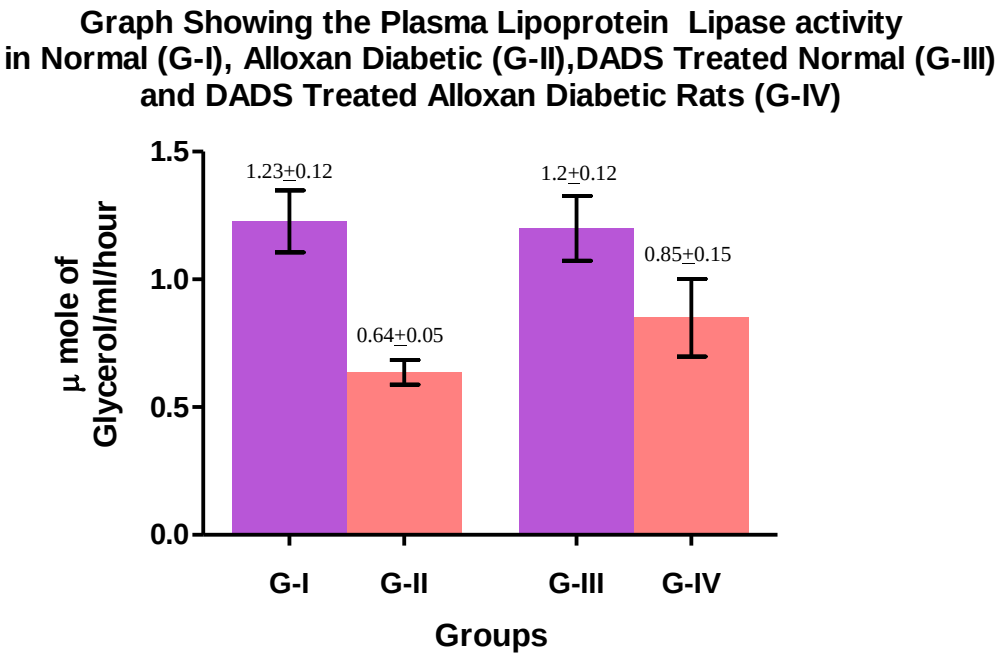


Figure – 17



iv) Oxidative stress and antioxidant status – Results observed in group II rats demonstrated a raise in liver tissue TBARS (p=0.29) and decrease in total thiols (p=0.006) when compared with the group I rats. Supplementation of DADS in group IV rats did not show any significant improvement either in TBARS or total thiol groups as compared to group II rats.

Table – 15 Showing changes in liver tissue Sulphydryl groups and TBARS in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	Sulphydryl Groups(µm/g)	1.09±0.13 [a]	0.75±0.18 [b]	1.06±0.15 [a]	0.86±0.11 [b]	6.448	0.0034
2	TBARS(µmMD/g)	4.97±1.65 [a]	6.14±1.8 [a]	5.04±1.58 [a]	5.55±1.93 [a]	0.5140	0.6776

Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a, b) are significantly different from each other ($p<0.05$).

v) Transaminases in Plasma and Liver tissue – In case of Liver markers, alloxan significantly elevated the plasma and liver tissue AST and ALT levels ($p<0.001$) in group II rats as compare to group I rats. However, treatment with DADS in group IV rats showed an improvement ($p<0.001$) in these transaminases as compared to the group II rats.

Table – 16 Showing changes in plasma and liver tissue AST and ALT in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	AST(U/L) (P)	28.5±6.8 [a]	58±4.7 [b]	31.5±6.9 [a]	38±9.2 [c]	17.79	<0.001
2	ALT(U/L) (P)	35.5±7.5 [a]	69±5.9 [b]	37.5±8.1 [a]	50.8±6.1 [c]	25.72	<0.001
3	AST(U/L) (T)	27±3.5 [a]	42.5±7.07 [b]	30.8±2.99 [a]	33.5±5.74 [c]	9.268	0.0005
4	ALT(U/L) (T)	41±5.1 [a]	54.6±5.0 [b]	39.4±7.8 [a]	47.4±3.28 [c]	8.013	0.0012

Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$).

5.4. To evaluate the effect of DADS on liver histopathology in alloxan induced diabetic rats:

Examination of H&E stained sections of the control group (group I) showed normal architecture. In diabetic rats (group II) liver tissue section showed distortion in the arrangement of cells around central vein, periportal fatty infiltration with focal necrosis of hepatocytes. In DADS treated normal rats (group III), architectural similarity was observed with the normal control group. In group IV rats (DADS treated diabetic rats), there was a reduction in the necrosis of hepatocytes, fatty infiltration and derangement of cells, in comparison with the group II rats.

Group I: Normal Control

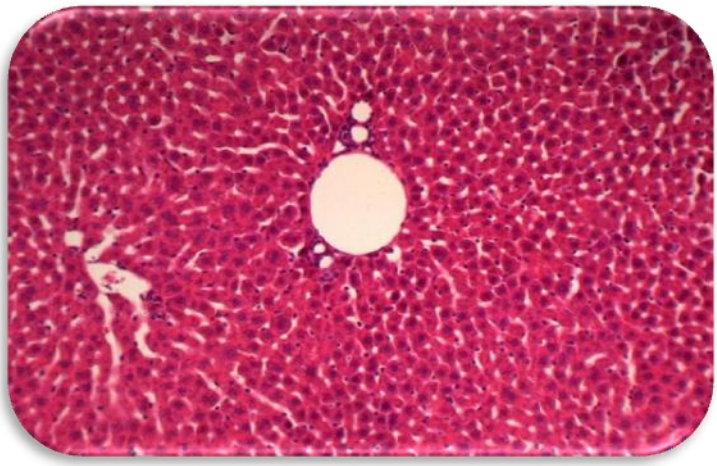


Figure – 18: Liver section of normal control rats (group I) (40x) showing hepatocytes and portal triad comprising (A) Hepatic artery, (B) Hepatic vein, (C) Bile duct.

Group II: Diabetic Control

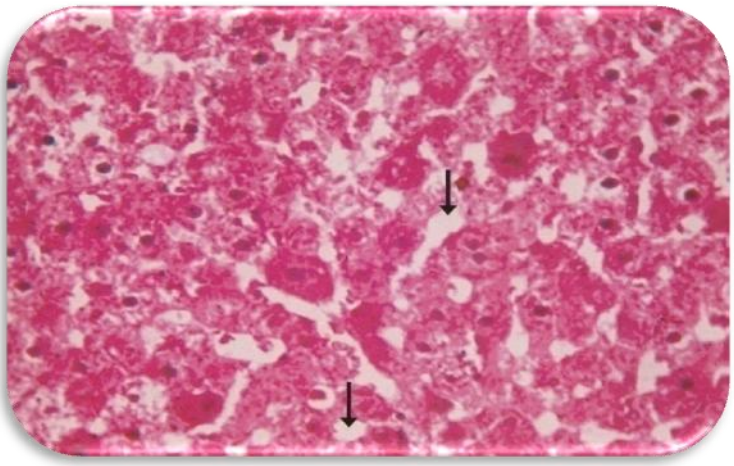


Figure – 19: Liver section of diabetic rats (group II) (40x) showing features of fatty liver (arrow marks indicate deposition of fat)

Group III: Normal rats treated with DADS

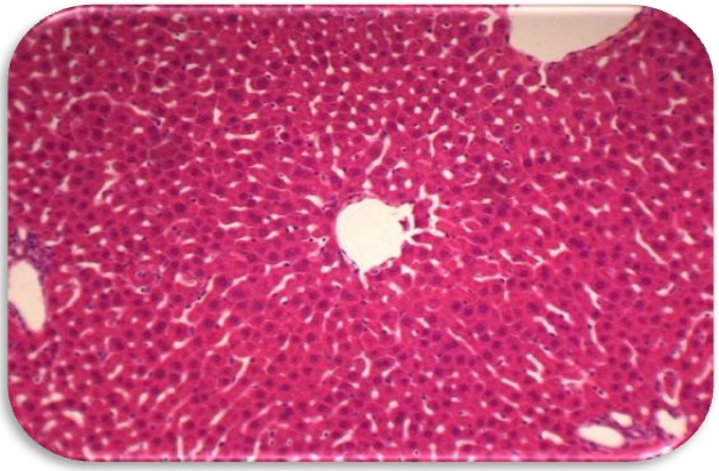


Figure – 20: Liver section of DADS treated normal rats (group III) (40x) showing normal hepatocytes.

Group IV: Diabetic rats treated with DADS

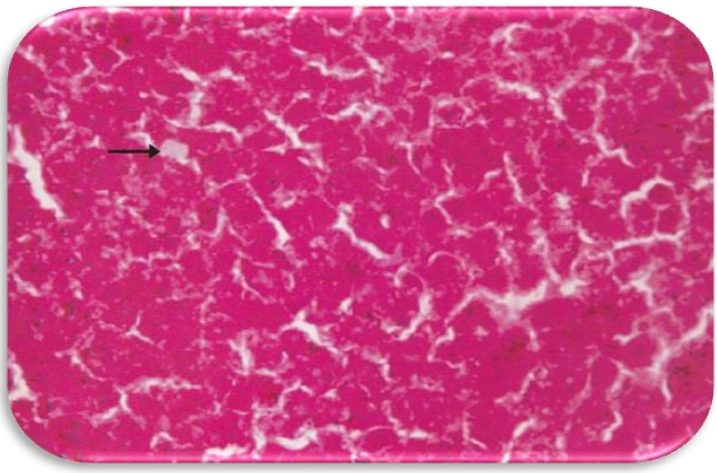


Figure 21: Liver section of DADS treated diabetic rats (group IV) (40x) showing hypolipidemic changes indicated by arrow marks.

Chapter 6

Discussion

Diabetes mellitus (DM) is a chronic metabolic syndrome affecting nutrient metabolism in general and glucose metabolism in particular. DM is characterized by persistent hyperglycemia and disturbance in the metabolism of carbohydrate, protein and fat associated with absolute or relative deficiency in insulin secretion and /or action (Ritz E *et al*., 2011).

Uncontrolled DM may lead to many acute and chronic complications. Acute complications include diabetic ketoacidosis and hyperosmolar hyperglycaemic state. Chronic complications include coronary heart disorder, dyslipidemia, retinopathy, nephropathy and neuropathy (American Diabetes Association, 2010). Dyslipidemia is a chronic complication associated with diabetes that results from insulin deficiency (Kasper DL *et al*., 2005). A similar picture can be seen in alloxan diabetic rats as it is known that alloxan induce profound beta cell damage of islets of langerhans, leading to insulin deficiency (Umesh CSY *et al*., 2004). Umesh CSY *et al* (2004) have demonstrated that in alloxan diabetic rat, the lipid levels in both liver and plasma rise by 48-55%. To counter regulate this diabetes induced dyslipidemia, many herbal preparations were evaluated since the ancient past (Ngugi MP *et al*., 2012).

Most diabetic patients initiate their treatment with dietary restrictions and exercise and remain unsuccessful in controlling diabetes through life style changes alone, prompting the need for a therapeutic management (Michael JF., 2007). Drugs such as biguanides, sulfonylureas, thiazolidines, statins are some of the first line therapeutic agents used in the management of diabetes. These drugs have both beneficial as well as adverse side effects (Chen ZC *et al*., 2001; Michael JF., 2007). Prolonged use of these drugs is associated with adverse side effects which outweigh the benefits of the drugs. Prolonged use of biguanides were reported to cause gastrointenstinal disorders and lacticacidosis, sulfonylureas have the

risk of hypoglycemic effect, thiazolidines are associated with hepatotoxicity (Michael JF., 2007) and statins were reported to increase the risk for myositis, myalgia, and liver damage (Beatrice AG *et al*., 2008).

To minimise the adverse side effects of these drugs, many medicinal plants were used in the past, which have hypoglycemic and hypolipidemic activities (Prakasham A *et al*., 2004; Yu-Yan Y *et al*., 2001). Studies conducted by the World Health Organisation (WHO) reported that 80% of the world's population relies on medicinal plants for their primary health care needs (Ngugi MP *et al*., 2012).

One of such medicinal plant, Garlic (*Allium* sativum linn) is known for its anti-hyperglycemic, anti-hyperlipidemic, anti-atherogenic properties (Vijay V *et al*., 2013). Many of these properties were attributed to the principle sulphur compound of garlic: Diallyl Disulphide (DADS) (Yu-Yan Y *et al*., 2001). However, some of the published reports failed in demonstrating the hypolipidemic effect of garlic (Superko HR *et al*., 2000; Jonathan LI *et al*., 1998). This discrepancy may be attributed to procedural/methodological shortcomings, such as: inappropriate methods of randomization, lack of dietary run in period, short duration, inappropriate modes of administration and inadequate statistical power (Neil HA *et al*., 1994; Neil HA *et al*., 1996; Silagy C *et al*., 1994; Warshafsky S *et al*., 1993). The present study was undertaken, to address these discrepancies and to determine the hypolipidemic effect of Diallyl Disulphide in alloxan induced diabetic rats.

6.1.1 Optimum effective dosage of DADS: Several clinical reports, including meta-analysis, have revealed a lipid lowering effect of garlic (Varsha G. 2013). However, more recent reports suggested that, not all the preparations may be hypolipidemic (Soni MSES *et al*., 2004; NMJ Contributors 2010). Although the exact reasons for this inconsistency remains unknown, it probably can be attributed to different garlic preparations (garlic powder, aged garlic extract, garlic oil, raw garlic), unknown active components and their bioavailability, different subject health status, gender differences, as well as the duration of different trails. In this context, need for standardised preparations of garlic with known active components are necessary as suggested by Yen PL *et al*., (2008). Earlier workers have shown that garlic and its extracts have significant hypoglycemic and hypolipidemic properties (Veena GR *et al*., 2012) and these actions were primarily attributed to garlic's principle organosulphur compound – DADS (Yu-Yan Y *et al*.., 2001). However, there is a visible scant of data regarding the usefulness of DADS in alloxan diabetic rats. Given this context, an array of experiments to establish the optimum effective dosage of DADS in regulating diabetes induced dyslipidemia in the present study. A set of specific biochemical parameters – plasma and liver tissue total lipids, total cholesterol, triacylglycerols and phospholipids have been chosen as markers for diabetes induced alterations in alloxan diabetic rats, who have been treated with various dosages of (50, 100, 150 mg/kg body weight) DADS for a period of 30 days. The results given in the table-11, clearly indicate that a 100 mg DADS per kg body weight is quiet satisfactory in regulating diabetes induced changes in these parameters. Considering this, we chose 100 mg of DADS per kg body weight as the optimum effective dosage in regulating diabetes induced dyslipidemic alterations in alloxan diabetic rats. The smaller reduction in lipid levels in group A indicate that a dosage of 50 mg/kg body weight of DADS was not sufficient to reduce lipid levels effectively and in group C dosage of 150

mg/kg body weight of DADS may have been linked to lesser tolerability to higher dosage, highlighting the importance of correct dosing and choice of active components. The dosage above 150 mg of DADS per kg body weight seems to be lethal as the rats did not survive up to 30 days. Moreover, lack of significant hypolipidemic activity at 50 and 150 mg/kg body weights underscore the utility of 100 mg/kg body weight dosage as optimum.

6.1.2 Gravimetric analysis: Results show loss in percent body weight among the group II rats. Alloxan induced insulin deficiency might be responsible for excessive burning of fat and loss of muscles causing decrease in percent body weight. DADS supplementation had a beneficial effect in reducing the loss of body weight in group IV rats.

A significant increase in hepato somatic index in group II rats as compared to group I rats indicates alloxan induced damage to the liver in group II rats. Similar observations were previously reported by Sunmonu TO *et al*., (2012). Bearing in mind that hepato somatic index represents the degree of damage to liver tissue, it is easy to infer that the DADS treatment might have reduced the hepato somatic index and damage to the liver among group IV rats.

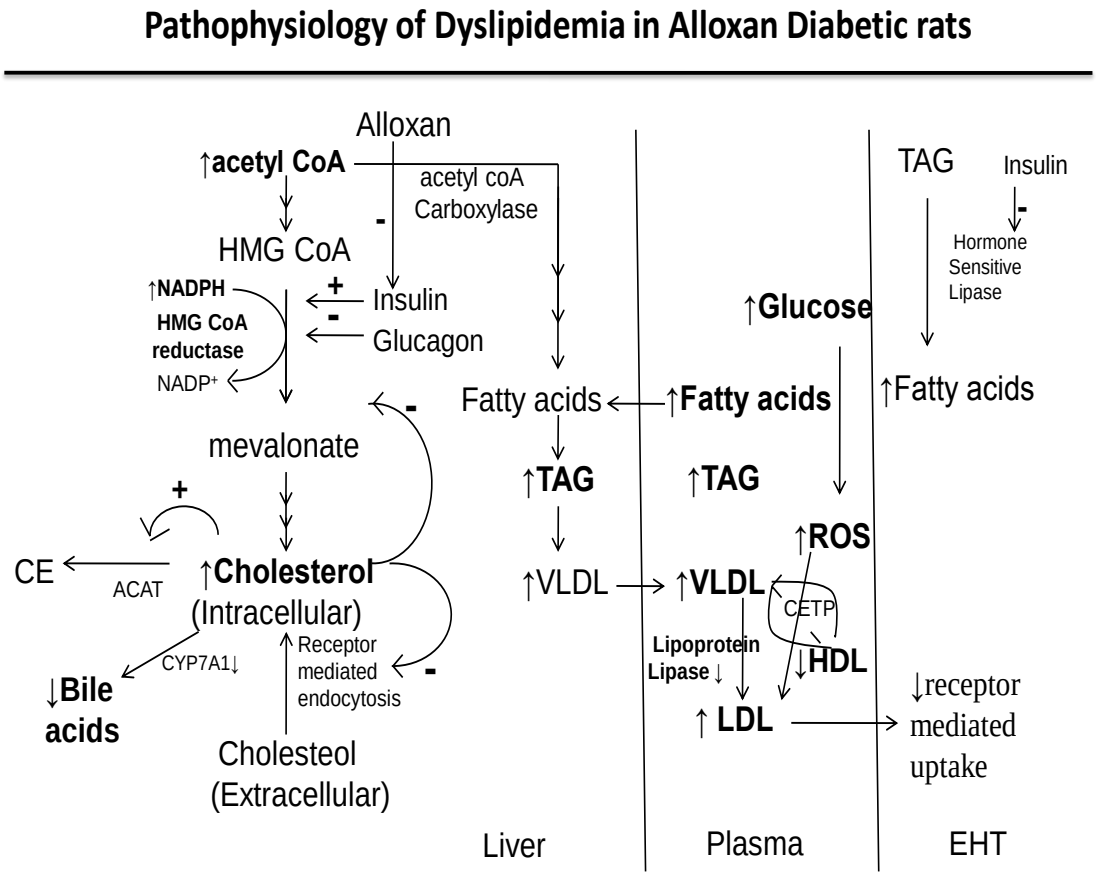
6.2 Effect of Diallyl disulphide on Plasma and Liver tissue lipids: The antihyperlipidemic effect of garlic and its organosulfur compounds were previously established in hyperlipidemic rats (Martha T *et al*., 2007). However, few clinical trials failed to approve this lipid lowering effect of garlic and its extracts (Superko HR *et al*., 2000; Jonathan LI *et al*., 1998), while many clinical trials approve this effect (Rizwan A *et al*., 2005). DM induces dyslipidemia and gross alterations in plasma and liver tissue lipid levels. A significant increase is observed in plasma total lipids, total cholesterol, triacylglycerols, phospholipids, free fatty acids, esterified fatty acids, total fatty acids, LDL-cholesterol, VLDL-cholesterol and liver tissue total lipids, total cholesterol, triacylglycerols, phospholipids and decrease in plasma HDL-cholesterol in alloxan induced diabetic rats (group II) as compared to normal rats (group I) is due to insulin deficiency caused by alloxan beta cell damaging effect (Rotimi SO *et al*., 2013) and is in agreement with earlier reports (Rotimi SO *et al*., 2013, Umesh CSY *et al*., 2004).

The increase in plasma and liver tissue total cholesterol might be due to the reduced catabolism of cholesterol (or) reduced activity of hepatic cholesterol 7 alpha hydroxylase, the rate limiting enzyme in bile acid synthesis from cholesterol (Szymanski *et al*., 1981). Increase in plasma cholesterol in diabetic rat can also be due to the decrease activity of LDL receptor of hepatocytes, which would reduce the synthesis of bile acid and increase HMG CoA reductase activity in liver. Increased plasma cholesterol levels might have even elevated the plasma LCAT activity significantly in alloxan induced diabetic rats (Emara, 1999).

Marked elevation of triglycerides in alloxan induced diabetic rats might be a consequence of either i) Over production of VLDL by the liver, which is attributed to increased availability of acetyl CoA – required for ATP generation and a substrate for the biosynthesis of triglycerides or ii) Defective removal of triglyceride rich lipoproteins from the

circulation, or both, the later possibility can be explained through decreased lipoprotein lipase activity, an insulin dependent enzyme involved in triglyceride removal (Yost *et al*., 1995). An increase in the plasma phospholipids in diabetic rats might be due to increased activity of choline phosphotransferase enzyme involved in phospholipids synthesis.

Figure – 22



Increase in the LDL cholesterol in diabetic rats may be related to increased degradation of IDL to LDL by the action of hepatic lipase or due to reduced catabolic rate of LDL cholesterol (Shepherd *et al*., 1980). Moreover, the production of LDL exceeds the capacity of LDL receptor uptake i.e., efflux of cholesterol from the liver is more than influx.

In alloxan induced diabetic rats, the decrease in HDL cholesterol is related to hypertriglyceridemia, which had lead to enhanced catabolism of HDL apo A-I fraction and this is possibly due to enhanced HDL cholesterol ester transfer to triglyceride rich lipoproteins (Li *et al*., 1994).

DADS (100 mg/kg body weight) significantly lowered the plasma and liver tissue lipids in diabetic rats (group IV) as compared to alloxan induced diabetic control rats (group II), which is in concordance with other reports (Varsha G 2013). DADS is a disulphide that undergoes reduction to its thiols similar to any other disulphide by using NADPH/NADH as follows –

$$C_3H_5\text{-}S\text{-}S\text{-}C_3H_5 + 3NADPH + H^+ \longrightarrow 2C_3H_7\text{-}SH + 3NADP^+ \quad \rightarrow I$$

Diallyl Disulphide

It is proposed that such a reaction of DADS with NADPH may reduce cellular levels of NADPH, hence lowers fatty acid and cholesterol synthesis as their synthesis requires sufficient supply of NADPH (Sunanda M *et al*., 2014). Further reduction in liver tissue lipids in part may also be due to the modulating effect of DADS, as it is known that DADS can undergo sulphydryl exchange reactions with thiol proteins and thiol enzymes as follows (Gebhardit R *et al*., 1996; Ziegler DM. 1985).

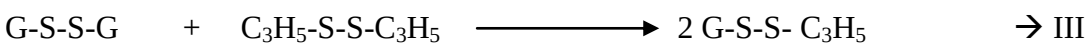
$$Enz\text{-}SH + C_3H_5\text{-}S\text{-}S\text{-}C_3H_5 \longrightarrow Enz\text{-}S\text{-}S\text{-}C_3H_5 + C_3H_5\text{-}SH \quad \rightarrow II$$

Such possible sulphydryl exchange reaction of DADS in reducing the activity of lipogenic enzymes such as – fatty acid synthase, HMG CoA reductase, glycerol phosphate dehydrogenase, could possibly be attributed to the lowered levels of liver tissue as well as plasma lipids in group IV rats as compared to group III rats.

The hypolipidemic action of DADS in alloxan induced diabetic rats was previously reported as a consequence of increase in cholesterol degradation to bile acids and neutral sterols, mobilization of triglycerides and increase in the catabolism of triacylglycerols by increased lipoprotein lipase activity (Rajasree *et al*., 1999). Moreover lipoprotein lipase activity is relevant to HDL cholesterol production. Previous studies demonstrated that enhanced lipolysis of triglyceride rich lipoproteins may lead to an increase in HDL cholesterol (Guillausseaun *et al*., 1992).

A significant increase in lipoprotein lipase and decrease in LCAT activities was recorded accompanied by the administration of DADS in diabetic rats. This decrease in LCAT activity might be attributed to the decreased cholesterol levels derived from VLDL (Heller *et al* 1981).

DADS can probably be involved in an exchange reaction with oxidised glutathione (G-S-S-G) as illustrated below.

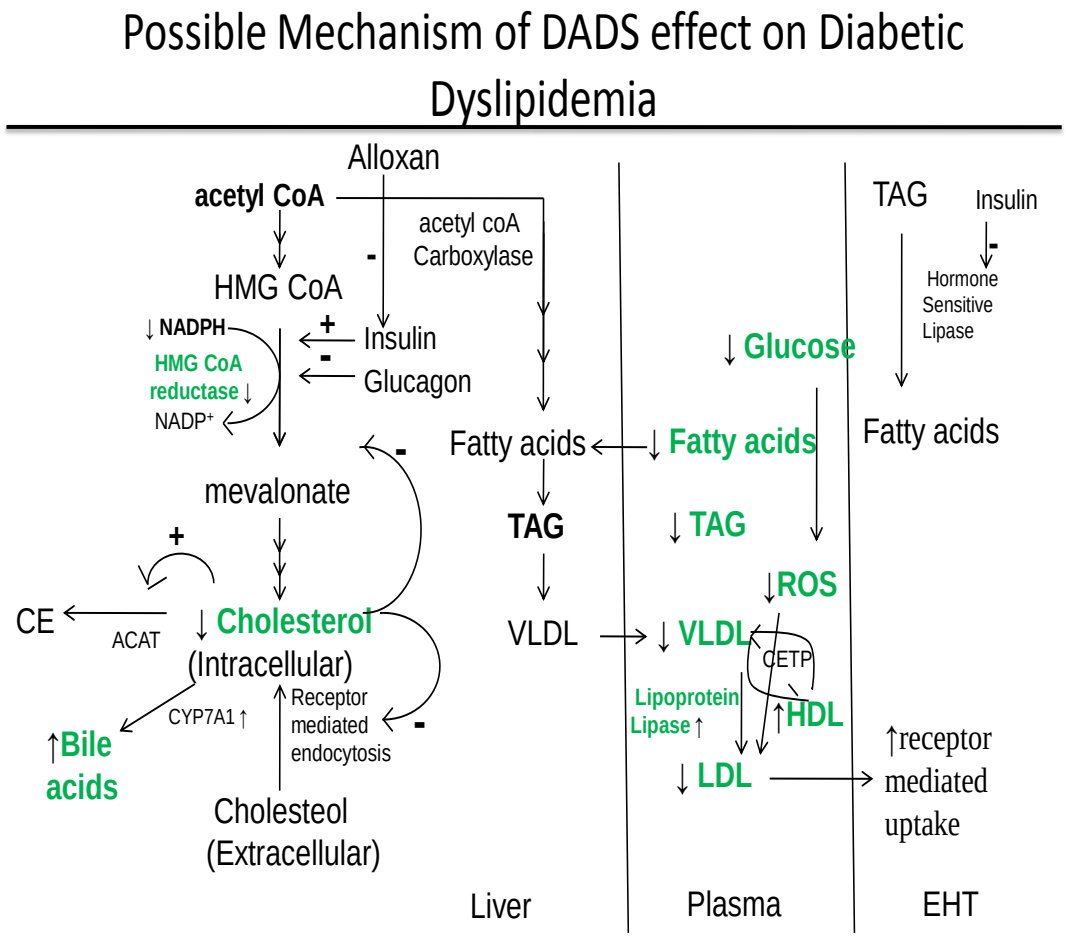


Oxidised Glutathione DADS

Thus, the inactivation of glutathione reductase in an exchange reaction with DADS and the utilization of oxidised glutathione, the substrate of the enzyme are accompanied by a decrease in the activity of the enzyme. DADS administration also significantly raised insulin levels in the blood (Raju P *et al*., 2011). The disulphide bonds in insulin may be reduced directly by reduced glutathione or in a reaction catalysed by liver glutathione-insulin transhydrogenase as shown below.

$$\underset{\text{Insulin}}{A\ (S\text{-}S)\ B} + \underset{\text{Reduced Glutathione}}{4\ GSH} \longrightarrow \underset{\text{Oxidised Glutathione}}{2\ G\text{-}S\text{-}S\text{-}G} + \underset{\text{Reduced Insulin}}{A\ (SH)_2 + B\ (SH)_2} \rightarrow IV$$

Figure – 23



The reduced glutathione is regenerated when the oxidised glutathione is reduced by glutathione reductase in the presence of NADPH.

$$G\text{-}S\text{-}S\text{-}G + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+ \rightarrow V$$

The degeneration of insulin can therefore be sustained by the increased availability of GSH. In the presence of DADS, reaction I reduces the concentration of NADPH and reaction III reduces the blood levels of GSSG and therefore, reaction V becomes insignificant. As GSH becomes unavailable, reaction IV becomes reduced and therefore insulin half life increases.

6.3 Effect of Diallyl disulphide on Liver tissue cholesterol turnover: The lipid of most health concern, cholesterol and a steroid is mainly synthesized in the liver starting from acetyl CoA through a series of reactions regulated by the key enzymes HMG CoA reductase (Russell ADB. 2008). Cholesterol, so synthesized is principally utilized for synthesis of bile acids apart from being converted to other useful products in the body (Chiang JY. 2013). In normal control rats, an intricate balance is maintained between the biosynthesis, utilization and transport of cholesterol, keeping its harmful deposition to a minimum. The multiple risk factor intervention trial (MRFIT) and the Framingham heart study have reported an increase in CAD risk by 3% in men and women with every milligram decrease in HDL levels (Schlant RC *et al*., 1994). Furthermore, cholesterol homeostasis is ensured by the coordinated interaction of LDL receptor expression, HMG CoA and LCAT activity.

Catabolism of cholesterol to bile acids is quantitatively the most important pathway of elimination of cholesterol from the body. It is said that changes in the rate of synthesis of bile acids are nearly paralleled by corresponding changes in the rate of cholesterol biosynthesis in the liver. Bile acids include primary bile acids – cholic acid, chenodeoxy cholic acid and secondary bile acids deoxycholic acid and lithocholic acid formed by the action of intestinal micro flora. These secondary bile acids are excreted through the fecal material. The amount of bile acids excreted in the faeces substantiates the amount of the cholesterol utilized through the bile acid pathway in liver (Murray K *et al*., 2000). Hence to assess the cholesterol turnover in the liver in the present study, liver tissue cholesterol content, the rate of activity of the enzyme HMG CoA reductase and the bile acid content of 24 hours fecal material was carried out. HMG CoA reductase activity was calculated as the ratio of HMG CoA/Mevalonate and an increase in the ratio indicated lowered activity where as a lowered ratio suggested an increased activity.

The results of the present experiment as depicted in graph-5.5 , clearly indicates that the HMG CoA reductase activity is significantly raised in alloxan diabetic rats (group II) as compared to normal control rats (group I) (Feingold KR *et al*., 1994) and significant lowered activity is seen in DADS treated alloxan induced diabetic rats (group IV) as compared to alloxan induced diabetic rats (group II) suggesting that DADS has a HMG CoA reductase suppressing action as shown by earlier workers (Lijuan L *et al*., 2002). Recent studies indicate that DADS even inhibits 4 alpha methyl oxidase, resulting in accumulation of linosterol and 4,4-dimethyl zymosterol, which then strongly promoted the feedback inhibition of HMG CoA reductase. By this effect DADS, is able to significantly reduce the biosynthesis of cholesterol in liver of alloxan induced diabetic rats.

A similar sulphydryl exchange reaction with cholesterol degrading enzymes (bile synthesizing enzyme – 7 α-hydroxylase) may promote the conversion of cholesterol into bile acids hence inducing a significant rise in fecal bile acids as seen in present study (Rajasree CR *et al*., 2008).

6.4 Effect of Diallyl disulphide on Plasma glucose and Lipoprotein lipase activity: Alloxan specifically damaging the beta cell of langerhans causes severe lack in insulin levels inducing a steep raise in plasma glucose levels. Elevation in blood glucose levels as seen in the present study is in agreement with previous reports (Mahmoud AA *et al*., 2009). It is seen in DADS treated alloxan diabetic rats (group IV), the levels of plasma glucose is significantly lowered as compared to group II rats. DADS can function as an effective hypoglycemic agent probably by decreasing cellular NADH/NADPH levels, hence resulting in a transient rise in NAD/NADP levels. This may enhance glucose utilising pathway. It is known that pyruvate dehydrogenase complex, alpha keto glutarate dehydrogenase complex, isocitrate dehydrogenase etc., are activated by NAD levels (Mallikarjuna Rao N. 2006). Insulin is a hypoglycemic hormone having alpha and beta chains interlinked by disulphide bridges. An NADPH dependent enzyme, insulinase or insulin transhydrogenase is involved in insulin degradation (Duckwort WC *et al*., 1988). A decrease in NADPH levels caused by DADS may limit insulinase action causing increase in half life of insulin. This leads to prolonged insulin action and hypoglycaemia.

DADS is a disulphide and similar to any other disulphide it may undergo sulphydryl exchange reaction with enzymes and proteins, as it known that disulphides can undergo such a reaction (Gebhardit R *et al*., 1996).

$$Enz\text{-}SH + C_3H_5\text{-}S\text{-}S\text{-}C_3H_5 \longrightarrow Enz\text{-}S\text{-}S\text{-}C_3H_5 + C_3H_5\text{-}SH$$

Such a sulphydryl exchange reaction with insulinase enzyme may delay insulin degradation and increasing its half life thus promoting hypoglycaemia. The glucose transporter molecules which are involved in glucose transport are further degraded by GLUT degrading systems or enzymes. A sulphydryl exchange reaction by DADS with GLUT

degrading systems may prolong the actions of GLUT molecules, hence more glucose is transported and utilised, hence favouring hypoglycaemia.

Marked hypertriglyceridemia observed in diabetic rats might be a consequence of decreased activity of lipoprotein lipase, an insulin dependent enzyme involved in triglyceride removal, as observed in the present study (Maximilian VE *et al*., 2004). DADS administration has increased the lipoprotein lipase activity in diabetic rats, which in turn can promote the catabolism of triglyceride rich lipoproteins (Hussein SA *et al*., 2004).

6.5 Effect of Diallyl disulphide on Oxidative stress and antioxidant levels: Studies suggest that oxidative stress is known to play a key role in the pathogenesis of diabetes mellitus (Ahmed RG. 2005). Alloxan, when administered intraperitoneally to albino rats, produce insulin dependent diabetes mellitus by selectively destroying the beta cells of islets of langerhans in the pancreas, as alloxan is a toxic analogue of glucose. Alloxan administration disproportionately produced free radicals like lipid peroxidation hydroperoxide, conjugated dienes by glucose oxidation, non-enzymatic glycation of proteins, oxidative degradation of glycated proteins and lipid peroxidation resulting in tissue damage (Sabu MC *et al*., 2004). Free radicals are very unstable molecules containing unpaired electrons, derived from the univalent reduction of oxygen and giving rise to numerous by products through their reactions with almost all unsaturated double bonds found in living cells (Sivakumar V *et al*., 2010). Free radicals are highly reactive and present challenges to the functions as decrease in membrane fluidity, loss of enzyme activity and damage to the membrane proteins leading to cell inactivation and hence cells have developed certain mechanisms to scavenge them (Dean RT *et al*., 1993). The protection of cell against free radicals can be accomplished through superoxide dismutase, catalase and glutathione peroxidase which are considered as primary anti-oxidant enzymes since they are involved in the direct removal of free radicals (Sivakumar V *et al*., 2010). Glutathione S-transferase and glutathione reductase are secondary anti-oxidant enzymes which help in the detoxification of reactive oxygen species by decreasing the peroxide levels and maintaining a steady supply of metabolic intermediates like glutathione and NADH for the primary antioxidant enzymes (Ghosal S *et al*., 2000). In the present study, the total TBARS levels are considered equivalent to total free radicals and total thiols as an approximate measure of total antioxidant mechanism. It is evident from these results that in alloxan induced diabetic rats, liver tissue

TBARS levels are significantly increased, where as liver tissue total thiols are significantly lowered (Yogesh M *et al*., 2013; Periyar SS *et al*., 2013). The liver tissue TBARS and total thiols given in table-15 show no significant change in group IV rats when compared to group II rats, suggesting DADS did not had any beneficial effect in reducing the oxidative stress.

6.6 Effect of Diallyl disulphide on Transaminases in plasma and liver tissue: Transaminases are the enzymes involved in the synthesis of non essential amino acids from non protein metabolites. These are pyridoxal phosphate dependent enzymes (Donald Voet *et al*., 2008). The most significant transaminases are aspartate transaminase (AST) and alanine transaminase (ALT).

The liver helps maintain blood glucose concentration in the fasting and postprandial state. Loss of insulin effect on the liver leads to glycogenolysis and an increase in hepatic glucose production. Up-regulation of sterol regulating element binding protein 1c, leading to increased lipogenesis in the liver causes increased intracellular availability of triglycerides, promoting fatty liver. The fatty liver state in diabetic rats is known to be directly toxic to hepatocytes. Putative mechanisms for elevated transaminases include cell membrane disruption at high concentration, mitochondrial dysfunction, toxin formation, and oxidative stress from lipid peroxidation, peroxisomal beta oxidation, recruited inflammatory cells and inhibition of key steps in the regulation of metabolism (Maddrey WC *et al*., 2007). The insulin deficit state is also characterised by an increase in proinflammatory cytokines such as tumour necrosis factor-alpha, which may also contribute to hepato cellular injury and rise in the plasma and liver tissues transaminases (Paul TG 2005). It has also been shown by other studies that both AST and ALT levels in liver and plasma are raised in diabetes mellitus, because of high rate of glycogenolysis, gluconeogenesis and liver cell destruction (Muhammad Zafal *et al*., 2009).

In the present study, a significant elevation in activity of plasma and liver tissue AST and ALT is observed in alloxan induced diabetic rats (group II) as compared to normal control rats (group I). Feeding optimum dosage of DADS (100 mg/kg body weight) to alloxan diabetic rats (group IV) for a period of 30 days significantly reduced the activity of

plasma and liver tissue AST and ALT levels as compared to group II rats (Sankaran M *et al*., 2010; Ohaeri OC. 2001), indicating the employed disulphide might have helped in suppressing the activities of AST and ALT, thus suppressing the gluconeogenesis and damage to the liver.

6.7 Effect of Diallyl disulphide on Liver Histopathology: Examination of H&E stained liver sections of control group showed normal architecture, including hepatic lobules with branching and anatomising cords of hepatocytes radiating from central vein. The cells appeared to be separated by the blood sinusoids.

H&E stained sections of alloxan induced diabetic rats showed a marked structural alteration characterized by degenerative changes in the hepatocytes and contained fatty vacuoles giving them foamy appearance. It could be due to increased influx of fatty acids into the liver induced by hypoinsulinemia and the low capacity of secretion of lipoprotein from liver resulting from a deficiency of apo B synthesis (Muhammad Z *et al*., 2009). This damage was partially reversed by DADS treatment and is similar to that observed by the administration of *vinca rosea* extract in alloxan induced diabetic rats (Ghosh *et al.,* 2001).

Chapter 7

Summary & conclusions

7.1 Limitations of the study:

1. Duration of the study was fixed to 30 days in the present study, which was a limitation of the study. By increasing or decreasing the duration of the study, the effectiveness of DADS could have been determined.
2. In the present study the toxic effects of DADS at a very high doses was not determined.

7.2 Summary and Conclusion:

1. The purpose of the study was to establish the optimum effective dosage of DADS and determine the hypolipidemic effect of DADS in alloxan induced diabetic rats.
2. A total of 42 healthy wistar strain male albino rats weighing between 200-250 grams were randomly selected. Of these, 18 rats were divided into three groups to determine the optimum effective dosage and remaining 24 rats were divided into four groups to determine the effect of DADS. Each group consisted of six rats.
3. The hypolipidemic effect was observed with and without the treatment of DADS for 30 days in normal and alloxan induced diabetic rats.
4. The following parameters were tested with and without the treatment of DADS for 30 days. Total lipids, total cholesterol, triacylglycerols, phospholipids, free fatty acids, esterified fatty acids, total fatty acids, LDL, HDL, VLDL-cholesterol, HMG CoA reductase activity, fecal bile acids, lipoprotein lipase activity, blood glucose, thiobarbituric acid reactive substances, total thiols, AST & ALT.
5. DADS significantly lowered the raised plasma and liver tissue lipids in alloxan induced diabetic rats. This hypolipidemic effect of DADS in alloxan diabetic rats may be due to lowered NADPH levels as the reduction of DADS to its constituent thiols requires NADPH.
6. The hypocholesterolemic effect of DADS in alloxan diabetic rats observed is due to partial inhibition of HMG CoA reductase as evidenced by increased levels of HMG CoA/mevalonate ratio.

7. The observed hypocholesterolemic action of DADS is partly due to increased catabolism of cholesterol to bile acids as evidenced by the increased excretion of fecal bile acids in alloxan diabetic rats.

8. DADS at the dose employed in present study, significantly lowers plasma glucose levels, probably by increasing glucose utilization.

9. DADS did not show a significant reduction in TBARS and improvement in total thiol groups in diabetic rats when compared to group II.

10. DADS show a decreased activity of transaminases in alloxan diabetic rat liver by suppressing the gluconeogenesis in the liver.

11. DADS may reduce the diabetes mellitus induced liver changes as evidenced by histological studies.

12. There was no significant difference between the normal rats and DADS treated normal rats in all the parameters assessed, proving the safety of DADS dose employed.

13. From these findings, we conclude that DADS treatment is an effective means to control diabetes induced dyslipidemia in wistar strain male albino rats. Even the blood glucose levels, transaminases and liver morphology were resorted by the treatment with DADS.

Future directions of the study:

1. Future studies are required to see the effectiveness of DADS by changing the duration of treatment.
2. Future studies are carried out to check the toxic effects of DADS at higher doses.
3. Future studies are carried out to find the mechanism by which DADS is beneficial.
4. Further research is carried out to find out more active compounds of garlic having the beneficial effects.

Chapter 8

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Chapter 9

Annexures

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Experimental Research

Biochemistry Section

Effect of Diallyl Disulphide on Diabetes Induced Dyslipidemia in Male Albino Rats

NAVEEN KUMAR SAMBU[1], R.T.KASHINATH [2], J.G.AMBEKAR[3]

ABSTRACT

Background: Diabetes Mellitus is a chronic metabolic disorder which may lead to various complications, the important being dyslipidemia leading to Coronary Heart Disorders (CHD), the major cause for morbidity and mortality in diabetic patients. Diabetes Mellitus could be treated by nutritional therapy/drug therapy and others. But the drug therapy would have its own limitations and side effects. To overcome from this an herbal extract is recommended, such as Diallyl Disulphide (DADS) a principle compound of Garlic oil.

Aim: To assess the hypolipidemic effect of Diallyl Disulphide (DADS) in alloxan induced diabetic rats.

Materials and Methods: Healthy adult wistar strain male albino rats weighing around 100-150 grams were randomly selected from the animal house at BLDE University's Shri B.M.Patil Medical College, Hospital and Research Centre, Bijapur, India. Diabetes was induced using alloxan and was treated with DADS. After a stipulated time the rats were anesthetised and sacrificed to collect the blood and liver tissue. Various Lipid parameters, HMG CoA Reductase, Fecal bile acids were estimated in the blood, feces and homogenised liver tissue using standard procedures.

Statiscal Analysis: One-way ANOVA followed by post-hoc t-test is done.

Result: There was significant decrease in the blood and liver tissue lipid parameters of DADS treated alloxan induced diabetic rats when compared to the alloxan induced diabetic rats.

Conclusion: From this study it can be concluded that the DADS a principle compound of garlic, definitely has the hypolipidemic effect in diabetic rats, which is reducing the morbidity in diabetic cases due to dyslipidemia without the adverse effects.

Keywords: Diabetes mellitus, Dyslipidemia, HMG CoA Reductase

INTRODUCTION

Diabetes Mellitus (DM) is a variable disorder of carbohydrate metabolism characterized by reduced insulin secretion (or) decreased glucose utilization [1]. Diabetes Mellitus may lead to many acute and chronic complications. Acute complications include diabetic ketoacidosis and hyperosmolar hyperglycaemic state. Chronic complications include coronary heart disorder, dyslipidemia, retinopathy, nephropathy and neuropathy [1]. Dyslipidemia is one of the major complications associated with DM [2]. If the lipid levels are not controlled may lead to coronary heart disorder [3].

Most diabetic patients start the treatment with diet and exercise but, unfortunately most patients are unsuccessful in controlling diabetes through life style change alone and require drug therapy [4]. Drugs like Biguanides, Sulfonylureas, Thiazolidines, Statins are some of the first medications used in the treatment of diabetes. These drugs have got beneficial as well as adverse side effects [4,5]. In the long run the adverse side effects outweigh the benefits of these drugs. Biguanides reported gastrointenstinal upset and lacticacidosis, Sulfonylureas have the risk of hypoglycemic effect, Thiazolidines are associated with hepatotoxicity [4] and Statins have the risk of myositis, myalgia, liver damage [6].

To substantiate the adverse side effects of these drugs many medicinal plants are in use, which have the hypoglycemic and hypolipidemic activities [7,8]. One among them is garlic (*Allium sativum linn*) known for its anti-hyperglycemic, anti-hyperlipidemic, anti-atherogenic properties [9,10] and many of these properties were attributed to the principle sulphur compound of garlic: Diallyl Disulphide (DADS). Few of the earlier studies fail to confirm the hypolipidemic effect of garlic [11,12]. The present study was under taken to determine the hypolipidemic effect of DADS in alloxan induced diabetic rats.

MATERIALS AND METHODS

Sigma Aldrich chemicals have supplied, Alloxan and Diallyl disulphide (DADS) required for the study. Healthy wistar strain male albino rats weighing around 100-150 gm were randomly selected from the animal house, BLDE University's, Shri B.M.Patil Medical College, Hospital and Research Centre, Bijapur, India, were used for the present study. The experiments were conducted in accordance with Committee for the purpose of Control and Supervision of Experimental Animals (CPCSEA), New Delhi and Institutional Animal Ethical Committee (IAEC) of Shri B.M.Patil Medical College, Hospital and Research Centre, Bijapur, India. These animals were divided into four groups of six rats in each group. Group I: Normal Control, Group II: Diabetic Control, Group III: DADS treated Normal rats, Group IV: DADS treated Diabetic rats. Group I and II rats are given 3ml of normal saline per kg body weight through gastric intubation for 30 days, stock lab diet and water was provided ad libitum. Group III and IV are given 100mg/kg body weight of DADS as 3ml of suspension per kg body weight through gastric intubation for 30 days, stock lab diet and water was provided ad libitum [13]. The study was conducted during the period July 2013 to November 2014.

Induction of diabetes: Induction of diabetes was done by intraperitoneal injection of freshly prepared aqueous alloxan monohydrate (150 mg per kg body weight). in sterile water to overnight fasted rats [14]. Later stock lab diet and water was provided ad libitum. The urine of the rats, which showed positive for sugar after alloxan treatment for 3 consecutive days, was labelled as diabetic rats. A day prior to animal scarification, each animal was kept in a separate cage and the fecal material of each animal was collected. Bile acids were extracted from this fecal material as per procedure [15] and the Bile acids content were estimated to assess cholesterol break down to bile acids.

On the completion of stipulated period, rats were anaesthetised and sacrificed. Blood was collected in heparinised tubes. Liver tissue was procured, then smoothly blotted it to dry, weighed and kept in clean dry beakers covered with aluminium foil.

Blood samples were employed for estimation of various lipid parameters - total lipids [16], total cholesterol [17], triacylglycerols [18], phospholipids [19], HDL cholesterol [17], free fatty acids [20], esterified fatty acids [21], total fatty acids, VLDL cholesterol and LDL cholesterol. One gram of the liver tissues was homogenized with 10ml of chloroform-methanol 1:1 (v/v) and centrifuged. The supernatant of chloroform-methanol extract was used for the estimation of lipid parameters - total lipids [16], total cholesterol [17], triacylglycerols [18] and phospholipids [19]. One gram of the liver tissue was homogenized with 10ml of phosphate buffer (pH-7.4) and centrifuged. The supernatant of phosphate buffer extract was employed for the estimation of HMG Co-A reductase activity [22].

Gravimetry

The body weight of all the animals of each group was recorded on the day 1 of the treatment and on the day of sacrifice. The liver weight was determined after dissecting out and blotting it dry in a single pan balance to evaluate the hepato - somatic index. Hepato - somatic index is the ratio of liver weight after dissection to body weight at the time of sacrifice.

STATISTICAL ANALYSIS

All the results are expressed as mean ± standard deviation. The statistical analysis was done using one-way analysis of variance (ANOVA) followed by post-hoc t-test to determine the significant difference between the groups. A p-value less than 0.005 were selected as the point of minimal statistical significance.

RESULTS

The results of the experiments conducted to assess the Diallyl Disulphide (100mg/kg body weight) induced changes in gravimetry, plasma and liver tissue lipid levels and cholesterol turnover are given in [Table/Fig-1-3].

Results show a significant decrease in final body weight and increase in hepato-somatic index in diabetic rats compared to normal controls. These changes were reversed by the administration of DADS to diabetic rats to [Table/Fig-1].

The plasma and liver lipids are significantly increased in diabetic rats compared to normal controls. DADS administration shows a hypolipidemic effect in diabetic rats to [Table/Fig-2]. [Table/Fig-3] depicts the cholesterol turnover, HMG CoA/Mevalonate ratio and fecal bile acids are significantly decreased in diabetic rats compared to normal controls. DADS administration has improved the HMG CoA/Mevalonate ratio and fecal bile acids in diabetic rats. DADS administration in normal rats has no significant effect in all the parameters when compared to normal controls [Table/Fig-1-3].

DISCUSSION

Dyslipidemia is a major complication associated with high rate of morbidity and mortality in diabetic patients [2]. Increase in plasma and liver lipids in alloxan diabetic rats was shown earlier by CS Yadav [23,24], the results depicted in [Table/Fig-2] agree with this and there is increase in plasma and liver lipids. This complication in diabetic patients can be treated with life style change but unfortunately most patients are unsuccessful in controlling through life style modification alone and require drug therapy [4], which have adverse side effects [4,7]. To substantiate the adverse effects of drugs, DADS a principle organosulphur compound of garlic is employed for its hypolipidemic effects, but the earlier works fail to confirm the hypolipidemic effect [11,12]. This inconsistency may be due to methodological shortcomings, such as mode of administration, short duration, and inadequate statistical power. In the present study feeding of 100 mg/kg body weight of DADS as 3 ml suspension for 30 days through gastric intubation shows a significant hypolipidemic and hypocholesterolemic effect [Table/Fig-2]. The results are in consideration with the earlier studies of hypolipidemic effect of DADS [25].

The most health concern lipid: Cholesterol is a steroid mainly synthesized in the liver from starting material acetyl CoA through a series of reactions regulated by the key enzymes HMG CoA

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	f-value	p-value
1	Initial Body weight (g)	260±9 [a]	257±16.8 [a]	251.5±6.9 [a]	253.5±9.3 [a]	0.7444	0.5388
2	Final Body weight (g)	276.6±11 [a]	238v18.1 [b]	266.6±8.1 [a]	242±7 [b]	15.38	<0.0001
3	% Body weight Change	6±1.34 [a]	-8.23±2 [b]	5.6±0.9 [a]	-4.7±1.8 [c]	120.3	<0.0001
4	Hepato-Somatic Index (g/Kg)	29±0.49 [a]	36± 1.2 [b]	30.5±1.8 [a]	33.±0.7 [c]	33.27	<0.0001

[Table/Fig-1]: Gravimetry: Changes in body weight and Hepato - Somatic index in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days
Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a,b,c) are significantly different from each other (p<0.05)

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	f-Value	p-Value
1	Total Lipids(mg/dl)	164± 18.8[a]	265±46.8 [b]	163.5±21.08 [a]	203.6±23.67[c]	14.83	<0.0001
2	Triacylglycerols(mg/dl)	60±4.0 [a]	90±9.1 [b]	49.8±6.3 [c]	64±7.4 [d]	33.09	<0.0001
3	Total Cholesterol(mg/dl)	58±4.5 [a]	100±6.1 [b]	53±4.8 [a]	77±5.41 [c]	89.33	<0.0001
4	Phospholipids(mg/dl)	31±4.1 [a]	66.4±3.8 [b]	30.5±3.2 [a]	42.6±5.3 [c]	83.24	<0.0001
5	Free Fatty acids(mg/dl)	4.66±0.64 [a]	6.96±0.61 [b]	4.4±0.3 [a]	5.8±0.39 [c]	26.35	<0.0001
6	Esterified Fatty acids(mg/dl)	65±9.74 [a]	90.2±9.39 [b]	68±16.1 [a]	82±12.1 [b]	5.149	0.0090
7	Total Fatty acids(mg/dl)	69.75±9.2 [a]	97.16±9.26 [b]	72.4±16.1 [a]	87.8±11.82 [b]	6.335	0.0037
8	HDL Cholesterol(mg/dl)	35±5.08 [a]	19±2.3 [b]	27.5±4.96 [c]	24±7.64 [b]	8.470	0.0009
9	LDL Cholesterol(mg/dl)	10.3±7.2 [a]	63±5.9 [b]	15.5±2.2 [a]	40.4±5.38 [c]	105.7	<0.0001
10	VLDL Cholesterol(mg/dl)	12.0±0.8 [a]	18.0±1.8 [b]	9.9±1.2 [c]	12.8±1.4 [d]	33.09	<0.0001
	Tissue						
1	Total Lipids(mg/g)	20±2.3 [a]	51±6.9 [b]	19.6±6.7 [a]	30±4.16 [c]	42.17	<0.0001
2	Triacylglycerols(mg/g)	14±1.7 [a]	44±2.9 [b]	12.8±3.4 [a]	32±8.2 [c]	53.33	<0.0001
3	Total Cholesterol(mg/g)	4.3±0.3 [a]	6.1±0.46 [b]	3.8±0.52 [a]	4.0±0.32 [c]	34.67	<0.0001
4	Phospholipids(mg/g)	0.96±0.21 [a]	2.48±0.23 [b]	1.0±0.2 [a]	1.55±0.36 [c]	32.90	<0.0001

[Table/Fig-2]: Changes in lipid profile in normal and alloxan induced diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days
Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a,b,c,d) are significantly different from each other (p<0.05)

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	f-value	p-value
1	Total Cholesterol (p)(mg/dl)	58±4.5 [a]	100±6.1 [b]	53±4.8 [a]	77±5.41 [c]	89.33	<0.0001
2	Total Cholesterol (t)(mg/g)	4.3±0.3 [a]	6.1±0.46 [b]	3.8±0.52 [a]	4.0±0.32 [c]	34.67	<0.0001
3	Liver HMG CoA / Mevalonate Ratio	4.5±0.96 [a]	3.78±0.61 [a]	4.83±1.4 [a]	7.8±1.00 [b]	16.35	<0.0001
4	Fecal Bile Acids (mg/24 hrs fecal matter)	1.2±1.28 [a]	0.67± 0.11 [b]	1.3±0.17 [a]	2.45±0.39 [c]	42.97	<0.0001

[Table/Fig-3]: Changes in cholesterol turnover in normal and alloxan induced diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days
Note: Each value is mean ± SD of 6 observations in each group. In each row values with different superscripts (a,b,c) are significantly different from each other (p<0.05)

reductase [26,27]. Cholesterol, so synthesised is mainly utilized for synthesis of bile acids apart from being converted to other useful products in the body [28]. The bile acids – includes primary bile acids – cholic acid, chenodeoxycholic acid and secondary bile acids - deoxycholic acid and lithocholic acid formed by the action of intestinal microflora. The secondary bile acids are excreted through the fecal material. The amount of bile acids excreted in the feces substantiates the amount of the cholesterol utilized through the bile acid pathway in liver. Hence, to assess the cholesterol turnover in the liver, in the present study, the liver tissue total cholesterol content, the rate of activity of the enzyme HMG CoA reductase and the bile acid content of 24 h fecal material was carried out. HMG CoA reductase activity calculated as the ratio of HMG CoA/ Mevalonate and an increase in the ratio indicates lower activity where as a lowered ratio suggests an increased activity. A significant raise in HMG CoA reductase activity and decrease in fecal bile acids was observed in Group II when compared to Group I. The HMG CoA reductase activity and fecal bile acids are reversed by DADS administration in diabetic rats.

CONCLUSION

From the findings of the present study it may be concluded that feeding of 100 mg/kg body weight of DADS for 30 days has hypolipidemic and hypocholesterolemic effect by inhibiting the activity of HMG CoA Reductase a key enzyme in cholesterol biosynthesis (Decreased synthesis) and by increasing the excretion of fecal bile acids, an only route for cholesterol excretion (Increased utilization) in alloxan induced diabetic rats.

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Effect of DADS on alterations of liver function in alloxan induced diabetic male albino rats

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Abstract

Introduction: Diabetes Mellitus is a syndrome characterized by a loss of glucose homeostasis from defective insulin secretion and its action, both resulting in impaired metabolism of carbohydrate, lipid and protein. Liver is one of the insulin dependent tissues which are severely affected in diabetes. The hypoglycemic effect of garlic has been reported. We aimed to study the effect of Diallyl Disulphide (DADS), a principle compound of garlic oil on liver function.

Aim: To assess the effect of DADS on alteration of liver function in alloxan induced diabetic male albino rats.

Materials and Methods: Healthy adult male albino rats weighing around 100-150 grams were randomly selected from the animal house at Shri B.M.Patil Medical College & Hospital, Bijapur. Diabetes was induced using alloxan and was treated with DADS. After a stipulated time the rats were anesthetised and sacrificed to collect the blood and liver tissue. Various parameters were estimated in the blood and homogenised liver tissue using standard procedures. Liver histological section were prepared and observed under the microscope.

Results: There was significant reversal of histological and biochemical changes in the liver of DADS treated alloxan induced diabetic rats when compared to the alloxan induced diabetic rats.

Statistics: One way ANOVA followed by post hoc't' test was done.

Conclusion- From the above findings it can be concluded that the DADS a principle compound of garlic, definitely has the hepatoprotective effect in diabetic rats, with least adverse effects.

Key words : Diallyl Disulphide (DADS), Diabetes Mellitus (DM), Hepatoprotective.

Introduction

Diabetes mellitus is a syndrome resulting from variable interactions of hereditary and environmental factors and characterised by abnormal insulin secretion or insulin receptor, affecting metabolism involving carbohydrate, protein and fats in addition to damaging beta cells of pancreas, liver and kidneys (1). Diabetes Mellitus could be treated by nutritional therapy/drug therapy and others. But the drug therapy would have its own limitations and side effects. The liver is insulin dependent tissue that plays a vital role in glucose and lipid homeostasis and is severely affected in diabetes (2). Insulin deficiency in diabetic rats lead to degenerative changes in liver causing an elevation in serum Aspartate transaminase (AST) and Alanine transaminase (ALT) and disturbances in antioxidant and free radical levels. Herbal extracts specifically extracts of *Allium sativum* (garlic) have been known to possess hypolipidemic as well as hypoglycemic actions (3,4), which is attributed to its organosulphur compound. The principle organosulphur compound is Diallyl Disulphide (DADS) (3, 4).

Aim

The present study was undertaken to assess the effect of DADS on alteration of liver function in alloxan induced diabetic male albino rats.

Materials and Methods

Alloxan and Diallyl disulphide (DADS) were procured from sigma Aldrich chemicals. All the other chemicals employed were of analytical grade. Healthy Wister strain male albino rats weighing around 100-150 grams were randomly selected from

the animal house, BLDE University's, Shri B. M. Patil Medical College, Hospital and Research Centre, Bijapur, India, for the present study. The experiments were conducted in accordance with Committee for the purpose of Control and Supervision of Experimental Animals (CPCSEA), New Delhi and Institutional Animal Ethical Committee (IAEC) of Shri B. M. Patil Medical College, Hospital and Research Centre, Bijapur, India. These animals were divided into four groups with six rats in each group as follows: Group I: Normal Control, Group II: Diabetic Control, Group III: DADS treated Normal rats, Group IV: DADS treated Diabetic rats. Group I and II rats were given 3ml of normal saline per kg body weight through gastric intubation for 30 days, stock lab diet and water was provided ad libitum. Group III and IV were given 100mg/kg body weight of DADS as 3ml of suspension per kg body weight through gastric intubation for 30 days, stock lab diet and water was provided ad libitum.

Study protocol

Induction of diabetes was done by intraperitoneal injection of freshly prepared aqueous alloxan monohydrate (150mg per kg body weight) (5) in sterile water to overnight fasted rats. Later stock lab diet and water was provided *ad libitum*. The urine of the rats, which showed positive for sugar after alloxan treatment for 3 consecutive days, was labeled as diabetic rats. On the completion of stipulated period, rats were anaesthetised and sacrificed. Blood was collected in heparinised tubes. Liver tissue was procured, then smoothly blotted it to dry, weighed and kept in clean dry beakers covered with aluminium foil. Blood samples were employed for estimation of various parameters – blood glucose by O-Toludine method (6), AST and ALT activities by Reitman and Frankel method (7). One gram of the liver tissue was homogenized with 10ml of phosphate buffer (pH-7.4) using potter Elvejham tissue homogenizer and the resultant mixture was centrifuged at 3000 rpm for 5 minutes. The clear supernatant of phosphate buffer extract was employed for the estimation of total thiols by nitroprusside method (8), AST and ALT activities by Reitman and Frankel method (7). One gram of the liver tissue was homogenized with 10ml of 5% cold TCA for 5 minutes using potter Elvejham tissue homogenizer and the resultant mixture was centrifuged at 3000 rpm for 5 minutes. The clear supernatant was employed for estimation of thio barbituric acid reactive substance (TBARS) levels (9). A part of liver tissue was fixed in 10% buffered formalin and embedded in paraffin. 5µm section were cut and stained with hematoxylin and eosin (H&E) (10, 11). The sections were examined under light microscope (10x & 40x) and photomicrographs were taken with connected camera.

Gravimetry

The body weight of all the animals of each group was recorded on the day 1 of the treatment and on the day of sacrifice. The liver weight was determined after dissecting out and blotting it dry in a single pan balance to evaluate the hepato - somatic index. Hepato - somatic index is the ratio of liver weight after dissection to body weight at the time of sacrifice.

Statistics

All the results are expressed as mean $\pm$ standard deviation. The statistical analysis was done using one way analysis of variance (ANOVA) followed by post hoc't' test to determine the significant difference between the groups. A p value less than 0.05 was selected as the point of minimal statistical significance.

Results

The results of the experiments conducted to assess the Diallyl Disulphide (100mg/kg body weight) induced changes in gravimetry, liver functioning, liver oxidative stress and liver histology are given in Table 1 to 3 and Fig. 1 to 4.

Table 1 shows a significant decrease in final body weight in group II when compared to group I (where there is increase in final body weight), suggesting loss of body weight in alloxan induced diabetic rats. No significant change in final body weight was observed in group I and III. An increase in final body

weight was observed in group IV compared to group II, indicating DADS administration improved diabetic body weight loss. Hepato - somatic index was increased significantly in group II when compared to group I while after the treatment with DADS in diabetic rats, hepato somatic index was decreased, indicating an improvement in liver recovery after treatment with DADS.

Examination of H&E stained sections of the control group (group I) showed normal architecture (Fig. 1a & 1b). In diabetic rats (group II) liver tissue section showed distortion in the arrangement of cells around central vein, periportal fatty infiltration with focal necrosis of hepatocytes were observed (Fig. 2a & 2b). In DADS treated normal rats (group III) the architecture was similar to that of normal control (Fig. 3a & 3b). In group IV, DADS treated diabetic rats, there was reduction in necrosis of hepatocytes, fatty infiltration and derangement of cells (Fig. 4a & 4b), when compared to group II.

Table 3 depicts a significant elevation in blood glucose in group II compared to group I, suggesting diabetes induced hyperglycemia. When group I was compared with group III no significant change was observed, suggesting normoglycemic effect of DADS on normal control rats. There was a significant reduction in blood glucose observed in group IV compared to group II, depicting the hypoglycemic effect of DADS on alloxan induced diabetic rats.

Table 2 and 3 showed a significant elevation in liver tissue and plasma AST and ALT in group II when compared to group I, suggesting diabetes induced liver derangement. When group I was compared with group III for the above said parameters, no significant change was observed. A significant lowering of liver tissue and plasma AST and ALT was observed in group IV when compared to group II, suggesting the liver tissue recovery with DADS treatment in alloxan induced diabetic rats.

Table 2 shows a reduction in liver tissue total thiols and increase in TBARS in group II compared to group I, depicting diabetes induced reduction in antioxidants and increase in free radicals. No significant change is observed in between group I and III. An increase in liver tissue total thiols and decrease in TBARS levels was observed in group IV compared to group II suggesting improvement in antioxidant levels upon treatment with DADS in alloxan induced diabetic rats.

Table 1 Gravimetry

Changes in body weight and Hepato Somatic index in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	Initial Body weight (g)	260+9 ^{a}	257+16.8^{a}	251.5+6.9^{a}	253.5+9.3^{a}	0.7444	0.5388
2	Final Body weight (g)	276.6+11^{a}	238+18.1^{b}	266.6+8.1^{a}	242+7^{b}	15.38	0.0000
3	% Body weight Change	6+1.34^{a}	-8.23+2^{b}	5.6+0.9^{a}	-4.7+1.8^{c}	120.3	0.0000
4	Hepato-Somatic Index (g/Kg)	29+0.49^{a}	36+ 1.2^{b}	30.5+1.8^{a}	33.2+0.7^{c}	33.27	0.0000

Note: Each value is mean $\pm$ SD of 6 observations in each group. In each row values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$).

Table 2 Tissue Biochemistry
Changes in liver tissue AST, ALT, Sulphydryl groups and TBARS in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	Sulphydryl Groups (μm/g)	$1.09+0.13^{a}$	$0.75+0.18^{b}$	$1.06+0.15^{a}$	$0.86+0.11^{b}$	6.448	0.0034
2	TBARS (μmMD/g)	$4.97+1.65^{a}$	$6.14+1.8^{a}$	$5.04+1.58^{a}$	$5.55+1.93^{a}$	0.5140	0.6776
3	AST(U/L)	$27+3.5^{a}$	$42.5+7.07^{b}$	$30.8+2.99^{a}$	$33.5+5.74^{c}$	9.268	0.0005
4	ALT(U/L)	$41+5.1^{a}$	$54.6+5.0^{b}$	$39.4+7.8^{a}$	$47.4+3.28^{c}$	8.013	0.0012

Note: Each value is mean ± SD of 6 observations in each group.
In each row values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$).

Table 3 Blood Biochemistry
Changes in blood glucose, AST and ALT in Normal and Alloxan induced Diabetic rats before and after treatment with DADS (100mg/kg body weight) for 30 days.

S.No	Parameter	Group I (n=6)	Group II (n=6)	Group III (n=6)	Group IV (n=6)	F Value	P Value
1	Blood Glucose (mg/dl)	$99.6+7.2^{a}$	$473+24.1^{b}$	$93.5+6.05^{a}$	$323+25.8^{c}$	580.1	0.0000
2	AST(U/L)	$28.5+6.8^{a}$	$58+4.7^{b}$	$31.5+6.9^{a}$	$38+9.2^{c}$	17.79	0.0000
3	ALT(U/L)	$35.5+7.5^{a}$	$69+5.9^{b}$	$37.5+8.1^{a}$	$50.8+6.1^{c}$	25.72	0.0000

Note: Each value is mean ± SD of 6 observations in each group.
In each row values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$).

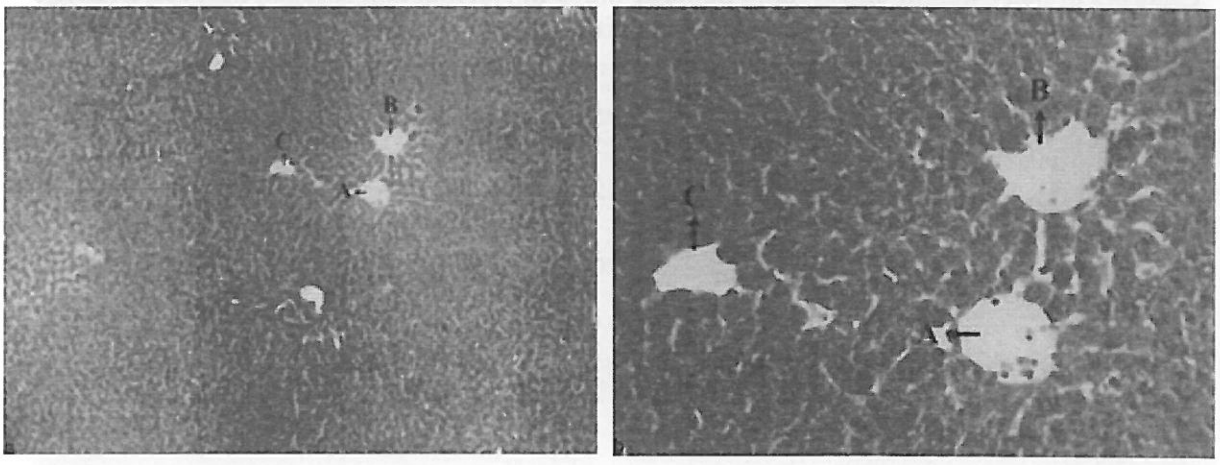

Fig.1 (a) Liver section of normal control group I (10x), (b) Liver section of normal control group I (40x) showing hepatocytes and portal triad comprising (A) Hepatic artery, (B) Hepatic vein, (C) Bile duct.

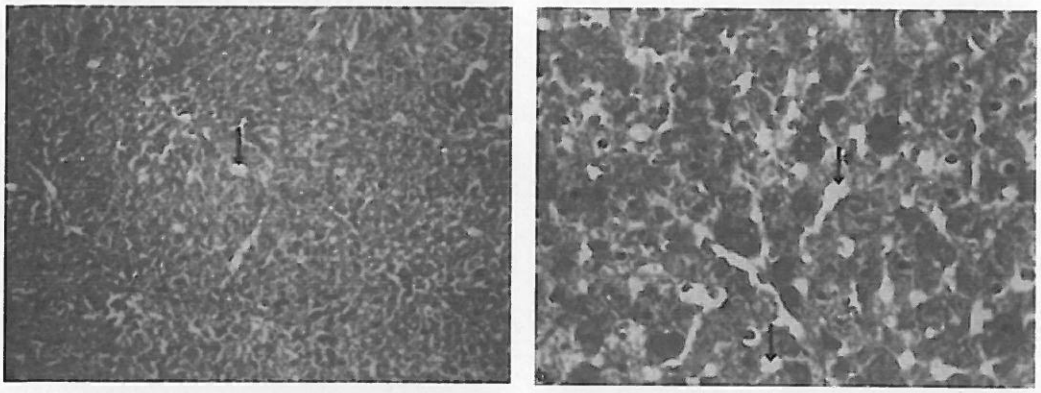

Fig.2 (a) Liver section of diabetic rat group II (10x), (b) Liver section of diabetic rat group II (40x) showing features of fatty liver (arrow marks indicate deposition of fat)

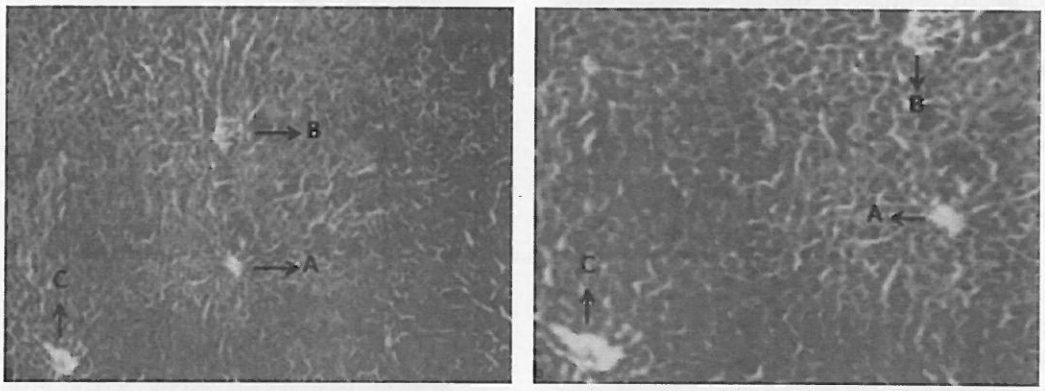

Fig. 3 (a) Liver section of DADS treated normal rats group III (10x), (b) Liver section of DADS treated normal rats group III (40x) showing normal hepatocytes and portal triad comprising (A) Hepatic artery, (B) Hepatic vein, (C) Bile duct.

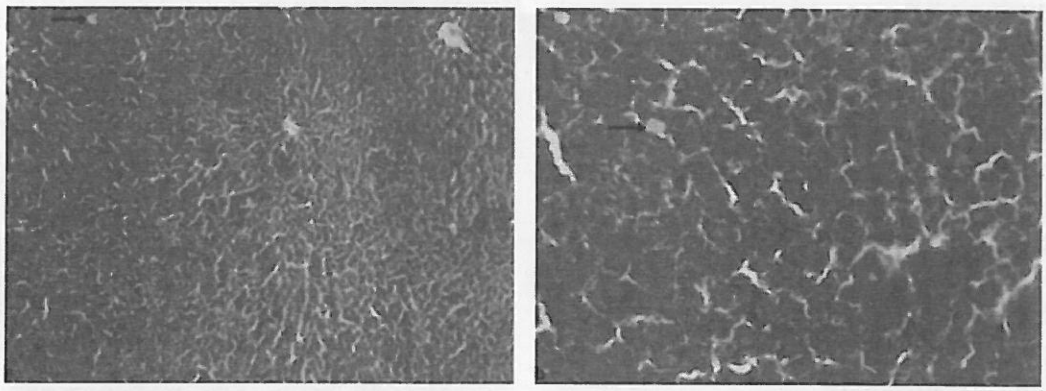

Fig. 4 (a) Liver section of DADS treated diabetic rats group IV (10x), (b) Liver section of DADS treated diabetic rats group IV (40x) showing hypolipidemic changes indicated by arrow marks.

Discussion

In this study a significant decrease in serum glucose was observed in DADS treated alloxan induced diabetic rats. In alloxan induced diabetic rats, i) the degenerative changes of liver histology were similar to the earlier observations (1, 12, 13, 14). ii) the absence of insulin showed a marked structural alteration in the liver histological sections. iii) the major alteration was periportal fatty infiltration and necrosis of hepatocytes. This damage is partially reversed by DADS treatment and is similar to that observed by *Vinca rosea* extract in alloxan induced diabetic rats by Ghosh *et al* (1). Prolonged hyperglycemia will produce reactive oxygen species (ROS), leading to increased oxidative stress and decreased antioxidant levels (15, 16). In our study in untreated diabetic rats, an increase in liver tissue TBARS was observed, which is considered as marker of oxidative stress and decrease in total thiol groups was observed, which is considered as marker for antioxidant levels. Upon treatment with DADS an improvement in TBARS and total thiol groups was observed. Liver cell destruction led impairment in permeability of liver cell membrane resulted in elevation of serum and liver tissue AST and ALT (17) in untreated diabetic rats. Our study showed that diabetic rats treated with DADS showed a reversal of histological and biochemical changes in the liver functioning.

Conclusion

In the present study, consumption of 100 mg/kg body weight of DADS reversed most of the histological and biochemical changes in liver of the diabetic rats. This effect was due to the hypoglycemic nature of the DADS. In addition, an associated increase in oxidative stress in diabetes was significantly reduced by DADS consumption. So we can conclude that DADS had a significant hepato-protective role in diabetic rats and offers promising perspectives deserve further investigations.

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