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 gastrointestinal disorders and lacticacidosis, sulfonylureas have the
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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**

**Pathophysiology of Dyslipidemia in Alloxan Diabetic rats**

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 –

\[
\text{C}_3\text{H}_5\cdot\text{S-S-C}_3\text{H}_5 + 3\text{NADPH} + \text{H}^+ \rightarrow 2\text{C}_3\text{H}_7\cdot\text{SH} + 3\text{NADP}^+ \rightarrow \text{I}
\]

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 sulphhydryl exchange reactions with thiol proteins and thiol enzymes as follows (Gebhardit R et al., 1996; Ziegler DM. 1985).

\[
\text{Enz-SH} + \text{C}_3\text{H}_5\cdot\text{S-S-C}_3\text{H}_5 \rightarrow \text{Enz-S-C}_3\text{H}_5 + \text{C}_3\text{H}_5\cdot\text{SH} \rightarrow \text{II}
\]

Such possible sulphhydryl 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 (Guillausseau *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.

\[
\text{G-S-S-G} + C_3H_5-S-S-C_3H_5 \rightarrow 2 \text{G-S-S- } C_3H_5 \rightarrow \text{III}
\]

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

\[
G-S-S-G + \text{NADPH} + H^+ \rightarrow 2 \text{GSH} + \text{NADP}^+ \rightarrow V
\]
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).

\[
\text{Enz-SH} + \text{C}_3\text{H}_5-\text{S}-\text{S}-\text{C}_3\text{H}_5 \rightarrow \text{Enz-S-S-C}_3\text{H}_5 + \text{C}_3\text{H}_5-\text{SH}
\]

Such a sulphhydryl 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 sulphhydryl 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).