4. DISCUSSION

The past two decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide (Amos et al, 1997; King et al, 1998). Increase in sedentary lifestyle, consumption of energy-rich diet, population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity has lead to an increase in the number of people suffering from diabetes. In view of its frequent clinical and epidemiological link with hypertension and arteriosclerosis, diabetes continues to be a health problem of paramount concern. There is no cure for diabetes but management and control are possible. Prolonged exposure to hyperglycemia is recognized as the primary casual factor in the pathogenesis of diabetic complications (Laakso M, 1999a; Grundy et al; 1999; DCCT, 1993). Degenerative changes in several tissues are of almost universal occurrence in diabetes mellitus sharing common etiology i.e., altered hormonal and biochemical parameters resulting from the absence of insulin.

Many studies on diabetic complications in tissues have been based on the hypothesis that similar mechanisms of cytopathology are involved even though the structural, biochemical and functional alterations are quite dissimilar in different organs and tissues (Parinandi et al, 1990). One possible mechanism is cellular damage from the cytotoxic oxygen free radicals. Free radicals have been implicated in the pathophysiology of diabetes (Wolff, 1993; Vlassara, 1994) and oxidative stress may be a common pathway linking diverse mechanisms of the complications of diabetes (Nishikawa et al, 2000). Thus various tissues in the diabetic state are more prone to oxidative damage and could result in various complications in long term diabetes implying that the restoration of antioxidant status is an important parameter to evaluate the effect of antidiabetic agent.

Normally there is a precise balance between the utilization of glucose by peripheral tissues and its production. This balance is severely affected in Type 1 diabetes due to insulin deficiency. Exogenous insulin is quite helpful in ameliorating these conditions. However, it fails to produce a well controlled glycemic condition in association with variable dietary intake and physical activity. Episodes of severe hypoglycemia leading to deleterious cerebral impact are common during insulin administration (McCall, 1992). Thus, there is utter urgency to develop antidiabetic drugs that are effective, safe, produce a well-controlled euglycemia and prevent the long-term complications. Since, diabetes associated tissue damage and its complications are primarily linked to the glucose homeostasis, reviving and maintaining euglycemia is of prime importance for any antidiabetic agent.
Presently, the medical fraternity has increasingly started using plants to overcome various illness and sufferings, mainly to obviate the profound side-effects encountered in usage of modern drugs. From the identified higher plants in the world, less than 1% has been screened pharmacologically and very few in regard to diabetes mellitus. Therefore, for treatment of diabetes, it is prudent to look for options in herbal medicine as well. Compounds with antidiabetic activities come from a range of chemical classes including polysaccharides and protein, flavonoids, steroids, terpenoids and alkaloids. Some compounds such as lectins and fiber are negatively correlated with the glycemic index. It is seen that certain resistant cases of diabetes who do not respond well to modern medicines such as chlorpropamide, tolbutamide and glibenclamide respond well when treated with herbal preparations alone or in combination with other oral hypoglycemic agents.

The current therapeutic agents used for diabetes have been discussed by Moller (2001) with their molecular targets, sites of action and adverse events occurring. Thus, it will be very significant to look for new and if possible, more effective and efficacious antidiabetic molecules from the vast reserves of phytotherapy. *Azadirachta indica* is one such plant that has been traditionally used in India, especially in the Ayurveda and Unani systems (Grover et al. 2002a; 2002b; Bhanwra et al, 2000). Reduced hyperglycemia is reported in streptozotocin induced diabetes when treated with leaf extract and the effect is possibly due to presence of flavinoid and quercetin (Chakraborty et al, 1989). A significant hypoglycemic effect was also observed with leaf extract and seed oil in normal as well as alloxan induced diabetic rabbits (Khosla et al, 2000). *A. indica* leaf extracts are known to reduce the elevated levels of serum aspartate aminotransferase, alanine aminotransferase and gamma glutamyl transpeptidase which are indicative of liver damage (Khosla et al, 2000). Also, leaf extract produces antiulcer effect in rats exposed to restraint-cold stress or ethanol orally by preventing mucus depletion and mast cell degranulation (Garg et al, 1993). These reports are suggestive of protective effect of *A. indica* against free radical induced tissue damage.

In the present study, antidiabetic potency of different *A. indica* extracts have been studied using alloxan induced diabetic rat model and compared their antioxidant potential for their ability to scavenge superoxide and hydroxyl radical and inhibit lipid peroxidation. The present study explored the possibility of using *A. indica* leaf extract (aq), bark extract (aq) and seed oil, and evaluated their effect on hyperglycemia, hyperlipidemia and altered oxidative status by studying major antioxidant enzymes- Superoxide dismutase, Catalase,
Glutathione peroxidase and Glutathione reductase, and evaluated the extent of damage by measuring the lipid peroxides in liver, kidney, heart, muscle and brain. Martini and Ursini (1996) reported that partial or complete inhibition of Glucose-6-phosphate dehydrogenase activity causes the buildup of glucose, reduction of NADPH and increase of advanced glycation end products (AGE) leading to highly significant increases in oxidative stress and cell death. The results provided strong evidence that G6PD is of central importance to cellular redox regulation. The enzyme has also been studied in the present dissertation. Earlier evidence suggested that there was an increase in the levels of PKC β2 in heart and muscle due to oxidative stress (Koya and King, 1998). This aspect has also been checked by measuring the protein levels of PKC β2 in heart and muscle. Moreover, expression of glucose transporter (GLUT 4) protein has also been monitored in membrane fractions of skeletal muscle.

In present study, a significant and potent hypoglycemic and hypolipidemic activity of A. indica was observed in diabetic rats. If the leaf extract (aq), bark extract (aq) or seed oil is to be used for a longer treatment period, it is necessary to find out if longer administration period would have any toxic effect. No toxic or lethal effect was observed when effective dosage of AILE, AIBE and AISO was administered in euglycemic groups for 14 days which indicates their safety margins. All the parameters estimated were normal even with two times of effective dose indicating that all the A. indica extracts can be safely administered to the experimental groups.

4.1 Effect of A. indica extracts on body weight and organ indices

In the present study, alloxan induced experimental diabetes in Wistar rats considered equivalent to Type 1 diabetes, has been undertaken to study the diabetes induced oxidative stress. The experimental diabetic animals showed characteristic symptoms of diabetes including hyperglycemia, glucosuria, polydipsia, polyurea and loss of body weight despite polyphagia. A decrease in liver weight and increase in kidney weight were observed in diabetic animals. The results obtained in the present study correlate well with the earlier studies. Glucose overutilization in kidney during diabetes causes an increase in glycogen, basement membrane and ribose 5-phosphate formation (Sochor et al, 1979; Steer et al, 1982). An increase in nucleic acid and protein synthesis occurs in the diabetic rat kidney, as has been reported earlier which correlates with kidney hypertrophy during diabetes (Cortes et al, 1981). However, these changes contrast sharply with the liver in which glycogen accumulation, nucleic acid and protein synthesis, and albumin synthesis
are all decreased in diabetes showing glucose underutilization (Khandelwal et al., 1978; Jefferson et al., 1980). In the present study, a significant fluid intake was observed in the experimental diabetic rats, which is associated with polydipsia and polyuria, characteristic features of Type 1 diabetes. Treatment with AILE, AIBE and AISO significantly revived the altered index of body weight and organ indices.

4.2 Effect of *A. indica* extracts on hyperglycemia

There was a significant increase of almost three fold in blood glucose levels of alloxan diabetic rats after 21 days of insulin withdrawal. Diabetic animals receiving three weeks of treatment with insulin showed a marked reduction in hyperglycemia. Treatment with AILE, AIBE and AISO for 21 days was found significantly effective in reviving the altered glycemia. In agreement with the present results, several studies have shown the hypoglycemic effect of *A. indica* leaf extract (Chattopadyay, 1999; El-Hawary and Kholief, 1990; Dixit et al., 1986). The mechanism of hypoglycemic action probably involves direct or indirect stimulation of insulin secretion. Glycosylated Hemoglobin content rather than fasting plasma glucose concentration is considered as a more reliable index of glycemic control in the management of diabetes mellitus (Canham and Lockett, 1980). The elevation in HbA1c level was significantly less in all the treated groups and the values were close to that of insulin treated diabetic group. Return of HbA1c to normal level after treatment is a clear indication that the diabetic state was well regulated after the treatment of diabetic animals with *A. indica* extracts.

The improvement in glucose homeostasis could reflect a consequent enhancement in the antioxidant status of the tissues as a result of antidiabetic treatment. This was examined by measuring the levels of antioxidant enzymes in different tissues of control, diabetic and diabetic animals treated with different *A. indica* extracts.

4.3 Effect of *A. indica* extracts on hyperlipidemia

It is well documented that in diabetes mellitus with only partial or improper control, one of the complications is the elevation of blood lipid levels (O’Brein et al., 1998; Nathan, 1993) which is seen in the present study also. Chronic hyperglycemia promotes the glycation of LDL-C and both these processes are believed to increase the atherogenicity of LDL-C (Rader, 2007). In people with type 1 diabetes having good glycemic control, plasma lipid and lipoprotein concentrations may be normal, but there may be oxidation and glycation of the lipoproteins, which may impair their function and/or enhance their atherogenicity (DCCT/EDIC, 2005). Albuminuria, which occurs frequently in the onset of
type 1 diabetes, is associated with increases in the levels of small, dense LDL particles and triglyceride-rich lipoproteins, and with a reduction in HDL levels (Jialal and Bajaj, 2009). In various rodent models (streptozotocin induced diabetic rats, hypertriglyceridaemic non-obese rats etc) it has been reported that α-glucosidase inhibitors may produce a dose dependent reduction in serum triglycerides, cholesterol and free fatty acid concentrations (Clissold and Edward, 1988). Clinical trials in which α-glucosidase inhibitors have been used in treatment of insulin dependent or non-insulin dependent diabetes mellitus have also shown that they reduce total serum triglycerides concentrations (Chiasson, 1998).

We have found that AILE, AIBE and AIS0 were effective in controlling the altered lipids levels, and total cholesterol (TC) and triglycerides (TG) were significantly reduced in all the treated groups. The reduction of total cholesterol and triglycerides values was much more than the fall produced by insulin treatment in diabetic rats. Treatment with insulin was not found significantly effective in correcting the altered TC and TG values as in accordance with the early reports (Sparks et al, 1986; Dunn, 1990; Friedberg et al, 1988). A noticeable feature is that the treatment of diabetic animals with AILE, AIBE and AIS0 for 21 days increased the HDLC level which is considered as good cholesterol; however no considerable improvement was observed in insulin treated group. Improvement in lipid profile is suggestive of the action of A. indica on enzymes of lipid metabolism.

4.4 Effect of A. indica extracts on antioxidant enzymes

The present study showed that 21 days of treatment with AILE, AIBE and AIS0 resulted in a marked reduction in hyperglycemia in diabetic animals and also improved the bodyweight. Therefore, after showing the improvement in glucose homeostasis in the treated animals, the metabolic consequences of this treatment in different tissues was further examined.

In diabetes, the persistence of hyperglycemia has been reported to cause not only increased production of oxygen free radicals through auto-oxidation and nonenzymatic glycation, but also for changes in tissue content (Wolff, 1987; Oberley, 1988). If the diabetic state is associated with a generalized increase in tissue oxidative stress (Jain and Palmer, 1997), it might be reflected in the changes in tissue antioxidant system. Therefore, the activities of some major antioxidant enzymes were measured in control and experimental rats. Diabetic rats showed altered levels of the antioxidant enzymes in different tissues studied, thus generating oxidative stress in these tissues. Under normal physiological condition there is a critical balance in the generation of oxygen free radicals and its
antioxidant defense systems used by organisms to deactivate and protect themselves against free radical toxicity (Sies, 1991). Impairment in the oxidant/antioxidant equilibrium in favor of the former provokes a situation of oxidative stress, which is known to be a component of molecular and cellular tissue damage mechanisms and contributes substantially to the pathogenesis of diabetic complications (Halliwell, 1994). In vitro and in vivo studies have reported that in a variety of tissues, hyperglycemia and possibly elevated free fatty acid levels result in the generation of oxygen free radicals and considerably increased oxidative stress (Evans et al, 2003; West, 2000; Mak et al, 1996).

Various tissues are more prone to oxidative damage and could result in many complications in long-term diabetes, implying that, the restoration of antioxidant status is an important parameter to evaluate the effect of antidiabetic agent. In the present study, major antioxidant enzymes, such as SOD, CAT, GPx, and GR, are studied to evaluate the antioxidant status in different tissues of A. indica treated diabetic rats. However the changes in their activities were complex, depicting a varied antioxidant enzyme level in different tissues during diabetes. In general muscle, brain and heart showed lower activities of all the antioxidant enzymes studied as compared to liver making these tissues more susceptible to oxidative damage by ROS.

### 4.4.1 Superoxide dismutase

The enzyme superoxide dismutase (SOD) is the cell’s first line of defense against the toxicity of superoxide radical and the subsequent radical derivatives (Fridovich, 1972; 1974). Although O$_2^-$ once formed, undergoes spontaneous dismutation to peroxide and oxygen, the presence of SOD increases the reaction rate by $10^9$ fold. The nonenzymatic dismutation of O$_2^-$ results in the production of singlet oxygen, and it has, therefore, been proposed that the function of SOD is to protect the aerobic cell from the toxic effects of not only O$_2^-$ but singlet oxygen and other reactive oxygen species (ROS) as well (Halliwell and Gutteridge, 1989). There are at least three SOD isozymes in the mammalian body; Copper zinc-superoxide dismutase (Cu/Zn SOD) in the cytosol of cells, manganese-superoxide dismutase (Mn-SOD) in the mitochondrial matrix, and extracellular Superoxide dismutase in the extracellular space (Marklund, 1984; Erlansson et al, 1990).

The result from the present study indicate that alloxan induced diabetes elicits tissue specific alteration in SOD enzyme activities. While liver, kidney, muscle and brain show significantly lowered levels of SOD, heart shows an increase in SOD activity. The results are in agreement with earlier published studies (Wohaib and Godin, 1987; Oberley,
DISCUSSION

Heart tissue has several features that make it vulnerable to damage from free radicals such as abundant mitochondria which may leak activated species. In this study we found that heart tissue exhibited different susceptibility to oxidative stress. The increased activity of SOD may be a manifestation of an adaptive response to compensate for increased oxidative stress. The decrease in the activity of SOD in liver, kidney, muscle and brain may be due to the inactivation or inhibition of SOD by increased production of ROS during diabetes (Van Dam et al, 1995; Kakkar et al, 1995).

The above results indicate that SOD exhibits a varied pattern of alterations. The paradigm of such alterations in the diabetic state suggests that these changes are adaptive in nature. Generally speaking the SOD activity is increased where it is present in relatively low amounts and where the oxidative stress is immense (e.g. in liver). However this cannot be held in case of muscle and brain where despite low levels of SOD, the activities are further dipped due to the diabetes-induced oxidative stress. It has been reported that SOD induction and consequently its activity progressively decreases. Furthermore, H₂O₂ has been shown to inhibit Cu/Zn SOD (Bray et al, 1974) and therefore the accumulation of H₂O₂ caused by low Catalase activity found in the diabetic groups could also explain the progressive decrease in SOD in later stages of diabetes. The results are in agreement with those of other authors (Wohaib and Godin, 1987; Oberley, 1988; Nonoda et al, 1993; Genet et al, 2002).

Treatment with AILE, AIBE and AISO for 21 days significantly reversed the lowered activities of SOD in liver, kidney and muscle, near to the activity in aged matched control. AILE treatment was most effective in correcting the variations in SOD activity. In liver which has the highest concentration of SOD activity, the altered level in the diabetic groups and the extent of reversal is further substantiated by the immunoblotting experiments. In diabetic state, 48% decrease in the levels of Cu/Zn SOD was observed in liver cytosol fraction. This clearly implies that the expression of SOD at protein level is altered during diabetes and the treatments are effective in restoring the protein levels of SOD. The restoration of the altered SOD activities could primarily be due the subsequent lowering of blood glucose levels. It has been postulated that excess free circulating glucose is toxic to the organism/cell and is the proximal source of increased oxidative stress in hyperglycemic condition (Baynes, 1991). Glucose has been demonstrated to get oxidized generating H₂O₂. Autoxidation of glucose is directly linked to protein glycation, which is another source of free radical production (Wells-Knecht et al, 1995). Thus the lowering of elevated blood glucose levels would prevent the subsequent glucose induced toxic effects.
4.4.2 Catalase

Hydrogen peroxide is damaging in living systems as it give rise to toxic hydroxyl radicals. Moreover, H$_2$O$_2$ has been implicated in the activities of cell signaling pathways. Studies have demonstrated that the exogenous H$_2$O$_2$ protein tyrosine kinase cascades are linked to growth and apoptosis (Allen and Tresini, 2000). Overall, H$_2$O$_2$ exerts deleterious effects on signal transduction (Lounsberry et al, 2000) and therefore its removal is vital for the proper functioning of cellular processes. The enzyme catalase (CAT) is believed to be the main regulator of hydrogen peroxide metabolism. Thus, CAT plays an important role in the scavenging activities of the antioxidant system. In animals, CAT is present in all major body organs and its activity in tissues varies greatly, being mainly concentrated in liver and erythrocytes. The brain, heart and skeletal muscle contain only low amounts of CAT. In tissues, it is mainly particle bound (in mitochondria and peroxisomes), whereas it exists in a soluble state in erythrocytes (Matkovics et al, 1982; Halliwell and Gutteridge, 1989).

In the present study, it was observed that the activity of CAT varied in different tissues. The results are in agreement with those of Genet et al, (2002) and Wohaieb and Godin (1987). As observed in the case of SOD, highest concentration of CAT is present in liver followed by kidney, muscle, heart and brain. The activity of CAT is reduced significantly in liver, kidney and brain in diabetic state whereas in skeletal muscle and cardiac tissue there is an increase in CAT activity. Reduced levels of CAT can be attributed to the decreased protein expression levels in the diabetic condition as reported recently in liver (Sindhu et al, 2004). The decreased activity may also be due to the partial inactivation of CAT by hydroxyl radicals and hydrogen peroxide (Hodgson and Fridovich, 1975; Pigeolet et al, 1990).

Heart tissue has several features that make it vulnerable to damage from free radicals such as abundant mitochondria which may leak activated species. In present study we found that heart tissue exhibited different susceptibility to oxidative stress. Perhaps due to the presence of large amounts of mitochondria, induction of CAT activity is overwhelmingly higher as compared to skeletal muscle tissue. In heart tissue, activity of SOD and CAT are synergistically increased. Increased activity of SOD in heart implies elevated production of H$_2$O$_2$ which if not effectively removed will result in tissue damage either directly or indirectly by forming hydroxyl radicals. Perhaps to counter the extremely high levels of SOD, there is induction of CAT activity. Skeletal muscle unlike liver and kidney has a weaker defensive capacity and even a moderate increase in O$_2$ concentration disturbs
this balance (West, 2000). This is obvious by the compensatory increase in the levels of CAT in skeletal muscle.

The exogenous supplementation of insulin (2U/day) considerably ameliorated the abnormalities in CAT activity in the diabetic tissues. Similarly, treatment with AILE, AIBE and AIS0 significantly controlled the change in CAT activity close to euglycemic condition in all the tissues studied namely, liver, kidney, heart, skeletal muscle and brain. The best reversal pattern was observed in the enzyme activity by AIBE treatment to the diabetic rats. The above results can primarily be due to the subsequent lowering of blood glucose levels by AILE, AIBE and AIS0. It has been postulated that excess glucose is toxic and the primary source of increased oxidative stress in hyperglycemic condition (Baynes, 1991) thereby generating high levels of H$_2$O$_2$. Thus, by lowering blood glucose levels the various antidiabetic treatments indirectly restored the euglycemic condition of the cell. It has already been reported that A. indica has hepato-protective effect against hydroxyl radical formation (Garg et al, 1993; Bhanwara et al, 2000).

4.4.3 Glutathione peroxidase

The enzyme glutathione peroxidase (GPx) has a key role in the enzymatic defense system against oxygen-derived free radicals (Raes et al, 1987). It detoxifies any H$_2$O$_2$ or any hydroperoxide utilizing reduced glutathione (GSH) as a reductant and results in the formation of H$_2$O$_2$ and oxidized glutathione (GSSG). In fact glutathione metabolism is one of the most essential antioxidative defense metabolisms (Sigalov and Stern, 1998; Rikans and Hornbrook, 1997). GPx is present both as selenoenzyme and as selenium independent form. There are five GPx isozyme found in mammals. Cytosolic and mitochondrial glutathione peroxidase (cGPx or GPx 1) reduces hydrogen peroxides and fatty acid hydroperoxides at the expense of glutathione (Asayama et al, 1986). It is specific for GSH as a hydrogen donor and is made up of four protein subunits, each of which contains one selenium atom at its active site. The GSH apparently reduces the selenium and the reduced form of the enzyme then reacts with hydrogen peroxide (Halliwell and Gutteridge, 1989).

GPx 1 and phospholipids hydroperoxide glutathione peroxidase (PHGPx or GPx4) are found in most tissues. GPx 4 is located both in cytosol and the membrane fraction. Hydrogen peroxide is produced within the cells as a result of various metabolic processes. Under normal conditions, it is mostly destroyed by CAT and in parts by GPx (Mavelli et al, 1982).

Although mammalian erythrocytes contain large amounts of CAT, the principal means of disposal of H$_2$O$_2$ in these cells has been considered to be the NADPH dependent
GR/GPx pathway (Cohen and Hochstein, 1963). Liver contains high concentration of both CAT and GPx. Thus, H$_2$O$_2$ produced in the peroxisomes is largely disposed of by CAT, whereas H$_2$O$_2$ arising from mitochondria, the endoplasmic reticulum or cytosolic enzymes such as SOD is acted upon by GPx (Halliwell and Gutteridge, 1989). The capacity of glutathione system to cope in other tissues depends on the activity of GPx, GR and the pentose phosphate pathway enzymes (Taylor and Agius, 1988; Halliwell and Gutteridge, 1989).

The diabetes is associated with generalized increase in tissue oxidative stress, which is reflected in the changes in tissue glutathione antioxidant system. In the present study, it was observed that there is a complex variation in GPx activity in the diabetic state. We observed that liver and muscle exhibit decreased activity in diabetic state while kidney, heart and brain showed an increase in GPx activity. GPx forms a key enzyme of glutathione antioxidant system and works in concert with oxidized and reduced glutathione. It has been reported that total glutathione level is significantly increased in kidney tissue, but a slight decrease in liver tissue and increase in cardiac tissue (Strother et al, 2001). GPx activity reveals a similar pattern as the levels of GSH in various tissues (Maritim et al, 2002). Given that GPx works together with GSH in the decomposition of hydrogen peroxides (Meister and Anderson, 1983), the induction of GPx activity and the accompanied increase in glutathione level may merely be a manifestation of an antioxidant response to the increased oxidative stress in diabetic state. Similar events may also occur in heart tissue which has also been reported to show increase in both total glutathione level and GPx activity (Strother et al, 2001).

Ulusu et al, (2003) reported that glutathione peroxidase (GPx) activity was increased by streptozotocin induced diabetes in brain. The enhance activity of GPx in brain is consistent with present study. The increased activity represents a compensatory mechanism to degrade H$_2$O$_2$ that is produced in excess during the metabolism of catecholamines. It has been proposed that diminished activity of SOD leads to the generation of superoxides which in turn deactivates selenium dependent GPx by its reaction with selenium at the active site of the enzyme (Prabhu, 2002). This might be the reason for decreased activity of GPx in liver and muscle. This decrease is in accordance with the findings of Genet et al, (2002) and Gumieniczek et al, (2001) respectively.

The treatment of experimental diabetic animals with insulin, AILE, AIBE and AISO separately, restored the altered activity GPx in liver, kidney, heart, muscle and brain.
However AILE treatment was found more effective in reversing the altered activity of GPx, than AIBE and AIISO. It is appropriate to say that the primary cause of the restoration of GPx activity could be the lowering of blood glucose levels. It has been postulated that excess glucose is toxic and is the proximal source of increased oxidative stress in hyperglycemic condition (Baynes, 1991). Glucose has been demonstrated to get oxidized generating H$_2$O$_2$. Thus, a curb in the H$_2$O$_2$ levels may cause a subsequent normalization of the enzyme activity. Garg et al, (1993) and Bhanwara et al, (2000) reported that A. indica leaf extract has hepato-protective effect against hydroxyl radical formation. The ability of A. indica to arrest the hydroxyl radical formation may also be a contributing factor in the restoration of the GPx activity.

4.4.4 Glutathione reductase

Glutathione reductase (GR) is an important enzyme required for maintaining high GSH/GSSG ratio (Carlberg I and Mannervick, 1985). The enzyme has ten cysteine residues per monomer that participate in catalysis. GPx works together with GSH in the decomposition of hydrogen peroxides (Meister and Anderson, 1983). The GSSG thus formed is reduced back to GSH in a secondary reaction catalysed by GR which utilizes NADPH as a cofactor. GR forms an inevitable part of glutathione antioxidant system and works in concert with GPx to maintain the redox state in the cell.

The present study demonstrates a complex nature of variations in GR activities in the diabetic rats. There is a decrease in the GR activity in liver and muscle whereas, kidney, heart and brain display increased activity of GR in alloxan diabetic rats. The observed pattern is in accordance with the GPx activity. The decreased GR activity in liver and muscle is in agreement with those of Wohaieb and Godin (1987) and Gumienczek et al, (2001). According to Kakarla (2005), reduced level of GR activity suggests an inadequate level of reducing equivalents (NADPH) and a failure to maintain GSH levels. It is possible that inability to maintain GSH could result from the loss of GR activity in these tissues. It has been reported that the loss of GR activity is due to its inhibition by glycation. The time dependent inhibition of bovine intestinal GR by glucose, glucose-6-phosphate and fructose suggest that these sugars bind non-enzymatically to this enzyme (Blakytny et al, 1992). An elevated GR activity in kidney and heart in diabetic rats had been reported earlier by Mak et al, (1996). Bhardwaj et al, (1998) reported increased activity of GR in different brain regions, such as cerebral hemisphere, cerebellum, brainstem, thalamus and hypothalamus after one and three months of streptozotocin induced diabetes in rats. The increase in
activity of GR may be attributed to the adaptive response against increased oxidative stress though a precise reason for the discrepancies in different tissues is not known.

In the present study, insulin supplementation to diabetic rats reversed the alterations in GR activities in all the tissues studied. Similarly, treatment with AILE, AIBE and AIso significantly restored the diabetes induced changes in GR activity and reverted the values back to euglycemic condition in all the tissues studied namely liver, kidney, heart and brain. However, in case of skeletal muscle, partial restoration of GR activity was observed in diabetic tissue. The best reversal pattern in GR activity was observed by AIBE treatment. The ameliorating effect may primarily be due to the glucose lowering ability of the various treatments. The beneficial effect of the extracts is likely to be due to their ability to improve the glucose metabolism by lowering the glucose concentration in the blood finally resulting in the reduction of free radicals production.

**4.4.5 Glucose-6-phosphate dehydrogenase**

Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme of the pentose phosphate pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the supply of reduced glutathione (GSH) in the cells that is used to scavenge free radicals which cause oxidative damage. The entire antioxidant system, as well as other reductant-requiring processes, relies on an adequate supply of NADPH because it is the principal intracellular reductant for all cells and G6PD is the principle source of NADPH. Therefore, a decrease in G6PD activity leads to decreased NADPH and makes cells very sensitive to oxidant damage. NADPH is used by the glutathione and thioredoxin systems to regenerate reduced forms that will then be used in antioxidant roles. Catalase does not use NADPH directly, but an essential allosteric binding site for NADPH maintains catalase in its most active tetrameric conformation and protects it against the toxicity of hydrogen peroxide (Kirkman, 1984).

In the present study, G6PD activity decreased in all the diabetic tissues studied, namely, liver, kidney, heart, skeletal muscle and brain. The results are in agreement with the previous reports (Diaz-Flores, 2006; Zhang et al, 2000). Decrease of G6PD causes the buildup of glucose and thus, an increase of advanced glycation end products (AGE). Martini and Ursini (1996) reported that partial or complete inhibition of G6PD activity led to highly significant increases in oxidative stress and cell death. Also, Rosenstraus and Chasin (1975), using a G6PD-deficient Chinese hamster ovary cell line, showed that G6PD deficient cells
were more susceptible to oxidative stress. On the other hand, any changes in G6PD activity will alter NADPH levels and thus impact the antioxidant system.

In the present study, insulin supplementation to diabetic rats reversed the alterations in G6PD activities in all the tissues studied. Similarly, treatment with AILE, AIBE and AISO significantly restored the diabetes induced changes in G6PD activity in liver and kidney. However, in case of cardiac tissue, skeletal muscle and brain, partial restoration of G6PD activity was observed after treatment. The best reversal pattern in G6PD activity was observed by AIBE treatment. The ameliorating effect may primarily be due to the glucose lowering ability of the various treatments. High glucose and diabetes decrease G6PD activity in endothelial cells, kidney, liver, and red blood cells, which leads to oxidative damage, cellular dysfunction, and organ damage (Diaz-Flores, 2006; Zhang et al, 2000). Zhang et al (2000) reported that G6PD activity was inhibited in endothelial cells exposed to high glucose and that this inhibition of G6PD occurred in part via phosphorylation caused by high glucose-induced protein kinase-A activation.

4.5 Effect of A. indica extracts on lipid peroxidation

In aerobic organisms, reactive oxygen species (ROS) are generated constantly during physiological or pathophysiological mitochondrial oxidative metabolism and are efficiently neutralized by cellular antioxidant defenses. An excessive and/or sustained increase in free radical production associated with diminished efficacy of the cellular defense systems results in oxidative stress, which occur in many unrelated pathological processes and may contribute significantly to disease mechanisms. The presence of oxygen free radicals and the simultaneous decline of antioxidative defense mechanism observed in diabetic patients could promote the development of late diabetic complications (Godin et al, 1988). It is generally agreed that lipid peroxidation is elevated in diabetes (Slatter et al, 2000; Chang et al, 2005). Among lipid peroxidation products, plasma MDA concentrations are the most frequently used biomarker for assessing in vivo oxidative stress in human subjects (Nielsen et al, 1997). In this study, oxidative modification of lipids were found in microsomal membranes of alloxan diabetic animals in which MDA levels were significantly higher than those of healthy age-matched controls, thereby showing them to be under oxidative stress.

Treatment of diabetic animals with AILE, AIBE and AISO significantly inhibit the lipid peroxide formation. A significant increase in the level of lipid peroxidation in diabetic rats could be due to reversal mechanism occurring in the diabetic condition like increased generation of free radicals by several means, including direct glucose autoxidation, non-
enzymatic protein glycation, activation of NADPH oxidases, nitric oxide synthase and xanthine oxidase (Wolff and Dean, 1987; Inoguchi et al., 2000; Desco et al., 2002). Furthermore, enhanced glucose flux through the polyol pathway leads to depletion of the NADPH available for glutathione reductase, thereby eliciting changes in the glutathione redox status (Williamson et al., 1993). According to Dominguez et al., (1998) and Ceriello et al., (2000), hyperglycemia has also been shown to disrupt intracellular antioxidant defense mechanisms. Another possible reason could be the increased production of hydroxyl radicals, which are stimulators of lipid peroxidation (Quinlan and Gutteridge, 1988).

*A. indica* have been shown to have protective action against hydroxyl radical formation (Garg et al., 1993; and Bhanwara et al., 2000). The present findings show the increased lipid peroxidation in tissues of the diabetic rats, and treatment with AILE, AIBE and AISO separately decreased the level of lipid peroxidation in all the tissues of diabetic rats studied. It may, therefore, be inferred that free radicals mediated lipid peroxidative injury, which plays a crucial role in the pathophysiology of diabetes, can be prevented and controlled by A. indica extracts.

### 4.6 Effect of *A. indica* extracts on GLUT 4 translocation

A key role of insulin is to facilitate the uptake of glucose from blood into muscle and adipose tissues (Kahn, 1996; Holman and Sandoval, 2001; Zorzano et al., 1996). In muscle, two glucose transporter isoforms are expressed—GLUT 1 and GLUT 4. The latter is quantitatively more abundant in adult rat muscle and is distributed among intracellular compartments in the basal state, from where it is translocated to the plasma membrane in response to insulin or exercise (Holman and Sandoval, 2001; Zorzano et al., 1996; Tomas et al., 2001). GLUT 1 is mainly located at plasma membrane (Zorzano et al., 1996) and is considered to be responsible for basal glucose transport across the membrane. GLUT 4 expression is down-regulated when there is relative insulin deficiency, such as in streptozotocin induced diabetes and in chronic fasting (Charron et al., 1999).

GLUT 4 protein levels were measured by immunoblot analysis in the membrane fraction of skeletal muscle. In agreement with the previous studies, GLUT 4 protein significantly decreased in the total membrane fractions of skeletal muscle of alloxan diabetic rats (Kahn and Cushman, 1987; Garvey et al., 1988). Since glucose transport in skeletal muscle occurs mainly through GLUT 4, the reduction in the GLUT 4 level results in decreased uptake of glucose and, therefore, contributes to the increased blood glucose levels in diabetic condition.
Treatment of diabetic rats with insulin, AILE, AIBE and AISO separately, restored the GLUT 4 levels close to the normal values. AILE and AIBE treatment showed better reversal than AISO after 21 days of treatment. The normalization of GLUT 4 level is an important parameter to evaluate the antidiabetic properties of AILE, AIBE and AISO, as one of the main reasons of hyperglycemia in diabetes mellitus is the decreased uptake of glucose by the insulin dependent tissues. The present study has shown that after 21 days of diabetes induction, the GLUT4 level reduced in both cytosolic and membrane fraction of skeletal muscle. This indicates that the deficiency of insulin in diabetic state decreased both expression and translocation of GLUT 4 in skeletal muscle tissues. Treatment with A. indica extracts partially revived the altered distribution and expression of GLUT4. Restoration of GLUT 4 levels would, therefore, enhance the uptake of glucose in skeletal muscle and thus helps in alleviating the hyperglycemic condition which in turn arrests all diabetic complications.

4.7 Effect of A. indica extracts on Protein Kinase C – β2 isoform

The protein kinase C (PKC) family of enzymes transduces myriad of signals promoting lipid hydrolysis. Signals that stimulate members of the large families of C-protein coupled receptors; tyrosine kinase receptors or non-receptor tyrosine kinases can cause diacylglycerol (DAG) production either rapidly by activation of phospholipase-C or more slowly by activation of phospholipase–D to yield phosphatidic acid and then diacylglycerol (Asaoka et al, 1992; Nishizuka, 1992; Nishizuka, 1995).

The increased level of PKC β2 in skeletal muscle and cardiac muscle was identified in the present study. The results are supported by various reports (Steiler et al, 2003; Inoguchi et al, 1992; Koya and King, 1998). Intracellular hyperglycemia increases the amount of DAG resulting in activation of PKC in cultured microvascular cells and in retina and renal glomeruli of diabetic animals (Koya and King, 1998). They seem to achieve this primarily by increasing de novo DAG synthesis from glycolytic intermediate dihydroacetone phosphate, through reduction of latter to glycerol-3-phosphate and stepwise acylation (Koya and King, 1998). Increased de novo synthesis of DAG activates PKC both in cultured vascular cells (Xia et al, 1998) and in retina and glomeruli of diabetic animals (Koya and King, 1998). The β- and δ- isoform of PKC are activated primarily but increase in other isoforms have also been found. The activation of PKC by hyperglycemia may be tissue specific, since it was noted in retina, glomeruli (Koya and King, 1998), aorta, heart (Inoguchi et al, 1992) and skeletal muscle (Steiler et al, 2003).
Hyperglycemia may also activate PKC isoforms indirectly through both ligation of AGE receptors (Portilla, 2000) and increased activity of polyol pathway, presumably by increasing ROS (Keogh et al., 1997). Oxidative stress may also be involved in the activation of DAG-PKC in vascular tissue (Nishikawa et al., 2000). This may be achieved by increasing de-novo synthesis of DAG, so the effect of hyperglycemia on PKC activation probably reflects increased DHAP levels resulting from inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by reactive oxygen species. Oxidants produced in the setting of hyperglycemia can activate PKC (Konishi et al., 1997). It has also been postulated that H$_2$O$_2$ induces oxidative modification of PKC, thereby activating the enzyme (Gopalakrishna and Anderson, 1989), but no substantial evidence is available for such modification.

The exogenous supplementation of insulin to diabetic rats considerably ameliorated the augmented levels of PKC $\beta$2 in the diabetic heart and muscle in experimental diabetic rats. Similarly, treatment with AILE, AIBE and AISO restored the levels back to euglycemic condition as demonstrated by immunoblots of heart and muscle. This could primarily be due to the subsequent lowering of blood glucose levels which reduces the oxidant levels. The lowered oxidative stress by these extracts may block the DAG synthesis that directly reduces PKC $\beta$2 levels. Several studies have shown that antioxidants such as vitamin E can inhibit PKC activation probably by decreasing DAG levels (Nishikawa et al., 2000; Kunisaki et al., 1996).

### 4.8 Effect of *A. indica* extracts on DNA degradation

Superoxide is an oxygen derived free radical that has been implicated in oxidative injury. Enzyme superoxide dismutase has been shown to both, ameliorate and exacerbate such oxidative injury (Chan et al., 1995; Bar-Peled et al., 1996). Oxidative stress is known to play an important role in the pathogenesis and complication of diabetes. One of the important fallbacks of oxidative stress is increased H$_2$O$_2$ production. It has already been reported that during vascular conditions such as atherosclerosis, circulating macrophages release large amount of H$_2$O$_2$. Apoptosis that occurs as a result of this contributes towards these pathologies (Geng and Libby, 1995). DNA fragmentation is one of the morphological changes during apoptosis.

All ROS have the potential to interact with cellular components including DNA bases or the deoxyribosyl backbone of DNA to produce strand breaks and damaged bases. The decrease in antioxidant enzyme activities and their protein levels leads to the accumulation of ROS during diabetes. These increased ROS cause damage to the vital processes of the cell.
like DNA degradation and induced apoptosis (Morel and Barouki, 1999). Murata (2004) showed that the concentration of many endogenous aldehydes such as 3-deoxyglucosone and glyceraldehyde (GA) increase under hyperglycemic conditions. GA has the strongest ability to damage DNA, and the addition of low concentrations of H$_2$O$_2$ markedly enhanced the DNA damage. Pathological condition such as diabetes, which increase the rate of H$_2$O$_2$ production with decreased antioxidant system will lead to the accumulation of H$_2$O$_2$ in tissues and cause DNA degradation (Burdon, 1995). It can, therefore be concluded that oxidative DNA damage by hyperglycemia-related generated aldehydes, especially GA, and marked enhancement of DNA damage by H$_2$O$_2$ may participate in diabetes associated long-term pathogenesis. Thus, the measurement of oxidative DNA degradation in a diabetic state can be a suitable marker for the evaluation of systemic oxidative stress in diabetic patients.

In the present study, DNA laddering method has been used to evaluate the degradation of genomic DNA of control, alloxan induced diabetic animals and diabetic rats treated with insulin, AILE, AIBE and AISO. Oxidative DNA degradation was observed in the diabetic animals when compared to the normal controls and results were in agreement with the earlier reports (Farhangkhoee et al., 2003; Andican and Burcak, 2005). Treatment of diabetic animals with insulin, AILE, AIBE and AISO for 21 days prevented genomic DNA degradation.

4.9 Therapeutic potential of A. indica

Majority of the metabolic derangements and clinical complications contributing to the morbidity and mortality of the diabetes mellitus may be prevented or reversed with an effective control of hyperglycemia. The excess amount of circulating free glucose and its over-utilization in insulin independent tissues leads to an increase in reactive oxygen species generation and subsequently cell membrane dysfunction. Insulin counters an array of hormons to regulate the glucose metabolism. Insulin regulates the uptake of glucose in peripheral tissues by recruiting the membrane vesicles containing the GLUT 4 transporter from intracellular storage sites to cell membrane, where it allows glucose to enter in cells by facilitative diffusion. Therefore, deficiency, absence or inactivation of insulin results in alteration in normal glucose metabolic pathway leading to the onset of hyperglycemia and subsequently causing diabetes mellitus.

The consequences of high levels of circulating glucose observed in uncontrolled diabetes, Type 1 and Type 2 are toxic to the cells. The high levels of glucose can lead to
activation of alternate pathways of glucose metabolism like sorbitol pathway. The major
toxic effect of high level of glucose is probably the glycation of protein and peptides. It is
believed that much of the neurological and circulatory defects in diabetes are due to
glycation. A number of drugs and insulin are used to treat diabetes, but none of them is
completely effective and without any side effects. Repeated insulin administration controls
the short term diabetic symptoms, but it fails to prevent the serious vascular and other
complications of diabetes. Moreover, exogenous insulin treatment fails to provide a well
controlled glycemc condition in association with variable dietary intake and variable
physical activity in Type 1 diabetes.

Episodes of severe hypoglycemia leading to a deleterious cerebral impact are
common during insulin administration (McCall, 1992). New drugs are coming up and the
management of diabetes is becoming easier. However, a number of people particularly in
the developing world have little or no excess to these new generation antidiabetic drugs.
Therefore, there is a need to make a therapy for diabetes, which is potent, safe and cost
effective. Several plants have been screened for the possible antidiabetic potential. The
results have been encouraging and though these natural products only partially reverse
hyperglycemia, no side effect has been reported with their use (Abdel-Berry and A-Hakiem,
2000; Flammang et al, 2005).

The present study showed that AILE, AIBE and AISO successfully attained
euglycemia and corrected the alteration in the metabolic pathways studied in the diabetic
rats. The alterations in the key enzymes of antioxidant defense system and membrane
transport system and membrane lipid peroxidation were normalized by AILE, AIBE and AISO
treatment. In addition to the above, the elevated levels of oxidative DNA damage in
diabetic rats were also prevented by the treatment with AILE, AIBE and AISO. There was a
substantial decrease in GLUT 4 protein expression and translocation in the heart and
skeletal muscle of diabetic rats, the same has been reported earlier (Kopp et al, 1997;
Koistinen et al, 2003). In the present results, after 21 days of treatment of diabetic rats with
AILE, AIBE and AISO, GLUT 4 levels were reverse to normal values. These beneficial effects
can be attributed to the hypoglycemic action of A. indica. The A. indica leaf extract are
known to possess hepato-protective activity against hydroxyl radical (Garg et al, 1993). The
aqueous extract of leaf also possesses potent immuno-stimulant activity as evidenced by
both humoral and cell-mediated responses (Sen et al, 1992; Ray et al, 1996). Results from
the present study also suggested an insulin secretion modulation in A. indica therapeutic
action. However, no detailed study is available to establish whether the plant extracts follow similar signaling biochemical routes such as those propose for insulin. Being a natural product with multitude of antidiabetic effects, *A. indica* can possibly be used as insulin replacement or an adjuvant in the management of both Type1 and Type 2 diabetes.

*A. indica* is the unique source of various types of compounds having diverse chemical structure. Very little work has been done on the biological activity and plausible medicinal applications of these compounds. More than 135 compounds have been isolated from different parts of *A. indica* and several reviews have also been published on the chemistry and structural diversity of these compounds (Kraus, 1995; Govindachari, 1992). Azadirachtin is a highly oxidized tetranortriterpenoid which boasts a plethora of oxygen functionality and comprising an enol ether, acetal, hemiacetal, and tetra-substituted oxirane as well as a variety of carboxylic esters. Priyadarshini et al. (2009) have reported that azadirachtin exhibit concentration-dependent anti-radical scavenging activity. In present study, considerable amount of azadirachtin has been observed in AILE, AIBE and AISO. However, a detailed study is required to ascertain its possible role in controlling diabetes induced oxidative stress.

Ethnomedical approach for diabetes is practical, cost effective and logical. Several modifications, improvement, sophistication and newer discoveries could contribute continuously to the type, quality, presentation and concept of medicinal preparation. The therapeutic use of development of human knowledge, scientists endeavor to isolate different chemical constituents from plant, put them to biological and pharmacological tests can be used to prepare modern medicines. *A. indica* may exhibit its therapeutic effects through modulation of insulin secretion. The present study showed the hypoglycemic and antioxidant properties of *A. indica*. A reduction in the production of free radicals and lipid peroxides formation by restoring the antioxidant enzymes was observed in the present study, which can beneficially prevent the diabetes associated tissue damage. As concluded from the studied parameters, AILE, AIBE and AISO were found effective in controlling the hyperglycemia induced oxidative stress and stabilizing the physiological parameters and antioxidant defense system. Further studies can ascertain which photochemical fraction is most efficacious in the treatment of diabetes. *A. indica* can further be explored for isolated compounds or herbal formulations for the development of integrated/alternative medicine to identify safe and effective drug for diabetes.