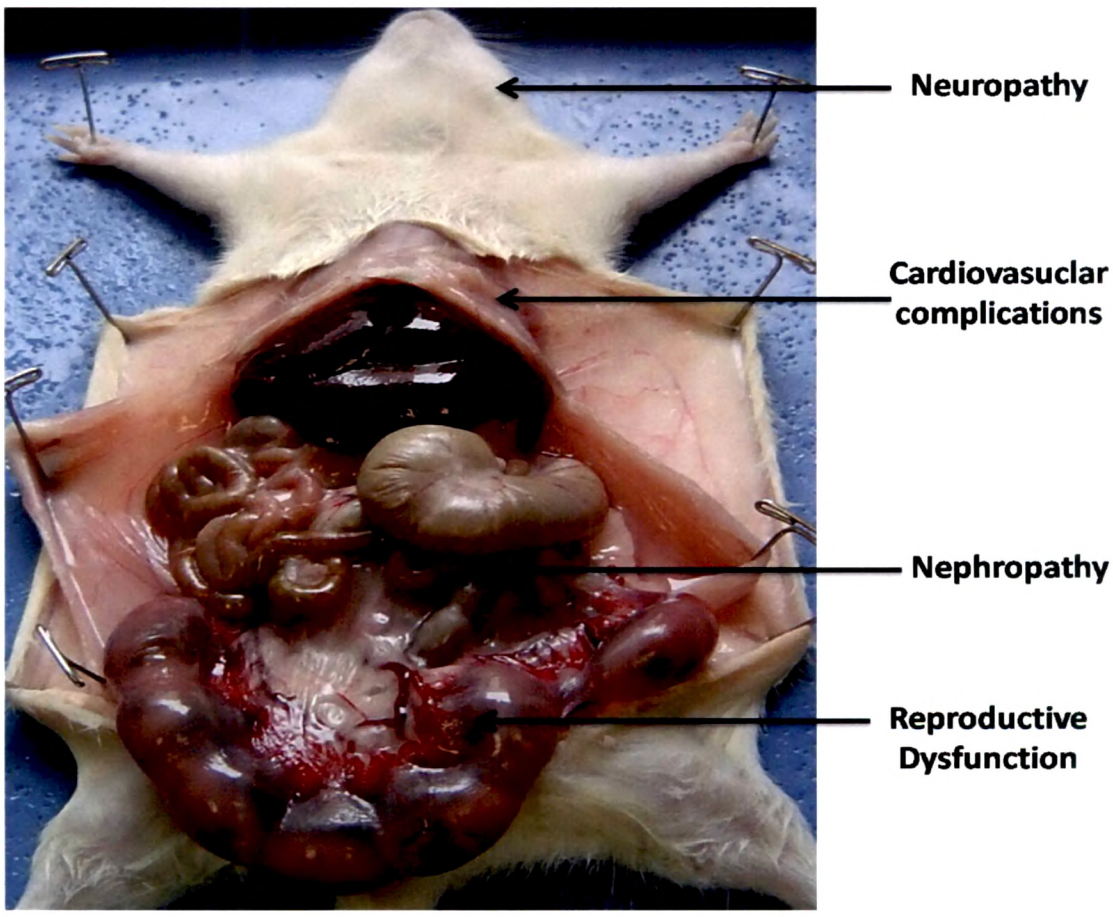
Chapter 6

Evaluation of efficacy of *E. littorale* methanolic extract in diabetic complications in alloxan- induced diabetic rat model.



Chapter 6

Evaluation of efficacy of *E. littorale* methanolic extract in diabetic complications in alloxan- induced diabetic rat model.

6A.1 Introduction

6A.2 Experimental design

6a.1 Diabetic Neuropathy

6b.2 Male Diabetic Reproductive Dysfunction

6c.3 Diabetic Cardiovascular Complications

6A.1 Introduction

6A.1.1 Microvascular and Macrovascular Complications of Diabetes

Microvascular complications in diabetes contribute to pathologic and functional changes in many tissues, including eye, heart, kidney, skin, and neuronal tissues. Based on the tissues affected, these changes are traditionally known as diabetic retinopathy, nephropathy, and neuropathy, respectively. In addition to these classic complications, pathologic changes in the microvessels of the myocardium reduce cardiac contractility, and ventricular dysfunction is often observed in diabetic patients. The development and progression of microvascular complications is associated closely with chronic hyperglycemia, a relationship supported by numerous clinical studies, such as the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study [The Diabetes Control and Complications Trial (DCCT)Research Group, 1993; The UK Prospective Diabetes Study (UKPDS) Group, 1998). Tight glycemic control is by far the most effective approach in the prevention of diabetic vascular complications.

6A.2. Pathophysiology

The association between hyperglycemia and diabetic complications is believed to be caused by the effect that high levels of glucose have on several metabolic pathways. Four specific pathways that have been identified are (DCCT Research Group, 1993) glycation of proteins, (UK Prospective Diabetes Study Group, 1998) the polyol or sorbitol pathway, (Koro et al., 2004) the protein kinase C pathway, and (Centers for Disease Control, 2006) the hexosamine pathway (Brownlee, 2001). These altered pathways form glucotoxins that cause alterations in gene expression and abnormal protein function. The effect is cellular dysfunction and damage, specifically in abnormal angiogenesis, abnormal cell growth and survival, hyperpermeability of cells, capillary basement membrane thickening, abnormal blood flow through the vasculature, increased leukocyte adhesions, and thrombosis (Sheetz and King, 2002). The role of oxidative stress

has been implicated as the unifying mechanism responsible for the microvascular complications of diabetes. All of the pathway defects are believed to lead to oxidative stress, which produces reactive oxygen species or free radicals that cause cellular damage (Brownlee, 2005). The polyol or sorbitol pathway metabolizes glucose into sorbitol via the enzyme aldose reductase. Aldose reductase uses nicotinamide adenine dicnucleotide in this pathway. Nicotinamide adenine dicnucleotide is also involved in the synthesis of nitric oxide, a key vasodilator in the microcirculation. When the amount of intracellular glucose increases, nicotinamide adenine dicnucleotide is diverted from its role in synthesizing nitric oxide. With less available nicotinamide adenine dicnucleotide in the cell, formation of nitric oxide decreases, causing vasoconstriction and decreased blood supply (Brownlee, 2001). Glycation of proteins produces advanced glycated end products (AGE). Circulating AGEs accumulate in the arterial walls, kidney mesangium, glomerulus, and basement membranes, leading to capillary basement membrane thickening. Low-density lipoproteins and immunoglobulins become trapped in artery walls, leading to oxidation and inflammation of the vessel wall. AGEs affect endothelial cells and stimulate macrophages to secrete factors that lead to enhanced cell permeability, increased fibroblast formation, and have procoagulant effects. The AGEs also cause the cells to become more rigid, impairing cell adhesion and axonal transport through the neuron (Feldman, 2005). AGEs in the kidney lead to leaking of protein through capillary basement membrane thickening, and alter function and structure of the microvessels. Protein kinase C is another glucose pathway that regulates vascular functions by specific enzymes. In the environment of hyperglycemia, the protein kinase C enzyme is activated and does not perform normally. This results in decreased amounts of nitric oxide leading to vasoconstriction. Growth factor-β1 and plasminogen activator inhibitor-1 are also increased (Brownlee, 2001). This can result in changes to the renal and retinal blood flow, contractility of vessels, vascular permeability, vascular inflammation, decreased fibrinolysis, and resultant vascular occlusion. Increased glucose flux through the hexosamine pathway causes a diversion in the pathway such that

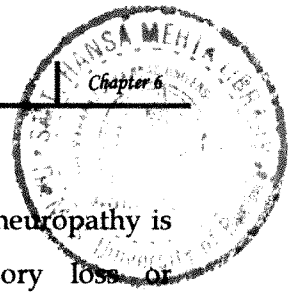
pathologic changes occur resulting in increased growth factor β1 and plasminogen activator inhibitor-1, both damaging to blood vessels. All of these pathway defects are believed to lead to oxidative stress. The free radicals produced by this affect the microvessels leading to the retina, kidney, and nervous system.

6A.2.1. Retinopathy

Diabetic retinopathy (DR) is an extremely common complication of diabetes affecting virtually all people with diabetes. Not all cases of DR result in blindness, although 33% to 87% of legal blindness occurring in people with diabetes is directly attributable to DR (Fong et al., 2004). The natural history of DR is a progression from mild nonproliferative DR, in which there is increased vascular permeability, to moderate to severe nonproliferative DR, to proliferative DR, which is characterized by growth of new blood vessels on the retina. Macular edema may also occur by leaking blood vessels, with retinal thickening. People with more severe disease can develop vitreous hemorrhage and retinal detachment. Because diabetes-related blindness is preventable, early detection and intervention is crucial to saving vision; there is ample evidence that demonstrates improvement with surgical treatments.

6A.2.3. Nephropathy

Diabetic nephropathy is a major microvascular complication with considerable medical and economic impact among persons with diabetes. Estimates of diabetic nephropathy defined by increased urinary albumin excretion in the absence of other renal diseases are approximately 40% of type 1 and type 2 patients with diabetes (Gross et al., 2005). Diabetic kidney disease is the most common cause of ESRD (or kidney failure requiring dialysis or transplantation) in the United States, Japan, and Europe (Rabkin, 2003) and is associated with increased cardiovascular mortality (Gross et al., 2005).



6A.2.4. Neuropathy

There are many types of diabetic neuropathy (peripheral polyneuropathy is the major form), characterized by distal, symmetrical sensory loss or sensorimotor dysfunction that often affects distal lower limbs (Harati, 1996). Several pathologic changes are noted, including the loss of nerve fibers, paranodal or segmental demyelination, axonal thickening, and endoneuronal capillary narrowing (Malik et al., 1993). Hyperglycemia and other metabolic derangement may damage neurons and nerve parenchyma (Sheetz and King, 2002). In addition, abnormalities in neurovascular blood flow may cause ischemic-neuronal damage (Sheetz and King, 2002). Biochemical changes akin to those seen in retinopathy and nephropathy (eg. activation of PKC, enhanced oxidative stress, formation of AGEs in neuronal tissues, and altered expression of neurotrophic factors such as nerve growth factor and insulin-like growth factors-1) have been suggested to contribute to the pathogenesis of diabetic neuropathy (Harati, 1996; Sugimoto et al., 2002). The vascular etiology of diabetic neuropathies is supported by multiple abnormalities in the neurovasculature, including the deposition of AGEs in the perineuronal vascular wall, basement thickening, endothelial cell swelling, loss of pericytes, reduced endothelial nitric oxide activity, capillary occlusion (Harati, 1996), and degeneration of blood vessels supplying neuronal tissues (Schratzberger et al., 2001). These changes eventually contribute to a hyperglycemia-induced decrease in neurovascular flow and subsequent hypoxic-ischemic damage (Cameron et al., 2001). Transfer of VEGF to neuronal tissue in experimental diabetic animal models has been reported to restore blood flow in neuronal tissues and rectify the conductivity of nerves that were impaired in diabetic states (Schratzberger et al., 2001), affirming the microvascular pathology nature of diabetic neuropathy.

6A.3. Macrovascular Complications of Diabetes

The central pathological mechanism in macrovascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body. Atherosclerosis is thought to result from chronic inflammation and injury

to the arterial wall in the peripheral or coronary vascular system. In response to endothelial injury and inflammation, oxidized lipids from LDL particles accumulate in the endothelial wall of arteries. Angiotensin II may promote the oxidation of such particles. Monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes. T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation. The net result of the process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Rupture of the lesion leads to acute vascular infarction (Boyle, 2007). In addition to atheroma formation, there is strong evidence of increased platelet adhesion and hypercoagulability in type 2 diabetes. Impaired nitric oxide generation and increased free radical formation in platelets, as well as altered calcium regulation, may promote platelet aggregation. Elevated levels of plasminogen activator inhibitor type 1 may also impair fibrinolysis in patients with diabetes. The combination of increased coagulability and impaired fibrinolysis likely further increases the risk of vascular occlusion and cardiovascular events in type 2 diabetes (Beckman et al., 2002). Diabetes increases the risk that an individual will develop cardiovascular disease (CVD). Although the precise mechanisms through which diabetes increases the likelihood of atherosclerotic plaque formation are not completely defined, the association between the two is profound. CVD is the primary cause of death in people with either type 1 or type 2 diabetes (Laing et al., 2003; Paterson et al., 2007). In fact, CVD accounts for the greatest component of health care expenditures in people with diabetes (Paterson et al., 2007; Hogan et al., 2002).

6A.4. Preventive strategies

Although there are specific treatment interventions for diabetic eye and kidney disease, the strategy common to all treatment interventions is control of blood glucose (hemoglobin A1c), blood pressure, and lipids. These have been labeled the ABCs (hemoglobin A1C, blood pressure, and cholesterol) of diabetes

management by the American Diabetes Association. In the Epidemiology of Diabetic Interventions and Complications study, individuals with type 1 diabetes who had experienced periods of good glycemic control but whose control had deteriorated somewhat years later still showed a sustained preventive effect of developing retinopathy and nephropathy (Writing team for the Diabetes Control and Complications Trial, 2002). Hypertension control has also been shown to decrease the risk for developing microvascular disease (The UKPDS Study Group, 1998) and there is evidence that links diabetic dyslipidemia with retinopathy and nephropathy (Jenkins et al., 2004). Smoking and poor metabolic control increase the risk of periodontal disease. Besides pharmacologic therapies, healthy lifestyle habits, such as healthy eating and being physically active, are mainstays of treatment. Diabetes self-management education is crucial to the prevention of diabetic complications.

6A.5. Experimental Design

6A.5.1. Induction of diabetes

Animals were fasted overnight and given injection of alloxan at a dose of 120mg/kg/ml body weight intraperitoneally. Animal were kept for blood glucose stabilization for 15 days and blood glucose levels were checked on the 15th day. Animals with blood glucose >200mg/dL were taken further for the experiment.

Animals were divided into 4 groups, minimum six animals in each:

Group-I : Control (C), injected intraperitonially (i.p) with saline and intragastrically (i.g) with 1% carboxymethylcellulose (CMC).

Group-II : Alloxan treated (i.p) and i.g with 1% CMC.

Group-III : Alloxan treated + EL treated (i.g).

Group-IV : Alloxan treated + Glib treated (i.g).

The *EL methanolic* extract was given at a dose of 2.5 g/kg B.wt/day via gastric intubation for 45 days (Maroo et al., 2003). The Glibenclamide (Glib) was

used as reference drug at a dose of 2mg/kg b.wt/day (Kavutcu et al., 1996) for 45 days. For the all the diabetic complication studies dose used was same. Dose selection was done based on previous studies of our lab. Different biochemical parameters were evaluated based on the requirement of the study.

6a. Evaluation of efficacy of *E. littorale* methanolic extract in diabetic neuropathy in rat model.

6a.1 Review of literature

6a.2 Experimental design

6a.3 Results

6a.4 Discussion

6a.1. Review of Literature

Diabetic neuropathy occurs in 50% of diabetic patients. These patients are suffering from severe and unremitting pain. Diabetic neuropathic patient generally complain about persistent burning or tingling sensation, usually in the legs and feet. Other symptoms include an inability to detect heat and cold, cutaneous hyperaesthesia, loss of vibration sensation, and paradoxically, the loss of pain perception. The pathophysiology of the condition remains unclear, although it has been associated with peripheral demyelination, a decrease in peripheral nerve conduction, and degeneration of myelinated and unmyelinated sensory fibres (Dyck et al., 1988).

The most important etiologic factors are poor glycemic control, and duration of the disease, with other risk factors like hypertension, age, smoking, hypoinsulinemia, and dyslipidemia (Shaw et al., 2003). Hyperglycemia can induce oxidative stress via glucose autooxidation and the subsequent formation of advanced glycation end products, disruption of the polyol pathway, altered eicosanoid metabolism, and decreased antioxidant defense (Greene et al., 1999; Cameron et al., 1996).

To combat oxidative stress in diabetic neuropathy antioxidants treatment has been tried in both animals and diabetic patients. Administration of the antioxidants vitamin C or α-lipoic acid, as well as free amino acids, improves responses to insulin and thus can provide additional benefit to the proposed reduction of oxidative stress in tissues (Paolisso et al., 1994; Jacob et al., 1995; Natarajan et al., 2002; Henriksen and Saengsirisuwan, 2003). Vitamin E decreases blood glucose in type 1 diabetic rats through an unknown mechanism (Nazaimoon and Khalid, 2002).

Diabetic neuropathic pain, an important microvascular complication of diabetes mellitus is recognized as one of the most difficult types of pain to treat.

The development of tolerance, inadequate relief and potential toxicity of classical antinociceptives warrant the investigation of the newer agents to relieve this pain. The aim of the present study was to explore the antinociceptive effect of EL and its effect on different biochemical parameters in sciatic nerve of diabetic neuropathic rat.

In traditional practice, medicinal plants are used in many countries to control DM. The National Center for Complementary and Alternative Medicine, established in 1998 by the United States Government; where development of herbal medicine is one of the important subjects of study (Yoon et al., 2004; Edwards et al., 2005).

6a.2. Experimental Design

Diabetes was induced by alloxan administration in the rats and treatment regimen was mentioned in section 6A.5.1 On 45th days after the treatment with EL and Glib animals were bled for blood glucose estimation. Their response to nociceptive response were evaluated with the help of tail-flick and formalin-induced flinch assay. Animals were sacrificed and their sciatic nerves were isolated for different biochemical parameters like aldose reductase, Na^+-K^+ ATPase and nonenzymatic and enzymatic antioxidant parameters were evaluated as per the procedure mentioned in chapter 2.

6a.3.Results

6a.3.1. Blood glucose Level

Alloxan-induced diabetic rats were hyperglycemic. Rats treated with EL for 45 days showed significant reduction in blood glucose levels by 54% (Fig 6a.1).

Figure 6a.1 Effect of EL on **blood glucose** levels of diabetic rats

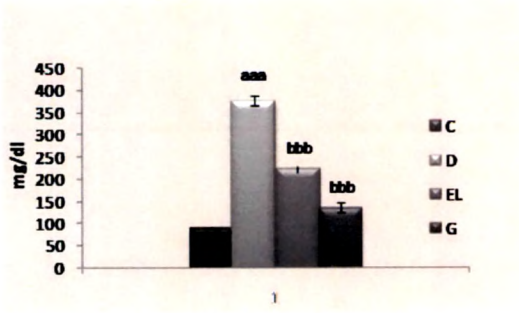


Fig. 6a.2 Effect of EL on **thermal nociception**

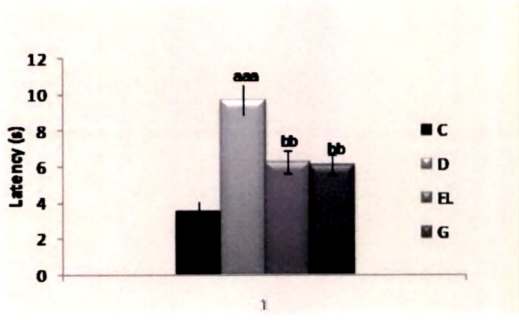


Figure 6a.3 Effect of EL on **formalin induced flinches**

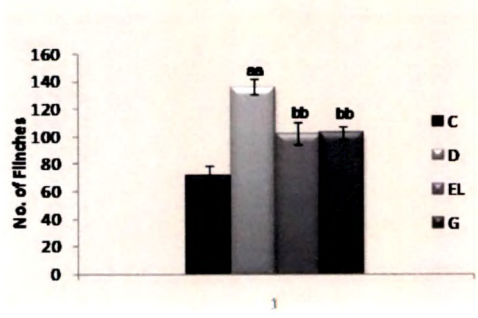


Figure 6a. 4. Effect of EL on sciatic nerve **aldose reductase** activity

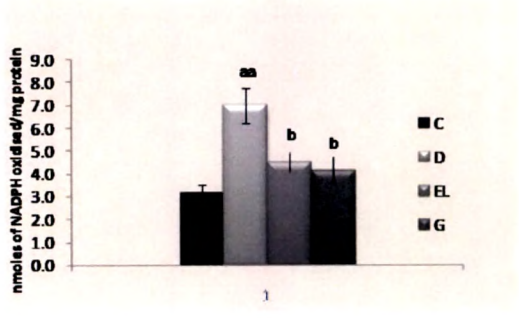


Figure 6a.5 Effect of EL on sciatic nerve **Na^+-K^+-ATPase** activity.

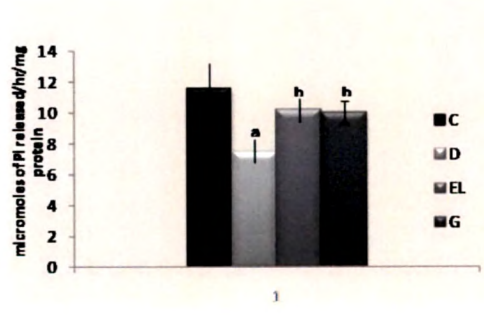
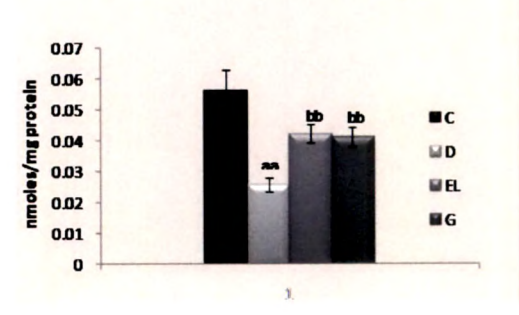


Figure 6a.6 Effect of EL sciatic nerve **GSH** content



Values are expressed as mean ± SEM (n=6 in each group). a, P<0.05, aa, P<0.01, aaa,P<0.001 vs. C, b,P<0.05, bb P<0.01, bbb, P<0.001 vs. D.

Figure 6a.7 Effect of EL on **LPO** of sciatic nerve

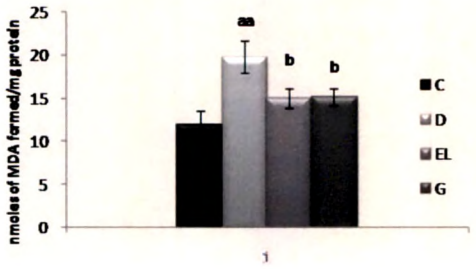


Figure 6a. 8. Effect of EL on **SOD** activity of sciatic nerve

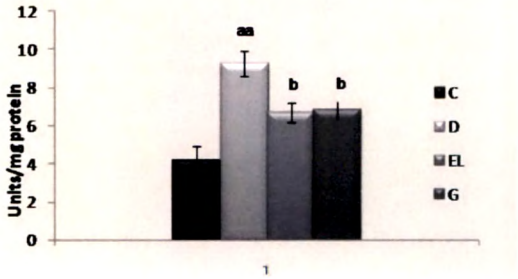


Figure 6a.9 Effect of EL on **CAT** activity of sciatic nerve

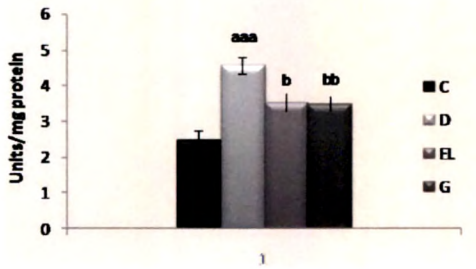
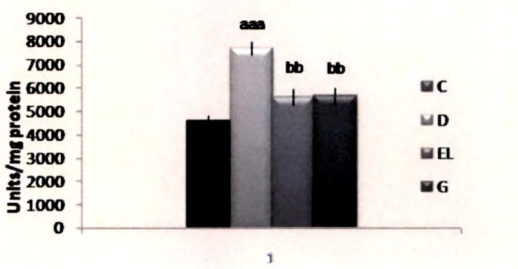


Figure 6a.10. Effect of EL on **GPx** activity of sciatic nerve



Values are expressed as mean ± SEM (n=6 in each group). a, P<0.05, aa, P<0.01, aaa,P<0.001 vs. C, b,P<0.05, bb P<0.01, bbb, P<0.001 vs. F.

6a.3.2. Nociceptive threshold

In the present study, thermal hypoalgesia occurred in alloxan-induced diabetic rats. Response to a thermal noxious stimulus of diabetic rats was prolonged significantly compared to normal rats (Fig 6a.2). Administration of EL prevented this elevation of tail-flick threshold by 56%. Injection of 5% formalin into the hind paw of control rats evoked a series of flinching responses of the afflicted paw. In diabetic animals it has been observed that number of flinches increased significantly as compared to control animals. Upon EL treatment noxious stimulus was reduced to 52% as reflected in decrease in number of flinches in 30 min (Fig 6a. 3).

6a.3.3. Aldose Reductase and Na-K ATPase activity from sciatic nerve

There was significant increase in aldose reductase enzyme activity in sciatic nerve of diabetic animals as compared to normal animals. Diabetic rats treated with EL showed decrease in aldose reductase activity by 64% (Fig 6a.4). Sciatic nerve Na-K ATPase activity (Fig.6a.5) was significantly reduced in diabetic rats as compared to normal control. This was largely corrected by EL treatment by 64%.

6a.3.4.Antioxidant parameters

The GSH contents (Fig.6a.6) of sciatic nerve was reduced to 54% in diabetic animals. The GSH level was significantly improved to 55% by EL treatment to diabetic rats. MDA, lipid peroxidation product significantly increases in sciatic nerve of diabetic rats as compared to normal rats. Diabetic rats treated with EL showed significant decrease in MDA levels by 62% (Fig 6a.7). Diabetic rats showed decrease in sciatic nerve SOD activity as compared to normal rats. EL treatment brings the activity of SOD to near normal by 66% (Fig6a.8). Similarly it has been observed that sciatic nerve Catalase (Fig 6a.9) and GPx (Fig 6a.10) activity increases significantly in diabetic rats as compared to normal control. Diabetic rats treated with EL showed decrease in these enzymes by 51% and 67% respectively.

6a.4. Discussion

Neuropathic pain is most common symptom associated with diabetic neuropathy. We evaluated the nociceptive response in alloxan-diabetic rats. The formalin test has received much attention as a proposed model of persistent pain which depends on sensitization in spinal cord dorsal horn (Coderre et al., 1990, 1993b, 1994) and brain (Coderre et al., 1993b). Formalin at high doses (5%) produced inflammatory response as compared to low dose (1%).

There are two main conclusions to be drawn from the obtained result; first, it clearly demonstrated an intensified nociceptive response in the formalin test in diabetic rats. It is a well-established fact that diabetic rats display exaggerated hyperalgesic behavior in response to noxious stimuli (Freshwater et al., 2002). STZ-diabetic rats have been increasingly used as a model of painful diabetic neuropathy to assess the efficacy of potential analgesic agents (Fox et al., 1999). Although evaluation of mechanisms causing these symptoms is complicated because of the overlap between the systemic effects of hyperglycemia and its toxic effects within the peripheral nervous system, but direct functional toxicity of hyperglycemia in the peripheral nervous system (Dobretsov et al., 2001), an increased activity of primary afferent fibers leading to an increased excitatory tone within the spinal cord, increased release of glutamate and activation of the NMDA receptor, reduced activity of both opioidergic and GABAergic inhibitory systems (Malcangio and Tomlinson, 1998), decreased activity of nNOS–cGMP system in neurons of dorsal root ganglion (Sasaki et al., 1998), altered sensitivity and responsiveness, possibly through the enhancement and/or deactivation of the endogenous Met-enkephalinergic system (Takeshita and Yamaguchi, 1998; Rutledge et al., 2002), and alterations in L-type Ca2+ channels (Gullapalli et al., 2002) could be involved in the modulation of nociception in diabetic rats.

Secondly, it was demonstrated that oral administration of methanolic extract of *Enicostemma littorale* at a dose of 2.5 g/kg for a period of 45 days could produce a significant analgesic effect in the formalin test in diabetic rats. On the other hand, Glibenclamide also significantly reduced the nociceptive score in the formalin test in diabetic rats. It has been known that central acting drugs like narcotics inhibit both phases of the formalin test equally (Shibata et al., 1989), while peripheral acting drugs like aspirin only inhibit the late phase (Rosland et al., 1990). Therefore, the effect of *Enicostemma littorale* extract in this study could be mediated through a central and/or a peripheral mechanism. One of the possible mechanisms that could partially explain the beneficial analgesic property of *Enicostemma littorale* extract may be attributed to its hypoglycemic

(Vijayvargia et al., 2000), and antioxidant (Maroo et al., 2003) effects. Since hyperglycemia in diabetic state could induce some functional alterations in the nervous system (Dobretsov et al., 2001). Swertiamarin one of the major constituent of EL possesses antiedematogenic activity in the carrageenan, formalin, and histamine-induced paw edema methods in rats and also showed in vitro antioxidant activities of E. axillare (Vaijanathappa et al., 2009). Studies by Sadique, inferred that E. littorale may exert its anti-inflammatory effect through inhibition of phospholipase A2 leading to restriction on the availability of arachidonic acid and resultant inhibition of PG biosynthesis and also possibly because of their beneficial effects on the stabilization of lysosomal membranes by corticosteroids.

There are many studies of hyper and hypoalgesia in STZ-induced diabetic animal models. While thermal hypo-algesia is reported in diabetic rats using the tail-flick test or the hot-plate test (Akunne et al., 1987; Apfel et al., 1994, Chu et al., 1986; Kolta et al., 1996; Levine et al., 1982; Calcutt et al., 2003), others have found hyperalgesia in diabetic animals (Forman et al., 1986; Ohsawa et al., 1999; Simon et al., 1981). Richard McCarty's findings provide support for the idea that hyperglycemia does contribute to a state of hyperalgesia in alloxan-diabetic rats. Diabetic animals, clearly demonstrated an increased sensitivity to pain when hyperglycemic and decreases when euglycemic in tail-flick experiment. In this study, we observed a reduction in tail flick latency in hot immersion test in diabetic rats, which indicates thermal hyperalgesia. We observed a significant increase in tail flick response latency for hot immersion tests following EL treatment and thus showed reduction in nociception. Similar study was conducted by Jaishree et al. (2009) reported that it is swersamarine one of the bitter principle present in the EL, have antinociceptive activity in three different animal models of pain sensation. They found that swertiamarin possess both peripheral and central antinociceptive activity, as we found with the methanolic extract of EL.

Another pathogenetic mechanism underlying the progressive nerve fiber loss seem to be multifactorial, including polyol pathway, glycation, reactive oxygen species, and altered protein kinase C activity (Brownlee, 2001).

The role of oxidative stress in diabetes and diabetic neuropathy has been strongly suggested. In diabetes, high glucose levels have been shown to stimulate ROS production in cultured vascular cells through PKC-dependent activation of NAD(P)H oxidase, (Inoguchi et al., 2000) which has also been linked to the increased production of AGE (Wautier et al., 2001). Increased formation of O_2^- in diabetes is also associated with the activation of xanthine oxidase in the liver and plasma of diabetic animals (Desco et al., 2002). In diabetes, the bioactivity and/or generation of nitric oxide (NO) by endothelial NO synthase is reduced (Huynh and Tayek., 2002) and this may be due, in part, to the quenching of NO by O_2^- to form ONOO–. Altered anti-oxidant enzyme levels have been reported in the diabetic condition (Low et al., 1997). In the present study, we observed increased lipid peroxidation and decrease SOD and catalase activity and increased GPx activity in diabetic nerves. Thus, we observed a significant increase in MDA levels and a reduction in the activity of anti-oxidant enzymes in diabetic rats. EL treatment significantly reduced MDA levels and increased the activity of anti-oxidant enzyme (SOD) in rats with diabetic neuropathy. Elevated GPx and Catalase activity in diabetic rats came back to normal upon EL treatment. Its beneficial effects in diabetic neuropathy are attributed to one of its major constituent swertiamarine. Its, anti-oxidant activity have been correlated with in vitro and in vivo antioxidant activity reported by different workers (Maroo et al., 2003a). The anti-oxidant property of EL may also increase energy metabolism and reduce the formation of AGE and inflammation, as reported for other anti-oxidants (a-lipoic acid, vitamin E, vitamin C and *N*-acetyl-l-cysteine). a-Lipoic acid has been shown to increase energy metabolism and myo-inositol levels in diabetic nerves (van Dam, 2002; Kishi et al., 1999). Vitamin C and vitamin E have been reported to decrease the formation of AGE (Singh et al., 2001) *N*-Acetyl-l-cysteine and α-tocopherol have been shown to reduce levels of pro-inflammatory

cytokines (interleukin, tumour necrosis factor-a), chemokines and C-reactive proteins in diabetic rats.(Sagara et al., 1996, Jialal et al., 2002). In addition, EL treatment reduces apoptotic cell death, which is usually a consequence of oxidative stress (as mentioned in chapter 4) (Martin, et al., 2005).

The glucose uptake into peripheral nerve is insulin independent therefore it is proportionate to ambient blood glucose concentration. The rate-limiting enzyme for polyol pathway is aldose reductase, which is expressed on Schwann cells. Excess glucose is shunted into the polyol pathway and is converted to sorbitol and fructose by the enzymes aldose reductase and sorbitol dehydrogenase respectively (Greene et al., 1992). The nerve cell membrane is relatively impermeable to sorbitol and fructose, which tend to accumulate within the nerve (Tornlinson et al., 1989). Fructose and sorbitol both being osmotically active compounds lead to increase in the water content in the nerves. Further the oxidation/reduction status of the cell is altered with loss of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and glutathione stores. It leads to a cascade of events like a reduced membrane Na^{+}-^{+}K ATPase activity, intra-axonal sodium accumulation which reduces nerve conduction velocity and brings about structural breakdown of the nerve (Greene et al., 1992). Myoinositol level is decreased because elevated levels of both glucose and sorbitol compete for the uptake of myoinositol in the tissues and cells (Winegrade et al., 1987). Moreover, reduced NADPH, a cofactor for the enzyme nitric oxide synthase, reduces nitric oxide formation leading to decreased vasodilatation that impairs blood supply to the nerve (Cameron et al., 1994).

In our study diabetic rats showed increase in AR activity in sciatic nerve. Increase in AR activity could be due to hyperglycemic condition or because of formation of carbonyl and lipid peroxidation products. EL and Glib treatment reduces the activity of AR. This may be due to the partial restoration of plasma insulin and decrease in hyperglycemic condition. These decrease in AR activity could be due to two reasons; due to hypoglycemic potential of EL (Vijayvargia et

al., 2000; Maroo et al., 2002, 2003a, 2003b) and secondly due to its aldose reductase inhibitory potential (Patel and Mishra, 2009). Restoration of AR activity towards normal condition, indirectly corrects disturbance in antioxidant status of the cell in terms of increase in GSH content and NADPH which is required as cofactor for nitric oxide synthesis. Thus it might be increasing neuronal blood flow, which got decreased in diabetic condition.

Na^+-^+K ATPase activity in diabetic rats was significantly reduced down to about half level of the control rats. EL treatment restored the Na^+-^+K ATPase activity. Na^+-^+K ATPase activity was possibly improved by inhibition of oxidative stress (Santini et al., 1996), also by amelioration of vascular function (Kihara et al., 1991) and by correction in AR activity upon EL treatment in diabetic rats. Hence, from the above reported results it is convincing to assume that the amelioration of hyperglycemic condition and oxidative stress using potent hypoglycemic and antioxidants can be beneficial in diabetic neuropathy.

Thus, this study supports the potential of EL use in treatment of diabetic complications. Results of our study demonstrate the protective effect of EL, on diabetic neuropathy in rat model, which may be a consequence to improved glycemic control and in the antioxidant defense system as well as due to improvement in the Na^+-^+K ATPase activity. Since, EL is already being used as folk medicine by the diabetic patients it may be evaluated for preventive and curative therapy in diabetic patients at risk of developing neuropathy.

To conclude, administration of methanolic extract of *Enicostemma littorale* could attenuate the hyperalgesic state of diabetic rats and this may be of potential benefit in painful diabetic conditions.

6b. Evaluation of efficacy of *E. littorale* methanolic extract in diabetic male reproductive dysfunction in rat model.

6b.1 Review of literature

6b.2 Experimental design

6b.3 Results

6b.4 Discussion

6b.1. Review of literature

Diabetes is associated with declining sexual function in male (Rehman et al., 2001) as well as female (Enzlin et al., 2002) individuals. In females, diabetic symptoms consistent with autonomic neuropathy associated with decreased subjective sexual arousal, has been documented (Tyrer et al., 1983). Sexual dysfunction has been shown to be frequently associated with diabetes in human male and experimental animals (Fairburn 1981; Steger et al., 1983; Calvo et al., 1984; Ficher et al., 1984). Several vascular and peripheral nervous system associated problems result from diabetes-induced changes. There is increasing evidence that suggests central nervous system-related changes in endocrine function and sexual arousal may also contribute to sexual dysfunction (McVary et al., 1997). Impotence, infertility and retrograde ejaculation have been described in diabetic men, but the etiology remains unclear. It is believed that pelvic autonomic neuropathy contributes to impotence and retrograde ejaculation in the male. Many studies have also documented abnormalities in testicular function and spermatogenesis in diabetic animals (Kuhn-Velten et al., 1984; Murray et al., 1983).

Experimentally-induced diabetes mellitus (DM) in male rats is often accompanied by a marked decrease in reproductive functions, including atrophy of accessory organs (Sing et al., 2005), reduced sexual behavior (Scarano et al., 2006), histo-architectural changes in the seminiferous tubules (Sing et al., 2005), decreased semen volume accompanied by reduced sperm motility (Calvo et al., 1984) and even infertility (Yoon et al., 2004). Studies on male rats with alloxan-induced DM have shown decline in testicular testosterone (T) concentration (Sanguinetti et al., 1995) and low levels of plasma gonadotrophins (Hutson et al., 1983; Seethalakshmi et al., 1987).

As traditional practice, medicinal plants have been frequently used in several countries to control DM. Many herbal products have been tested for the

treatment of reproductive dysfunctions in diabetic state. Formulated drug MTEC, which is a combination of *Musa paradisiaca, Tamarindus indica* LINN, *Eugenia jambolana* and *Coccinia indica,* has a significant protective effect on testicular dysfunctions in streptozotosan (STZ) induced diabetic rats (Mallick et al., 2007). Thai plant, Red Kwao Krua (*Butea superba*), has been popular among Thai males for the purpose of rejuvenation and increasing sexual vigor (Suntara, 1931). Clinical trials of *Butea superba,* for erectile dysfunction (ED) have revealed the plant extract to be effective without apparent toxicity (Cherdshewasart & Nimsakul, 2003). *Cnidium monnieri* with its vasodilatory effect on animal corpus cavernosum (Chiou et al., 2001) is another promising candidate plant for treatment of ED. White Kwao Krua (*Pueraria mirifica*) has also been used for a long time, as herbal medicine for its effect on reproductive physiology (Jones & Pope GS, 1960; Benson et al., 1961). Its clinical applications have been well studied (Muangman & Cherdshewasart, 2001).

In light of this, we have explored the possible protective role of *Enicostemma littorale* Blume, having good hypoglycemic, antioxidant potential (Maroo et al., 2003a) on diabetes induced male reproductive dysfunction.

6b.2. Experimental design

Establishment of the model was mentioned in section 6A.5.1. After the completion of the treatment period weight of all the animals were taken. Animals were sacrificed and all the reproductive organs were taken out and their individual weights were recorded. Following the experimental regime as discussed previously, rats were sacrificed by decapitation on 45th after the treatment. Blood from orbital sinus was collected just prior to decapitation, in clean, dry eppendorfs containing anticoagulant. The clear plasma was removed after centrifugation at 1500 ×*g* for 15 min at 4 °C and stored at −80 °C until the assay of hormone. Plasma samples were also assayed for blood glucose and glycosylated hemoglobin measurement.

Serum concentrations of testosterone was measured with commercially available kit (Immunotech, France), following the radioimmunoassay (RIA) with a testosterone I^{125}. Radio activity was determined by gamma scintillation counter. Sample preparation was carried out using the method described by Tohda et al., 2001.

The testis, cauda epididymis, prostate gland and seminal vesicle were immediately excised out and processed for various biochemical estimations. Testis was used for the measurement of activities of 17β-and 3β-hydroxy steroid dehydrogenase key steroidogenic pathway enzymes as per method of Shivanandappa and Venkatesh, 1997. Cauda epididymis was separated from testis and were put into 2 ml pre-warmed PBS, pH 7.4. Sperm were allowed to diffuse after the epididymal tubule was pierced with a scalpel blade and sperm was forced out so as to enable maximum mature spermatozoa to be diffused out, not forcing out excess material, i.e., immature cells. The dish was shaken gently and, after 5 min of dispersion, an aliquot of sperm was used for sperm count, viability and motility. An aliquot of sperm was diluted 1:100 with fixative (10% formalin in PBS, pH 7.4) and counted using a haemocytometer. Sperm viability was performed by the eosin nigrosin staining. One drop of semen was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of mixture on a clean glass slide and allowed to air dry. The prepared slide was examined using a phase contrast microscope. Pink-stained dead sperm were differentiated from unstained live sperm, and there numbers were recorded following the method of (Eliasson, 1977). Epididymal sperm motility was evaluated in the PBS, pH 7.4. A 50 µl aliquot was diluted 20 times in PBS 37 °C, and transferred to a glass slide. Under a light microscope (10X magnification), a random field was chosen, and sperm classified as motile or immotile. Sperm motility was expressed as the percentage of motile sperm per field. The seminal vesicle and part of prostrate tissue were removed, weighed and stored at -20 °C to determine the content of fructose following the method of (Motoshima and Settlage, 1978).

Oxidative-stress related parameters such as lipid peroxidation (LPO) and reduced glutathione (GSH) were assayed in testis and cauda epididymis following standardized protocols which are described in **Chapter 2.** Biochemical parameters such as Vitamin C content was measured, from prostate and epididymis tissue while acid phosphatase activity was measured from only prostate tissue, Fructose content in seminal plasma, and prostate were assayed following standardized protocols and the details of the same are discussed in **Chapter 2.** Aldose reductase enzyme activity was measured from testies, epididymis, semial vesicle and prostate tissue.

6b.3. Results

6b.3.1. Blood glucose level

Diabetic rats suffering from hyperglycemic condition, when treated with EL and Glib for 45 days, showed significant decrease of 49 and 70% respectively in blood glucose levels, approaching normal levels (Fig. 6b.1B).

6b3.2. Relative organ body weight and body weight

Diabetic animals showed significant decrease in weight of testis, epididymis, prostate and seminal vesicle as compared to control animals. Animals treated with methanol extract of EL and standard drug Glib for 45 days resulted in significant recovery in organ weight. (Table 6b.1). Diabetic animals showed significant decrease in body weight as compared to normal control while EL and Glib treated rats showed improvement in body weight (Fig. 6b.1A).

6b.3.3. Testicular $\Delta^5$3β-HSD and 17 β-HSD

There was significant decrease in the activities of Δ^5,3β-HSD and 17β-HSD in alloxan-induced diabetic rats as compared to control. EL administration markedly increased 3β-HSD and 17 β-HSD activities by 69 and 38% respectively which were similar to the results for Glib treated animals where 73 and 35% increase in activities were observed respectively (Fig. 6b.2A).

Table 6b.1: Effect of EL and Glibenclamide treatment on organ weight of male diabetic rats

Tissues (Weight in gms)	Control	Diabetic	EL treated	Glib treated
Testis	2.85 ± 0.13	1.53 ± 0.08 [a]	2.32 ± 0.11[b]	2.43 ± 0.086[b]
Epididymis	0.73 ± 0.02	0.38 ± 0.02[a]	0.60 ± 0.03[b]	0.63 ± 0.01[b]
Prostate	0.46 ± 0.013	0.22 ± 0.015[a]	0.36 ± 0.015[b]	0.36 ± 0.012[b]
Seminal Vesicle	0.60 ± 0.013	0.36 ± 0.009 [a]	0.49 ± 0.014 [b]	0.49 ± 0.01 [b]

Values are expressed as mean ± SEM (n=6 in each group). a, $p<0.05$, aa, $p<0.01$, aaa,$p<0.001$ vs. C, b,$p<0.05$, bb $P<0.01$, bbb, $p<0.001$ vs. D.

6b.3.4. Plasma testosterone level

Testosterone level of plasma decreases significantly in diabetic animals while EL treated and Glib treated animals showed an increase of 66% and 76% respectively in plasma testosterone levels, when compared to diabetic animals (Fig. 6b.2B).

6b.3.5. Sperm count, viability, morphology and motility of sperms

Epididymal sperm count, sperm viability and motility decreases significantly in diabetic animals. EL treated animals demonstrated considerable restoration in sperm count, sperm viability and sperm motility by 26%, 61% and 50% respectively, while Glib treated animals showed improvement in the above parameter by 34%, 65% and 55% respectively. Diabetic animals were having abnormality in sperm morphology as compared to control animals, while animals treated with EL and Glib showed significant amelioration in sperm morphology by 58% and 46% respectively (Table 6b.2).

Table 6b.2: Effect of EL and Glibenclamide treatment on sperm parameters of male diabetic rats

Parameters	Control	Diabetic	EL treated	Glib treated
Sperm Count (in millions)	48.2 ± 3.3	14.4 ± 2.2 [a]	23.3 ± 1.7 [b]	25.8 ± 2.4[b]
%Sperm Viability	83 ± 4.27	41.8 ± 1.85[a]	67±3.78[b]	68.4±4.69[b]
% Sperm Motility	68.4 ± 2.4	41.8 ± 1.9 [a]	55 ± 2.1 [b]	56.4 ± 2.3[b]
Sperm Morphology (% Normal Sperm)	79.0 ± 2.4	54.0 ± 2.9 [a]	68.6 ± 2.5 [b]	65.4 ± 2.6 [b]

Values are expressed as mean ± SEM (n=6 in each group). a, p<0.05, aa, p<0.01, aaa,p<0.001 vs. C, b,p<0.05, bb P<0.01, bbb, p<0.001 vs. D.

6b.3.6.Epididymal LDH activity and prostatic ACP activity

The LDH activity was significantly diminished in epididymis of diabetic animals. Animals treated with EL and Glib increased LDH activity by 65 and 75% (Fig. 6b.3). There is significant decrease in prostatic ACP activity in diabetic animals as compared to normal animals. Animals administered with EL demonstrated significant increase in prostatic ACP activity by 61%. Upon Glib treatment, ACP activity increase significantly by 63% (Fig. 6b.4).

6b.3.7.Aldose reductase activity in testis, epididymis, seminal vesicle and prostate

There was significant increase in aldose reductase enzyme activity in all four tissues namely, testis, epididymis, seminal vesicle and prostate in diabetic rats as compared to control rats. Diabetic rats treated with EL showed decrease in aldose reductase activity in above tissues by 53%, 63%, 51% and 61%

respectively. Diabetic rats treated with Glib showed decrease in enzyme activity by 56%, 74%, 49% and 70%, respectively in all above tissues (Fig. 6b.5A).

6b.3.8. Fructose content in seminal vesicle and prostate

There was significant increase in fructose content in prostate and seminal vesicle in diabetic animals. Treatment with EL significantly decreased fructose levels by 53% and 61% respectively in both the tissues, while upon Glib treatment fructose levels decreases by 39% and 64% respectively in both the tissues (Fig. 6b.5B).

6b.3.9. Prostate and epididymis vitamin C content

Diabetic animals showed decreased vitamin C content in prostate and epididymis tissues as compared to control group. Upon EL treatment vitamin C content had significantly increased in prostate and epididymis tissues by 55% and 50% respectively, while Glib increased vitamin C content by 59% and 56% in prostate and epididymis respectively. (Fig. 6b.6A)

6b.3.10. LPO levels in Testies and epididymis

MDA, lipid peroxidation product significantly increased in testis and epididymis of diabetic rats as compared to normal rats. Diabetic rats treated with EL showed significant decrease in MDA levels by 62% and 67% in these tissues respectively. Diabetic rats treated with Glib also showed decrease in MDA levels in these tissues by 59% and 64% (Fig. 6b.6B).

6b.3.11. GSH levels in testis and epididymis

There was significant decrease in GSH content in testis and epididymis of diabetic rats. EL treated rats showed significant increase in GSH content by 51% and 57% in both the tissues respectively. Standard drug Glib treated rats also showed significant increase in GSH content by 54% and 63% respectively (Fig. 6b.7).

Figure 6b.1: Effect of EL treatment on **body weight** and **blood glucose** levels

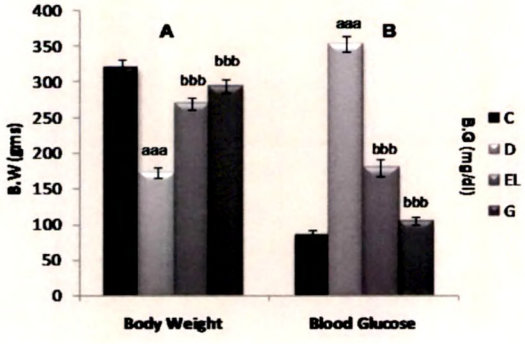


Figure 6b.2: Effect of EL treatment on activities of androgenic key enzymes, Δ^5,*3β-HSD* and **17 β-HSD** and **plasma testosterone** levels

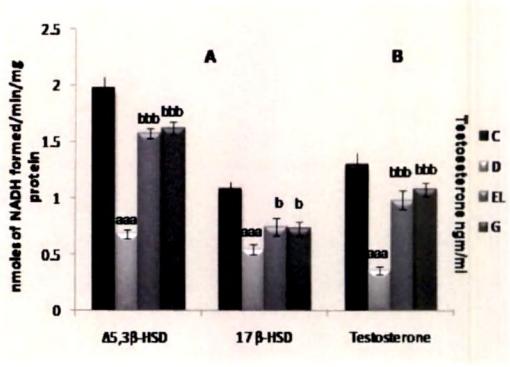


Figure 6b.3: Effect of EL treatment on epididymal **lactate dehydrogenase** enzyme activity

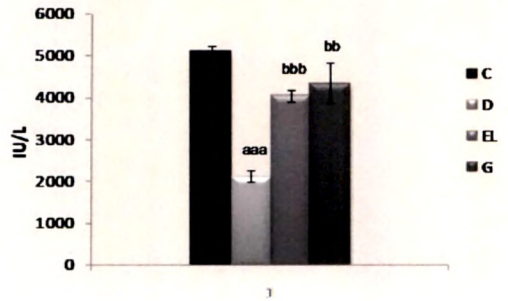


Figure 6b.4: Effect of EL treatment on **prostatic acid phosphatase** activity

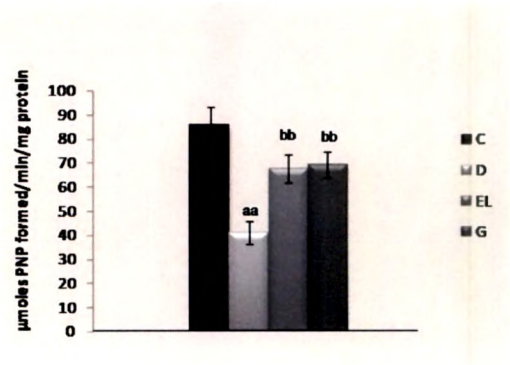


Figure 6b.5: Effect of EL treatment on **aldose reductase** activity and **fructose** content in male reproductive tract

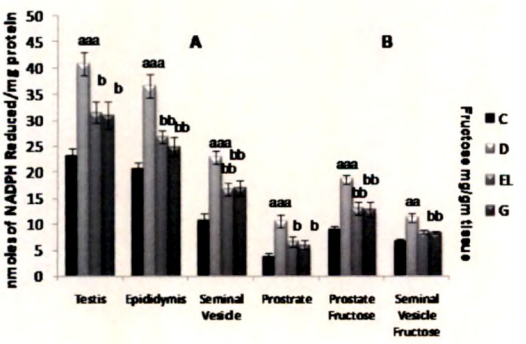
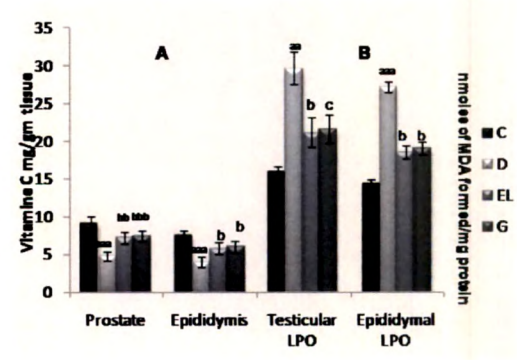
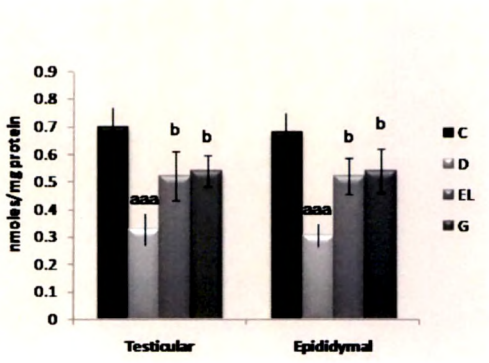


Figure 6b.6: Effect of EL treatment on **Vitamins C** and **LPO** levels in reproductive tissues



Values are expressed as mean ± SEM (n=6 in each group). a, p<0.05, aa, p<0.01, aaa,p<0.001 vs. C, b,p<0.05, bb P<0.01, bbb, p<0.001 vs. D.

Figure 6b.7: Effect of EL treatment on prostatic and epididymal **GSH** content



Values are expressed as mean ± SEM (n=6 in each group). a, p<0.05, aa, p<0.01, aaa,p<0.001 vs. C, b,p<0.05, bb P<0.01, bbb, p<0.001 vs. D.

6b.4. Discussion

Diabetes mellitus, a multifaceted multiorgan disorder declared as a disease of complication, is prevalent globally and has been projected to become one of the world's main disablers and killers within next 25 years (Hakim & Goldstein, 1996). Diabetes also leads to development of reproductive disorders that, results into male and female infertility (Rehman et al., 2001; Enzlin et al., 2002). The present work confirms the male reproductive disorders in diabetic state as proposed by others along with protective effect of herbal medicine Enicostemma littorale (Zimmet et al., 2001).

Rats treated with alloxan showed hyperglycemic condition as compared to control group. Rats treated with EL decreases blood glucose level. This could be due to increase in serum insulin levels, which decrease blood glucose levels as shown in our previous reports (Maroo et al., 2003a). Improvement in serum insulin levels on EL treatment is due to insulinotropic activity of EL (Maroo et al., 2002).

It has been observed that weight of reproductive organ were associated with metabolic alterations (including decreased testosterone levels) caused by the diminution of serum testosterone levels (Seethalakshmi et al., 1987). Earlier, insulin therapy was shown to restore body weight and the weight of most reproductive organs, but does not significantly improve weight of prostate. It has been unclear whether or not the changes in accessory sex organ weight in diabetes mellitus results from the alteration of glucose metabolism or a secondary decrease in androgen status. Observation that testosterone is needed to restore prostate weight under conditions of hyperglycemia indicate that diabetic rats are androgen deficient (Seethalakshmi et al., 1987). Administration of high doses of STZ to male rats induces a decrease in testicular testosterone production (Sanguinetti et al, 1995). This decrease may be the result of both, a decrease in the total number of Leydig cells and the rhythm of androgen biosynthesis by the remaining functional cells (Orth et al, 1979; Paz & Homonnai, 1979; Hurtado de Catalfo et al, 1998).

After administration of EL and Glib, a recovery in reproductive organ weight observed may be due to elevation in plasma testosterone levels as a result of increased activities of key androgenic enzymes i.e Δ^5,3β-HSD and 17 β-HSD as well as due to recovery of serum insulin levels, which has a positive role in testicular testosterone synthesis (Sudha et al, 1999a).

Seminal vesicle and prostate secrete acid phosphatase (Tenniswood, 1976; Vanha-Perttula et al., 1972) and are shown to be under the control of androgens (Cunha et al., 1987; Famy et al., 1980). STZ-induced diabetes resulted in a significant reduction in serum testosterone, prolactin, T3 and T4 levels (Seethalakshmi et al., 1987; Sudha et al., 1999b; Ikeda et al., 2000; Mitsuma & Nogimori, 1982; Jolin, Oritiz-Caro, 1985). Thus, the decreased activity of acid phosphatases may be the consequence of diabetes-induced low levels of circulating testosterone. The present investigation showed a significant decrease in prostatic acid phosphatase activity of diabetic rats. In accordance with the

earlier study of Seethalakshmi et al. (1987). A significant increase in the prostatic and epididymal acid phosphatases activity also correlates with increased plasma testosterone, in EL and Glib-treated diabetic rats.

In polyol pathway, glucose is first reduced to sorbitol by aldose reductase (AR) and the resulting sorbitol is subsequently oxidized to fructose by sorbitol dehydrogenase (SDH). Enhanced AR activity also causes a reduction-oxidation (redox) imbalance by altering the NADPH:NADP ratio (Bravi et al., 1997) and triggers damage to cells with a low redox capacity, shown in pancreatic β-cells (Hamaoka et al., 1999). Active steroid hormones and their metabolites are also substrate of AR (Wermuth & Monder, 1983; Warren et al., 1993). AR detoxify carbonyl compounds caused by the glycation reaction, such as MG and 3-deoxyglucosone (Vander et al., 1992); and highly toxic compounds produced by lipid peroxidation, such as 4-hydroxynonenal and acrolein (Kolb et al., 1994; Vander et al., 1995). Under hyperglycemic conditions, dysfunction occurs in the male reproductive tract, including testis (Sexton & Jarow, 1997), and abnormal sorbitol accumulation has been demonstrated in accessory glands (Paz et al., 1980). In our study diabetic rats showed increase in AR activity in testis, epididymis, seminal vesicle and prostate. Increase in AR activity could be due to hyperglycemic condition or because of formation of carbonyl and lipid peroxidation products, as mentioned earlier. These results are in accordance with studies of Sexton & Jarow et al., (1997), where hyperglycemia has been responsible for sorbitol accumulation and reproductive dysfunction. Alloxan-induced diabetic rats also showed an increase in fructose concentration in prostate as well as in seminiferous tubules as compared to control. It has been shown that the synthesis and secretion of fructose in ventral prostate is under the control of androgens and estrogens (Grayhack, 1965). It is suggested that the diabetes-induced decrease in circulating testosterone (Seethalakshmi et al., 1987; Sudha et al., 1999; Ikeda et al., 2000) may be responsible for the impaired secretion of fructose leading to its accumulation in ventral prostate. In accordance

with the present study, Ford and Hamilton (1984) have reported similar increase in fructose concentration in the coagulating gland of alloxan-diabetic rats.

EL and Glib treatment reduced the fructose concentration in prostate and seminal vesicle. This may be due to the partial restoration of plasma testosterone and AR activity towards normal. These decrease in AR activity could be due to two reasons; due to hypoglycemic potential of EL (Vijayvargia et al., 2000; Maroo et al., 2002, 2003a, 2003b) and secondly due to its aldose reductase inhibitory potential (Patel and Mishara., 2009).

It is well established that oxidative damage to testicular male germ cells induced by diabetes, various xenobiotics, products of abnormal metabolism, or ROS can result in testicular dysfunction leading to infertility (Seethalakshmi et al., 1987; Sanguinetti, 1995). Moreover, Cameron et al., 1985, defined increasing tubule wall thickness, germ cell depletion and sertoli cell vacuolization in diabetic human testicular biopsies and in diabetic rats.

In addition, numerous epidemiological and experimental evidences have emphasized a potential relationship between oxidative damage in testis/sperms and testicular dysfunction leading to infertility (Orth, et al., 1979; Paz & Homonnai, 1979). Our results revealed that alloxan administration in rat causes significant oxidative impairments in the male reproductive milieu in diabetic state. Oxidative stress was evident in terms of enhanced MDA levels and decreased GSH content in testis and epididymis of diabetic rats. Generation of oxidative stress is also evident by decrease in vitamin C content in testis and prostate tissue in the diabetic rats. This could be due to the participation of ascorbic acid in scavenging of ROS formed in diabetic condition and could lead to the reduction in ascorbic acid level. The same trends were observed in prostate (Seethalakshmi, 1987; Orth, et al., 1979). Ascorbic acid is also involved in steroidogenesis of the gonads has been reported (Agrawal & Laloraya, 1977; Datta & Sanyal, 1977; Chinoy, et al., 1982). Possibility for the corrective role of EL

and Glib may be due to improvement in the testicular and epididymal GSH content and also by increase in Vitamin C content of testis and prostate tissue. EL and Glib are having hypoglycemic effect and thus reduces increased blood glucose levels in diabetic rats. However our previous report on antioxidant potential of EL further supports reduction in oxidative stress being generated by hyperglycemic condition (Maroo et al., 2003a). The improvement in vitamin C levels in testis correlates with the increase in steroidogenic enzyme activity and also correlates with plasma testosterone levels as well as with improved Δ^5,3β-HSD and 17 β-HSD enzyme activities in EL and Glib treated diabetic rats.

Diabetic rats showed decreased epididymal sperm count, viability and motility. The decrease in sperm count is likely due to the influence of severe hyperglycemia in late stages of spermatogenesis, possibly through an increase in ROS. The consequences of such oxidative damage could include loss of motility due to lipid peroxidation indicated by increased MDA levels in testis and epididymis (Oehninger et al., 1995; Sikka, 2001; Aitken & Sawyer, 2003). After 4 weeks of STZ treatment, a significant increase in degenerated germ cells at various stages of development is observed (Orth et al., 1979; Sanguinetti et al., 1995). Furthermore, Soudamani et al. also found that STZ induced diabetes has detrimental effects on the maintenance and establishment of fully differentiated epididymal epithelium during sexual maturation. Thus, increase in abnormal sperm count in diabetic rat can be due to effect at spermatogenesis stage. Diabetic rats also showed decrease epididymal sperm LDH activity. In the absence of LDH no lactate utilisation can take place, which leads to reduced motility and sperm survival. Diabetic rats treated with EL and Glib showed improvement in epididymal sperm count, viability, and motility. This improvement could be due to decrease in oxidative stress and improvement in LDH activity.

Thus hypoglycemic and antioxidant potential of EL plays protective role in reproductive dysfunction and thus can be used for the management of male diabetic reproductive dysfunction.

6c. Evaluation of efficacy of *E. littorale* methanolic extract in cardiovascular complications in rat model.

6c.1 Review of literature

6c.2 Experimental design

6c.3 Results

6c.4 Discussion

6c.5 Summary of chapter

6c.6 References

6c.1. Review of literature

Diabetes mellitus has a major impact on cardiac morbidity and mortality and cardiovascular diseases now account for 80% of all diabetic deaths (WHO Library Cataloguing). A number of studies have been published concerning impaired cardiac function in diabetes. Myocardial performance has been reported to be altered in both clinical and experimental diabetes (Norton et al., 1996; Cai et al., 2002; Price et al., 2003). The activities of membrane-bound enzymes play a major role in many of the complications of diabetes and in the development of diabetic vascular complications (Kiziltunc et al., 1997; Jain et al., 2000). The abnormalities in Na^+-K^+-ATPase and Ca^{2+}-ATPase activities with accompanied increase in base line sodium and calcium concentration are well documented in cardiac dysfunction in diabetes (Dhalla et al., 1998; Pekiner et al., 2002; Ramasamy et al., 1999). Diabetes-induced hyperlipidemia (Kuwahara et al., 1997), oxidative stress (Ziegelhoffer et al., 1997) and protein glycation (Flecha et al., 1990) seem to be the major contributing factors associated with abnormal membrane-bound enzyme activities resulting in cardiac dysfunction. Normalization of membrane-bound enzymes was proposed to be one of the important mechanisms for the protection of diabetic heart (Ramasamy et al., 1990). Previous studies have reported that supplementation with antioxidants prevents inhibition of membrane bound ATPases activity caused by hyperglycemia (Pekiner et al., 2002).

In addition to the 'classic' risk factors, like hypertension and dyslipidaemia, several factors play a role in the accelerated atherosclerosis observed in diabetic patients, such as endothelial dysfunction, increased propensity for thrombosis and impaired fibrinolysis, and increased platelet aggregation (Knobler et al., 1998; Lorenzi & Cagliero, et al., 1991; Carmassi et al., 1992; Ceriello 1993; Tschoepe et al., 1993). Damage to the endothelium plays an important role in the development and progression of atherosclerosis (Lorenzi and Cagliero et al., 1991; Fortes et al., 1983; Glasser et al., 1996; Quyyumi 1998). Endothelium-

derived factors are involved in the regulation of blood coagulation and fibrinolysis such as von Willebrand factor (vWF), tissue factor (TF) and tissue factor pathway inhibitor (TFPI), thrombomodulin and plasminogen activator inhibitor-1 (PAI-1). Several of these are altered in diabetes (Carter et al. 1997; Leurs et al., 1997; Juhan-Vague and Alessi 1991; Galajda et al., 1997; Gruden et al., 1995; Plater et al., 1996). Disturbances in endothelial function may lead to initial platelet adhesion and subsequent platelet aggregate formation, while the altered platelet metabolism and changes in intraplatelet signalling pathways contribute to the overall increased platelet hyperactivity (Mazzanti and Mutus 1997; Huszka et al., 1997).

Conventional treatment includes Beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, calcium channel antagonist, diuretics, angiotensin-receptor antagonist, antiplatelet treatment, lipid lowering drugs and good glycemic control by anti-diabetic drugs. Thus for the treatment of cardiovascular complications, drugs having hypolipidemic, antioxidant and hypoglycemic activity would be a better choice as compared to classical treatment. Complementary and alternative medicines have been used to treat cardiovascular diseases. Green tea corrects dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca2+-ATPase and Na+-K+-ATPase activity in the heart of streptozotocin-diabetic rats (Velayutham et al., 2006). Daming capsule (DMC), a traditional Chinese formula can prevent elevated diastolic and systolic function of diabetic heart with the improvement in dislipidemia (Jing et al., 2009). Red wine consumption (300 ml) during a meal was associated with significant preservation of plasma antioxidant defenses and reduction of both LDL oxidation and thrombotic activation in Type 2 diabetics, thereby preventing cardiovascular diseases in diabetic patients (Ceriello et al., 2001). Daily oral feeding of garlic extract increased cardiovascular functions in STZ rats, prevented abnormality in lipid profile and increased fibrinolytic activities with decreased platelet aggregation. Plasma insulin level increased with concomitant decrease in plasma glucose levels. In addition, daily oral feeding of the same dose for 16

weeks showed anti-atherosclerotic effects in STZ-diabetic rats. Thus, garlic may prevent diabetic cardiovascular complications (Grover et al., 2002; Patumraj et al., 2000). As indicated from above herbal medicine which is having good hypoglycemic, antioxidant, anti-hyperlipidemic and antithrombotic activity can be a good drug candidate for the prevention of diabetic cardiovascular diseases. Similar activities of *Enicostemma littorale* extract have been observed in chapter 3 & 4, which lead us to evaluate its efficacy in prevention of diabetic cardiovascular complications in diabetic rat model.

6c.2. Experimental design

Establishment of the model was mentioned in section 4.2. After the completion of the treatment period weight of all the animals were taken. We have evaluated cardiac dysfunction in diabetic rats after the period of 6 weeks, after induction of diabetes; because in many studies the duration of diabetes to induce cardiac dysfunction in experimental rats was found to be 6 weeks (Velayutham et al., 2006; Paulson, 1997).

Following the experimental regime as discussed previously in section 4.2, rats were sacrificed by decapitation on 45th after the treatment. Blood from orbital sinus was collected just prior to decapitation, in clean, dry eppendorfs containing anticoagulant (selection of the anticoagulant was based on the parameters to be checked or without anticoagulant (for serum preparation). The clear serum/plasma was removed after centrifugation at 1500 ×*g* for 15 min at 4 °C and the assay of enzymes LDH, CK-MB, and SGOT were carried out. Plasma samples were also assayed for blood glucose and glycosylated hemoglobin, lipid profile measurements. For platelet aggregation study, plasma reach in platelet and plasma poor in platelet were prepared as per the procedure mentioned in chapter 2. Blood coagulation parameters like PT and APTT were also evaluated from the plasma samples.

Heart tissue was homogenized and mitochondrial and post-mitochondrial fractions were prepared. Post-mitochondrial fraction was used for the estimation of Na^+-K^+- ATPase and Ca^{2+}-ATPase activity. Both the fractions were also used for the evaluation of antioxidant parameters like SOD, GPx, GSH and also for lipid peroxidation a marker of oxidative stress. Entire study was carried out 3 times to evaluate different parameters each time.

6c.3. Results

6c. 3.1. *Heart and Body weight (table will be included)*

Diabetic rats showed a significant reduction in body weight and heart weight whereas the ratio of heart weight to body weight in diabetic rats was increased compared to control rats (Table 6c.1.). EL extract as well as Glib treatment normalized the ratio of heart weight to body weight in rats with diabetes mellitus.

Table 6c.1: *Effect of EL treatment on body weight, heart weight and heart/b.wt ratio of diabetic rats.*

Groups	Body Weight (gm)	Heart Weight (mg)	Heart/Body Wt. Ratio	Heart Rate (Beats/min)
C	308 ± 7	653 ± 8.04	2.13 ± 0.06	340 ± 13
D	169 ± 6^{aaa}	511 ± 7.51^{aaa}	3.03 ± 0.06^{aaa}	255 ± 8^{aaa}
D+EL	235 ± 8^{bbb}	593 ± 5.82^{bbb}	2.54 ± 0.08^{bb}	305 ± 6^{bb}
D+G	242 ± 9^{bbb}	597 ± 5.99^{bbb}	2.49 ± 0.11^{bbb}	307.2 ± 5^{bb}

Values are expressed as mean ± SEM (n=6 in each group). a, $p<0.05$, aa, $p<0.01$, aaa, $p<0.001$ vs. C, b, $p<0.05$, bb $p<0.01$, bbb, $p<0.001$ vs. D.

6c. 3.2. *Blood glucose and glycosylated hemoglobin levels*

Diabetic rats were hyperglycemic; when treated with EL and Glib for 45 days, showed significant decrease in blood glucose levels by 61% and 73%

respectively, approaching towards normal levels (Fig. 6c.1). Diabetic rats showed 83% increase in glycosylated hemoglobin (GlyHB) levels as compared to normal control rats. EL and Glib treatment reduces GlyHB levels by 54% and 60% respectively (Fig. 6c.2).

6c. 3.3. *Lipid profile*

Diabetes causes dyslipidemia, which was clearly shown by the alloxan-induced diabetic rats with increased in serum cholesterol levels by 92%, serum triglycerides by 110%, LDL cholesterol by 177%, VLDL cholesterol by 111% and decrease levels of HDL cholesterol by 46%. Both extract and standard drug Glib treatment to diabetic rats for 45th days showed significant amelioration in lipid profile. *E. littorale* treatment showed a decrease of 50%, 63%, 48% and 63% in serum cholesterol, serum triglycerides, LDL cholesterol, VLDL cholesterol and an increase of 57% in HDL cholesterol levels in diabetic rats respectively. Glib treated diabetic rats showed a decrease of 42%, 57%, 42% and 57% in serum cholesterol, serum triglycerides, LDL cholesterol, VLDL cholesterol and an increase of 64% in HDL cholesterol levels respectively (Fig. 6c.3; 6c.4). Lipid lowering effect of EL and Glib was quit comparable in alloxan-induced diabetic dyslipidemia.

6c. 3.4. *Serum CK-MB, LDH and SGOT activity*

Serum CK-MB, LDH and SGOT activity increased by 164%, 141% and 53% in the untreated diabetic rats as compared to the control (C) (Fig. 6c.5; 6c.6; 6c.7). EL treatment attenuated the increase in plasma CK-MB, LDH and SGOT activity by 54%, 67% and 44% respectively. Similarly, Glib treatment attenuated the increase in CK-MB, LDH and SGOT by 52% and 63% and 45% respectively.

Figure 6c.1: Effect of EL treatment on **blood glucose** levels.

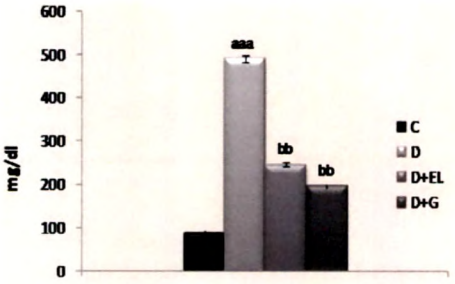


Figure 6c.2: Effect of EL treatment on **glycosylated hemoglobin** levels.

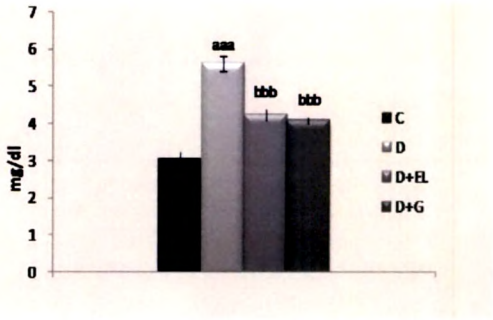


Figure 6c.3: Effect of EL treatment on **lipid profile**.

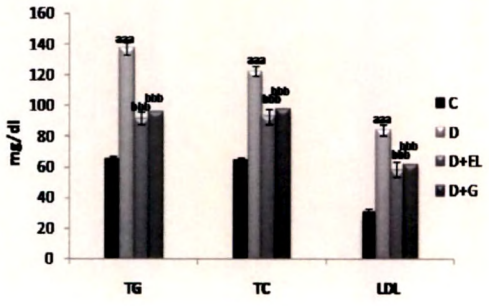


Figure 6c.4: Effect of EL treatment on **lipid profile**.

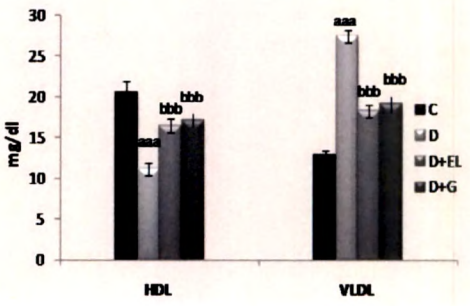


Figure 6c.5: Effect of EL treatment on serum **CK-MB** activity.

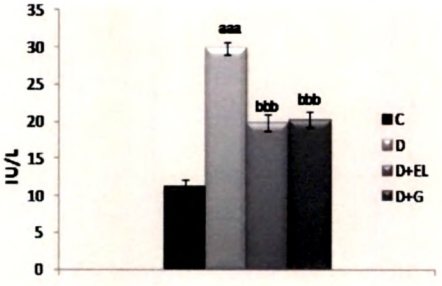
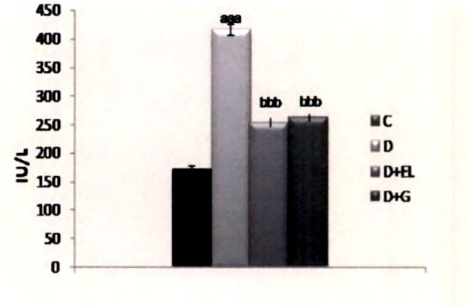


Figure 6c.6: Effect of EL treatment on serum **LDH** activity.



Values are expressed as mean ± SEM (n=6 in each group). a, $p<0.05$, aa, $p<0.01$, aaa, $p<0.001$ vs. C, b, $p<0.05$, bb $p<0.01$, bbb, $p<0.001$ vs. D.

Figure 6c.7: Effect of EL treatment on serum **SGOT** activity.

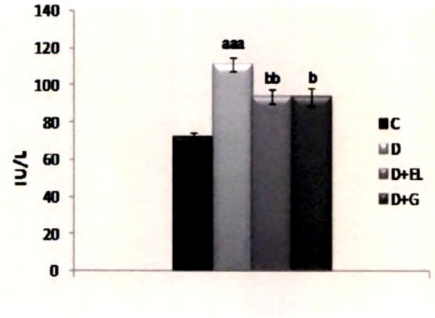


Figure 6c.8: Effect of EL treatment on **plasma prothrombine** and **partial activated thromboplastine time**.

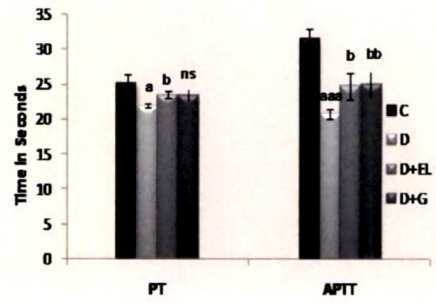


Figure 6c.9: Effect of EL treatment on **platelet aggregation**.

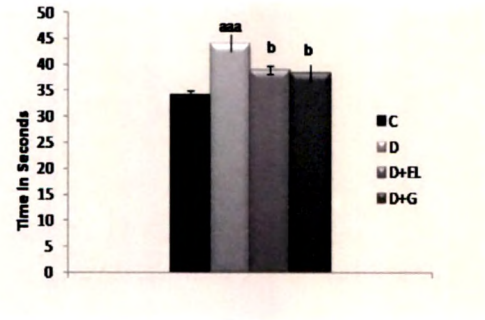


Figure 6c.10: Effect of EL treatment on **platelet counts**.

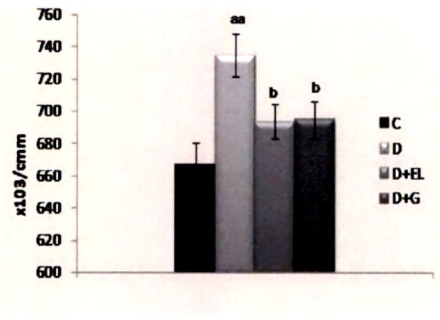
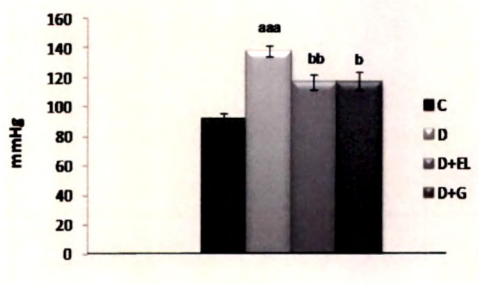


Figure 6c.11: Effect of EL treatment on **systolic blood pressure**.



Values are expressed as mean ± SEM (n=6 in each group). a, $p<0.05$, aa, $p<0.01$, aaa, $p<0.001$ vs. C, b, $p<0.05$, bb $p<0.01$, bbb, $p<0.001$ vs. D.

Figure 6c.12: Effect of EL treatment on **blood glucose** levels.

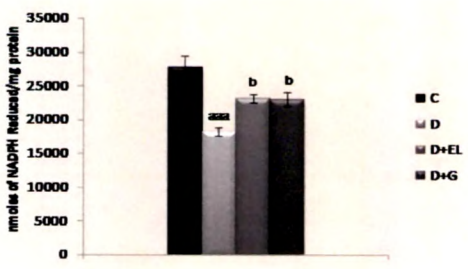


Figure 6c.13: Effect of EL treatment on heart **Na^+-K^+-ATPase**.

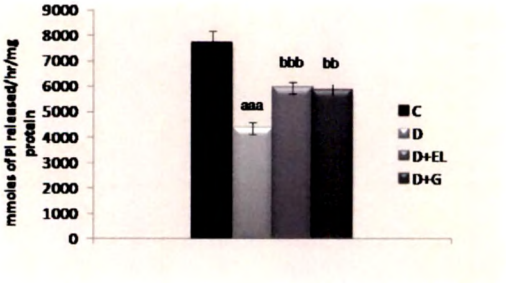


Figure 6c.14: Effect of EL treatment on **lipid peroxidation**.

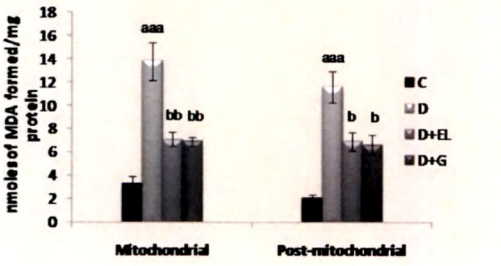


Figure 6c.15: Effect of EL treatment on **GSH** content.

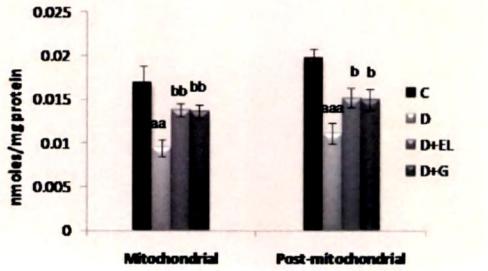


Figure 6c.16: Effect of EL treatment on **SOD** activity.

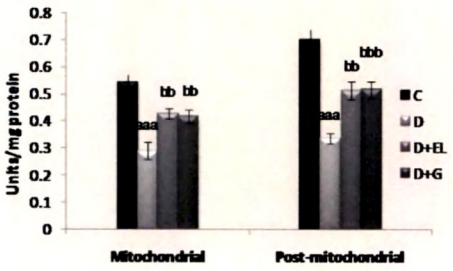


Figure 6c.17: Effect of EL treatment on **GPx** activity.

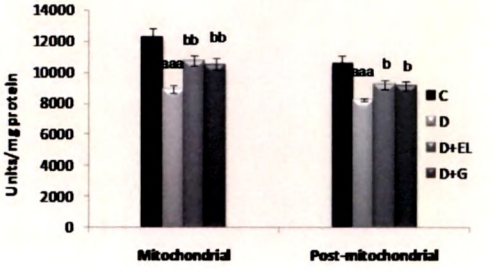
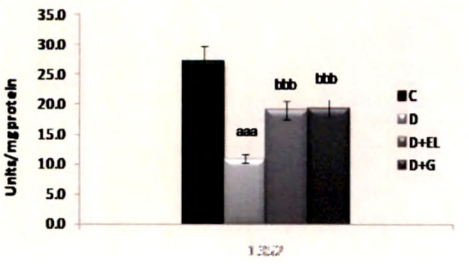


Figure 6c.18: Effect of EL treatment on **Catalase** activity.



Values are expressed as mean ± SEM (n=6 in each group). a, $p<0.05$, aa, $p<0.01$, aaa, $p<0.001$ vs. C, b, $p<0.05$, bb $p<0.01$, bbb, $p<0.001$ vs. D.

6c. 3.5. *Platelet aggregation, platelet count and blood clotting time (PT & APTT)*

Untreated diabetic rats showed 29% increase in platelet aggregation time, while 13% and 33% decrease in prothrobin time (PT) and activated partial thromboplastin time (APTT). Treatment with EL to diabetic rats for 45 days ameliorates platelet hyperaggregability by 52% and increases PT, APTT by 46%, 37% respectively (Fig. 6c.8; 6c.9). Diabetic rats treated with Glib for 45 days also showed 57% reduction in platelet hyperaggregability, increase 44% in PT and 39% in APTT. Diabetic animals also showed increase in platelet count by 10%. Platelet count decreases with EL and Glib treatment by 61% and 59% respectively (Fig. 6c.10).

6c. 3.6. *Systolic blood pressure and heart rate*

Untreated diabetic rats showed increase in systolic blood pressure by 49% as compared to control rats (Fig. 6c.11). EL treatment for 45 days, bring down systolic B.P by 46%, while glib treatment decreases it by 45%. Thus, both the treatments individually showed comparable effect in reducing systolic B.P. Heart rate was found to be significantly lower in diabetic rats as compared to controls. Chronic treatment with EL and Glib to diabetic rats exhibited significant increase in heart rate as compared to diabetic control animals (Table 6c.1.).

6c. 3.7. *Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase activity in heart tissue*

There was significant decrease in Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase enzyme activity by 34% and 43% respectively in heart tissue of untreated diabetic rats as compared to control rats. Diabetic rats treated with EL and Glib for 45 days showed increase in Ca^{2+}-ATPase activity by 52% and 51%, while improves Na^{+}-K^{+}-ATPase activity by 48% and 45% respectively (Fig. 6c.12; 6c.13). EL and Glib treatment showed similar effect on both the ATPase activity.

6c. 3.9 *LPO, GSH and antioxidant enzyme activities in mitochondrial and post-mitochondrial fractions of heart tissue*

Diabetic rats showed 316% increase in MDA level and 44% decrease in GSH level in mitochondrial fraction (Fig. 6c.14; 6c.15). This indicates generation of oxidative stress in this fraction. Mitochondrial SOD and GPx activity decreases by 46% and by 27% in these rats. Diabetic rats treated with EL decreases MDA content by 64%, while increases GSH content by 58% in this fraction. This treatment also increases SOD, GPx activity by 54% and 56% respectively (Fig. 6c.16; 6c.17). Similarly, Glib treated diabetic rats showed decrease in MDA level by 66%, while increase GSH level by 56% in this fraction. SOD, GPx activities, in these rats were improved by 50% and 48% respectively.

Post-mitochondrial fraction of heart tissue of diabetic rats also showed generation of oxidative stress, by increase in the lipid peroxidation level by 455%, and decreasing GSH levels by 44%. It also showed decreases antioxidant enzymes, SOD, GPx and CAT activities by 52%, 28% and 60% respectively (Fig. 6c.16; 6c.17; 6.18). Diabetic rats treated with EL showed reduction in oxidative stress by decreasing lipid peroxidation level by 49% and increasing GSH level by 46% in post-mitochondrial fraction. It also improves antioxidant enzyme activities. It increases SOD, GPx and CAT activities by 48%, 55% and 50% respectively (Fig. 6c.16; 6c.17; 6.18). Glib treated diabetic rats also showed similar effect by decreasing lipid peroxidation and increasing GSH content by 52% and 44% respectively. Antioxidant enzymes SOD, GPx and CAT activities were also improved upon Glib treatment by 49%, 53% and 52% in this fraction.

6c.4 Discussion

In the present investigation, we found that alloxan-produced cardinal signs and characteristics of diabetes, viz. hyperglycemia, dyslipidemia, and cardiovascular alterations like bradycardia, hypertension, and hypertrophy of heart. These results are consistent with those reported by others (Umrani and

Goyal, 2002). Elevation of blood glucose for a longer time causes non enzymatic glycosylation of vital body proteins which leads to the thickening of capillary basement membrane thickening along with atherosclerosis (Pershadsingh et al., 2003).

The increase in heart to body weight ratio in diabetic rats is an indicative of cardiac hypertrophy, which is due to accumulation of cholesterol, triglycerides, phospholipids and glycated protein in the myocardium (Christopher et al., 2003). EL and Glib treatment to diabetic rats significantly reduced this ratio which could be due to improved glycemic control and hypolipidemic activity shown by EL and Glib in diabetic rats. Previous studies indicate that EL could prevent the hyperlipidemia-induced by Fructose rich diet in rats (as mentioned in chapter 3) as well as with high fat diet (Vihas et al., 2005). The potential hypolipidemic effect of EL is well documented (Vihas et al., 2005; Vaidya et al., 2009) and is shown in the present study.

Lactate dehydrogenase and creatinine kinase levels are reported to be increased 12–24 h after a myocardial infarction (Howard-Alpe et al., 2006). LDH levels are also reported to increase in type 2 diabetic patients and may serve as a cardiovascular risk-related marker for the same (Huang et al., 2006). Further, increased serum CK-MB and LDH levels in diabetic rats indicate cardiac damage (Hagar, 2002). In our study, we also found significant rise in LDH and CK-MB levels in alloxan-diabetic rats as compared to normal rats. Treatment with EL and Glib significantly reduced LDH and CK-MB levels, which further substantiates its beneficial effect in reducing the cardiovascular risk in diabetes mellitus.

The abnormalities in Na^+-K^+-ATPase and Ca^{2+}-ATPase activities are well documented in cardiac dysfunction in diabetes (Dhalla et al., 1998; Pekiner et al., 2002). In the present study, Ca^{2+}-ATPase activity was depressed in diabetic rats. Impaired calcium homeostasis was reported in diabetic cardiomyopathy and other complications of diabetes mellitus (Golfman et al., 1996; Hattori et al., 2000).

Abnormal Ca^{2+}-ATPase activity and intracellular calcium levels were reported as important mechanisms responsible for the cardiac dysfunction exhibited by type 1 diabetic animal (Golfman et al., 1996). The increased intracellular concentration of calcium may be explained by the osmotic activity of high glucose (cell shrinkage), demonstrated to activate G proteins, most likely through a stretch receptor, which in turn stimulates calcium channels (Smogorzewski et al., 1998). Diabetes-induced hyperlipidemia alters the membrane phospholipids and fatty acids and shown to depress membrane bound enzyme activities, which influence intracellular calcium metabolism resulting in cardiac dysfunction (Kuwahara et al., 1997).

Reactive oxygen species formed in diabetes attack the membranes of intracellular organelles and reported to lead to a decrease in cardiac Ca^{2+}-ATPase activity (Ziegelhoffer et al., 1997). A decrease in ATPase enzyme activity in any diabetic tissue could be due to excessive non-enzymatic glycation of the enzyme itself (Flecha et al., 1990). Thus, the hyperlipidemia, oxidative stress and protein glycation seem to be the major contributing factors associated with abnormal calcium homeostasis in diabetic animals. Similarly, Na^+-K^+-ATPase activity plays a major role in many of the complications of diabetes and in the development of diabetic vascular complications (Kiziltunc et al., 1997; Jain and Lim, 2000). Hence, in the present study, the observed decrease in the activity of Na^+-K^+-ATPase may be considered as an index of cardiovascular complications induced by diabetes.

Membrane fluidity has a strong influence on important membrane functions such as the conformation and thus the activity of membrane associated abnormal metabolism (Rizvi and Zaid, 2005). Inhibition of myocardial Na^+-K^+-ATPase, as observed in diabetics, results in increased baseline sodium concentration. Normalization of Na^+-K^+-ATPase was proposed to be one of the important mechanisms of protection in heart from diabetic animals (Ramasamy et al., 1999).

Numbers of dietary compounds have been shown to influence membrane characteristics such as fluidity, stability and susceptibility to membrane oxidative damage (Peck et al., 1994; Gutteridge and Haliwell, 1994). Studies have reported that supplementation with antioxidants prevents lipid peroxidation, protein glycation and inhibition of Na^+-K^+-ATPase and Ca^{2+}-ATPase activity caused by hyperglycemia (Jain and Lim, 2000; Pekiner et al., 2002; Jain et al; 2001).

In our study we observed that EL treatment ameliorates Na^+-K^+-ATPase and Ca^{2+}-ATPase in heart, which could be due to improvement in glycemic control, dislipidemia and glycation of proteins, as well as oxidative stress parameters responsible for alteration of these ATPase activities. Similarly Glib treatment also showed ameliorating effect in above enzyme activity.

In our study, blood pressure and heart rate of alloxan-diabetic animals was found to be significantly higher as compared to nondiabetic animals. EL and Glib prevented the rise in blood pressure in diabetic animals. A number of factors are involved in the pathogenesis of hypertension in diabetes mellitus such as sodium retention, ECF volume expansion, altered activity of the sympathetic nervous system, and rennin angiotensin system, increased vascular reactivity toward noradrenaline and angiotensin II (Ramos, 1980). As we have mentioned in chapter 3.2 that, EL treated animals showed amelioration in Kidney Na-K ATPase activity, which is responsible for retention of sodium in body and leads to hypertension. Bradycardia has been frequently observed in STZ-diabetic rats (Zicha et al., 1989). The development of STZ-induced bradycardia has been attributed to a down regulation of myocardial beta adrenoceptors and increase in circulation and heart levels of catecholamines (Savaress and Berkowitz., 1979). In the present investigation diabetic animals were found to have bradycardia compared to control animals. EL and glib treatment did produce significant change in heart rate.

Among various other factors responsible for decrease in cardiac function hyperlipidaemia and atherosclerosis also appear to be of prime importance. It has been well documented that diabetes mellitus is associated with changes in lipid metabolism. Rats treated with STZ have increased plasma levels of triglycerides, cholesterol, free fatty acids and phospholipids (Rodrigues et al., 1986). In the present study, alloxan-induced diabetic animals showed a hypoinsulinaemia (Maroo et al., 2003a) state which may be responsible for the rise in triglyceride levels. Insulin has an inhibitory action on HMG-CO-A reductase, would therefore be responsible for the elevation of cholesterol levels. In the present investigation it was observed that triglyceride, cholesterol and LDL cholesterol levels are elevated in diabetic rats. EL and Glib treatment reduced triglyceride, cholesterol and LDL in diabetic animals. The possible mechanism involved in above changes by EL and Glib may be the improvement in hypoinsulinaemia state in diabetic animals. The HDL which leads to treatment leads to correction in diabetic dislipidemia and possibly responsible for preventing atherogenic condition in treated diabetic rats.

The pathogenic factors contributing to vascular complications associated with diabetes are not fully understood. Platelet hypersensitivity, endothelial cell dysfunction and alterations in coagulation mechanisms have been observed in diabetic patients and are implicated as possible factors. Many investigators have shown that diabetic patients have enhanced platelet function and hypercoagulability (Bell, 1996). Diabetes is associated with increased risk for atherosclerosis and its thromboembolic complications (Pyorala et al., 1987). Atherosclerosis is contributed to platelets through their effects on vessels by materials released from the platelets, which interact with injured or altered vessels (Moore, 1985). Platelets from diabetic patients and animals are known to be hypersensitive to agonists (Wincour et al., 1986; Wincour, 1992). In diabetes, platelets are activated by a number of mechanisms, including activated arachidonate pathway and increased TXA2 formation, which could contribute to enhanced atherosclerosis and vascular complications (Winocour, 1993). Indeed,

platelet microthrombi have been reported to occur more readily in diabetic patients and animals (Williams et al., 1980; Honour and Hockaday, 1976) Prevention of platelet activity, therefore, should provide effective prophylactic and/or therapeutic means of treating such complications of diabetes.

One of the objective of this study was to ascertain whether EL could produce antithrombotic effect in alloxan-induced diabetic rat, and the result from this study indicate a potential use of EL as a antithrombotic agent in diabetes. In this study, we found that EL extract significantly reduced the platelet aggregation as well as platelet count in diabetic animals and thus showed it's antithrombotic effect. This could be due to hypolipidemic activity of EL as it is known that hypercholesterolaemia and hypertriglyceridemia are responsible for platelet hyperaggregability (Aoki et al., 1997). Patients with hypercholesterol-aemia have elevated levels of β-thromboglobulin and other markers of platelet activation compared to age-matched control subjects. Aoki et al. (1997) demonstrated that platelet-dependent thrombin generation was increased in patients with hypercholesterolemia and in patients with hypercholesterolemia plus hypertriglyceridemia compared with patients with hypertriglyceridemia and control subjects. EL also has HMG-COA inhibitory activity responsible for decrease in hypercholesteromic condition (Vasu et al., 2005) and thus improvement in platelet aggregation activity.

In humans with diabetes mellitus, many studies showed disturbances of hemostatic and fibrinolytic mechanisms, namely, activation of blood coagulation (Ceriello et al., 1994) and hypofibrinolysis (Garcı´a-Frade et al., 1990). Hyperglycemia is regarded as one key causal factor in the development of diabetic vascular complications. A large body of evidence converges to point to glycation as one key molecular basis of diabetic complications due to hyperglycemia (Carr, 2001; Gugliucci, 2000). Our results are in accordance with above facts. In the present study, the coagulant properties were assessed by APTT and PT using rat plasma. In the screening test for the coagulation pathway,

the prothrombin time (PT) and activated partial thromboplastin time (APTT) were shorter in alloxan-induced diabetic rats (Acang & Jalil, 1993). Protective effect of EL and Glib was observed in diabetic rats by bringing PT and APTT time towards normal values and indicates that correction in hyperglycemic state as well as reducing glycation of protein (as indicated by glycosylated hemoglobin) in these rats, could be the possible mechanism for the amelioration in anticoagulant state. Thus, in the present study, EL extracts displayed well anticoagulant and platelet antiaggregatory effects in alloxan-induced diabetic rats.

Another possible mechanism for the development of cardiovascular disease is ROS generation. Hyperglycemia enhances the production of reactive oxygen species (ROS) by auto-oxidation of glucose, oxidation of glycosylated proteins, and also enhances the production in mitochondria (Gillery et al., 1988; Hunt et al., 1990; Wolff and Dean, 1987). Activation of protein kinase C and the enhanced polyol pathway may also increase ROS (Aronson & Rayfield, 2002). Hyperglycemia stimulates the mitochondrial electron transport system, which increases the production of ROS (Bellin et al., 2006; Wolf, 2004). Mitochondrial DNA deletion has been observed in the hearts of patients with diabetes mellitus (Takeda et al., 1993). Mutations of mitochondrial DNA lead to impairment of energy production and the radical scavenging system in mitochondria.

In present study we tried to understand the role of mitochondrial and post-mitochondrial oxidative stress in the development of cardiac dysfunction and to understand the role of antioxidant treatment as protective measure. We found increased level of MDA and decreased activities of CAT, GPx and SOD, in the cardiac tissue of diabetic rats compared with the control rats in both mitochondrial and post-mitochondrial fraction. MDA, a routine index of lipid peroxidation, increased in diabetes mellitus, which implies that hyperglycemia induces peroxidative reactions in lipids. The decrease of CAT, SOD and GPx activity in diabetic heart tissue suggests increased oxidative stress due to chronic

exposure to glucose, which may be an important mediator for any possible tissue damage in alloxan-induced diabetes. The level of MDA was reduced markedly upon EL and Glib treatment, as well as the activity of GPx, CAT and SOD in the cardiac tissue was elevated by EL and Glib treatment in diabetic rats in both mitochondrial and post-mitochondrial fractions.

It is quite evident from results that decreased GSH content and antioxidant enzyme activities were almost similar in both the fractions of heart tissue of untreated diabetic rats. This suggests that generation of oxidative stress in both the compartments were comparable and is equally responsible for hyperglycemia induced damage to the heart tissue and thus responsible for the development of disease condition. EL and Glib treated diabetic rats showed, comparable improvement in antioxidant defense system of mitochondrial and post-mitochondrial fraction of the heart tissue. This study also indicates that efficacy of EL and Glib treatment is comparable with respect to the improvement in antioxidant defense system of heart mitochondrial and post-mitochondrial fractions.

In conclusion, our data suggest that EL prevents not only the alloxan-induced metabolic abnormalities, but also cardiovascular complications as evident from the reduction in cholesterol, triglyceride, LDH, CK-MB, Na^+-K^+-ATPase, Ca^{2+}-ATPase platelet hyperaggregability, PT, APTT, and oxidative stress in both mitochondrial and post-mitochondrial fractions of heart tissue which are the symptoms of congestive heart failure. Glib treatment had also shown similar efficacy in preventing diabetic cardiovascular complication.

6c.5. Summary of the chapter

Neuropathy is another diabetic microvascular complication undertaken for the study. Animals were made diabetic with the help of diabetogenic compound alloxan. Animals with hyperglycemic condition for 45 days developed neuropathic symptoms. These animals showed thermal hypoalgesia and hyperalgesia in formalin induced paw irritation test. Biochemical parameters were evaluated in sciatic nerve. Polyol pathway marker AR activity was high in the sciatic nerve while Na-K ATPase activity was low. Oxidative stress was also high in this nerve. EL treatment reduces the blood glucose level and there by decreases flux of glucose into polyol pathway. EL also increases insulin secreation from remaining islets. EL treatment also reduces oxidative stress in the sciatic nerve by increasing the activity of antioxidant enzymes and protects nerves from damage. EL extract is having AR inhibitory activity also which helps in ameliorating the disease condition. It is reported by others that EL is having anti-nociceptive activity. Thus EL have complete package of insulin screatogouge activity, antioxidant and hypolipidemic required for the better drug candidate for diabetes and diabetic complications.

As EL extract is efficacious in preventing diabetic neuropathic condition, we hypothesized that it should also protect diabetic animals from reproductive dysfunctions as peripheral neuropathy is one of the causative factors for its development. Male as well as female diabetic patients suffer from reproductive dysfunction. In male it causes erectile dysfunction and infertility. Prolog hyperglycemic condition for 45 days leads to atrophy of reproductive organs in alloxan-induced diabetic animals. It causes decreased steroidogenesis marked by decreased 17β-HSD and 3β-HSD activity in testis and leads to decreased serum testosterone levels, which is required for spermatogenesis. AR activity was high in testis, epididymis, seminal vesicles and prostate tissue, while fructose content increases in prostate and seminal vesicles. GSH level were less and lipid peroxidation levels were high in testis and epididymis indicating generation of oxidative stress in these tissues. Prostate and epididymal Vit C content was

decreased due to oxidative stress. Vitamin C is also responsible for steroidogenesis thus decreased vitamin C may lead to low steroidogenesis. Steroidogenesis takes place in testis while sperm maturation takes place in epididymis. Oxidative stress in these two tissue leads to decreased spermatogenesis, sperm viability and sperm maturation in diabetic rats. One group of diabetic animals was treated with methanolic extract of EL for 45 days. These rats showed improved testicular enzyme activities of 17β-HSD and 3β-HSD evident by increased serum testosterone level. Decreased AR activity could be due to inhibitory effect of EL as shown by others and also by decreased glucose flux through this pathway. EL treatment also decreases levels of glycosylated hemoglobin indicating reduced level of AGE formation and its related metabolic derangements. Thus our study on efficacy of EL on male reproductive dysfunction suggest that EL can prevent atrophy of reproductive organs by reducing oxidative stress as well as increasing serum testosterone level required for the growth and maintenance of these organs. Hence, it increases spermatogenesis and prevent sperm function abnormalities.

Above studies indicated protective effect of EL on microvascular complications in diabetic rats. Another study was carried out to evaluate the efficacy of EL on macrovascular complications of diabetes that is cardiomyopathy. Diabetic rats after 45 days of hyperglycemic condition showed the sign of cardiovascular complications like dislipidemia, increased enzymatic serum marker of cardiac function, increased platelet hypersensitivity, blood coagulation abnormalities, depressed cardiac Na^+-K^+; Ca^{2+}-ATPase activity, bradycardia, hypertension and generation of oxidative stress in heart tissue. Diabetic animals treated with methanolic extract of EL correct, all metabolic abnormalities as well as vascular abnormalities. It also improves antioxidant status equally in both mitochondrial and post-mitochondrial compartment of heart tissue. Thus, EL has good efficacy in protecting diabetic rats from development of cardiovascular complications.

Efficacy of EL in different disease condition was comparable to standard drugs glibenclamide and rosiglitazone. This could be because both the standard drugs are having good antioxidant activity along with hypoglycemic/insulin sensitizing activity.

Conclusively our study indicates that EL is having good hypoglycemia, hypolipidemic, antioxidant, insulin secreatogouge and insulin sensitizing activity. These activities can improve insulin resistance, microvascular and macrovascular complications of diabetes in rat models.

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