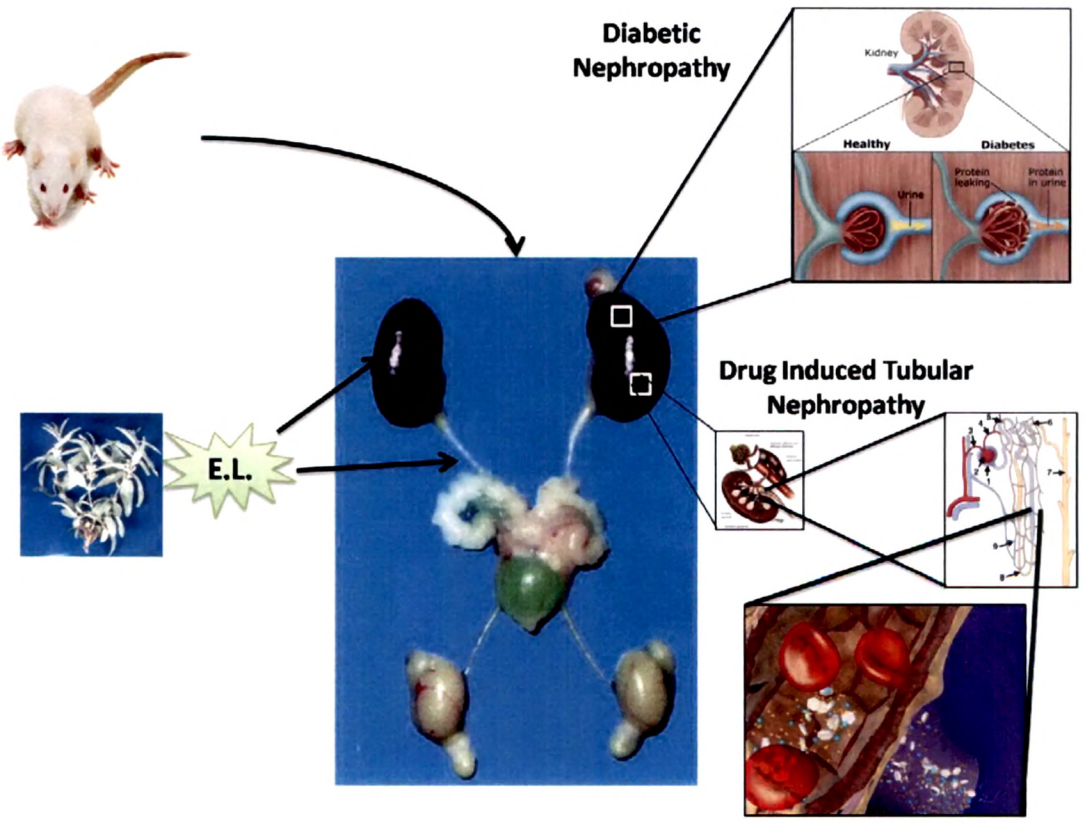
Chapter 5

Evaluation of efficacy of *Enicostemma littorale* methanolic extract in nephropathic condition in rat models.



Chapter 5 Evaluation of efficacy of *E. littorale* methanolic extract in nephropathic condition in rat models.

5A.1 Introduction

5A.2 Experimental design

5a.1 Gentamicin-Induced nephrotoxicity

5b.1 Diabetic nephropathy

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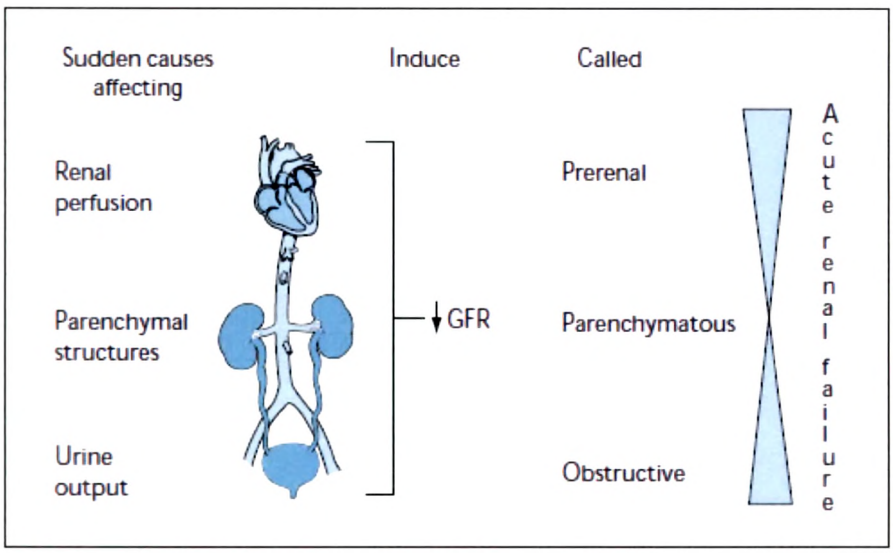
5A.1 Introduction

There are many causes more than fifty that can trigger pathophysiological mechanisms leading to acute renal failure (ARF). This syndrome is characterized by a sudden decrease in kidney function, with a consequence of loss of the hemostatic equilibrium of the internal medium. The primary marker is an increase in the concentration of the nitrogenous components of blood. A second marker, oliguria, is seen in 50% to 70% of cases. Depending on the localization or the nature of the renal insult, ARF is classified as prerenal, parenchymatous, or obstructive (postrenal), (Fig. 5A.1).

Figure 5A.1: Classification of acute renal failure



During the last years, acute tubulointerstitial nephritis is increasing in importance as a cause of acute renal failure. For decades infections were the most important cause. At present, antimicrobials and other drugs are the most common causes. Mechanisms of drug-induced tubulointerstitial disease generally fall under one of two categories. First, there is disease mediated by inflammation of the interstitium and tubules that is commonly referred to as acute interstitial nephritis (AIN). Acute interstitial nephritis usually occurs on an allergic basis in

an idiosyncratic and non-dosedependent manner. The second pathomechanism of drug-induced tubulointerstitial disease is toxic acute tubular necrosis (ATN) whereby the pharmacologic agents or their derivatives act as direct tubular toxins. Toxic ATN is at least in part dose-dependent and is characterized by tubular injury in the absence of significant inflammation.

5A.1.1. Acute interstitial nephritis (AIN)

Acute interstitial nephritis may occur as an adverse reaction to many different drugs. Patients present with acute renal failure (ARF) following exposure to a medication (Ditlove et al., 1977; Pirani et al., 1987). Commonly implicated drugs include β-lactam antibiotics (including cephalosporins), other antibiotics (sulfonamides, rifampin, vancomycin, ciprofloxacin), non-steroidal anti-inflammatory drugs, phenytoin, and diuretics (furosemide, thiazides). Pathologic findings in AIN include interstitial inflammation, edema, and tubulitis. In addition to lymphocytes (mainly T cells) and mono- cytes, the interstitial infiltrate typically includes eosinophils. Importantly, interstitial granulomas are commonly seen in AIN and may represent the predominant pattern of injury (bgranulomatous interstitial nephritis) (Border et al., 1974).

5A.1.2. Acute tubular necrosis (ATN)

The role of the proximal tubule in concentrating and reabsorbing the glomerular filtrate renders it vulnerable to toxic injury. Toxic ATN has a predilection for the proximal tubule and is caused by a wide variety of substances including heavy metals, organic solvents, pigments, and multiple classes of drugs. Therapeutic agents commonly implicated in toxic ATN include aminoglycoside antibiotics, amphotericin B, cisplatin, anesthetic agents (methoxyflurane), calcineurin inhibitors, mannitol, and radiocontrast media. The development of ARF is often dose-dependent and may develop rapidly following exposure to certain therapeutic agents such as Aminoglycoside antibiotics (Matushima et al., 1998), (Fig. 5A.2).

Figure 5A.2: Most common causes of tubulointerstitial nephritis.

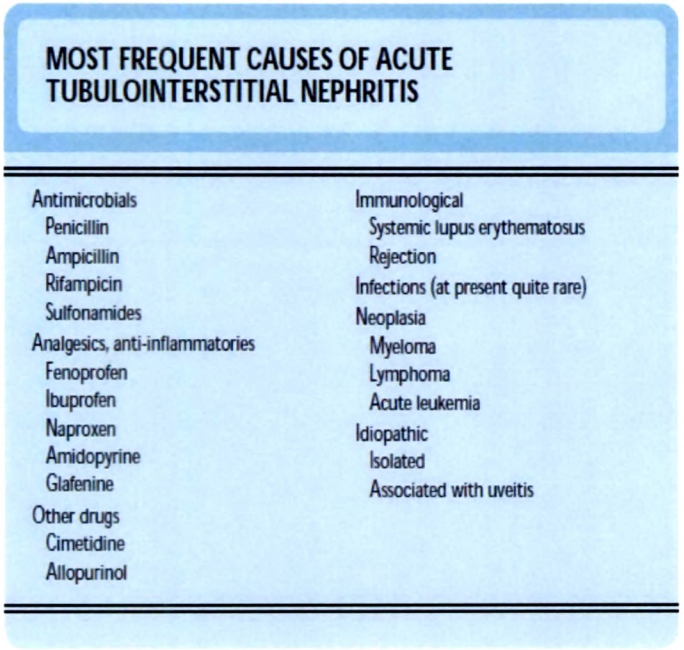
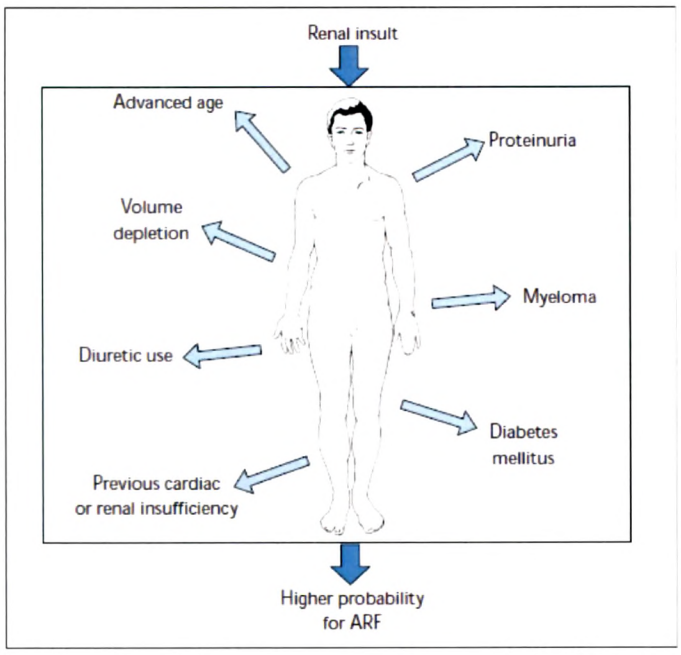


Figure 5A.3: Factors that predispose to acute renal failure (ARF)



Diabetic nephropathy is one of the major "microvascular"complications of diabetes (Fig. 5A.3). The renal lesions, whether related to type 1 or 2 diabetes

mellitus, are similar (Fioretto and Mauer, 2007). Various cells involved include glomerular podocytes, mesangial and endothelial cells, tubular epithelia, and interstitial fibroblasts and vascular endothelia. The pathophysiologic changes in diabetic nephropathy include hyperfiltration and microalbuminuria followed by worsening of renal functions associated with cellular and extracellular derangements in both the glomerular and tubulo-interstitial compartments (Reddy, 2004). They include hyperplasia/ hypertrophy of various cell types of the glomerulus and tubules, associated with thickening of glomerular and tubular basement membranes, and expansion of tubulo-interstitial and mesangial compartments (Mason and Wahab, 2003). Other changes include hyalinization of arterioles and at times thickening of branches of intrarenal arteries that leads to impairment in "autoregulation" of glomerular microcirculation, which apparently could amplify the renal damage.

5a. Evaluation of efficacy of *E. littorale* methanolic extract in Gentamicin-induced nephropathy in rat model.

5a.1. Review of literature

5a.2. Experimental design

5a.3. Results

5a.4. Discussion

5a.1. Review of literature

Aminoglycoside antibiotics, especially gentamicin (GM) are widely applied in veterinary and human clinical practices for treatment of life-threatening gram negative infections. These Aminoglycoside antibiotics also cause drug induced nephrotoxicity in 10–20% of therapeutic courses. Therefore, the clinical use of these drugs is limited by the development of nephrotoxicity. GM induced nephrotoxicity is characterized by tubular necrosis, without any morphological change in glomerular structures (Eisenberg et al., 1987; Cuzzocrea et al., 2002; Pedraza-Chaverri et al., 2003). Rats with GM-induced nephrotoxicity provides an excellent model of acute renal failure and to test the protective effects of chemical compounds, plant extracts or drugs GM-induced nephrotoxicity is an ideal model (Ali, 1995). The detailed mechanism by which GM-induces nephrotocitiy is not very well understood; however, reactive oxygen species have been shown to be involved in pathogenesis (Walker & Shah, 1987; Mazzon et al., 2001; Cuzzocrea, 2002; Morales et al. 2002).

GM has been shown to generate superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO) production from renal mitochondria (Yang 1995; Walker et al., 1999). In addition to generation of free radicals it also increases (Guidet and Shah, 1989), lipoperoxidation (Pedraza-Chaverrí, 2000; Ali, 2002), protein nitrosylation and carbonyaltion (Mazzon, et al., 2001; Maldonado, et al., 2003), and reduces glutathione content (Sener, et al. 2002) in renal cortex from GM-treated rats. Morever, the administration of several compounds with antioxidant properties, reactive oxygen species (ROS) scavengers, and/or antioxidant enzymes were able to ameliorate the severity of GM-induced renal damage by reducing oxidative stress (Pedraza-Chaverrí, et al., 2000; Ali, 2002; Morales, et al., 2002; Sener, et al., 2002; Ali, 2003; Maldonado, et al., 2003). In addition, kidney is deficient in the antioxidant enzymes Mn-superoxide dismutase (Mn-SOD) (Al-Majed et al., 2002; Pedraza-Chaverrí, et al., 2000), glutathione peroxidase (GPx) (Pedraza-Chaverrí, et al., 2000),

glutathione reductase (GR) (Maldonado, et al., 2003), and catalase (Pedraza-Chaverrí, at el., 2000) and thus more vulnerable to ROS in GM-treated rats.

Plants with potent antioxidant activity, has been shown to protect against gentamicin-induced nephrotoxicity. In the light of this, we have explored the possible protective role of *Enicostemma littorale* Blume, having good antioxidant potential (Maroo et al., 2003) on gentamicin-induced oxidative stress in kidney, causing nepherotoxicity.

5a.2. Experimental design

5a.2.1. Animals and treatment

Male Charles Foster rats (body weight 200–250 g) were used for the study. They were allowed *ad libitum* access to water and food. During the study, rats were maintained in stainless steel metabolic cages with a 12-h light/dark cycle to collect 24 h urine at the end of the study. All the animal studies were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Rats were divided into four groups of six rats each as follows:

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

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

Group-III : GM + EL treated with EL and with GM. EL was given i.g at a dose of 2.5 g of methanol extract/kg body wt/day (Maroo et al., 2003).

Group-IV : GM + Vit C treated with Vit C and with GM. Vitamin C was given i.g at a dose of 600mg/kg b.wt/day.

The onset of damaging renal function induced by GM occurs after 5–8 days' treatments between of 80 and 150 mg kg^{-1}. In this study, GM was injected intraperitoneally at the dose of 80 mg kg-1, for eight successive days, which is well

known to cause significant nephrotoxicity in rats (Abdel-Gayoum et al., 1994, Kumar et al., 2000; Ali et al., 2004).

The *Enicostemma littorale* extract was given at a dose of 2.5 g/kg b. wt/day via gastric intubation for 11 days (Maroo et al., 2003). The Vit C, potent antioxidant, was used as reference drug at a dose of 600 mg/kg b.wt/day (Kavutcu et al., 1996) for 11 days. EL and Vit C treatment was started three days prior to the 8 days gentamicin treatment and continued till the end of gentamicin treatment.

At the end of experiment final weight of all animals were taken and urine collection has been done for 24 hrs with the help of metabolic cages. Blood samples were collected at the end of treatment on 9th day. The animals were sacrificed, kidney dissected out for estimation of various parameters as mentioned below. Change in body weight was monitored before and after the treatment. From blood sample creatinine and blood urea nitrogen levels were estimated for kidney dysfunction. Various non-enzymatic and enzymatic antioxidant parameters along with oxidative stress markers were estimated from mitochondrial and post-mitochondrial fractions, to understand the contribution of both the compartments for the development of disease condition and also to understand the role of antioxidant treatment in the prevention of disease condition.

5a.3. Results

5a.3.1. Body weight and urinary volume

Body weight among different treated group did not change significantly from the control group (Table 5a.1). Urinary volume increased significantly in the GM group (Table 5a.1) as compared to control group. In EL and VC treated rats, urine volume was significantly reduced as compared to GM treated rats (GM+EL and GM+VC groups). In fact, EL and VC treated rats showed decrease in the urine volume to the level of the control.

5a.3.2. Serum creatinine and urea levels

Serum creatinine level increased by 368% and blood urea nitrogen level (BUN) increased by 176% in the GM group compared to the control (C) group (Table 5a.2). EL attenuated the increase in creatinine by 60% and 63% fold in BUN level. Similarly, VC attenuated the increase in creatinine and BUN level by 55%.

Table:5a.1. Effect of EL treatment on GM-induced changes body weight and urine volume

Groups	Body Weight (gms)	Urine Volume (ml/24hrs)
C	272 ± 8.33	5.8 ± 0.24
GM	267 ± 6.63	15.6 ± 1.18^{aaa}
GM+EL	270 ± 8.22	7.3 ± 0.39^{bbb}
GM+VC	272 ± 7.66	6.9 ± 0.25^{bbb}

Values are expressed as mean ± SEM (n=6 in each group). aaa, $p<0.001$ vs. C, bbb, $p<0.001$ vs. GM.

Table:5a.2. Effect of EL treatment on GM-induced changes in Creatinine, BUN and tubular necrosis.

	Creatinine (mg/dl)	BUN (mg/dl)	Tubular Necrosis
C	0.76 ± 0.04	21.5 ± 1.08	—
GM	3.57 ± 0.14^{aaa}	59.3 ± 2.31^{aaa}	+++
GM+EL	1.88 ± 0.11^{bbb}	35.3 ± 2.06^{bbb}	++
GM+VC	2.02 ± 0.08^{bbb}	38.7 ± 1.31^{bbb}	++

Values are expressed as mean ± SEM (n=6 in each group). aaa, $p<0.001$ vs. C, bbb, $p<0.001$ vs. GM.

5a.3.3 Lipid peroxidation, reduced glutathione and antioxidant enzymes activities in mitochondrial and post-mitochondrial fractions of kidney tissue.

MDA is a marker of oxidative stress. GM treated group showed 159% and 123% increase in MDA levels of mitochondrial and post-mitochondrial fraction respectively. Thus the content of LPO is higher in mitochondrial fraction than in post-mitochondrial fraction (Fig. 5a.2). The EL treated group showed 80% and 63% decrease in mitochondrial and post-mitochondrial MDA levels respectively. However, the VC treated group decreases MDA levels by 77% and 64% in mitochondrial and post-mitochondrial respectively. Decrease in MDA levels were more in rats treated with EL compared to VC treated rats.

The GSH content in the post-mitochondrial fraction was relatively higher as compared to mitochondrial fraction in control group. GM treated rats showed decrease in GSH by 50% in mitochondrial fraction and 33% in post-mitochondrial fraction (Fig. 5a.3). The EL treated group showed 75% and 88% improvement in GSH content of mitochondrial and post-mitochondrial fractions respectively. Similarly, VC treated group showed 62% and 89% increase in mitochondrial and post-mitochondrial GSH content. Treatment with EL and VC were having similar effect on GSH content of mitochondrial and post-mitochondrial fractions.

SOD activity was higher in post-mitochondrial fraction as compared to mitochondrial fraction in control rats. The activity of SOD decreases by 61% in mitochondrial fraction, while decreases by 36% in post-mitochondrial fraction (Fig.5a.1). The EL treated group showed 78% and 66% increase in mitochondrial and post-mitochondrial SOD activity, respectively. Whereas VC treated group showed 73% and 57% increase in mitochondrial and post-mitochondrial SOD activity. EL treated rats showed little bit more increase in post-mitochondrial SOD activity as compared to VC treated rats, but having similar effect on mitochondrial SOD activity. GPx activity in the mitochondrial fraction was relatively higher than the post-mitochondrial fraction in control rats (Fig. 5a.4). Mitochondrial GPx activity decreases by 60%, while post-mitochondrial activity decreases to 43% in GM-treated rats. The EL treated group showed increase in GPx activity by 80% and 113% in mitochondrial and post-mitochondrial fraction respectively. However, the VC treated group showed 82% and 101% increase in mitochondrial and post-mitochondrial GPx activity. Effect of EL and VC on GPx activity of mitochondrial and post-mitochondrial compartments was comparable. Catalase activity decreases 42% in post-mitochondrial fraction in GM treated group as compared to control group (Fig 5). The EL treated group showed 95% increased in activity, while the VC treated group showed 93% increase in CAT activity. EL and VC have similar effect on post-mitochondrial CAT activity.

5a.3.4. Histopathological analysis

Rats treated with GM showed sever tubular necrosis (Table 5a.2). Treatment with GM alone caused a marked vacuolization and necrosis (+++) in proximal tubular epithelial cells. Treatment with EL (++) and VC (++) significantly decreases GM-induced tubular necrosis.

5a.4. Discussion

Previous studies with EL in our lab, has shown that methanolic extract of EL showed significant antioxidant activity *in-vitro* and *in-vivo* (Maroo et al. 2003). As ROS play very important role in development of pathogenic condition of many

diseases, like diabetic nephropathy. We hypothesized EL extract will protect rats from GM-induced nephrotoxicity, which is mainly caused by generation of ROS.

Figure 5a.1: Effect of EL on kidney SOD levels in GM-induced nephrotoxicity.

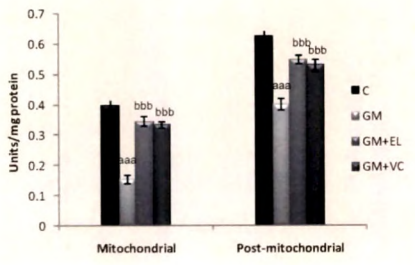


Figure 5a. 2: Effect of EL on kidney LPO levels in GM-induced nephrotoxicity.

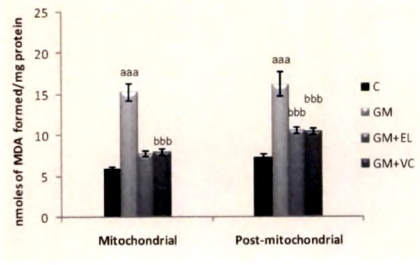


Figure 5a.3: Effect of EL on kidney GSH levels in GM-induced nephrotoxicity.

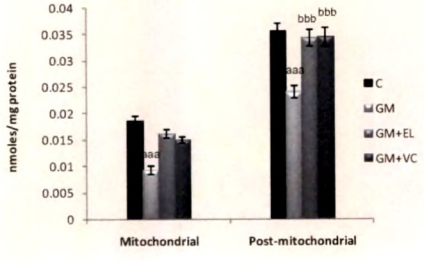


Figure 5a.4: Effect of EL on kidney GPx activity in GM-induced nephrotoxicity.

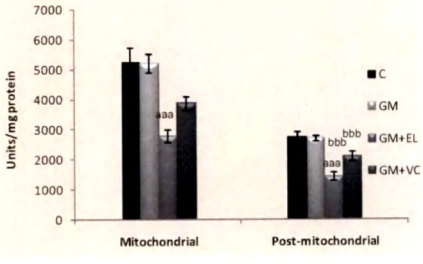
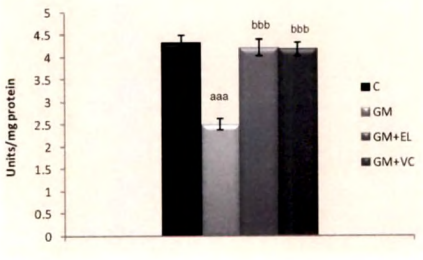


Figure 5a.5: Effect of EL on kidney Catalase activity in GM-induced nephrotoxicity.



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.

The results of the present study indicate that GM administration brought about a significant increase in BUN and serum creatinine levels, indicating kidney dysfunction. The results are in agreement with the reports of K.Vijay Kumar, et al. (2000). These alterations in biochemical parameters were well correlated with the renal histological score of GM treated rats.

Reactive oxygen species including hydroxyl radical have been implicated in the etiology of GM gentamicin-induced nephrotoxicity. Walker and Shah (1987) showed that GM enhances the generation of hydrogen peroxide by renal cortical mitochondria and that iron chelators and hydroxyl-radical scavengers protect against GM-mediated renal damage. It has been also demonstrated generation of hydrogen peroxide by rat kidney cortex and glomeruli (R Guidet and Sudhir V. Shah, 1989). Treatment with SOD or hydroxyl radical scavenger, dimethyl-thiourea (DMTU), two methods for reducing reactive oxygen metabolites, significantly lessened the GM-induced reduction in GFR (Toshiaki Nkajima, et al. 1994). Salvatore Cuzzocrea et al. 2002 demonstrate that daily administration of M40403, low molecular weight synthetic manganese containing superoxide dismutase mimetic, which selectively removes superoxide to gentamicin-treated rats, resulted in an almost complete normalization of kidney functions.

Our results show that GM-induced oxidative stress as demonstrated by significant elevation in lipid peroxidation and decrease in GPx and SOD activities both in mitochondrial and post-mitochondrial fraction. Lipid peroxidation levels were higher in mitochondrial fraction as compared to post-mitochondrial fraction. Similarly, decrease in mitochondrial SOD and GPx activity was higher in mitochondrial fraction as compared to post-mitochondrial fraction. The depletion in GSH content was higher in mitochondria fraction as compared to post-mitochondrial fraction. Decreased GSH status might be responsible for the observed decrease in GPx activity. Even post-mitochondrial CAT activity decreased significantly. Decrease in GPx and CAT activity indicates decrease efficiency in H_2O_2 scavenging activity in kidney tissue of GM treated rats, which might be responsible for increase in lipid peroxidation levels in both the fractions. Hence, above results clearly

indicates higher oxidative stress in mitochondrial fraction as compared to post-mitochondrial fraction in GM-induced nephrotoxicity. Histipathological changes observed in GM treated rat well correlates with GM-induced oxidative stress.

Oral administration of EL and Vit C was found to have a protective potential against GM-induced nephrotoxicity. Treatment with EL and Vit C showed increase in SOD and GPx activity, in both the fractions, along with increase in the GSH content, which is a primary antioxidant defense system of the cell. Amelioration in antioxidant defense system in both mitochondrial and post-mitochondrial fractions, leads to decrease in lipid peroxidation levels. However, the improvement in antioxidant defense system was more in mitochondrial fraction than in post-mitochondrial fraction which is a major site of ROS generation. In the present study, EL and Vit C treatment showed comparable protective effect on GM-induced nephrotoxicity. However, EL treatment showed better ameliorating effect on certain biochemical parameters as compared to Vit C treatment.

In summary, the present study provides evidence that co-administration of EL along with GM attenuates the increase in lipid peroxidative damage, restores antioxidant status, markers of renal injury and histopathological alterations. The present findings demonstrate that EL possesses significant therapeutic effects and it is a promising candidate for chemoprevention of GM-induced renal damage.

Previous studies in our lab, has clearly showed hypoglycemic and hypolipidemic activities of EL in diabetic rats and newly diagnosed diabetic patients. Present study indicates that oxidative stress is responsible for the development of GM-induced nephrotoxicity and antioxidant activity of EL alone is able to protect against GM-induced oxidative stress in nephrotoxicity. On the basis of this study we hypothesize that EL might protect against diabetic nephropathy as its antioxidant effect alone is able to protect against GM-induced oxidative stress in nephrotoxicity. Hypoglycemic and hypolipidemic activities of EL along with its antioxidant activity will be having better protection against diabetic nephropathy as hyperglycemic,

hyperlipidemic and severe oxidative stress are hallmark in the development of diabetic nephropathy.

Chapter 5b. Evaluation of efficacy of *E. littorale* methanolic extract in diabetic nephropathy in rat model.

5b.1. Review of literature

5b.2. Experimental design

5b.3. Results

5b.4. Discussion

5b.5. Summary

5b.6. Bibliography

5b.1. Review of literature

At least 25% of patients with Type 1 diabetes mellitus (DM) and 30% to 40% of patients with Type 2 DM eventually develop diabetic nephropathy that may progress to end-stage renal disease (ESRD) (United States Renal Data System, 2000; Ritz et al., 1997). As Type 1 DM usually manifests early in life, ESRD may develop at a younger age producing an additional burden for patients and health services (Mauer et al., 2001). Several pathways are thought to be involved in the pathogenesis of hyperglycemia-induced complications in DM such as activation of protein kinase C isoforms, increased formation of advanced glycation end products (AGEs), and increased flux through the aldose reductase pathway (Ishii et al., 1996). Interestingly, the excessive production of reactive oxygen species (ROS) has been suggested as a common product of all these pathways that can result in the DM-associated complications (Ishii et al., 1996).

The extent of derangements in the cellular elements of various target organ systems affected by chronic hyperglycemic injury ultimately dictate the morbidity and mortality in patients with diabetes mellitus (Le Roith et al., 2004). The complications related to cellular derangements in most of the target organs appear to be similar in type 1 and 2 diabetes mellitus, although the pathogenetic mechanisms for the development of hyperglycemia in the two types are quite dissimilar, that is, the former is characterized by absolute insulin deficiency and the latter by relative insulin deficiency with insulin resistance (Brownlee, 2000; Sheetz and King, 2002). In either case, the major organs affected in chronic hyperglycemic injury include the kidney, retina and nervous tissue, and the disease processes affecting them are described as nephropathy, retinopathy and neuropathy, respectively; and these collectively are referred to as the microvascular complications of diabetes. The term 'microvascular' encompasses renal glomerular capillaries, small retinal blood vessels and vasa nervosum, implying that the cells primarily affected are mesenchymal-angioblastic derivatives, i.e., endothelium. However, other derivatives of angioblasts, e.g., the

renal mesangium and retinal pericytes, are also known to be the targets of chronic hyperglycemic injury. In addition, literature data suggest that the diabetic milieu can conceivably also modulate the biology of mesodermally derived cells, i.e., skeletal muscle, as well as those derived from the endodermal or ectodermal lineage, e.g., glomerular podocytes and renal tubular epithelia. Moreover, taking into account the pathobiology of the 'caudal regression syndrome' seen in the offspring of severely affected diabetic mothers it would seem that the cells derived from all the embryonic germ layers are directly or indirectly susceptible to hyperglycemic injury (Chugh et al., 2003).

Among various target organ injuries, diabetic nephropathy has been the most extensively investigated, and it is one of the common metabolic disorders leading to chronic progressive renal failure (Reddy, 2004; Parving et al., 2000). The annual incidence of this disease has more than doubled in the past decade to reach almost 50% of all end-stage renal diseases. Initial stages of diabetic nephropathy are characterized by hyperfiltration and microalbuminuria, later followed by declining renal functions that are reflected by marked cellular and extracellular changes in the glomerular and tubulo-interstitial compartments. The glomerular changes include hyperplasia/ hypertrophy of mesangial and endothelial cells with thickening of glomerular basement membrane and expansion of the mesangium and alterations in the podocytes. Similar changes occur in the tubular-interstitial compartment that includes thickening of tubular basement membranes, interstitial fibrosis and hyalinization of the arterioles with effacement of their endothelia. In aggregates, it seems that all the cellular elements of the kidney, i.e., endothelial, epithelial, mesangial, podocytes and interstitial cells, are affected to varying degrees by hyperglycemia. Conceivably, upon glucose entry the subsequent intracellular signaling events in all the renal cells may be similar, with the expected variations depending on the expression of a given molecule in a particular cell, e.g., aldose reductase in the tubular cells and protein kinase C (PKC)-α and -β isoforms in the glomeruli. There are a number of intracellular events that occur in the presence of high-glucose ambience, which

include increased flux of polyols (Dunlop, 2000), and hexosamines, formation of advanced glycation end products (AGEs) (Soulis-Liparota et al., 1991), and reactive oxygen species (ROS), activation of PKC (Xia et al., 1994), transforming growth factor (TGF)-β (Cooper et al., 1999), Smad-mitogen-activated protein kinase (MAPK) (Dunlop and Muggli, 2000; Haneda et al., 1997), and G-protein signaling, altered expression of cyclin kinases, their inhibitors and of matrix proteins, matrix-degrading enzymes, metalloproteinases, and their inhibitors. Possibly, these events are interconnected at various levels of different signaling pathways with certain common denominators and telescopic end results, i.e., increased extracellular matrix (ECM), a hallmark of diabetic nephropathy responsible for end-stage renal failure (Mason and Wahab, 2003).

Diabetic nephropathy occurs as a result of an interaction between haemodynamic and metabolic factors mentioned above (Cooper, 2001). Haemodynamic factors that contribute to the development of diabetic nephropathy include increased systemic and intraglomerular pressure, as well as activation of vasoactive hormone pathways including the renin angiotensin system (Zatz et al., 1986) and endothelin (Hargrove et al., 2000). In combination, these pathways ultimately lead to increased renal albumin permeability and extracellular matrix accumulation, resulting in increasing proteinuria, glomerulosclerosis and ultimately tubulointerstitial fibrosis (Fig. 5b.1).

5b.1.1 Management of diabetic nephropathy

The United Kingdom Prospective Diabetes Study (UKPDS) (Holman et al., 2008; Bilous, 2008), STENO-2 (Gaede et al., 2001), and ADVANCE studies (Patel et al., 2007; Group et al., 2008; Zoungas et al., 2009) all demonstrated that tight control of blood glucose level, blood pressure (and lipids in STENO-2) significantly reduced incidence and progression of diabetic kidney disease.

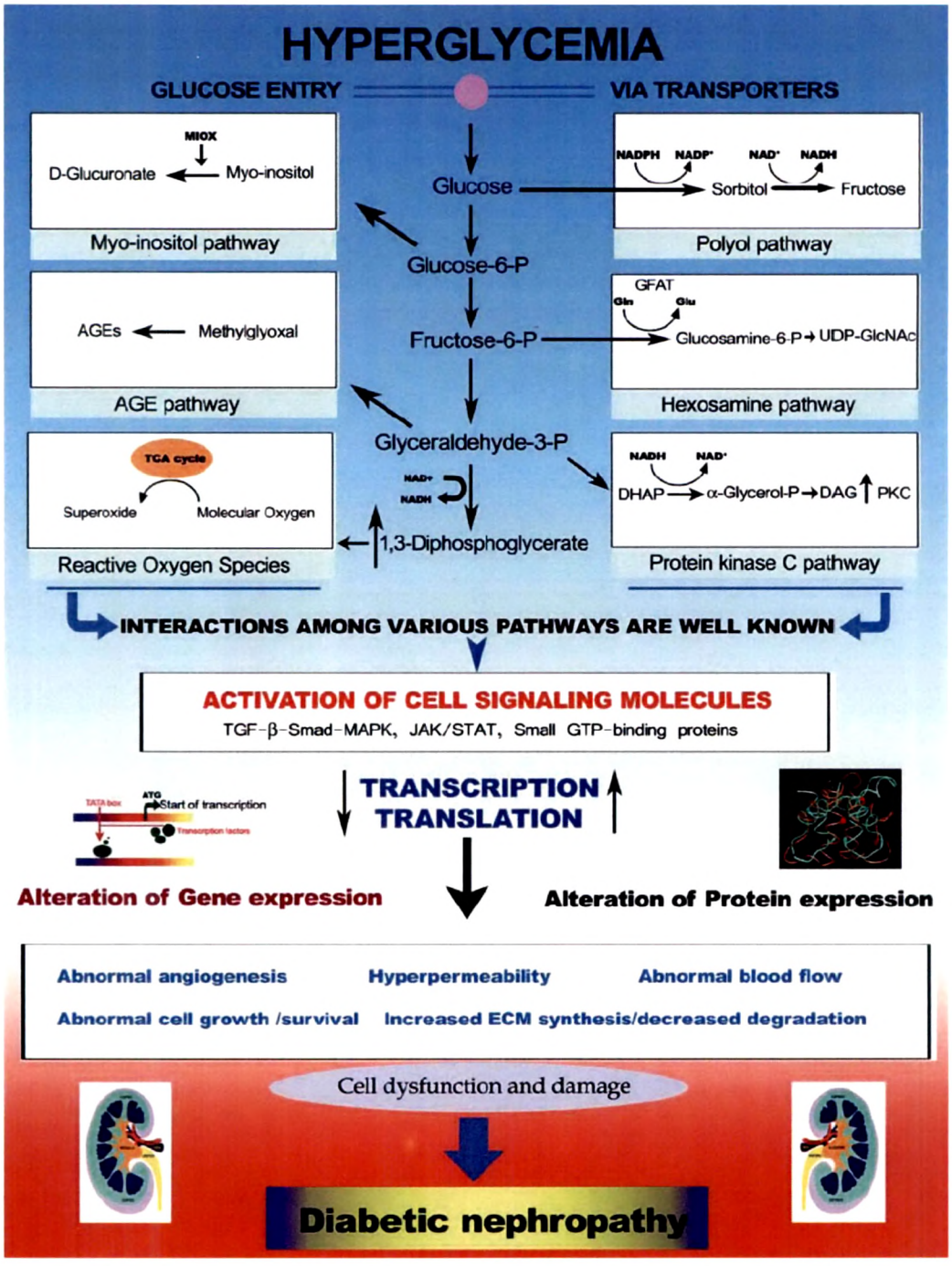


Figure 5b. 1: An overview depicting activation of different signaling pathways by high glucose ambience with altered expression of various genes and cellular dysfunctions leading to diabetic nephropathy.

Source: Kanwar et al., 2005.

In people with type 2 diabetes, inhibition of the renin-angiotensin-aldosterone system using an angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) decreased the progression from normoalbuminuria to microalbuminuria (Ruggenenti et al., 2004), reduced the progression from microalbuminuria to macroalbuminuria (Parving et al., 2001), and slowed the development of ESRD (Lewis et al., 2001). Thus, the use of an ACEI or ARB is now standard therapy for patients with diabetic nephropathy as well as glucose, lipid and blood pressure control. Effective management using evidence based therapies is tackling diabetic kidney disease. The next step is development of new therapies. Numerous novel target sites such as TGF-β, AGEs, PKC, Rho-kinase, NADPH oxidase, PARP and ET-1 have been identified to play a pathogenic role in the progression of DN. The pharmacological agents inhibiting these target sites have been noted to halt the progression of DN. However, further clinical studies are needed to illuminate their therapeutic potential in treating diabetic patients with nephropathy (Table 5b.1).

Recently, numerous potential herbal interventions have been identified to have ameliorative effect in the progression of DN (Table 5b.2).The administration of Hashimi-jio-gan has been shown to provide renoprotection as it reduces proteinuria and prevents glomerulosclerosis, tubulointerstitial fibrosis and mesangial matrix expansion by decreasing the AGE formation and sorbitol levels, and inhibiting the renal lipid peroxidation in diabetic rats with nephropathy (Yokozawa et al., 2002). Quercetin, an antioxidant was noted to reduce proteinuria, serum creatinine, blood urea nitrogen and oxidative stress by inhibiting the renal peroxidation in diabetic rats with nephropathy (Anjaneyulu and Chopra, 2004).

In a clinical study, treatment with leaf extract of *Ginkgo biloba* produced renoprotective action in diabetic patients with early stages of nephropathy by reducing albuminuria and N-acetyl-beta-D-glucosaminidase (Zhu et al., 2005). Administration of *Salvia miltiorrhiza* has been noted to possess renoprotective

effect as it decreases urinary albumin excretion rate by reducing the renal expression of TGF-β, CTGF, fibronectin and PAI-1 in diabetic rats with nephropathy (Liu et al., 2005).

Table 5b.1: Potential target sites for diabetic nephropathy

S. no.	Target sites	Therapeutic interventions
1	ACE inhibition	Captopril, lisinopril, imidapril, ramipril, perindopril, cilazapril, benazapril, trandolapril, enalapril and fosinopril
2	Blockade of AT_1 receptor	Losartan, irbesartan, olmesartan and candisartan
3	Aldosterone antagonism	Spironolactone, eplerenone and FAD286
4	Calcium channel blockade	Nicardipine, isradipine, nitrendipine, mibefradil, verapamil, lacidipine, nifedipine, amlodipine and diltiazam
5	TGF-β inhibition	Anti-TGF-β2 IgG4, murine (1D11), recombinant hepatocyte growth factor (HGF), circular antisense TGF-β oligodeoxynucleotides (ODNs) and soluble human TGF-β type II receptor (sTβRII.Fc)
6	ACE inhibition	OPB-9195, ALT-946, ALT-711, aminoguanidine, TM2002 and LR-90
7	PKC inhibition	Ruboxistaurin
8	Renin inhibition	Aliskiren
9	Rho-kinase inhibition	Fasudil
10	Fibrotic inhibition	Tranilast and SMP-534
11	NADPH oxidase inhibition	Apocynin
12	PARP inhibition	INO-1001 and PJ-34
13	ET_A receptor antagonism	Avosentan
14	$ET_{A/B}$ receptor antagonism	CPU-0213
15	Aldose reductase antagonism	Fidarestat
16	Ligands of PPAR-α	Fenofibrate and gemfibrozil
17	Ligands of PPAR-γ	Rosiglitazone and pioglitazone

Chronic treatment with curcumin, the active principle of turmeric (*Curcuma longa*), has been noted to attenuate the renal dysfunction by reducing proteinuria and inhibiting the renal peroxidation in diabetic rats with nephropathy (Sharma et al., 2006). Treatment with gui qi mixture (Haung qi and Dang gui) has been noted to afford renoprotective effect as it reduced albuminuria, serum creatinine and blood urea nitrogen levels by inhibiting the overexpression of Ang-II and TGF-β in diabetic rats with nephropathy (Zhang et al., 2006). Treatment with resveratrol has been noted to attenuate the renal dysfunction by reducing proteinuria, serum creatinine, blood urea nitrogen and inhibiting the renal peroxidation in diabetic rats with nephropathy (Sharma et al., 2006).

Table 5b.2: Potential herbal interventions to have ameliorative effect in the progression of diabetic nephropathy.

S. no.	Herbal interventions	Therapeutic outcome
1	Hashimi-jio-gan	Reduces proteinuria and prevents glomerulosclerosis, tubulointerstitial fibrosis and mesangial matrix expansion by decreasing the AGE formation, sorbitol levels and inhibiting the renal lipid peroxidation in diabetic rats with nephropathy [122].
2	Quercetin	Reduces proteinuria, serum creatinine, blood urea nitrogen and oxidative stress by inhibiting the renal peroxidation in rats with diabetic nephropathy [123].
3	*Ginkgo biloba*	Reduces albuminuria and N-acetyl-beta-D-glucosaminidase in patients with diabetic nephropathy [124].
4	*Salvia miltiorrhiza*	Decreases urinary albumin excretion rate by reducing the renal expression of TGF-β, CTGF, fibronectin and PAI-1 in diabetic rats with nephropathy [125].
5	Curcumin	Reduces proteinuria and oxidative stress by inhibiting the renal peroxidation in rats with diabetic nephropathy [126].
6	Cui Qi mixture (Haung qi and dang gui)	Reduces albuminuria, serum creatinine and blood urea nitrogen levels by inhibiting the overexpression of Ang-II and TGF-β in diabetic rats with nephropathy [127].
7	Resveratrol	Reduces proteinuria, serum creatinine, blood urea nitrogen and oxidative stress by inhibiting the renal peroxidation in rats with diabetic nephropathy [128].
8	Dang gui buxue tang	Reduces albuminuria by inhibiting the overexpression of TGF-β in renal cortex [129].
9	KIOM-79	Reduces proteinuria and attenuates glomerular hypertrophy by inhibiting the overexpression of TGF-β, type IV collagen and VEGF and glomerular AGE formation in experimental diabetic rats with nephropathy [130].
10	Garlic (*Allium sativum*) and ginger (*Zingiber officinale*)	Reduces serum glucose, proteinuria, glomerular hypertrophy, capsular space shrinkage, glomerular and microvascular hyalinization in diabetic rats with nephropathy [131].
11	Berberine (main constituents of Coptidis rhizoma and Cortex phellodendri)	Inhibits fibronectin and collagen synthesis partly via modulating p38MAPK signal pathway in rat glomerular mesangial cells exposed to high glucose [132].
12	Grape seed proanthocyanidin extracts	Prevents extracellular matrix accumulation and occurrence of albuminuria by reducing AGEs and renal expression of RAGE and CTGF in rats with diabetic nephropathy [133].
13	Halofuginone, an analogue of febrifugine	Reduces mesangial expansion, renal oxidative stress and overexpression of the renal fibronectin by downregulating TGF-β expression in db/db mice with nephropathy [134].
14	Green tea (*Camellia sinensis*)	Reduces albuminuria, renal expression of collagen IV and renal oxidative stress by downregulating the expression of NADPH oxidase in Spontaneously hypertensive rats (SHR) with diabetic nephropathy [135].

Source: Balakumar et al., 2009.

Administration of Dang gui buxue tang, a traditional Chinese herbal preparation, has been noted to retard the progression of DN by inhibiting the overexpression of TGF-β in the renal cortex of diabetic rats with nephropathy

(Zhang et al., 2006). Treatment with ethanolic extract of Korean herbal preparation named KIOM-79 has been shown to prevent the induction and progression of DN as it reduces proteinuria and attenuates glomerular hypertrophy by inhibiting the overexpression of TGF-β, type IV collagen and VEGF and glomerular AGE formation in type 2 diabetic goto-kakizaki rats (Kim et al., 2007). Treatment with garlic (*Allium sativum*) and ginger (*Zingiber officinale*) has been shown to attenuate the progression of DN by reducing serum glucose, proteinuria, glomerular hypertrophy, capsular space shrinkage, glomerular and microvascular hyalinization in diabetic rats with nephropathy (Al-Qattan et al., 2008). Berberine, a main constituent of *Coptidis rhizoma* and *Cortex phellodendri*, was noted to possess renoprotective effect by inhibiting fibronectin and collagen synthesis partly via modulating p38MAPK signal pathway in the rat glomerular mesangial cells exposed to high glucose (Liu et al., 2009).

Administration of grape seed proanthocyanidin extract produced renoprotective effect as it prevents extracellular matrix accumulation and occurrence of albuminuria by reducing AGEs and renal expression of RAGE and CTGF in rats with DN (Li et al., 2009). Halofuginone, an analogue of febrifugine has been noted to suppress the progression of DN by reducing mesangial expansion, renal oxidative stress and overexpression of renal fibronectin by down regulating TGF-β expression in db/db mice with nephropathy (Sato et al., 2009). Recently, treatment with green tea (*Camellia sinensis*) was noted to possess renoprotective effect as it reduces albuminuria, renal expression of collagen IV and renal oxidative stress by down regulating the expression of NADPH oxidase in diabetic spontaneously hypertensive rats (Ribaldo et al., 2009). Taken together, above studies suggest that herbal drugs may provide a new therapeutic advancement for treating diabetic patients with nephropathy. One such herbal medicine *Enicostemma littorale* Blume have good antidiabetic activity. Our lab has shown that it is having good hypoglycemic, hypolipidemic and antioxidant activity in diabetic animals as well as newly diagnosed NIDDM patients. In chapter 5a we had demonstrated that antioxidant potential of methanolic extract

of EL could able to prevent drug-induced nephrotoxicity in rats. Thus lead us to evaluate its efficacy in rat model of diabetic nephropathy.

5b.2. Experimental design

5b.2.1 *Extract preparation*

Methanolic extract was prepared according method described by Maroo et al., 2002 (as mentioned in chapter 2 section 2.3).

5b.2.2 *Animals and treatment*

As mentioned in chapter 2 section 2.2

5b.2.2.1 *Surgical procedure for unilateral nephrectomy*

Rats were anesthetized under continuous ether anesthesia and maintained till the end of surgical procedure. Animal were laid down on the back and abdominal cavity was cleaned with antiseptic (Dettol). About 2cm cut was made with surgical blade on left side of abdominal cavity. Left kidney was located and taken out from the abdominal cavity. Renal artery and vein was ligated with suture to prevent blood loss. Kidney was de-capsulated. Internal and external suture were taken. Neosporin (antibiotic) powder was spreaded on the wound. A known analgesic, novalgin and oxytetracyclin antibiotic was given intramuscularly for 4 days to relive animal from pain and infection and monitored for renal function.

5b.2.2.2 *Induction of diabetes*

After 4 days of recovery after surgery, 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. Blood glucose levels were checked on 15th day. Animals with blood glucose >200mg/dL were taken further for the experiment.

Animals were divided into 5 groups, minimum six animals in each:

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

Group-II : Unilateral Nephrectomized (X) treated i.p with saline and i.g with 1% CMC.

Group-III : Unilateral Nephrectomized (XD) + Alloxan treated (i.p) and i.g with 1% CMC.

Group-III : XD + Alloxan treated + EL treated.

Group-IV : XD + Alloxan treated + Glib treated.

Studies showing acceleration of diabetic glomerulopathy in rats following uninephrectomy at 1.5 months to 3 months (Steffes et al., 1978) and with hypertension (Mauer et al., 1978) indicate that haemodynamic perturbations can modulate the rate at which the secondary complications of diabetes become manifest in the kidney.

Normal control, nephrectomised control and diabetic control received 1% CMC 10ml/kg B.W daily for 45 days. Blood was collected on the 45th day, from orbital sinus and blood glucose, Gly Hb, creatinine, platelet aggregation, prothrobin time (PT) and activated partial thromboplastin time (APTT) were analyzed. Weight of animals was recorded on 0th and 45th day of experiment. Animals were placed in metabolic cages for urine collection for 24hrs before the end of the experiment. One set of animals were kept daily in animal restrainer for acclimatization, for blood pressure measurement by tail-cuff method. The animals were sacrificed and weight of the kidney tissue was also recorded thereafter and antioxidant enzymes, reduced glutathione, lipid peroxidation levels were measured from mitochondrial and post-mitochondrial fraction of kidney tissue. Activities of Na^{+}-^{+}K ATPase and polyol pathway enzyme, aldose reductase were also evaluated from the kidney tissue.

The *Enicostemma littorale* 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.

5b.3 Results

5b.3.1. Body weight, Relative organ weight and urinary volume

Body weight decreased in untreated diabetic rats and EL and glib treatment restores body weight towards normal condition (Table 5b.3). Urinary volume increased significantly in the diabetic group (Table 5b.3) as compared to control and nephrectomized group. In EL and Glib treated rats, urine volume were significantly reduced as compared to XD untreated rats bring down almost near to the level of the control rats. Diabetic animals showed significant increase in kidney weight, as compared to XD control animals. Animals treated with methanol extract of EL and standard drug Glib resulted in significant recovery in organ weight (Table 5b.3).

5b.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 60 and 85% respectively, approaching towards normal levels (Fig. 5b.2). Diabetic rats showed 80% increase in glycosylated hemoglobin (GlyHB) levels as compared to normal control rats. EL and Glib treatment reduces GlyHB levels by 52% and 62% respectively (Fig. 5b.3).

5b.3.3. Serum creatinine and urea (BUN)

Serum creatinine levels increased by 147% and blood urea nitrogen levels (BUN) increased by 143% in the XD group compared to the control (C) and X group. EL attenuated the increase in creatinine by 58% and 47% in BUN levels.

Similarly, Glib attenuated the increase in creatinine by 56% and 55% in BUN levels (Fig. 5b.4 & 5b.5).

5b.3.4. Hypolipidemic parameters

Diabetes causes dyslipidemia which was clearly shown by the alloxan-induced diabetic rats with increased 79% serum cholesterol levels, 103% serum triglycerides, 194% LDL cholesterol, 103% VLDL cholesterol and 35% decreased levels of HDL cholesterol. Both extract and standard drug Glib treatment in XD rats for 45th day showed significant amelioration in lipid profile as compared to untreated diabetic rats. EL extract showed a decrease of 58%, 65%, 50% and 65% in serum cholesterol, serum triglycerides, LDL cholesterol and VLDL cholesterol and an increase of 48% in HDL cholesterol levels in treated diabetic rats, whereas the Glib treatment showed a decrease of 54%, 58%, 47% and 58% in serum cholesterol, serum triglycerides, LDL cholesterol and VLDL cholesterol and an increase of 44% in HDL cholesterol levels respectively (Fig. 5b.6 & 5b.7). Lipid lowering effect of EL and Glib was quit comparable in alloxan-induced diabetic dyslipidemia.

5b.3.5. Platelet aggregation and blood clotting time (PT & APTT)

Untreated XD rats showed 23% increase in platelet aggregation time, while 15% and 33% decrease in prothrobin time (PT) and activated partial thromboplastin time (APTT). Treatment with EL to XD rats for 45 days ameliorates platelet hyperaggregability by 69% and increases PT, APTT by 75%, & 41% respectively. XD rats treated with Glib for 45 days also showed 77% reduction in platelet hyperaggregability, 65% in PT and 42% in APTT (Fig. 5b.8 & 5b.9).

5b.3.6. Systolic and diastolic blood pressure

Nephrectomized control rats were having almost same systolic and diastolic B.P as compared to normal rats with both intact kidneys. Untreated XD rats showed increase in systolic as well as diastolic blood pressure by 33% and 17% as

compared to X rats. EL treatment for 45 days brings down systolic and diastolic B.P by 66% and 68% while glib treatment decreases it by 60% and 77%. Both the treatments individually showed comparable effect in reducing systolic and diastolic B.P. Glib treatment to XD rats was little more effective for reducing diastolic B.P as compared to EL treatment (Fig. 5b.10).

5b.3.7. Aldose reductase and Na^+-^+K ATPase activity in kidney tissue

There was significant increase in aldose reductase enzyme activity in kindney of untreated XD rats as compared to control rats. XD rats treated with EL and Glib for 45 days showed decrease in aldose reductase activity by 52% and 65% respectively (Fig. 5b.11). Glib treatment showed slightly more reduction in AR activity as compared to EL treatment. Similarly XD rats showed 60% increase in Na^+-^+K ATPase activity as compared to X rats. Upon EL and Glib treatment for 45 days to XD rats decreases Na^+-^+K ATPase activity by 58% and 57% respectively (Fig. 5b.12).

5b.3.8. LPO, GSH and Antioxidant activity in mitochondrial and post-mitochondrial fraction of kidney

XD untreated group showed 98% and 123% increase in MDA levels of mitochondrial and post-mitochondrial fraction respectively. Thus the content of LPO is higher in post-mitochondrial fraction than in mitochondrial fraction (Fig. 5b.13). The EL treated group showed 51% and 57% decrease in mitochondrial and post-mitochondrial MDA levels respectively. However, the Glib treated group showed 49% and 55% decrease in mitochondrial and post-mitochondrial MDA levels. Decrease in MDA level was almost similar in rats treated with EL and Glib treated rats. The GSH content in the post-mitochondrial fraction was relatively higher as compared to mitochondrial fraction in control group. XD untreated rats showed decrease in GSH, 50% in mitochondrial fraction and 55% in post-mitochondrial fraction (Fig. 5b.14). The EL treated group showed 56% and 53% increase in GSH content of mitochondrial and post-mitochondrial fractions respectively. Similarly, Glib treated group showed 48% and 47% increase in

mitochondrial and post-mitochondrial GSH content, respectively. Treatment with EL and Glib were having similar effect on GSH content of mitochondrial and post-mitochondrial fractions.

SOD activity was higher in post-mitochondrial fraction as compared to mitochondrial fraction in control rats. The activity of SOD decreases 46% in mitochondrial fraction, while decreases 50% in post-mitochondrial fraction (Fig. 5b.15). The EL treated group showed 48% and 56% increase in mitochondrial and post-mitochondrial SOD activity, respectively. Whereas Glib treated group showed 43% and 46% increase in mitochondrial and post-mitochondrial SOD activity. EL treated rats showed almost equivalent efficacy in improving SOD activity both in mitochondrial and post-mitochondrial fraction. Same is the case with standard drug Glib, having same effect in both the fraction (Fig. 5b.15). GPx activity in the mitochondrial fraction was relatively higher than the post-mitochondrial fraction in control rats (Fig. 5b.16). Both mitochondrial and post-mitochondrial GPx activity decreases by 47% in XD untreated rats. The EL treated group showed 45% and 54% increase in mitochondrial and post-mitochondrial GPx activity, respectively. However, the Glib treated group showed 42% and 56% increase in mitochondrial and post-mitochondrial GPx activity. Efficacy of EL was comparable with Glib, on GPx activity of mitochondrial and post-mitochondrial fraction (Fig. 5b.16). Catalase activity decreases 58% in post-mitochondrial fraction in untreated XD group as compared to control group (Fig. 5b.17). The EL treated group showed 67% increase in activity, while the Glib treated group showed 54% increase in CAT activity. EL have slightly better ameliorating effect on catalase activity as compared to Glib treated rats.

Table 5b.3: Effect of EL and Glib treatment on body weight, kidney weight and urine volume in diabetic nephropathic rats.

	Body Weight (gms)	Kidney Weight (gms)	Urine Volume (ml/24hrs)
C	341 ± 11	0.84 ± 0.027	7.200 ± 0.75
X	343 ± 8	1.14 ± 0.043	7.783 ± 0.88
X+D	174 ± 8 ^{aaa}	1.37 ± 0.029 ^{aa}	30.17 ± 1.48 ^{aaa}
X+D+EL	259 ± 9 ^{bbb}	1.26 ± 0.024 ^{ns}	12.70 ± 1.00 ^{bbb}
X+D+G	272 ± 7 ^{bbb}	1.25 ± 0.025 ^{ns}	13.08 ± 1.27 ^{bbb}

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. XD. Kindney Weight : aaa, $p<0.001$ vs. X, Ns vs. XD.

5b.4. Discussion

Chronic hyperglycemia accelerates the activation of the formation of advanced glycation endproduct, oxidative stress, the polyol pathway, and PKC pathway. These metabolic factors are synergistically correlated with one another, and great efforts have been made to identify therapeutic agents from herbal medicines or nutraceuticals to treat diabetes and its complications because of their absence of toxic and/or side effects based on the long history of ethnopharmacological evidence (Cooper, 2001; Yokozawa et al., 2004; Yamabe et al., 2007). In the present work, efficacy of methanolic extract of EL was evaluated against diabetic complication and its efficacy was compared with standard drug glibenclamide in alloxan-induced diabetic rats in this study. Diabetic rats with long-term moderate hyperglycemia, however, do not develop characteristic glomerular lesions of human diabetic nephropathy and, in fact, develop only minimal glomerular injury even after 1 year of diabetes. Although the diabetic rat with moderate hyperglycemia may be useful to study the mechanisms of glomerular hyperfiltration in early diabetes, it may not be an appropriate model of renal failure in IDDM. Therefore, the development of an appropriate animal model which reflects the properties of human diabetic nephropathy seems essential.

Figure 5b.2: Effect of EL treatment on **blood glucose** levels.

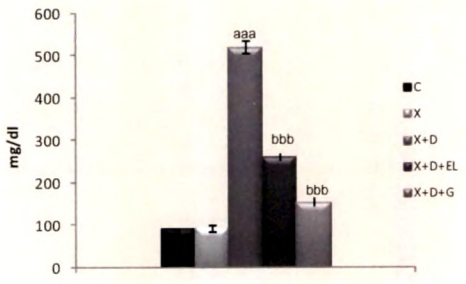


Figure 5b.3: Effect of EL treatment on **glycosylated hemoglobin** levels.

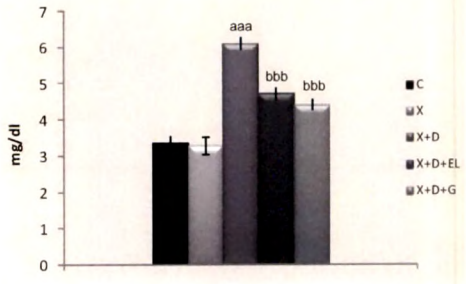


Figure 5b.4: Effect of EL treatment on **Creatinine** levels.

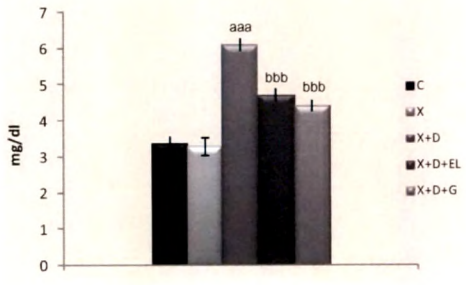


Figure 5b.5: Effect of EL treatment on **Urea** levels.

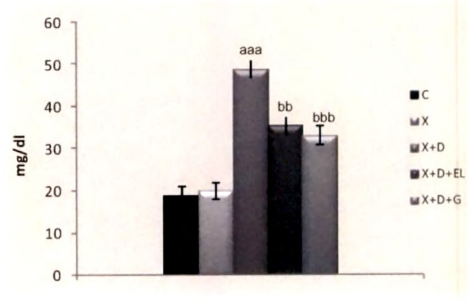


Figure 5b.6: Effect of EL treatment on **TG, TC** and **LDL** levels.

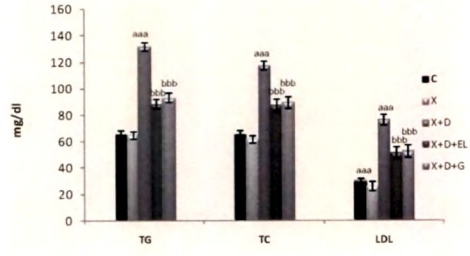
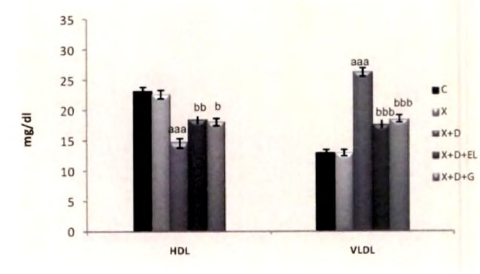


Figure 5b.7: Effect of EL treatment on **HDL** and **VLDL** levels.



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.

Figure 5b.8: Effect of EL treatment on **platelet aggregation**.

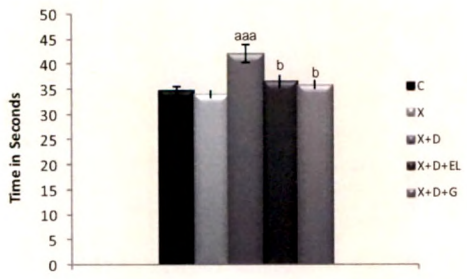


Figure 5b.9: Effect of EL treatment on **PT, APTT.**

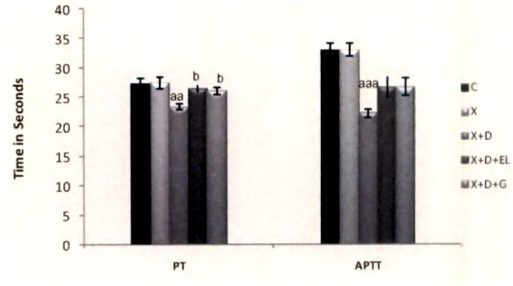


Figure 5b.10: Effect of EL treatment on **SBP** and **DBP**.

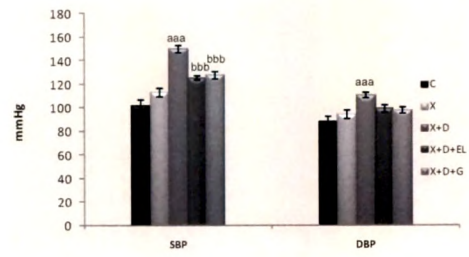


Figure 5b.11: Effect of EL and on **AR** activity.

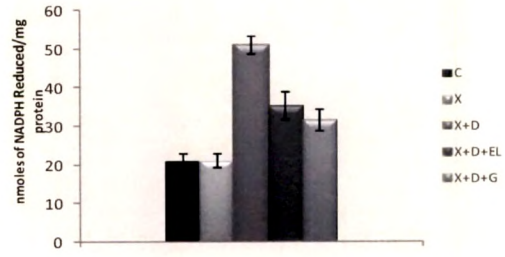


Figure 5b.12: Effect of EL treatment on **Na-K ATPase** activity.

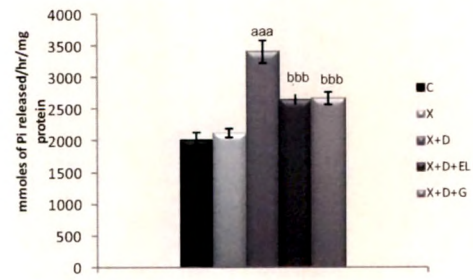
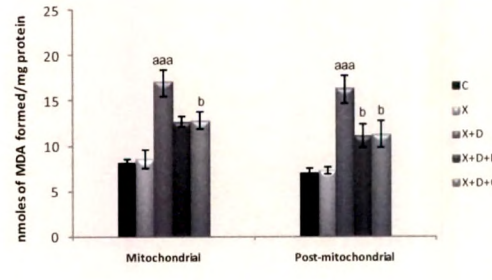


Figure 5b.13: Effect of EL treatment on **lipid peroxidation**.



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.

Figure 5b.14: Effect of EL treatment on **GSH** content.

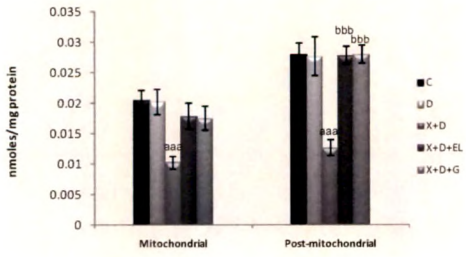


Figure 5b.15: Effect of EL treatment on **SOD** activity.

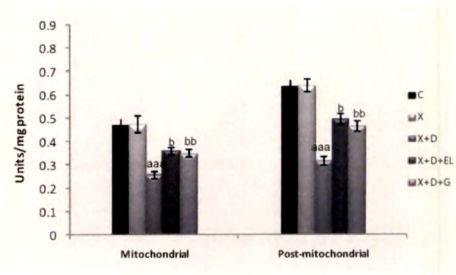


Figure 5b.16: Effect of EL treatment on **GPx** activity.

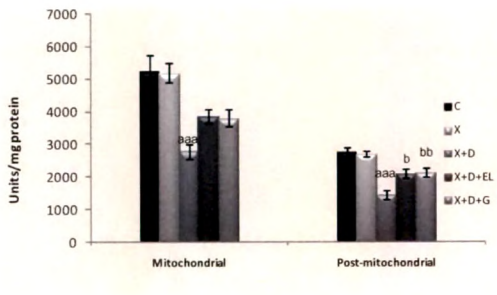
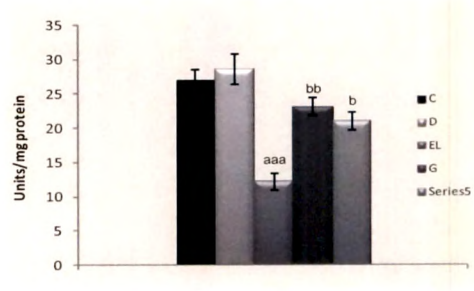


Figure 5b.17: Effect of EL treatment on **catalase** activity.



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. F.

In the present study, biochemical alterations found in nephrectomized diabetic rats which had been injected with alloxan were basically consistent with the clinical features of patients with diabetic nephropathy. Removal of one kidney changes the structure and function of the remaining kidney. Enlargement of both glomerular and tubular components increases the size of the remaining kidney. Changes in glomerular haemodynamics following uninephrectomy in the rat include increased glomerular capillary pressure, elevated transcapillary hydraulic pressure and increased glomerular filtration rate per nephron (Azar et al., 1977; Peters, 1978).

Insulin-dependent diabetes mellitus in man causes increased renal size and elevated glomerular filtration rate (Mogensen, 1976). Diabetic rats also have large kidneys with enlarged glomeruli and tubules (Seyer-Hansen et al., 1980). Further, micropuncture studies performed shortly after the onset of diabetes in rats demonstrate alterations in glomerular haemodynamics closely resembling those which follow uninephrectomy (Hostetter et al., 1981). Studies showing acceleration of diabetic glomerulopathy in rats following uninephrectomy (Steffes et al., 1978) and with hypertension (Mauer et al., 1978) indicate that haemodynamic perturbations can modulate the rate at which the secondary complications of diabetes are manifested in the kidney.

In the present study, we have shown that alloxan-injected nephrectomized (XD) rats demonstrate typical characteristics of diabetes mellitus, such as hyperglycaemia, polyuria, increase glycosylated hemoglobin levels and an increase in urinary volume. With the diabetes mellitus along with unilateralnephrectomy, there is a subsequent increase in serum creatinine and urea levels. It has also been observed that increased blood urea nitrogen and serum creatinine in XD rats indicates progressive renal damage, which is taken as an index of altered GFR in diabetic nephropathy (Yokozawa et al., 2001). Nepherctomized and diabetic nephrectomized rats showed compensatory renal growth following uninephrectomy, which involves hypertrophy of the proximal tubule in rats (Seyer-Hansen et al,. 1980). This renal growth can be prevented by insulin treatment (Mogensen and Andersen., 1973) and is thus due to the metabolic derangement of diabetes.

There was a strong relationship between glucose and glycosylated protein (r=0.982) in diabetic nephropathy. Pathophysiologically, the increased level of non-enzymatic glycosylation may alter the structure-function relationships of the capillary filtration barrier in the glomerulus, as suggested by Brownlee et al. (1984) and Soulis-liparota et al. (1995).

Patients with diabetic nephropathy often have multiple lipoprotein abnormalities (Shoji et al., 2001). In patients with microalbuminuria and overt proteinuria, increased plasma levels of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and triglycerides are usually found. However, the plasma level of high-density lipoprotein (HDL) is lower than those patients with normoalbuminuria. Dyslipidemia may also cause or exacerbate diabetic nephropathy by alterations in the coagulation fibrinolytic system, changes in membrane permeability, damage to endothelial cells, and increased atherosclerosis (Misra et al., 2003).

Increased spontaneous whole blood platelet aggregation has been reported in diabetes mellitus with microvascular complications as nephropathy, retinopathy and neuropathy (Chitre and Velaskar et al., 1988; Cho et al., 1992). We also found increased platelet aggregation upon ADP challenge. Our findings are supported by Cho et al. (1992), who found that the frequency of overt nephropathy, defined as albumin excretion above 200 mg min^{-1}, was more common in the group of patients with the highest quartile of platelet count.

In present study we investigated that long term treatment with EL and glib would ameliorate blood coagulation abnormalities in XD rats. EL and glib treatment lead to increase in PT and APTT activity towards normal value. These results suggest that EL and glib ameliorated the hypercoagulation of diabetic state. Furthermore, EL and glib treatment significantly reduced ADP-induced platelet aggregation.

Diabetic nephrectomized rats showed dsylipidemia, increased platelet count, platelet aggregability and hypercoagulation of blood, which is indicator for the development of micro-vascular complication in these rats, which is responsible factor for development of diabetic nephropathic condition as we have discussed above.

Our results also showed that untreated diabetic animals had a significant increase in systolic and diastolic blood pressure when compared with controls, 6 weeks after induction of DM. Hypertension is frequently seen in Type 1 DM and its pathogenesis is multifactorial. For example, alterations in the renin-angiotensin system, decreased availability of nitric oxide (NO), dyslipidaemia, as well as increased production of ROS may be involved (Haidara et al., 2006).

Considerable interest has focused on the enzyme aldose reductase (AR) as a candidate gene product. AR catalyzes the NADPH-dependent reduction of sugar aldehydes to their corresponding sugar polyols. The polyol pathway is present in lens (Bekhor et al., 1990; Carper et al., 1990), eye (Ludvigson and Sorenson, 1980), nerve (Nishimura et al., 1988), and kidney (Ludvigson and Sorenson, 1980; Kikkawa et al., 1987) and has been implicated in the pathogenesis of cataract formation (Kinoshita, 1965; Robinson et al., 1983), retinopathy (Kinoshita, 1979), neuropathy (Gabbay, 1973), and nephropathy (Bank et al., 1989a; Bank et al., 1989b; Goldfarb et al., 1986). Studies in rats with streptozotocin-induced diabetes have linked the polyol pathway to the morphological and hemodynamic changes characteristic of diabetic nephropathic condition (Kaneko et al., 1990; Tilton et al., 1989). The polyol content of glomeruli is increased 10-fold 6 weeks after the induction of STZ-induced diabetes (Byer-Mears et al., 1984). Administration of AR inhibitors (ARI) decreases the glomerular hyperfiltration and mesangial expansion observed in STZ-induced diabetic rats (Itagaki et al., 1994; Cunningham et al., 1994; Pedersen et al., 1991). Human studies also support a role for AR in the pathogenesis DN (Bank et al., 1989a; Bank et al., 1989b; Goldfarb et al., 1986; Lee et al., 1995; Itagaki et al., 1994; Cunningham et al., 1994; Pedersen et al., 1991; Passariello et al., 1993). The administration of ponalrestat (Pedersen et al., 1991) and tolrestat (Passariello et al., 1993) to IDDM patients decreases the glomerular filtration rate and the urinary protein excretion rate. ARI administration normalizes RBC sorbitol *in vivo* in patients with IDDM (Cunningham et al., 1994).

The above findings have indicated that AR inhibitors can effectively prevent and delay the development and progression of diabetic nephropathy in a diabetic animal model. This suggests that AR activation is involved in the pathogenesis of diabetic complications, including diabetic nephropathy. In present study, we observed that in alloxan-induced diabetic rats with DN, AR activity was increased significantly. EL treatment, significantly decrease/inhibited the increase in AR activity in DN rats. 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). Similarly glib treated DN rats showed decrease in AR activity, which might be responsible for recovery of renal function in DN rats.

Intracellular accumulation of sorbitol interferes with the uptake and metabolism of myo-inositol and decreases the activity of Na^+-K^+-ATPase, leading to dysfunction of renal tubular reabsorption (Dunlop et al., 2000). Insulin and catecholamines are the principal mediators of acute hormonal control of Na^+-K^+-ATPase (Clausen and Everts, 1989). In our study, diabetic rats had decreased level of Na^+-K^+-ATPase in the kidney tissues, which resembles the report by Kjeldsen et al. (1987). This might be associated with the deficiency of insulin as insulin administration partially restored Na^+-K^+-ATPase (Gupta et al., 1996). The oxidative damage of tissue lipids and proteins also causes Na^+-K^+-ATPase inactivation. Na^+-K^+-ATPase is rich in thiol groups and oxidation of thiol groups has been reported to inhibit enzyme activity (Unlucerci et al., 2001). Treatment with EL and glibenclamide restored Na^+-K^+-ATPase. This may be due to insulin secretory effect along with decreasing peroxidative damage to membrane lipids as reported by others (Ramesh and Pugalendi, 2006; 2005a; 2005b) as well as amelioration in kidney AR activity.

The "unifying hypothesis" described by Brownlee (discussed earlier) suggests that the generation of mitochondrial ROS is the primary initiating event in activating a number of other pathways implicated in the development of the complications of diabetes (Brownlee et al., 2005). There remains debate, however, as to whether oxidative stress is an important early link between hyperglycemia and complications, or just a byproduct of various pathogenic mechanisms. Because of the ability of ROS to directly oxidize and damage DNA, protein, and lipids, it is believed that these species play a key direct role in the development of late diabetic complications (Rosen et al., 2001; Nishikawa et al., 2000). In addition to the ability to directly inflict macromolecular damage, ROS function as signaling molecules and can induce a number of stress-sensitive pathways that cause cellular damage.

In diabetic cells, the amount of glucose being oxidized in the Krebs tricarboxylic acid cycle is increased, which pushes more electron donors (NADH and FADH2) into the electrontransport chain, leading to an increase in ROS generation (Brownlee, 2001; Du et al., 2001; Nishikawa et al., 2000; Pamplona et al., 2007). Conversely, in insulin resistance, the increased free fatty acid (FFA) flux from adipocytes into arterial endothelial cells results in increased FFA oxidation by mitochondria (UK Prospective Diabetes Study Group, 1998; Brownlee, 2005). This suggests that excessive FFA oxidation reinforces the mitochondrial overproduction of ROS by high glucose, thereby worsening the oxidative stress.

Electron transfer through Complexes I, III, and IV generates a proton gradient. Much of the energy of this voltage gradient is used to generate ATP during oxidative phosphorylation (OXPHOS). During OXPHOS, a low proportion of molecular oxygen is converted to superoxide and subsequently hydrogen peroxide and the hydroxyl radical, which, under normal conditions, are scavenged by antioxidant enzymes, including mitochondrial MnSOD (SOD2) or glutathione peroxidase (GPx). Damaged or dysfunctional mitochondria,

however, overgenerate superoxide radicals, creating a state of redox imbalance (Pitkanen and Robinson, 1996).

The generation of intracellular AGEs can disturb redox homeostasis by modifying protein and enzyme structure and function. For example, glycation of antioxidants such as copper and zinc-containing SOD (CuZn-SOD or SOD1) contribute to the decline in antioxidant activity (Fujii et al., 1996). Whereas oxidative stress can augment the formation of AGEs through glycoxidation, AGEs can also lead to enhanced formation of free radicals, both directly through catalytic sites in their molecular structure (Yagihashi et al., 1997) and via stimulation of membrane-bound NAD(P)H oxidase through the RAGE receptor (Wautier et al., 2001).

In our study the superoxide overproduction was observed in the context of decreased activity of MnSOD in renal mitochondria from diabetic nephrectomized rats. Mitochondrial GPx activity also decreased in these rats along with decrease in GSH levels where as MDA levels were increased. One of the mechanisms of generation of thiobaarbituric acid reactive substance (TBARS) is AGE-RAGE interaction (Schmidt et al., 1995; Schmidt et al., 1992; Wautier et al., 1994). Indeed, the effect of this interaction was inhibited by antioxidants such as N-acetylcysteine, probucol or vitamin E (Schmidt et al., 1995; Wautier et al., 1994; Wautier et al., 1996).

Furthermore, we specifically highlighted the importance of the cytosolic compartment in facilitating mitochondrial generation of ROS. It is well documented that hyperglycemic condition leads to over production of cytosolic H_2O_2. Upon exposure of H_2O_2 to kidney mitochondria releases cytochrome C and brings about apoptosis only in groups cultured in high glucose environment (Liu et al., 1996). In our study, cytosolic compartment showed decreased SOD, CAT and GPx enzyme activity in diabetic state. Attenuation in anti-oxidative mechanisms could be through glycation of the scavenging enzymes including

SOD and catalase. Increased content of MDA and decreased content of GSH suggests that over-oxidative stress was implicated in impairment of renal function in these XD rats. Drel et al. (2006) has also demonstrated that renal hydrogen peroxide overproduction and lipid peroxide accumulation occur at very early stages of STZ-diabetes.

There are several mechanisms whereby hyperglycaemia may bring about oxidative stress via changes in glutathione metabolism (Bayraktutan, 2002). Excessive glucose oxidation overloading the mitochondrial electron transport chain is thought to be the main source of O^{2-} putting demands upon the glutathione pool. Hyperglycaemia also results in increased flux through the polyol pathway causing NADPH depletion, impaired GR activity and a decrease in the GSH:GSSG ratio. There is increased formation of advanced glycation end-products which are known to bind to RAGE receptors thereby generating ROS and depleting glutathione (Deuther-Conrad et al., 2001; Loske et al., 1998).

The decrease in GSH content might be the reason for decrease in GPx activity in both mitochondrial as well as post-mitochondrial compartment in the kidney tissue of untreated diabetic rats. Glutathione parameters also appear to be deranged in patients with diabetic nephropathy. A recent study on diabetic patients with microalbuminuria, noted the study group to have significantly lower red blood cell GSH and GPx levels than diabetic subjects without microalbuminuria (Ozdemir et al., 2005). Our results also correlate with this study. It is clear from the above that there is a strong theoretical basis for believing that the therapeutic elevation of intracellular glutathione levels would be beneficial in patients with diabetes.

Looking at the non-enzymatic and enzymatic antioxidant parameters in our study in both mitochondrial and post-mitochondrial compartment of kidney tissue, clearly demonstrated that generation of oxidative stress in both the

compartment is equally responsible for development of diabetic nephropathic condition.

The changes of mitochondrial and post-mitochondrial SOD, GPx activity, GSH and MDA content in rats with DN were reversed by EL treatment, indicating that antioxidant effect could be one of the mechanisms by which EL ameliorated the kidney dysfunction in alloxan-induced diabetic rats. Amelioration in oxidative stress upon EL and Glib treatment could be due to its hypoglycemic activity, as hyperglycemic is the main causative factor for oxidative stress generation in addition to its antioxidant activity. It also reduces AR activity and thus brings back the concentration of cytosolic NADPH, which is required to maintain the GSH/GSSG pool. Protection by EL is comparable with standard drug treatment. Protection offered could be due to hypoglycemic, hypolipidemic and antioxidant activity of EL.

5b.5 Summary of the chapter

Drug induced nephrotoxicity is a leading cause of end stage renal failure in humans. Efforts are being made to find suitable drug candidate which can counteract drug induced nephrotoxicity. In present study nephrotoxicity was induced by aminoguanoside class of antibiotic drug gentamicin in rats. Gentamicin administration leads to generation of oxidative stress in kidney tissue and leads to tissue damage. Present study had been conducted to understand the role of oxidative stress generation in mitochondrial as well as post-mitochondrial fraction of kidney tissue and whether antioxidant treatment prevents drug induced kidney failure or not. Our results demonstrated that mitochondria generate more oxidative stress as compared to post-mitochondrial fraction in GM treated rats. Damage to kidney tissue had been confirmed by high plasma creatinine and urea levels as well as histopathological score in GM treated rats. Treatment with EL ameliorates antioxidant defense system of mitochondrial as well as post-mitochondrial fraction but the improvement was more in

mitochondrial fraction as compared to post-mitochondrial fraction which is similarly demonstrated by potent antioxidant Vitamin C. Thus, suggesting that antioxidant like EL with the use of aminoguanoside class of antibiotic drugs; can prevent its side effect on kidney tissue.

Diabetic nephropathy is another cause for end stage renal failure. Prolonged hyperglycemic condition leads metabolic alterations like generation of oxidative stress, AGE formation, and increased polyol pathway activity. These metabolic alterations are responsible for the development of diabetic complications like retinopathy, nephropathy, neuropathy etc..In present study we evaluated the efficacy of EL extract in diabetic nephropathic condition in rats. Alloxan-induced diabetic rat along with nephrectomy (XD) develops nephropathic lesions in short time of 6 weeks, similar to human DN. These rats showed increase serum levels of renal function markers and confirms the establishment of DN condition. Other physiological changes observed in DN rats were dislipidemia, increased platelet count, platelet hyperaggregation, increased blood clotting time, which are responsible for the development of microvascular complications. Dislipidemia and altered platelet function are responsible for the development of atherosclerotic condition leading to elevated blood pressure, which indicates peripheral vascular resistance. These vascular alterations were responsible for the development of DN in XD rats. The present study was undertaken, also to understand the role of mitochondrial and post-mitochondrial oxidative stress in the development of disease condition and also to evaluate the efficacy of antioxidant drug as preventive measure. Another metabolic alteration found in the XD kidney tissues were higher AR activity and lowered Na^+-K^+ ATPase activity. Diabetic nephropathic rats treated with EL extract had improved kidney metabolic alteration like AR activity, Na^+-K^+ ATPase activity. EL treatment not only reduces blood glucose levels but it also corrects dislipidemia, blood clotting paramenters. Thus decreases blood pressure Na^+-K^+ ATPase platelet functionality along with blood clotting parameters. Indirectly EL treatment decreases the development of blood pressure. EL treatment reduces AR activity

by improving glycemic condition as well as by direct inhibition (demonstrated by others). Oxidative stress, in mitochondria as well as in post-mitochondrial fraction was reduced almost equally upon EL and Glib treatment and reduces tissue damage done by oxidative stress. Thus our results conclusively demonstrated that EL extract efficaciously prevents development of diabetic nephropathy; 'a microvascular complication'.

5.6 References

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