Therapeutic potential of green tea (*Camellia sinensis*) extract on renal damage in diabetic nephropathy rat model

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Animals approved: 36 FEMALE ALBINO WISTAR RATS

Remarks if any: - One animal from each group will be sacrificed for the study, 30 animals will be rehabilated, Protocol no 5 and 6 will be shared.

Dr. Monica Gulati	**Mrs. Yogyata Vaidya**	**Mr. Bimlesh Kumar**
Biological Scientist, Chairperson IAEC	Scientist, COD Pharmacology	Scientist In-Charge of Animal House

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List of abbreviations

Abbreviations	
AGEs	Advanced glycation end products
ACE	Angiotensin converting enzyme
ARBs	Angiotensin receptor blockers
BMI	Body mass index
CaMKK	Ca2+/calmodelin-dependent protein kinase
CML	Carboxy methyl lysine
CMP	Cytosine monophosphate
DAG	Di acyl glycerol
DBP	diastolic blood pressure
DM	diabetes mellitus
DN	Diabetic nephropathy
EGCG	Epigalocatechin galate
eNO	endothelial nitric oxide
ESRD	End stage renal disorder
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FBS	Fasting blood sugar
GAPD	Glycer aldehyde 3-phosphate dehydrogenase
GBM	Glumerular basement membrane
GFR	Glumerular filtration rate
Glx	Glyoxalase
HSP	Hexose amine synthesis pathway
IP	Intra peritoneal
IV	Intra venous
MGO	Methyglyoxal

NAD	Nicotin amide adenine dinucleotide
OGTT	Oral glucose tolerance test
PKC	Protein kinase C
RAGE)	Receptors of advanced glycated end products
RAS	Renin angiotensin system
RBS	Random blood sugar
ROS	Reactive oxygetn species
RRT	Renal replacement therapy
SD	Superoxide dismutase
SPB	Systolic blood pressure
STZ	Streptozotocin
TAC	Total anti-oxidant capacity
TCA	Tri carboxylic acid
TGF	transforming growth factor
UDP	Uridine di phosphate

Abstract

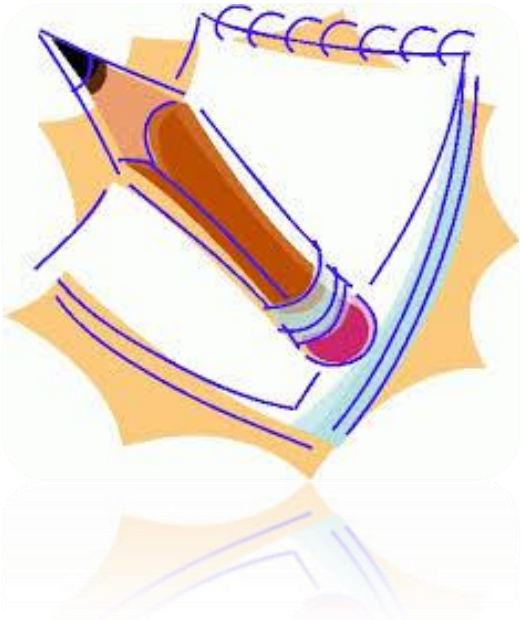
The study was aimed to evaluate the therapeutic potential of green tea extract (GTE) on histoarchitecture of diabetic nephropathy (DN). The Sprague Dawley female rats were selected as experimental models. Diabetes was induced by single IP injection of Streptozotocin 45mg/kgbw which represent type 1 Diabetes. Animal were divided into four groups consisted of six animals each. Animals were fed with normal laboratory rat chow and fresh deionized water (to control group) and 200mg/kgbw /day of green tea extract dissolved in drinking water (instead of water to experimental group). Blood samples were taken on 7th and 14th day of the study and animal were sacrificed on the second sample collection. Serum was tested for all the parameters toward development of DN and for all changes indicating alteration of renal histoarchtecture. In the first panel (parameters toward development of DN)of tests serum glucose, lipid profile, total anti-oxidant capacity while as part of second(indicating abnormality of kidney) serum albumin, non- protein nitrogen compounds, serum electrolyte, urea clearance, urinary salbumin excretion and histological studies of the kidney were performed.

Blood glucose, total cholesterol and triglyceride level of a diabetic control group was increased at a significant level of $p<0.01$, $p<0.05$ and $p<0.05$ respectively, supplementation of GTE reduced the levels significantly ($p<0.05$), TAC was decrease in diabetic animal at significant level ($p<0.01$) and following therapy it improved significantly ($p<0.05$). Serum urea level was significantly increased by diabetes ($p<0.01$) and treatment with GTE decreased the level of diabetic group ($p<0.05$) while did not affect the level in normal control. Serum creatinine level remained unchanged without considering muscle mass, and urea clearance was significantly improved in the experimental group. Level of sodium was dropped because of hyperglycemia and supplementation of GTE was proved to improve the situation at a significant level ($p<0.01$). Serum potassium level was increased by DM but till the end of study its level remained as such in the experimental group. Significant polyuria was present in both experimental and diabetic groups. Prompt albuminurea was seen in diabetic control group and the level was significantly less in experimental group.

H&E stain of renal tissue did not show any change in 14 days study but all factors of developing DN were increased in the diabetic control group while a significant improvement was seen following supplementation of GTE, indicating the strong therapeutic and preventive potential of GTE in diabetic nephropathy.

CHAPTER 1

INTRODUCTION



Introduction

By gaining advances in human life day by day the life of human being has become easy and comfortable but side by side with developments in science, technology and sanitary life style, the health problems especially chronic degenerative and metabolic disease is also getting increased.

Diabetes mellitus is one of such problems with multiple etiologies and serious life threatening co morbid complications. The most serious complication of diabetes mellitus is diabetic nephropathy, the main cause of end stage renal disorder (ESRD) and RRT. Thou there are multiple hypoglycemic medicine and also commercial forms of insulin are available but still $1/3^{rd}$ of DM type 1 and $1/6^{th}$ of DM type 2 develops diabetic nephropathy, account for about $1/3^{rd}$ of total ESRD most of which undergo renal replacement therapy. That's why medical science is trying to develop new hypoglycemic agents to use them as medicine or to include them in the daily diet and consequently the control of glycaemia and prevention of diabetic nephropathy will be done more efficiently. Green tea is part of such agents that have attracted the interest of scientists for its anti-oxidant, anti-inflammation and anti-hyperglycemic properties. There are lots of works have been done on green tea extract and diabetes but detailed study related to renal histology and histochemical changes in diabetic rat using GTE is still in infancy. Therefore, it has been decided to work on streptozotocine induced diabetic rat models to evaluate the therapeutic potential of green tea extracts on renal damage of diabetic nephropathy.

1.1Diabetic Nephropathy: Characteristics and etiology

The range of influence of metabolic disorder and other chronic degenerative disease is being wider day by day and the age of incidence and diagnosis of the disease is rapidly decreased [1]. Metabolic syndrome is characterized by a group of symptoms indicating an abnormality such as obesity, hypertension, dyslipidemia, glucose intolerance and insulin resistance. This syndrome is also considered as "diabesity ″ and is a call for the incidence of DM [2].

Metabolic disorders are interred connected with each other. Obesity is recognized as a major cause of hypertension and essential hypertension is usually followed by insulin resistance [3]. Insulin resistance as well as with high risk of hypertension occurs at early stages of type 2 and later on in type 1 DM. Furthermore, the risk of development of type 1 DM is greater in patients with uncontrolled blood pressure as compare to the controlled blood pressure [4].

Though there are multiple antihypertensive agents available but still maintaining of blood pressure at normal range is difficult especially in the case of hypertension associated with DM [5].

Patient with hypertension, DM or both are at high risk of developing a wide range of chronic complications of inclusive nephropathy and cardiovascular disease [6, 7]. More ever different modifiable risk factors like obesity, blood glucose and lipid level, blood pressure and smoking are known for their association with bad image of poor renal and cardiovascular complications [8] . It also should be known that association of DM and hypertension worsens the situation of enhancing their secondary complication and thus puts salt to the wound of social dysfunction and premature death [9].

DM is a major health problem characterized by hyperglycemia with main etiology of insufficient insulin secretion, insulin action or both. About 6.6% of the world population suffers from this disease and it is estimated that the number will be increased up to 7.8% of global population in the next 20 years. As DM is known as an important risk factor for the development of chronic complications in vital and target organs for example cardiovascular disease, nephropathy, retinopathy and neuropathy, different research groups are working on comprehension of these mechanisms and also on treatment and prevention of these complications [10].

Diabetic nephropathy is the most serious secondary complication of DM with high rate of mortality and is the main cause of ESRD in adults [11]. About 30% of diabetic patient (either type 1 or type 2) develops diabetic nephropathy [12] and in the case of poor

control of blood pressure and urinary albumin excretion, they lead to ESRD about 80% of which have hypertension which contributes in worsening of renal disease [13].

In DM type 2 hypertension is the part of syndrome characterized by insulin resistance, hyperuremia, dyslipidemia, obesity and atherosclerosis [14] though the exact cause and explanation of condition is still unknown but according to many researchers. Insulin resistance plays important role in the development of syndrome [15], but in the case of DM type 1 hypertension develops secondary to the occurrence of DN [16]. The link between glycemia level and development of hypertension has been proved through landmark follow up by DCCT (Diabetes Control and Complication Trail), according to that on 8^{th} year of follow up there was 10% decline in development of hypertension in intensified insulin regimen treatment group as compare to the conventionally treated group. Likewise there were beneficial effects on renal consequences of first group [5].

1.2 **Phases of development of DN**

DN develops in 5 following characteristic phases.

1. Plasma flow of kidneys increases along with an increase in GFR, hypertrophy of kidney and also renal hyper-filtration occurs.
2. Renal parenchymal changes and normo-albuminuria, mesangial expansion and thickening of basement membrane occur.
3. Early hypertension and micro-albuminuria
4. Observable albuminuria
5. End stage renal disorder [17]

All of the above disorder contributes in generating of cell injury and therefore apoptosis of podocytes, extracellular proteins accumulate in tubular interstitial and in glomerular region [18].

1.3Mechanism of development of DN

Persistent hyperglycemia has a strong relationship with development of DN. various mechanisms of involvement of hyperglycemia in the development of DN are proposed.

1. Activation of oxidative stress by high concentration of glucose and production of ROS
2. Production AGEs (advanced glycated end products)
3. Activation of PKC (protein kinase C), proinflimmatory transcription factor NF-κβ, transforming growth factor (TGF) and RAS (renin angiotensin system) [19].

1.3.1 ROS

Mechanism of production in DM and their role in development of DN: Free radical production due to hyperglycemia and its important role in induction of cellular oxidative damage has been known [20]. Its role in production of diabetes associated macro vascular complication is also approved. In constant high glucose concentration, there is overproduction of mitochondrial superoxide which is blame to be responsible for the hyperglycemia induced apoptosis [21]. More interestingly the production of oxidative stress and also role of cellular apoptosis was high in intermittent high glucose level as compare to constant glucose [22]. Piconi L *et al.* (2006) by observing enhancement of apoptosis of cells by adding of 8 OHdG and nitrotyrosine and reversed in apoptosis due to SOD and MnTBAP the SOD mimetic and mitochondrial electron transporter complex II inhibitors, revealed that the major oxidative stress due to high glucose level is mitochondrial based [23].

All pathways responsible for production of secondary complication of DM like AGEs formation, RAGE ligand binding, specific inhibitors of aldose reductase activity, activation of protein kinase C and hexose amine flux are correlated to the production of high level of superoxide induction in mitochondrial electron transport chain due to hyperglycemia [24].Superoxide then can be converted into different other free radicals which may be more reactive and causes cellular damage by various mechanisms [25].In ETC transfer of electrons take place through complex I, II and IV and expel protons to intermembrane space thus the proton gradient generates which activates ATP synthase or complex V to bring back protons to matrix via inner membrane. In the case of higher concentration of glucose or in diabetic cells there is high level of pyruvate generated from glucose and oxidized in TCA cycle providing more NADH and $FADH_2$ as electron donors into the electron transport chain, increasing the voltage gradient of mitochondrial

membrane till reaching of critical threshold. This is the point where complex III is blocked and become unable to transfer electrons. Electrons go back to coenzyme Q which can donate just one electron to molecular oxygen therefore it generates superoxide as shown in Figure (1). Mitochondrial superoxide oxide dismutase catalyzes this superoxide and yields H_2O_2 which then be converted to water and O_2 by other enzymes. The *ex-vivo* studies of arterial endothelial cells showed that hyperglycemic conditions increase the electron gradient up to the threshold level and thus increase the production of ROS, which then can produce dynamic changes in mitochondrial morphology. The fluctuation in ROS was prevented by inhibition of mitochondrial fission [26]. As previously described hyperglycemia induce oxidative stress occurs in mitochondria and the pathway that are activated by SOD and are responsible for hyperglycemia induced cellular damage are NADPH oxidases, uncoupled eNOS and redox changes [27].

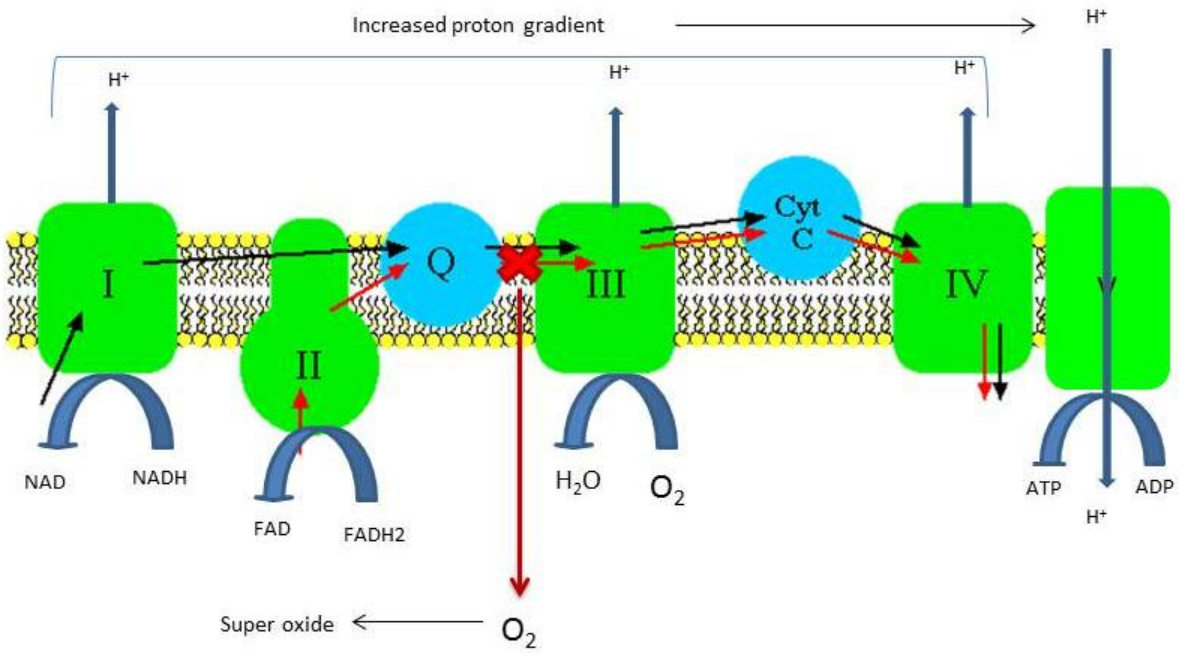


Figure 1: Production of superoxide in ETC

Mitochondrial superoxide activates hyperglycemic induced damaging pathways. In diabetic patients, diabetic animal models and hyperglycemic cells the decrease is seen in the activity of GAPDH. Mitochondrial superoxide is considered as a main reason [28]

and the condition leads to increase the level of upstream glycolytic intermediates. As methylglyoxal is generated non-enzymatically from glyceraldehyde 3P, so it activates AGEs pathway, increases the expression of RAGE and also activates the ligand S100 calgranuline and HMG B1 [29]

1- High level of glyceraldehyde 3P also activates PKC pathway because glyceraldehyde 3P produces DAG which is the physiologic activator of PK-C pathway
2- Blockage of GAPDH further increases the level of fructose 6P which then undergo in hexose amine pathway and produces UDP- N acetyl glucose amine by the help of GFAT.
3- At last inhibition of GAPD causes increased concentration of glucose inside the cell which then is consumed through polyol pathway by using NADPH as reducing equivalent.

1.3.2 Advanced glycated end products:

Advanced glycated end products are the products of non-enzymatic reaction of reducing sugars and amino group of proteins, lipids and nucleic acids. AGE produces via a series of reactions in which Amadori products and Schiff bases produces prior to AGE [19].This reaction was first described in early 1900 when the development of brown color was observed by heating of amino acids with reducing sugars, the reaction was known as Mailard reaction [30, 21].The production of advanced glycation end products require couple of weeks therefore glycation affects long-lived proteins for example structural components of basement membrane or connective tissue matrix in which collagen is the most affecting protein but myaline, complent C3, fibrinogen , tubulin and plasminogen activator factor could also be affected [31, 32]. Exception is uremia where even short lived compounds like nucleic acids and lipids are also affected.

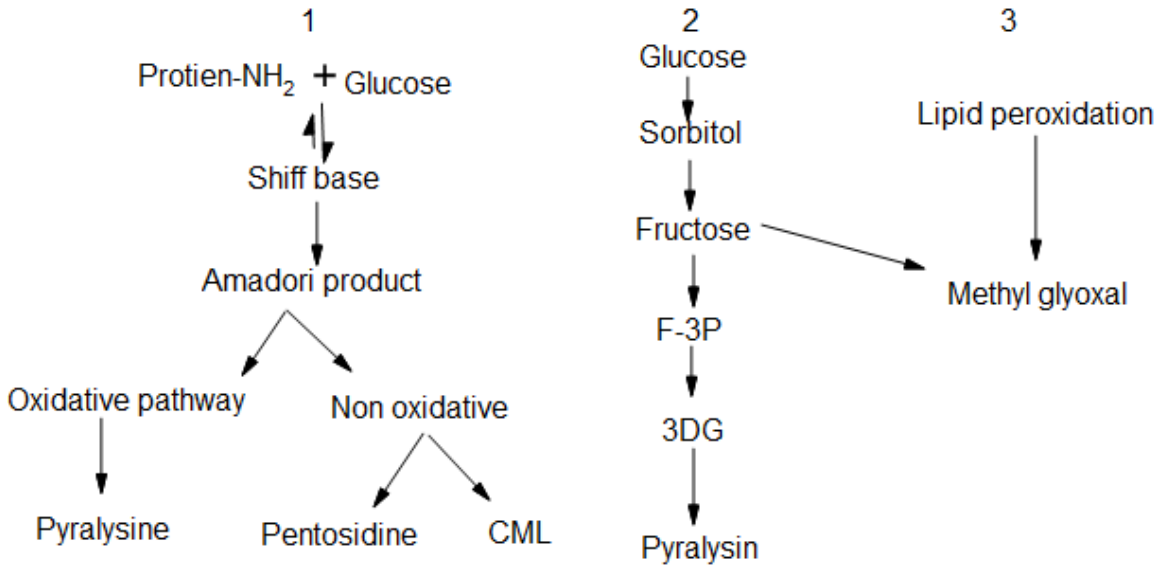


Figure 2: formation of AGE from glucose and incorporated polyol pathway

(1, 2, 3 indicates 3 major pathways of producing AGEs)

In early stages of Millard reaction the rate of reaction is concentration dependent therefore the rate is improved in DM [33, 34]. The process of glycation by glucose is slower but the rate is faster in the case of Glucose-6-P and fructose [35] the rate is accelerated by the presence of transitional metals while it is inhibited by reducing agents such as vit C [36] and green tea [37] Glycoxidation is another phenomena which is used when glycation occurs along with oxidation, pentosidine and N^{ε}-[Carboxy methyl lysine] (CML) are examples of such reaction. In Millard reaction the production of dicarbonyls or oxoaldehydes is of clinical importance. These compounds produce during Amadori rearrangement as reaction intermediates. Methyglyoxal (MGO) and 3-deoxyglucose (3DG) are good examples of such intermediates [38].

Methyl glyoxal with a strong electrophilic nature is considered as a toxic compound in high concentrations, and can cause cell death [39]. It is constituted by non-oxidative mechanisms, non-enzymatically in an aerobic glycolysis, it is also produced from poly unsaturated fatty acids [40] methylglyoxal also produce during the fragmentation of triose phosphate, catabolism of threonine and ketone bodies [41]. It is related to the dihydroxy aceton phosphate an intermediate of glycolytic sequence. After detoxification

it is converted to the lactate (figure) [42]. Methylglyoxal is eletctrophyl in nature so it results with neucleophilic centers of macromolicule for example DNA, RNA and proteins. Furthermore, it reacts with the side chain of amino acids lysine, cysteine and arginine; it also binds with guanine base and to a lesser extends with adenine and cytosine [43]. It is suggested that the cytotoxic characteristic of methylglyoxal is due to its inhibitory action of DNA replication [44]. Mutagenic characteristic of methylglyoxal was proposed by Marnet et al.; when he observed mutagenic changes in *Salmonella typhimurium* cells by converting arabinose sensitive strains to arabinose resistant strains [45].

1.3.2.1 Production of methylglyoxal

Methylglyoxal can be produced enzymatically and also non-enzymatically. The latter route is toxicologically more important.

1.3.2.2 **Enzymatic production of methylglyoxal:**

There are three kinds of enzymes that are responsible for methylglyoxal production

1. Methylglyoxal synthase: Enzyme which participates in glycolytic bypass
2. Cytochrome P450IIE1 isozymes participates in acetone metabolism
3. Amino acid oxidase: responsible for amino acid breakdown [46]

Methylglyoxal synthase is found in prokaryotes and also in mammalian [47] the enzyme was first purified from E-coli [48] and its main and important characteristic is its inhibition by inorganic phosphate (Pi). As per its dependence, on intracellular phosphate its role in controlling of glycolysis has been become obvious [49].

Production of methylglyoxal from acetone needs cytochrome P450IIE1 subfamily. Acetone first converts to acetol and uses NADPH+H and then it turns into methylglyoxal [50].

Third enzymatic production of methylglyoxal is from amino acetone which is the product of threonine and glycine metabolism [46]. Amino acetone oxidase is the enzyme of amino acid oxidase superfamily, needs cofactor having carbonyl and is inhibited by semicarbazide, is responsible for conversion of aminoacetone to methylglyoxal.

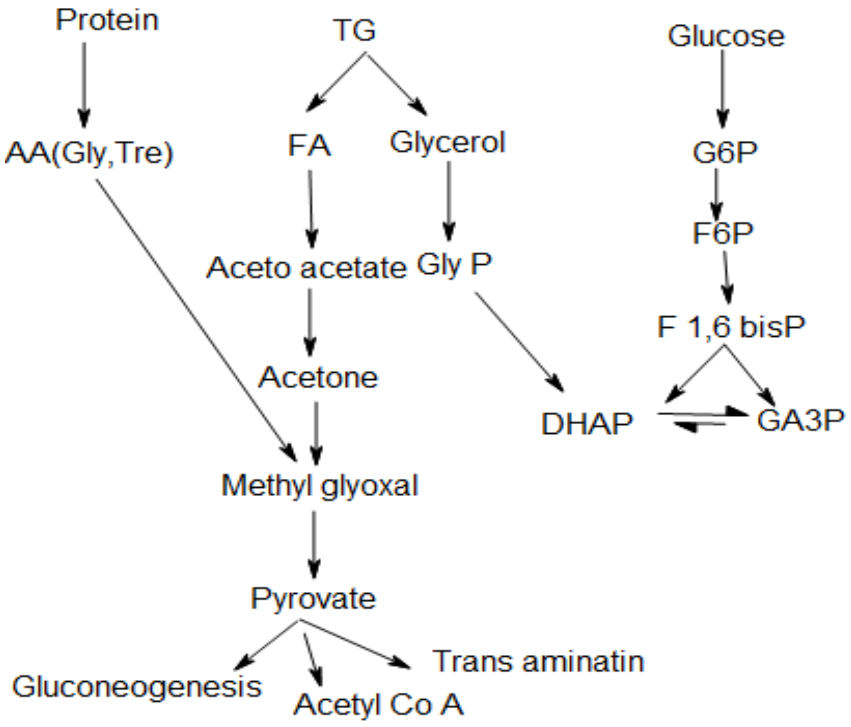


Figure 3: metabolic pathways of methyl glyoxal metabolism

1.3.2.3 Methylglyoxal detoxification and protective mechanisms:

Detoxification of methylglyoxal is taken place by Glyoxalase II (Glx I/Glx II) which is glutathione dependent and is kinetically the most important one [39].In this system, there are two enzymes, Glyoxalase I which is a metaloprotein and acts upon the product of glutathione and methylglyoxal and catalysis the isomerization reaction of hemimercaptal into SD lacto glutathione [46]. The latter is then split into GSH and D- methylglyoxal lactate by glyoxalase II which is not a metaloenzyme [51]. α-oxoaldehyde dehydrogenase is another enzyme responsible for the degradation of methylglyoxal [52] both NAD+ and NADP+ can be used as coenzymes of this enzyme [46]. Bacterial cells apart from this enzyme have intrinsic capability to withstand toxicity of by their capacity of DNA repair mechanism and also their membrane permeability [39].

1.3.2.4 Non-enzymatic methyl glyoxal production

Non-enzymatic dismutation of triose and triose phosphate into methyl glyoxal:

The idea of formation of methylglyoxal from glyceraldehyde and dihydroxy acetone, without the involvement of any enzyme was generated at the middle of 19s but it was under controversies till more than 30 years, at last in 1990 the idea was proven and the exact mechanism was suggested as: first substrate is deproteinized and endothelial phosphate is generated then the phosphate group is released and enol form of methyl glyoxal is generated [53].

Formation of methyl glyoxal from acetoacetate: acetoacetate is converted into methyl glyoxal in the presence of molecular oxygen and myoglobin, presence of free radicals and manganese, are important in the synthesis process. Cytochrome C and hemoglobin are also considered as catalysts, but their catalytic power is less than myoglobin [46].

1.3.2.5 **3-Deoxyglucasone:**

3-Deoxyglucasone is another important advanced glycated end product, presence and also involvement of which is reported in diabetic microangiopathies such as retinopathy, nephropathy and neuropathy [54] .

3-Deoxyglucasone forms of two distinct pathways.

1- Polyol pathway: 3-deoxy glucasone is generated after the hydrolysis of fructose 3-phosphate.
2- Maillard reactions: In this pathway first the enzymatic glycation of amino group of proteins is occurred and Amadori products are yielded and there after a multiple dehydration reactions and subsequent rearrangements, the 3-Deoxyglucasone and other highly reactive carbonyl compounds are produced. 3DG is able to react with free amino group and AGEs such as pyrraline and CML is generated [55].

By controlling the level of blood glucose with anti-hyperglycemic agents the reduction was seen in 3DG level, the level of CML was also decreased but the level of pyrralysin was not affected. Which indicate more involvement of 3DG in the production of CML rather than in pyrraline [56].

Apart from the potential of 3-DG in producing of advanced glycation end products, it has some unique biological activities [57] it is accepted that the level of plasma 3DG increases in DM and also it is consider that plasma 3DG plays role in producing the complication of DM [58]. Moreover it has been revealed that 3-DG is associated with the pathology and bioactivities of senile disease [59].

As the role of MG and other AGEs in the induction of insulin resistance is proved so the studies are going on to reveal the interrelation of 3DG in DM, aging and senile disease [60]. Although its role in the inhibition of glucose uptake by the liver due to decreased activity of glucose 6-P dehydrogenase and hexokinase by 3DG is reported [61] and also its effect on producing glucose intolerance and insulin resistance has also been proved in healthy mice [62]

We observed that methylglyoxal, 3 deoxyglucasone and glyoxal could be produced in all stages of glycation (figure 1) [63] in the early stages by degradation of Schiff base or glucose and in intermediate stages from Amadori products like fructoseamine. So α-oxoaldehyde is considered as important focal points of formation of AGE fron gloucose in classical Millard reaction, in polyol pathway and also by *in- vivo* factors such as lipid peroxidation and catabolism of ketone bodies and threonine [19].

Accumulation of carbonyl precursor of glyoxidation product such as pentosidine and CML or dicarbonyl precursor (precursors of methylglyoxal and deoxyglucasone) and lipoxidation products are known as carbonyl stress [64] . The condition of carbonyl stress is observed in DM and uremia and is considered as an important factor of producing and accelerating vascular damage in both conditions [65].

1.3.2.6 **Glycated hemoglobin:**

Glycated hemoglobin is an indicator of glycemia level from 6 weeks to 12 weeks. AGE are deemed to produce over a long period of time so it is not AGEs instead it is Amadori product [66].

1.3.2.7 **Cross linking induced by AGE**

Though the AGEs are differ chemically from each other but they have some consequences of covalent cross linking of proteins. Usually stable and long lived proteins such as collagen undergo cross linking process. The main mechanism of cross linking is not fully understood but according to researchers the lysine residue of the protein is likely to be involved in [67] The mechanism of physiological cross linking of proteins such as collagen in which enzyme lysyl oxidase is involved, is well understood. But there is not any evidence to prove such mechanism in accelerated cross linking situation like in DM [67].Pathological crosslinking of proteins causes stiffness of proteins as well the process of removal by proteolytic mechanism becomes slower and thus the tissue remodeling process is also affected. All of these changes occur in aging and DM accelerates their rate [68]. The evidence of these process are delivered by the observation of crosslinks between AGE pyraline and pentosidine by immunostaining in immunohistological studies of diabetic nephropathy and also by the correlation between accumulation of AGE and stiffness of aorta in the postmortem reports of humans [69].

Cross linking leads to atherosclerosis, thickening of basement membrane of capillaries and sclerosis of renal glomeruli. The mechanism of developing atherosclerosis is not only by cross linking but they also trap lipoproteins and thus hampers the efflux of cholesterol from vessel walls and causes macro-vascular disease [19].

1.3.2.8 **Interaction of AGE with their receptors:**

Many receptors of AGE are known which include oligosaccharyl transferase 48(AGE-R1), Macrophage scavenger receptor type I and II, 80 K-H phosphoprotein (AGE-R2), receptor for AGE (RAGE) and galectin (AGE-R3) [70]. The receptors are present in different cells such as microglia, macrophages, podocytes, endothelial cells, monocytes, astrocytes and smooth muscle cells [71]. In DM, the expression of some receptors increased for example expression of RAGE is increased in endothelial cells of kidney. Galactine is increased in kidney of diabetic patients. The RAGE receptor which is present in endothelial cells is the well characterized, the member of immunoglobulin superfamily, multiligand in nature and plays the role of scavenger and also as a mediator

of cellular signaling [72].According to in-vitro observation it is revealed out that when AGE-RAGE complex is produced on macrophage and microglia, it creates oxidative stress and causes activation of a free radical sensitive transcription factor NF-χB then modulates transcription factor for VCAM-1, thrombodeline, tissue factor and indotheline [19].

As the non-enzymatic glycation in non-diabetic individual also occur but the harmful complication are mostly seen in the case of DM because for producing of complication the rate of accumulation of AGE is more important than the concentration of AGE, this was proved by comparative study of young diabetic patient with micro-vascular complication who had less concentration of AGE accumulation with older age group having more AGE accumulation but without any complication [72].

1.3.3 **Activation of polyol pathway:**

The first enzyme in this pathway is aldose reductase that is present in cytosol and is in monomeric form. It catalyzes the NADPH dependent reduction of glucose and other carbonyl compounds [73]. Affinity of aldose reductase is low (high km value). So in normal glucose concentration, very small level of glucose is catalyzed by this pathway but in diabetes the intracellular glucose concentration increases which undergo catalyzes by polyol pathway in higher rate concomitant with depletion of NADPH reservoirs. In the second step of polyol pathway fructose is produced from the oxidation of sorbitol and NADH is used as coenzyme in this reaction [74].

Different mechanisms are proposed to explain the harmful effects of hyperglycemic induced flux of polyol pathway include the hyper osmolarity induced by sorbitol, decrease in cellular NADPH level, decreased activity of Na^+/K^+ ATPase and an increase in NADH/NAD^+ ratio. As the diffusion of sorbitol across the cell membrane is not easy so it can cause osmotic damage to micro-vascular cells. Although concentration of sorbitol in diabetic cells is too low to cause damage, the other suggested mechanism is a decrease in activity of Na^+/K^+ ATPase which occurs as a result of Protein Kinase C (PKC) activation. As oxidation of sorbitol increases the ratio of NADH/NAD^+ which further causes inactivation of GADP and thus production of AGEs including DAG take

place and it is previously described the latter is a potent activator of PKc. Though hyperglycemia causes increase in NADH/NAD^+ ratio, but the absolute concentration of NAD+ is decreased because of its utilization in the synthesis of PARP (poly ADP ribose polymerase) that is activated by ROS [75].

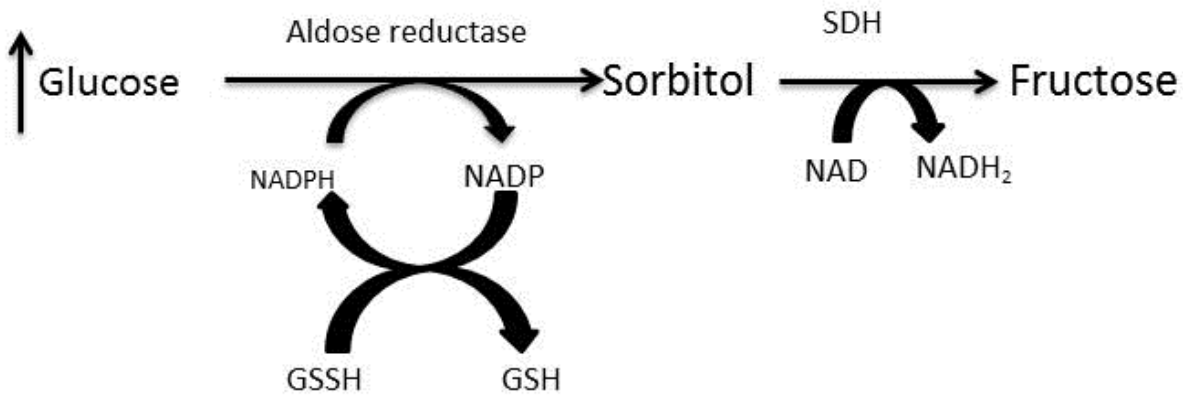


Figure 4: Production of ROS

The most deleterious mechanism is the consumption of $NADPH^+$ for the formation of sorbitol. That causes oxidative stress and hampers the process of reduction of oxidized glutathione which is ADP dependent [75].

1.3.4 **Activation of protein kinase C:**

Protein kinase C subfamily consists of eleven isoforms. Nine of which are activated by DAG. Studies in cultured micro-vascular cells and retinal and renal cells of diabetic mice have demonstrated that the high level of glucose in the intracellular environment causes production of DAG at a higher rate. This mechanism was reviled by *denovo* production of DAG from reaction of dihydroxy acetone phosphate to Glycerol-3 P and its subsequent acylation [76].

DAG was seen to activate β and δ isoforms of PKC *ex-vivo* in vascular cultured cells and *in-vivo* in retinal and glomerular cells of diabetic mice. Other isoforms were also activated for example α and β isoforms were reported in glomerular cells and ε and β

isoforms were activated in retinal cells [76, 77]. Protein kinase C can also indirectly be activated by hyperglycemia induced ROS through activation of polyol pathway and ligation of AGE receptor [75].

In the early stages of experimental diabetes the renal and retinal blood flow abnormalities were mediated by PKC-β isoforms may be due to stimulating the activity of endotheline - 1 or depressing of nitric oxide. The decrease in nitric oxide synthesis in diabetic animal models is present in glomerular cells. Furthermore, PKC inhibit mRNA for the synthesis of endothelial nitric oxide (eNO) synthesis. And in glomerular cells of hyperglycemia induced PKC also increase endotheline-1 stimulated MAP kinase activity [78].

Apart from induction of abnormalities in blood flow and its permeability, PKC contributes to expression of TGF β1, type IV collagen and fibronectin in both glomeruli of diabetic rats and cultured mesangial cells [79]. Furthermore, PKC is also responsible for fibrinolytic inhibitor PAI-1, activation of NF-κB in the regulation of membrane associated NAD(P)H dependent oxidases [80].

1.3.5 **Activation of Hexose amine pathway and its effects**

Activation of hexose amine pathway can also be contributed to the production of diabetic complications [81] and induction of diabetic nephropathy. In this pathway fructose 6-P undergo catalysis by GFAT to provide Glucose amine 6-P a substrate for the synthesis of UDP-Glc NAc which is then utilized for the formation and synthesis of proteoglycan and O-linked glycoproteins.

HSP is considered as a part of the glycolytic pathway, normally about 3% of glucose is utilized via this pathway [82]. First step in HSP pathway is rate limiting and is catalyzed by a Glutamine: Fructose-6Phosphate amidotransferase, in result the fructose-6P and glutamine are converted to Glucose amine 6P and glutamate. Subsequently GlcN-6P is metabolized to CMP-syalic acid, N-acetyl galactose amine (UDP-GalNAC) and N-acetyl glucose amine, glycolipids, essential building blocks of the glycosyl side chains of glycoproteins, gangliosides and proteoglycans. Among these metabolites, UDP-GlcNAc has attracted more interest because:

1. Its quantity is higher as compare to other metabolites of HSP
2. It regulates the entry of glucose into HSP by a feedback mechanism by binding to GFAT allosterically.
3. It plays a role of obligatory substrate for muscular and cytosolic enzyme O-Glc NAC transferase, an enzyme responsible for post translational modification of proteins by transferring of N-acetyl gluseamine to O-linkage of serine or threonine residue of specific proteins [83]

GlcNAc modification has a regulatory function and usually they are found adjacent to phosphorylation site [84]. This type of acylation have functional significance for different proteins including transcription factors c-myc, Sp1, CMP responsive element binding protein, pancreatic duodenal home box-1, enzymes of cytosol and nucleus, RNA polymerase II and glycogen synthase, IRS 1 & 2 and Glu 4 [85].

Studies have shown that HSP can cause insulin resistance and glucose amine that too enters HSP after the catalyzation by GFAT also causes insulin resistance but in lower concentration [86].The role of HSP in the development and pathogenesis of renal and vascular complication in diabetic patients is proved by remarkable evidences. As in diabetic nephropathy the initial stage is the accumulation of extracellular matrix in glomerular region which is promoted by persistent hyperglycemia in diabetic experimental models and diabetic patients [85]. McClain et al suggested that in hyperglycemic conditions HSP affects vascular smooth muscles genes in smooth muscle cells [87]. After that it becomes clear that for the effect of high glucose concentration, synthesis of transforming growth factor β is compulsory [88].

Recently the mechanism of stimulation of TGF-β1 is suggested as, the sequence of promoter region of TGF-β1 homologs the glucose response elements in the gene of proteins contribute in glucose metabolism and regulated by glucose for example pyruvate kinase. GREs than by binding with stimulatory factors USF-1 and 2 enhance the expression of TFG-β1. In hyperglycemic state over expression of TGF-β1 take place which then stimulate the expression of USF-1 and 2, thus upregulate TGF-β1's promoter activity [89]. Apart from GRE there are two other protein binding sites in promoter

region which are activated by MAP kinase and PKC, that are also dependent to a high glucose concentration [89].

1.4. Histopathological changes in DN

In DN extracellular matrix and its normal components like collagen (type IV and VI), fibronectin and laminin accumulate in higher quantity that leads to thickening of GBM (the first sign of DN occur after 1.5-2.5 year of onset of DM) [90], thickening of tubular basement membrane and mesangial expansion. The latter is usually seen in all type 1DM patients with renal insufficiency which is also called diabetic glumerulosclerosis [91].

Diabetic glumerulosclerosis is characterized by nodular lesions that consist of mesangial expansion that makes fibrilar mesangial zones, where the mesangial nuclei accumulates around nodules and compress the glomerular capillaries. Increased collagen contents in fibrilar regions are seen in the patients when GFR decrease [92].

After few years of onset of DM, hyalinosis occur in afferent and efferent arterioles. In these situations exudate lesions composed of complement, fibrinogen, immunoglobulins, albumin and other plasma proteins replaces endothelial smooth muscles. These types of vascular lesions further contribute in development and worsening of the glumerulosclerosis. Such lesions can also occur in sub endothelial cells of glomeruli and parietal surface of Bowman's capsule [93].

Patients with end stages of disease with prominent proteinuria show abnormality in glumerulotubular junction (GTJA). These abnormalities are classified according to the severity of disease as normal tubular glomeruli, atrophic tubular glueruli and atubular glomeruli. Atrophic tubular glomeruli is further divided into short ATs and long ATs, in first one atrophy occur just in first few cells while in second one the longer part of PCT cells are atrophic. In atuublar glomeruli there is open blood circulation and no tubular attachment, these glomerulus are nonfunctional [94].

Tip lesions are seen in all forms of GTJA, they are reported in all short ATs, 82% of ATs without observable opening, in 64% of long ATs and in 9% of normal tubular glomeruli while presence of such lesions are not reported in normal individuals. Furthermore

severity of GTJA are strongly associated directly with the excretion of protein in urine and inversely related to GFR [95].

In all renal disorder with proteinuria injury, detachment or effacement of podocyte is seen. In DN also defect of podocytes play important role in the progression and worsening of disease. In DN width of podocyte foot process increased which decreases the area of slit pore length and thus causes proteinuria. Changes in shape of podocytes are documented in all T1DM young patients with normoalbuminuria [96]. Increased albuminuria worsens detachment of podocytes that can lead to decrease in number of podocytes [92]. Different studies showed decreased number of podocytes in DN patient suggesting the role of podocytes in the development of overt DN [97].

1.5Therapeutic approach to DN:

By getting deep information about the mechanisms of development and pathogenesis of diabetic nephropathy scientist are trying either to decrease the level of causative agents or block the leading pathways toward diabetic nephropathy. And thus the main therapeutic agents and strategies are suggested as follows.

Table 1: Mechanisms for therapy of DN

Mechanism	Treatment
Metabolic	
✓ Hyperglycemia	Insulin
✓ Increase glucose derived protein	Aminoguanidine ,AGE cross link breakers
✓ Polyol pathway	Aldose reductase inhibitors
Mechanical/Hormonal	
✓ Elevated systemic blood pressure	Anti- hypertensive drug
✓ Increased intraglumerular pressure	ACE inhibition, lower protein diet
✓ Increased vasoactive hormones	ACE inhibition, Angiotensin VI antagonist
	ET receptor antagonist
Intramediate pathways	
✓ Growth factors eg: TGFβ, TGF	Antibodies
✓ Protein kinase C dependent	PKC β inhibitors

1.5.1 **Glycemic control**

Hyperglycemia is considered as a major cause of development of diabetic nephropathy in both type 1 and type 2 diabetic patients [98]. According to several research groups including the Diabetic Control and Complication Trail, intensified glycemic control can prevent the incidence and development of microalbuminuria and also overt proteinuria in type 1 diabetic patients [99]. The 6 year study done by Ohkubo et al 1995 in Japanese patient with type 2 DM, multiple insulin therapy showed marked decrease in development of diabetic nephropathy [98] Diabetic Control and Complication Trail (DCCT) has suggested that by controlling intensively glycemic level(goal HbA1c< 6.5% and mean achieved Hb≈7%) in both type 1 and type 2 diabetic patients marked reduction in the development of micro-vascular complications like retinopathy, nephropathy and neuropathy is seen [100]. In UK, a 10 year study was done on the newly diagnosed patients with type 2 DM, in whom the intensified glycemic control, showed a 25% decrease in the rate of progression and development of secondary diabetic complications as compare to standard therapy. Study which was done by Vijan S *et al*. 1997 showed that glycemic control in type 2 DM is more beneficial in prevention of development of secondary complications than in type 1 DM [98]. However some controversial studies are also present like according to the research done by DCCT and Micro albuminuria study group, intensified blood glucose control was not able to decrease the rate of progression from microalbuminuria to macroalbumiuria in type 1 diabetic patients [99, 101]. But glycemic control along with blood pressure control in type 1 diabetic patients was reported to prevent the worsening of renal function [102].

In type 2 diabetic patient, the role of strict glycemic control is less studied but there are reports on some hypoglycemic agents for example Rosiglitazone is reported beneficial in decreasing the UAE rate as compare to the Glyburide [103].Use of metformin due to the risk of lactic acidosis is inhibited in patients with high level of creatinine [100] in these patients use of drugs independent from renal excretion are safe, for example Repaglinde and Nataglinide but Sulfonuria and its derivatives will worsen the condition [104]. However in the study for type 2 diabetic patients with exogenous insulin should be

administered because of low production of endogenous insulin in response to insulin secretagougs [100].

1.5.2 Intensive blood pressure control

Hypertension is a common problem of diabetic patients; about 40% of type 1 and 70% of type 2 diabetic patients are with normo-albumiuria [105] thus the hypotensive agents are reported to significantly decrease the risk of development of micro and macro vascular complications [100]. The study in UKPDs has shown that a decrease of 10mmHg (from 154 to 144 mmHg) reduces the risk of development of DN to 29%. As hypertension is consider critical to renal function so control of blood pressure with any of hypotensive agent may be beneficial [106] but RAS blockers either ACE inhibitors or ARBs despite of their anti-hypertensive characteristics are preferred due to the role of this system in the pathogenesis of diabetic nephropathy and their effect in decreasing intraglumerular pressure that results little passage of proteins to proximal tubules [107].

Though the preventive effects of ACE inhibitors has not been defined yet but a 3 year study in normotensive, normo-albuminuric type 1 diabetes showed delay in progression of DN about 24% in type 2 diabetic patients and also Ramipril was reported to decrease the urinary albumin excretion rate, thus ACE inhibitors can be beneficial agents in prevention of developing DN [100].The meta analysis of evaluation of 12 trails containing 698 non-hypertensive type 1 diabetic patients showed ACE inhibitors are not only beneficial in decreasing the chance of progression from micro-albuminuria to macro-albuminuria but they are also beneficial in increasing the chances of regression from micro-albuminuria to normo-albumiuria [108]. Furthermore, ARBs are proved to be efficient in prevention of the development of micro-albuminuria to macro- albuminuria in type 2 diabetic patients, treated with Irbastan 300mg/dl which showed a 70% decrease in development of diabetic nephropathy [109].

1.5.3 Novel therapy

1. Strategies to block AGE formation: The AGE can be blocked by several pathways. Most of therapeutic strategies till now, work on the inhibition of synthesis of AGEs. Aminoguanidine is the most studied AGE blocker; it is a

neucleophilic compound by interacting with the intermediates of AGEs inhibits the process of cross linking [110]. The studies done in diabetic animal model have shown the efficacy of aminoguanidine in the attenuation of the signal transduction, over expression of growth factor, structural and functional alteration of diabetic nephropathy [111]. The study done by Ateon a pharmaceutical company responsible for this research has concluded the significant reduction in the albuminuria following the administrating of aminoguanidine [112]. But there was no statistical significant change in GFR level. Some other AGE inhibitors that require further studies for clinical use are ALT 486, NNC 39-0028 and OPB 9195

2. Cross link breakers: other proposed strategy of therapy is the breaking of cross linking, the idea was developed when phenacylthiazolium(PBT) a cross link breakers was discovered [113]. But the study conducted on diabetic rats did not proved the efficacy of PBT in the treatment of DN. Another cross link breaker is ALT 711 which is able to inhibit and also to improve age related stiffness of myocardium and has also been shown to significantly beneficial to reduce blood pressure, UAE and renal lesions [114, 115].
3. Receptor blocker: Shmidt and collegues suggested another therapeutic strategy by administration of soluble, extracellular domain of RAGE (sRAGE) that was able to bind with AGE and thus inhibiting its receptor, subsequent gene activation and underlying pathophysiology [116].
4. Protein kinase C inhibitors: As the number of pathogenic pathways toward DN is activated by PKC the inhibition of PKC can be efficient therapeutic strategy in the management of DN on other side there are several isoforms of enzyme performing different function, therefore while inhibiting the specific isozyme related induction of DN (PKC-β) should be targeted [117].Inhibitors of PKCβ ameliorate glomerular lesions thus normalize GFR and inhibit protein excretion in diabetic rat models [118]. Till now the known inhibitor of PKC is LY333531 that was able to reduce UAE and GFR in diabetic rat models. That also resulted in

attenuation of mesangial expansion, reduction of collagen and expression TGF-β expression in Ren-2 diabetic rats, even in the presence of hyperglycemia.

5. Inhibition of vasopeptidase: The important vasopeptidases that contribute to the control of blood pressure are RAS, Kalikarin-Kinin system and natriuretic peptide system. All of these systems together play role in modulating the vascular tone, water and salt balance and have growth factor like activity. Interrelation of these systems is also important in the development of hypertension and renal complication. ACE and neutral endopeptidase have structural similarities and both are zinc containing cell surface peptidases therefore can be inhibited by single inhibitor. The inhibition of both systems can lead to better control of blood pressure. Furthermore, other vasoactive peptidases are also reported to be affected by changes in these systems. For example the degradation of bradykinin is inhibited by ACE inhibitors and NEP inhibitors reduce the endotheline and potentiate the natriuretic effect of adrenomodeline [119].

   Several preclinical and clinical studies are going on dual ACE/NEP vasopeptide inhibitors like Omapatriate the phase II and III studies for which are completed [120]. SA7060, MLD100240,MLD100173, Fasidotril, Sampatrilat, Alanopril, CGS30440 and S21402 [121].Number of studies are done on animal models to evaluate the comparative action of dual VPIs and ACE inhibitors. In one study the S21402 dual VPIs was compared with ACE inhibitors and in result the S21402 was found more efficient in controlling of blood pressure while the effect on AER was similar [122]. The other study was done on semi nephrectomized mice with some characteristics of DN, to study the effects of CGS 30440 and Omaptrilate, both of these VPIs were able to significantly reduce proteinuria [123].

6. Miscellaneous therapeutic strategies: Overdose of thiamin and its derivative Benfothiamin due to decrease oxidative stress, PKC and protein glycation is reported to slow down the development of microalbuminuria in diabetic nephropathy [124]. The administration of heparin glycosaminoglycan apart from the beneficial effects of PKC inhibitors also decrease the accumulation of tubular and glomerular matrix and inhibit the synthesis of PKC mRNA [125].

Pimagedine a second generation of AGE inhibitor and Suldexide a glycose amino glycan also have beneficial effects in the decreasing of the urinary albumin excretion and in normalization of GFR in diabetic rat models. Taniguchi K *et al* 2013 suggest the role of Src kinase in collagen accumulation and PP2 by inhibition of Src kinase that leads to the inhibition of collagen IV accumulation, high glucose induced phosphorylation of proteins and the pathological mechanisms of diabetic nephropathy thus Src inhibitors were suggested as a novel therapeutic targets for diabetic nephropathy [126].

Role of PG in the pathogenesis and development of DN is not clear but there is higher amount of PG in the kidney of patient and also diabetic animal models with diabetic nephropathy. Makino *et al* 2002 showed that administration of selective antagonists of PGE receptor EP-1 subtype was able to selectively prevent development of diabetic nephropathy in STZ induced diabetic rats, Which was able to decrease massengial expansion, ameliorate glomerular hypertrophy, inhibit up regulation of fibronectine and transcriptional growth factor β1 (TGF-β1) in messengia cells cultured in high glucose concentration. According to this study the role of PG-EP1 system in the development of diabetic nephropathy become clear. Makino *et al* 2002 also explained that aspirin a non - selective prostaglandin synthase inhibitor and EP-1 antagonist both decreases messengial expansion but aspirine is not able to inhibit glumerular hypertrophy and proteinuria while EP-1 inhibitor is able to produce these changes suggesting that the mode of action of these drugs may be different and suggests the novel therapeutic strategy [127]. Studies conducted on STZ induced diabetic rat models have suggested that in renal mitochondria high level of SO is produced along with the post transcriptional modification of mitochondrial compexIII. Thus Chacko BK *et al* revealed out that in $Ins2^{+/\_AKitaJ}$ mice targeted antioxidant therapy with mitochondria –targeted ubiquinone (Mito Q) was able to prevent and treat diabetic nephropathy [128]. However the studies in human being are still needed to approve the effect of these novel drugs.

1.5.4 Herbal therapeutic agents:

Since ancient years medical plants play an important role in the treatment and prevention of disease. For the treatment of diabetes mellitus and for prevention of occurrence of diabetic induced secondary micro and macro vascular complication certain medical plants are known. That contributes not only at therapeutic level side by side with pharmacological medicines also helps financially diabetic patient because of low cost and ease of use.

Well-known plants containing antidiabetic characteristics that were approved as hypoglycemic agents by researchers were listed by Bnouham M *et al* (2006) [129].They are *Momordica charantic L, Ficus bengalensis L, Polygala seneg L, Gymnema sylvestre R, Opuntia streptocautia Lem, Alium sativum, Aloe, Artemisia*

A list of Indian medicinal plants owing antidiabetic activity was proposed by Modak *et al*. 2006 containing *Allium sativum, Trigonella foenum graecum, Tinospora cordifolia, Eugenia jambotana, Pterocarpus marsupium, Withiana somnifera, Phyllanthus armus, Momordica charanhia Ocimum sanctum, Camella sinenses* [130]

Among antidiabetic plants *Camella sinenses* or green tea has attracted the great interest of scientist worldwide for its therapeutic application here I will try to shed light on green tea's characteristics and therapeutic usage.

CHAPTER 2

GREEN TEA

2.1 General information

Green tea is the most consuming beverage after water worldwide. It is obtain from plant of Theacea family, *Camellia sinensis*. Green tea is obtained by non- fermenting process of plant leaves while black tea is fermented part of same pant. Tea is the infusion of plant leaves, cultivation of plants takes place approximately at 30 countries.

2.2 Scientific classification:

Kingdom: *Plantea*

Order: *Ericales*

Family: *Theacea*

Genus: *Camellia*

Species: *Camellia sinensis* [131]

2.3 Morphological characteristics of plant

It is shrub or average tree. Flowers of plant are yellow in color, 3 flowers per cluster or solitary and axillary, leaves of plant are long, serrated and young leaves contain white hair at their underside. Edges of leaves are epileptic, oblong or oblong-epiliptic, branches are glaborous and grayish yellow when they are young and then the color is changed to purplish red [132].

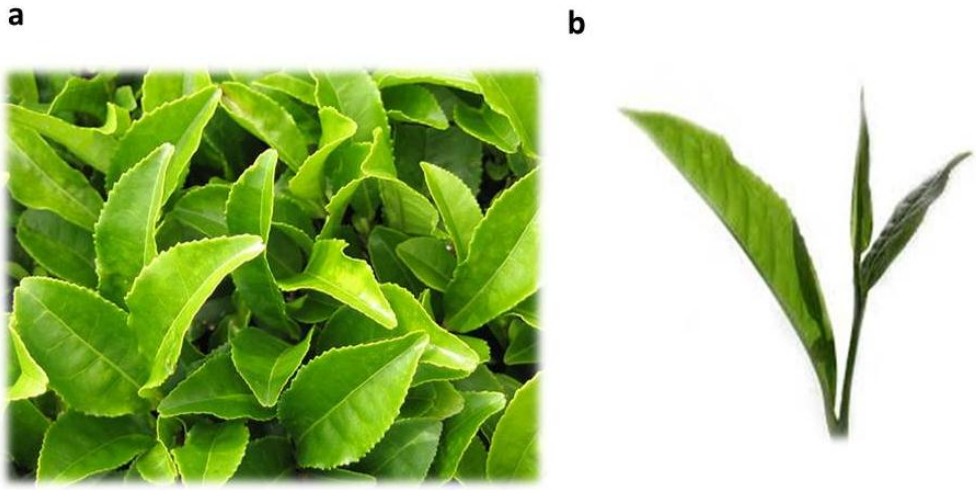


Figure 5: leaves of Camellia sinensis a: front view of leaves b: side view

2.4 Chemical constituents

There are about more than 4000 bioactive chemicals in green tea the most important and major part, account for approximately 30% of the dry weight of green tea is polyphenols include flavanols, flavndiol, phenolic acid and flavonoid; alkaloids (3-4% of fresh leaves) consist of theobromine, theophylline and cafein, carbohydrates, amino acids, volatile organic compounds, trace elements and fluride [133]. The major part of phenolic compounds which are also responsible for major health benefits of plant, are flavanols known as catechins consist of (-) –epigalocatecines, (EC), (-)-epicatechin gallete (ECG), (-) –epigalocatechine galate (EGCG). The most active is EGCG [134]. Derivatives of green tea is usually in powder form and varies in the percentage of its constituents (0.4-10)% caffeine and (45-90)% cathechines [135]. Amount of cathechines in green tea is dependent on the variety, conditions of growing and origin [136]. As these compounds are relatively unstable, it is hard enough to keep their concentration stable during experimental periods [137] therefore in *in-vivo* studies the exact administered dose usually remained unknown [138].

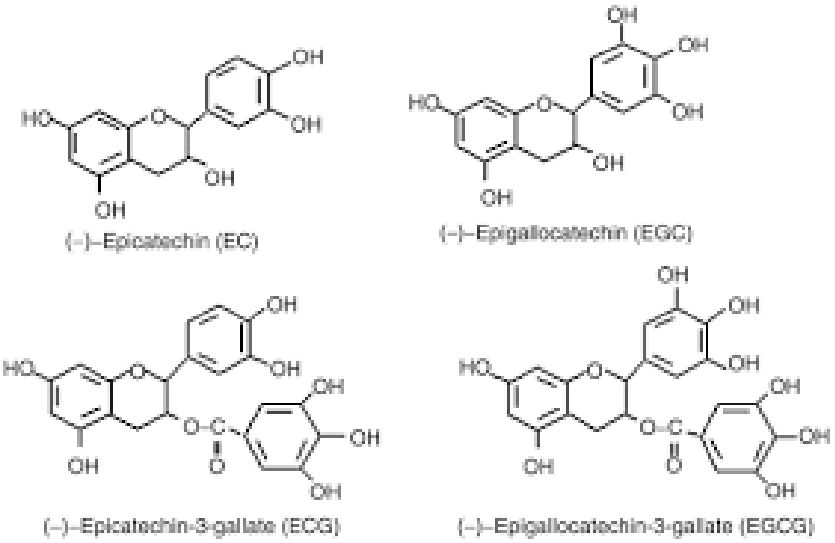


Figure 6: Catechines of Camellia sinesis

Table 2: Composition of green tea

Composition of green tea	
Polyphenols (30% of dry weight)	Flavandiol, Phenolic acid, Flavonoids,Flavanols
Proteins (15-20 % of dry weight)	Mostly important enzymes
Amino acids(1-4% of dry weight)	5-ethyl glutamine(thianine), glutamic acid, serine, aspartic acid, tyrosine, valine, leucine, threonine, arginine, lysine
Carbohydrates (5-7% of dry weight)	Cellulose, Pectin, Glucose, Fructose, Sucrose
Mineral and trace elements	Calcium, Magnesium,Chromium,Manganese, Iron, Copper, Sodium, Phosphorus, Cobalt, Strontium, Nickel, Potassium, Aluminum
Vitamins	Vit B, Vit C, Vit E
Alkaloids (3-4% of fresh leaves)	Caffeine, Theophylline, Theobromine
Pigments	Chlorophyll, Carotene
Volatile compounds	Aldehydes, Alcohols, Esters, Lactones

2.5 Health benefits:

2.5.1 Anti-Aging activity:

Responsible mechanism for phenotypic changes and senile disease for example Alzheimer's disease, Parkinson's disease, cancer and diabetes is free radical generation and increase in anti-oxidant/oxidant ratio [139].Thus the anti-oxidant effect of green tea was proved by its protecting effect of aged mice from ethanol induced oxidative stress [140] however green tea was not able to increase the life expectancy at significant level in mice.

2.5.2 Alzheimer's disease

Studies which are done on cultured cells and animal models reveal that EGCG present in green tea due to its anti-oxidant property protects beta amyloid induced toxicity in culture hippocampal neuron and it will target other potential pathways associated with Alzheimer's disease [141].

2.5.3 Antiparkinson activity:

According to an epidemiological study green tea was able to decrease the prevalence of Parkinson's disease about 5-10 fold in Asian population [142]. As per results of experimental studies the anti-oxidant and iron chelating characteristic of EGCG is proved due to which it is able to prevent accumulation of alpha-synuclein and iron in MPTP treated mice [143].Furthermore, administration of EGCG is reported to preserve dopamine level and prevent loss of dopaminergic neurons in substantia nigra [144].

2.5.4 Anti-stroke activity:

A meta-analysis of recently published literature showed that consumption of green or black tea about 3 cups or more daily can decrease the chance of stroke up to 21% [145].

2.5.5 Cardiovascular disease:

Green tea due to owing antioxidant properties is able to reduce the peroxidation of LDL, the underlying cause of arteriosclerosis and CVD [146]. It is reported to be beneficial in controlling of blood pressure in hypertensive mice [147]. As most of CVD are associated with dysfunction of endothelium due to oxidative and the anti-oxidant potential of green

tea is approved by many literatures, so consumption of green tea seems to beneficial in prevention of coronary artery disease [146]. The lowering of cholesterol level and reduction in the development of atherosclerosis is studied and proved in mice and rabbits respectively. Studies in human are not easy task but epidemiological studies showed significantly beneficial effects of green tea in prevention of CVD [131].

2.5.6. Anti-cancer activity:

Green tea have potential role in prevention of cancer. Most of anticancer activities of green tea are mediated by its polyphenolic compounds [148]. The process of apoptosis is the wanted process for the control of unregulated growth of cells and the EGCG from green tea is reported to induce cell cycle arrest and apoptosis on many cancer cell with leaving normal cells unaffected [149] this result was verified in study of different cells including prostat, skin, colon and pancreas [150]. As there is strong link between regulation of cell cycle and induction of cancer, so regulation of cell cycle can be efficient target of cancer management. Numbers of factors of cell cycle progression are known to be affected by EGCG, the primary event is consider the direct inhibition of cyclin dependent kinase. Furthermore, expression p27 and p21 is induced by EGCG while the phosphorylation of retinoblastoma and expression of cyclin D1 is decreased. Primary events of green tea for induction of apoptosis are considered as activation of caspase-3, cleavage of poly (ADP) ribose polymerase and nuclear condensation. Moreover, Bax oligomerization and mitochondrial membrane depolarization for the release of cytochrome C into cytoplasm in implemented by EGCG. The H_2O_2 generated from EGCG is claimed to be responsible for the induction of apoptosis. The anti-apoptic function of tea polyphenol is proved by binding to the BH3 pocket of anti-apoptic Bcl-2 family proteins.

Modulation of cell signaling by EGCG is mediated by:

- ✓ Targeting NF-κB/p65 component of NF-κB complex an oxidative stress sensitive transcription factor and a target for the management of cancer.
- ✓ MAPKs pathway which is implicated in cell proliferation, differentiation and death is also inhibited by EGCG.

- ✓ EGCG also have inhibitory effects on epidermal growth factor receptor mediated signal transduction pathway, the over expression of which causes neoplastic changes in tumor cells.
- ✓ By inhibition of IGF-1 mediated signaling pathway
- ✓ By inhibition of vascular endothelial growth factor, a mitogen of endothelial cells and responsible for tumor induced angiogenesis
- ✓ By inhibition of matrix metalloproteinase, the enzyme that play important role in tumor invasion and metastasis.

Experimental studies on mice showed the preventive effects of green tea after giving carcinogens to the laboratory mice [151]. Cathechines of tea are reported to promote destruction of leukemic cells and also inhibit tumor cells proliferation [152]. Studies in Chinese population showed that development of gastric cancer and chronic gastritis is inversely proportional to green tea consumption [131]. Apoptosis of cervical cancer was seen followed by consumption of green and black tea [131].Because of anti-oxidant, anti-apoptic and anti angiogenic property of green tea and EGCG, they are able to inhibit the initiation, promotion and progression of cancer cells, these effects are also proved in animal models.

2.5.7 Anti-diabetic activity:

The anti-diabetic effects of green tea and its polyphenols is reported by several studies [153]. It is reported that green tea has insulin enhancing and also insulin mimicking activities [154]. EGCG is believed to inhibit sodium dependent glucose transporter (SGLT1) and thus regulates blood glucose level [155]. GTPs due to their anti-oxidant properties are reported to reduce glutathione and superoxide dismutase activity [156], the characteristic that is believed to inhibit the development of secondary diabetic complications.

In study done by Sabu *et al* administration of GTPs to diabetic rats were shown to reduce blood glucose, blood lipid and lipid peroxidation, enhance antioxidant level and ameliorate renal and hepatic tissue damage induced by STZ and diabetes [157]. Study done in db^{+}/db^{+} mice and healthy human volunteers has shown promotion of glucose

metabolism and oral glucose tolerance [158]. EGCG of green tea has reported to decrease glucose production *ex-vivo* in hepatoma cells of H4IIE rats by mimicking insulin action, reducing the expression of gene for phosphenoyl pyruvate kinase, an important glucogenic enzyme and increasing the tyrosine phosphorylation of insulin [159].

CHAPTER 3

REVIEW OF LITERATURE

3.1 Studies on animal models

Gomes A. *et al,*. 1995: The study was conducted on STZ induce diabetic rat models to investigate the anti-hyperglycemic effects of black tea extract (*Camellia Sinensis(L.)O. Kuntze (Theaceae)* and in result it was proved that black tea extract significantly reduces blood glucose level of diabetic rats and have curative and preventive effects in diabetic rat models. [160]

Yen G-C and Chen H-Y 1995: Yen studied the relation between antioxidant and anti-mutagenic effects of different tea extracts including green tea, black tea, oolong tea and pouching tea. In result they conclude that all of extracts have anti-oxidant activity. They scavenge 65-75% of superoxide, 30-50% hydrogen peroxide and 100% hydroxyl radical at a dose of 1mg, 400μg and 4mg respectively. The extract also exhibited 50-70% scavenging effect on a diphenyl-β-picryl hydrazyl radical. The anti-mutagenic effect of extracts was proved by inhibiting the action of 5 indirect mutagens each Trp-P-1, AFB1, IQ and Glu-P-1. The reducing power was as semi-fermented > non-fermented> fermented. There was good correlation between the anti-oxidant and anti-mutagenic effects of tea extracts. [161]

Kao Y-H *et al,*. 2000: The study was aimed to observe the effect of EGCG on modulation of endocrine system. The study was designed as administration of pure catechines of green tea by IP route and then the level of hormones were determined. Both sexes of rats and also lean and obese rats were used in analysis, after seven days of IP injection of EGCG and other catechines the body weight, level of food intake, insulin, ILGF-1, leptin, estradiol, LH, testosterone and lipid level of blood was determined. Furthermore the weight of androgen sensitive organs for example prostate, ovary and uterus, androgen non-sensitive organs like kidney and liver were also measured. At result effects of EGCG was gender independent but was dose dependent and lean and obese animal were also affected equally indicating that the EGCG affects metabolic pathways independent of leptin receptors. Food intake, body weight, level of circulatory testosterone was decreased. Any toxic effect on kidney or liver was not observed. In this study it was concluded that the inhibitory effect of green tea on prostate and breast cancer

may be due to the changes of endocrine systems followed by parenteral administration of EGCG [162].

Sabu M.C *et al,*. 2002: Have conducted a study on green tea polyphenol to analyze its antidiabetic and anti-oxidant activity. This study was done on aloxan induced diabetic rat models, according to this study administration of green tea polyphenol to normal rats (500mg/kg bwt) was found to increase glucose tolerance at significant level ($P<0.005$). Serum glucose level of alloxan induced diabetic rats was also decreased following the administration of green tea polyphenols at dose 100mg/kgbwt. Level of liver glycogen which was decreased by alloxan administration was improved at significance level of ($P<0.001$). Elevation in the level of hepatic and renal marker enzymes induced by alloxan was reversed at significant level of ($P<0.001$). The level of serum lipid peroxidation was decreased significantly ($P<0.001$), SOD and glutathione were increased ($P<0.001$), level of catalase was remained unchanged. The anti -oxidant activity of green tea poly phenols was also checked *in-vitro* 50 % of superoxide, hydroxyl and lipid peroxide was scavenged at the concentration of 10, 52.5 and 136 μg/ml respectively. [163]

Wu L-Y *et al,*. 2004: Liang *et al* have performed a research to evaluate the effect of green tea on fructose rich diet fed rats which resembles the type 2 diabetes mellitus, in this study the rats were divided into 3 groups each group containing 8 animals, 1st group was fed with standard chow and water, 2nd group with high fructose diet and third group was fed with high fructose containing diet+ green tea (0.5g of lyophilized green tea dissolved in 10ml of deionized distilled water), after 12 weeks at the end of study the OGTT was performed by taking blood from tail's vein and the animals where dissected, levels of glucose, TG, Cholesterol, lipoproteins and insulin were checked in blood sample and the level of glucose transporter I and IV were checked in adipose tissues, in result blood glucose level, blood insulin level and blood pressure were increased in high fructose rich diet fed group while the decrease was seen in insulin stimulated glucose uptake and insulin dependent glucose transporter (Glu IV) and insulin binding to adipocytes and in group fed with fructose rich diet+ green tea all the above metabolic defects were improved and according to this study the hypoglycemic effect of green tea

may be due to the increase in expression of Glu IV glucose transporter following administration of green tea, was proved. [164]

Wu L-Y *et al,*. 2004: The study was aimed to evaluate the effect of green tea on blood glucose and sensitivity of insulin in *Sprague Dawley* rats and they achieved the result that supplementation of 0.5g of lyophilized green tea dissolved in 100 ml deionized water was able to increase the sensitivity of insulin and insulin sensitive glucose uptake by adipocytes [165].

Mustata GM *et al,*. 2005: In this study paradoxical effects of green tea and antioxidant vitamins were analyzed in diabetic rat models. Diabetic rats were treated with anti-oxidant vitamins, tap water and green tea extract for 12 months. At the end of study, body weight of diabetic rats was decreased and level of glycated lysine was increased in plasma, tendon and aorta at significant level of $p<0.001$ but these changes were not seen in groups treated with green tea or vitamins. Level of glutathione in RBCs and hydroperoxide in plasma was improved following therapy by vitamins ($p<0.05$) and also green tea ($p<0.001$). Moreover green tea was also able to ameliorate lenses crystalline fluorescence at 370/440nm, but this effect was not observed by vitamins. Activity of complex III, dinitrophenol dependent respiration and NADP dependent respiration was improved by green tea. The effects on nephropathy were noted at marginal level may be because of mild induction of nephropathy. Level of glycation was increased by diabetes and after green tea supplementation either it remain as such or even worsened. Thus at the end of study it was concluded that green tea reduces oxidative stress while increases carbonyl stress of diabetic patients [166].

Yamabe N *et al,*. 2006: The study was conducted on diabetic rat models with subtotal nephropathy along with intraperitoneal administration of STZ to see the therapeutic potential of Epigalocathechine on renal damage. In this study the one half of left and total of right kidney was dissected and the STZ was administered to rats. The epigalocathechines was given to rats by oral gavage at concentration of 25, 50 and 100mg/kg bw, after 50 days of therapy the analysis was done and the results showed that epigalocatechine treated groups were with lower kidney weight as compare to control

diabetic nephropathy rats. Although the level of serum creatinine and glycated proteins were slightly decreased but serum glucose level, lipid peroxidation, urinary proteins, advanced glycated end products,protein expression and other pathologic conditions were significantly decreased following epigalocathechine administration. This study showed that epigalocathechines are able to ameliorate oxidative stress due to glucose toxicity in diabetic patient. [167]

Wolfram S *et al,*. 2006: In this study the anti-diabetic effects of EGCG extracted from green tea was analyzed on type 2 diabetic mice models and in hepatoma cels of H4IIE rat. EGCG was given to diabetic mice models and ADF rats for 2 weeks and then glucose tolerance test and gene expression of metabolic enzymes were analyzed. At result OGTT was improved, insulin secretion was enhanced and TG level of plasma was reduced significantly. In H4IIE cells gene expression of gluconeogenesis, TG, FA and cholesterol biosynthesis were downregulated followed by treatment with EGCG. The mRNA expression of glucokinase was upregulated in liver of diabetic mice and mRNA of phosphoenoyl carboxykinase was decreased in H4IIE cells by EGCG. In this study the beneficial effect of EGCG on lipid and carbohydrate metabolism was proved [168].

Babu *et al,*. 2006: The study was aimed to analyze the effect of green tea on oxidative stress in heart and aorta of diabetic rats. In this study diabetes was induced by STZ in rats and after 6 weeks of diabetes, therapy was started orally with EGCG for four weeks. Analysis was done after scarifying of animals; level of glutathione was decreased in diabetic rats. Treatment with GTE caused reduction in lipid peroxidation and increase level of glutathione. The study suggested that the ameliorating effect of oxidative stress due to green tea is not its effect on anti-oxidant enzymes but it may be because of direct anti-oxidant effects of green tea [169].

Babu *et al,*. 2007: The study was conducted to assess the attenuation of green tea on diabetes induced collagen linking Maillard-type fluorescence in the heart of streptozocin induced diabetic rats. Green tea treatment was started after six weeks of induction of diabetes with 300mg/kgbw/day and the result was assessed after 4 weeks of therapy. AGE cross linking of collagen and collagen content, glycaemia level and activity of heart

marker enzymes were determined. In the observation of results due to diabetes the activity of marker enzymes was reduced in cardiac tissue while increased in serum and treatment by green tea was successful in rectifying of the situation. There was no change in the glycogen content of cardiac tissue between groups but the solubility of collagen and Maillard type flurescence of collagen indicationg the cross linking of collagen and degree of advanced glycation end products respectively, was significantly ($p<0.05$) increased in diabetic group and by the administration of green tea the level of Maillard type florescence was decreased at significant level of $p<0.05$. The level of collagen solubility was increased (41 ± 1.04) indicating the decrease in the formation of AGEs and collagen crosslinking. Thus the therapeutic effect of green tea on cardiovascular complication of DM was proved [170].

Collin Q-F *et al,*. 2007: The study was conducted to know the molecular mechanisms of inhibitory effect of EGCG on hepatic gluconeogenesis. In this study the isolated hepatocytes from mice liver were exposed to different concentrations of EGCG and it was revealed out that EGCG inhibits gluconeogenesis at dose of $\leq 1\mu M$ and at this dose any toxics effects of EGCG are not observed but it showed toxic effects at dose of 10 $\mu M$and grater concentrations. EGCG at $\leq 1\mu M$ did not activate insulin pathway but it induce its inhibitory effects on gluconeogenesis via activation of 5'-AMP activated protein kinase pathway. Activation of 5 AMPK was via Ca2+/calmodelin-dependent protein kinase (CaMKK). Moreover the mechanism of activation of AMPK and inhibition of gluconeogenesis are reported as via production of ROS which is robust activator of CaMKK [171].

Kim J *et al,*. 2007: The study was based on hypothesis that EGCG may cause vasodilation via stimulation of NO production. The analysis was done by acute intra-arterial administration of EGCG *ex-vivo* to the mesenteric vascular bed isolated from WKY rats. In result vasorelaxation was occurred which was dose dependent. The action was inhibited by Wortmanin (Phosphatidyl inositol 3 kinase inhibitors), L-NAME (NO synthase inhibitor) and PP2 (inhibitor of PKC family). Furthermore bovine aortic endothelial cells (BAEC) were also treated with EGCG (50μM) and stimulation of NO

was seen that was also dose dependent and was inhibited by PP2, L-NAME and Wortanin. For understanding the specific pathway of stimulation of NO by EGCG, first Fyn was knockdown with siRNA which also inhibited the stimulatory action of EGCG on NO production and phosphorylation of eNOs and Akt. Furthermore the EGCG increased the intracellular H_2O_2, and N-acetyl cysteine was reported to inhibit the EGCG phosphorylation of Akt, phosphatidyl inositol 3-kinase and Fyn. In this study it was concluded that EGCG have vasodilatory action which is mediated by Fyn and H_2O_2 [172].

Haidari F. *et al,*. 2012: The basic aim of this study was the evaluation of antidiabetic activity of green tea and its effect on hepatic and serum antioxidant level. The study was done on rats which were made diabetic by single IP injection of STZ (55mg/kg bw). Green tea was administered at dose of 200mg/kg as its alcoholic extract by oral gavage. After 4 week therapy the rats were dissected and their fasting blood glucose level, serum and hepatic anti-oxidant level were checked. The result proved that green tea extract at dose of 200mg/kg bw of green tea was able to decrease significantly ($p<0.05$) blood glucose level. Serum and hepatic MDA (malondialdehyde) level was increased and to decrease the total antioxidant capacity at significance level ($p<0.05$). Thus the study showed that green tea has anti –hyperglycemic and anti-oxidative effects which can be used as prophylactic treatment agent for diabetes and its complication. [173]

Kang *et al,*. 2012: The study was done on db/db mice model and MDCK cells to evaluate the preventive effects of *Camellia Sinensis var. assamica* on diabetic nephropathy. In this study MDCK cells were incubated with 1mM oxalate to induce oxidative stress and cytotoxicity and then these cells were divided into different groups with different concentrations of CSVA and the control group without CSVA. The experiments were also done *in-vivo* on male db/db diabetic mice models. The mice were divided into three groups, control group was fed AIN-93G *ad libithum* and the experimental groups fed AIN-93G, libithum + 10% fermented CSVA and AIN-93+ 10% non- fermented CSVA respectively.

The analysis was done after 14 weeks of therapy following scarification. Serum glucose, 24h urine chemistry and renal morphology were assessed. In result the level of MTT and MDA in CSVA treated groups were decreased and level of cells was increase as compare to control group. *In-vivo* study showed less amount of urine micro-albumin in group 3 (fed AIN-93G + 10% non- fermented CSVA) and more foot processes were preserved in group 2 and 3 [174].

Nasri *et al,*. 2013: Nasri *et al* studied the preventive effects of green tea extracts on contrast media induced acute renal injury and in result green tea was able to prevent renal damage and decrease blood creatinine level at significant level of $p<0.05$ [175].

Tsuneki H. *et al,*. 2014: The study was conducted to study the effect of green tea extract on glucose metabolism in human volunteers, db+/db+ diabetic mice and STZ induced diabetic mice, the result proved the anti-diabetic effect of green tea. Glucose metabolism was promoted in healthy volunteers after administration of 1.5g of oral glucose and OGTT test but the basal glucose level was not changed significantly, green tea also showed hypoglycemic activity in db+/db+ diabetic mice and STZ induced diabetic mice. In this study the effect of green tea on diabetes modified proteins was also studied, the modification induced by DM was not reversed but the level of 4211(4212) Da protein which decreases in DM was further decreased by green tea administration. [158]

3.2 Studies in Human

Dulloo et al 1999: study was aimed to determine the efficacy of green tea extract rich in caffeine and catechin polyphenols in increasing of the 24 hour fat oxidation and expenditure of energy in humans. The study was designed to be conducted on 10 healthy, non-smokers, mild or non -obese and consuming normal diet. They were treated on 3 separate occasions with green tea extract containing 90mg epigallocatechine and 50 mg caffeine, 50mg caffeine and placebo at all therapeutic stages normal diet was given to individuals. Their 24h energy expenditure (EE) and respiratory quotient (RQ) and urinary excretion were measured. In result green tea extract was reported to increase EE 4% at $p<0.01$ and decrease RQ by 0.03% (from 0.88 t0 0.83) at significant level of $p<0.001$. Urinary nitrogen level remained unchanged while level of urinary epinephrine was higher than placebo.(40% at significant level of $p<0.05$). Treatment with caffeine had no effect in all of above mentioned markers [176].

Jatoi et al 2003: A phase II trial of green tea therapeutic effect in androgen independent metastatic carcinoma was studied. In this study 42 patients with noticeable progression of prostatic specific antigen (PSA), who were not receiving any other medication. Patients were treated with 6 g of green tea orally in 6 divided doses. And at the end of one month of PSA was increased by 43% and in 69% of patient grade 2, 6 episode of grade 3 and one episode of grade 4 toxicity was observed. After getting results it was concluded that green tea has limited anti-tumor activity and it was suggested that for androgen independent prostate carcinoma, patients should try other more beneficial therapeutic strategies [177].

Mackenzie et al 2007: The study was performed on 49 type 2 diabetic patients with average age of 65 years and 6 year of diabetic history. The study was designed for 3 months as double blind, a placebo controlled group, 375mg and 750mg not taking insulin. In result there was not significant changes in the glucose level of green tea treated and controlled group [178].

Nagao et al 2007: Effect of green tea extract on reduction of body fat and risk of cardiovascular disease was analyzed. In this study the double blind assay was performed

on Japanese obese individuals. A test group containing 123 subjects was treated with 583mg catechins while control group with 117 subjects consumed 96mg cathecheins for 12 weeks. Data was adjusted according to age and gender. BMI, body weight, ratio of body fat, mass of body fat, visceral fat area, waist circumference and hip circumference were decreased more in high catechines taking group. Effect of decreasing of blood pressure and LDL-cholesterol was also significant and no adverse effects were seen. This study suggested the capacity of green tea catechines in decreasing body weight and reducing risk of cardiovascular disease [179].

Fukino et al 2008: The study was conducted to examine the effect of green tea powder on glucose abnormalities. The study was designed to be conducted on volunteers living in Shizako Prefecture with FBS level $\geq 6.1 mmol/l$ and RBS$\geq 7.8 mmol/l$. Number of participants was 49 men and 11 women that were devided in 2 early and later intervention groups. To early intervention group green tea extract powder containing 456mg catechines was given for 2months and then for two other months treatment with green tea was restricted. For later intervention group treatment was started after two months from the starting of study and continued for 2 months. At the end of study there was significant reduction in $HbA1_C$ level during intervention period while changes in body weight, BP, serum lipids and CRP were not significant [180].

Brown et al 2008: The study was purposed to examine the effect of EGCG on insulin resistance and metabolic risk factors associated to insulin in humans. In this study 88 obese or overweight male subjects were included. To test group capsules containing 40mg EGCG and to control group at same quantity lactose as placebo was given twice per day for eight weeks. OGTT, BMI, lipid profile, percentage of body fat, waist circumstance and blood pressure were evaluated before and after treatment period. Mood was evaluated per week according to check list prepared by the university of Wales institute of Science and Technology. At result there was no change in insulin sensitivity, insulin secretion and glucose tolerance between test and control group. Reduction in DBP was significant but other metabolic risk factors were remained unchanged. Furthermore, green tea consuming group was with more positive mood as compare to control group. At

conclusion the positive effect of green tea on mood and CVS was proved but for understanding of detailed mechanisms further research was proposed [181]

Makino KC et al 2009: The effect of green tea catechin consumption on exercise induced abdominal fat in overweight and obese individual was studied. The study was conducted for three months on 103 individuals. EGCG 635mg with 39mg caffeine and 39mg caffeine without catechines (as control beverage) were randomly given to participants. Normal energy intake, 180 min walk with moderate exercise and 3 supervised sessions of exercise were maintained by participants. At the end of study weight loss of catechine consuming group was tend to decrease more than control group ($p=0.079$). the percentage of changes in serum TG level, total abdominal fat area and subcutaneous fat area was more than control group at significant of $p=0.023$, $p=0.013$ and $p=0.019$ respectively, thus the enhancing effect of green tea catechine on exercise induced fat loss and plasma hypolipidemic activity was proved [182].

Hsu et al 2011: A randomised double blind study was performed to investigate the effect of decafeinated green tea extract on type 2 diabetic obese patients. The study was conduccted for one year on 68 type 2 diabetic patients aged from 20-65 years. The subjects were assigned randomely to be treated with 1500mg of green tea or placebo for 16 weeks. Results were on the base of homeostatic model assessment for insulin resistance. FBS, HbA1c and antropometic measurement were done at the end of 16 weeks. In result there was no statistically significant changes in any of variable between control and test group but reduction in HbA1c level, waist circumstance and HOMA-IR level was significant within groups consuming green tea extract. Level of ghrelin was also significantly increased within test group. According to this study there was no statistically significant change in variables between groups while singnificant improval was seen within groups as compare to the baseline situation. But for confirmation of

effects of green tea on type 2 obese diabetic patient further research was suggested [183]

CHAPTER 4

STUDY DESIGN

4.1 Hypothesis

Green tea supplementation will be able to reduce the rate of incidence and also the progress of DN and thus increase the life expectancy of diabetic patients.

4.2 Objectives:

1) To carry out kidney function study biochemically in diabetic rat models following administration of GTE
2) To study the histopathology of kidney and restoration of histo-architecture of kidney in diabetic rat using GTE
3) To evaluate the preventive effects of GTE on development of DN by analyzing total anti-oxidant capacity

4.3 Plane of work:

This project was planned for 2 month of practical work but because of late providing of animals and limited duration of semester the practical part was shortened to two weeks. The protocol was made for 36 animal divided into six groups of six animals each, but because of mortality of animals in acclimatization period study was arranged into 4 groups of six animals. For this reason we purchased water soluble GTE and gave it in drinking water to rodents.

Table 3: time line for 4th semester

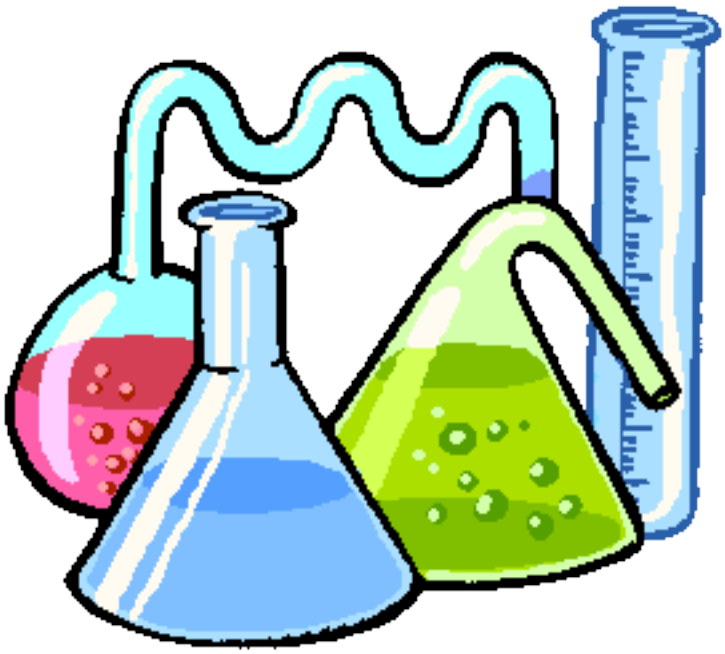
Month	Months			
	0-1	1-2	2-3	3-4
Introduction				
Review of literature				
Validation of tests				
Practical work part of research and analysis of data				

Table 4: Time line for practical part of research

Test	Weeks			
	0-1	1-2	2-3	3-4
Time of acclimatization				
Induction of diabetes and determination of blood glucose				
Lipid profile of experimental groups				
Estimation of diagnostic and Membrane bound enzymes (related to kidney)				
Electrolyte assay				
Kidney profile test, Protein estimation and Kidney tissue sampling for histopathological analysis				
Analysis of data and application of statistical tools				
Report Writing				

CHAPTER 3

MATERIAL AND METHODS



5.1 Plant material

Green tea (*Camellia sinensis*) extract was purchased from Blue Berry, a new venture of Vijay group Mumbai, India. The extract was in spray dried powder form product, with 90% polyphenol/40% EGCG.

5.2 Animals and diet

Female Sprague-Dawley rats weighing 220-320g and 2.5 -3 moth of age were obtained from NIPER, Mohali, Punjab. They were kept in animal house of university, department of Pharmacy, school of applied medical science, lovely professional university Jalandhar, Punjab. Three animals were housed per cage in an air-conditioner room at 25-27°C, temperature, relative humidity of about 55-60% and 12h light-dark cycle. The rats were maintained according to the guide line of IAEC (Institutional Animal Ethics Committee) with having free access to normal rat chow and clean deionized drinking water. We used rice husk as bedding material for rats and changed it daily. Handling of animal was done as per good laboratory practice but over handling was avoided. After giving of 10 days acclimatization period the experiment was started which lasted for three weeks.

5.3 Drugs and chemicals

STZ was obtained from Pharmaceutics laboratory, school of Pharmaceutical Science, Lovely Facultey of Applied Medical Sciene. Stains of histopathology were provided by histopathology laboratory, department of Paramedical Sciences, School of Applied Medical Sciences. All assay kits were purchased by university. All chemicals were analytical grade and were used as such without any testing.

5.4 Treatment

a. Animals were divided into four groups of six animals randomly and were treated as table 5.Diabetes was induced by IP injection of STZ, and green tea extract was given orally in drinking water.

Table 5: Groups of animal for treatment

Groups	Treatment	Sex	Required animal	Dose/route
I	Non-diabetic rats + Normal diet	Female	6	Nil
II	Non- diabetic rats + Green tea extract	Female	6	200mgkg^{-1} bw; Orally
III	Diabetes induced rats + Normal diet	Female	6	STZ (45mgKg^{-1}bw)
V	Diabetes induced rats + Green tea extract	Female	6	STZ (45mgKg^{-1}bw) + GTE orally 200mgkg^{-1} bw

b. Control and observation of physiological condition of rats

The following characteristic were measured before of diabetes and then on 7th and14th day of induction of diabetes.

- ✓ Behavior of animal
- ✓ Motility and responses
- ✓ Weight
- ✓ Body temperature
- ✓ Blood biochemical parameters

5.5 Induction of diabetes:

Rats were kept for overnight fasting (for 18h fasting). STZ was dissolved in sterile 0.2-0.3 ml of 50 mM sodium citrate solution (pH 4.5) containing STZ. Instead of drinking water a 50 ml of 2% glucose solution per cage was given to rats [184].

5.5.1 Materials:

- ✓ Alloxan monohydrate

- ✓ Sterile normal saline
- ✓ 70% alcohol
- ✓ Sterile cotton
- ✓ Syringe: 1cc
- ✓ Needle: 22-30G
- ✓ Glucometer

5.5.2 Procedure*:*

To avoid any secondary problem good restrain and good injection technique is helpful. The animal was gently grabbed over the shoulder to cross the legs over the chest for avoiding getting bit. The injection was performed at lower right quadrant to prevent hitting internal organs such as bladder, liver and cecum (Figure 13).

Maximum volume: 10ml/kg

5.5.2 Mechanism of STZ

STZ enters to the beta cells of pancreas vai (GLUT2) and there it causes alkylation of the DNA. Following the damage of DNA poly ADP ribosylation is activated. This is more important for the induction of damage itself. Ribosylation of poly ADP depletes cellular ATP and NAD^+. Enhanced ATP dephosphorylation after the treatment of STZ supplies a substrate for xanthine oxidase and in result radical of superoxide is generated. Consequently, H_2O and OH^- are produced. Furthermore, STZ liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the STZ action, B cells undergo the destruction by necrosis [185]**.**

5.5.3 Diagnosis of diabetic rats

72 h after the administration of STZ blood sample collected from tail vein was determined for blood glucose level. Glycaemia level was determined using glucometer. The lower part of tail or the palm of the back foot was disinfected by 70% alcohol, and local anesthesia was applied, a drop of blood was collected by pricking the site. Glycaemia level of 250mg/dl or more was considered as diabetic.

5.6. Biochemical test:

5.6.1 Blood collection:

Blood sample was collected on the 7th and 14th day of induction of diabetes and treatment with GTE. As per general guidelines for collecting of blood sample from rat, for maintaining of optimal health condition the volume of blood should be at a lower normal range only for healthy animal it can be at maximum level. Approximate volume for rats is 10% of total blood volume of rat [186]. The total blood volume of rat is 55-70 ml/kg of body weight and 5.5-7.0ml/kg of body weight. The blood was collected from lateral tail vein and retro-orbital route.

a) Material

- ✓ Ether
- ✓ 70% alcohol
- ✓ Syringe: 1cc
- ✓ Needle: 21-25G
- ✓ Heat source
- ✓ Sterile capillary tubes with 0.5mm diameter
- ✓ Gel containing vacutainer

b) Procedure

1. For doing a successful IV puncture in tail vein the following steps were followed:

I. Animal was anesthetized by ether.

II. Tail of animal was warmed by applying compress of warm water. The veins were located at both sides of the tail and the needle was inserted at lower portion of the tail approximately 1/3rd part of tail from the top (Figure 16).

III. Before inserting, the needle was kept parallel to the tail and the bell of needle was up side (Figure 7).

Figure 7: Position of needle for vein puncture

IV. After collecting the required amount of blood, needle should was removed and pressure was applied on the area to stop bleeding.

2. Collection of blood by retro-orbital route: However retro-orbital route is considered as a category 2 procedures but as per study design we needed high volume of blood from a live animal so we too took blood by this route (Figure 16).

I. Animal was anesthetized by ether.
II. A sterile glass capillary tube was penetrated through retro-orbital plexus.
III. About 7.5% of total blood volume was collected either in Eppendrof's tube or gel containing vacutainer.
IV. From each eye the blood was collected only once.
V. The animal was under observation for 24 h for the presence of any complication.

The blood was transferred to the yellow top colored vacutainer and allowed to clot. The serum was separated by centrifuging at 2500rpm for 10 minutes. Serum was transferred by 1.0 ml pipette to Eppendorf tubes and following tests were done on it by using commercial analytical kits.

5.6.2 Determination of blood glucose

Principle of test:

Glucose present in the sample is oxidized by glucose oxidase enzyme and yields H_2O_2 and gluconate. H_2O_2 then is coupled with 4 amino antipyrine and phenol in the presence of peroxidase thus the quininimine dye produces the intensity of which is observed at 546nm against reagent blank and is directly proportional to the H_2O_2 which in turn is directly proportional to the level of glucose in the sample [187].

Requirements:

- ✓ Semi auto analyzer(Photometer 5010 V5+)/Colorimeter (LT-12)
- ✓ Auto pipettes of 10-100µl and 100-1000 µl
- ✓ Test tubes
- ✓ Glucose assay kit

Procedure

For each assay of tests one blank test tube, one standard test tube and tubes for samples were taken and labeled accordingly, and then pipetting was done as follow:

Table 6: Pipetting procedure for glucose determination

Particular	**Blank**	**Test**	**Standard**
Reagent	1000µl	1000µl	1000µl
Distilled water	10µl		
Serum/Plasma		10µl	
Standard			10µl

The contents were mixed and incubate at room temperature (RT) for 10 min and the absorbance was measured at 546nm.

Calculation

The concentration of glucose of samples was measured by formula:

$$concentration\ of\ sample = \frac{absorbance\ of\ test}{absorbance\ of\ standard} \times concetration\ of\ standard$$

Linearity of test:

The test is linear up to 500mg/dl of glucose concentration in a sample.

5.6.3 Determination of Lipid profiles

Requirements

- ✓ Semi auto analyzer (Photometer 5010 V5+)/Colorimeter (LT-12)
- ✓ Auto pipettes of 10-100µl and 100-1000 µl
- ✓ Test tubes
- ✓ Total cholesterol assay kit
- ✓ Triglyceride assay kit

5.6.3.1Determination of total cholesterol

Total cholesterol of serum was by (CHOD/PAP) method.

Principle: Cholesterol esterase present in reagent hydrolyses cholesterol esters. Free cholesterol is then oxidized and hydrogen peroxide is generated. Which then reacts with 4-amino antipyrin and phenol, by the help of catalytic action of peroxidase a red colored compound is generated. The intensity of color is directly proportional to the concentration of cholesterol in serum sample [188].

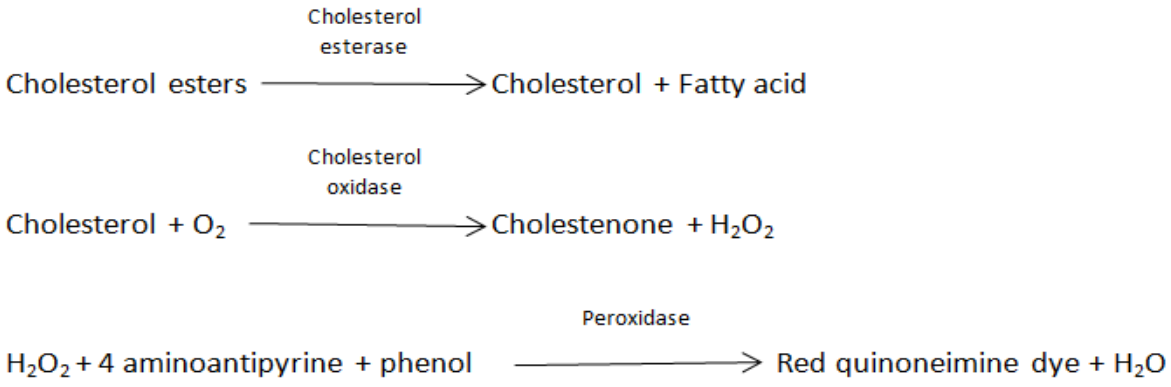


Figure 8: Principle of determination of glucose by glucose oxidase method

Procedure:

Test tubes for blank, standard and tests were labeled and pipetted as follow.

Table 7: Procedure for determination of total cholesterol

Particular	Blank	Test	Standard
Reagent	1000µl	1000µl	1000µl
Distilled water	10µl		
Serum/Plasma		10µl	
Standard			10µl

The contents of test tubes were mixed well and incubated at RT for 15 min. The absorbance of standard and tests was measured against reagent blank at 546nm.

Calculation:

$$concentration\ of\ Cholesterol = \frac{abs\ of\ sample}{abs\ of\ standard} \times conctration\ of\ standard\ (200\ mg/dl)$$

Linearity: The procedure was linear up to 750 mg/dl.

5.6.3.2 Triglycerides:
Serum triglycerides were evaluated using Erba commercial kit with GPO/PAP method.

Principle: Triglycerides of serum are hydrolysis by lipoprotein lipase present in reagent into glycerol and free fatty acids. Glycerol kinase converts glycerol into glycerol 3 phosphate by consumption of one molecule of ATP. The product is then oxidized by glycerol phosphate oxidase and H_2O_2 is produced which further react with 4-aminoantipyrin in the presence of peroxidase and a colored complex is produced [189].

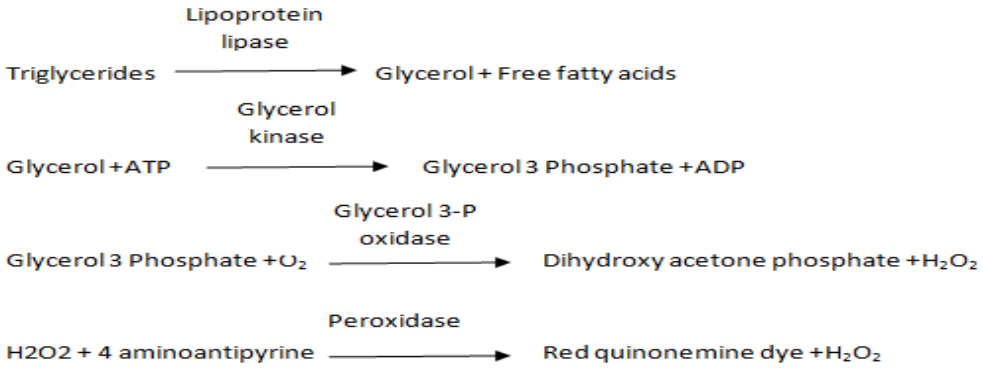


Figure 9: Principle of determination of serum triglyceride level

Procedure: Test tubes are labeled as blank, standard and test. And the reagents are added as follow.

Table 8: Pipetting procedure for determination of triglycerides

Addition sequence	**Blank**	**Standard**	**Test**
Reagent	1.0 ml	1.0 ml	1.0 ml
DW	100µl		
Standard		100µl	
Serum			100µl

Test tubes were allowed to incubate for 15 min at RT and absorbance of test and standard was measured against reagent blank.

Calculation:

$$TG\left({}^{mg}/_{dl}\right) = \frac{Abs\ T}{Abs\ S} \times 200$$

Linearity: The method is linear up to 1000mg/dl.

5.6.4 Determination of Proteins

5.6.4.1 Estimation of total protein:

Total protein of serum sample was determined by the Biuret method using Erba company's commercial kit.

Principle: Proteins react with cupric ions in an alkaline medium and produce a blue colored complex. The intensity of color is directly proportional to the concentration of proteins in the sample [190].

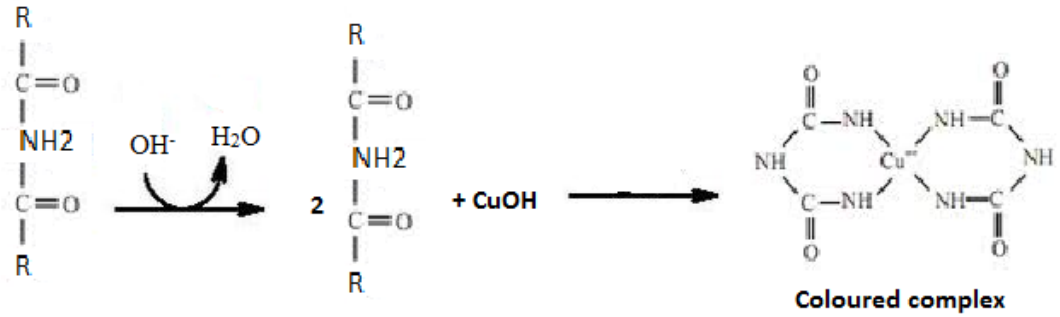


Figure 10: Principle of determination of total protein

Procedure: Test tubes were labeled as blank, standard and test and pipetting was done as follow.

Table 9: Procedure for determination of serum total protein

Additional sequence	**Blank**	**Standard**	**Test**
Reagent	1 ml	1 ml	1 ml
Distilled water	0.1 µl		
Standard		0.1 µl	
Serum			0.1 µl

The contents of tubes were mixed well and incubated for 15 minutes at RT. The absorbance was seen at 546 nm.

Calculation:

$$Total\ protein\left({}^{g}/_{dl}\right) = \frac{Abs\ T}{Abs\ S} \times 6$$

Linearity: The procedure was linear up to 15 g/dl.

5.6.4.2 Albumin:

Serum was measured by BCG (bromocresol green) method using a commercial assay kit of Erba company.

Principle: A green colored complex is produced by the binding of albumin of serum and bromocresol green of reagent in buffered medium. The intensity of color is directly proportional to the amount of albumin in serum sample [190].

Procedure: Dry and clean test tubes were labeled as standard, test and blank and pipetting was done as follow:

Table 10: Procedure for determination of serum albumin

Additional sequence	**Blank**	**Standard**	**Test**
Reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	10 µl		
Standard		10 µl	
Sample			10 µl

The contents were mixed well and incubated for one minute at RT. The absorbance was measured at 630 nm.

Calculation:

$$Albumin\left({}^{g}/_{dl}\right) = \frac{Abs\ T}{Abs\ S} \times 4$$

Linearity: The procedure was linear up to 7 g/dl.

5.6.4.3 Calculation of Albumin/Globulin ratio:

For the estimation of Alb/Glu ratio, the concentration of globulins was established from the following equation and then the ration was measured [191].

$$Glubolin = Total\ protein - Albumin$$

$$Alb/_{Glu}\ ratio = \frac{Alb}{Glu}$$

5.6.5 Estimation of Non-Protein nitrogen substances

Requirements:

- ✓ Semi auto analyzer (Photometer 5010 V5+)
- ✓ Auto pipettes of 10-100µl and 100-1000 µl
- ✓ Test tubes
- ✓ Assay kit for estimation of urea
- ✓ Assay kit for estimation of creatinine

5.6.5.1 Urea estimation:

Urea was measured by Berthelot method. In this method urea present in serum is hydrolysis to ammonia and CO_2 by urease present in the reagent. Generated ammonia then reacts with ketoglutarate and NADH to form glutamate and NAD. Rate of oxidation of NADH which is proportional to the concentration of urea is measured as a decrease in absorbance in fixed time [192].

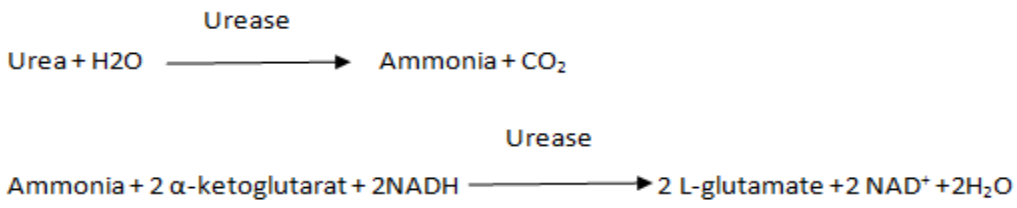


Figure 11: Principle of determination of blood urea

Procedure: Dry and clean test tubes were labeled as below.

Table 11: Addition sequence for determination of urea

Addition sequence	
Working reagent	1.0ml
Urea standard/serum	0.01ml

The mixture was mixed well and the absorbance was taken after 30 sec and then after 60 sec.

Calculation: Changes in the initial and later absorbance were measured and the value of urea was calculated according to the following equation.

$$urea\ in\ {}^{mg}/_{dl} = \frac{\Delta Abs\ T}{\Delta Abs\ S} \times 40$$

Linearity: The test was linear up to 250mg/dl.

5.6.5.2 Creatinine:
Creatinine was measured by the method of alkaline pictrate.

Principle: Creatinine present in serum reacts with picric acid in alkaline medium and an orange colored complex is generated. The intensity of produced color is directly proportional to the concentration of creatinine in the sample [193].

Procedure: Equal volume of R1 (picric acid) and R2 (alkaline solution) were mixed. Two test tubes were labeled as standard and test and then pippeting was done as below.

Table 12: Procedure for the determination of serum creatinine

Addition sequence	**Test**	**Standard**
Reagent	1ml	1ml
Serum	0.1ml	
Standard		0.1 ml

The contents of test tubes were mixed well and the initial absorbance was taken after 30sec and later absorbance was taken after 60sec.

Calculation: Level of creatinine was measured according to the formula.

$$creatinine\ in\ {}^{mg}/_{dl} = \frac{\Delta AT}{\Delta AS} \times 2$$

Linearity: This method is linear up to 20mg/dl.

5.6.6 Estimation of Electrolytes:

5.6.6.1 Estimation of sodium*:*

Sodium was determined by the commercial kit of BEACON Company for in vitro serum determination.

Principle: In this method the chromogen compound present in the reagent was undergone to react with serum sodium and the chromophore was produced the concentration and absorbance of which was directly proportional to the concentration of sodium in serum sample [191].

Procedure: After the immediate centrifugation of blood and separation of serum from blood, dry and clean test tubes were labeled as standard, test and blank and reagents were added as follow:

Table 13: Procedure for the determination of serum sodium level

Additional sequence	**Blank**	**Standard**	**Test**
Reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	10 µl		
Standard		10 µl	
Serum			10 µl

The contents of tubes were mixed well and incubated for 5 minutes at RT and the absorbance was seen at 630 nm.

Calculation:

$$concentration\ of\ sodium\ ({}^{mEq}/_{L}) = \frac{Abs\ T}{Abs\ S} \times 150$$

Linearity: The procedure was linear up to 80mEq/L.

5.6.6.2 Estimation of Potassium*:*

Potassium level of serum sample was determined by using the commercial kits of BEACON Company for determination of in vitro serum potassium.

Principle: Potassium of serum was brought to react with sodium tetraphenol boron prepared in a special buffer and a colloidal suspension was formed. The amount of turbidity of suspension was directly proportional to the concentration of potassium in serum sample [191].

Procedure: A serum sample free of hemolysis was separated immediately from RBCs and was pippeted in labeled test tubes as follow:

Table 14: Procedure for the determination of serum potassium level

Additional sequence	**Blank**	**Standard**	**Test**
Reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	10 µl		
Standard		10 µl	
Serum			10 µl

The mixtures were mixed well, incubated at RT for 5 minutes and the absorbance was seen at 630nm against a reagent blank.

Calculation:

$$concentration\ of\ potassium\left({}^{mEq}/_{L}\right) = \frac{Abs\ T}{Abs\ S} \times 5$$

Linearity: The procedure was linear up to 7mEq/L.

5.6.7 Estimation of antioxidant level:

Serum total anti-oxidant capacity was measured by determination of DPPH free radical scavenging capacity of the serum sample. For this purpose, we analyzed serum sample by a slight modification of Chrzczanowicz *et al*. method [194].

Requirements:

- ✓ Auto pipettes (10 – 100)µl and (100 – 1000) µl
- ✓ Cooling high speed microfuge
- ✓ Spectrophotometer
- ✓ Acetonitrile (9.5mmol/l)
- ✓ Methanol
- ✓ DPPH (1mmol/l sol in methanol)

Principle of test: DPPH is a colored compound with maximum absorbance at 517nm. There is one unpaired electron on nitrogen base of this compound. Whenever DPPH comes in contact with any anti-oxidant it reduces by accepting electron and forms 1,1 diphenyl, 2 picryl hydrazine. The latter produced compound does not have absorbance at 517nm, so the decrease in absorbance is directly proportional to the radical scavenging activity of compound.

Procedure:

1. Deproteinization of serum: 200 µl of serum was mixed with 200 µl of 9.5mmol/l acetonitrile. The mixture was incubated at RT for two minutes and then centrifuged at 4˚C, 9500g for 10 minutes.

2. Four test tubes were taken and labeled as blank, standard, test and sample blank. The pipetting was done as follows (this step was modified according to the size of cuvette).

Reagent blank: 2.25 ml methanol+ 75 µl acetonitrile + 600 µl methanol

Standard: 2.25 ml methanol +600 µl DPPH +75 µl acetonitry

Test: 2.25 ml methanol +600 µl DPPH +75 µl deproteinized serum

Sample blank: 2.25 ml methanol+ 75 µl deproteinized serum + 600 µl methanol

The contents were mixed well and incubated at RT for 30 minutes. The absorbance was measured at 517nm against a reagent blank.

Calculation:

$$Abs_{sample} = Abs_{\ test} - Abs_{sample\ blank}$$

$$\%\ radical\ scavenging\ activity = \frac{Abs_{stand} - Abs_{sample}}{Abs_{standard}} \times 100$$

5.7 Histological studies:

The animals were scarified after 14 days of treatment; blood was collected by cardiac puncture. The organs were taken and fixed with 10% formalin. On the next day grossing was done and samples were fixed in 10% formalin for 24 h. The tissues were dehydrated and processed with three changes of pure acetone and then with three changes of pure chlorophorm. Impregnation and embedding were done with paraffin wax and 5µm sections were cut down by microtome. Sections were rehydrated with graded ethanol series. Sections were stained with H&E stain. Rehydration through graded ethanol and clearing with Xylene was done. Stained sections were mounted with DPX and assessment under the light microscope at 10x lens was done [195].

5.8 Statistical tool:

Results are presented as $mean \pm 2SEM$. Data were analyzed by using unpaired, one tailed Student's t-tes. MS – Excel software was used for the application of these tools. p value of <0.01 and 0.05 were considered as significantly important for comparison.

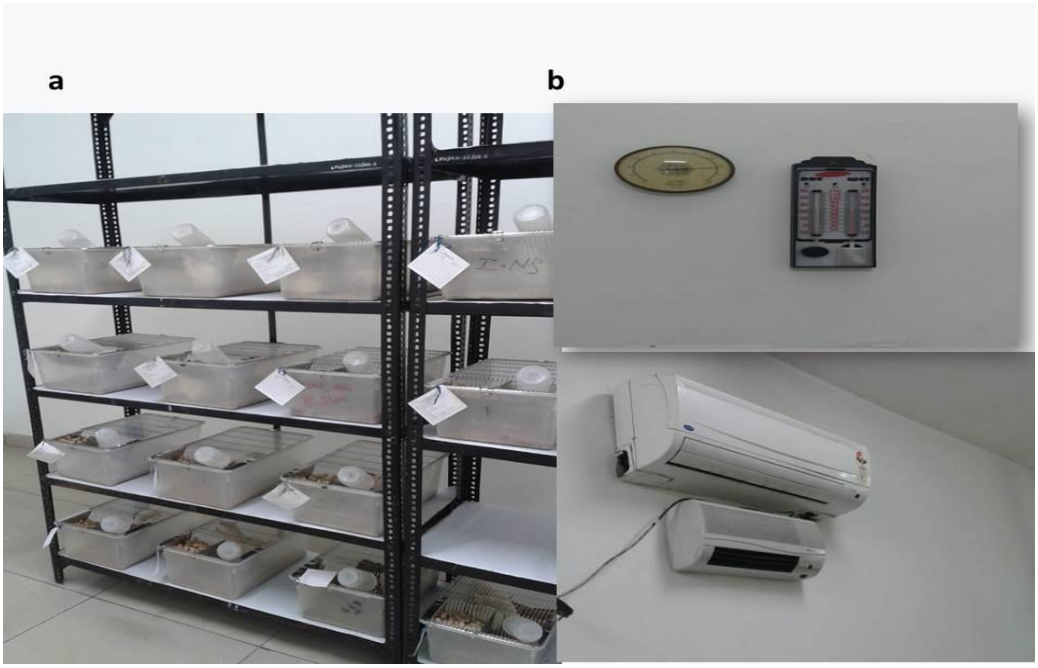


Figure 12: animal house

a:cages of rats with proper labeling b: facilities of animal house for control of room temperature

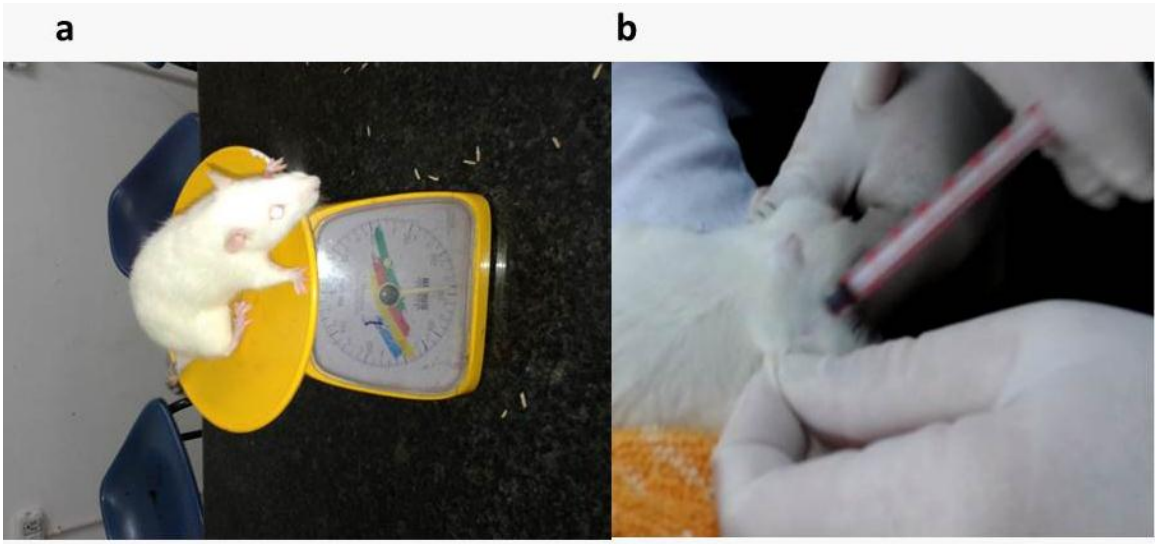


Figure 13: weighing and IP injection to rat

Figure 14: Determination of FBS by glucometer

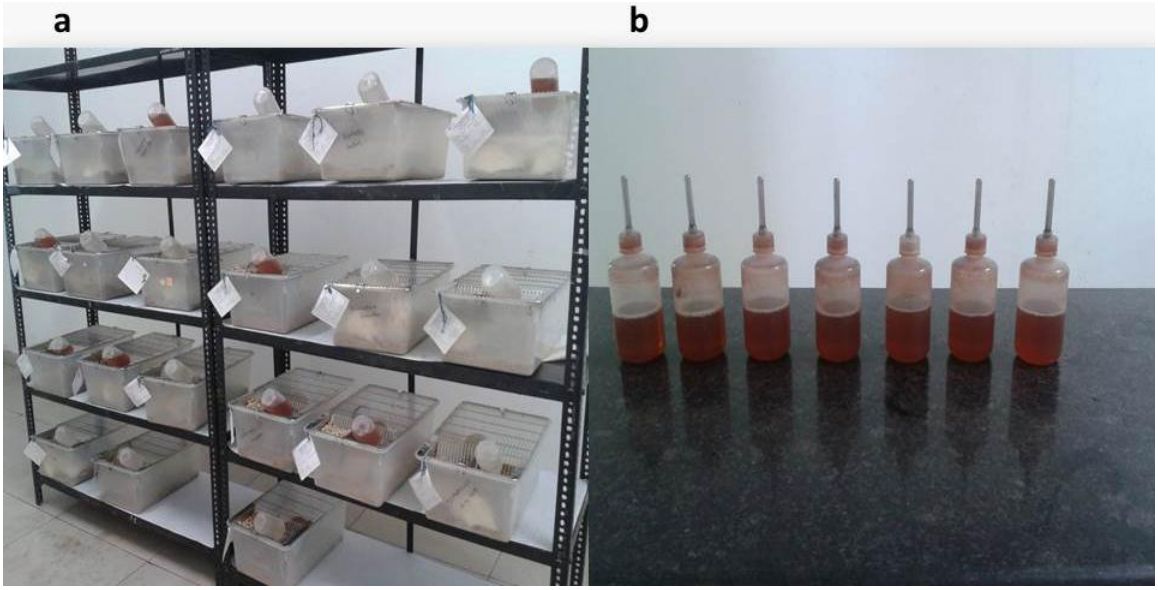


Figure 15: supplementation of GTE to rats

a: cages of rats supplemented by GTE b: GTE dissolved in drinking water of rats

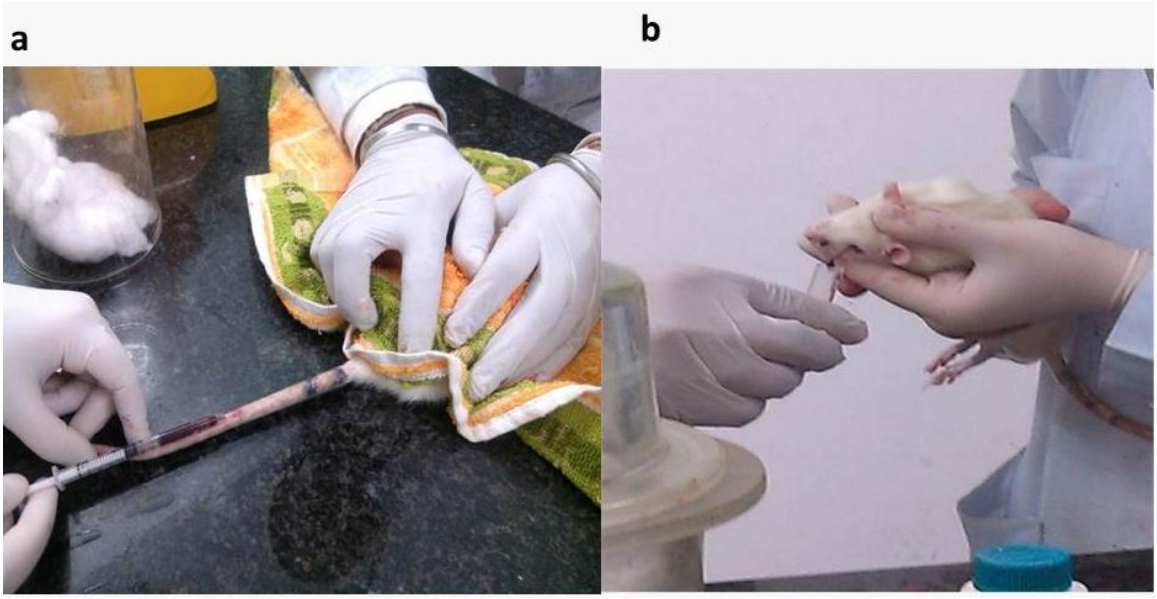


Figure 16: Blood collection

a: blood collection from tail vein b: blood collection by retro-orbital route

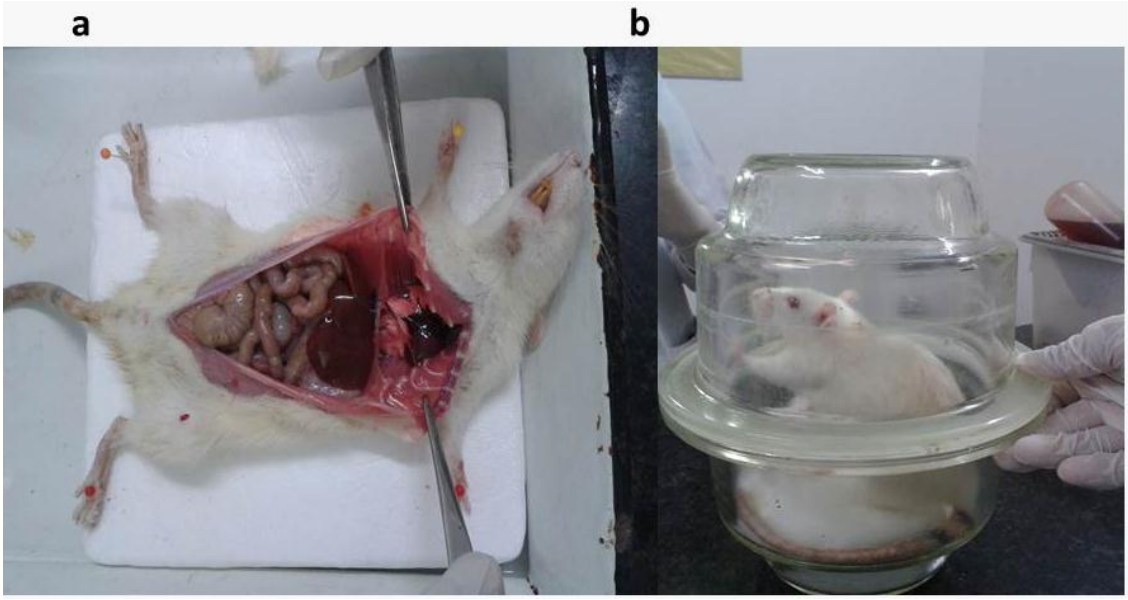


Figure 17: dissection of rats

a: dissected rat b: anesthetizing of rat before dissection

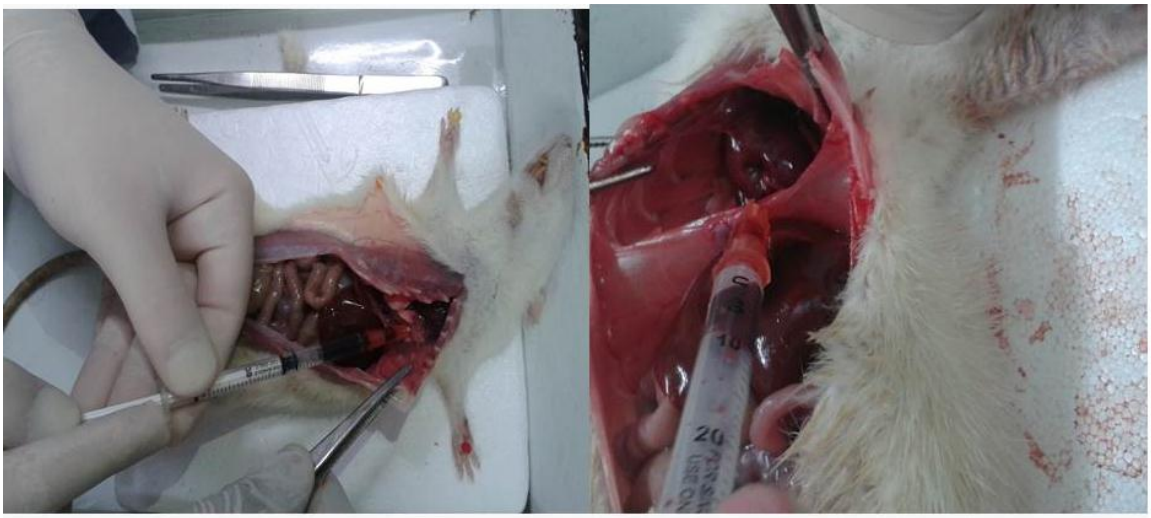


Figure 18: collection of blood by cardiac puncture

Figure 19: biochemical tests on serum samples

CHAPTER 6

RESULTS

6.1Body weight:

Body weights of all groups are showed in Table 15 (

Figure 20). Body weight of diabetic control group was reduced significantly ($p<0.01$) as compare to its initial weight. Weight of normal control was increased in 14 days at significant level of $p< 0.05$, while normal group treated with green tea extract remained unchanged. Weight of diabetic group treated with green tea was also reduced.

6.2 Blood Glucose Level:

Blood glucose level of all four groups is shown in Table 16 (Figure 21) and their comparison during experimental duration is presented in graph 2. Fasting blood sugar level of normal control was in the limit of 70- 110 mg/dl, similar to FBS level of humans. On the third day of IP administration of STZ to diabetic control and experimental group the FBS level of the animal was more than 250 mg/dl, but after compensation of animals the level decreased at level of 130 or more. FBS level of diabetic group remained high throughout the experiment. Administration of green tea extract was able to control sugar level of a diabetic animal significantly. However, the difference between sugar level of diabetic control and the treated group was not significant on the 7th day but on the 14th day the FBS level of the treated group was decreased at a significant level of $p<0.05$.

6.3 Lipid Profile:

6.3.1: Total Cholesterol:

Level of serum cholesterol in the control group was in the range of 80 – 90 mg/dl(Table 17,Figure 22). The level of serum cholesterol was increased in diabetic control in the first week but not at a significant level. However, level of cholesterol was increased in the diabetic group in the second week of the experiment at a significant level ($p<0.05$). The level of cholesterol in the treatment group was not significantly increased and there was significant difference between cholesterol level of diabetic and treatment group at the end of study. We also compared the serum cholesterol level of normal control and normal control treated with GTE to estimate the effect of GTE on the cholesterol level of the normal individual. In first week of our experiment there was not any change in

cholesterol level of normal group following GTE treatment but in second week cholesterol was significantly ($p<0.05$) decreased.

6.3.2: Triglyceride:

Normal range for serum triglyceride level was estimated according to the range of serum triglyceride in the normal control group. The levels obtained from normal control at different time interval were in the range of 40-50mg/dl. Level of triglyceride of a control group treated with GTE was decreased at a significant level at the end of experiment. The level of TG of diabetic animals increased during this period. The difference was not significant at the first sample collection but till the end of experiment the serum level of TG increased significantly ($p<0.05$) by diabetes. Treatment of green tea was able to decrease the TG in both normal and diabetic groups at a significant level of $p <0.01$ and $p < 0.05$ respectively. Table 18 (Figure 23) indicates results of serum TG level in all four groups.

6.4 Serum Proteins:

Normal range for total protein according to the level of the normal control group and the method of estimation was (6-8) g/dl. Concentration of serum albumin was in the range of 2.5-5 g/dl for the normal control group. And thus calculated Alb/Glu ratio for the normal group was about 1.5-3. In the first week of our experiment, there was not any change in total as well as in albumin level of all groups. In the second week the decline in serum protein in all groups was seen, but the level of proteins was remained in the normal range. Level of albumin and Alb/Glu ratio of normal control and normal control treated with GTE was in normal range. Albumin level of diabetic and diabetic group treated with GTE was low or at lower normal range. The Alb/Glu ratio of diabetic control and diabetic group treated with GTE was considerably ($p<0.01$) less than normal groups. There was no significant change between diabetic control and diabetic group treated with GTE. (Table 19,Figure 24, Figure 25, Figure 26)

6.5 Non protein nitrogen compounds

6.5.1 Creatinine:

Serum creatinine level of the normal group was within range of (0.5-1.5) mg/dl. We compare the creatinine level of experimental groups with this limit to estimate renal function of the animal. Changes in creatinine level during the experimental time period were not significant. The results of serum creatinine are given in Table 20(Figure 27).

6.5.2: Serum Urea level:

Normal range for blood urea level was considered less than 35mg/dl as per literatures and values obtained from the normal control group. After induction of diabetes, the urea level of the diabetic group was increased at a significant level of $p<0.05$ in our both experimental analysis. Urea level of the treated group was also significantly higher than the normal group but difference between diabetic control and diabetic group treated with GTE was also significant ($p< 0.05$).Table 21, Figure 1

6.5.3 Urea clearance:

Diabetic control and treatment group produce polyuria; there was no significant difference in urine volume between both these groups. Urine volume of normal group treated with green tea was also increased but not significantly. Urea clearance was significantly decreased in diabetic control group ($p < 0.05$). Clearance was improved by treatment with GTE in the experimental group but the level of improvement was not in 95%CI yet ($p=0.06$). Continuation of therapy with GTE may be beneficial. Results of urea clearance are shown in Table 22(Figure 29).

6.7 Electrolytes:

6.7.1 Sodium:

In the present study serum sodium level of normal control was within 135-145mEq/l that was according to our environment, method or instruments of analysis. So we compared the experimental groups with this range. Level of sodium of diabetic control was decreased at a significant level ($p<0.05$). There was no any significant change in serum

sodium level of diabetic group following GTE supplementation as compare to normal control and the situation was improved significantly ($p<0.05$).Table 23,Figure 30

6.7.2 Potassium:

Potassium level of normal control was within (2-3) mEq/l. In diabetic group level of potassium increased significantly ($p< 0.050$) while change in animal treated with GTE was not significant. There was not any change in normal control followed by GTE administration. Table 24, Figure 1

6.8 Total anti -oxidant capacity:

Total anti- oxidant capacity of normal control according to the modified method and the instruments that we used was in the range of (30-45) %. Differences in all other groups were compared with this range. TAC in diabetic control group was considerably decreased as compare to normal group. Free radical scavenging activity was improved following consumption of GTE by the normal group ($p =0.05$). TAC of experimental group (D+GT) was still significantly ($p <0.01$) less than normal control, but as compare to diabetic group the improvement was significant ($p <0.05$).Table 25, Figure 32

6.9 Histology:

H&E stain of renal tissue did not show any mesangial expansion in diabetic control and also in experimental group. Kimmel Willson noduls were not observed in any group till the end of study. Any observable change in glomerular basement membrane was not reported. However, the kidney of paralyzed animal due to hyperglycemia showed signs of inflammation and the postmortem analyze of diabetic animal showed tubular secretion and fatty changes. Figure 39,Figure 39,Figure 42

6.10 Tables of results

Table 15: weekly body weight of all groups

Groups	**7th day**	**14th**	***14th day***
N	236 ± 10	243 ± 10	*s*249 ± 10
N+GT	247.5 ± 10	245 ± 10	243.8 ± 10
D	236.6 ± 10	210.8 ± 10	183.3 ± 10
D+GT	230 ± 10	208 ± 10	186.6 ± 10
N: Normal control N+GT: Normal control treated with GTE *D: Diabetic control D+GT: Diabetic group treated with GTE*			

Table 16: FBS level on 7th and 14th day

Groups	**7th day**	**14th**
N	78.6 ± 18	79 ± 16
N+GT	84 ± 13	77 ± 14
D	151 ± 15	154 ± 12
D+GT	140 ± 5	122 ± 6

Table 17: Serum cholesterol level

Groups	**7th day**	**14th**
N	**86. 6 ± 4**	**86. 6 ± 4**
N+GT	**83 ± 6**	**70 ± 5**
D	**94 ± 13**	**96 ± 7**
D+GT	**83 ± 7**	**85 ± 4**

Table 18: Serum TG level of all groups

Groups	7th day	14th
N	49 ± 4	49.5 ± 6
N+GT	44 ± 5	40 ± 5
D	60.5 ± 6	79 ± 15
D+GT	75 ± 13	56 ± 24

Table 19: Results of total protein, Albumin and Alb/Glu ration

Groups	Total Protein		Albumin		Alb/Glu ration	
	7th day	14th day	7th day	14th day	7th day	14th day
N	7.5 ± 2	6.5±1	5 ± 1	4.3 ± 1	2.7 ± 0.5	2 ± 0.6
N+GT	7.3 ± 2	6.5 ± 1	4.7 ± 1	4.2 ± 1	2.5 ± 0.5	2 ± 0.5
D	7.1 ± 2	6.6 ± 1	4.8 ± 1	3.5 ± 1	2.6 ± 0.5	1.1 ± 0.5
D+G	7.2 ± 2	6.2 ± 1	4.8 ± 1	3.2 ± 1	2.4 ± 0.5	1. ±0.5

Table 20: Serum Creatinine level

Groups	7th day	14th
N	0.9 ± 0.5	0.91 ± 0.5
N+GT	0.87 ± 0.5	0.82 ± 0.5
D	0.97 ± 0.4	1.08 ± 0.45
D+GT	0.86 ± 0.5	0.86 ± 0.5

Table 21: Serum urea level

Groups	7th day	14th
N	30 ± 6	30 ± 6
N+GT	30 ± 6	30 ± 6
D	144 ± 20	154 ± 17
D+GT	95 ± 24	83 ± 10

Table 22: Comparative result of urea clearance

Group	Urine Volume (ml/h)	Urea Clearance	Urinary Protein
N	1.2 ± 4	7 ± 2	Nil
N+GT	1.5 ± 4	7 ± 2	Nil
D	5 ± 1.5	0.39 ± 0.04	+++
D+GT	5 ± 1.4	0.56 ± 0.13	+/++

Table 23: Result of serum sodium level

Groups	7th day	14th
N	139 ± 5	140 ± 5
N+GT	137 ± 5	138 ± 5
D	131 ± 4.5	135 ± 3
D+GT	140 ± 4	139 ± 4.7

Table 24: serum level of K+

Groups	7th day	14th
N	3.6 ± 0.9	3.66 ± 0.9
N+GT	3.5 ± 0.9	4 ± 1.1
D	4.1 ± 0.8	4.1 ± 0.7
D+GT	3.4 ± 0.7	4.04 ± 0.9

Table 25: TAC capacity of serum

Groups	DPPH scavenging capacity (%)
N	39 ± 5
N+GT	44.5 ± 3
D	21.2 ± 3
D+GT	26.4 ± 4

6.11 Graphical presentation of results

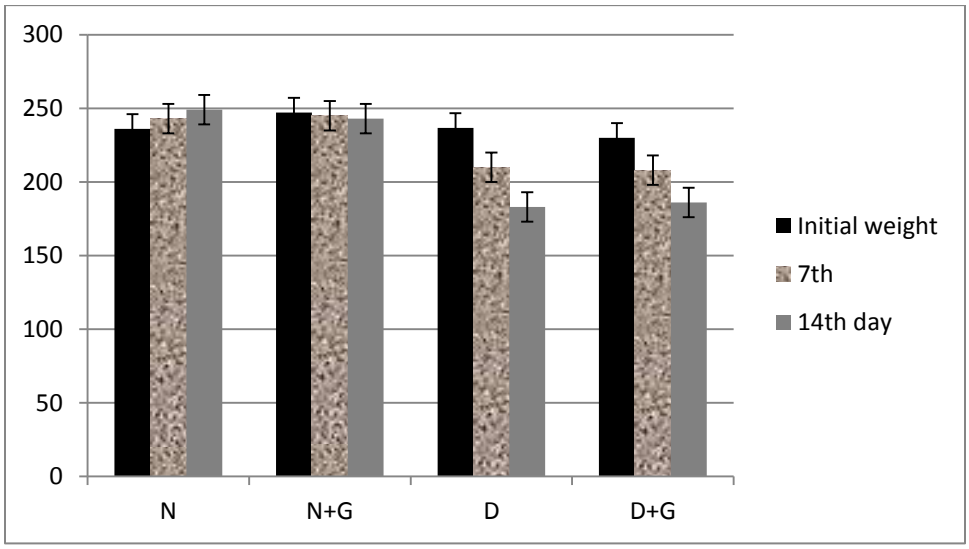


Figure 20: effect of DM, and GTE on body weight

Weight of diabetic control decreased (p<0.05). GT supplementation in normal rats inhibits weight gain and there was not any effect of green tea on weight of diabetic animals.

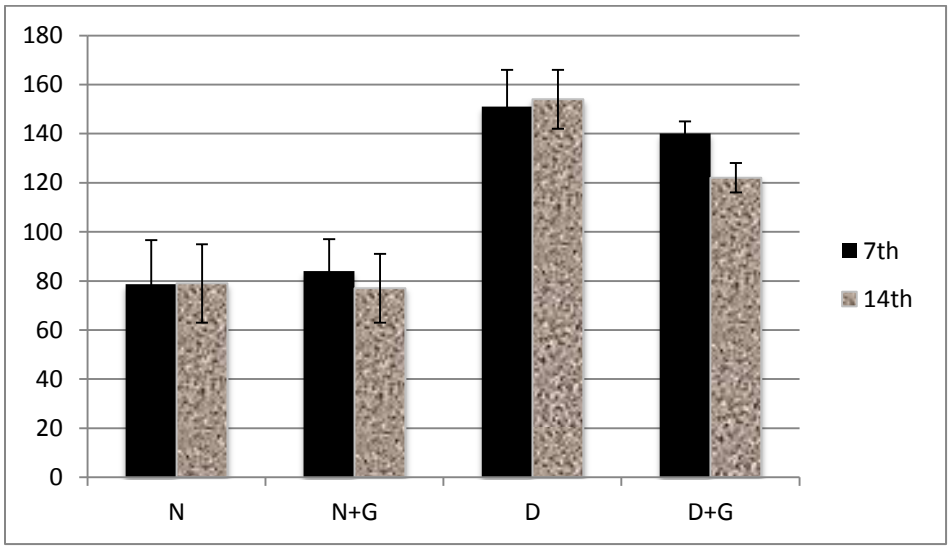


Figure 21: effect of DM and green tea on serum glucose level

.DM increased blood glucose level significantly (p<0.01). Green tea supplementation decrease blood glucose of diabetic rats significantly (p<0.01) while did not affect blood glucose level of normal animals.

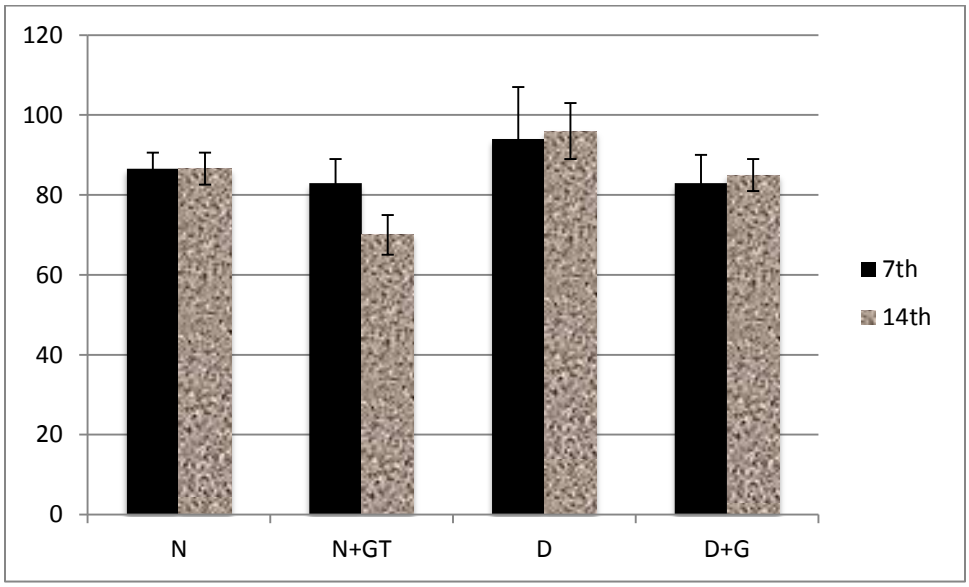


Figure 22: effect of DM and GTE on serum cholesterol level.

Serum cholesterol of diabetic animal was significantly increased in 14 days. GTE significantly decreased serum cholesterol level of normal and diabetic animal ($p<0.05$).

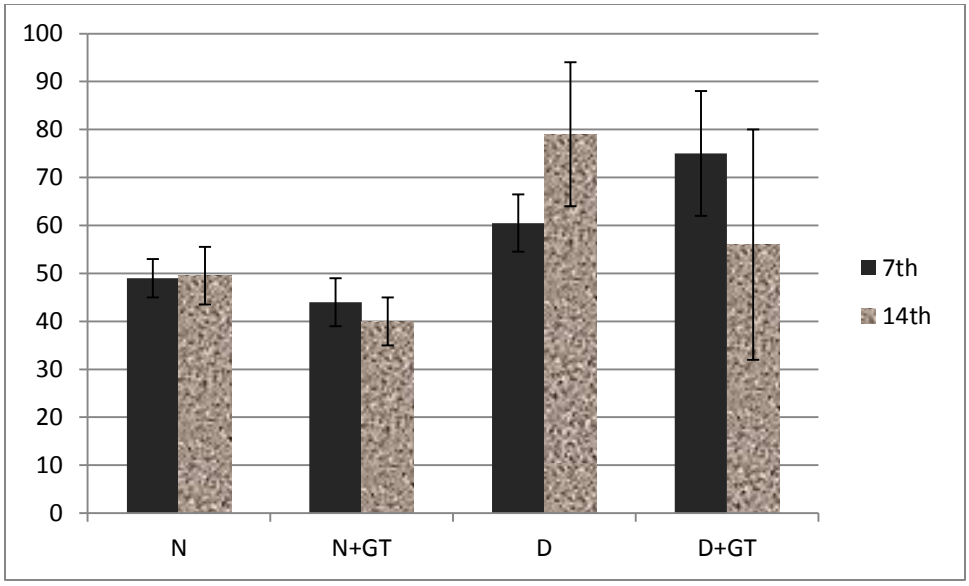


Figure 23: Effect of DM and GTE on serum TG level. Cholesterol of diabetic control group was increased significantly ($p<0.05$). Supplementation of GTE significantly decreased TG level in normal and also in diabetic animals at significant level of $p<0.01$ and $p<0.05$ respectively.

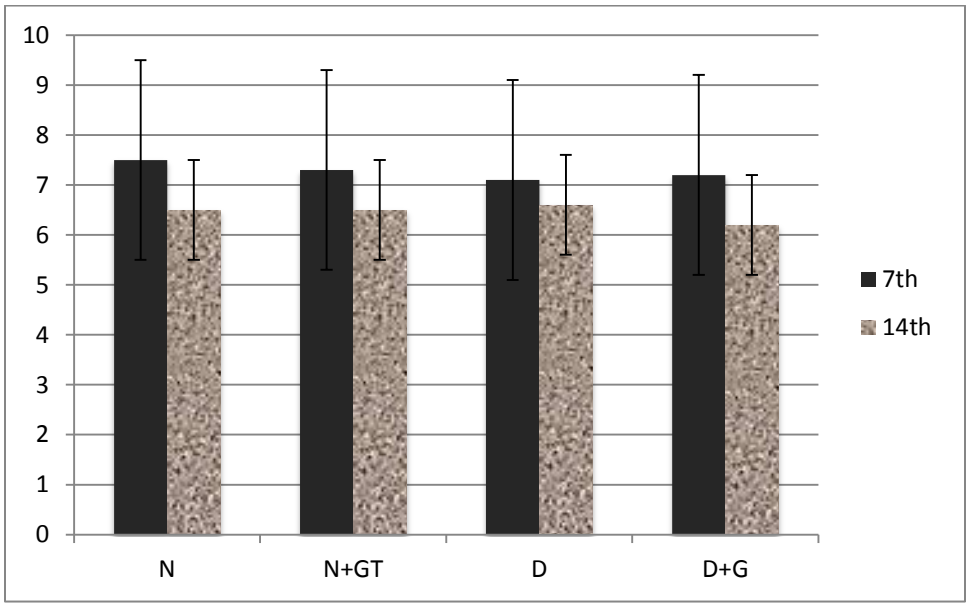


Figure 24: Effects of DM and GTE on serum total protein.

Any significant difference in the level of total protein was not observed.

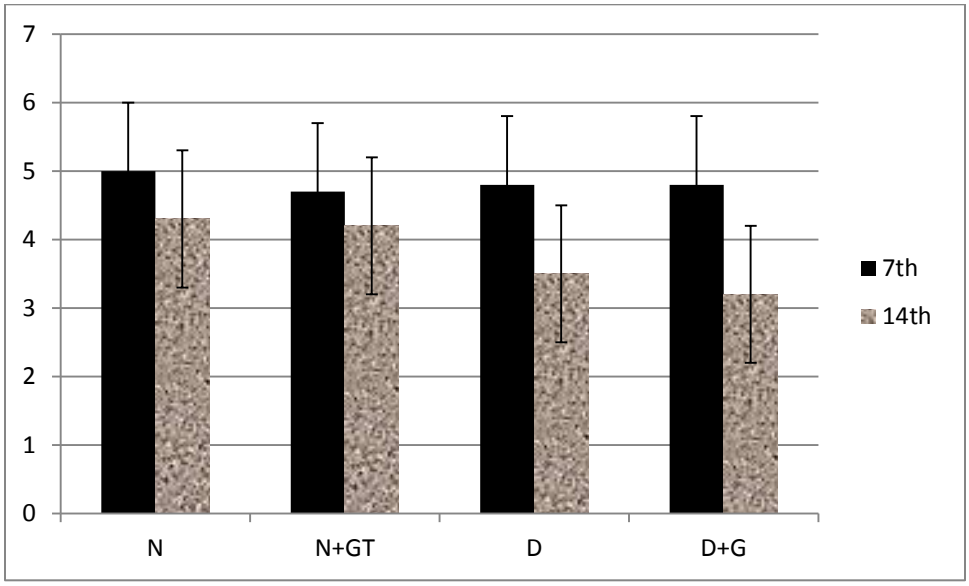


Figure 25: effect of DM and GTE on serum albumin level.

Serum albumin was significantly decreased in diabetic groups ($p<0.05$). There was not any effect of GTE on albumin level of normal and diabetic animals.

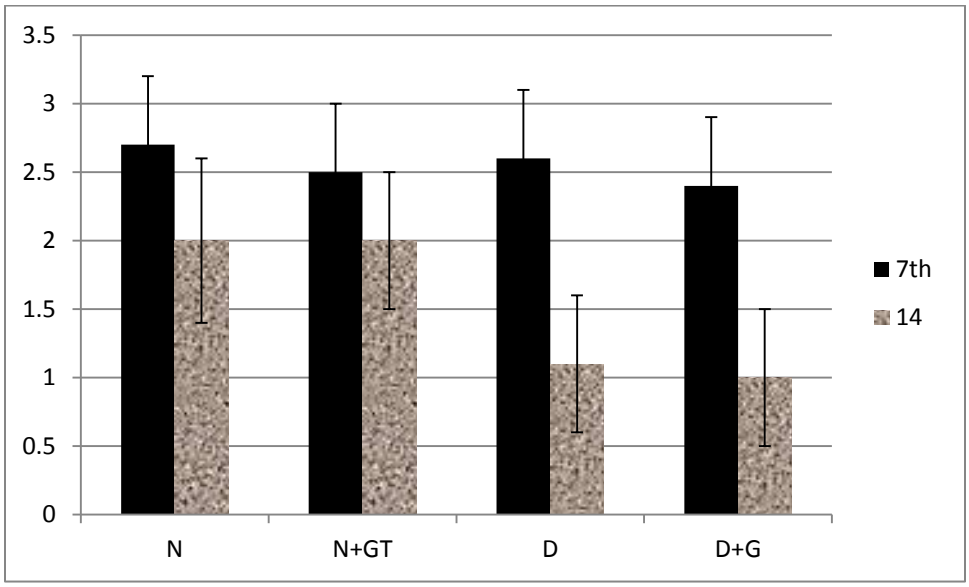


Figure 26: Effect of DM and GTE on Alb/Glu ratio.

Ratio was decrease in diabetic groups significantly ($p<0.05$). There was not any effect of GTE on Alb/Glu ration of normal and also diabetic animals.

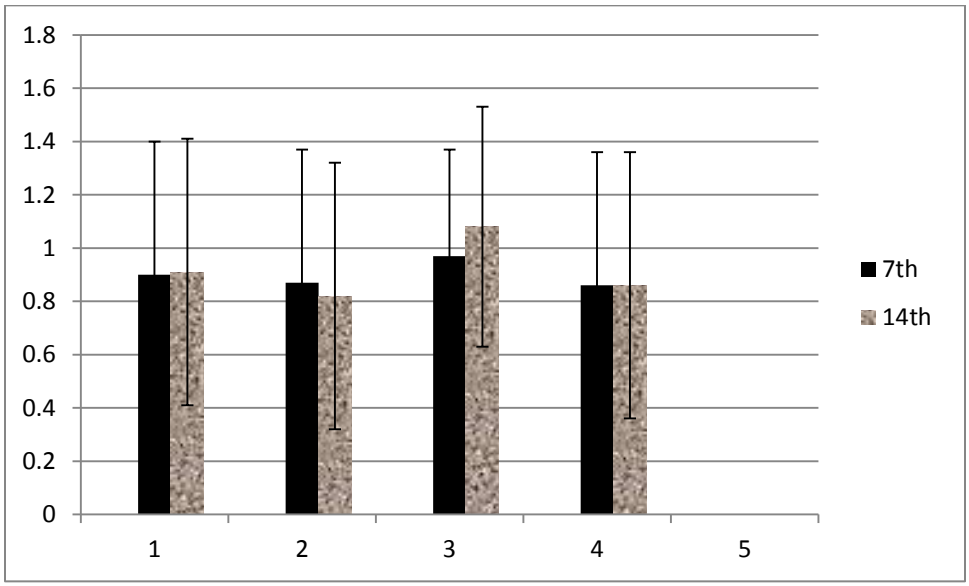


Figure 27: Effect of DM and GTE on serum creatinine level.

Serum creatinine was not changed in any group till the end of study.

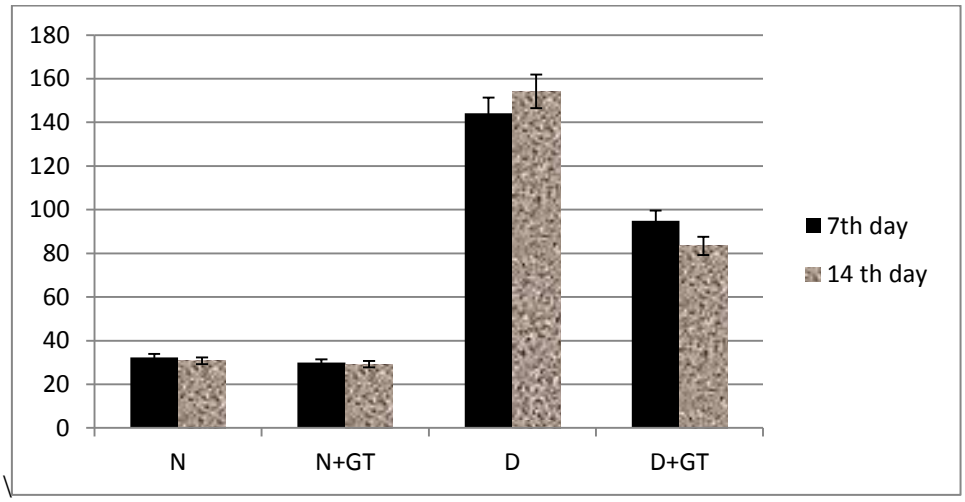


Figure 28: effect of DM and GTE on serum urea level.

Serum urea was significantly ($p<0.05$) increased in diabetic group. GTE decrease urea level of diabetic animal at significant level of ($p<0.05$) while did not affect urea level of normal group supplemented with GTE.

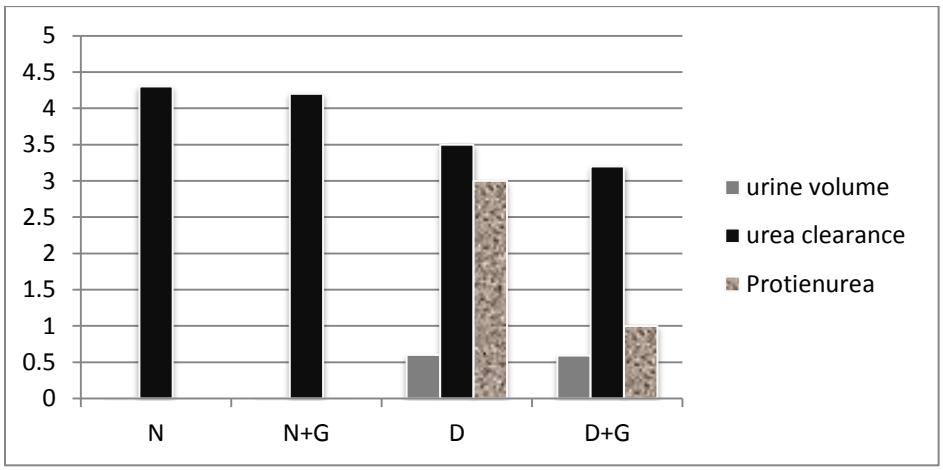


Figure 29: effect of DM and GTE on urea clearance and urine parameters.

Urine volume of diabetic groups was increased ($p<0.01$). There was not any difference between urine volume of diabetic and didactic group taking GTE. Urea clearance of diabetic group was decreased significantly ($p<0.05$). Supplementation of GTE improved clearance but not significantly.

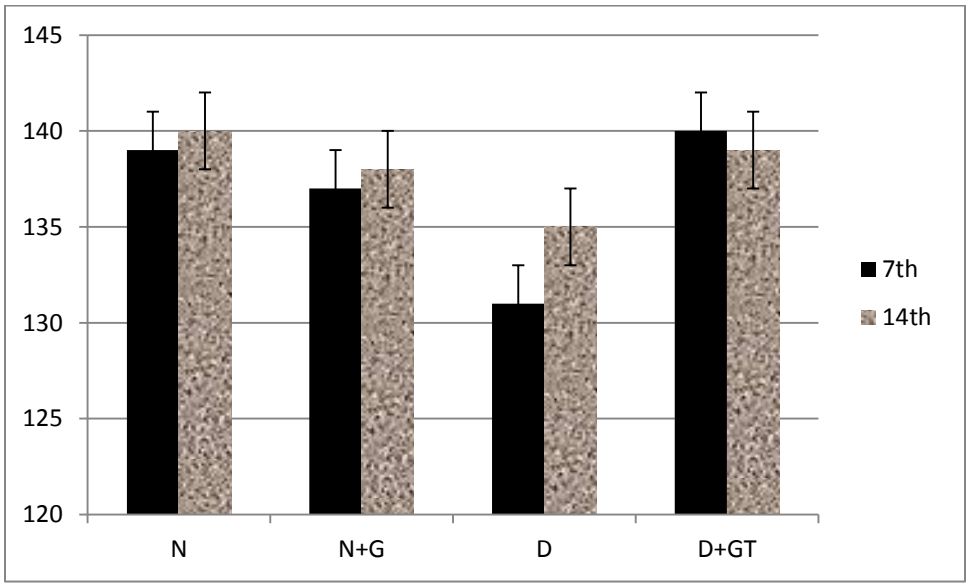


Figure 30: effect of DM and GTE on serum sodium level.

Serum sodium level was significantly ($p<0.05$) decreased in diabetic animal. Supplementation of GTE caused significant ($p<0.05$) improvement in sodium level of diabetic animal. It did not affect sodium level of normal animals.

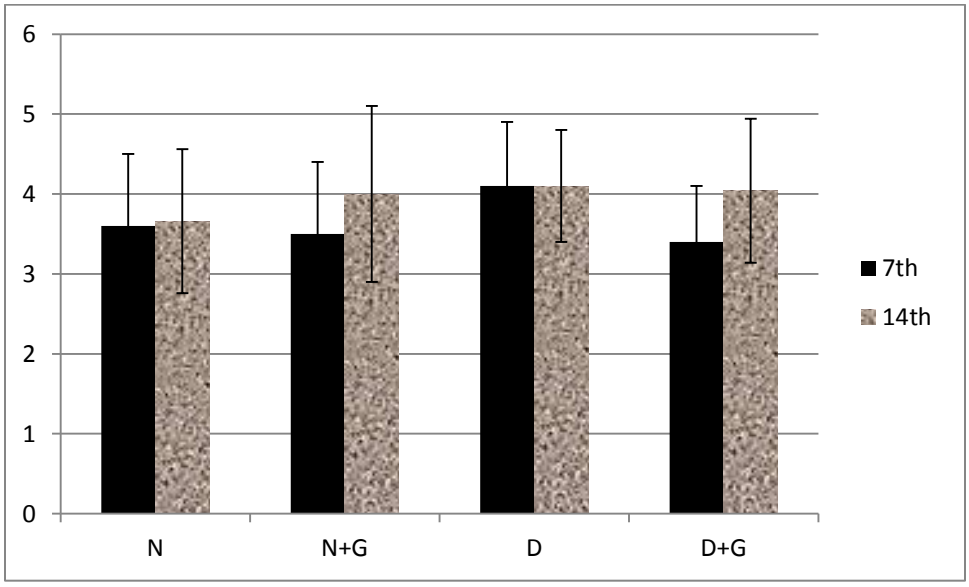


Figure 31: effect of DM and GTE on serum K+ level.

Serum K+ was increased in diabetic control group significantly ($p<0.05$). There was not any effect of GTE on Serum K+ of normal and diabetic groups.

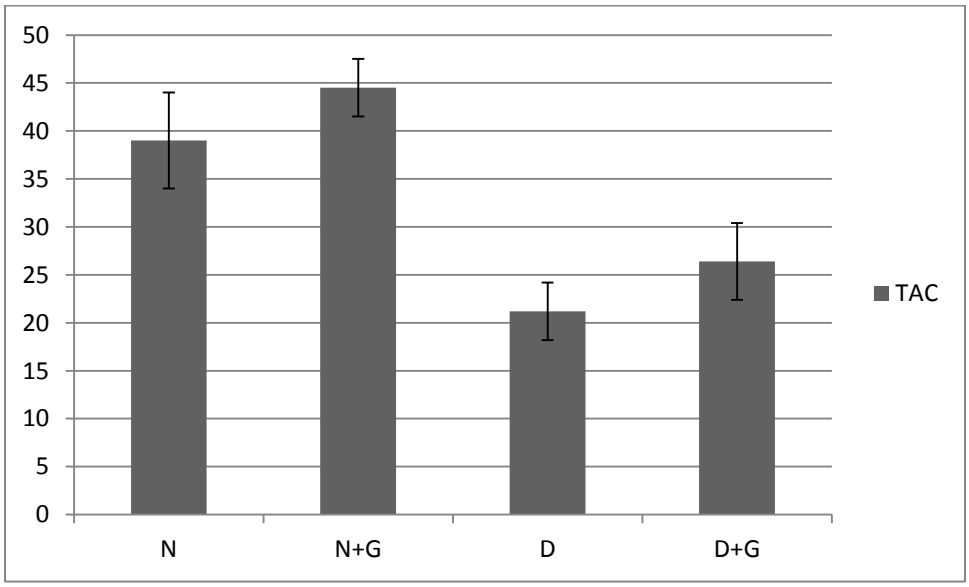


Figure 32: effect of DM and GTE on TAC of serum.

TAC was decreased by DM ($p<0.01$). TAC improved by GTE in normal individual ($p=0.05$) and in diabetic group as compare to diabetic control by significant level of ($p<0.05$

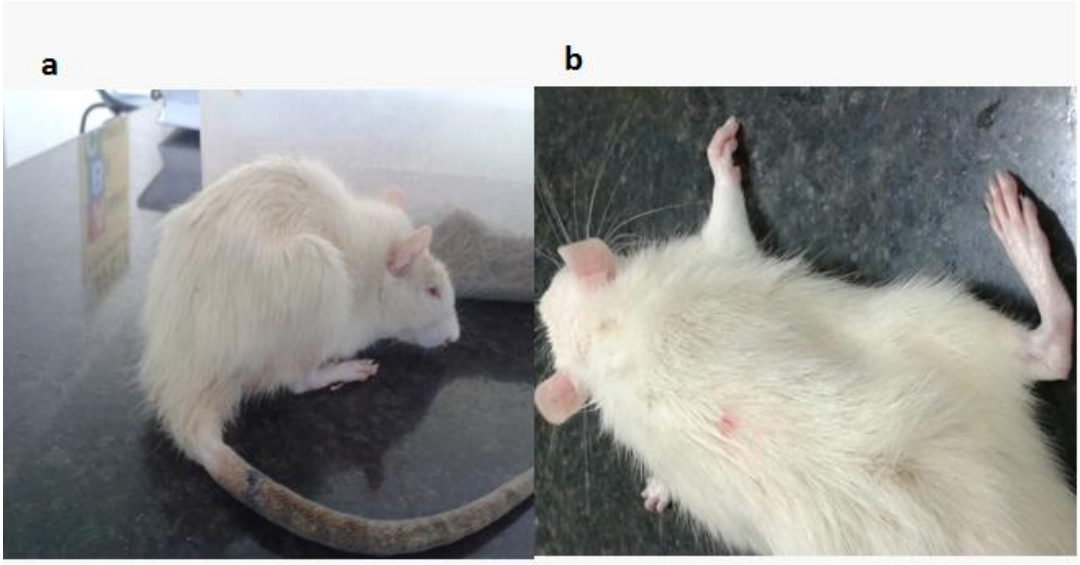


Figure 33: morphology of diabetic rat

a: diabetic rat on 14th day of diabetes b: paralyzed rat due to acute hyperglycemia

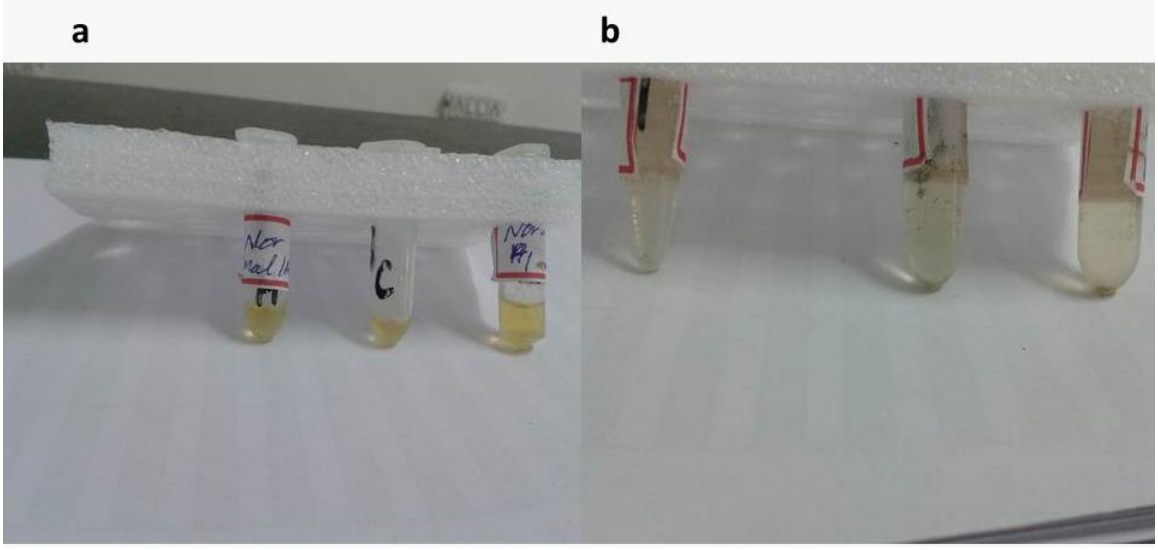


Figure 34: urine samples of rats

a: urine sample of normal rate in 15 minutes b: urine sample of diabetic rats

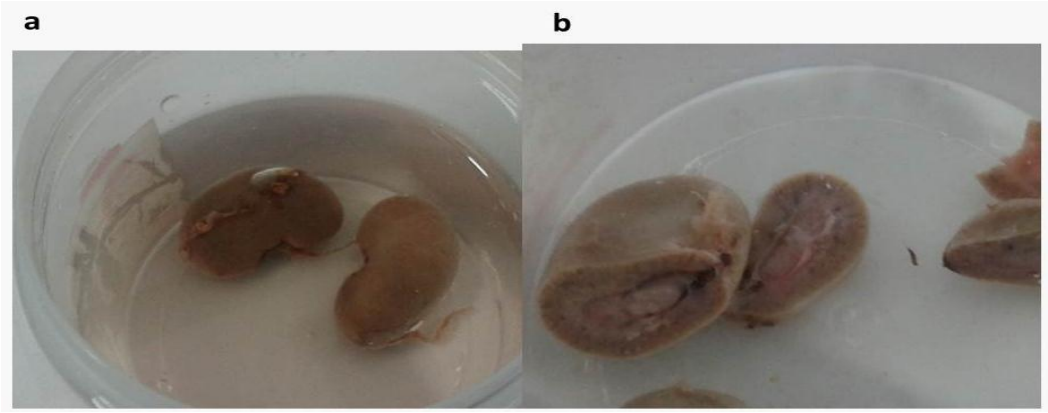


Figure 35: morphology kidney of normal anim

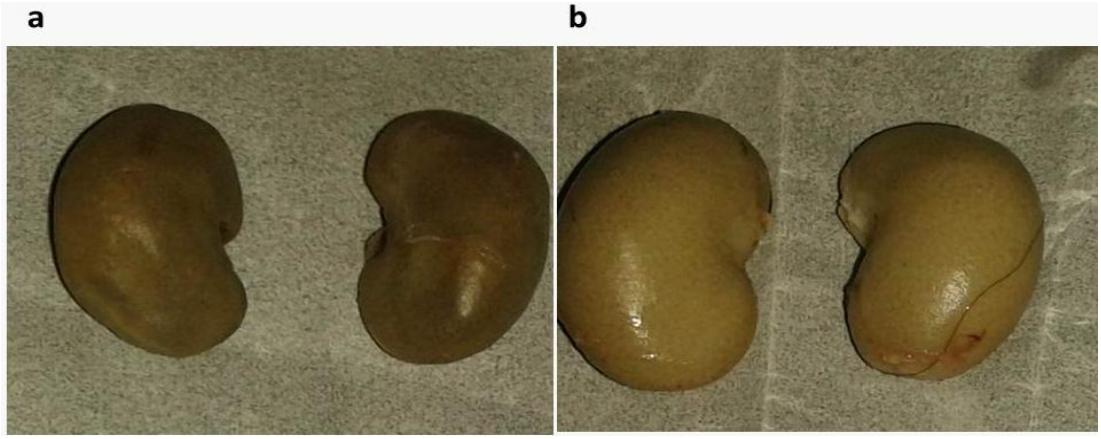


Figure 36: postpartum samples of kidney

a: kidney of diabetic rat b: kidney of normal rat

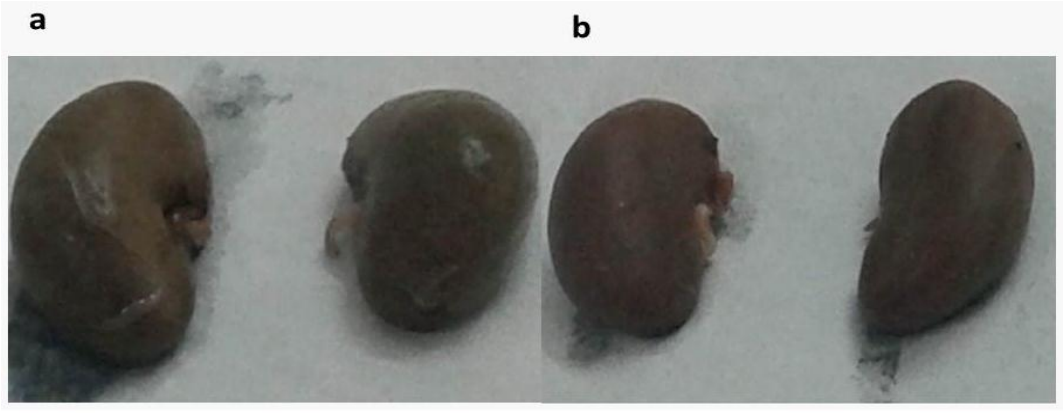


Figure 37: kidney of control and experimental group

a: kidney of experimental animal(D+GT) b: kidney of diabetic animal

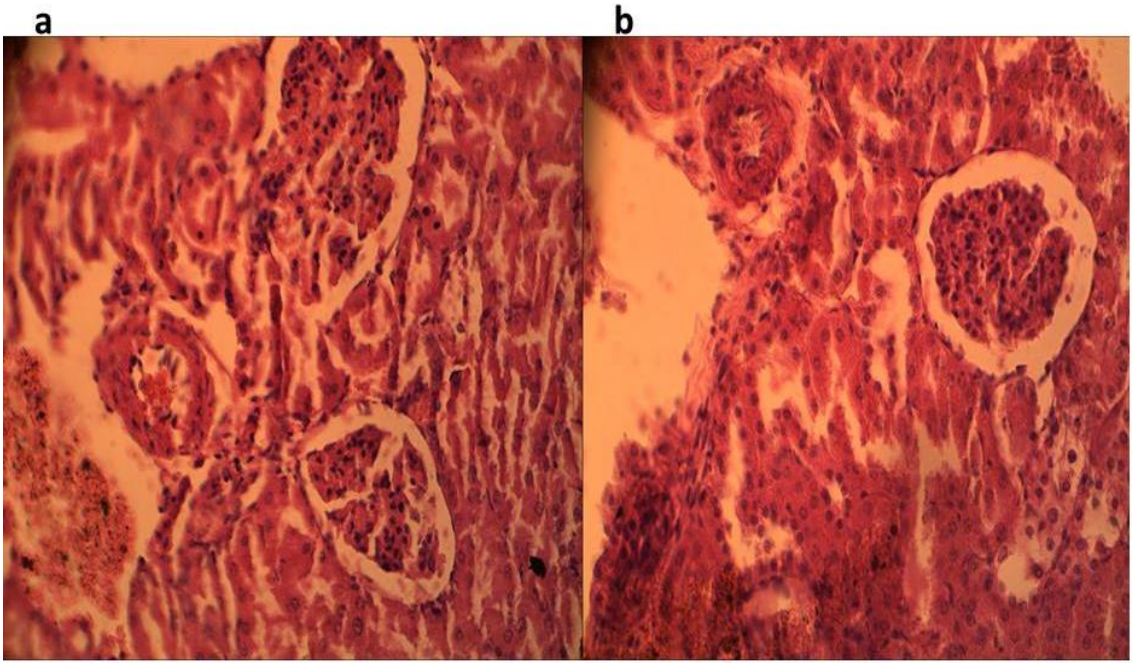


Figure 38: kidney tissue of normal and normal rat treated with GTE (100x,H&E stain)

a:tissue section of normal rat b: tissue section of normal rat treated with GTE

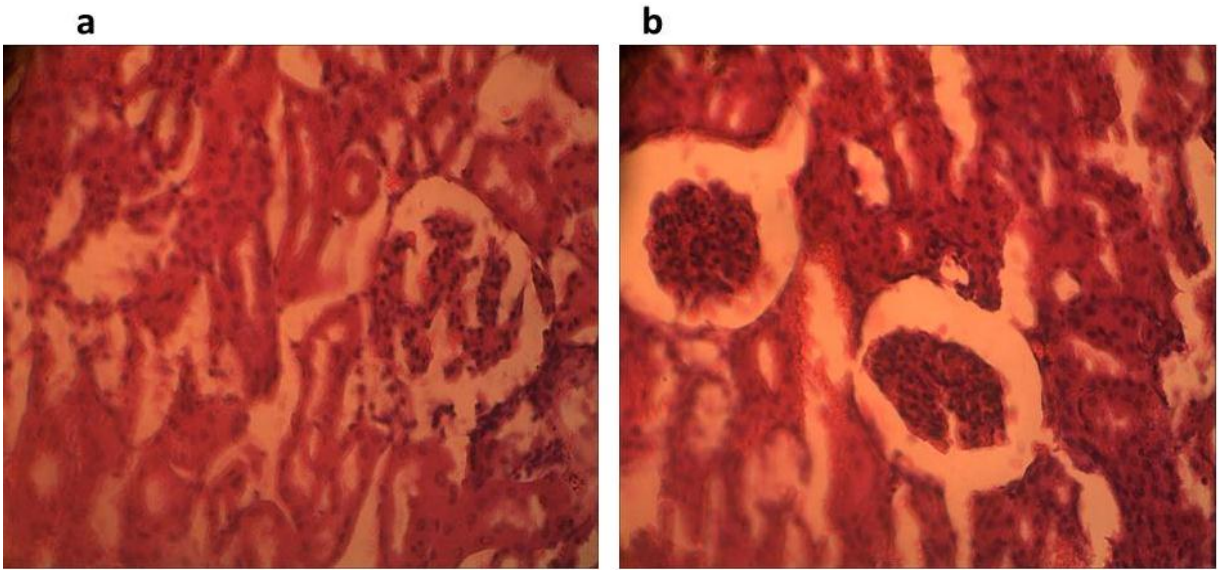


Figure 39: kidney tissue of diabetic and diabetic +green tea (100x,H&E stain)

a: kidney of diabetic rat (normal view) b: kidney of diabetic rat treated with GTE (normal view)

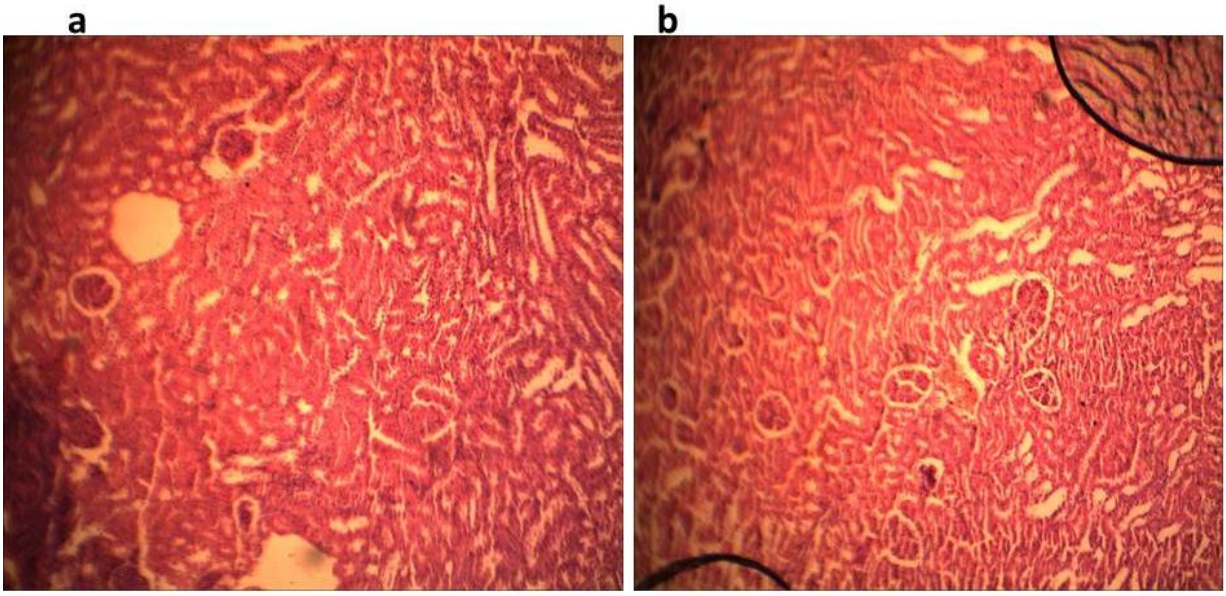


Figure 40: kidney tissue of normal and normal rat treated with GTE (10x,H&E stain)

a:tissue section of normal rat, normal view

b: tissue section of normal rat treated with GTE, normal view

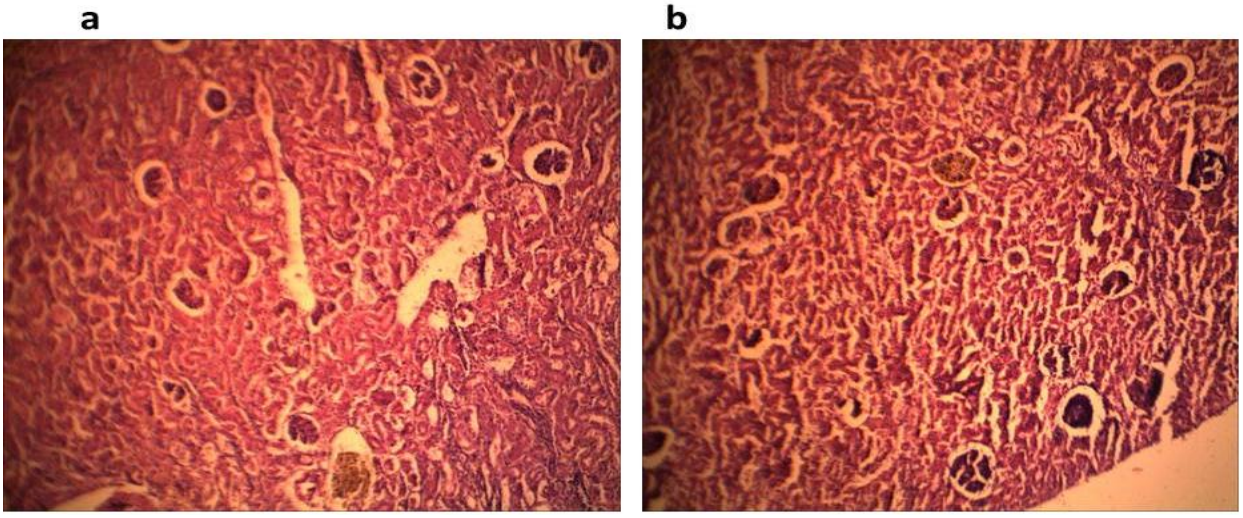


Figure 41: kidney tissue of diabetic rats (10x,H&E stain)

a: tissue section of paralyzed rat due to hyperglycemia showing signs of inflammation

b: tissue section of diabetic rat normal view

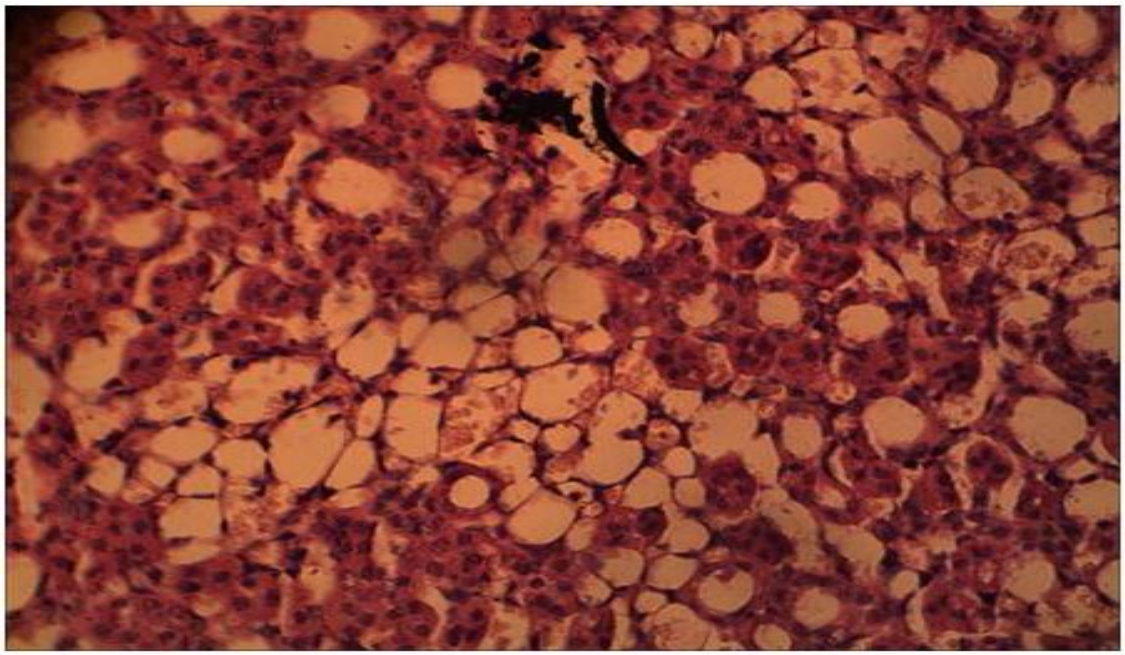


Figure 42: autopsy section of diabetic rat (10x, H&E stain

CHAPTER 7

DISCUSSION AND CONCLUSION

7.1 Discussion

DM is the most common metabolic disorder in developing and also in developed countries. With sanitary life style, lack of literacy and natural food products the burden of diabetes and the rate incidence of its complications is being increased. DN is the major complication of DM and is the main cause of ESRD.

Alteration of glycemic level, blood pressure, electrolyte disturbances and dyslipidemia are considered the main causes of DN. Biochemical changes of diabetes produce unique changes in the kidney.

Polyphenols of dietary and plant component have attracted more interest for their anti-diabetic characteristic. Present study was aimed to evaluate the therapeutic potential of green tea extract with a high percentage of polyphenols (90% ECCG) on renal damage in diabetic nephropathy. Similar works were previously conducted by Yamabe N *et al* (2006) [167] and Haidari F. *et al* (2012) [173] but the therapeutic effect of high percentage of GTE in as a drinking beverage on histoarchitecture is analyzed for the first time.

Insulin promotes lipogenesis and inhibits lipolysis so in lack of insulin, fat reservoirs are depleted. That is the cause of weight loss in type 1 diabetic patients [196]. Studies conducted by Mustata GM *et al* (2005) [166] and Babu PV *et al* (2006) [197] showed the reversion of weight loss followed by treatment with GTE in diabetic rat models. But in our study we did not find any significant change in weight loss between diabetic and treated groups.

Effects of weight loss and energy expenditure by administration of green tea was proved by Dulloo *et al* (1999) [176] and Nagao *et al* (2007) [179]. In our study, we did not see weight loss of normal animal treated with green tea but administration of green tea significantly inhibited weight gain of these animal. And the long term administration may decrease body weight. The differences may be because of sensitivity of species to GTE and the duration of study. The previous results were obtained from human studies and for 12 weekly treatments while our study was on SD rats for 14 days.

Anti-diabetic and hypoglycemic activity of green tea poly phenols was demonstrated by Sabu MC *et al*. (2002) [163], Liang –Yi Wu *et al.* (2004) [165]and Wolfram S *et al.*

(2006) [168] .Present work also expressed support for the anti-hyperglycemic effect of GTE ($p<0.01$). Suggested mechanisms for this effect of GTE are the inhibition of sodium dependent glucose transporter in intestinal epithelial cells [155], increase in sensitivity of insulin and insulin sensitive glucose transporter [165] and increase in expression of Glu IV transporter [164] .

Hyperlipidemia is a common complication of diabetes. In type 1 diabetes lack of insulin is expected to result in decrease in the activity of lipoprotein lipase, simultaneously circulating concentration of chylomicron and VLDL increases. Furthermore, in the deficiency of insulin, synthesis of proteoglycans which are responsible for the association of remnants with hepatic cells is also affected. Therefor the process of removal of VLDL and chylomicron remnants by the liver is hampered [198] .

Moreover, higher concentration of TG rich lipoprotein in plasma leads to increase the activity of HDL for removing circulatory TG via CETP mediated pathway. The latter mechanism is responsible for decrease HDL-cholesterol concentration; simultaneously LDL-Cholesterol increases [198].

Total serum cholesterol and triglyceride lowering property of green tea were demonstrated by Kao *et al* (2005) [199] and the inhibition of pancreatic lipase and thus prevention of dietary lipid absorption by green tea was suggested by Ikea I *et al* (2005) [200].

Effects on serum lipid level of our study were in favor of described studies. There was significant difference between triglyceride and cholesterol level of diabetic control and diabetic animal treated with GTE ($p<0.05$). Serum triglyceride of normal group treated with GTE also decreased significantly as compare to normal control. However, level of cholesterol of normal group remained unchanged may be because of short duration of the study.

In DM especially in case of the early stages of DN, acute phase proteins increase but the level of albumin decreases. Hypoalbuminemia is also a call for the starting and development of further vascular complications. There are growing evidences that indicate the involvement of immunological mechanisms and inflammatory process in the development of DN.

DM causes significant changes in the metabolism of proteins, catabolism of plasma and liver proteins increases that elevate the level of blood urea and nitrogen balance goes toward negative. Significant decrease in body weight and a decrease in muscle mass is also sign of protein catabolism. Suggested mechanisms for all of these alterations in protein metabolism suggest negative correlation between protein biosynthesis and insulin concentration. Proteolysis enhanced and the uptake of amino acids by cells is decreased. The overload of metabolites such as urea and glucose at one hand and the glycosylation of proteins at another hand cause proteinuria.

In the present study, we observed similar changes in our diabetic control group. However, the concentration of serum total protein of all groups was decreased in the second week of our experiment. The possible reason for this change may be the blood withdrawal for experiment in the first week (as the reversion of blood parameters to normal level need at least two weeks). To differentiate the pathological decline in serum proteins from physiological change, we calculated the Alb/Glu ratio. There was not any significant change in Alb/Glu ratio of normal groups in the second week as compare to the first week. But the ratio was significantly decreased in both diabetic control and diabetic treated with GTE, from the first week as well as from normal groups in the second week of observation.

All of above changes indicate the presence of inflammation and the development of DN in our model animals. Till the second week of treatment we did not observe any improvement in the diabetic group treated with GTE.

As mentioned above lack of insulin in type 1 diabetes mellitus leads to proteolysis and subsequently production of urea at a higher rate. Level of creatinine can also be increased in DN, but it is better to calculate the level of creatinine according to the body surface area. Because of a drop in muscle mass can overlap improper excretion of creatinine. So for the evaluation of kidney status we preferred to measure urea clearance and eGFR. By comparing urea clearance of diabetic groups with normal group we found a significant difference ($p<0.01$). Clearance of treated diabetic group with GTE as compare to diabetic control was enhanced but the level of improvement was not statistically significant. Serum creatinine level of all groups was approximately same but we analyzed the results

of serum creatinine according to their body weight and surface. Thus we estimate the proportional eGFR of all groups. According to which we suggest same results of urea clearance with serum creatinine level and eGFR because there was a significant difference between normal and diabetic groups while a body surface area of diabetic control and diabetic group treated with GTE was not significantly different.

Alteration of serum electrolyte level is one of the most common features of DM and is also the contributing cause toward the development of DN. The most known mechanism for imbalance of electrolytes level is the decrease in the activity of Na+/K+ ATPase and hyperosmolarity [201].

Na+/K+ ATPase is responsible for the maintaining the balance of sodium and potassium ions across the cell, that transfers 3 ions of sodium to outside the cells while allows tow ions of potassium to the inside compartment of cells. Activity of this enzyme is dependent to insulin which can be the cause of the electrolyte imbalance in DM type 1 and development of diabetic vascular complications.

The role of hyperosmalarity in alteration of serum electrolytes was suggested by Katz *et al*. (1973), according to him for each 100mg/dl rise in blood sugar can decrease 2-8mEq/L of serum sodium concentration because of water shift from cells [202]. In the case of hyperosmolar diabetic coma, even complete depletion of serum sodium is observed [203].

Babu et al 200 has found that green tea extract increases the Na+/K+ ATPase activity [204] . Results of the present study were also in favor of motion. The concentration of sodium ion in the serum of diabetic rats was increased by significant interval ($p<0.05$) while serum sodium level of the treated group with GTE remained normal. The level of serum potassium in the treated group remained unchanged.

Oxidative stress is the well-known mechanism of diabetes and hyperglycemia for the development of micro vascular complication including DN. In DM higher concentration of glucose produces more pyruvate and subsequently NADH and FADH in ETC. Which increases the voltage gradient of the mitochondrial membrane, blocks complex III of

ETC and generates superoxide. Superoxide than can be converted into several more reactive free radicals [23].

Overproduction of ROS causes the alteration of the balance of pro-oxidants and anti-oxidants. Free radical interacts with nucleic acids, proteins and lipids, which produces marked changes in normal physiology and histology of the organ. [205].All other pathways, for example production of DAG, PKC, Hexose amine pathway, and polyol pathway have link with ROS production.

Thickening of glomerular basement membrane and expansion of mesangial extracellular matrix occurs. A change of basement membrane causes hyperfilteration and elevates glomerular hydrostatic pressure. All these mentioned changes lead to microalbuminurea. Expansion of mesangial matrix, encroach glomerular capillaries and narrowing of lumen occurs that decrease the surface for the filtration. Tubule intercial fibrosis also occurs by the same mechanism [206].

Mesangium expands due to accumulation of excess amount of matrix proteins either because of excess production or decrease in turnover of these proteins. Studies have shown that hyperglycemia, AGE and glycated albumin causes the upregulation of genes responsible for the mesangium expansion such as collagen IV, fibronectin and TGF-β [207].

Direct *in vitro* and also *in-vivo* and also indirect anti-oxidant effects of green tea have been proved. EGCG by owing the galloyl group on its B and D ring have the potent anti-oxidant activity. They scavenge ROS and produce more stable phenolic radicals. The positive correlation between green tea and in vitro anti-oxidant ability such as FRAP (Ferric reducing anti-oxidant power), ORAC (oxygen radical absorbing capacity) and DPPH scavenging is observed [208].

Direct *in-vivo* antioxidant capacity of green tea was proved by the measuring of AOC of plasma by TEAC method following administration of green tea.Furthermore, the indirect anti-oxidant effect of green tea was also proved by observing increased activity of phase II anti-oxidant enzymes in mouse liver, small intestine and skin of mice followed GTE treatment [208]. The elevation of glutathione and decrease in peroxidation of lipid following treatment by GTE was also reported. The inhibition effects of green tea on the

formation of AGE are proved by inhibition of Maillard type reaction in vitro and by attenuation of Maillard type fluorescence in the heart of diabetic rats treated with GTE [170]. All of these effects suggest the protective effect of green tea that were proved in the present study practically.

In our study the TAC determined by DPPH radical scavenging capacity was increased in normal animal treated with GTE significantly ($p<0.05$) administration of GTE to diabetic rats was also able to decrease the oxidative stress of diabetic animal at significant level ($p<0.05$).

Further we analyzed the level of therapeutic effects of GTE on renal tissue damage by analyzing urinary albumin (as an indicator of early renal tissue damage) and histological studies of kidney tissue. Level of urinary albumin in diabetic control group was significant (+++) while albuminuria of the treated group was significantly inhibited (++).

Alteration of renal histo architecture is a long term defect. So by visualizing the histology of kidney remarkable changes were not observed in diabetic kidney so we were not able to demonstrate the therapeutic effects of GTE on renal histo-architicture. However, while the observation of morphological characteristics there were noticeable changes in the kidneys of normal, diabetic and diabetic group treated with GTE.

7.2 Conclusion

The study was conducted to evaluate the therapeutic potential of *Camellia sinensis* on histoarchitecture of DN. In this study, all the underlying mechanism and biochemical pathways toward DN were studied. Alteration of blood parameters due to early changes in renal tissue in DN and analyzed and the effect of GTE on those parameters were evaluated. At the end of study, we found out that persistent hyperglycemia and oxidative stress that leads to production of ROS and AGEs and activation of PKC and polyol pathway.

Oral supplementation of polyphenol rich (90%) GTE at a dose of 200mg/kgbw in drinking water was able to

- ✓ Decrease glucose level of diabetic rat models while did not have any effect on normal groups
- ✓ Reduce oxidative stress and enhance TAC
- ✓ Prevent diabetes induced hyperlipidemia
- ✓ Regulate serum electrolyte level and prevent hypoglycemic induced hyponatremia
- ✓ Decrease blood urea level and improve urinary urea clearance

By having all of above mentioned characteristics we can conclude that the use of GTE can be useful to prevent development of DN and diabetes induced changes in renal histoarchitecture and thus can be helpful in increasing the life expectancy of diabetic patients.

7.3 Future prospective:

A long term study should be designed on animal diabetic models as well as on human suffering from diabetes to evaluate the therapeutic potential of GTE on renal histoarcheticture in DN.

CHAPTER 8

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