

**Influence of Green Tea (*Camellia sinensis*) Extract
on Liver Function in Streptozotocin Induced
Diabetic Wistar Rats**



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Thesis Submitted to
Lovely Professional University, Punjab
in partial fulfillment of the requirements for the degree
of
Master of Science
in
Clinical Biochemistry

Submitted by
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Under the Supervision of
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LOVELY PROFESSIONAL UNIVERSITY, PUNJAB, INDIA
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CERTIFICATE

This is to certify that the present thesis entitled "**Influence of Green Tea (*Camellia sinensis*) Extract on Liver Function in Streptozotocin Induced Diabetic Wistar Rats**" is the outcome of the original piece of work carried out by **Mr. Nommanudien Naibkhil** (Registration No: **11313093**) himself under my guidance and the contents of his thesis did not form a basis of the award of any previous degree to him and to the best of my knowledge to anybody else also. The thesis has not been submitted by the candidate for any research degree in any other University.

The dissertation is fit for submission to the partial fulfillment of the conditions for the award of **M.Sc. in Clinical Biochemistry**. Further, certified that the candidate in habit and character is a fit and proper person for the award.

(Dr. Pranay Punj Pankaj)

Supervisor

DECLARATION

I, **Nommanudien Naibkhill**, hereby declare that the dissertation, entitled “**Influence of Green Tea (*Camellia sinensis*) Extract on Liver Function in Streptozotocin Induced Diabetic Wistar Rats**”, submitted to the Lovely Professional University, Punjab in partial fulfillment of the requirements for the award of the Degree of **Master of Science in Clinical Biochemistry** is a record of original project work done by me during Jan- May 2015 under the supervision and guidance of Dr. Pranay Punj Pankaj and it has not formed the basis for the award of any Degree/Diploma/Associateship/ Fellowship in any other university.

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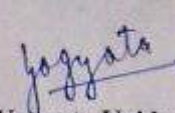
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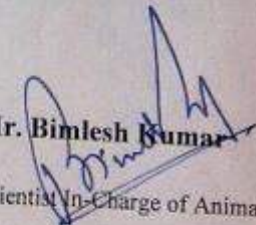
Remarks if any: - One animal from each group will be sacrificed for the study, 30 animals will be rehabilitated, Protocol no 4 and 7 will be shared


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Dedicated to

I am spiritually dedicating all my works and
degree to my only and best brother
“Anamudien Naibkhill”, my parents and my
country.

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Nommanudien Naibkhil

Table of Contents

• Abstract	d
• List of abbreviations	e
• List of tables	g
• List of figures	i
Chapter-I	
1. Introduction	1
1.1. Diabetes	1
1.1.1. Type-1 diabetes	2
1.1.2. Type-2 diabetes	4
1.2. Green Tea	7
1.2.1. Chemical composition of green tea	8
1.2.1.1. Minerals	9
1.2.1.2. Flavonoids	9
1.2.1.3. Methylxanthins	12
1.2.1.4. Protein and amino acids	13
1.2.1.5. Enzymes	13
1.3. Liver	14
1.3.1. Histology of liver	15
1.3.2. Function of the liver	15
1.3.3. Liver function tests	16
1.4. The chemistry of super oxides and oxidative stress	18
1.4.1. Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and Its complications	22
Chapter-II	
2. Review of literatures	26
2.1. Anti-diabetic effect of green tea	26
2.2. Hepatotoxic effects of green tea	31
2.3. Hepatoprotective effect of green tea	34
2.4. Effect of green tea on cytochrom P450	41
2.5. Antioxidative effect of green tea	43
2.6. Pro-oxidant properties of green tea extract	46
2.7. Association of liver damage/impaired functions and diabetes mellitus	50
Chapter-III	
3. Methods and materials	56
3.1. Plant materials	56
3.2. Experimental animals	56

3.3. Chemicals and instruments	57
3.4. Induction of diabetes	58
3.5. Blood sample collection	58
3.6. Experimental design	60
3.7. Biochemical analysis	61
3.7.1. Fasting blood sugar (FBS)	61
3.7.2. Total serum protein	63
3.7.3. Serum albumin and A:G ratio	64
3.7.4. Serum triglyceride	65
3.7.5. Serum total cholesterol	66
3.7.6. Serum bilirubin	67
3.7.7. SGOT	69
3.7.8. SGPT	71
3.7.9. Alkaline phosphatase	72
3.7.10. Total Anti-oxidant Capacity	73
3.8. Histopathological tests	75
3.9. Statistical analysis	76
Chapter-IV	
4. Result analysis	78
4.1. Morphological analysis	78
4.2. Biochemical parameters	80
4.2.1. Body weight	80
4.2.2. Blood glucose	81
4.2.3. Serum total protein	82
4.2.4. Serum total albumin	84
4.2.5. Serum triglyceride	87
4.2.6. Serum total cholesterol	88
4.2.7. Serum bilirubin	90
4.2.8. SGPT	93
4.2.9. SGOT	95
4.2.10. ALP	96
4.2.11. Serum total anti-oxidant capacity	97
4.3. Histopathological analysis	101
Chapter-V	
5. Discussion, Summary and Conclusion	103
5.1. Discussion	104
5.1.1. Effect of GTE on body weight	104
5.1.2. Effect of GTE on serum glucose level	104
5.1.3. Effect of GTE on serum total protein and albumin	106
5.1.4. Effect of GTE on serum triglyceride and serum total cholesterol	106
5.1.5. Effect of GTE on serum bilirubin level	108

5.1.6. Effect of GTE on SGPT, SGOT and ALP	109
5.1.7. Effect of GTE on total antioxidant capacity	110
5.1.8. Histopathological analysis	111
5.2. Summary	112
5.3. Conclusion	118
Chapter-VI	
6. References	120
Chapter-VII	
7. Appendages	140
7.1. Poster presentation	141
7.2. Poster presentation certificate	142
7.3. Abstract published	143

Abstract

Diabetes mellitus is a chronic metabolic disorder causing disturbances in carbohydrate, fat, and protein metabolism, characterized by hyperglycemia, polydipsia, polyphagia, and polyuria. DM is associated with induced risk of other comorbid conditions such as generalized atherosclerosis and other cardio-vascular diseases, peripheral vascular disease, hypertension, retinopathy, cerebro-vascular disease, nephropathy, and liver disease. Elevation of oxidative stress in DM is thought to be the key mechanism of pathogenesis and complication of DM. consumption of antioxidants reduce the risk of DM complication. Green tea is widely consuming beverage after water globally. It possesses more than 4000 bioactive components. One third of its component is polyphenols which contains mainly catechins exhibiting strong antioxidative effects. To evaluate effect of green tea extract on diabetic liver function, 4 groups of albino wistar rats (each contains 6 animals) were included in this experiment. Normal control group maintained as reference range for biochemical and histopathological parameters. Other normal group were giving daily fresh 0.2% aqueous green tea extract (having 90% catechins) solution instead of drinking water for 14 days. Diabetic controls were induced by 45mg/kg BW IP injection of streptozotocin to evaluate the effect of diabetes on liver and as well as to compare changes occur by GTE. The 4th group were diabetic animals drunk daily fresh 0.2% aqueous green tea extract (having 90% catechins) solution instead of drinking water for 14 days. Body weight gaining was significantly inhibited by GTE in “N+GTE” as compared to “NC” while no significant changes observed between “DC” and “D+GTE”. “DC” group showed significant increased in level of glucose, TG, T.Chol, bilirubin, SGPT, SGOT, and ALP, and significant decrease in body weight, albumin, total antioxidant capacity. GTE significantly reduced the elevated glucose, TG, and T.Chol in diabetic groups in 14 days while no significant changes occurred in elevated level of, bilirubin, SGPT, SGOT and ALP. GTE extract also did not show any significant effect on decreased serum albumin level during 14 days of treatment in diabetic animals. GTE significantly induced total anti-oxidant capacity in “D+GTE” and “D+GTE” groups. Histological analysis of liver tissue showed fatty vacuolation in diabetic tissues while the fatty degeneration degree was lesser in “D+GTE” group.

List of Abbreviations

- ADH: Antidiuretic hormone
- AGEs : Advanced glycation end products
- ALT: Alanine transaminase
- AMI Acute myocardial infarction
- AST: Aspartate transaminase
- BTE: Black tea extract
- CAT: Catalase
- CD4⁺: Cluster of differentiation-4
- CD8⁺: Cluster of differentiation-8
- CG: Catechin gallate
- CYP-450: Cytochrome P-450
- D.Bil Direct bilirubin
- D+GTE: Diabetic + green tea extract
- DAG: Diacylglycerol
- DC: Diabetic control
- DM: Diabetes mellitus
- DNA: Deoxyribonucleic acid
- EC: Epicatechin
- ECG: (-)-epicatechin-3-gallate
- EGC: Epigallocatechin
- EGCG: Epigallocatechin gallate
- ER: Endoplasmic reticulum
- ETC: electron transport chain
- FA: Fatty acid
- FBG: Fasting blood glucose
- FPIR: first-phase insulin response
- GCG: Gallocatechin gallate
- GGT: Gama-glutamyl transferase
- GI: Gastro-intestinal
- GLUTs: Glucose transporters
- GPO: glycerol-3-Phosphato-oxidase
- GSH: Reduced glutathione
- GSH-Px: Glutathione peroxidase
- GSSG: Oxidized glutathione
- GST glutathione S-transferase
- GTE: Green tea extract
- GTP: Green tea polyphenols
- HLA: Human leukocyte antigen
- IAA: insulin auto-antibodies
- ICA: Islet cells antibodies
- IFG: impaired fasting glycemia
- IGF-1: Insulin-like growth factor 1
- IGT: Impaired glucose tolerance
- IL-6: Interleukin-6
- IP: Intra-pretoane

- IRS-1: Insulin receptor substrate-1
- LDH: Lactate dehydrogenase
- LFTs: Liver function tests
- MDA Malondialdehyde
- MDH: Malate dehydrogenase
- MHC-1: class-1 major histocompatibility complex
- N+GTE: Diabetic+green tea extract
- NAD⁺: Nicotinamide adenine dinucleotide (oxidized form)
- NADH: Nicotinamide adenine dinucleotide (reduced form)
- NADP⁺: Nicotinamide adenine dinucleotide phosphate (oxidized form)
- NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)
- NAFLD: Non-alcoholic fatty liver disease
- NC: Normal control
- NO: Nitric oxide
- RET: Reverse electron transport
- RNA: Ribonucleic acid
- RNS: Reactive Nitrogen species
- ROS: Reactive Oxygen Species
- SGLT: Sodium-dependent glucose transporter
- SGOT: serum glutamate-oxaloacetate transaminase
- SGPT: serum glutamate-pyruvate transaminase
- SMRs: Standardized mortality ratios
- SOD: Superoxide dismutase
- SREBP-1c: Sterol Regulatory Element-Binding Protein-1c
- T.Bil total bilirubin
- T.Chol total cholesterol
- T1DM: Type-1 diabetes mellitus
- T2MD: Type-2 diabetes mellitus
- TAC: Total antioxidant capacity
- TF: Theaflavin
- TF3: Theaflavin digallate
- TNF- α : Tumor necrosis factor alpha
- XO: Xanthine oxidase
- Υ -PGA : Poly- Υ -glutamic acid

List of Tables

#	Table No.	Title	Page No.
1	Table 1-1	Classification of diabetes mellitus	1
2	Table 1-2	Risks of T1DM in first-degree relatives, proband diagnosed before 20 years of age	3
3	Table 1-3	Genetic loci involved in T1DM	4
4	Table 1-4	Different tests with the region of sampling used for demonstrating of type 2 DM	6
5	Table 1-5	Clinical feature of Type 1 & 2 diabetes mellitus	7
6	Table 1-6	Scientific classification of green tea plant	7
7	Table 1-7	Electrolyte composition of GTE	
8	Table 1-8	Structure of flavonoids	10
9	Table 1-9	Chemical composition of the tea leaf	13
10	Table 3-1	Experimental design	61
11	Table 3-2	Procedure for glucose estimation	62
12	Table 3-3	Procedure for total protein estimation through biuret kits	63
13	Table 3-4	Procedure for estimation of albumin	64
14	Table 3-5	Procedure for triglyceride estimation through beacon assay kits	66
15	Table 3-6	Procedure for serum total cholesterol estimation	67
16	Table 3-7	Procedure of reagent making for estimation of Total bilirubin and direct bilirubin through Diazo kit method of Pearlman and Lee	68
17	Table 3-8	Procedure for Bilirubin estimation by Diazo kit method of Pearlman and Lee	68
18	Table 3-9	Procedure for estimation of SGOT through IFCC method, kinetic	70
19	Table 3-8	Procedure for SGPT estimation through IFCC method, kinetic. After maxing well, aspirated	71
20	Table 3-11	Procedure for estimation of serum ALP	72
21	Table 3-12	Procedure for TAC using DPPH	74
22	Table 4-1	Body weight on zero, 7 th and 14 th days	80
23	Table 4-2	Comparison of body weight among the groups on zero, 7 th and 14 th days	80
24	Table 4-3	Fasting blood glucose level on 7 th and 14 th days	81
25	Table 4-4	Comparison of fasting blood glucose levels among the experimental groups after 7 th day and 14 th day of GTE treatment	82
26	Table 4-5	Serum total protein levels of each group on 7 th and 14 th days	83
27	Table 4-6	Comparison of serum total protein level among the groups on 7 th and 14 th days	83

28	Table 4-7	Comparison of serum albumin levels of each group between 7 th and 14 th days	85
29	Table 4-8	Comparison of serum albumin levels among experimental groups after 7 th and 14 th days	85
30	Table 4-9	Albumin:globulin ratio of 7 th and 14 th days	86
31	Table 4-10	Comparison of serum A:G ratio among groups on 7 th and 14 th days	87
32	Table 4-11	Comparison of Serum triglyceride levels of each group between 7 th and 14 th days	87
33	Table 3-12	Comparison of serum triglyceride levels among the groups on 7 th and 14 th days	88
34	Table 4-13	Comparison of serum total cholesterol levels of each group between 7 th and 14 th days	89
35	Table 4-14	Comparison of serum total cholesterol levels among experimental groups on 7 th and 14 th days	89
36	Table 4-15	Comparison of serum total bilirubin levels of each group between 7 th and 14 th days	90
37	Table 3-16	Comparison of serum total bilirubin levels among experimental groups on 7 th and 14 th days	91
38	Table 3-17	Comparison of serum direct bilirubin levels of each group between 7 th and 14 th days	92
39	Table 4-18	Comparison of serum direct bilirubin levels among experimental groups after 7 th and 14 th days	92
40	Table 3-19	Comparison of SGPT levels of each group between 7 th and 14 th days	93
41	Table 4-20	Comparison of SGPT levels among experimental groups on 7 th and 14 th days	94
42	Table 4-21	Comparison of SGOT levels of each group between 7 th and 14 th days	95
43	Table 4-22	Comparison of SGOT levels among experimental groups on day 7 th and 14 th	95
44	Table 4-23	Comparison of ALP levels of each group between 7 th and 14 th days	96
45	Table 4-24	Comparison of ALP levels among experimental groups on day 7 th and 14 th	97
46	Table 4-25	TAC after 14 th days of GTE supplementation	98
47	Table 4-26	Comparison of TAC among experimental groups after 14 days of GTE treatment	98
48	Table 4-27	Data of all estimated parameters on 7 th and 14 th days of experiment	99

List of Figures

#	Figure No.	Title	Page
1	Figure 1-1	Different countries producing tea 1996 (Douglas, A. <i>et al.</i> , 1997)	8
2	Fig 1-2	<i>skeletal structure of flavonoids</i>	9
3	Fig 1-3	Major tea catechins	10
4	Fig 1-4	the major tea flavonols	11
5	Fig 1-5	The Bisflavanols chemical structure	12
6	Fig 1-7	Chemical structure (left) (+) catechin, and the (right) (-) epicatechin	13
7	Fig 1-8	Liver blood vessels	14
8	Figure 1-9	Overview of mitochondrial ROS production	21
9	Fig 1-10	common pathways of oxidative stress contribution to development of diabetes complication	22
10	Fig 2-1	General proposed anti-diabetic mechanism of GT	30
11	Figure 2-2	The chelating reaction of catechins with metals (Anna <i>et al.</i> , 2014).	43
12	Figure 2-3	<i>In vivo</i> ROS formation and its clearance. GSSH (glutathione disulfide), GSH (glutathione).	45
13	Figure 2-4	The possible mechanism of DNA damage by quercetin copper complexes	47
14	Fig. 2-5	Proposed mechanism of ROS generation by auto-oxidation of EGCG	48
15	Figure 2-6	Role of EGCG, superoxide, and ferric ion in production of oxidative stress, EGCG dimer, and EGCG-cysteine conjugates (EGCG-SR).	50
16	Fig 3-1	Room environment for keeping of rats	56
17	Fig 3-2	STZ chemical structure	57
18	Fig 3-3	IP injection of STZ	58
19	Fig 3-4	Blood collection from the tail by 1 ml syringe, blood collection from the eyes of rats by capillary tubes	59
20	Fig 3-5	Rats were anesthetized with di-ethyl ether before eyes blood collection and dissection	59
21	Fig 3-6	Dissection and blood collection from the heart of the animals after anesthesia	60
22	Fig 4-1	Diabetic rat (left), diabetic + GTE (right).	79
23	Fig 4-2	Normal animal during weighing	59
24	Fig 4-3	Body weight in different experimental groups on zero, 7 th and 14 th days	81
25	Figure 4-4	FBG level in different groups on 7 th and 14 th days of experiment	82
26	Figure 4-5	Serum total protein level on 7 th and 14 th days	84
27	Fig 4-6	Serum total protein levels on 7 th and 14 th days of	84

		experiment	
28	Fig 4-7	Serum albumin levels in different groups on 7 th and 14 th days	86
29	Fig 4-8	Serum triglyceride levels in different groups on 7 th and 14 th day	88
30	Fig 4-9	Serum total cholesterol levels in different groups on 7 th and 14 th days	90
31	Fig 4-10	Serum total bilirubin levels in different groups on 7 th and 14 th day	91
32	Fig 4-11	Serum direct bilirubin levels in different groups after 7 th and 14 th days of experiment	93
33	Fig 4-12	Serum GPT levels in different groups on 7 th and 14 th day of experiment	94
34	Fig 4-14	ALP levels in different groups on 7 th and 14 th days of experiment	97
35	Fig 4-15	Total anti-oxidant capacity of experimental groups on 14 th day	98
36	Fig 4-16:	Microscopic appearance of liver tissue belongs to the “normal control” group (H&E stain, 40x)	101
37	Fig 4-17	Microscopic appearance of liver tissue belongs to the “normal+GTE” group (H&E stain, 100x)	101
38	Fig 4-18	Microscopic appearance of liver tissue belongs to the “diabetic control” group showing fatty vacuolation of liver and mild inflammation (H&E stain, 40x).	102
39	Fig 4-19	Microscopic appearance of liver tissue belongs to the “diabetic+GTE” group showing mild fatty vacuolation (H&E stain, 40X).	102



Chapter-I

Introduction

Page: 1- 24

1. Introduction

1.1 Diabetes

Hyperglycemia is the word used for elevation of plasma glucose level above the normal range. Diabetes is a chronic metabolic disorder causing disturbances in carbohydrate, fat, and protein metabolism (betteridge, 2004), characterized by hyperglycemia, polydipsia, polyphagia, and polyuria. Insulin is secreted from β -cell of pancreas in response to hyperglycemia in healthy people. Insulin increases cell membrane permeability to glucose in liver, muscle, and adipose tissues, alters the glucose metabolic pathways. Brajendra, *et al.*, (2006) reported total diabetic population 19.4 million and estimated to reach 60 million by the year 2025.

Diabetes is classified into two main classes, diabetes insipidus which is not dependent on carbohydrate while it depends on ADH, and diabetes mellitus which depends on carbohydrate metabolism. Diabetes mellitus is described as a group of metabolic diseases characterized by hyperglycemia (fasting blood sugar $>126\text{mg /dL}$) resulting from defects in insulin secretion, insulin action, or may be both. The National Diabetes Data Group in 1979 developed a classification of diabetes mellitus into type 1 (insulin dependent DM) and type 2 (insulin independent DM), while other classes of DM are exist (Table 1-1, National Diabetes Data Group, 1979; Bishop, Fody, and Schoeff, 2012).

Table 1-1: Classification of diabetes mellitus

DM classification	Pathogenesis
Type 1	<ul style="list-style-type: none"> • β-cell destruction • Absolute insulin deficiency • Autoantibodies (islet cell autoantibodies, insulin autoantibodies, glutamic acid decarboxylase autoantibodies, tyrosine phosphatase IA-2 & IA-2B autoantibodies).
Type 2	<ul style="list-style-type: none"> • Insulin resistance with an insulin secretory defect • Relative insulin deficiency
Other	<ul style="list-style-type: none"> • Associated with secondary conditions (genetic defects of B-cells function, pancreatic disease, endocrine disease, drug or chemical induced, insulin receptor abnormalities, and other genetic syndrome).
Gestational	<ul style="list-style-type: none"> • Glucose intolerance during pregnancy • Due to metabolic and hormonal changes.

Both types of DM (type 1 & 2) are associated with induced risk of other comorbid conditions such as generalized atherosclerosis and other cardio-vascular diseases, peripheral vascular disease, hypertension, retinopathy, cerebro-vascular disease, nephropathy, and liver disease (Clark and Lee, 1995). Renal involvement association with DM is counted about 25-45% of both type diabetics (Geoffry and Cooper, 2003).

1.1.1. Type 1 Diabetes Mellitus

T1DM is an organ-specific chronic autoimmune disease which depends on insulin (requiring life-long insulin therapy). T1DM which accounts for 10% of all primary diabetes is the result of antibodies-mediated destruction of pancreatic β -cells which leads to insulin deficiency and hyperglycemia (Harrison *et al.*, 1990; Bach, 1994). It is one of the most common childhood chronic diseases with prevalence rates of about 0.2-0.5% (LaPorte, *et al.*, 1995). Micro- and macrovascular complications are responsible for increased morbidity and mortality rates of T1DM (Geoffry and Cooper, 2003). It is reported that 40% of T1DM occurs in age >20 years (Laakso and Pyorala 1985), while most of the cases occur in childhood or young adults with normal or low body weight (Geoffry and Cooper, 2003). Both Insulin and C-peptide—is produced in an equimolar ratio with insulin by pancreatic β -cell, are reduced in plasma. In contrast, insulin resistance is existing with relatively milder insulin deficiency mostly in obese individuals, while insulin and C-peptide are higher in T2DM than in T1DM (Geoffry and Cooper, 2003). T1DM is not always due to islet cells antibodies (ICA), therefore it subclassified into two other classes, type-1a which β -cells destruction is due to ICA, while type-1b β -cells destruction is not due to ICA. Typ-1b which is present in minority of the people usually Asian or African descent, displays strong inheritance, but no association with HLA, clinically shows episodic ketoacidosis, insulin deficiency without presence of islet autoantibodies (Geoffry and Cooper, 2003).

Inuslitis (the lymphocyte-rich inflammatory infiltrate in the pancreatic islets) observed in autopsy studies of pancreas consists mainly $CD8^+$ and variable numbers of $CD4^+$ cells, and also macrophages (Itoh *et al.*, 1993; Foulis *et al.*, 1996). Induce expression of MHC-1 (class-1 major histocompatibility complex) molecules and other inflammatory markers such as adhesion molecules, Fas (CD95) and interferon- α are also associated with insulitis (Hanafusa *et al.*, 90). Detection of insulitis in T1DM is a direct evidence for the immunopathogenesis. The ICA reaction is included responses of auto-antibodies against the β -cell proteins, IA-2 and glutamic acid decarboxylase, while insulin auto-antibodies (IAA) are not part of ICA but they are important markers for type-1DM (Verge *et al.*, 1998; Geoffry and Cooper, 2003). About >95% of T1DM patients have one of the above mentioned antibodies, while <1% of normal subjects may have them (Geoffry and Cooper, 2003). ICA can be detected about 30 months before any other biochemical abnormalities suggesting β -cells failure (Gorsuch, *et al.*, 1981). All auto-antigens related to the diabetes are intracellular, so the antibodies against them can not bind to the surface of living β -cells, therefore, they do not mediate directly β -cell destruction. Islet autoantibodies can be found many years before clinical symptoms of diabetes appear (Geoffry and Cooper, 2003). Risk of

development of diabetes within 5-years in people with ICA-positive is 85% when the first-phase insulin response (FPIR) is <50 mU/L, 48% when the FPIR is between 50 to 100 mU/L and 17% when the FPIR is more than 100 mU/L (Bingley, 1996), so, the FPIR and progression to the diabetes is very strongly associated. Impaired FPIR is the first detectable metabolic defect, followed by impaired glucose tolerance (IGT) and/or impaired fasting glycemia with β -cell destruction before diabetes appear (Geoffry and Cooper, 2003). Tuomilehto, J., *et al.*, (1991) reported that about 13% of children of first-degree relatives primarily had markers of β -cell autoimmunity (ICA or IAA), while the percentage is reduced to 6-7% at the ages of 5-9 years, and the cumulative incidence of T1DM reached to 2-3% at age of 10 years. Hence it can be hypothesized that progress of T1DM development in some children may be very slow, or even may recover from autoimmunity. It also has been reported that some of patients with T2DM showed positive β -cell autoimmunity (Gorsuch *et al.*, 1981; Humphrey *et al.*, 1998). They may have slow progress in T1DM which terms as latent autoimmune diabetes in adults (LADA) (Tuomi *et al.*, 1993).

Incidence of T1DM in monozygotic twins is about 30-40% and in siblings 5%. These findings suggested role of genetic factors in development of the T1DM, from other side it also shows the involvement of non-genetic/environmental factors. It has been reported that several genetic loci (polygenic) are involved in transmission of T1DM. Heterogeneity (polygenic or non-mendelian inheritance) contribution in T1DM made it complex genetic diabetes (Geoffry and Cooper, 2003).

Table 1-2: Risks of T1DM in first-degree relatives, proband diagnosed before 20 years of age

Parents	2.2 ± 0.6%
Children	5.6 ± 2.8%
Siblings	6.9 ± 1.3%
HLA nonidentical sibling	1.2%
HLA Haploidentical	4.9%
HLA identical sibling	15.9%
Identical twin	30-40%

Source: *Geoffry Boner and Mark E Cooper, 2003*

It is also reported that genetic factors, outside the MCHs are also involved in T1DM development. Non-MHC genes are thought to be 50% responsible for susceptibility of diabetes (Table 1-3, Lucassen and Bell 1995).

It is hypothesized that some of the viral infections such as picornavirus (Onkamo P., *et al.*, 1999), rubella virus, rotavirus, herpesvirus, influenza virus, mumps, retrovirus (Ginsberg-Fellner F., *et al.*, 1984), and measles may also involved in β -cell autoimmunity. It is also hypothesized that exposure to cow's milk protein may results cross-reaction the β -cells of pancreas antigens which may leads to T1DM (*Geoffry and Cooper, 2003*).

Table 1-3: Genetic loci involved in T1DM

Associated	Locus	Chromosome
Confirmed polymorphism demonstrated (gene)	IDDM1 (HLA) IDDM2 (insulin)	6p21 11p15.5
Confirmed analysis demonstrated (linkage)	IDDM4 IDDM5 IDDM6 IDDM8 IDDM12 (CTLA-4)	11q13 6q25 18q21 6q27 2q33
Suggestive (less evidence)	IDDM3 IDDM7 IDDM10 IDDM11 IDDM13 IDDM18	15q26 2q31 10p11.2-q11.2 14q24.3-q31 2q34 5q33-34

There is no difference between male and female for risk of developing T1DM (Karvonen *et al.*, 1993), while studies showed that there is high risk of T1DM in female non-obese diabetic mice (Gale and Gillespie, 2001). Diabetic mother and diabetic father can provide different risk for development of diabetes in offsprings. Offspring of diabetic father may have risk of about 1 in 40 while offspring of diabetic mother may have risk of 1 in 66 for development of T1DM (Geoffry and Cooper, 2003). Studies in Finland also suggested that risk of T1DM is dependent on the sex of offspring also (Onkamo *et al.*, 1999). The offspring with same sex to the diabetic parent is less likely to develop diabetes. So, the father may transmit more the disease to daughter. Mortality rate in female diabetic patients is relatively more than male diabetic patients (Geoffry and Mark Cooper, 2003).

Studies between 1960 and 1996 in 37 countries showed that the prevalence of T1DM is increased in low and high risk population (Tuomilehto *et al.*, 1995) approximately 3-4%, which most of them were within 5-10 years of olds. Incidence of T1DM showed 40% increased in 2010 than 1998. The highest number of T1DM patients is in Fenland and the lowest are Tanzania, Korea and Mexico City (Geoffry and Cooper, 2003).

Ludvigsson, *et al.*, (1989) reported seasonal variation in the onset of T1DM in many countries, with slightly decrease in incidence in warm seasons then colder seasons specially in countries with well demarcated seasons. These changes are mostly revealed in peripuberatl children then younger children (Joner, G. and Sovik, O., 1989).

1.1.2. Type 2 Diabetes Mellitus

Formerly also known as non-insulin dependent DM is the most common type of DM with frequency of 150 million individuals affected in 2000, and predicted to reach double in number by 2025 (Geoffry and Cooper, 2003). T2DM accounts for 80-90% of diabetes

globally (Johne E. Gerich, 2013). It is characterized by hyperglycemia as result of disordered insulin action (resistance) with an insulin secretory defect. Diabetes type 2 can be associated with defects at different points such as decrease in receptor concentration, reduction in kinase activity, decrease in translocation of glucose transporters, IRS-1 & -2, PI-3-K, and intracellular enzymes' activities (Brajendra *et al.*, 2006). The specific genetic and biochemical abnormality reason of insulin resistance and insulin secretion are not well understood yet. T2DM is mostly found in elders but in youth and children also can be found. Mostly this resistance does not cause absolute insulin deficiency. Most of the patients with T2DM are obese or possess higher percentage of fat distribution in abdominal parts. T2DM is often kept undiagnosed for several years and the risk is increased in obesity, increase in age, and lack of physical activities. The onset of disease is mostly in adulthood with milder symptoms than T1DM, and ketoacidosis seldom occurs. Patients with T2DM are more prone to go into a hyperosmolar coma and are more prone to develop macro- and microvascular complications (Bishop, Fody, and Schoeff, 2012). Insulin resistance in T2DM occurs in muscle, adipose tissues and liver. There may be relative or absolute deficiency of insulin secretion, but the insulin concentration would be high or normal at onset of the disease and with increasing duration of the disease a progressive deterioration of pancreatic β -cell function occurs. This disease may be occurring for many years before, but symptoms and complications may reveal later. Half or more patients with T2DM may remain undiagnosed unless systemic screening for diabetes is performed. T2DM is associated with common diabetic symptoms such as polydipsia, polyuria, polyphagia, and weight loss (Geoffry and Cooper, 2003). It has been shown that T2DM has strong familial aggregation (Newman *et al.*, 1987; Sakul, *et al.*, 1997; John E. Gerich, 2013). Genetic susceptibility is thought to be the prerequisite of T2DM development, but environmental factors are also responsible for clinical expression of it (Geoffry and Cooper, 2003). Recently two genes which increase susceptibility to T2DM are identified, NIDDM1 (Hanis, C., *et al.*, 1996) and NIDDM2 (Mahtani M., *et al.*, 1996), as well as others are PPAR gamma (Altshuler *et al.*, 2000), FABP2 (Baier *et al.*, 1995), PPP1R3 (Xia *et al.*, 1998), and adiponectin (Kondo *et al.*, 2002). A parent or sibling with T2DM increases the risk of occurrence from 2 to 6 folds. T2DM inheritance is polygenic (several abnormal genes are required for development of the disease). Genetic controls the insulin sensitivity and insulin secretion which is very important in pathogenesis. Most of the T2DM are obese (especially obesity in intra-abdominal region, Geoffry and Cooper, 2003). Some of the tests help in diagnosis of T2DM demonstrated in table 1-4.

Sex differences do not have significant effect on prevalence of T2DM, while by increasing age the risk for prevalence of T2DM is also increased. In developing countries, prevalence of T2DM is seen mostly in younger adult years, but major increase occurs in prevalence of T2DM in the age group 45-70 years at developed countries (King H., *et al.*, 1998). In recent years many reports of the occurrence of T2DM have been seen in childhood and adolescence (Dean *et al.*, 1992; Kitagawa *et al.*, 1998; Dabelea *et al.*, 1998; Fagot-Campagna *et al.*, 2000).

Table 1-4: Different tests with the region of sampling used for demonstrating of type 2 DM

Type of tests showing DM	Venous whole blood mmol/L (mg/dL)	Capillary whole blood	Venous plasma
Fasting or 2 hrs post-glucose impaired glucose tolerance	≥6.1 (110)	≥6.1 (110)	≥7.0 (126)
Fasting (if measured) and 2 hrs post-glucose impaired fasting glycemia	≥10.0 (180)	≥11.1 (200)	≥11.1 (200)
Fasting (and if measured)	<6.1 (110)	<6.1 (110)	<7.0 (126)
2 hrs post-glucose	6.7-9.9 (120-179)	7.8-11.0 (140-199)	7.8-11.0 (140-199)
Fasting (and if measured)	5.6-6.0 (100-109)	5.6-6.0 (100-109)	6.1-6.9 (110-125)
2 hrs post-glucose	<6.7 (120)	<7.8 (140)	<7.8 (140)

Environmental factors also may have role in prevalence and incidence of T2DM. Infants born with low weight have more chance to exhibit T2DM in adulthood (Hales and Barker, 1992; McCance *et al.*, 1994; Lithell *et al.*, 1996; Lindsay and Bennett, 2001). Children of diabetic pregnancies also may have high risk of T2DM in adulthood. Infants who are feeding by mother's milk are at low risk T2DM in their future than infants who were bottle-fed (Pettitt *et al.*, 1997; Young *et al.*, 2002). Obesity and physical inactivity are the major risk factor for T2DM, and upper body/central obesity is a higher risk factor than generalized obesity. High fat containing foods with low fiber and high refined carbohydrate content increase the risk of this disease (West KM, 1978). People consuming high fat foods are in high risk of T2DM (Reid *et al.*, 1971). Several studies showed association of some inflammatory markers such as C-reactive protein, TNF- α , IL-6, γ -globulin, and adiponectin with T2DM. Many of these markers are products of adipocytes (Geoffry and Cooper, 2003). Insulin resistance is also associated with other clinical issues such as micro-albuminuria, increased blood pressure, low HDL-cholesterol concentration, obesity and hypertriglyceridemia (Groop *et al.*, 1999). Mostly T2DM occurs in individuals with prolonged insulin resistance which show normal glucose tolerance and elevated insulin concentration (Geoffry and Cooper, 2003). Insulin resistance is also found in the non-diabetic siblings and children of Type 2-diabetic patients which increase the risk of developing of T2DM in future (Haffner *et al.*, 1986; Lillioja *et al.*, 1987; Forsblom *et al.*, 1995). Thus, defects in insulin action along with insulin secretion in individuals with normal glucose tolerance predict the occurrence of T2DM (Geoffry and Cooper, 2003). Impaired glucose tolerance (IGT) and impaired fasting glycemia (IFG) are two clinical stages for high risk of developing T2DM and in the development of T2DM respectively (Bennett *et al.*, 1982; Saad *et al.*, 1988; Gavin *et al.*, 1997; World Health Organization: Geneva, 1999). About 50% of people with IGT and IFG are developing T2DM within 5 years (Gibir *et al.*, 1997; Shaw *et al.*, 1999; De Veigt *et al.*, 2001). Risk of T2DM is decreased by lifestyle interventions such as reducing the weight, increasing physical activity and controlling the diet (Geoffry and Cooper, 2003). The major causes of morbidity

and mortality of T2DM is the complication induced due to diabetic condition. The common complications are coronary heart disease and other cardiovascular disease, stroke, retinopathy, neuropathy, and nephropathy which are more occurred in T2DM patients than non-diabetic patients with same age and sex. Nephropathy and retinopathy association is lesser in T2DM than other types of DM, while coronary heart disease and stroke have 2-4 folds higher risk in T2DM than non-diabetic people. T2DM patients have 1.5 to 2 times higher mortality rates than non-diabetic individuals in the same age and sex (Kleinman *et al.*, 1988; Gu *et al.*, 1998; Roper *et al.*, 2001).

Table 1-5: Clinical feature of Type1 & 2 diabetes mellitus

	Type-1 Diabetes Mellitus	Type-2 Diabetes Mellitus
Age at diagnosis	Usually less than 30 years	Usually above the 30 years
Weight	Normal or thin	Overweight
Ketoacidosis	Susceptible	Not susceptible
Plasma insulin and C-peptide	Reduced	Normal or induced
Islet auto-antibodies	Present	Absent

1.2. Green Tea

Green tea, native from china, is the infusion of *Camellia sinensis* the most widely consuming beverage after water, globally. *Camellia sinensis* is a plant in the family of Theaceae. Different types of tea such as green tea (GT), black tea (BT), white, oolong, and twig tea (kukicha) are obtained from *Camellia sinensis* through different procedures (Sabu *et al.*, 2010; Parmar *et al.*, 2012). Tea plant is cultivated almost in around 30 tropical and subtropical countries around the world. The scientific classification of tea plant is showed in table 1-6 (Anand *et al.*, 2012; Parmar *et al.*, 2012):

Table 1-6: Scientific classification of green tea plant

Classification	
Kingdom	Plantae
Subkingdom	Supermatophyta
Superdivision	Spermatophyte
Division	Magnoliopsida
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Theales
Family	Theaceae
Genus	Camellia L
Species	Sinensis
Binomial name	Camellia sinensis L.

Common Names in different countries, Afghanistan: Chaai, United State: Tea Russia: Chai, India: Chha, Italy: Te, China: Cha, Africa: Itye, England: Tea plant (Parmar *et al.*, 2012). It is small tree with height of almost less than 2 meters and strong taproot, possesses yellow-white flowers which consist 7-8 petals and 2.5-4 cm diameter. Leaves have 4-15 cm long and 2-5 cm wide. The young and light green leaves are preferred for tea production which may have short hairs on the underside. Different leaf age may have different chemical composition which may produce different tea qualities. Commonly the bud and first 2-3 leaves are collected for processing of tea production which this process may repeated every 1-2 weeks (Parmar *et al.*, 2012). White tea is the least processed tea which may have the highest catechins following by green tea. It is estimated that 3 billion Kg of tea is produced and consumed annually that 78% is black tea mostly utilized in western countries, 20% green tea mostly used in Asian countries, and 2% is oolong produced and consumed in southern china (Milind *et al.*, 2012; Anand *et al.*, 2012).

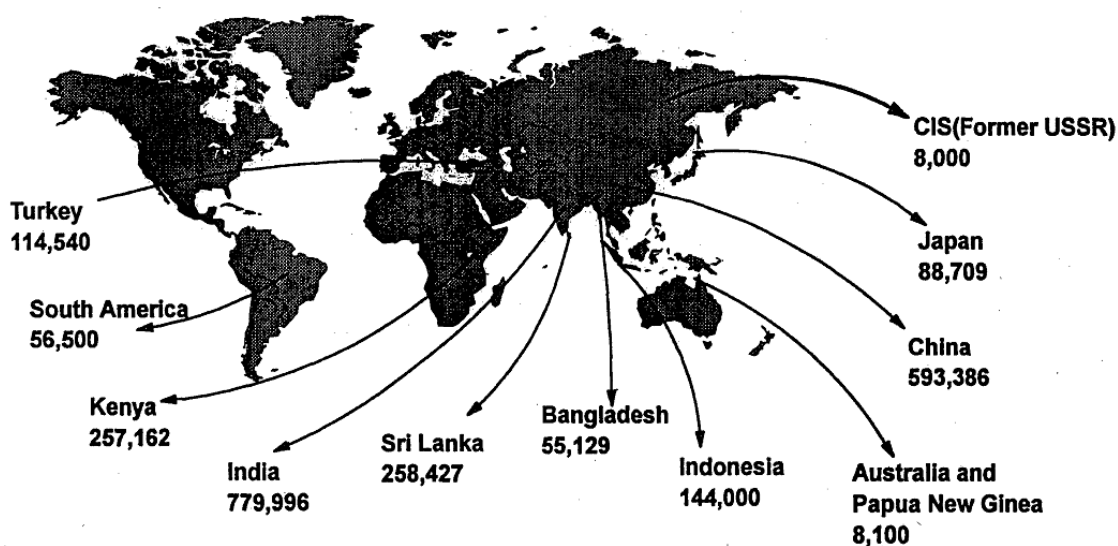


Figure 1-1: Different countries producing tea 1996 (Douglas, A. *et al.*, 1997)

1.2.1. Chemical Composition of Green Tea

Tea composition differs with season, climate, variety, horticultural practices and the age of the leaf. GT with having more than 4000 bioactive components possesses complex chemistry in such as polyphenols, alkaloids, amino acids, glucides, proteins, volatile compounds, minerals and trace elements (Stagg *et al.*, 1975; Marcia *et al.*, 2007; Tariq *et al.*, 2010). Some components of GT is much interested for human health like fluoride, caffeine, minerals, trace elements like chromium, and manganese, and polyphenols (Powell *et al.*, 1998; Cabrera *et al.*, 2003; Hope *et al.*, 2006).

1.2.1.1. Minerals

GTE contain several minerals which are beneficial for human health (Table 1-7). GTE have 2-3 times much more potassium than sodium which is helpful for lowering blood pressure (Marcia *et al.*, 2007).

Table 1-7: Electrolyte composition of GTE

#	Chemical	Amount	References
1	K	92- 259 mg/L	Powell <i>et al.</i> , 1998; Mastuura <i>et al.</i> , 2001; Fernandez <i>et al.</i> , 2002; Marcia <i>et al.</i> , 2007
2	Na	33.9 mg/kg to 1760 mg/kg	Fernandez <i>et al.</i> , 2001; Marcia <i>et al.</i> , 2007
3	Ca	1.9– 3.5 mg/L	Mastuura <i>et al.</i> , 2001; Marcia <i>et al.</i> , 2007
4	F	0.8- 2.0 mg/L	Fung <i>et al.</i> , 1999; Behrendt <i>et al.</i> , 2002; Marcia <i>et al.</i> , 2007
5	Fe	0.03– 0.17 mg/L	Mastuura <i>et al.</i> , 2001; Fernandez <i>et al.</i> , 2002; Marcia <i>et al.</i> , 2007
6	Al	1-2.5 mg/L	Marcia <i>et al.</i> , 2007
7	Mn	0.42-2.1 mg/L	Marcia <i>et al.</i> , 2007
8	Cr	Not detectable	Marcia <i>et al.</i> , 2007
9	Si	Not detectable	Marcia <i>et al.</i> , 2007

Approximately 34% of GT fluoride is retained in the oral cavity. So, GT is an effective vehicle by delivering fluoride helping to prevent dental decay (Sampson *et al.*, 2001). About 40% of Manganese is in the form of potentially bioavailable, so 225 ml (a cup) of GT may deliver 10% of routine dietary intake of Manganese (Powell *et al.*, 1998). Although tea plants possess substantial amount of aluminium, but is not included in dietary sources of "Al" associated with increased risk of Alzheimer's disease (MacKay and Blumberg, 2000). Aluminium has poor bioavailability, like iron, due to action of polyphenols present in green tea which bind to trivalent metals and inhibit their intestinal absorption (Marcia *et al.*, 2007).

1.2.1.2. Flavonoids

Flavonoids, the largest class of polyphenols with low molecular weight, may be defined as a class of polyphenols possessing compounds that have 2 benzene rings linked by 3 carbon chain and one oxygen bridge (Figure 1-2) (Cook and Samman, 1996)

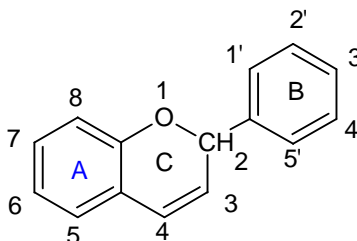


Fig 1-2: skeletal structure of flavonoids (Source: Cook and Samman 1996).

The biochemical activities of different flavonoids are dependent on their chemical structure which vary by relative orientation of various radicals on the molecule and accordingly classified to flavones, flavonols, flavanones, flavanols (catechins), isoflavones, anthocyanins, chalcones and dihydroflavonols. Flavonols and flavanols are the main classes found in tea which make 16-30% of the dry weight of fresh leaves. Catechins (flavan-3-ols) are the predominate form having di- or tri-hydroxyl groups on B ring and the meta-5,7-(OH)₂ on the A ring. When 3 (OH) groups are attached on the "B" ring the compound is called as gallo catechins (Lunder, T., 1989). Tea tannins' four major catechins are: (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin gallate (EGCG) and (-)-epigallocatechin (EGC) (Liss, *et al.*, 1976; Harborne, 1986; Pierpoint, Muramatsu, *et al.*, 1986; W.S. 1986; Harborne, 1988; Hodnick, 1988; Graham, 1991; Balentine, 1997; Harbowy and Balentine 1997). EGCG is the predominant make more than half of all tea catechins (Muramatsu, *et al.*, 1986; Coultate, 1990). Flavonols like kaempferol, myricitin, quercetin and their glycosides may also find in GT (Balentine, *et al.*, 1997; Del Rio *et al.*, 2004). The higher antioxidant activity of GT observed when there is more EGCG and EGC (Toashi, G.T. *et al.*, 2000). Solid water-soluble extract of tea possess 2-3% flavonol glycosides, while the flavonol aglycones are not present in significant amount due to their poor water solubility (Harbowy, and Balentine, 1997; Balentine, 1997). Many flavonoids naturally exist in the form of flavonoid glycosides (KiJhnau, 1976; Havsteen, 1983) and mostly these carbohydrates are D-glucose, galactose, L-rhamnose, arabinose, lignin, and glucorhamnose (Habsteen, B. 1983). Flavonoids may exist in the form of mono-, di-, or oligomeric (Harborne, 1988). Tannins (polymeric compounds) are classified in to two main groups based on their structure, condensed and hydrolysable (Brune, *et al.*, 1989). Condensed tannins are the polymers of flavonoids (Middleton, and Kandaswami, 1993) and hydrolysable tannins possess gallic acid, or similar compounds, esterified to a carbohydrate (Brune, *et al.*, 1989). Galloyl groups possess iron chelating properties, so it is believed to interfere with Fe-absorption *in vivo* (Brune, *et al.*, 1989).

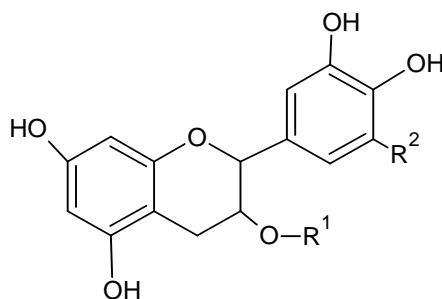


Fig 1-3: Major tea catechins

Name	Abbreviation	R1	R2
Epicatechin	EC	H	H
Epicatechin gallate	ECG	gallate	H
Epigallocatechin	EGC	H	OH
Epigallocatechin gallate	EGCG		gallate OH

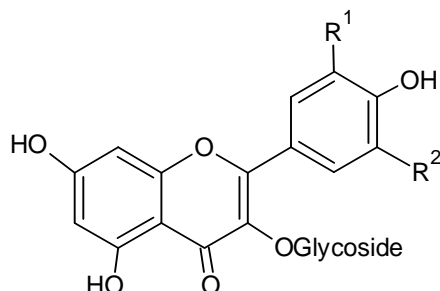


Fig 1-4: the major tea flavonols

		R1	R2
Kaempferol glycoside	KaG	H	H
Quercetin glycoside	QuG	OH	H
Myricetin glycoside	MyG	OH	OH

Table 1-8: Structure of flavonoids

Flvnonoid	Total No. of OH groups	Position of OH groups	Substitutions on the generic structure	Position of the substitutions
Myricetin	6	3,5,7,3',4',5'		
Gossypetin	6	3,5,7,8,3',4'		
Quercetagen	6	3,5,6,7,3',4'		
Quercetin	5	3,5,7,3',4'		
Morin	5	3,5,7,2',4'		
Robinetin	5	3,7,3',4',5'		
Myricetrin	5	5,7,3',4',5'	O-Ru	3
Rutin	4	5,7,3',4'	O-Ru	3
Kaempferol	4	3,5,7,4'		
Quercetrin	4	5,7,3',4'	O-Rh	3
Fisetin	4	3,7,3',4'		
Datiscetin	4	3,5,7,2'		
Rhamnetin	4	3,5,3',4'	O-Me	7
Tamrixetin	4	3,5,7,3'	O-Me	4'
Silybin	3	3,5,7	O-ligo-O	4'
Galangin	3	3,5,7		
Kaempferide	3	3,5,7	O-Me	4'
Diosmin	2	3,3'	O-Ru,)-Me	5,4'
Robinin	2	5,4'	O-Gal-Rh, Rh	3,7'
Troxenutin	1	5	O-Ru, O-He	3,7,3',4'
3-OH-flavone	1	3		

Rh=rhamnose, Lig= lignin, Ru=rutinose, He=hydroxyethyl, Me=methyl, and Gal=galactose

Recently, by developing of new and more sophisticated identification and separation techniques, more catechins and related compounds identified in tea while little quantitative data reported (Hashimoto and Nonaka, 1989), such as methylated catechins and catechin digallate. Other novel group of compound has been identified called chalcon-flavans-bimolecular combinations of a catechin attached to chalcone derivatives (Harlod and Graham,

1992). Bisflavanols (Theasinensins), the dimeric gallocatechins linked by C-C bonds on the "B" rings, are also found in GT leaf (Hashimoto, and Nonaka, 1989) showed in figure 1-5.

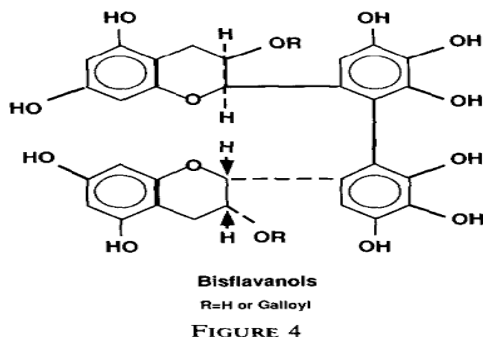


Fig 1-5: The Bisflavanols chemical structure.

Dimeric proanthocyanidins which are considered as condensation products of catechins, linked by C-C bonds between pyran ring and an "A" ring (Hashimoto, and Nonaka, 1989). Delphinidin and cyanidin (monomeric anthocyanidins) are the flavones equivalents of epigallocatechin and epicatechin respectively (Harlod, and Graham, 1992) (Figure 1-6)

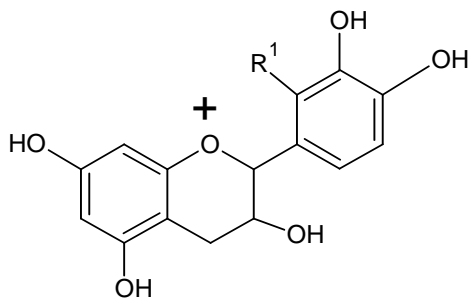


Fig 1-6: general structure for, Cyanidin R=H, and Delphinidin R=OH

1.2.1.3. Methylxanthines

Tea leaves contains about 2.5-4.0% (dry weight basis) caffeine, and smaller amount of methylxanthine theobromine. Presence of theophylline in tea leaves also has been reported (Michl, and Haberler, 1954), however, it is usually not detectable in tea beverage (Hicks, *et al.*, 1996) and also does not form by normal biosynthetic pathway of methylxanthines (Ashihara, *et al.*, 1997). 180 ml of GT contains 60 mg of caffeine while 180 ml of coffee possess 100 mg of caffeine. Available decaffeinated tea contains less than 5 mg of caffeine in 180 ml (Douglas, A. *et al.*, 1997).

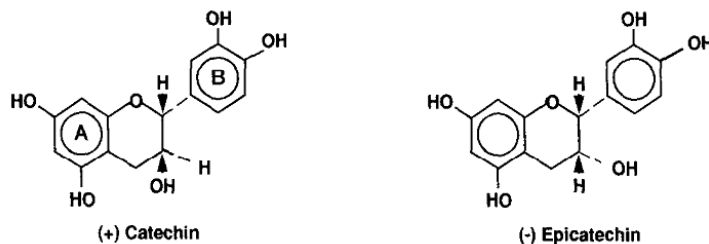


Fig 1-7: left, (+) catechin, and the right (-) epicatechin

1.2.1.4. Protein and Amino acids

GT beverage contains about 17% w/w nitrogenous materials (about 6% w/w protein, and about 1% w/w amino & nucleic acids). Side of other normal complement of amino acids, theanine (N-methylated derivative of glutamine), an unusual and unique amino acid, also exist in a quantity of approximately 3% w/w in GT beverages (Sanderson, 1972; Eden, 1976; Liang, *et al.*, 1990; Harlod, and Graham, 1992). Trigalloylglucose also has been identified in GT leaf (Hashimoto, and Nonaka, 1989).

1.2.1.5. Enzymes

The most important enzymes in GT are those responsible for the biosynthesis of tea flavonoids, and for the conversion of fresh GT leaf to manufactured commercial tea, such as polyphenol oxidase-involved in the formation of black tea polyphenols (Gregory, and Bendall, 1966; Sanderson, 1972; Eden, 1976; Liang, *et al.*, 1990). Peroxidase (POD) is also present in GT leaf (Bokuchava, 1950; Roberts, 1952) catalyzes the decomposition of H₂O₂ to water, and organic peroxide species to the alcohol. Catalase enzyme is more active in tea and removes peroxides rapidly, hence limiting the activity of POD in fermentation reactions (Balentine, 1997; Harbowy, and Balentine, 1997).

Table 1-9: Chemical composition of the tea leaf

Component	% of dry weight
Flavanols	25
Flavonols and flavonol glycosides	3
Phenolic acids and depsides	5
Other polyphenols	3
Caffeine	3
Theobromine	0.2
Amino acids	4
Organic acids	0.5
Monosaccharides	4
Polysaccharides	13
Cellulose	7
Protein	15
Lignin	6
Lipid	3
Chlorophyll and other pigments	0.5
Ash	5
Volatiles	0.1

Source: Douglas, *et al.*, 1997.

Carotenoids also present in GT but in low level which make precursors of tea aroma. P-carotene, lutein, neoxanthin, and violaxanthine have been identified (Venkatakrisna, *et al.*, 1976).

Volatile components are also exist in fresh green tea with very small amount which more than 60 of them including alcohols, esters, carbonyls, cyclic compounds and acids have been identified (Yinfang *et al.*, 1982)

1.3. Liver

Liver, the largest gland in the body and second largest single organ, is located in the upper-right part of abdominal cavity. Mainly it lies in the right hypochondrium and in the epigastrium, as well as part of it is continued into the left hypochondrium inferior to diaphragm, and into the right lateral region. Liver weighs about 1.5 kg which accounts for approximately 2.5% of adult body weight. In the late fetus it is approximately of normal adults' liver weight (5% of total fetus body weight). The liver is almost but not completely covered by visceral peritoneum. This non-covered part is called falciform ligament which divides the liver into two lobes (left and right lobes). Right lobe is the larger one which considered by many anatomists to has quadrate lobe and a posterior caudate lobes, and the left lobe is the smaller one. Each lobe receives its own primary branch of the hepatic portal vein and artery and is drained by its own hepatic dust. Left and right hepatic ducts are joined and form the common hepatic duct. Hepatic artery—a branch of the celiac trunk, is responsible for delivery of oxygenated blood to the liver which enters to the liver at the porta hepatis and divides into 2 main branches to supply blood to the right and left lobes of liver. Portal vein transfer blood from GI tract to the liver with divided into right and left branches at porta hepatis (Figure 1-8). Blood from the liver is drained out through number of hepatic veins into the inferior vena cava.

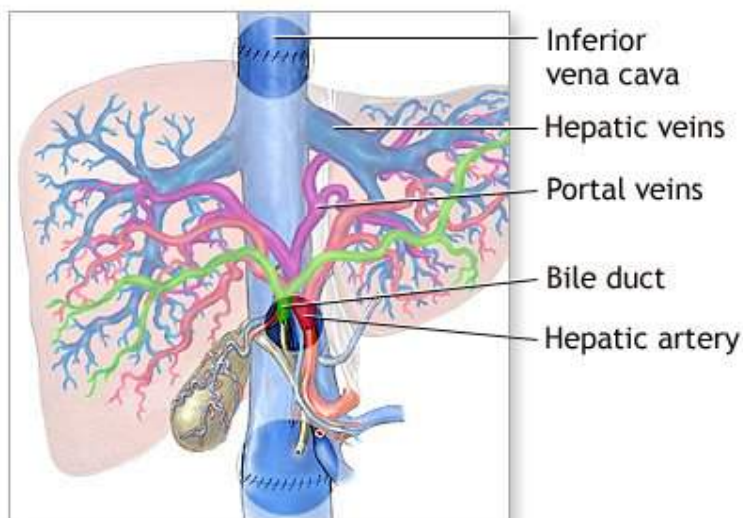


Fig 1-8: Liver blood vessels
 Source:http://www.pennmedicine.org/encyclopedia/em_PrintPresentation.aspx?gclid=100090&ptid=3 (accessed on 25th April- 2015)

1.3.1. Histology of the liver

Lobes of liver are consist of many functional unites called lobules. Each lobules is a hexagon structure having made of hepatocytes (specialized epithelial cells)—arranged in irregular, branching, and interconnected plates around the each central vein. Sinusoids are the highly-permeable capillaries located in the liver lobules. Kupffer cells (stellate reticuloendothelial cell) are fixed phagocytes present in sinusoids, which destroy worn-out WBCs and RBCs, bacteria, and other foreign materials in the venous which blood comes from GI tract.

1.3.2. Function of the liver

- Production and secretion of bile, to facilitate the digestion and absorption of fats from intestine.
- Carbohydrate metabolism: liver is one of the important organs in maintaining of blood glucose level. Glycogen which are stored in liver break down and release glucose to circulation to maintain blood glucose level in the case of hypoglycemia. Liver can also produce glucose from amino acids and lactic acid, as well from other sugars such as galactose and fructose. While in the case of hyperglycemia, liver can convert glucose to glycogen and triglycerides for storage.
- Lipid metabolism: liver can store TGs in hepatocytes, break down FAs to generate ATPs, synthesize cholesterol, synthesize lipoproteins for transportation of FAs, TGs, and cholesterol, and produce bile salt by using cholesterol.
- Protein metabolism: liver remove the amino group (deamination) from amino acids so the amino acid can convert to carbohydrates or fats or can be used for ATP production. NH₃ is converted to urea through urea cycle in liver to eliminate toxic NH₃ from the body. Hepatocytes also produce most of plasma protein such as albumin, alpha and beta globulins, prothrombin, and fibrinogen and some other clothing factors.
- Processing of drugs and hormones: liver has the ability of detoxification of some substance such as alcohol and excrete drugs such as erythromycin, sulfonamides and penicillin into bile. Liver can also metabolize thyroid hormones and steroid hormones.
- Excretion of bilirubin.
- Synthesis of bile salts: bile salts are synthesized by use of cholesterol in liver and used for emulsification and absorption of lipids in small intestine.
- Storage: liver can storage the glycogen, some vitamins such as A, B12, E and D, minerals such iron and copper.
- Phagocytosis: the kupffer cell in liver phagocytize the old RBCs, WBCs, and some bacteria.
- Activation of Vitamin D. skin, liver and kidneys are the organs responsible for activation process of Vit.D.

(Inderbir Singh , 2002; Keith and Arthur, 2006; Gerard J. Tortora and Bryan, 2006; MN Chatterjea and Rana, 2012; Taylor and Barbara, 2013).

1.3.3. Liver function tests

Liver function tests are categorized based on the main function of liver.

1.3.3.1. Tests based on the abnormalities of bile pigment metabolism

- **Conjugated, un-conjugated and total bilirubin estimation in blood.**
In haemolytic jaundice there is an increase in un-conjugated bilirubin, in obstructive jaundice conjugated bilirubin is increased, and in hepatocellular jaundice or damage either or both may be present in higher level in blood.
- **Bile pigments in Urine/Faeces**
Bilirubin can be found in the urine the case of obstructive jaundice, conjugated bilirubin may pass through glomerular filtration process. Bacteria present in intestine reduce the bilirubin so normally bilirubin is not present in faeces, but when found may be due to rapid movement of materials along the intestine, or in very young infants due to lack of intestine flora, or patients used gut sterilizing antibiotics, or biliverdin is found in meconium.
- **Urinary and Faecal Urobilinogen**
Normal range of urobilinogen in faeces is 50-250 mg/day. It increase in haemolytic jaundice and is decreased or absent in obstructive jaundice. Average 0.64 mg/day urobilinogen and maximum 4 mg/day is present in urine. No urobilinogen is found in urine in the case of obstructive jaundice while in haemolytic jaundice it increases. It also increase in the case of hepatic parenchyma damage.

1.3.3.2. Tests based on liver's part in carbohydrate metabolism

- **Glucose tolerance test**
It does not have much value in liver disease.
- **Galactose tolerance test**
Impaired liver cannot convert galactose to glucose.
- **Fructose tolerance test**
Impaired liver cannot convert fructose to glucose
- **Epinephrine tolerance test**

1.3.3.3. Tests based on changes in plasma protein

- **Total plasma protein, albumin, globulin and A:G ratio**
This test provides useful information about chronic liver diseases. Liver is site of albumin synthesis and also some of α - and β -globulins. In obstructive jaundice they are normal, while in advanced parenchymal liver disease and in cirrhosis of liver the albumin level is decreased and globulins are often increased. In infectious hepatitis total protein may be normal but in early stage β -globulins and later stages γ -globulins show elevation.
- **Estimation of plasma fibrinogen**
- **Flocculation tests**
- **Amino acids in urine**

1.3.3.4. Tests based on abnormalities of lipids

- **Cholesterol-cholesterol ester ratio**

Normal serum cholesterol level is 150-205 mg/dl with approximately 60-70% as ester form. In obstructive jaundice total cholesterol is increased but the chol:cholesterol is not changed, while in parenchymatous liver disease there is no increase or even reduce in total cholesterol level with always reduced ester-fraction.

1.3.3.5. Tests based on the detoxicating function of the liver

- **Hippuric acid test of quick**

1.3.3.6. Tests based on the excretory function of liver

- **BSP retention test**
- **Rose-Bengal dye test**
- **Bilirubin tolerance test**

1.3.3.7. Tests based on formation of prothrombin by liver

- **Determination of prothrombin time.**

1.3.3.8. Tests based on amino acid catabolism

- **Determination of blood NH₃**
- **Ammonia tolerance test**
- **Determination of glutamine in CS fluid**

1.3.3.9. Tests based on drug metabolism

- **MEGX test**
- **Antipyrine breath test**

1.3.3.10. Tests based on liver enzymes activities

Number of enzymes are used to demonstrate the liver function which divided into two groups, most commonly estimated (serum transaminases and serum alkaline phosphatase), and not routinely estimated.

- **Serum Transaminases (amino transferases)**

SGOT (serum glutamate-oxaloacetate transaminase) or AST (Aspartate transaminase) normal range in human is 4-17 IU/L and SGPT (serum glutamate pyruvate aminotransferase) or ALT (alanine transaminase) normal range is 3-15 IU/L. these enzymes can be found in most of the tissues with higher amount of SGOT in heart muscles, and both in higher amount in liver whereas SGPT (serum glutamate-pyruvate transaminase) is dominant. Liver diseases are associated with elevation level of both, but SGPT higher than SGOT. Serum levels of these two enzymes are more elevated in hepatocyte involvement then bile ducts.

- **Serum alkaline phosphatases (ALP)**

This enzyme is present in most of the tissues and organs but higher level can be found in bones and liver, following by small intestine, kidney and placenta. Reference range

for ALP is 3-13 KA units/100 ml which equals to 23-92 IU/L. Serum level of ALP is more elevated in obstruction (sinusoidal ducts disorders) than hepatocytes.

- **Enzyme which are not estimated routinely**

These enzymes are serum 5'-nucleotidase, serum lactate dehydrogenase, serum cholinesterase, serum ornithine carbamoyl transferase, serum leucine amino peptidase, serum hydroxyl butyrate dehydrogenase, serum aldolase and phosphohexose isomerase, serum sorbitol dehydrogenase, and serum γ -glutamyl transferase.

(MN Chatterjea and Rana, 2012; Bishop, Fody, and Schoeff, 2012)

1.4. The chemistry of superoxides and oxidative stress

The phrase of Reactive Oxygen Species (ROS) is used to describe a variety of molecules and free radicals derived from molecular oxygen such as superoxides, hydrogen peroxide and hydroxyl radical. Molecular oxygen (bi-radical) in the ground state possesses two unpaired electrons in the outer shell, known as a triplet state, and contains parallel spin. This molecule reacts only with one electron at a time, therefore, it is not very reactive with two electrons in a chemical bond. When this molecule is excited and one of the two unpaired electrons changes its spin, results to generation of a powerful oxidant and these two electrons with opposite spin can react very quickly with other pairs of electron (especially double bonds). Superoxide anion ($O_2^{\bullet-}$), the result of reduction of oxygen by one electron at a time and the initial product of the electron transport chain (ETC), is a mediator in oxidative chain reactions and the precursor of most ROS. Superoxide anion goes under dismutation, either spontaneously or by superoxide dismutase enzymes, and produces H_2O_2 . This H_2O_2 further may be fully reduced and produce water by the mean of catalase or glutathione peroxidase or partially reduced and produce hydroxyl radical (OH^{\bullet}), one of the strongest oxidants in nature, in presence of reduced transition metals (reduced copper or iron) (Cristina Camello-Almaraz, *et al.*, 2006; Julio, F. Turrens., 2003). Superoxide anion may react with radical such as nitric oxide (NO^{\bullet}) and produces a very powerful oxidant (peroxynitrite) (Beckman & Koppenol, 1996; Radi *et al.* 2002b). The NO^{\bullet} derived oxidants are called reactive nitrogen species (RNS) (Julio, 2003).

In vivo, $O_2^{\bullet-}$ production can be enzymatically or non-enzymatically. NADPH oxidase in cell membrane of macrophages, endothelial cells, polymorphonuclear cells (Babior, 2000 and 2002; Vignais, 2002), and CYP450-dependent oxygenases (Coon *et al.* 1992), and the conversion of xanthine dehydrogenase to xanthine oxidase are the enzymatic sources of $O_2^{\bullet-}$ and H_2O_2 formation, hence they also provide the source of OH^{\bullet} . The non-enzymatic production of $O_2^{\bullet-}$ includes the direct transfer of a single electron to O_2 by a prosthetic groups (e.g., flavins or iron sulfur cluster), or reduced coenzymes, or by reduced xenobiotics (Julio, 2003).

Respiratory complex I & III which are located at the inner membrane of mitochondria are the main generating sources of superoxide anions as side product of electron transport during

oxidative phosphorylation. Mitochondria are involved in production of ATP by oxidative phosphorylation, maintenance of cellular redox status, regulation of apoptosis, and ROS formation (Dikalov, 2011).

Complex I releases the $O_2^{\cdot-}$ into the matrix and complex III releases $O_2^{\cdot-}$ into both the matrix and the intermembranous space (Nicholls and Budd, 2000; Chen *et al.*, 2003) and it can produce $O_2^{\cdot-}$ in both forward and reverse electron transfer which succinat-dependent reverse electron transport is more efficient than NADH-dependent forward (Panov *et al.*, 2007). $O_2^{\cdot-}$ is formed by complex III during cycling of the electron acceptor ubiquinone, which can donate electrons to the molecular oxygen in both faces of inner mitochondrial membrane (Julio, 2003; Cristina *et al.*, 2006). Electron transfer through the mitochondria respiratory chain is highly involved in ATP synthesis, which basically electrons are supplied by NADH at complex I and by $FADH_2$ at complex II which in turn reduced the complex IV. Complex I, III and IV also pump the protons from matrix in to the intermembrane space at the same time. Complex V uses the proton motive force across the inner mitochondrial membrane and produces ATP (MN chatterjea and Rana, 2008; Sergey and Zoltan, 2013). Leakage of electron from ETC to O_2 leads to production of $O_2^{\cdot-}$. Superoxide anion is produced from reaction of O_2 with fully reduced FMN in complex I, and the proportion of fully reduced FMN is set by the NADH/NAD⁺ ratio (Michael, 2009), so inhibition of ETC by mutation, damage, loss of cytochrome c, ischaemia or by more formation of NADH due to low ATP demand and low respiration rate will induce the NADH/NAD⁺ ratio and cause $O_2^{\cdot-}$ formation (Hansford *et al.*, 1997; Boveris *et al.*, 1997; Kushnareva *et al.*, 2002; Liu *et al.*, 2002; Kudin *et al.*, 2004; Kussmaul and Hirst 2006).

There are three modes of operation which may lead to efflux of $O_2^{\cdot-}$ and H_2O_2 . First, when there is high NADH-to-NAD⁺ ratio in the matrix of mitochondria which may cause more electron flow and electron leakage (Kudin *et al.*, 2004; Kussmaul and Hirst 2006), the second, when there is high level of reduced coenzyme Q pool in association with maximal Δp and less or no ATP formation (Hansford *et al.*, 1997; Korshunov *et al.*, 1997; Lambert and Brand, 2004), and the third one is the normal function of mitochondria which synthesis ATP, or when uses Δp for other function (e.g., thermogenesis) which H_2O_2 efflux in this mode is negligible in compare with two other modes (Michael, 2009). Complex I is the main source when electron transport rate and ATP synthesis are low and NADH-to-NAD ratio is high (Cristina *et al.*, 2006). MnSOD (Manganese containing SOD) quickly dismutates the superoxide anions in the mitochondrial matrix, while Zn/Cu-SOD (Zinc or Copper containing SOD) dismutates the intermembranous space superoxide anions to H_2O_2 (Julio, 2003) and when the concentration of $O_2^{\cdot-}$ is high enough in the mitochondrial matrix, part of that transfer to the intermembranous space and cytoplasm through anion channels (Han *et al.*, 2003; Aon *et al.*, 2004). The rate of $O_2^{\cdot-}$ formation by ETC is increasing when the electron flow is down which may lead to increase of concentration of electron donor (R^{\cdot}) or when the concentration of oxygen is induced (Julio *et al.* 1982). The energy which release from the electron flow in ETC is converted to a H^+ gradient via the inner mitochondrial membrane and consecutively this gradient is dissipated by the complex V (ATP synthase complex). H^+ movement via ATP synthase is ceased In the absence of ADP and H^+ gradient causes slow

electron flow and the ETC becomes more reduced and results induced $O_2^{\cdot-}$ formation (Boveris *et al.* 1972; Julio, 2003). Several factors are affecting the rate of $O_2^{\cdot-}$ production such as concentration of enzyme or protein [E]-responsible for electron transfer, able to react with O_2 to form $O_2^{\cdot-}$, the proportion of this enzyme in a redox form which can react with O_2 , concentration of local $[O_2]$, and the second-order rate constant (k_E) for the reaction of that electron carrier with O_2 to form $O_2^{\cdot-}$. The concentration of enzyme may vary with organism, age, state, tissue and hormonal status. Changes in local $[O_2]$ concentration have little direct effect on mitochondrial function, but instead affect $O_2^{\cdot-}$ production. When $[O_2]$ is increased above the normal atmospheric level of 21% O_2 , $O_2^{\cdot-}$ and H_2O_2 generation also may induce in mitochondria, and $O_2^{\cdot-}$ and H_2O_2 generation is decreased when $[O_2]$ is lowered below that of air-saturated medium (Alvarez *et al.*, 2003; Kudin *et al.*, 2004; Hoffman *et al.*, 2007). While other study showed that rate of H_2O_2 production did not vary when $[O_2]$ reduced from $\sim 200\mu M$ to $\sim 5\mu M$ but below $5\mu M$ the rate of H_2O_2 production varied (Hoffman *et al.*, 2007). Relationship of $O_2^{\cdot-}$ and H_2O_2 production with $[O_2]$ concentration is very important because extracellular $[O_2]$ varies with physiological state (Skulachev, 1996). All $O_2^{\cdot-}$ are not converted to H_2O_2 in vivo, as some of them react with NO^{\cdot} or with some other electron acceptors (Packer *et al.*, 1996; Szabo *et al.*, 2007). All H_2O_2 are not survive to move outside the mitochondria, owing to some matrix peroxidases including catalase (Radi *et al.*, 1991; Salvi *et al.*, 2007), peroxiredoxins 3 & 5 (Rhee *et al.*, 2001), and glutathione peroxidases 1 & 4 (Imai and Nakagawa, 2003) which consume H_2O_2 (Zaccarato *et al.*, 2004; Andreyev *et al.*, 2005; Hurd *et al.*, 2005). Probably peroxiredoxins have the greatest significance (Rhee *et al.*, 2001). Many mitochondrial manipulations have effect on the activity of H_2O_2 degradation system such as GSH/GSSG ratio linked through glutathione peroxidase (Imai and Nakagawa, 2003) and thioredoxin 2 oxidized/reduced ratio linked through peroxiredoxins (Holmgren, 1985; Rhee *et al.*, 2005; Hurd *et al.*, 2005; Rigobello *et al.*, 2005; Hurd *et al.*, 2005) and these are in turn affected by the NADPH/NADP⁺ ratio which is regulated with the activity of the transhydrogenase and activity of NADP⁺-dependent isocitrate dehydrogenase (Sazanov and Jackson 1994; Rydstrom, 2006). The other mechanism, complex I produces extensive $O_2^{\cdot-}$ by RET (Reverse electron transport) under high Δp condition with electron supply to the CoQ pool from α -glycerophosphate, succinate or fatty acid oxidation at isolated mitochondria of heart, liver, brain and muscle (Votyakova and Reynolds, 2001; St-pierre *et al.*, 2002; Lui, *et al.*, 2002; Adam-vizi and Chinopoulos, 2006). Other different sites which produce $O_2^{\cdot-}$ or H_2O_2 are also exist in mitochondria, such as those sites interact with matrix NADH pool and those other sites which are connected to the CoQ pool within the inner membrane (Michael, P. Murphy. 2009). When NADH/NAD⁺ ratio is high due to low respiratory chain activity or due to RET, it may leads to $O_2^{\cdot-}$ production at other sites which are connected to the NADH, in addition to the complex I (Michael, 2009). Therefore when the NADH/NAD⁺ ratio is high, not only complex I but other enzymes such as α -ketoglutarate dehydrogenase, and perhaps some other enzymes linked to the NADH pool, contribute to $O_2^{\cdot-}$ production in mitochondria (Michael, 2009). In addition to the complex I & III discussed above, there are several other enzymes which may contribute to $O_2^{\cdot-}$ or H_2O_2 production. Some of them have no connection with NADH or CoQ pool, instead they receive electrons from NADPH pool such as adrenodoxin reductase/adrenodoxine/cytochrome P450 system in the mitochondrial matrix

(Hanukoglu *et al.*, 1993; Hanukoglu, 2006; Michael, 2009). Conditions such as slow respiration, ischaemia or damage to the respiratory chain which may lead to build up of NADH induce the mode I production of $O_2^{\bullet-}$ which may occur when cytochrome c releases in apoptosis and after inhibition of respiration by NO^{\bullet} at cytochrome oxidase (Michael, 2009).

Oxidative stress is the term used to describe various harmful processes due to excessive formation of ROS and/or RNS along with decreased or limited antioxidant defenses. Over production of ROS and RNS lead to more generation of free radicals which indiscriminately target DNA (Richter *et al.*, 1988; LeDoux *et al.* 1999), proteins (stadtman and Levine, 2000), polysaccharides (Kaur and Halliwell, 1994) and lipids (Rubbo *et al.*, 1994).

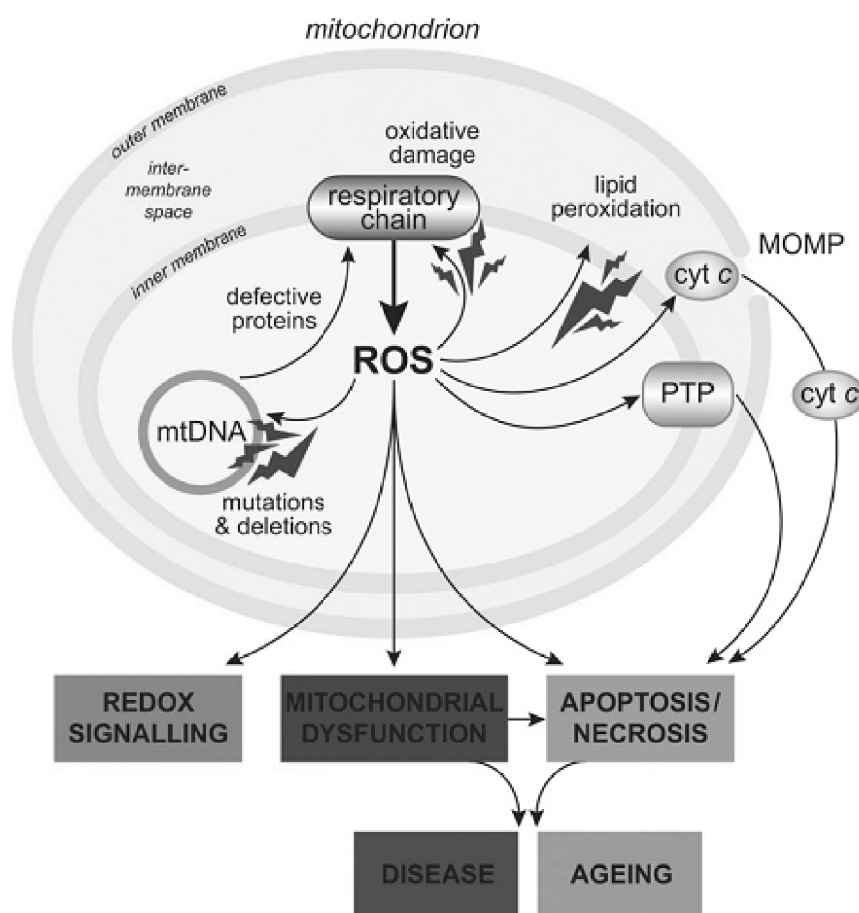


Figure 1-9: Overview of mitochondrial ROS production

ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions, including the tricarboxylic acid cycle, fatty acid oxidation, the urea cycle, amino acid metabolism, haem synthesis and FeS centre assembly that are central to the normal operation of most cells.

1.4.1. Impact of ROS in the Pathogenesis of Diabetes Mellitus and Its Complications

Mitochondria is the main and primary source of ATP production, disturbance of its function may cause several disease such as Friedreich ataxia, Parkinson, pathophysiology of aging, diabetes and its complications, and Huntington disease (Takeshi and Eiichi, 2007). It also has been found that mitochondrial mutation was associated with maternally inherited diabetes mellitus (Kadowaki *et al.*, 1994). Studies showed that dysfunction of mitochondria in β -cells of pancreas damages secretion of insulin in response to induced glucose level (Takeshi and Eiichi, 2007). It is also has been reported that there is link between impaired mitochondrial oxidative phosphorylation in liver and muscle and diabetes type 2 (insulin resistance diabetes, Petersen *et al.*, 2003; Petersen *et al.*, 2004). Experiment on animal models revealed that induction DM altered mitochondrial respiration and disrupted energy production in liver, diaphragm, skeletal muscle and heart. Administration of insulin restored function of mitochondria (Vester and Stadie, 1957; Hall, 1960a and 1960b; Robertson *et al.*, 1992; Tomita *et al.*, 1996). Numerous in vivo and in vitro studies revealed elevation of oxidative stress in DM and it is thought to be the key mechanism of pathogenesis and complication of DM (Baynes JW. 1991; Giugliano *et al.*, 1996; Green *et al.*, 2004; Hinokio, *et al.*, 1999; Oberley, 1988; Oberley 1998; Suzuki, *et al.*, 1999; Wolff *et al.*, 1991; Takeshi and Eiichi, 2007). Different mechanism contribute in development of oxidative stress in diabetes which are included non-enzymatic glycosylation (glycation), auto-oxidative glycosylation, metabolic stress (resulted from changes in energy metabolism), alterations of sorbitol pathway activity, alteration in the level of inflammatory mediators and antioxidant defense system, and ischemic reperfusion injury and hypoxia are caused localized tissue damage (John, 1991).

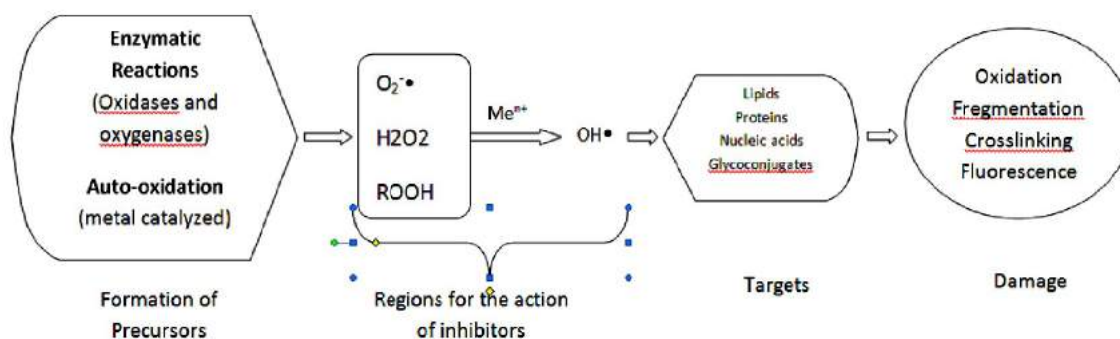


Fig 1-10: common pathways of oxidative stress contribution to development of diabetes complication.

Damages due to oxidative stress are not reversible. The kinetic of turnover of the molecules such damaged proteins, lipids, and RNA is the only way of limiting the accumulation of oxidative damages, while only DNA can be repaired (John, 1991). Modification of extracellular proteins such collagens, crystallins, liminin, myelin sheath proteins and elastins, and as well as structural changes in cells with high level of these proteins are associated with development of diabetic complication such as microangiopathy, cataracts, nephropathy and atherosclerosis (John, 1991). Structural changes are associated with functional and

morphological alteration of collagen-rich tissue and cells such as thickness of basement membrane, decreased joint mobility, impaired wound healing, and alteration in vascular permeability in diabetic subject (John, 1991). It is thought that protein potential exposure to the oxidative damage is enhanced by protein glycation which supported by in vitro studies showed glycation of protein was accompanied by oxidative fragmentation of the protein and peroxidation associated lipids (Wolff, 1987; Hicks et al., 1988; Hunt et al., 1990; John, 1991). Significant changes have been seen in metabolism and structure of lipids in diabetes which are associated with oxidation of lipids of plasma lipoproteins and cell membrane lead to vascular disease (Lyons, 1991; John, 1991). Diabetes is accelerated the atherosclerotic disease of arteries which supply blood to brain, heart and lower parts. Cardiomyopathy is one of the major diabetic complication (Ferdinando and Michael, 2010). Five major mechanisms are suggested for hyperglycemic-induced damage: 1). Induced flux of glucose and other sugars through the polyol pathway; 2). Induced intracellular formation of advanced glycation end products (AGEs); 3). Induced receptor expression of AGEs and its activating ligands; 4). Protein kinase C (PKC) isoforms activation; and 5). Increased activity of hexosamine pathway (Ferdinando and Michael, 2010). All these mechanism are stimulated by mitochondrial ROS over formation. Family of aldo-keto reductase enzymes use a wide variety of carbonyl compounds as substrates and reduce them to respective sugar alcohols (polyols) by consuming NADPH. Aldose reductase is one of these enzymes, converts glucose to sorbitol which further then oxidized to fructose by sorbitol dehydrogenase by use of NAD⁺ as co-enzyme (Ferdinando and Michael, 2010). Some tissues such as retina, lens, nerve, vascular cells and glomerulus which possess insulin-independent GLUTs, are rich in aldose reductase enzyme (Ramasamy, and Goldberg, 2010) and intracellular glucose increases parallel with hyperglycemia. Rise in aldose reductase activity increases NADPH consumption; whereas, decreased NADPH reduces GSH regeneration which GSH is an important scavenger of ROS. Hence rise in ROS induces oxidative stress (Ferdinando and Michael, 2010). It is reported that over expression of aldose reductase in diabetic mice induced atherosclerosis and decreased glutathione regeneration regulatory genes (Vikramadithyan et al., 2005). In diabetic vascular cells, it is suggested that glucose is not the substrate of aldose reductase because lesser affinity of enzyme to it, but glycolytic metabolites of glucose such as glyceraldehydes-3-phosphate could be the physiologically relevant substrate because of high affinity of enzyme to them (Zhang et al., 2003; Ferdinando and Michael, 2010). AGEs are the products of reaction between proteins and glucose or other glycating compounds derived from glucose or from increased fatty acid oxidation such as 3-deoxyglucosone, glyoxal and methylglyoxal (Candido, 2003, Wautier and Schmidt, 2004;.). Advanced glycation end products (AGEs) are found in higher level in diabetic retinal vessels, and renal glomeruli (Brajendra et al., 2006). Three general mechanisms have been suggested for harmful effect of AGEs on target cells: AGEs modify the intracellular proteins and alter their functions; AGE precursors modify the extracellular matrix components which in turn they interact abnormally with the receptors of matrix proteins (integrins) on cells and with other matrix components; and the 3rd mechanism suggest is that AGE precursors-modified proteins interact with AGE-receptors on mesangial cells, endothelial cell, and macrophages, including receptor-mediated formation of reactive oxygen species (Brajendra et al., 2006). The

pleiotropic transcription factors are activated by AGE receptor ligation causing changes in gene expression and other cellular signaling affairs for example activation of some kinases such as mitogen-activated protein (MAP) kinase and protein kinase C (PKC) which finally lead to cellular dysfunction (Ishii et al., 1996; Brajendra et al., 2006). PKCs are intracellular signaling molecules involved in regulation of many vascular functions such permeability, endothelial activation, growth factor signaling and vasodilator release. Nine of eleven isoforms of PCK are activated by Diacylglycerol (DAG)—a lipidic second messenger. Induced level of intracellular glucose increases amount of DAG (because ROS inhibits the GAPDH enzyme) in retina and glomeruli in diabetic subjects which leads to more activation of PKC results retinal and renal blood flow abnormalities (Brajendra et al., 2006; Ferdinando and Michael, 2010). Hyperglycemia also may cause flux of fructose-6-P (derived from glycolysis) to hexosamine pathway. Increased conversion of F6P to glucoseamine-6-P leads to over transcription of TGF (transforming growth factor)- α and $-\beta 1$, and PAI (plasminogen activator inhibitor)-1 (Brajendra et al., 2006; Ferdinando and Michael, 2010). All above mechanisms are stemmed from a single hyperglycemia-induced process known as overproduction of superoxide by mitochondrial ETC (Ferdinando and Michael, 2010).



Review of
Literatures

Chapter-II

- Anti-diabetic effect of green tea
- Hepatotoxic effects of green tea
- Hepatoprotective effect of green tea
- Effect of green tea on cytochrom P450
- Antioxidative effect of green tea
- Pro-oxidant properties of green tea extract
- Association of liver damage/impaired functions and diabetes mellitus

2. Review of Literatures

2.1. Anti-diabetic Effect of GT

Tea polyphenols especially EGCG and ECG inhibited the sodium-dependent glucose transporter -1 (SGLT-1) in brush-border membrane vesicles of rabbit small intestine. It was suggested one of the possible mechanism of anti-diabetic/lowering glucose effect of GT. It was revealed that ECG inhibited the SGLT-1 in a competitive way, but itself did not transport via SGLT-1. Tea polyphenols (e.g., ECG) interacted with glucose transporter as antagonist-like molecule (Yoko Kobayashi, *et al.*, 2000).

GTE also have shown inhibitory effect on α -amylase in intestine. Studies on EC, EGC, ECG, EGCG, catechin, GC, CG, GCG, theaflavin (TF1), theaflavin digallate (TF3), theaflavin monogallates (TF2A & TF2B) revealed that EC and EGC including their isomers had insignificant effect on the activity of α -amylase, while others showed inhibitory effect with the most stronger inhibitory effect by TF3 followed by TF2A, TF2B, TF1, CG, GCG, ECG, and EGCG (Yukihiko & Miwa, 1990). They have suggested existence of 3,4,5-(OH)₃-benzoyl moiety at 3-OH and the stereostructure of B-ring are essential for their inhibitory effect, and the inhibitory potency of theaflavins was increased as the number of galloyl moiety increased with the strongest inhibitory effect of TF3 followed by TF2 and TF1 (Yukihiko & Miwa, 1990). As α -amylase has important role in digestion of starch so, inhibition of that could be one of the other mechanisms of anti-hyperglycemic effect of green tea.

They have investigated more to found the effect of GT and BT on intestinal sucrase and α -glucosidase (Miwa & Yukihiko, 2014). Their finding presented EGCG, TF3, ECG, TF2A, TF2B the most potent sucrose inhibitors respectively. While the inhibitory effect of TF1 and non-gallated catechins were weaker. Simple catechins had the strongest inhibitory activity (85.3%) among the all simple polyphenols such as catechins, thearubigin and theaflavins, as well as stronger (1.7 time) than tannic acid. Similarly esterified (gallated) polyphenols showed stronger inhibitory activity on α -glucosidase enzyme with the most potent inhibitory effect of TF3 followed by TF2B, TF2A, ECG, EGCG. Differently, in the case of α -glucosidase, simple theaflavins showed stronger inhibitory effect (84.6%) among all crude polyphenols. They have reported that 3,4,5-trihydroxybenzoyl moiety (galloyl group) at 3-position of the A-ring shows important role in the inhibitory potent of these catechins (Miwa & Yukihiko, 2014). These findings suggest that GT may prevent digestion of carbohydrate in GI tract.

Similarly other studies also showed inhibition of starch and sucrose digestion by GT catechins *in vivo*. Different concentration of catechins suppressed the elevation of plasma glucose level within 2 hours after administration of starch or sucrose in rats. The reason behind it was inhibition of α -amylase and sucrase enzymes (Matsumoto, *et al.*, 1993).

GTP enhanced insulin activity. The context was studied by Richard and Marilyn (2002), who found that GT, Black, and oolong teas increased the activity of insulin around 15-folds *in vitro* in an epididymal fat cell assay. They have found EGCG the most potent insulin-enhancing component of GT and oolong tea, while in black tea, the chromatogram corresponded to several undefined regions in addition to EGCG, theaflavins and tannins. Catechin, epicatechin and caffeine did not show significant insulin-enhancing properties. Addition of 5gm of 2% milk/cup reduced the potency of GT to enhance insulin activity, and addition of 50gm milk decrease about 90% the insulin-potentiating activity, while addition of lemon did not affect in this context (Richard and Marilyn, 2002). Finding by Md. Shahidul Islam and Haymie Choi (2007) regarding effect of GT on insulin was more interesting. They have reported that lower dose of GT (0.5%) is insulinotropic while higher dose (2.0 %) is hyperglycemic.

Gomes *et al.*, (1994), reported the preventive and curative effects of green and black teas on streptozotocin (STZ)-induced diabetic rats. Black tea showed stronger curative effect and GT showed more preventive effect. Both decreased the elevated serum glucose level in diabetic rats.

Ki-Cheor *et al.*, (2013), studied differences between effect of GTE and GTE co-administrated with poly- γ -glutamic acid (γ -PGA) which inhibits absorption of gallated catechins (GCs) in intestine, on obese type2-diabetic mice. None of the drugs showed any effect in non-diabetic mice after 4 weeks, while in *db/db* mice body fat gain and weight gain were significantly reduced and glucose intolerance was ameliorated after treating with GTE+ γ -PGA in comparing with control group. Histopathological analysis revealed decreased incidence of fatty liver and reduced pancreatic islet size in GTE+ γ -PGA-treated *db/db* mice. Treatment with combination of GTE+ γ -PGA was useful tool for preventing both obesity and obesity-induced type2-diabetic than GTE or γ -PGA alone. As the GCs such EGCG and ECG are the major active components of GT (Park *et al.*, 2009), in most cells they inhibit the function of plasmalemmal glucose transporters, such SGLTs in the intestine and kidney, GLUTs in liver, adipocytes, and skeletal muscle, therefore, block the entry of glucose into the cells (Yoko *et al.*, 2000; Ki-Cheor *et al.*, 2013). They have suggested GTE+ γ -PGA did not alter NPY expression and food intake, instead reduced intestinal uptake of calories.

GTE protected rat liver against STZ (75mg/kg BW)-induced diabetes. Diabetic rats showed significant elevation in liver MDA, serum AST, ALT, ALP, and bilirubin (liver injury biomarkers), and significantly decreased the serum albumin, SOD, GSH, GSH-Px, and CAT contents of liver. Histopathologically changes were in association with biochemical findings. Treatment with 1.5% of GTE for 8 weeks decreased the elevated liver MDA, serum AST, ALT, ALP, and bilirubin in STZ-induced diabetic rats compared with diabetic control group and increased the reduced serum albumin, SOD, GSH, GSH-Px, and CAT contents of liver. There were no histopathological changes in normal control group, while diabetic rat showed fatty changes in centrilobular portions of their liver, but diabetic group treated with 1.5% GTE did not showed considerable fatty changes in their liver tissues (Ali Akbar *et al.*, 2012). They have also reported that GTE (1.5%) significantly reduced the blood glucose level in healthy normal rats and also caused significant hypoglycemic effect in diabetic rats.

However, it is in opposition of finding by Fatemeh Haidari *et al.*, (2013) who suggested no changes in level of serum glucose in normal non-diabetic control group after administration of 100 and 200 mg/kg BW of GTE for 4 weeks.

Similarly, Fatemeh Haidari *et al.*, (2013) have induced diabetes in rats by IP injection of 55 mg/Kg streptozotocin and demonstrated the effect of green tea extract (100 and 200 mg/Kg BW) which was administrated through oral gavages, after duration of 4 weeks. They have found that 200 mg/kg dose of GTE significantly decreased the serum glucose levels, hepatic and serum MDA concentration and elevated the TAC (total antioxidant capacity) in diabetic rats and also they have found increased level of hepatic TAC in normal rats after GT supplementation and no significance changes in serum glucose level of normal non-diabetic control group.

It has been reported that aqueous solution of GTP inhibited lipid peroxidation, scavenged OH• and superoxide radicals *in vitro*. 10 mg/ml, 52.5 and 136 mg/ml concentrations were required to inhibit 50% of superoxide, OH and lipid peroxide radicals respectively. Results showed that GTP (100 mg/kg BW) significantly reduced serum glucose level in alloxan induced diabetic rats, while 500 mg/kg BW GTP increase glucose tolerance test at 60 min in normal rats. Administration of 50 and 100 mg/kg BW (GTE) for 15 days reduced the serum glucose level 29 and 44% respectively in alloxan-induced diabetic rats. GTP also decreased the elevated level of liver and kidney enzyme (injury biomarkers) and alloxan-induced lipid peroxidation, and increased the reduced liver glycogen. They have found that GTP improved the SOD and GSH levels, while no change was observed in CAT, LP, GSH-Px levels in alloxan treated rats (Sabu *et al.*, 2002).

Effect of 50 and 100 mg/kg BW of aqueous GTE (rich in catechism) and BTE (rich in theaflavins and thearubigins), administrated for 28 consecutive days to alloxan-induced diabetic male wistar albino rats, studied by Gamal Ramadan *et al.*, (2009). 120 mg/kg BW alloxan monohydrate was injected subcutaneously to damage the β -cells of pancreas through induction of oxidative stress to produce diabetes type 1. Standard rodent food pellets contained 2% w/w cholesterol to induce hyperlipidaemia, obesity and diabetes type 2. After 4 weeks of daily (orally) administration of GTE and BTE, results showed alleviation in most signs of the metabolic syndrome such as dyslipidaemia, hyperglycaemia and liver functions which were induced by alloxan and high-cholesterol diet. Both teas significantly controlled the body weight complete or partial dose dependent. High doses of GTE and BTE reduced the spleen:body weight ratio and increased lymphopenia (Gamal *et al.*, 2009). The mechanism behind anti-obesity and hypolipidemic effects of GTE and BTE is thought to be due to reducing digestive enzymes activity, reducing lipogenic activity, inducing lipolytic activity, inducing fat oxidation and thermogenesis, modulation the activity and expression of lipoproteins, reducing the numbers of adipocytes and preadipocytes (Yung-Hsi *et al.*, 2006).

Studies by Kao *et al.*, (2000) revealed that EGCG IP injection to rats significantly decreased food intake. Additionally they have found that EGCG also decreased serum glucose level, body weight, blood level of insulin, leptin, insulin-like growth factor 1 (IGF-1), testosterone, estradiol, triglyceride and cholesterol, as well as growth of ovary, uterus and prostate. Similar

result have found in thin and obese male Zucker rats which was suggesting that effect of EGCG was no dependent of an intact leptin receptor. So EGCG my interfered leptin-independent appetite control pathway (Kao *et al.*, 2000). Decreasing food intake could be one of the possible mechanisms of hypoglycemic and anti-diabetic effect of GTE.

GTE (especially EGCG) could prevent *in vitro* cytokines-induced destruction of RINm5F (an insulinoma cell line). The possible mechanism was suggested down-regulation of NO synthase through inhibition of NF-kB activation. *In vivo* studies on autoimmune diabetes type-1 induced by multiple low doses of streptozotocin showed that EGCG (100 mg/day/kg for 10 days) also prevent β -cells and reduced the increased serum glucose. EGCG ameliorated the reduction of islet mass caused by MLD-STZ, therefore suggested that EGCG were able to prevent the onset of diabetes induced by multiple low doses of STZ by protection of pancreatic islets (Song *et al.*, 2003).

Studies by Liang-Yi *et al.*, (2004) showed that GTE ameliorated the insulin resistance through induction of GLUT-IV expression in adipocytes fructose-fed rat model. Fructose-rich diet provided pathological resembling type-II DM, an insulin resistance and hypertension. Adipocytes from epididymal fat pads of all rats isolated and tested for insulin bindings, glucose intake and glucose transporters. Results showed that hyperglycemia, hyperinsulinemia, hypertension were developed, while insulin stimulated glucose uptake and insulin binding to adipocytes and GLUT-4 were significantly reduced in fructose-fed group. The fructose+GT-treated group revealed improvement in all of these metabolic defects.

GTE decreased adipose tissue weight without any change in other tissues, body weight, and food and water intake. It was confirmed by treating rats with GT instead of drinking water for three weeks, and the result showed reduced plasma level of cholesterol and free fatty acids (FFA). GT significantly decreased the glucose uptake along with decrease in translation of GLUT-4 in adipose tissue, while it oppositely increased glucose uptake by skeletal muscle cells through increasing GLUT-4 translation. GT also suppressed the expression of peroxisome proliferator-activated receptor γ and the activation of sterol regulatory element binding protein-1 in adipose cells. Finally the anti-obesity action of GT could be due to the modulation of glucose uptake in skeletal muscles and adipose tissues, suppression of the expression of, and/ or activation of adipogenesis-related transcription factors (Hitoshi *et al.*, 2004).

It has been reported that EGCG has the similar cellular effect of insulin such as reductive effect on the gene expression of rate-limiting gluconeogenic enzymes of cell culture system. *In vivo* studies showed that GTE reduced the level of mRNA of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (gluconeogenic enzymes) in mouse liver (Oyama, *et al.*, 2004).

From all above research results we can conclude that the mechanisms of anti-diabetic (anti-hyperglycemic) of GTE are as following:

- decreasing the appetite,

- inhibiting α -amylase, sucrase and α -glucosidase in intestine,
- Reducing carbohydrate absorption from intestine by decreasing SGLTs,
- Reducing oxidative stress in liver, pancreas and other cells,
- Prevent β -cells of pancreas destruction and improve their damage,
- By affecting immune system can prevent β -cells from autoimmune type-1 diabetes
- Increasing insulin sensitivity, and insulin binding to adipocytes,
- Inducing IGF-1,
- Increasing glucose uptake by muscle cells by increasing GLUT-4,
- Down-regulating the levels of mRNA for gluconeogenic enzymes (PEPCK & G-6-Pase) in liver, and
- Decreasing adipose mass and resistin and blood lipids.

Several mechanisms are suggested for the action of green tea EGCG on diabetes shown in figure 2-1.

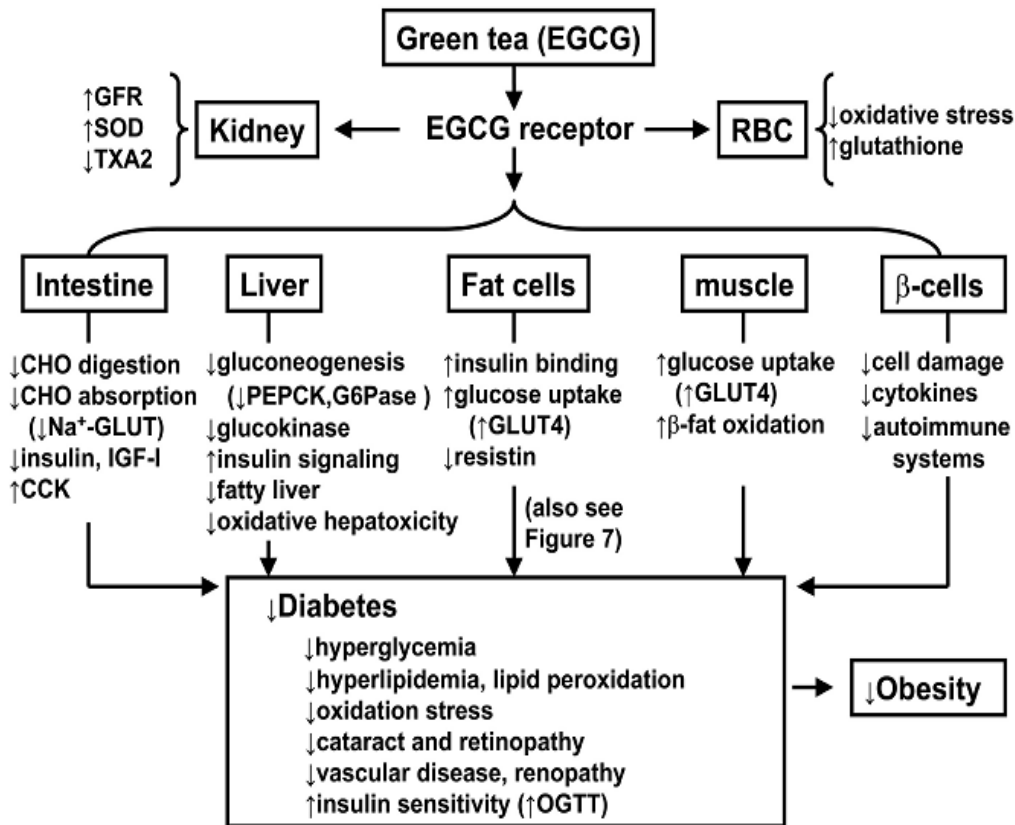


Fig 2-1: General proposed anti-diabetic mechanism of GT.

Source: Yung-Hsi *et al.*, 2006

2.2. Hepatotoxic Effect of GT

Naturally derived antioxidants or alternative antioxidants may be effective, but does not always guarantee safety when not used in recommended dose. 100 ml of green tea contains 50-100 mg of polyphenols (Luo *et al.*, 1997) which may cause several adverse events such as hepatic failure when taken frequently for prolonged period (Gloro *et al.*, 2005; Javaid and Bonkovsky, 2006; Jimenez and Martinez, 2006; Bjornsson and Olsson, 2007).

Different doses (1%, 1.5% and 3%) of green tea extract (GTE) was given instead of drinking water for 25 days to study histo-architecture and function of rat liver. 1% GTE showed hydropic degeneration, larger and cloudy liver cells with deposition of glycogen in the cytoplasm, congested hepatic arterioles and the leucocytes were infiltrated in the portal part of the hepatic tissue. Ultramicrograph, revealed several secondary lysosomes, several lipid droplets, lesser granular endoplasmic reticulum profile, some imprecise mitochondria and marginization of nuclei in some hepatocytes (Amal A.A El Daly 2011). Treatment of 1.5% GTE resulted in some hepatocytes destruction, congestion of liver arteriols, deposition of fibrin in hepatic stroma, deposition of more glycogen in the cytoplasm, chromosomal damage, mutation and necrosis of liver and kidneys (Elasrag, 2010; Amal, 2011). Ingestion of higher dose (3% GTE), revealed lesser discharge of glycogen in liver cells cytoplasm than lower dose. It was confirmed by electron microscopy which showed destruction of mitochondrial cristea with decrease in granular endoplasmic reticulum profiles in rats' liver, especially treated with higher doses of GTE (Amal, 2011). Similarly significant increase of phospholipid in cytoplasm after GTE consumption were also reported in this context (Khan, S.A., et al., 2009; Amal A.A. Daly et al., 2011). There was contradictory in the finding which suggested that higher doses of the tea catechin cause hepatotoxicity (Galati *et al.*, 2006; Isbrucker *et al.*, 2006) on the other hand it was considered safe in a wider range of do doses (Bonokovsky, 2006, Federico et al., 2007 and Sarma et al., 2008). Galati *et al.*, (2006) reported about cytotoxicities of major tea phenolic compounds on isolated rat hepatocytes and found epigallocatechin-3-gallate to be the most cytotoxic followed by propyl gallate, Epicatechin-3-gallate, Gallic acid and epigallocatechin and epicatechin. One of the possible mechanisms of hepatocyte cytotoxicity may be potential collapse of mitochondrial membrane by ROS formation (Galati *et al.*, 2006).

Similarly green tea and its polyphenols at higher doses were found to be toxic by various researchers (Mc Cormick *et al.*, 1999; Johnson *et al.*, 1999; Stratton *et al.*, 2000; Chang *et al.*, 2003). Severe hepatic necrosis resulted to death in female swiss-webster mice after EGCG administration (Goodin and Rosengren 2003). Po C. Chan et al., (2010), demonstrated the hepatotoxiciy and hepatic necrosis of GTE at doses of 500 mg/Kg and 1000 mg/Kg for 14 weeks in rats and mice. Serial solutions containing 0, 62.5, 125, 250, 500 and 1000 mg/kg GTE were tested. Dose of 250 mg Kg⁻¹ BW and higher were capable to decrease total protein and albumin significantly. Increased mortality and hepatosomatic index were also seen in mice. Jimenez and Martinez, (2006), confirmed concentrated GTE poses risk to liver when taken for long duration.

In vitro test also showed reduced cell viability of rat hepatocytes when treated with high concentration of EGCG (Galati *et al.*, 2006; Schmidt *et al.*, 2005) and it also caused kidney, Gastro-intestinal and liver toxicity (Isbrucker *et al.*, 2006).

The possible mechanism may be disturbance in carbohydrate metabolism that lead to mitochondrial toxicity and formation of ROS (Reactive Oxygen Species) by catechins of GTE (Galati *et al.*, 2006).

Several cases of GTE consumption induced hepatotoxicity have been reported in this context. Results of 34 cases of hepatitis after the consumption of some preparations containing green tea have been reviewed which showed elevation of ALT and AST (up to 140 fold ULN), ALP levels (>8.3 fold ULN), GGT (394 U/L) and bilirubin (25 fold ULN). Such cases were categorized as hepatocellular (62.50 %), cholestatic and mixed (each 18.75%). The time of onset of related pathology; 25% of the cases were ≤ 4 weeks and 70% were \leq months. The histological examination showed inflammation, cholestasis, steatosis and necrosis. Serological analysis also showed negative results for hepatitis (A, B, C), autoimmune reactions and alcoholism which had been excluded. Repairing of hepatotoxicity were observed in 29 cases after stopping the consumption of herbal products containing GT or its components (Gabriela *et al.*, 2009).

An 81 year old Italian woman was taking one tablet of Epinerve per day for one month (made up of dry aqueous green tea extract) which was containing 90% of EGCG for treatment of glaucoma, reported for hepatotoxicity (Gabriela *et al.*, 2009). Clinical features included severe asthenia, jaundice, dark urine, pale face, nausea and vomiting. ultrasonography of abdomen confirmed alteration of hepatic parenchyma cells, bile duct dilation and pancreatic changes. Biochemical parameters such as ALT, AST, PT/INR (prothrombin time/international normalized ratio), total bilirubin and direct bilirubin showed the values 2368 U/L, 1996 U/L, 47%/1.51, 21.8 mg/dL and 12.10 respectively.

A 72 year old woman having acute jaundice reported elevated total bilirubin, AST and ALT (> 700 U/L) with negative Virologic and serologic markers of hepatic viruses. She was using supplemented diet for treatment of glaucoma, Epinerve (2 tablets per day) containing 90% EGCG and luteinofa (one tablet per day) containing 10 mg lutein and 12 mg Vit. E, for 3 months. It was suggested that catechins and their gallic acid esters (particularly EGCG) are responsible for hepatotoxicity (Goodin *et al.*, 2006).

Females are more prone to hepatotoxicity due to green tea as reported by *Gabrialia Mazzanti et al.*, (2009). Such findings were also confirmed using animal model where swiss Webster femal mice were more susceptible to EGCG toxicity comparing with males (Goodin *et al.*, 2006).

Studies also confirmed that 400 mg EGCG/day for 60 days caused hepatotoxicity in a male patient. He was using some other nutritions which are reported safe for liver. During this time he has lost 56 pounds. After liver function tests, the patient was diagnosed having acute liver injury and possible impending liver failure. The liver markers improved after stopping the

consumption of green tea fat burner and required treatment has been done (Shreena *et al.*, 2013).

Exolises (a hydro-alcoholic extract of *C. sinensis*) induced acute liver damage in 13 cases. (Felix *et al.*, 2010).

Green tea induced hepatotoxicity may also depend on fasting/feeding condition. In a study it has been found that plasma level of EGCG was found to be lower in pre-fed dogs (19.8 ng h/mL) than in fasted dogs (205.7 ng h/mL) following 2 weeks of 300 mg. Kg⁻¹day⁻¹. There is no adverse effect seen when 500 mg/Kg⁻¹day⁻¹ of EGCG for 13 weeks administrated to pre-fed dogs, but the same amount led to death in fasted dogs in the same duration (Isbrucker *et al.*, 2006). Similar study on healthy individuals revealed that the plasma C_{max} of free EGCG in fed-individuals were 5 times lower then who were administrated in fasting condition after giving of 800 mg Polyphynol E (a decaffeinated extract of GT having 60% EGCG, Chow *et al.*, 2005).

Hepatotoxicity of green tea also depends on the rout of administration. AST, ALT, ALP and the liver weight were increased after dietary administration of 3500 mg/Kg of GTE containing 55.3% catechins for 90 day in rats (Takami, S. *et al.*, 2008 (33), conversely, intraperitoneal low doses of EGCG (50 mg/Kg) caused severe hepatic necrosis and 20% mortality in mice and rats with elevation of plasma ALT, and 150 mg/Kg resulted death (Goodin *et al.*, 2006); at dose of 100 mg/Kg of EGCG in less than one day (Galati *et al.*, 2006).

Hepatotoxicity of green tea also depends on the dose of its administration. Dietary supplementation of 500 mg/Kg/day EGCG for duration of 13 weeks decreased fibrinogen and increased bilirubin, while 2000 mg/Kg of EGCG in a single dose through oral rout was found to be toxic (Isbrucker *et al.*, 2006). Experiments in rat liver cells revealed toxicity of high concentration of GTE & single tea phenolics. The least cytotoxic component was Epicatechin, while the most cytotoxic component was EGCG (Schmidt *et al.*, 2005; Galati *et al.*, 2006). The mechanism for cytotoxicity by green tea was associated with ROS (Reactive Oxygen Species) formation and decrease of GSH. Cytotoxicity by EGCG was due to its metabolite, o-quinone. This metabolite is detoxified by NAD(P)H:quinone oxidoreductase 1 (NQO1), thus EGCG cytotoxicity and ROS formation was significantly increased by dicumarol, a NQO1 inhibitor. It has been found that treatment with entacapone, a catechol-O-methyltransferase inhibitor, caused in ROS synthesis and EGCG cytotoxicity (Galati *et al.*, 2006). Catechol-O-methyltransferase caused methylation at the 4' – and 4"- hydroxyl groups, both possible locations for formation of quinone and redox cycling, hence protects the cells from EGCG-induced oxidative stress & hepatotoxicity (Sang *et al.*, 2005a). It has been demonstrated that 200 and 400 mg/Kg IP dose of EGCG caused the formation of EGCG- 2'-cysteine and EGCG- 2" – cysteine (two cysteine conjugates of EGCG). This conjugate is formed from the EGCG quinone by reacting of EGCG quinone with sulfhydryl group of cysteine and other –SH containing molecules like GSH (Sang *et al.*, 2005b). Besides, daily injection of 40 mg Kg⁻¹ intraperitoneal (IP) of EGCG for 40 days to treat lung tumor- bearing nude mice, increased phosphorylated histone 2AX expression; a marker of DNA damage,

and expression of metallothionein; a marker of response to oxidative stress, in the liver and as well as tumors of mice relative to vehicle-treated (Lambert *et al.*, 2007).

Mostly suspected hepatic reaction is attributed to the catechins, especially EGCG and its metabolites, so dietary supplements of GT with higher level of catechins should be utilized carefully. Rout of administration of green tea affects the bioavailability of catechins, likely it is lower after oral administration. However fasting condition and repeated administration of green tea may increase the bioavailability of catechins even up to the toxic doses. Catechins are capable to induce oxidative stress in the liver. Therefore, it is better to avoid consumption of GTE in fasting condition, as well as concentrated GTE, and also to avoid more repeated consumption of GTE. 4- 5 cups of GT is recommended per day as a safe and useful beverage, more than 9-10 cups can cause liver toxicity.

2.3. Hepatoprotective effect of GT

GTE protected rat liver against STZ (75mg/kg BW)-induced diabetes. Diabetic rats showed significant elevation in liver MDA, serum AST, ALT, ALP, and bilirubin (liver injury biomarkers), and significantly decreased the serum albumin, SOD, GSH, GSH-Px, and CAT contents of liver. Histopathologically changes were in association with biochemical findings. Treatment with 1.5% of GTE for 8 weeks decreased the elevated liver MDA, serum AST, ALT, ALP, and bilirubin in STZ-induced diabetic rats compared with diabetic control group and increased the reduced serum albumin, SOD, GSH, GSH-Px, and CAT contents of liver. There were no histopathological changes in normal control group, while diabetic rat showed fatty changes in centrilobular portions of their liver, but diabetic group treated with 1.5% GTE did not showed considerable fatty changes in their liver tissues (Ali Akbar Abolfathi, *et al.*, 2012). They have also reported that GTE (1.5%) significantly reduced the blood glucose level in healthy normal rats and also caused significant hypoglycemic effect in diabetic rats. However, it is in opposition of Fatemeh Haidari *et al.*, (2013) who found no changes in level of serum glucose in normal non-diabetic control group after administration of 100 and 200 mg/kg BW of GTE for 4 weeks (Ali Akbar Abolfathi, *et al.*, 2012).

Similarly, Fatemeh Haidari *et al.*, (2013) have induced diabetes in rats by IP injection of 55 mg/Kg streptozotocin and demonstrated the effect of green tea extract (100 and 200 mg/Kg BW) which was administrated trough oral gavages, after duration of 4 weeks. They have found that 200 mg/kg dose of GTE significantly decreased the serum glucose levels, hepatic and serum MDA concentration and elevated the TAC (total antioxidant capacity) in diabetic rats and also they have found increased level of hepatic TAC in normal rats after GT supplementation and no significance changes in serum glucose level of normal non-diabetic control group.

Sabarimuthu Darlin Quine *et al.*, (2005) conducted a study to reveal the antioxidant effect of (-)-epicatechin in STZ-induced diabetic rats. 15 and 30 mg/kg I.P administration of (-)-epicatechin for a period of 35 days to diabetic rats, significantly reduced the blood glucose, hydroperoxides, thiobarbituric acid reactive substance (TBARS), and significantly induced the activity of catalase and concentration of GSH, SOD and GPx (glutathione peroxides).

STZ (streptozotocin) causes oxidative stress which leads to endothelial dysfunction, activation of the NADPH oxidase, and increased vascular superoxide production (Hink et al., 2001). Lowered level of glutathione in diabetic tissues consider as indicator of increased oxidative stress (McLennan, et al., 1991), other study by (Matkovics., et al., 1998) also demonstrated decrease GSH in diabetic liver, heart and kidney tissues, (-)-epicatechin improve this condition.

These studies observed that concentration of HPs (hydroperoxides) are increased in STZ-induced diabetic kidney, liver and heart (Kamalakkannan and Stanely, 2003), possibly it is caused by decrease in activity of enzymatic antioxidants (Henning and Chow, 1988). (-)-Epicatechin reduced HPs levels in STZ-induced diabetic tissues.

SOD is responsible to reduce superoxide radical to H₂O₂ and O₂, catalase and GPx are responsible for reduction of H₂O₂ (Eriksson and Borg, 1991; Gutteridge, 1995; Izuka, et al., 1992). Reduction in activity of these enzymes leads to formation of H₂O₂ and superoxide ions which then can form OH⁻ radicals. Therefore, more accumulation of free radicals in diabetic tissues is due to the reduced activity of these enzymatic antioxidants, it is also reported by other researchers (Kamalakkannan and Stanely, 2003). (-)-epicatechin therapy induces the activity of these enzymatic antioxidants in diabetic cells.

Juskiewicz *et al.*, (2008) reported that the higher concentration of glucose, the higher level of lipid peroxidation and the lower level of total antioxidant are interrelated. It is confirmed that there is direct relationship between diabetes and oxidative stress condition and the major mechanisms included in increased oxidative stress biomarkers in diabetes are protein glycation, glucose oxidation, formation of advanced glycated end- products and the polyol pathways.

Bioactive component of GTE increased the activity of glutathione reductase, glutathione peroxidase and the contents of GSH, and reduced significantly in lipid hydroperoxides (LOOH), 4-HNE (4-hydroxynonenal) and MDA (malondialdehyde), in liver. Vit. A concentration also increased by 40%. All above parameters had minor changes in serum. But increase in total antioxidant status and decrease in MDA & 4-HNE strongly marked in blood serum, when 2 active components of green tea (Epicatechin & EGCG) were administrated. EGCG is the most protective agent comparing with others (E. Skrzaydlewska.J *et al.*, 2002).

Ju- Hua Chen, *et al.*, (2004) have studied the effect of green tea polyphenols (pure form of EGCG) on liver injury induced by carbon tetrachloride (CCl₄) in mice. CCl₄ caused a severe liver necrosis associated with elevation in lipid peroxidation, nitric oxide synthase mRNA & protein, nitric oxide radical and nitrotyrosine. EGCG administration caused a dose-dependent reduction in all biochemical and histological variables of liver injury which occurred in CCl₄ treated mice (5). In this study they showed EGCG patently inhibits hepatocellular toxicity in a well-characterized murine model of liver injury. Formation of trichloromethyl free radical is the initial step in liver injury caused by CCl₄, and subsequently, activation of kupffer cells of liver with release of pro-inflammatory mediators is considered to be a key event in liver

toxicity (6). Protective effect of EGCG against CCl₄-induced toxicity is mediated through the scavenging of Nitric oxide (NO) expression and reducing of the production of pro-inflammatory mediators- resulting from induction of iNOS (5). NO reacts with superoxide anions and produces peroxynitrite, which is a strong oxidative species, able to nitrating the tyrosine residues of different proteins, which causes the formation of nitrotyrosin. Nitrotyrosine which is increased in CCl₄-induced mice significantly reduced by EGCG treatment (5). Other pathways of tissue injury mediated by NO and peroxynitrite include inactivation of proteinase inhibitors, inhibition of mitochondrial respiration, and formation of free radicals (34). Several mediators are implicated in toxin- induced liver injury such as eicosanoids, inflammatory cytokines, and ROS (13, 14). Recent studies indicate significant role of NO in the pathogenesis of toxin-induced liver injury (7-9). NO as second messenger is synthesized by NOS (NO synthases), and mainly in normal liver it is synthesized by the endothelial NOS (Constitutive NOS isoform) (10). Pro-inflammatory cytokines and mediators can induce the inducible isoform of NOS (iNOS) (19). Inhibition of iNOS in endotoxemia models leads to liver damage, which is thought to be a beneficial role for nitric oxide (NO) (20, 21). Side of protective effect of NO, harmful effect of NO in liver injury also reported (19). Ju- Hua Chen, et al., 2004, they have evaluated that inhibition of nitric oxide (NO) production as a possible mechanism of green tea polyphenols protective effect in toxin-induced liver injury.

Shafaq Noori *et al.*, (2004) have conducted study to evaluate hepatoprotective effect of green tea and coffee for duration of 8 weeks against CCl₄-induced liver cirrhosis in male albino rats. CCl₄, 0.8 mg/kg, Coffee, 1%, and green tea, 5% were used and ALT, ALP, total and direct bilirubin, tissue SOD, tissue catalase and tissue MDA were used as biochemical markers, as well histopathological examination also performed. The results showed that coffee and green tea improved the changes and restored liver enzymes and antioxidants.

Mechanism of CCl₄-liver intoxication is due to cytochrome P450 activation of CCl₄ to CCl₃ free radical within the endoplasmic reticulum membrane. This free radical is highly reactive molecule and react with –SH group, protein-thiol and reduces GSH, thus leads to lipid peroxidation of membrane and causes necrosis of the cell (Kyung *et al.*, 2007).

Randall J. Ruch, *et al.*, (1989) have found that GTA (0.05 – 50/tg/ml) preventing the killing of hepatocytes (measured by LDH release) by 1-10mM paraquat and 0.8-40/xg/ml glucose oxidase (GO) in a concentration dependent fashion. Paraquat (5 mM), GO (0.8/tg/ml) and Phenobarbital (500 mg/ml) were inhibiting the intercellular communication which inhibited by GTA (50/tg/ml). Moreover, 50/tg/ml of GTA had preventive effect on inhibition of intercellular communication in human keratinocytes by 100 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Two possible mechanism by which tumor promoters may generate promoting effect are cytotoxicity and inhibition of intercellular communication, which prevented by GTA. It is suggested that GTA is a protective agent for oxygen radical-generating hepatotoxicants with ability to prevent liver cells death and the loss of intercellular communication induced by PQ and GO. Green tea antioxidants are preventive agents against oxygen radical and hydrogen peroxide- induced cytotoxicity and inhibition of intercellular

communication in cultured mouse hepatocytes (B6C3F1) and NHEK cells (Human keratinocytes).

Hesham A. El-Bashbishy (2005) have evaluate the antioxidant capacity of GTE against TAM-induced liver injury. Tamoxifen (TAM)-is used for breast cancer treatment with having hepatic carcinogenesis effect, 45 mg/Kg/day intraperitoneally injected for 7 successive days. GTE (1.5%) was orally giving 4 days before to 14 days after tamoxifen intoxication as source of drinking water. The model of tamoxifen- intoxication showed reduction in Glutathion-S-transferase, SOD, glutathione peroxidase and catalase (antioxidant enzymes) and induction in thiobarbituric acid reactive substance (TBARS), SGPT and SGOT levels. GTE induced the antioxidant enzymes and reduced TBARS and liver transaminases in TAM-intoxicated rats. High dose of TAM inhibits HMP shunt strongly in rats' liver, so NADPH is decreased leads to lipid peroxidation.

In oxidative stress, regeneration of protective and antioxidants such as GSH is impaired and hepatic peroxides are increased (*sun et al., 1998*) (18). It was also reported that there is observed reduction in antioxidant defenses in liver due to liver damage (*saven et al., 2004*).

Puming He *et al.*, (2001) have conducted *in vivo* experiment to evaluate hepatoprotective effect of GT against lipopolysaccharid-1-D-galactosamine- induced liver injury in 7 week old male rats. They have found that 30 or 35 g/Kg of GTE added in normal diet for 14 days or 0.4- 1.2 g/Kg of GTE forced-fed 1.5 hour before drugs injection, significantly decreased liver transaminases (ALT and AST) and suppressed LPS-GaIN- induced liver injury. All 5 types of GTE (with different solvent) exhibited suppressive effects but the caffeine containing fraction showed the strongest protective effect against LPS-GaIN-induced liver injury. While caffeine alone (2 mg/Kg) added to the diet for 14-days strongly suppressed LPS-GaIN-induced liver injury. So, it is suggested that the protective effect of green tea against LPS-GaIN-induced liver is belonged to the caffeine. Dietary GT did not suppressed the serum TNF-a (tumor necrosis factor – alpha) which was enhanced by LPS-induced liver injury, but suppressed the concentration of interleukins (IL-1b, 2, 4, 6 & 10) and interferon-g (IFN-g) in plasma. Dietary GT was able to suppress the TNF-a-GaIN-induced liver injury and apoptosis. In contrast, serum concentration of TNF-a, IFN-g and interleukins (IL-1b, 2, 4, 6 & 10) were suppressed by dietary caffeine in LPS-induced liver injury. It is suggested that suppression of LPS-GaIN-induced liver injury by green tea is mainly via the scavenging of TNF-a-induced apoptosis of liver cells, rather than via decreasing production and release of TNF-alpha.

In contrast, Yang *et al.*, (1998) reported that high serum TNF-a concentration and mortality induced by LPS (40 mg/Kg body weight) suppressed by single force-feeding of catechins in mice.

Ueda and Yamazaki, (1997) reported that GTE or caffeine is able to suppress the systemic inflammation which was induced by muramyl dipeptide derivative and OK-432, a bacterial infection, in mice. This report indirectly supports hepatoprotective effect of caffeine and GT by suppressing inflammation (finding of Puming He *et al.*, 2001). They have found that chloroform-soluble fraction of GT (contains 90% caffeine) as forced-fed alone to mice,

suppressed the elevated plasma Tumor Necrosis Factor- α concentration induced by muramyl dipeptide derivative and OK-432, whereas plasma TNA- α concentration was stimulated by water-soluble fraction of Gt. The results show that GT have both TNF- α increasing and TNF- α decreasing agents, and the caffeine is one of the Tumor Necrosis Factor- α decreasing component of GT (Puming He *et al.*, 2001).

Hesham A. El-Beshbishy *et al.*, (2001) have studied the hepatoprotective effect of green tea polyphenols against azathioprine (AZA)-induced livery injury. AZA (50 mg/Kg) as single i.p was administrated to adult male rats and GTP (100mg/Kg/day and 300 mg/kg/.day) were orally administrated 7 days prior to AZA injection for 21 consecutive days. Results showed that GTP significantly prevented the induction of SGOT, SGPT, serum ALP, prevented the reduction of liver GSH, GSSG, GPx, and catalase and accumulation of MDA. GTP also normalized hepatic CAT, TAA, caspase-3, TNF- α and serum total protein in AZA-intoxicated rats. GTP prevented apoptosis induced by AZA in livery injury. Data indicated that protective effect of GTP was through antioxidant, anti-apoptotic and anti-inflammatory mechanisms. In this study they have demonstrated that GTP (both 100mg/kg and 300 mg/kg) decreased AZA-evoked elevation of Serum Tumor Necrosis Factor-alpha (TNF- α) which is in opposition to Puming He *et al.*, (2001), and in association to Ueda and Yamazaki, (1997).

Sang Il Lee *et al.*, (2008) evaluated the effect of green tea on cultured HepG2 treated with lethal dose of ethanol and the GGT was measured as a marker. EGCG (20 μ M- 2mM) found the effective protective, and the caffeine and L-theanine had no effects on ethanol cytotoxicity. It was suggested that EGCG inhibiting GGT activity to attenuate ethanol-induced liver injury. GGT the plasma membrane enzyme catalyses extracellular GSH breakdown (Whitfield, 2001). The products are interred to the cell to be used for GSH synthesis. GGT activity demand is increased while intracellular GSH is decreased by ethanol toxicity therefore GGT plays an important role in intracellular glutathione (GSH) homoeostasis (Zhang *et al.*, 2005; Chinta *et al.*, 2006). In this study, acivicin, a GGT inhibitor also protected cells against the ethanol-induced cytotoxicity without altering the GSH level inside the cells same as EGCG. It needs more studies to find how GGT inhibitors do not affect intracellular GSH (Sang Il Lee *et al.*, 2008).

There are other reports to support hepatoprotective properties of green tea against ethanol toxicity (Arteel *et al.*, 2002; Skrzydlewska *et al.*, 2002; Baltaziak *et al.*, 2004).

Ojo, O., *et al.*, (2006) have evaluated the protective effect of 100 mg/kg B.w of green tea and lemongrass on paracetamol (2 g/kg)-induced liver injury in wister albino rats. They found that green tea extract and lemongrass significantly prevented the depletion of vit.C and attenuated the induction of serum MDA and CAT through their antioxidative effect. They also prevented the elevation of cholesterol/phospholipid ratio by paracetamol. They have suggested that green tea and lemongrass are able to protect the hepatocytes from paracetamol-induced lipid peroxidation by antioxidative effect, therefore, eliminating the harmful effects of toxic metabolite of paracetamol.

Several compounds such as some clinical useful drugs may cause cellular damage through their metabolites after activation to higher reactive substance like free radicals, nitrenes and carbenes (Gupta *et al.*, 2004). Hepatotoxicity of paracetamol is also because of formation of its toxic metabolites by act of hepatic CYT p450 (Savides and Oehme, 1983). N-acetyl-P-benzoquinoneimine, active metabolite of paracetamol (Vermeulen *et al.*, 1992), is detoxified by conjugation with GSH and forms mercapturic acid (Moore *et al.*, 1985). When the rate of N-acetyl-P-benzoquinoneimine formation increased then the detoxification rate by GSH, causes oxidation of macromolecules for instance lipids, -SH groups of proteins and alters calcium homeostasis (Ojo, O., *et al.*, 2006).

In a study on 18,815 individuals ages between 40- 69 years who participated for health checkup survey in 1993 and 1994, followed for demonstration of liver cancer in 2006 in the Japan Public Health Center. They examined whether green tea consumption decrease the risk of liver cancer in patients with hepatitis virus infection. The results showed coffee consumption decrease the risk of liver cancer after either HCV or HBV infection, but GT may not decrease this risk. Vitamin C component of GT, with antioxidant potential, also stimulates iron absorption from food (Lynch SR., 1997). Vit.C is directly associated with ferritin, which is a measure of body iron store, and coffee intake has inversely association with ferritin (Fleming DJ., *et al.*, 1998; Fleming DJ., *et al.*, 2002). Excess iron intake is associated with hepatic fibrosis (Philippe MA., *et al.*, 2007), therefore, more consumption of green tea is harmful for liver because of having vit.C which leads to more uptake iron (Manami inoue *et al.*, 2009).

Noritaka Kagaya *et al.*, (2002) conducted experiment to evaluate hepatoprotective effect of green tea, especially catechins, against hepatotoxins on primary cell cultured of rat hepatocytes. Rubratoxin B and Bromobenzene cause apoptosis and necrosis, respectively. EGCG and EGCg-3W-OMe [(*l*)-epigallocatechin-3-(3W-O-methyl) gallate] suppressed the morphological changes and cell death induced by bromobenzene. Also they protected apoptotic cell death occurred by rubratoxin B. So, catechins are suppressors of cytotoxin-induced cell death.

Some chemicals like bromobenzene through formation of free radical reactions cause necrosis and finally lead to liver damage (Thor and Orrenius, 1980; Lau and Monks, 1988). Bromobenzene is metabolically activated by cytochrom P450 and leads to formation of intermediate metabolites which are induced covalent binding, lipid peroxidation and enzyme inactivation, which finally lead to necrosis and cell death (Thor and Orrenius, 1980 (56); Lau and Monks, 1988); Lauriault *et al.*, 1992). It was considered that catechins suppress the CYPs-induction or scavenge the reactive intermediates to protect the cells against bromobenzene intoxication. In this study they have examined expression of some CYPs (1A1, 3A2, 2B1/2 and 2E1) which were same under both conditions, EGCG-present and EGCG-nonpresent, in bromobenzene -treated cells. So it shows that catechins did not alter the expression of CYPs, but act through scavenging of reactive intermediates. They have demonstrated that EGCG scavenges the DPPH (1,1-diphenyl-2-picrylhydrazyl), a stable free radical.

Rubratoxin B induces apoptosis but the mechanism has not been well known yet. Caspase-3 activity is increased in rubratoxin B-treated cell death, and the results showed that EGCG decreased the activity of caspase-3. In contrast, EGCG increase the activity of caspase-3 in cancer cells. These two opposite behavior of EGCG on caspase-3 is not clarified, which needs more investigation. They also found that EGCG did not protect HL-60 human promyelotic leukemia cells against rubratoxin B. So, having ability of protecting normal liver cells and inducing apoptosis in cancer cells make the EGCG a safe antitumor medicine (Noritaka Kagaya *et al.*, 2002). EGCG-3W-OMe also showed equal hepatoprotective effect to EGCG.

Induction of cytochrome P450 2E1 is a pathway of ethanol-produced oxidative stress. Accumulation of PUSFA (poly unsaturated fatty acids) and iron contributes to hepatotoxicity in the ethanol treated intragastric infusion model. This study evaluated the effect of EGCG on CYP2E1-induced cytotoxicity in HepG2 cell with CYP2E1 overexpressing (E47 cells). Arachidonic acid+ iron (AA+Fe) produce synergistic toxicity in E47 cells by CYP2E1 activity and oxidative stress. EGCG found that had no effect on CYP2E1 activity, but reduces oxidative damage through scavenging of ROS, preservation of intracellular glutathione, and decreasing lipid peroxidation. So, EGCG is a useful chemical to prevent toxicity of various hepatotoxins which are activated to reactive intermediates by CYP2E1 (Jose M. *et al.*, 2004). Ethanol is produced its toxicity through production of ROS (Arteel, 2003), by induction of CYP2E1 which leads to oxidative stress in the liver (Morimoto *et al.*, 1993; Tsukamoto, 2000). Since a-tocopherol, an effective antioxidant, inhibitor of lipid peroxidation, and DFO, an iron chelator agent, are analogous of the protective EGCG (Jose M. *et al.*, 2004).

Iron and other transition metals are powerful catalysts for free radical formation and lipid peroxidation, and PUSF like AA (arachidonic acid) provide basic substrate of these reactions (Wilhelm, 1990). Reports demonstrates that polyphenols like EGCG containing a catechol-type structure are strong free radical inhibitors and transition metals chelators (usually iron) (Rice-Evans, *et al.*, 1996; van Acker, *et al.*, 1996; Guo, *et al.*, 1999). It is also reported that EGCG protects lead-exposed HepG2 cells toxicity and lipid peroxidation damage (Chen, *et al.*, 2002).

Jan Frank *et al.*, (2008) conducted a study to assess effect of the daily GTP consumption on CVD risk biomarkers, and liver function in healthy men. Subjects consumed 2 capsules of GTP, before each principal meal (6 capsules/day; containing green tea extract equivalent to 714mg/d GTP) for a period of 3 weeks; placebo was also used by other group. The finding of this study showed that daily consumption of high amount of GTP for 3 weeks is safe in healthy men and also does not effect on CVD risk biomarkers, just total:HDL cholesterol ratio was decreased. Routing consumption of high amount of GTP; 6-8 cups of green tea, for a period of 3 weeks did not affect bilirubin, ALT, AST and GGT (liver biomarkers), and Albumin, Uric acid, urine creatinine and urea (kidney biomarkers) in healthy men. They placebo capsules did not have only GTP, but had similar dose of caffeine to GTP capsules. They daily amount of caffeine was equal to the one cup; 200 ml, of coffee (91). Consumption of daily GTP did not affect fasting blood lipids (HDL cholesterol, total cholesterol, NEFA

and TAG), and glucose in healthy individuals. Finding of this study which GTP did not affect plasma tocopherol and urinary CEHC concentration in men is in opposition to the results of previous studies which they suggested that α -tocopherol is increased in catechin- & epicatechin- fed rats (Frank *et al.*, 2006), and the finding decrease of α -tocopherol lymphatic absorption in GTE-fed rats (Ikeda *et al.*, 1992).

Obesity, insulin resistance, chronic inflammation, and other obesity related metabolic abnormalities may increase the risk of liver carcinoma. IGF (insulin-like growth factor)/IGF-1R (IGF-1 receptor) axis abnormal activation is also concerned in obesity related liver tumorigenesis. This study involved in assessment of EGCG effect on progress of DEN (diethylnitrosamine)-induced liver tumorigenesis in C57BL/KsJ-db/db obese mice. The male db/db mice were received 40 ppm DEN for a period of 2 weeks and then were given 0.1% EGCG as drinking water for 34 weeks. The result showed EGCG significantly scavenged the development of hepatocyte adenoma. It is observed that EGCG inhibited the phosphorylation of ERK (extracellular signal-regulated kinase), IGF-1R, GSK-3 β (glycogen synthase kinase-3 β), AKt, JNK (c-Jun NH 2 -terminal kinase), and Stat3 proteins in hepatocytes of experimental mice. EGCG also decreased the level of serum IGF-1, IGF-2, Insulin, free fatty acid, and TNF- α , and as well as decreased the expression of interleukins (IL-6, IL-1 β , and IL-18mRNAs) and TNF- α in the liver. Improvement of liver steatosis and activation of AMP-activated kinase protein also performed by EGCG (Masahito Shimizu *et al.*, 2011).

2.4. Effect of Green Tea on Cytochrome P450 Enzymes

Preclinical study consisting of limited sample size and number of medication, on urine and plasma metabolites of medications processed by cytochrome P450 in GT consumers comparing with non-consumers of GT, has been found insignificant differences (Muto *et al.*, 2001). 42 non-smoking healthy individuals after 4 weeks washout period (no use of GT and its products) received the cocktail of CYT-P metabolic probe medicines (Caffeine, Dextromethorphan, Losartan and Buspirone) were evaluated for the activity of CYP1A2, CYP2D6, CYP2C9 and CYP3A4 respectively. There were no significant differences in phenotypic indices of CYP1A2, CYP2D6, and CYP2C9, but demonstrating a small decrease in CYP3A4 activity after four weeks consumption of GT catechin. Urine and blood samples results showed consumption of 800 mg epigallocatechin gallate/day for a period of 4 weeks on an empty stomach did not significantly effect on disposition of drugs metabolized by Cytochrome- P (H-H. Sherry Chow *et al.*, 2006).

Flavonoids, ECG ((-)-epicatechin-3-gallate) and tangeretin, effects on CYP 450 1a was dose dependent. They have inhibited EROD (7-ethoxyresorufin-o-deethylase; 450 1a) activities at higher concentration while in lower concentration moderate activation was seen in liver. Activity of 450 2B, 2E1, and 3A enzymes were only moderately inhibited in rat and human liver. Tangeretin inhibited the EROD (450 1A2) in a competitive manner while it inhibited NIFO (P450 3A4) in an uncompetitive manner in human liver microsomes (Obermeier *et al.*, 1995). Study by Wang *et al.*, (1988) showed no activation of EROD at lower concentration of green tea flavonoids occurred. Cytochrome P450 1A (EROD) involved directly in

biotransformation of non-O-PCBs (non-ortho-polychlorinated biphenyls, Ishida *et al.*, 1991), led to formation of hydroxylated PCB metabolites which have been found to bind to plasma carrier protein transthyretin led to interfering plasma transportation of thyroid, and also interfered plasma transporter of retinol, thus increased the circulation by elimination of thyroxine and retinol (Brouwer, 1987; Lans *et al.*, 1993). ECG, the most potent EROD activator with half-maximal activation about 250 times less than the IC₅₀ is much more stimulator than it is inhibitor (Obermeier *et al.*, 1995). GT flavonoids including quercetin may either be able to bind to P450 system in such a way so as to facilitate the binding of substrate, or bind to reductase and either facilitate the electron transfer to P450 or facilitate the binding of P450 to reductase (Buening *et al.*, 1981; Sausa and Marletta, 1985, Siess *et al.*, 1989; Obermeier *et al.* 1995).

Activation of two major P450 isoforms (CYP3A4 and CYP2D6) involved in metabolism of about 70% of medications (Zanger and Eichelbaum, 2000; Wrighton and Thummel, 2000) is unlikely to be significantly altered by 884 mg of catechin per day (Jennifer *et al.*, 2004). *In vitro* and *in vivo* animal studies showed green tea flavonoids may inhibit the activity of steroid-5-alpha-reductase, phase II conjugation enzymes and CYP2B1 (Huynh and Teel, 2002; Lu *et al.*, 2003b; Hiipakka *et al.*, 2002).

Tea polyphenols have shown selective inhibitory effects on microsomal enzyme activities like GTP as well as (-)-epicatechin derivatives which have stronger inhibitory effect on cytochrome P450-dependent monooxygenase as comparing to NADPH-Cytochrome c reductase, and EH (Zhi, V. wang. *et al.*, 1987). Since these phenolic compounds have the ability to generate semiquinones and quinines which undergo reversible transformation, impair the electron flow from the reduced pyridine nucleotide to the cytochrome P540 protein; therefore inhibit NADPH-cytochrome c (Zhi *et al.*, 1987). GTPs can also inhibit P450-dependent 7-ethoxycoumarin-O-deethylase, 7-ethoxyresorufin-O-deethylase and aryl hydrocarbon hydroxylase activities in a dose-dependent manner (Mukhtar *et al.*, 1992).

GTPs interact with microsomal cytochrome P-450 enzymes and impair electron transfer which may lead to inhibition of activation of mutagens and carcinogens (Zhi *et al.*, 1987).

Since most of carcinogens especially those possess polyaromatic hydrocarbons with benzo- α -pyrene as a prototype, are activated by cytochrome P450-dependent monooxygenase system and other enzyme to ultimate carcinogenic metabolites which are capable of binding to DNA and cause carcinogenesis (Conney, 1982). Mechanisms by which plant phenols apply their anticarcinogenic and antimutagenic effects are believed to be through either activation or inhibition of certain P450 enzymes leading to decreased formation of ultimate carcinogen compounds (Das *et al.*, 1985; Das *et al.*, 1987; Obermeier *et al.* 1995) or scavenging of the reactive molecular species of carcinogenic metabolites (Wood *et al.*, 1982; Sayer *et al.*, 1982).

The inhibitory effect of catechins is due to the galloyl or (-OH) groups attached to the skeleton structure of catechin (Mukhta *et al.*, 1992), and the inhibitory potent of (-)-epicatechin derivatives is dependent on the substitution at the 2, 3-positions of 5,7- (HO)₂-

benzoflavon, and on the number of (OH) groups present in these substituents (Zhi *et al.*, 1987). The hydrogen bonds binding force between the hydroxyl group of phenolic compound and ketoimide group of protein may also play a role in the inhibitory effect (Phillips and Langdon, 1962).

2.5. Anti-oxidative effect of green tea

2.5.1. Antioxidant actions and free radical scavenging properties of catechins

Cells under oxidative stress may produce reactive oxygen species (ROS) includes hydroxyl free radicals and superoxides, and reactive nitrogen species (RNS) includes peroxynitrite and nitric oxide (NO) (Mattivi *et al.*, 2002). These free radicals may damage cells by altering DNA, proteins and lipids by lipid peroxidation that finally lead to apoptosis and cell death (Lazarus *et al.*, 1999). Several *in vivo* studies showed that catechins possess free radical scavenging properties and serve as antioxidants. Catechins are able to scavenge hydroxyl group, superoxides (Ruch *et al.*, 1989; Guo *et al.*, 1996; Kashima, 1999; Nanjo *et al.*, 1999; Zhao *et al.*, 2001), 1,1-diphenyl-2-picrylhydrazyl radical ($C_{18}H_{12}N_5O_6^\bullet$, Sawai *et al.*, 1998; Nanjo *et al.*, 1999; Zhao *et al.*, 2001), peroxy radicals (Sang *et al.*, 2003), NO (Kelly *et al.*, 2011), singlet oxygen, carbon-center free radicals, and lipid free radicals (Guo *et al.*, 1996; Zhao *et al.*, 2001), and peroxynitrite by inhibiting the nitration of tyrosine (Pannala *et al.*, 1997). It also have capability of chelating the metal ions such as Cu^{+2} and Fe^{+3} to form inactive compound which further prevent damaging form free radicals (Figure 2-1, Guo *et al.*, 1996; Kashima 1999; Grinberg *et al.*, 1997; Seeram *et al.*, 2002). They possible mechanism may be Ultrarapid transfer of electron from catechins to ROS-induced radical sites on DNA (Anderson *et al.*, 2001), or by forming of stable semiquinone free radicals, hence, inhibit deaminating ability of free radicals (Guo *et al.*, 1996). After reaction of catechins with free radicals, catechins are oxidized to form dimerized product which shows increased iron-chelating and superoxide scavenging potential (Yoshino *et al.*, 1999).

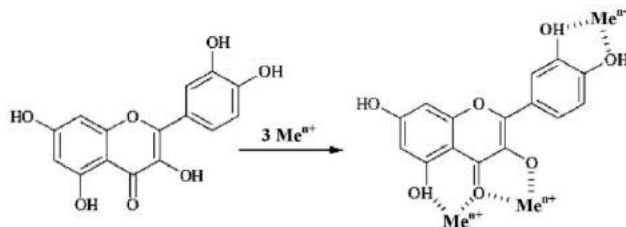
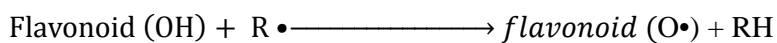


Figure 2-2: The chelating reaction of catechins with metals (Anna *et al.*, 2014).

Catechins showed higher antioxidant effects than other antioxidants (e.g., α -tocopherol (Terao *et al.*, 1994; Hashimoto *et al.*, 2000), vitamin E & C (Zhao *et al.*, 1989). The free radical scavenging potency of catechins is dependent on their chemical structure (gallate esterified at the position 3 of the C-ring, the 3, 4-dihydroxyl group (catechol group) on the B-ring and "OH" groups at the position 5th and 7th of A-ring). Hydroxyl groups of B-ring are electron donor for radicals (Anna *et al.*, 2014). The existence of double bond between C-2

and C-3 and 4-oxo group in the C-ring may cause dislocation of an electron in the B-ring which may lead to antioxidative properties of aromatic unit. Phenoxyl radicals are created during reaction of the compounds with free radical, and stabilized by the effect of the aromatic ring resonance (Anna *et al.*, 2014). Presence of OH groups near C-3 & C-5 along with 4-oxo groups in A-ring and C-ring leads to maximum free radical scavenging effect (Anna *et al.*, 2014). Several studies showed that free radical scavenging potency of EGCG and ECG is higher than other catechins (Guo *et al.*, 1996; Pannala *et al.*, 1997; Nanjo *et al.*, 1999; Lotito *et al.*, 2000; Nie *et al.*, 2000; Hashimoto *et al.*, 2000; Zhao *et al.*, 2001) due to presence of C-ring gallate group. Galloylation of catechins increase the phospholipid/water partition coefficients which affects the properties of the phospholipid bilayers of membranes and increase solubilization leads to increase the antioxidative properties of these kinds of catechins (Caturla *et al.*, 2003). There is slight difference between antioxidant activity of EGCG and ECG due to only difference in the structure, which is the existence of O-hydroxyl group at the position 5 of the B-ring of EGCG and also it depends on the model used and the free radical involved. Observations showed that catechin possesses more "OH" group (such as EGCG & ECG) has the more effective free radical scavenging properties (Zhao *et al.*, 2001). The A-ring is also important in antioxidant activity that decarboxylated and oxidized following reaction with H₂O₂ and produced different reaction products for EGCG and EGC (Zhu *et al.*, 2000). Flavonoids are oxidized by free radicals and make more stable compounds with less radical reactivity which stabilize the reactive oxygen species.



R• is the free radical and O• is the oxygen free radical (Robert *et al.*, 2001).

Polyphenols may exhibit their antioxidative activity by following mechanism (Lotito *et al.*, 2000; Skrzydlewska *et al.*, 2002; Anna, 2014):

- Direct scavenging of ROS lead to interruption of reactions which may lead to lipid peroxidation,
- Inhibition of enzymes involved in promoting ROS formation such as xanthine oxidase, cyclooxygenase I & II, ascorbic acid oxidase, protein kinase C, lipoxygenases, cAMP phosphodiesterase and Na⁺/K⁺ ATPase.
- Indirectly inducing endogenous antioxidant enzymes such as catalase, SOD, glutathione peroxidase and reductasae.
- Scavenging of metal radical formation by chelating.
- Directly preventing decrease of endogenous antioxidants such as α-tocopherol & β-carotene.

Catechins also have the ability to indirectly induce the endogenous antioxidants to decrease the oxidative damage. Orally administration of GTE showed induced levels of catalase, superoxide dismutase (SOD), glutathione peroxidase and reductase (Skrzydlewska *et al.*, 2002). Catechins can also directly prevent depletion of endogenous antioxidants (such as α-tocopherol & β-carotene) by lipid peroxidation through AAPH (Lotito *et al.*, 2000). Similarly, study on human who were drinking 2 cups of GT for 42 days showed elevation in

endogenous plasma antioxidants, and reduction in peroxides and oxidative stress-induced damage which was a back up for the animal studies (Erba *et al.*, 2005). In a crossover clinical trial, GTE induced activity of plasma antioxidants and reduced oxidative damage. Tea catechins directly scavenged the free radicals, and inhibited the ROS accumulation through inhibiting xanthine oxidase (XO)—an enzyme catabolizes the purines and produces uric acid and ROS (Aucamp *et al.*, 1997), where up-regulation of XO may lead to gout and oxidative stress. EGCG inhibited the XO as same potency as allopurinol (the drug of choice for gout), so it is suggesting an effective mechanism for prevention of free radical formation (Brad *et al.*, 2006).

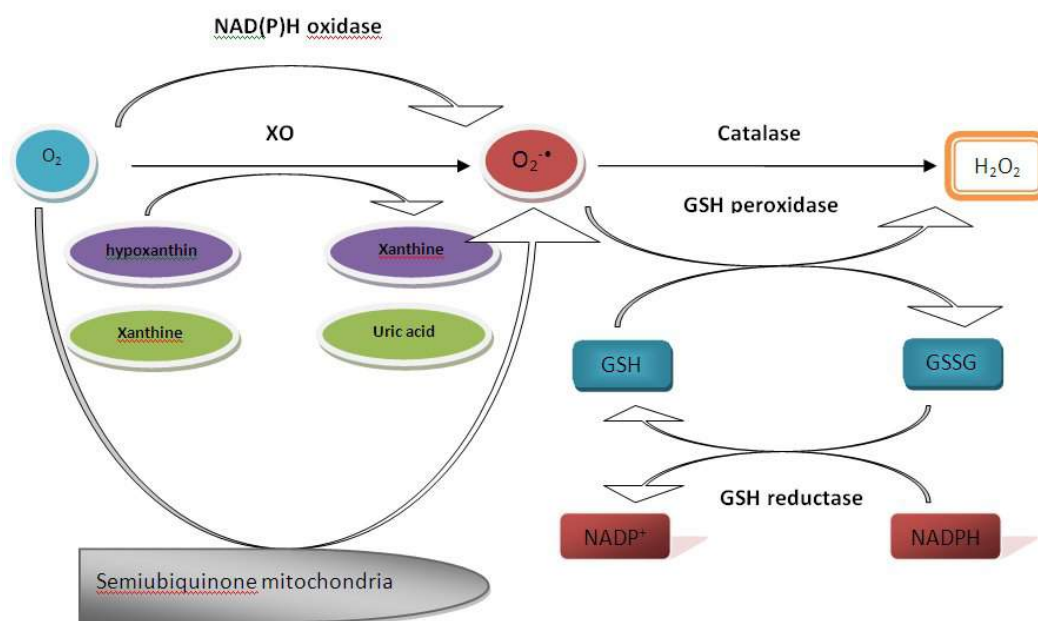


Figure 2-3: *In vivo* ROS formation and its clearance. GSSH (glutathione disulfide), GSH (glutathione).

Even the glucuronide metabolite of catechins (B-ring o-methylated catechins) has antioxidant activity but little than its parent compound due to its reduced H-donating properties (Harada *et al.*, 1999) but still can protect cells against toxicity as effectively as its parents compounds (Schroeter *et al.*, 2001). Hence it can be suggested that catechins protect cells against cytotoxicity by other ways also in addition to the free radical scavenging mechanism.

2.5.2. Prevention of oxidative stress induced lipid peroxidation by catechins

Catechins are able to protect cells from oxidative stress—induced lipid peroxidation and DNA deamination. It is proved that GTE reduced the lipid peroxidation markers in serum, brain and liver, as well as malondialdehyde, 4-hydroxynonenal and lipid hydroperoxides in rats (Skrzydowska *et al.*, 2002). Catechins also protect cells against oxidative stress lipid peroxidation-initiators such as 6-hydroxydopamine (Nie *et al.*, 2000; Jin *et al.*, 2001; Levites *et al.*, 2002a; Levites *et al.*, 2002b; Nobre *et al.*, 2003), t-butylhydroperoxide (Saffari and Sadrzadeh, 2004), H_2O_2 (Lee *et al.*, 2003; Koh *et al.*, 2003), 3- hydroxykynurenine (Jeong *et*

al., 2004), UVB-radiation (Katiyar *et al.*, 2001), lead (Chen *et al.*, 2002; Chen *et al.*, 2003) and iron (II/III) (Guo *et al.*, 1996; Lee *et al.*, 2003).

2.5.3. Catechins inhibit and stimulate apoptosis

Catechins at low dose inhibit apoptosis while at higher doses stimulate apoptosis. EGCG showed evidence of altering several apoptotic pathways to protect cells against oxidative stress to maintain cell survival. Reports have been shown that EGCG, after exposure of PC12 cells to H₂O₂, inhibited different points of apoptotic chain such as caspase 3, glycogen synthase kinase-3 pathway, poly-ADP-ribose-polymerase cleavage, cytochrome c release, and altered cell signaling through activation of the phosphatidyl inositol-3 kinase/Akt pathway (Koh *et al.*, 2003). Other study revealed that EGCG inhibited the apoptosis and caspase-3 activity in SH-SY5Y neuroblastoma human cells after exposure to 3-hydroxykynurenine (Jeong *et al.*, 2004). EGCG and its methylated metabolite (EGCG-3U-OMe) protected hepatocytes against necrosis and apoptosis induced by rubratoxin B and bromobenzene cytotoxins through suppressing caspase-3 activity (Kagaya *et al.*, 2002), furthermore, GTP prevented hepatocytes from Azathioprine-induced apoptosis and normalized the elevated caspase-3 (Hesham *et al.*, 2002). Catechins through altering the expression of proapoptotic and antiapoptotic genes can also modulate the cell apoptosis. When the antiapoptotic genes (Bcl-2, Bcl-w & Bcl-xL) induced to protect SH-SY5Y cells from 6-OHDA-induced apoptosis, EGCG prevented the expression of proapoptotic genes (Bad, Mdm2 and Bax), and restored extracellular signal-related kinases 1/2 pathways and the protein kinase C which let to cell survival promotion (Levites *et al.*, 2002b). Other studies showed that EGCG at lower dose (1-10 µM) has anti-apoptotic effect which reduced Bad, Bax and caspase-6, while in higher doses (50-500 µM) has pro-apoptotic effect through inducing Bad, Bax, Fas, gadd45 and caspase-6, and reducing Bcl-2, Bcl-xL (Mandel *et al.*, 2004). EGCG also increased apoptosis and caspase-3 in cancer cells (Noritaka *et al.*, 2002). Hence, the effect of catechins on apoptosis is divergent, at low doses showed antiapoptotic effect; while in higher concentration induced apoptosis, in normal cells inhibit apoptosis while in cancer cell stimulate apoptosis pathways.

2.6. Pro-oxidative properties of catechins

Although catechins have antioxidative and antiapoptotic effects, but several studies also showed that catechins contain prooxidant properties. At low doses (1-50 µM) *in vitro*, catechins are antiapoptotic and antioxidant, while at higher doses (100- 500 µM), catechins are oxidant and apoptotic. Human isolated DNA were exposed to EGCG and EGC (200 µM), oxidative damage has been induced due to H₂O₂ (Szeto and Benzie 2002). It has been shown that GTE (10-200 µg/ml) and 20-200 µM of EGCG deteriorated the oxidant activity, and oxidative stress, cytotoxicity and genotoxicity induced by H₂O₂ in RAW 264.7 macrophages (Elbling *et al.*, 2005). It also has been demonstrated that catechins, particularly EGCG (100 µM) increased the oxidative damage due to generation of hydroxyl radical and hydrogen peroxide in presence of Cu⁺² and Fe⁺³, occurred following of DNA exposure to 8-oxy-7,8-dihydro-2V-deoxyguanosine (Furukawa *et al.*, 2003; Oikawa *et al.*, 2003; Azam *et al.*, 2004).

Metal complex of quercetin have higher antioxidative properties than simple quercetin (Zhou *et al.*, 2001; Rubens and Wagner, 2004; Armida *et al.*, 2005). While mutagenic and carcinogenic effect of it are belonged to production of ROS (Das *et al.*, 1994; Duthie *et al.*, 1997; Pamukcu *et al.*, 1980). The mutagenic effect of flavonoids is due to ROS formation via oxidation and redox cycling (Tan *et al.*, 2007). It has been suggested that prooxidative effect of some flavonoid which may damage DNA is the result of their combination to the metals (Masataka *et al.*, 1999; Pattubala *et al.*, 2004).

Quercetin has opposite effects on DNA damage induced by Cupric ion + H₂O₂ where at low concentration of cupric ion (=25 μM) it decreased the DNA damage, while at higher concentration of cupric ions (>50 μM) it promotes the DNA damage (Osakao *et al.*, 2005; Tan *et al.*, 2007). Metal ions are able to react with O₂^{•-} and H₂O₂ to produce strong reactive species (e.g., •OH) and metal-oxygen complex which may lead to DNA damage (Kennedy *et al.*, 1997; Galaris and Evargelous, 2002; Tan *et al.*, 2007).

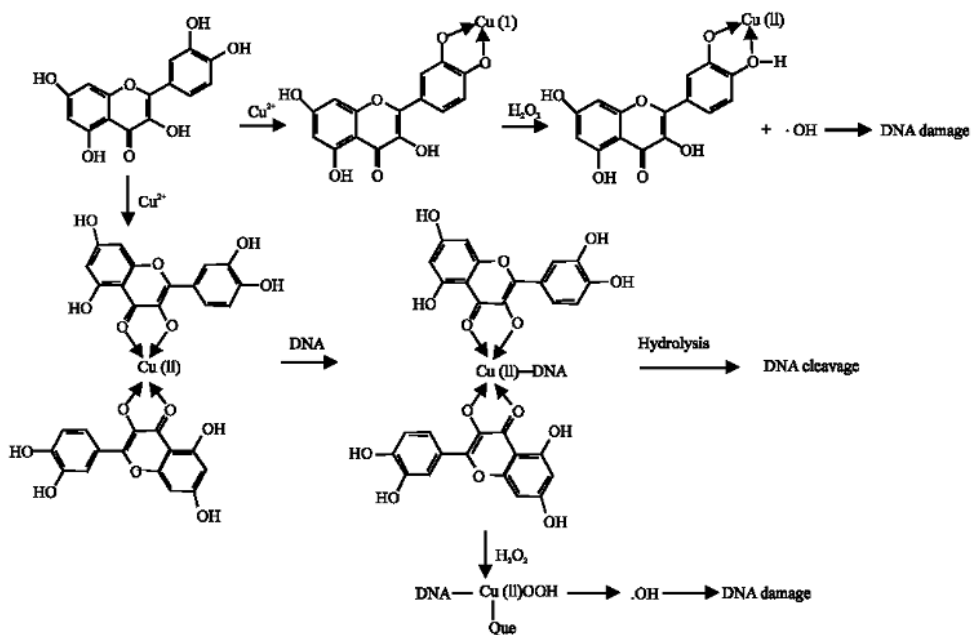
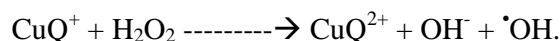


Figure 2-4: The possible mechanism of DNA damage by quercetin copper complexes

Cu²⁺ bound to 3',4'-(OH)₂ groups and reduced to Cu¹⁺ which further react with H₂O₂ and produce •OH as following reaction:



Q= Quercetin (Tan *et al.*, 2007).

Quercetin protects DNA through scavenging of ROS either as free molecule or at the site of binding of quercetin to DNA (Naruto *et al.*, 1999; Alina and Juan, 2001).

Copper-oxidized catechins were stronger prooxidants than unoxidized, reported by Azam *et al.*, (2004). Galati *et al.*, (2006) reported about cytotoxicities of major tea phenolic compounds on isolated rat hepatocytes, and EGCG found to be the most hepatocytotoxic followed by propyl gallate, ECG, gallic acid and EGC, and epicatechin. One of the possible mechanisms of cytotoxicity may be potential collapse of mitochondrial membrane by ROS formation (Galati *et al.*, 2006). Similarly other researchers (Mc Cormick *et al.*, 1999; Johnson *et al.*, 1999; Stratton *et al.*, 2000; Chang *et al.*, 2003) also considered toxicity of green tea and its polyphenols at higher doses. Severe hepatic necrosis resulted to death in female swiss-webster mice after EGCG administration (Goodin and Rosengren, 2003). Po C. Chan *et al.*, (2010), demonstrated the hepatotoxicity of GTE at dose of 500 mg/Kg and hepatic necrosis at dose of 1000 mg/Kg in duration of 14 weeks in rats and mice. They tested effect of 0, 62.5, 125, 250, 500 and 1000 mg/kg GTE and found significantly decrease in total protein and albumin at dose of 250 mg/kg and higher. Mice showed more mortality and increased hepatosomatic index (liver: body weight ratios). There is no doubt that green tea concentrated extract poses risk to liver when taken for long duration (Jimenez and Martinez 2006).

Cell viability of rat hepatocytes got reduced in *in vitro* test also after treatment with high concentration of epigallocatechin gallate (EGCG) (Galati *et al.*, 2006; Schmidt *et al.*, 2005) and it also caused kidney, Gastro-intestinal and liver toxicity (Isbrucker *et al.*, 2006).

The possible mechanism may be disturbance in carbohydrate metabolism lead to mitochondrial toxicity and formation of ROS (Reactive Oxygen Species) by catechins of GTE (Galati *et al.*, 2006).

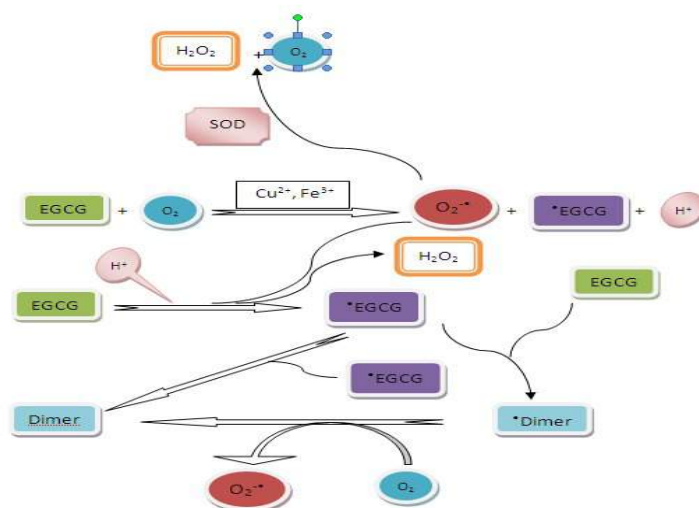


Fig. 2-5. Proposed mechanism of ROS generation by auto-oxidation of EGCG

In cell culture medium with neutral or slightly alkaline pH, $O_2^{\bullet-}$ and $\bullet EGCG$ (EGCG radical) were formed after oxidation of EGCG by molecular oxygen probably with catalytic mediation of Cu^{2+} and Fe^{3+} . Superoxide anion then may react with another EGCG molecule to produce $\bullet EGCG$. Two EGCG radical may react with each other to produce an EGCG

dimer, or •EGCG may react with EGCG to produce EGCG dimer radical, which can react with O₂ to generate O₂^{•-} and EGCG dimer (Shengmin *et al.*, 2005).

2.6.1. The anti-inflammatory properties of green tea through Nitric oxide synthase pathway

Although Nitric oxide (NO) performs several useful functions *in vivo*, such as accounts for the activity of endothelium derived relaxing factor which leads to vasodilation via stimulation of the soluble guanylate cyclase which causes relaxing of vascular smooth muscles (Moncada *et al.*, 1989 & 1991), while the excess amount of NO can be cytotoxic and cytostatic (Moncada *et al.*, 1991; Stefanovic-Racic *et al.*, 1993). It may cause direct toxicity by reacting with mitochondrial iron-sulphur proteins (Hibbs *et al.*, 1988), or interact with free radicals. NO may also form by macrophages activated by cytokines and lipopolysaccharide (LPS) (Hibbs *et al.*, 1990). NO synthase synthesizes the NO from the guanidine terminal nitrogen atom of the L-arginine (Palmer *et al.*, 1988). Still two types of NO synthase has been identified, constitutive, a Ca²⁺/calmodulin-dependent in response to receptor stimulation releases nitric oxide for short time, and the other (Ca²⁺-independent) is found in activated macrophages and polymorphonuclear leukocytes (PMNs), is inducible, once expressed may synthesize the NO for long period (Moncada *et al.*, 1991). NO reacts with superoxide radical (O₂^{•-}) and produces peroxynitrite (ONOO⁻) (Huie and Padmaja, 1993; Beckman *et al.*, 1990; Koppenol *et al.*, 1992). Peroxynitrite can have direct cytotoxic affect and it may generate different products such as nitronium ion (NO₂⁺) and hydroxyl radicals (OH[•]) (Beckman *et al.*, 1990; Radi *et al.*, 1991; Koppenol, *et al.*, 1992; schiropoulos *et al.*, 1992; Beckman *et al.*, 1994; Van der Vliet *et al.*, 1994). Further, peroxynitrite causes nitration of aromatic amino acids. Presence of nitrated aromatic amino acids may be considered as marker of peroxynitrite-induced damage (NO[•]-dependent) *in vivo* (schiropoulos *et al.*, 1992; Beckman *et al.*, 1994; Van der Vliet *et al.*, 1994). It was often suggested that NO participate in joint damage (Farrell *et al.*, 1992; Stefanovic-Racic *et al.*, 1993; McCartney-Francis *et al.*, 1993; Palmer *et al.*, 1993; Oyanagui, 1994) and inhibition of NO[•] synthase suppressed the arthritis in mice (McCartney-Francis *et al.*, 1993).

Catechins showed anti-inflammatory properties by inhibition of carrageenin-induced edema (Matsuoka *et al.*, 1995), however the exact mechanism of this properties is not much clear. One of the suggested mechanism can be the inhibition of NO and decreasing of NO synthase activity (Chan *et al.*, 1995; Nagai *et al.*, 2002; Tedeschi *et al.*, 2004; Suzuki *et al.*, 2004; Sutherland *et al.*, 2005). Catechins (with EGCG being the most effective) of GTE can directly scavenge the NO and peroxynitrite (Paquay *et al.*, 2000). Catechins through inhibiting of neuronal nitric oxide synthase (nNOS) scavenge inflammatory effect of NO in neurons. Experiments in mouse peritoneal calls revealed that EGCG inhibited the nNOS activity which was stimulated by interferon g (IFN-g) and lipopolysaccharide (LPS) (Chan *et al.*, 1997). Studies suggested that inhibition of iNOS which was induced by LPS, IFN-g, tumor necrosis factor- α (TN- α) and interleukin-1 is also a possible mechanism of anti-inflammatory effect of EGCG and other catechins (Chan *et al.*, 1997; Lin YL, 1997; Paquay *et al.*, 2000; Tedeschi *et al.*, 2004). Ju- Hua Chen *et al.*, (2004) showed that EGCG reduced

the elevation of lipid peroxidation, nitric oxide synthase mRNA & protein, nitric oxide radical and nitrotyrosine in carbontetrachloride-induced liver injury. It is suggested that inhibition of iNOS by catechins is not a direct mechanism but they inhibit the binding of nuclear factor- κ B (NF- κ B) to the promoter of the iNOS gene and hence inactivating it (Lin YL, 1997), while Tedeschi *et al.*, (2004) revealed that GTE inhibit iNOS by down-regulated the DNA binding activity of transcription factor signal transducer and activator of transcription-1, but not through decreasing NF- κ B. In contrast, other study showed that ECG improved the quality of scarring by induced cyclooxygenase (COX)-2 and iNOS in a model of wound healing (Kapoor *et al.*, 2004). EGCG also induced the activity of endothelial NOS (eNOS) which is involved in production of NO and vasorelaxation, in rat aortic rings (Lorenz *et al.*, 2004).

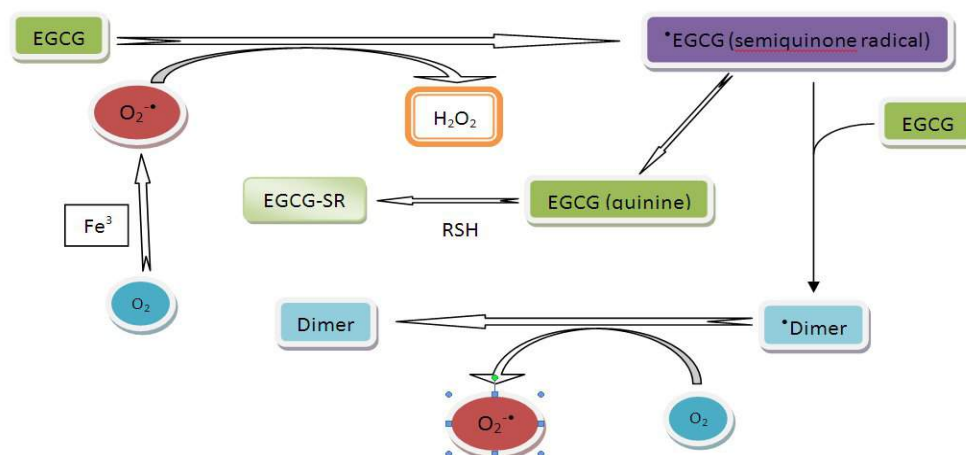


Figure 2-6: Role of EGCG, superoxide, and ferric ion in production of oxidative stress, EGCG dimer, and EGCG-cysteine conjugates (EGCG-SR).

2.7. Association of liver disease and DM

It has been known that the patients with diabetes have high prevalence of liver disease and also patients with liver disease possess high risk of diabetes (Trombetta *et al.*, 2007).

In a 5 years study on 7,148 known Type-2 diabetic patients (3,782 female and 3,366 male), it has been reported that 1,550 diabetic subjects had died. They highest standardized mortality ratios (SMRs) in diabetic cohort were for diabetes and liver cirrhosis. Results showed more than 2 folds of dying risk from liver cirrhosis in the Verona diabetic cohort than general population. The observed:expected ratios were decreased in both sexes as age progressed, with highest mortality risk in patients in the first tertile of time since diagnosis. Perhaps, alcohol consumption also may have contribution. Alcoholic patients with liver disease often show reduced glucose tolerance. Similarly Balkau *et al.*, 1991 & 1992 showed high risk of death because of alcohol-related diseases in diabetic patients. Hepatitis is the other possible contributor for increased risk of cirrhosis in diabetic cohorts. In this study possibilities of alcohol and virus infection were not considered (Roberto *et al.*, 1999).

Similarly other study reported 12.1% hepatopathies in 3,554 diabetic patients of study. Incidence in men was higher than women. Among hepatopathies, fatty degeneration of the liver (28.8%) and the cirrhosis (17.4%) were most frequent. They have showed there is no correlation to the duration of the diabetes (Bauch *et al.*, 1975).

Foster and colleagues (1980), assessed the liver function tests in 60 unselected diabetic out-patients which were stabilized on insulin or oral hypoglycemic agents. GGT and ALP enzymes were elevated occasionally, but mostly not more than twice the ULN. They have suggested that there is no any correlation between measures of diabetic control and result of LFTs. In this study 20% of diabetic patients had evidence of gall stones (a prevalence more than the expectation from the community), and 23% showed abnormal liver ultrasound echo pattern (perhaps indicative of fatty infiltration of the liver). There was only a minimal rise in plasma ALT and ALP concentrations in these patients with abnormal echo pattern. So, they have suggested functionally abnormal liver disease is not common amongst stabilized diabetic patients. While disturbance of LFTs have reported frequently in uncontrolled diabetic patients admitted to hospital, notably increase in plasma ALP and GGT concentrations and reduced in plasma albumin.

293 patients with liver disease (100 with non-autoimmune chronic active hepatitis, 50 with cirrhosis, 25 with chronic persistent hepatitis, and 118 with hepatoma) reviewed for prevalence and characteristics of glucose intolerance and diabetes. 44% of patients with severe chronic active hepatitis, 8% of patients with chronic persistent hepatitis and mild chronic active hepatitis, 15% of patients with hepatoma, and 40% of patients with cirrhosis had diabetes (FBG>140 mg/dL on two separate occasions), while only 30% of diabetic patients showed chronic hepatitis or cirrhosis. Chronic active hepatitis and cirrhosis showed similar glucose tolerance test and was characterized by normal basal glucose level and basal hyperinsulinemia, but increased blood glucose following glucose loading, and insulin resistance (Kingston *et al.*, 1984).

In other study, 100 consecutive patients were assessed for correlation of liver disease and diabetes. The degree of fatty metamorphosis and fibrosis was analyzed in 3 separate categories by the glycemic status of the patient. 46% of patient had normal glucose tolerance, 23% had impaired glucose tolerance, and 31% had non-insulin dependent diabetes mellitus, which severity of fatty metamorphosis was increased from normoglycemic obese to the diabetic obese patient. More glycogen nuclei and PAS-positive blood vessels were found in diabetic obese patients than simple obese patients. So, the distribution of significant liver histopathology in the morbidly obese patient was correlated in severity with the degree of abnormal glycemic status (Silverman *et al.*, 1990).

Non-alcoholic fatty liver disease (NAFLD) is very common in patients with T2DM. NAFLD occurs in 49-62% of patients with T2DM, while 18-33% of patients with NAFLD have T2DM or impaired fasting glucose (more than 6.0 mmol/L) (Browning *et al.*, 2004; Gupte, *et al.*, 2004; Fan *et al.*, 2005; Jimba, *et al.*, 2005; Briohny and Leon, 2010). Study on 438,069 patients showed that adults with newly diagnosed diabetes (excluding patients with pre-existing liver or alcohol-related disease) are at higher risk of advanced liver disease (also

known as diabetic hepatopathy) with incidence rate of serious liver disease 8.19 per 10,000 person-years, while among people without diabetes it was 4.17 per 10,000 person-years (Porepa *et al.*, 2010). Patients with T2DM and NAFLD have more severe metabolic disturbances in compare with those with T2DM without NALFD, as well as they have greater insulin resistance and visceral adiposity, severe dyslipidemia, and greater levels of inflammatory markers including interleukin-6 and tumor necrosis factor (Banerji *et al.*, 1995; Kelley *et al.*, 2003; Toledo *et al.*, 2006; Smith & Adams, 2011). A community-based study on 337 diabetic subjects revealed that occurrence of NALFD in diabetic patients more likely to be in younger, and females. Association of NAFLD in diabetic patients increase risk of death 2.2 folds compared with those non-NAFLD diabetic patients (Leon *et al.*, 2010). The fourth most common cause of death among patients with T2DM includes chronic liver disease and/or hepatocellular carcinoma which have occurring rate of 1:20 deaths (Smith & Adams, 2011). Risk of liver disease-related death is directly related to the severity of DM, which those patients using oral hypoglycemic medicines are associated with 4.9-fold induced risk, and patients using insulin are associated with 6.8-fold induced risk of death if compare with those dependent on diet control alone (de Marco, *et al.*, 1999). T2DM in association with obesity and age was demonstrated as risk factor for the development of advanced fibrosis in NAFLD, which accelerated the rate of hepatic fibrosis progression (de Marco, *et al.*, 1999).

Not only T2DM is associated with NAFLD, T1DM is may also associated with NAFLD. Up to 44% of adult patients with T1DM showed altered liver tests or have been diagnosed with NAFLD (de Marco *et al.*, 1999; Rashid & Roberts, 2000; Manton *et al.*, 2000; Leeds *et al.*, 2009; Targher *et al.*, 2010). Glycogenic hepatopathy which may occur in T1DM patients typically happens with insulin administration in past poor glycemic control in young children. Mostly this condition leads to Mauriac syndrome—presents with hepatomegaly, induced transaminases, delayed puberty and growth retardation (de Marco *et al.*, 1999; Torbenson *et al.*, 2006).

Insulin resistance disturbs the balance between factors which ameliorate lipid synthesis and factors which favor hepatic lipid accumulation including lipid influx and *de novo* lipid biosynthesis, such as lipid oxidation and lipid export. Lipolysis in peripheral adipocytes is increased in insulin resistance condition which may lead to a net influx of free fatty acid into the liver (Adams *et al.*, 2005). Hyperinsulinemia and hyperglycemia promoted *de novo* hepatic lipogenesis through up regulating of several key lipogenic transcription factors such as SREBP-1c (Sterol Regulatory Element-Binding Protein-1c) and carbohydrate response element-binding protein—also known as MLXIPL (Browning *et al.*, 2004). Insulin-mediated activation of SREBP-1c induced the levels of malonyl-CoA which resulted inhibition of intrahepatic lipid utilization for energy (Browning *et al.*, 2004). Apolipoprotein B—the major carrier protein of VLDL synthesis reduced which led to reduced hepatic lipid export in VLDL in NAFLD-patients (Charlton *et al.*, 2002). Studies on mice showed that accumulation of triglyceride in liver may cause hepatic steatosis, but lack hepatic and systemic insulin resistance (Monetti *et al.*, 2007), while it has been shown that free fatty acids trigger lipotoxicity in liver via multiple complex pathways as well as due to FA metabolites

including diacylglycerols and ceramides which induce ER stress and apoptosis (Neuschwander-Tetri, 2010). Hepatic steatosis by interfering in tyrosine phosphorylation of insulin receptor substrates 1 & 2 through the JNK1 pathway may lead to hepatic insulin resistance (Schattenberg et al., 2007). Degree of hepatic inflammation and fibrosis in patients with NAFLD is closely related to the levels of visceral adipose tissue (van der Poorten *et al.*, 2008) and in comparison with subcutaneous adipose tissue, visceral adipose tissue cause more insulin resistance with containing greater number of large adipocytes, greater lipolysis rates, and higher number of inflammatory cells (Ibrahim, 2002).

However the majority of NAFLD patients are asymptomatic, but some of them may report malaise, abdominal discomfort or fullness (Angulo, P., 2002), and hepatomegaly. Some children with insulin resistance may present acanthosis nigricans (Smith, B. W. & Adams, L. A., 2011). Liver enzymes has been shown normal up to 78% of patients with NAFLD, hence, they are insensitive parameter for diagnosis of NAFLD (Browning, J. D. *et al.*, 2004; Torres, D. M. & Harrison, S. A., 2008). It was approved by a study on cohort of Italian patients who had normal ALT but had either elevated ferritin or persistent steatosis on ultrasound scan, and 59% had NASH (nonalcoholic steatohepatitis) on liver biopsy (Fracanzani, A. L. *et al.*, 2008). In patients without or with minimal fibrosis the ratio of AST:ALT is usually less than one, however it will be reversed in the case of cirrhosis (Angulo, P., *et al.*, 1990). Studies on 48 young NAFLD male patients with normal fasting blood glucose levels, showed impaired glucose tolerance test in 33% and diabetes mellitus in 6% of these patients (Yun, J. W. *et al.*, 2009). Hepatocellular triglyceride is accumulated in macrovesicles or microvesicles in all NAFLD cases. Fibrosis is typically predominant but with progressive disease, it develops between the hepatic vein and portal tract and go on to form nodules and cirrhosis. It is thought that many cases of cryptogenic cirrhosis can be reflecting cases of 'burn-out' NASH cirrhosis, as by disease progress steatosis diminishes and even may become absent in patients with cirrhosis (Powell, E. E. *et al.*, 1990). Hepatosclerosis (sinusoidal basement-membrane thickness and fibrosis in the absence of NAFLD and mostly it is associated with microvesicular disease) and glycogenic hepatopathy (abundance of cytoplasmic and nuclear glycogen with severe hepatocyte swelling) are found in patients with T1DM and NAFLD (Torbensohn, M. *et al.*, 2006; Harrison, S. A., *et al.*, 2006; Targher, G. *et al.*, 2010).

Impaired glucose tolerance occurs in 60-80% of cirrhotic patients while frank diabetes mellitus occurs in 10-30% of patients. Most of patients with cirrhosis exhibit induced peripheral plasma insulin levels and severe insulin resistance (Alexander *et al.*, 1994). Insulin resistance is occurred in the early stage of liver disease, and factors other than liver dysfunction such as portsystemic shunting and nutritional status will play role in the progress to overt diabetes mellitus (Alexander *et al.*, 1994).

Different kinds of liver disease are reported in patients with T2DM including abnormal liver enzymes, NAFLD, hepatocellular carcinoma, cirrhosis, and finally acute liver failure. Unexplained association of diabetes with hepatitis-C is also reported (Keith *et al.*, 2007). The prevalence of diabetes in cirrhotic patients has been reported almost from 12.3-57% (Trombetta *et al.*, 2007). The ALT level elevation is common in patients with T2DM, showed

by studies on 3,701 T2 diabetic patients which ALT level was increased above the upper limit of normal in 2-24% of subjects (Belcher and Scherthaner 2005; Keith *et al.*, 2007).

Several studies has been suggested 4-fold induced prevalence of hepatocellular carcinoma in type 2 diabetic patients and vies versa, increased in prevalence of diabetes in patients with hepatocellular carcinoma (Keith *et al.*, 2007). Studies on 153,852 diabetic patients showed overall standardized incidence ratio (SIR) of 2.5 for liver cancers. They have found that men were in higher risk than women. They have reported 4-fold increased risk of primary liver disease in hospitalized diabetic patients. Smaller increases have been seen in risk of cancers of gallbladder, extra-hepatic bile ducts, and amulla of vater (Adami *et al.*, 1996). T2DM is characterized by insulin resistance, hyperinsulinemia, and induced growth factor production. Therefore, hepatocytys are directly faced to higher level of insulin (Adami *et al.*, 1996). Insulin and/or its precursors interact with hepatocytys and stimulate mitogenesis or carcinogenesis (Tabor, 1994; Kahn and White, 1995; Russell and Van, 1995). Higher plasma insulin concentration in men than women can be reason for higher risk of liver cancer in diabetic men than diabetic women (Ferrara A., *et al.*, 1984; McKeigue *et al.*, 1993). In some study cohort members, T2DM may be secondary to progressive liver disease such as alcohol-induced liver disease that can reduce insulin sensitivity and secretion (Kingston *et al.*, 1984; Allison *et al.*, 1994; Petrides, 1994).

Similarly studies on 2061 hepatocellular carcinoma subjects against 6183 non-cancer controls showed that proportion of HCC patients with diabetes (43%) was significantly greater than non-cancer controls (19%). Diabetes showed 3-folds higher risk of HCC than other risk factors such as HBV, HCV, haemochromatosis, and alcoholic liver disease, so diabetes is an independent risk factor for HCC. In this study a significant positive interaction between HCV and diabetes was also detected. Diabetes in the presence of other risk factors including HBV, HCV, haemochromatosis, and alcoholic liver disease, increased the risk of developing HCC beyond the risk associated with diabetes in the absence of these risk factors (Davila *et al.*, 2005). Diabetes has been identified as a risk factor for NAFLD and its severe form NASH, whereas NASH has been implicated as cause of both cryptogenic cirrhosis and HCC (Caldwell *et al.*, 1999; Bugianesi *et al.*, 2002).

Diabetes is also associated with high risk of chronic liver, including fatty liver and cirrhosis, mostly among obese individuals (Nagore and Scheuer, 1988; Silverman *et al.*, 1990). Since, gallbladder emptying is reduced in diabetic patients, especially when it is associated with autonomic neuropathy, promoting stasis and bacterial overgrowth which in turn promote the formation of stones. Although other mechanisms may be required for cancer initiation than increased mitotic activity which result mechanical trauma, infection, and chronic inflammatory process which have been implicated in biliary carcinogenesis (Preston-Martin *et al.*, 1990; Ekbohm *et al.*, 1993; Farber, 1995, Adami *et al.*, 1996).

Chapter-III

Methods & Materials



Chapter-III

Methods and Materials

3. Methods and Materials

3.1. Plant Material

Green tea extract was procured from Blue Berry Agro, Mumbai, India. It contained 90% catechins. 0.2% aqueous solution of the extract powder was freshly prepared every morning and supplemented instead of drinking water to two groups of animals (Normal + GTE and Diabetic + GTE). The amounts were calculated to provide a 200 mg/kg BW of GTE for each animal. GTE was kept in dry and cold place.

3.2. Experimental Animals

36 Sprague dawley male rats were procured from NIPER, Mohali, Punjab, having weight between 190-310 gm, and age 2.5-3 months. 24 of them were used in experiment the rests were kept for backup in the case of mortality. They were kept in animal house of university (Lovely Professional University, Jalandhar, Punjab, India) under standard condition and acclimatized for 10 days before experimental period. Each 3 rats were placed in one polypropylin cage—having rice husk as bedding material (the cages were changed each 2 days), and normal diet (standard chow) and water were provided (*ad-libitum*). Room was facilitated with good ventilation system; fans and Air conditioner were used to maintain room temperature between 25 and 30° C with relative humidity 45-55%. 12 hours light and dark



Fig 3-1: Room environment for keeping of rats

cycle was maintained. Animal handlings were performed as per standard laboratory practice.

Ethical certificate was received from IAEC (Indian Animal Ethics Committee), attached in appendages.

3.3. Chemicals and Instruments

90% GTE was purchased from Blue Berry Agro, Mumbai, India. STZ was received from university chemical store. Diagnostic assay kits were purchased from local market belonged to following companies: triglyceride, cholesterol, albumin, and urea (*Beacon Diagnostics PVT, LTD*, India), glucose, total protein, total and direct bilirubin, SGPT, SGOT and ALP (ERBA diagnostic kit, Transasia Bio-Medicals LTD, India). Diethyl ether, ethanol, acetone, chloroform, xylene, paraffin wax, heamatoxylin, eosin, DPX, formalin, DPPH, acetonitrile, standard rats' chow were provided from the chemical store of the university. The instruments used are semi-autoanalyzer, colorimeter, water bath, float bath, microtome, glucometer, light microscope, UV-spectrophotometer (UV-1800, Shematzu), digital balance and refrigerator.

3.4. Induction of Diabetes

For induction of diabetes type-1 we have used 45 mg/kg BW of streptozotocin (STZ) (Wisetmuen *et al.*, 2013). STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose, figure 3-0) is synthesized by streptomycetes achromogenes, used for induction of both T1DM and T2DM. STZ enters to β -cells of pancreas through GLUT2 and leads to changes of DNA (Szkudelski T., 2001). Alkylation of DNA as a main reason of cell death by STZ has been reported (Delaney *et al.* 1995, Elsner *et al.* 2000). As well as NO contribution in STZ-induced DNA damage reported by Morgan *et al.* (1994) and Kröncke *et al.* (1995). While not NO is the only molecule responsible of cytotoxicity induced by STZ, the increased formation of superoxide anions by mitochondria and increased activity of xanthine oxidase are the other purposed mechanisms of STZ-induced cytotoxicity. As a result of superoxide anion generation, hydrogen peroxide and hydroxyl radicals are formed, lead to β -cells damage and insulin formation and secretion impair (Szkudelski T., 2001).

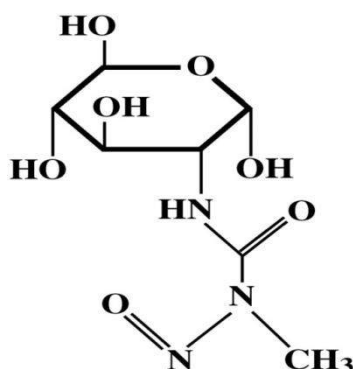


Fig 3-2: STZ chemical structure

Animals were kept in overnight fasting condition with free access to water, then T1DM was induced by IP injection of STZ (45 mg/kg body weight of animal). After 3 days the FBG level was checked and $\text{FBG} \geq 200 \text{ mg/dL}$ was considered as diabetes. Although the FBG level was decreased to around 150 mg/dL, but in compare to normal control the FBG level was high.



Fig 3-3: IP injection of STZ

3.5. Blood sample collection

Four types of blood collection have been done. Tail blood collection by syringe (figure 3-4), tail prick for glucometer, cardiac puncture (figure 3-6), and eyes puncture (orbital venous plexus, figure 3-4). Tail samples were not in sufficient amount so only performed used only while estimating blood glucose was required. Sufficient amounts of blood were collected from eyes after giving anesthesia with di-ethyl ether (figure 3-5). Blood was collecting in gel tubes without anticoagulant and immediately centrifuged at 3000 rpm for 10 min to separate the serum.



Fig 3-4: Blood collection from the tail by 1 ml syringe, blood collection from the eyes of rats by capillary tubes.



Fig 3-5: Rats were anesthetized with di-ethyl ether before eyes blood collection and dissection.

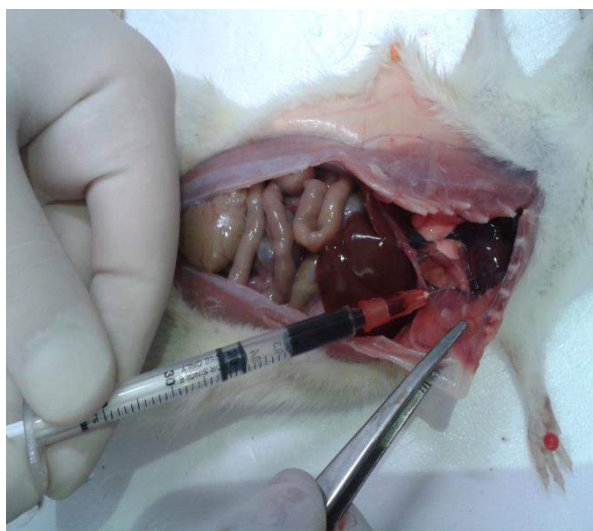


Fig 3-6: Dissection and blood collection from the heart of the animals after anesthesia.

3.6. Experimental Design

The experimental rats were divided into 4 groups of 06 animals each. First group was the “Normal control”. They were not treated neither STZ nor green tea. Status of each biomarker was compared with this group. The second group, “Normal + GTE” is non-diabetic group which are treated with 0.2% aqueous GTE (having 90% catechins) solution as drinking water. The 3rd group, “Diabetic control” animals were injected streptozotocin (45 mg/kg BW) and server as “diabetic control”. The 4th group, “Diabetic + GTE” animals were injected STZ (45 mg/kg BW)), and after diabetes induced, treated with 0.2% aqueous solution of GTE (90%) instead of drinking water for 14 days (table 3-1) . The parameters were analyzed after 7 and 14 days of GTE supplementation. Results were compared between “NC” and “N+GTE”, “NC” and “DC”, and “DC” and “D+GTE”. At the end of experiment one animal from each group was dissected for further tissue processing and histopathological analysis.

This study initially was designed to evaluate effect of 2 months GTE supplementation, but unfortunately due to late providing of animals by university, the experiment converted to 14 days. The total experimental protocol was maintained for 14 days after induction of diabetes.

Table 3-1: Experimental design

Groups	Groups	Sex of animal	Required animal	Dose (mg.kg ⁻¹ bw)/ route
I	Non-diabetic rats with Normal diet (Normal control)	Male	6	Nil
II	Non- diabetic rats + green tea extract (Normal+GTE)	Male	6	0.2% aqueous green tea extract (90%) orally instead of drinking water
III	Diabetes- induced rats + normal diet (Diabetic control)	Male	6	Single IP dose of STZ (45mg Kg ⁻¹ bw)
IV	Diabetic animal + green tea extract (Diabetic+GTE)	Male	6	Single IP dose of STZ (45mgKg ⁻¹ bw) , +0.2% GTE aqueous solution instead of drinking water for 14 days

3.7. Biochemical analysis

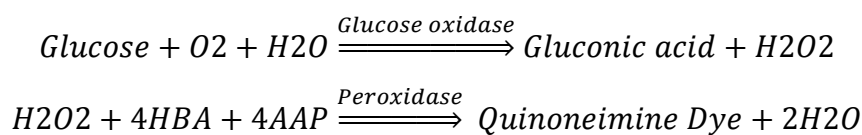
3.7.1. Fasting Serum Glucose

After 3 days of STZ injection, fasting blood glucose was checked. FBG \geq 200 mg/dL was considered as hyperglycemia and diabetes. Later FBG was estimated on 7th and 14th days of GTE supplementation in all groups. FBG was estimated through GOD-POD kit method (*Erba Mannheim Ltd*), and glucometer.

Methodology:

Determination of glucose in blood using glucose oxidase with an alternative oxygen receptor Trinder, P., (1969).

Principle of the test:



4AAP: 4-aminoantipyrine

4HBA: 4-hydroxy benzoic acid

The intensity of the pink color (quinoneimine dye) is proportional to the concentration of glucose present in the sample and photometrically measured at 520 nm.

(Aumiller *et al.*, 2014).

Reagent composition:

Reagent 1: glucose reagent

- Glucose oxidase: 20,000 IU/L
- Peroxidase: 3250 IU/L
- 4-aminoantipyrine 0.52 mmol/L
- 4HBA: 4-hydroxy benzoic acid: 10 mmol/L
- Phosphate buffer: 110 mmol/L

Ph: 7.0±0.2 at 25 °C.

Reagent 2: glucose standard

- Glucose standard: 100 mg/dL (5.55 mmol/L)

Procedure:

Blood sample was collected in non-coagulant tubes and left for 10 min to be clotted. Then centrifugation at 3000 RPM for 10 min performed and the serum was separated.

Table 3-2: Procedure for glucose estimation

Pipette into tubes marked	Bank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	-	-
Standard	-	10 µl	-
Test	-	-	10 µl

Test contents were mixed well and incubated for 15 min at 37 °C. The absorbance of tests and standards were estimated against blank at 500 nm in colorimeter and UV-visible spectrophotometer (UV-1800 Shimadzu). Calculation was done through following formula:

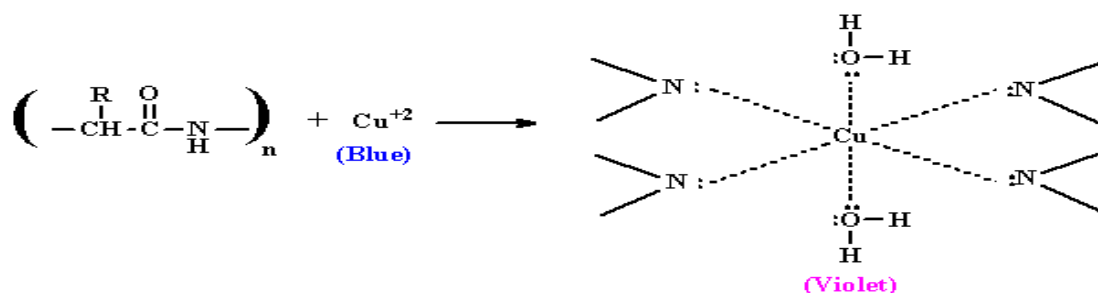
$$\text{Glucose } \left(\frac{\text{mg}}{\text{dL}} \right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} * \text{Concentration of Standard } \left(\frac{\text{mg}}{\text{dL}} \right)$$

Data were compared with normal control group.

3.7.2. Serum Total Protein

Serum total protein was estimated on 7th and 14th days of GTE supplementation. It was estimated by biuret kit method (*Erba Mannheim Ltd.*).

Principle of the test:



The intensity of violet color is proportional to the concentration of protein present in sample.

(Tietz, N.N. (Ed), 1986)

Reagent composition:

Reagent 1: total protein reagent

- CuSO₄ 19 mmol/L
- K⁺-Na⁺- tartarate: 43 mmol/L
- KI: 30 mmol/L
- NaOH: 600 mmol/L

Reagent 2: total protein standard

- Protein standard: 6.0 g/dL

Procedure:

Blood sample was collected in non-coagulant tubes and left for 10 min to be clotted. Then centrifugation at 3000 RPM for 10 min performed and the serum was separated.

Table 3-3: Procedure for total protein estimation through biuret kits

Pipette into tubes marked	Bank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	20 µl	-	-
Standard	-	20 µl	-
Test	-	-	20 µl

Test tubes were mixed well and incubated for 15 min at 37 °C. The absorbance of tests and standards were estimated against blank at 520 nm in colorimeter and at 546 nm in UV-visible spectrophotometer (UV-1800, Shimadzu). Calculation was done through following formula:

$$\text{Total Protein } \left(\frac{\text{g}}{\text{dL}}\right) = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} * \text{Concentration of Standard } \left(\frac{\text{g}}{\text{dL}}\right)$$

Data were compared with normal control group.

(Zawada *et al.*, 2009; Sood R., 2009).

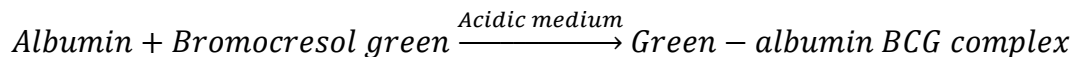
3.7.3. Serum Albumin and Albumin:globulin ratio

Serum Albumin was estimated on 7th and 14th days of GTE supplementation. It was estimated using Beacon diagnostics PVT.LTD assay kit.

Methodology

The test was based on BCG (bromocresol green) described in ().

Principle



(Sood R., 2009).

Reagents

Reagent 1: BCG reagent
 Reagent 2: Albumin standard 4 gm/dL

Procedure

Blood sample was collected in non-coagulant tubes and left for 10 min to be clotted. Then centrifugation at 3000 RPM for 10 min performed and the serum was separated.

Table 3-4: Procedure for estimation of albumin

Addition sequence	Blank	Standard	Test
BCG reagent	1 ml	1 ml	1 ml
Standard	-	10 µl	-
Serum	-	-	10 µl

Test tube contents were mixed well and incubated for 1 minutes at room temperature. The Abs of std and test were measured against the reagent blank at 620 nm.

Calculation

$$\text{albumin concentration } \left(\frac{\text{gm}}{\text{dL}}\right) = \frac{\text{Abs.T}}{\text{Abs.Std}} * 4$$

(Zawada *et al.*, 2009; Sood R., 2009)

Globulin value (gm/dL)= serum total protein-Albumin.

3.7.4. Serum triglyceride

Serum TG (fasting samples) was measured on 7th and 14th days of green tea supplementation in fasting condition. It was estimated using Beacon diagnostics PVT.LTD assay kit.

Methodology

The test was based on GPO/ POD method (Sood R., 2009).

Principle

Triglyceride → glycerol + free fatty acids

glycerol + ATP → glycerol - 3 - P + ADP

glycerol - 3 - p + O₂ → DHAP + H₂O₂

H₂O₂ + phenolic chromogen → red color compound

Contents

Reagent 1: Triglyceride enzyme reagent

Reagent 2: Triglyceride standard 200 mg/dL

Procedure

Initially all the reagent brought to working temperature.

Table 3-5: Procedure for triglyceride estimation through beacon assay kits

	Blank	Standard	Test
Enzyme reagent	1 ml	1 ml	1 ml
Standard solution	-	10 µl	-
Sample	-	-	10 µl

The test tube contents mixed well and incubated for 10 minutes at 37°C. The absorbance of standard and sample was measured against the reagent blank at 505 nm.

Calculation

$$\text{concentration of TG } \left(\frac{mg}{dL} \right) = \frac{\text{Abs. of sample}}{\text{Abs. of standard}} * 200$$

(Zawada *et al.*, 2009; Sood R., 2009).

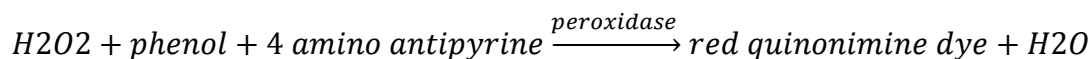
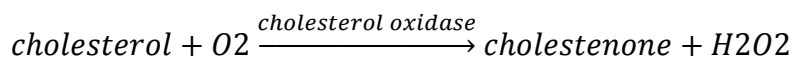
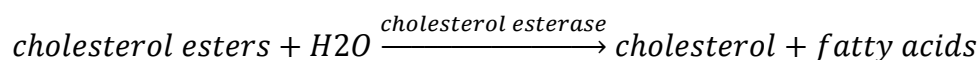
3.7.5. Serum total cholesterol

Serum total cholesterol (fasting sample) was estimated on 7th and 14th days of GTE treatment. It was estimated using Beacon diagnostics PVT.LTD assay kit.

Methodology

The kit was established based on CHOD/POD method. (Sood R., 2009).

Principle



Contents

- Reagent 1: cholesterol enzyme reagent
- Reagent 2: cholesterol standard 200 mg/dL
- Reagent 3: cholesterol precipitating reagent

Procedure

Total cholesterol was estimated as following procedure

Table 3-6: Procedure for serum total cholesterol estimation

Adding materials	Blank	Standard	Test
Enzyme reagent	1 ml	1 ml	1 ml
Standard solution	-	10 µl	-
Sample serum	-	-	10 µl

The test tube contents mixed well and incubated for 37 °C for 5 minutes. The absorbance of the standard and blank measured against the reagent blank at 505 nm.

Calculation

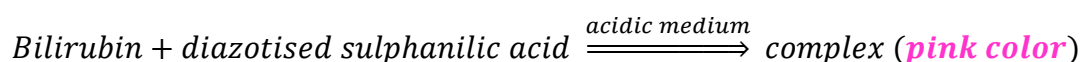
$$\text{cholesterol} \frac{mg}{dL} = \frac{Abs.T}{Abs.S} * 200$$

(Zawada *et al.*, 2009; Sood R., 2009).

3.7.6. Serum bilirubin

Serum bilirubin was estimated on 7th and 14nd days of GTE supplementation. It was estimated by Diazo kit method of Pearlman and Lee (*Erba Mannheim Ltd.*).

Principle:



Direct (conjugated) bilirubin is water soluble and directly can act with sulphanilic acid in acidic medium, while indirect (unconjugated) bilirubin reacted with sulphanilic acid after addition of surfactant (methanol).

Reagent composition

Reagent 1: total bilirubin reagent

- Surfactant: 1.00%
- HCl: 100 mmol/L
- Sulphanilic acid: 5 mmol/L

Reagent 2: direct bilirubin reagent

- Sulphanilic acid: 10 mmol/L
- HCl: 100 mmol/L

Reagent 3: Sodium Nitrite reagent

- Sodium Nitrite: 144 mmol/L
-

Reagent preparation

Working reagents were prepared as following manner (table 3-7)

Table 3-7: Procedure of reagent making for estimation of Total bilirubin and direct bilirubin through Diazo kit method of Pearlman and Lee

Test	Volume of working reagent	Add		
		Reagent 1	Reagent 2	Reagent 3
Bilirubin total	10 ml	10 ml	-	0.2 ml
	25 ml	25 ml	-	0.5 ml
	50 ml	50 ml	-	1.0 ml
	100 ml	100 ml	-	2.0 ml
Direct bilirubin	10 ml	-	10 ml	0.1 ml
	25 ml	-	25 ml	0.25 ml
	50 ml	-	50 ml	0.5 ml
	100 ml	-	100 ml	1.0 ml

Procedure:

Initially all reagents brought to room temperature and followed bellow procedure.

Table 3-8: Procedure for Bilirubin estimation by Diazo kit method of Pearlman and Lee

Reagents	Blank	Standard	Test
Working reagent	500 µl	500 µl	500 µl
Distilled water	25 µl	-	-
Standard	-	25 µl	-
Test	-	-	25 µl

The test tube contents mixed well and incubated for 5 minutes at 37 °C for total bilirubin and direct bilirubin estimation, and the absorbance was observed at 546 nm in uv-visible spectrophotometer against blank. Calculation was done according to the following formula:

$$\text{Total bilirubin } \left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\text{Abs.of test}}{\text{Abs.of standard}} * \text{concentration of standard } \left(\frac{\text{mg}}{\text{dL}}\right)$$

$$\text{Direct bilirubin } \left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\text{Abs.of test}}{\text{Abs.of standard}} * \text{concentration of standard } \left(\frac{\text{mg}}{\text{dL}}\right)$$

Indirect bilirubin = Total bilirubin – direct bilirubin

Or according to the factor, total bilirubin (mg/dL) = Abs.t *factor (23) and direct bilirubin= Abs.t* 23

(Zawada *et al.*, 2009; Sood R., 2009).

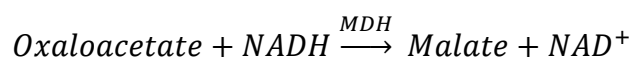
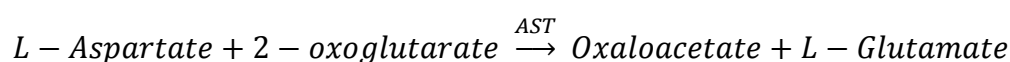
3.7.7. Serum Glutamate-Oxaloacetate Transaminase

Serum SGOT was estimated on 7th and 14th days of GTE treatment. It was estimated by IFCC kit method (*Erba Mannheim Ltd.* Diagnostic assay kits).

Methodology:

Kinetic method recommended by International Federation of Clinical Chemistry (IFCC).

Principle:



AST: Aspartate aminotransferase

MDH Malate dehydrogenase

LDH: Lactate dehydrogenase

Reagent composition:

Reagent 1: SGOT reagent

2-oxoglutarate	12 mmol/L
L-Aspartate	200 mmol/L
MDH	≥ 545 u/L
LDH	≥ 909 u/L
NADH (yeast)	≥0/18 mmol/L
Tris buffer	80 mmol/L (pH=7.8 ± 0.1 at 25°C)
EDTA	5.0 mmol/L

Assay Procedure:

The reagents were attained 37 °C before use.

Table 3-9: Procedure for estimation of SGOT through IFCC method, kinetic.

Pipette	Volume
Working reagent	1000 µl
Test	100 µl

Calculation:

The measured absorbance is converted into international units (IU) of activity as following:

$$\frac{IU}{L} = \frac{\left(\frac{\Delta A}{\text{min.}}\right) * T.V.* 10^3}{S.V.* \text{Absorptivity} * P}$$

T.V. Total reaction volume (µl)

S.V. Sample volume (µl)

Absorptivity = m.molar absorptivity of NADH at 340 nm (6.22)

P cuvette lightpath (1 cm)

And activity of AST is calculated through ($\Delta\text{Abs}/\text{min} * 1768$). SGOT activity was estimated by semi-autoanalyzer machine using *Erba Mannheim Ltd.* Assay kit.

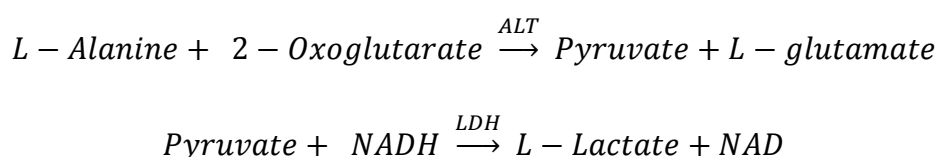
3.7.8. Serum Glutoamte-Pyruvate Transaminase

Serum SGPT was estimated on 7th and 14th of GTE treatment. It was estimated using IFCC kit method, kinetic (*Erba Mannheim Ltd.* Assay kit) by semi-autoanalyzer.

Methodology:

Kinetic method recommended by International Federation of Clinical Chemistry (IFCC). (Sood R., 2009)

Principle:



Reagent composition:

Reagent 1:	SGPT reagent
L-Alanine	500 mmol/L
NADH (yeast)	0.18 mmol/L
LDH	≥ 1820 IU/L
2-oxoglutarate	12 mmol/L
Tris buffer	80 mmol/L (pH 7.5 ± 0.1 at 25°C)

Assay Procedure:

Table 3-8: Procedure for SGPT estimation through IFCC method, kinetic. After maxing well, aspirated.

Pipette	Volume
Working reagent	1000 µl
Test	100 µl

Test tube contents mixed well and aspirated to semi-autoanalyzer machine.

Calculation:

The measured absorbance is converted into international units (IU) of activity as following:

$$\frac{IU}{L} = \frac{\left(\frac{\Delta A}{\text{min.}}\right) * T.V.* 10^3}{S.V.* \text{Absorptivity} * P}$$

T.V.	Total reaction volume (μl)
S.V.	Sample volume (μl)
Absorptivity	= m.molar absorptivity of NADH at 340 nm (6.22)
P	cuvette lightpath (1 cm)

And activity of ALT is calculated through ($\Delta\text{Abs}/\text{min} * 1768$). SGPT activity was estimated by semi-autoanalyzer machine, using *Erba Mannheim Ltd.* Assay kit.

(Zawada *et al.*, 2009; Sood R., 2009)

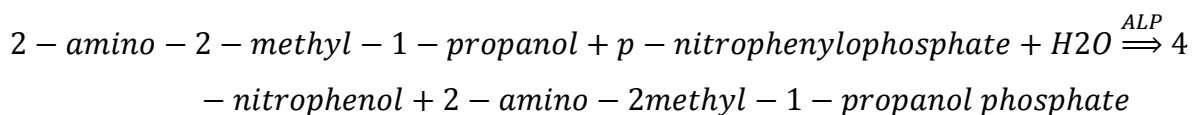
3.7.9. Alkaline Phosphatase

Serum ALP was estimated on 7th and 14th days of GTE treatment. It was estimated by IFCC kit method, kinetic (*Erba Mannheim Ltd.* Diagnostic assay kits).

Methodology:

Kinetic method recommended by International Federation of Clinical Chemistry (IFCC).

Principle:



The rate of 4-nitrophenol formation is directly proportional to the ALP activity.

Reagent composition:

- AMP: 350 mmol/L
- Mg⁺²: 2.0 mmol/L
- Zn⁺²: 1.0 mmol/L

- HEDTA: 2.0 mmol/L
- P-nitrophenyl phosphate: 16.0 mmol/L

Procedure:

Table 3-11: Procedure for estimation of serum ALP

Reagent	1000 µl
Sample	20 µl

Test tube contents were mixed well and absorbance was observed at 0 min., 1min. and 2 min. and the result was calculated by following formula:

$$ALP \text{ activity } \left(\frac{IU}{L} \right) = \frac{\Delta A}{min} * factor (2764)$$

ΔA= difference between absorbances. Mean was taken for differences of absorbance between 0-1 min. and 1-2 min. The result was compared with normal control group. However, we measured serum ALP levels by semi-autoanalyzer instrument, and *Erba Mannheim Ltd.* Assay kit.

(Zawada *et al.*, 2009; Sood R., 2009).

3.7.10. Serum Total Anti-oxidant Capacity

TAC was measured by method reported by Jacek *et al.*, (2008) and Gawron *et al.*, (2008) with some modifications. DPPG is used as main component of this test. DPPH (2,2-Diphenyl-1-picryl-hydrazyl) radical is a relatively stable component with a peak absorbance at 517 nanometer, and has an unpaired valence electron at one atom of a nitrogen bridge. DPPH is acting as donor of electron and/or hydrogen radical while reacting with a variety of anti-oxidant, leads to formation of 1,1-diphenyl-2-picryl-hydrazine and lose its absorbance at 517 nm (Jacek *et al.*, 2008). Because of stability of DPPH in alcohol, the procedure is performed in either methanol, ethanol or other organic solvents.

Procedure

Deproteinization of serum

200 µl of serum was taken in a dry and clean eppendorf tube, and 200 µl of acetonitrile was added, let at room temperature for 2-4 minutes and then centrifuged at 9450 G for 10 minutes at +4°C. After centrifugation supernatants were separated immediately for further process.

DPPH scavenging process

Table 3-12: Procedure for TAC using DPPH

Reagents	Standard	General blank	Test	Control blank (H)
Methanol	2.25 ml	2.85 ml	2.25 ml	2.85 ml
DPPH (1 mmol/L in methanol)	600 µl	-	600 µl	-
Acetonitril	75 µl	75 µl	-	-
Deporteinized serum	-	-	75 µl	75 µl

Contents of the test tubes mixed well and incubated for 30 minutes at room temperature. Absorbances were measured at 517 nm. To exclude (subtract) the absorbance of methanol and acetonitril, absorbance of general blank was made auto-zero. Standard tube color intensity was considered as 100% and the absorbance was measured. The color intensity of other test tubes was compared with standard. As serum anti-oxidants act with DPPH and decrease the level of it, so the color intensity were decreased, while the absorbances were higher than standard. It showed that other components of serum might have light absorbance at 517 nm. To solve this issue we used control blank (H) which was made for each serum sample separately. Each sample absorbance was subtracted by its own control blank-H and the result was put in formula.

$$Abs.T_b = Abs.T_a - B_H$$

$$\%Inhibition = (Abs.Std - Abs.T_b)/(Abs.std) * 100$$

3.8. Histopathological test

Livers were freshly separated from animals after dissection, blood washed out by normal saline, feature of the livers were examined and placed in 10% formalin solution for fixation purpose at least for 24 hours, grossing was performed to make the organ smaller pieces. Feature of grossing area was observed and again placed into 10% formalin at least for 24 hours more. At least after 48 hours of fixation tissue processing was performed. Common method of tissue processing was used.

Tissue processing:

- **Dehydration**

50% Ethanol – 1 Hour,

70% Ethanol – 1 Hour,

90% Ethanol – 1 Hour,

95% Ethanol, two changes, 1 hour each,

Abs. Ethanol, 3 changes, 1 hour each,

- **Clearing**

Xylene, two changes, 1 hour each.

- **Infiltration and**

Paraffin wax, two changes, 1 hour each.

- **Embedding**

Embedding was performed by paraffin wax.

Section cutting and staining process

Sections were cut by microtome in 8-9 micrometer diameter. Sections were attached to glass slide by the help of tissue floatation bath and egg albumin. Slides were deparaffinized by slide warmer and xylene. Sections were rehydrated through a graded ethanol series, stained

with Haematoxylin-Eosin (HE) and dehydrated through a graded alcohol, cleared in xylene and mounted with DPX for histological assessment under the light microscope (Bankroft, 6th edition (2008). Theory and practice of histological techniques.).

3.9. Statistical analysis

Methods used for analysis of data were unpaired student's t-test (one tail), Coefficient of variance (CV) used to compare whether how much GTE supplementation affected the variation of data among the groups. Results presented as means±SD. MS-Excel software program used for all statistical analysis. $P \leq 0.05$ is considered as significant differences while $P > 0.05$ was consider insignificance.



Chapter- IV

Result Analysis

Page 78-102

Chapter IV

Result Analysis

4. Result

The animals (Albino wistar rats) were acclimatized for about 10 days after arrival and kept in standard environment. After acclimatization period rats were classified into 4 different groups (normal control, normal + GTE, diabetic control, and diabetic + GTE). Diabetes was induced in diabetic and diabetic+GTE groups by IP single injection of 45 mg/kg BW STZ. After 3 days of STZ injection, fasting blood glucose levels were estimated and rats with ≥ 200 mg/dL of FBS considered as diabetic animals. Further, 0.2% aqueous solution of GTE (having 90% catechins) was provided as drinking water for 14 days to “normal + GTE” and “diabetic +GTE” groups. Biochemical parameters were estimated in serum. Results presented in mean \pm SD. To exclude or differentiate the effect of other factors than effect of GTE, we initially compared results of 7th and 14th days within each group, and then to know the effect of GTE we compared results of 7th and 14th days among the different groups. “NC” group was compared with “N+GTE” and “DC” groups, and “DC” group was compared with “D+GTE” group. After 14 days treatment with GT, one animal was dissected under diethyl-ether anesthesia and the liver was removed immediately washed with normal saline, morphological features were observed and placed in 10% formalin for further analysis.

4.1. Morphological analysis

Animals induced diabetes were looking different. Hairs of the body in diabetic patients were more loser and thin. The back bone of diabetic animals were looking like kyphosis during setting position. Eyes of the diabetic animals were looking smaller in compared to normal animals. Color of legs and faces of diabetic animals were looked changed. Motility of diabetic animals was decreased and breathing rate was induced. Polydipsia was considered significantly in diabetic animals. Liver showed mild inflammation. While some changes in “D+GTE” animals were not considered in compared to “DC”, such as eyes were looked normal and motility of “D+GTE” was better than “DC”.



Fig 4-1: Diabetic rat (left), diabetic + GTE (right).



Fig 4-2: Normal animal during weighing

4.2. Biochemical Parameters

4.2.1. Body weight

Each animal was weighted in 0th day (day of administration of STZ), 7th day (after treating with GTE), and 14th day (after treating with GTE). Body weights were compared within each group (between zero, 7th and 14th days) to exclude or differentiate the effect of other factors than effect of GTE (table 4-1).

Table 4-1: Body weight on zero, 7th and 14th days

Groups	0 day (mean±SD)	7 th day (mean±SD)	14 th day (mean±SD)	Difference of 14 days	Remarks (changes between zero and 14 days)
Normal	236 ± 10	243±17.5	249± 11.1	P< 0.05	significant increase
Normal+GTE	247 ± 18	245±22	243±19	P> 0.05	insignificant decrease
Diabetic control	236 ± 25	210±24	183±25	P< 0.05	Significant decrease
Diabetic+GTE	230 ± 15	208±30	186±36	P< 0.05	Significant decrease

P-value calculated for differences between zero day and 14th day.

“Normal control” group weight was significantly (P< 0.05) increased within 14 days. Body weight was decreased in “Normal + GTE” but insignificantly. In both groups, “diabetic control” and “diabetic + GTE” body weights were significantly decrease (P< 0.05) in 14 days.

For knowing whether green had significant affect on body weight, body weights were compared among the different groups (table 4-2, Figure 4-3).

Table 4-2: Comparison of body weight among the groups on zero, 7th and 14th days.

Days	0 day	P-value	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	236 ± 10/247 ± 18	P> 0.05	243±17/ 246±22	P> 0.05	Insignificant increase	249± 11/ 243±19	P> 0.05	Insignificant decrease
Normal/ Diabetic control	236 ± 10/236 ± 25	P> 0.05	243.33 ±17/ 210±24	P< 0.05	Significant decrease	249.16± 11/ 183±25	P< 0.05	Significant decrease
Diabetic/ Diabetic+GTE	236 ± 25/ 230 ± 15	P> 0.05	210±24/ 208±30	P> 0.05	Insignificant decrease	183±25/ 186± 36	P> 0.05	Insignificant increase

No significant differences have been observed on zero day among the compared groups, while on 7th and 14th days some significant changes observed. Body weight was increased in “NC” as compared to “N+GTE” group on both 7th and 14th days but insignificantly. Body

weight in “DC” was significantly ($P < 0.05$) decreased as compared to “NC” group on both 7th and 14th days. Body weight of “D+GTE” had no significant ($P > 0.05$) differences with “DC” group, while significantly decreased as compared to “NC” group.

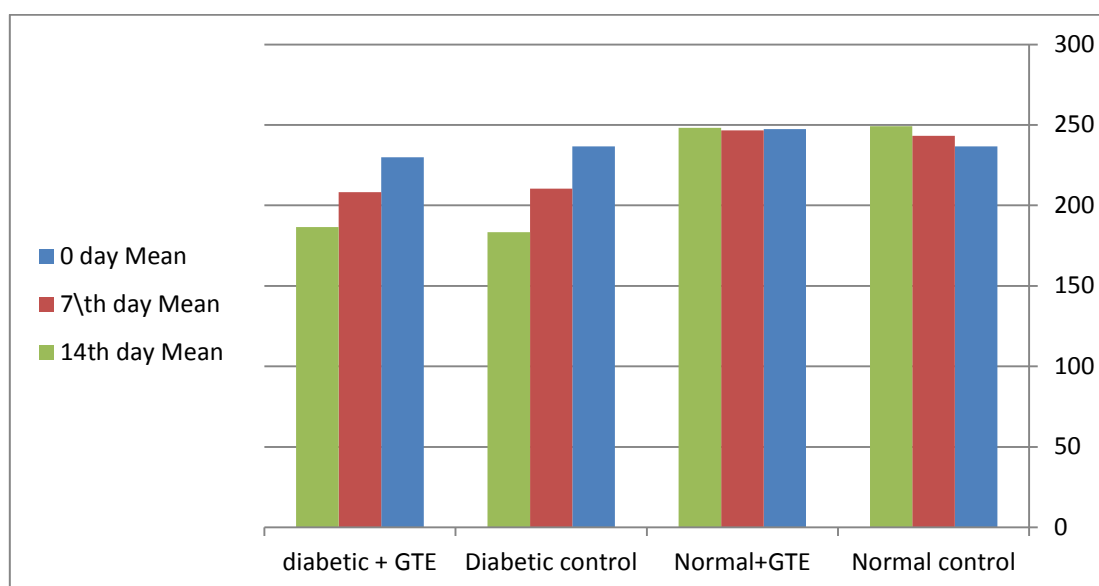


Fig 4-3: Body weight in different experimental groups on zero, 7th and 14th days.

4.2.2. Fasting Blood Glucose

FBS was estimated after an overnight fasting condition. Glucometer and GOD-POD kit (*Erba Mannheim Ltd*) were used. Glucose level above the 200 mg/dL was considered as diabetes. FBG level of 7th and 14th days were compared in each group (table 4-3), as well as compared among the groups to reveal whether the changes are significant or not (Table 4-4, Figure 4-4). Results showed in mean \pm SD.

Table 4-3: Fasting blood glucose level on 7th and 14th days

Groups	7 th day (mean \pm SD)	14 th day (mean \pm SD)	P-value	Remarks
Normal	78.61 \pm 18.87	79.78 \pm 16.5	P> 0.05	insignificant increase
Normal + GTE	84.16 \pm 13.3	77.65 \pm 14.06	P> 0.05	insignificant decrease
Diabetic control	151.07 \pm 15.10	154 \pm 12.08	P> 0.05	insignificant increase
Diabetic + GTE	140.33 \pm 5.5	122 \pm 5.79	P< 0.05	significant decrease

FBG levels were estimated in each group on 7th and 14th days. There were no significant differences ($P > 0.05$) in FBG level of normal animals between 7th day and 14th day of experiment. Similarly there were no significant differences ($P > 0.05$) in FBG levels of "N+GTE" group and "DC" on 7th and 14th days of experiment, while significant differences ($P < 0.05$, decrease) were in FBG level of "D+ GTE" between 7th and 14th days of experiment.

FBS levels were compared among the groups in 7th and 14th days to observe the effect of GTE on FBG in diabetic and normal subjects (table 4-4, Figure 4-4).

Table 4-4: Comparison of fasting blood glucose levels among the experimental groups after 7th day and 14th day of GTE treatment

Groups	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal + GTE	78.61±18.87/ 84.16 ± 13.3	P> 0.05	Insignificant increase	79.78 ± 16.5/ 77.65 ± 14.06	P> 0.05	Insignificant decrease
Normal/ Diabetic control	78.61±18.87/ 151.07± 15.10	P< 0.05	Significant Increase	79.78± 16.5/ 154 ± 12.08	P< 0.05	Significant increase
Diabetic/ Diabetic+ GTE	151.07± 15.10/ 140.33 ± 5.5	P> 0.05	Insignificant decrease	154± 12.08/ 122 ± 5.79	P< 0.05	significant decrease

On 7th and 14th days, there were no significant differences (P> 0.05) in FBG between normal and normal + GTE groups, while there were significant differences (P< 0.05) between normal group and diabetic control group on 7th day as well as 14th day. There were no significant differences (P> 0.05) in FBG levels between “DC” group and “D+GTE” in 7th day, while FBG was significantly reduced (P< 0.05) in “D+GTE” as compared to “DC” group after 14 days.

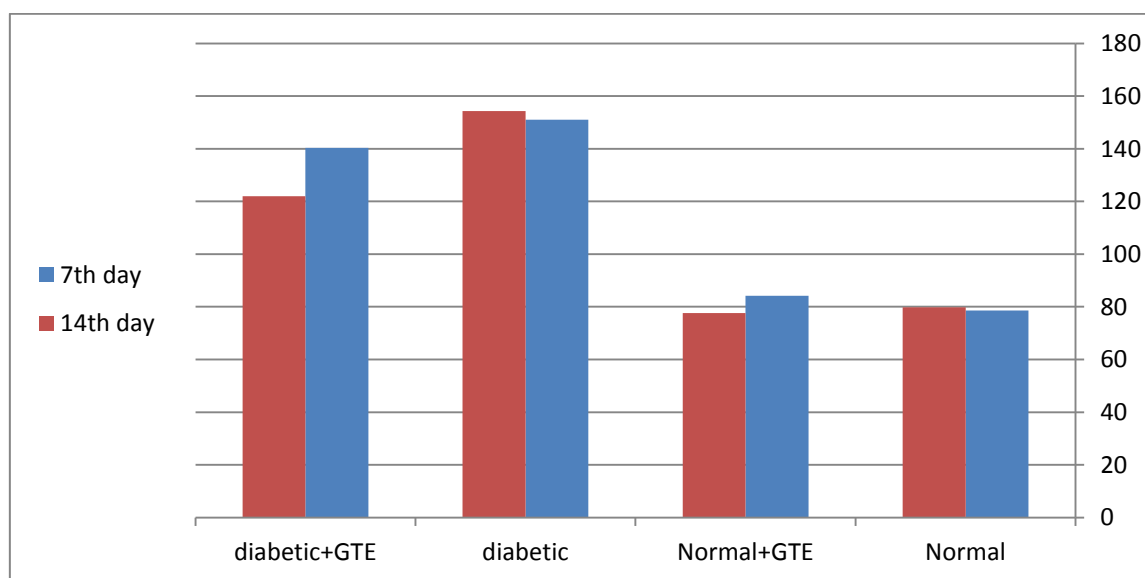


Figure 4-4: FBG level in different groups on 7th and 14th days of experiment.

4.2.3. Serum Total Protein

Serum total protein (STP) was estimated on 7th and 14th days after diabetes induced. It was estimated by biuret kit method (*Erba Mannheim Ltd.*). STP level was estimated within each

groups (table 4-5) and between the groups (table 4-6) to be sure the differences is due to diabetes or GTE. Data showed in table 4-5 and 4-6, figure 4-5.

Table 4-5: Serum total protein levels of each group on 7th and 14th days

Groups	7 th day (mean ± SD)	14 th day (mean ± SD)	P-value	Remarks
Normal	7.84 ± 0.09	6.52 ± 0.6	P< 0.05	Significant differences
Normal + GTE	7.32 ± 0.46	6.45 ± 0.43	P< 0.05	Significant differences
Diabetic control	7.45 ± 0.57	6.71 ± 0.48	P< 0.05	Significant differences
Diabetic + GTE	7.2 ± 0.7	6.25 ± 0.2	P< 0.05	Significant differences

STP showed significant decrease (P< 0.05) between 7th and 14th days of experiment in each group. Other factors such as environmental and diet may be involved. To evaluate the effect of diabetes and GTE, results compared among the groups (table 4-6, figure 4-5).

Table 4-6: Comparison of serum total protein level among the groups on 7th and 14th days

Groups	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal + GTE	7.84 ± 0.09/ 7.32 ± 0.46	P> 0.05	No significant differences	6.52 ± 0.6/ 6.45 ± 0.43	P> 0.05	Insignificant decrease
Normal/ Diabetic control	7.84 ± 0.09/ 7.45 ± 0.57	P> 0.05	Insignificant decrease	6.52 ± 0.6/ 6.6±0.48	P> 0.05	No significant differences
Diabetic/ Diabetic+GTE	7.45 ± 0.57/ 7.2 ± 0.7	P> 0.05	Insignificant decrease	6.6±0.48/ 6.25 ± 0.2	P> 0.05	Insignificant differences

As data shows there was no significant differences (P> 0.05) between “NC” and “N+GTE” groups, and between “NC” and “DC” group both after 7 and 14 days of experiment. Similarly we did not observed statistically significant differences between “DC” and “D+GTE” groups while STP level was decreased slightly in “D+GTE” as compared to “DC” group after 7 and 14 days.

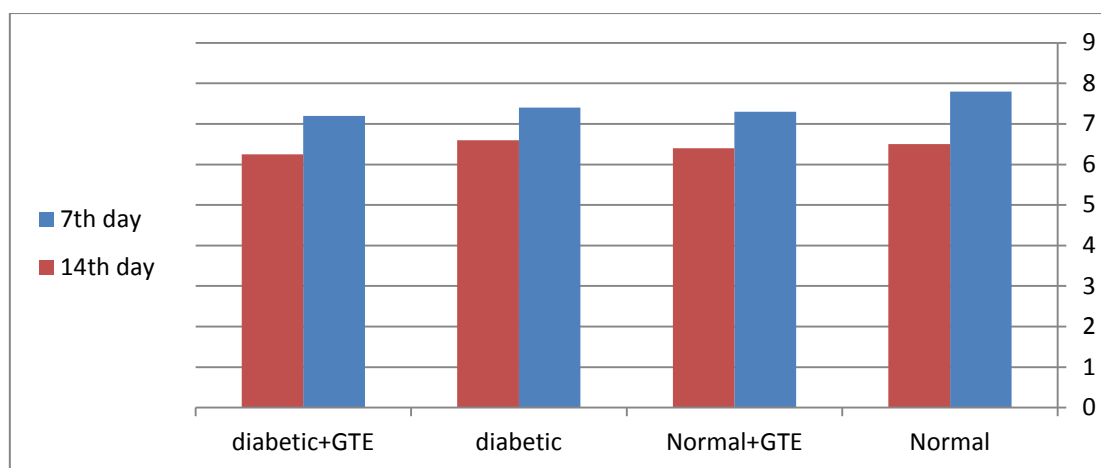


Figure 4-5: Serum total protein level on 7th and 14th days.

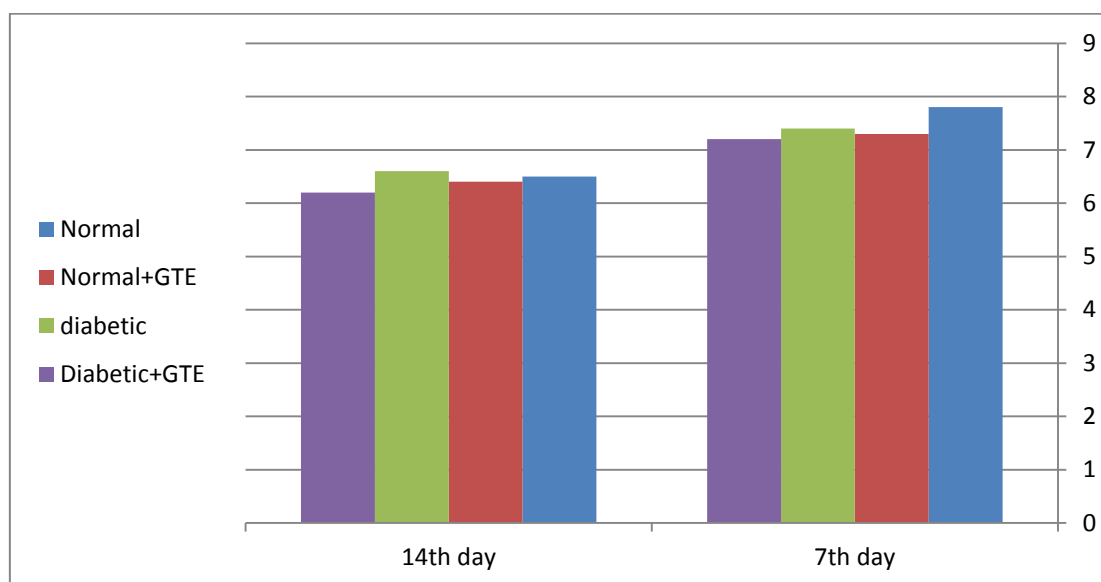


Fig 4-6: Serum total protein levels on 7th and 14th days of experiment

4.2.4. Serum Albumin and Albumin:globulin ratio

As serum albumin is synthesized in liver, we measure its amount in serum to evaluate liver status in diabetic, and diabetic treated animal in compare to the normal groups. Serum albumin was estimated by BCG (bromocresol green) method, (*Beacon diagnostic PVT. LTD. Assay kits*). To exclude non-volunteer factors initially we compared result of 7th and 14th days within each group (table 4-7), and to examine the effect of diabetes and GTE treatment on serum albumin level, results were compared among the groups after 7th and 14th days of GTE treatment (table 4-8, figure 4-7). To know effect of GTE on serum albumin level, “N+GTE” group compared to “NC” group and to consider effect of diabetes on serum albumin, “DC” group was compared with “NC” and finally for understanding the effect of GTE on serum albumin of diabetic subjects, “D+GTE” group was compared with “DC” group. Results showed in mean±SD. Statistical tools were applied.

Table 4-7: Comparison of serum albumin levels of each group between 7th and 14th days

Groups	7 th day (mean ± SD)	14 th day (mean ± SD)	P-value	Remarks
Normal	5.11±0.45	4.38±0.18	P< 0.05	Significant decrease
Normal + GTE	4.74±0.22	4.24±0.15	P< 0.05	Significant decrease
Diabetic control	4.85±0.49	3.5±0.2	P< 0.05	Significant decrease
Diabetic+ GTE	4.8±0.29	3.31±0.17	P< 0.05	Significant decrease

Serum albumin levels were significantly decreased (P< 0.05) in each group between 7th and 14th days of experiment. It is similar to serum total protein level which decreased in all groups. The reason behind that may be environmental factors, dietary factor, or renal disease. For demonstration of diabetes and GTE we have compared the result among the groups after 7 and 14 days (table 4-8, figure 4-7).

Table 4-8: Comparison of serum albumin levels among experimental groups after 7th and 14th days

Groups	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal + GTE	5.11±0.45/ 4.74±0.22	P> 0.05	No significant differences	4.38±0.18/ 4.24±0.15	P> 0.05	No significant differences
Normal/ Diabetic control	5.11±0.45/ 4.85±0.49	P> 0.05	No significant differences	4.38±0.18/ 3.5±0.2	P< 0.05	Significant differences
Diabetic/ Diabetic+GTE	4.85±0.49/ 4.8±0.29	P> 0.05	No significant differences	3.5±0.2/ 3.31±0.17	P> 0.05	No significant differences

Serum albumin had no significant differences (P> 0.05) observed between “NC” and “N+GTE” groups on both 7th and 14th days. “DC” group showed significant decrease (P< 0.05) in serum albumin level on 7th and 14th days as compared to the “NC” group. While there were no significant differences between “DC” and “D+GTE” groups on 7th and 14th days.

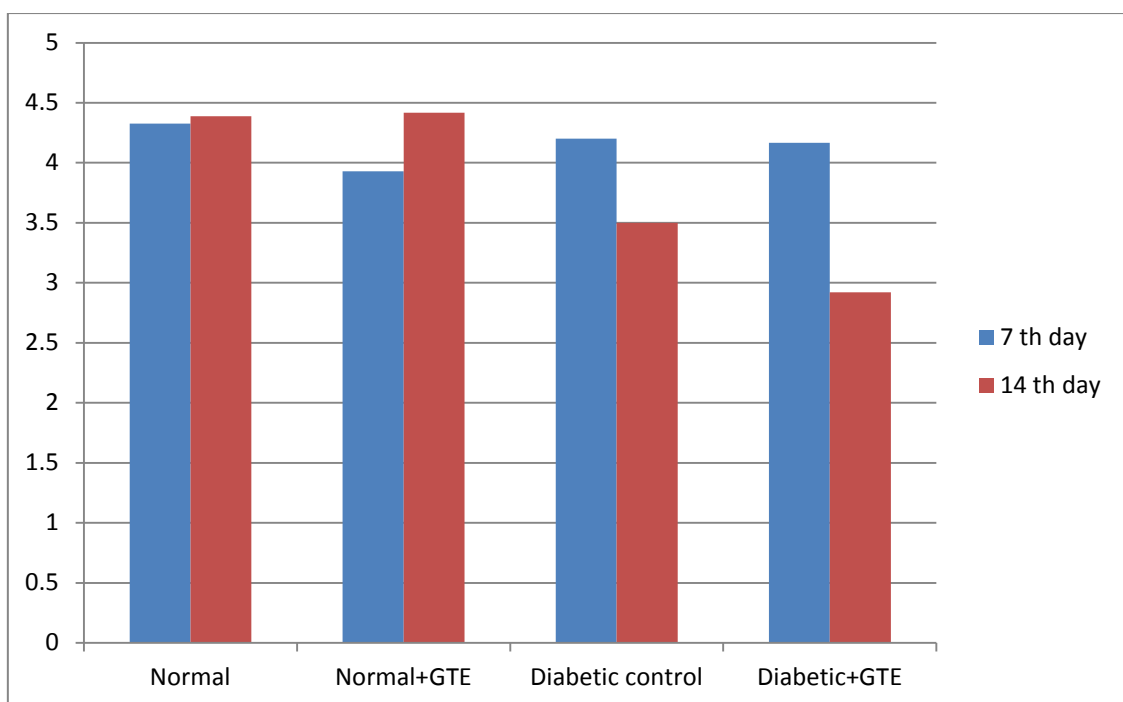


Fig 4-7: Serum albumin levels in different groups on 7th and 14th days

Normal serum albumin:globulin ratio differs from 2.5:1 to 1.2:1 (Harold Varley, 4th Edition, 2002). Albumin-globulin ratio is compared in table 4-9.

Table 4-9: Albumin:globulin ratio of 7th and 14th days

Groups	7 th day	14 th day	P-value	Remarks
Normal control	5.1: 2.7	4.3: 2.1	P< 0.05	No significant differences
Normal + GTE	4.7: 2.6	4.2: 2.2	P< 0.05	No significant differences
Diabetic control	4.8: 2.6	3.5: 3.2	P> 0.05	Significant decrease
Diabetic+GTE	4.8: 2.4	3.3: 2.9	P> 0.05	Significant decrease

Serum A:G ratio was not significantly differed between 7th and 14th days in “NC” and “N+GTE” groups, while the serum A:G ratio significantly decreased in “DC” and “D+GTE” after 7 and 14 days of GT treatment. The effect of diabetes and GTE on A:G ratio was examined by comparing different groups among each other (table 4-10).

Table 4-10: Comparison of serum A:G ratio among groups on 7th and 14th days

Groups	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	5.7:2.7/ 4.7:2.6	P> 0.05	No significant differences	4.3:2.1/ 4.2:2.2	P> 0.05	No significant differences
Normal/ Diabetic control	5.7:2.7/ 4.8: 2.6	P< 0.05	Significant decrease	4.3:2.1/ 3.5: 3.2	P< 0.05	Significant decrease
Diabetic/ Diabetic+GTE	4.8:2.6/ 4.8: 2.4	P> 0.05	No significant differences	3.5:3.2/ 3.3: 2.9	P> 0.05	No significant differences

A:G ratio was significantly decrease in “DC” as compared to “NC” on both 7 and 14th days, while no significant changes observed between “DC” and “D+GTE” after on 7th and 14th days of GT treatment. No significant changes considered between “NC” and “N+GTE” groups.

4.2.5. Serum Triglyceride

Serum TG was estimated on 7th and 14th days of experiment through GPO/POD method, (*Beacon diagnostic PVT.LTD.* assay kits). To exclude or differentiate the effect of other factors than effect of GTE, we initially compared results of 7th and 14th days within each group (table 4-11), and then to know the effect of GTE and diabetes we compared results of 7th and 14th days among the different groups (table4-12).

Table 4-11: Comparison of Serum triglyceride levels of each group between 7th and 14th days

Groups	7 th day (mean ± SD)	14 th day (mean ± SD)	P-value	Remarks
Normal	49 ± 4	49.3 ± 6	P> 0.05	Insignificant increase
Normal + GTE	44 ± 5	40.1 ± 5	P> 0.05	Insignificant decrease
Diabetic control	60.3 ± 5.9	78.8 ± 14	P< 0.05	Significant increase
Diabetic+ GTE	75.1 ± 13.3	55.6 ± 24.4	P< 0.05	Significant decrease

There were not significant differences (P> 0.05) in normal control group between 7th and 14th days, similarly in normal + GTE group also, while there were significant increase (P< 0.05) in serum TG level of diabetic control group between 7th and 14th days. While significant decrease observed in serum TG of “D+GTE” group between 7th and 14th days. Values compared among the groups to evaluate the effect of GTE and diabetes on serum TG level (Table 4-11).

Table 3-12: Comparison of serum triglyceride levels among the groups on 7th and 14th days

Days	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	49 ± 4/ 44 ± 5	P> 0.05	Insignificant decreases	49.3 ± 6/ 43.66 ± 4.92	P> 0.05	Insignificant decrease
Normal/ Diabetic control	49 ± 4/ 60.3 ± 5.9	P< 0.05	Significant differences	49.3 ± 6/ 78.8 ± 14	P< 0.05	Significant increase
Diabetic/ Diabetic+GTE	60.3 ± 5.9/ 75.1 ± 13.3	P> 0.05	No significant differences	78.8 ± 14/ 55.6 ± 24.4	P< 0.05	Significant decrease

Differences were significant (P < 0.05) between “NC” group and “DC” in both days (7th and 14th). Serum TG level in “DC” group was increased significantly on 14th day as compared to “NC”, while had not significant differences with diabetic+GTE group on 7th day, while serum TG level in “D+GTE” group was significantly decreased on 14th day as compared to “DC” group.

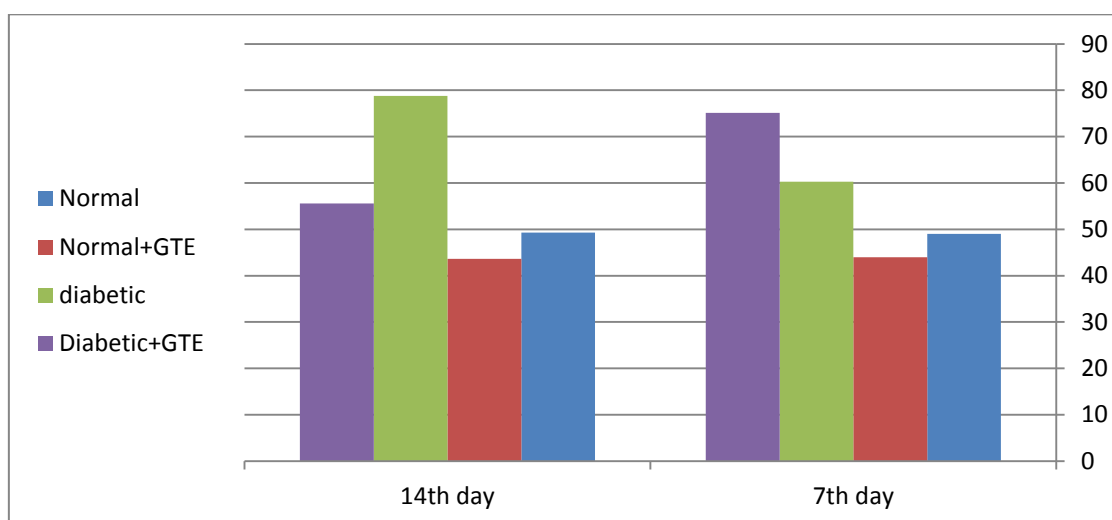


Fig 4-8: Serum triglyceride levels in different groups on 7th and 14th day

4.2.6. Serum total cholesterol

Serum TC was estimated on 7th and 14th days of experiment through CHOD/POD method, (*Beacon diagnostic PVT.LTD.* assay kits). To exclude or differentiate the effect of other factors than effect of GTE and diabetes, we initially compared results of 7th and 14th days within each group (table 4-13, and then to know the effect of GTE and diabetes we compared results of 7th and 14th days among the different groups (4-14, figure 4-9).

Table 4-13: Comparison of serum total cholesterol levels of each group between 7th and 14th days

Groups	7 th day (mean±SD)	14 th day (mean±SD)	P-value	Remarks
Normal	86.60±3.55	86.76±5.87	P> 0.05	Insignificant increase
Normal + GTE	82.9±6.20	70.58±5.26	P< 0.05	Significant decrease
Diabetic control	94.13±13.99	96.5±7.66	P> 0.05	Insignificant increase
Diabetic+ GTE	83.16±7.37	85±4.84	P> 0.05	Insignificant increases

Only significant (P< 0.05) changes (decrease) in total cholesterol level observed in normal + GTE compared with normal control group on 14th day of GT treatment, while no significant differences observed in 7th day. T.Chol level was insignificantly increased in “DC” and “D+GTE” groups on 14th day as compared to 7th day. CV for “D+GTE” was 5.6% while for “DC” it was 7.45% on 14th day. Data were compared among the groups on 7th and 14th days of GTE treatment to clarify the effect of diabetes and GTE supplementation on serum T.Chol levels (table 4-14, figure 4-9).

Table 4-14: Comparison of serum total cholesterol levels among experimental groups on 7th and 14th days

Days	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	86.60±3.55/ 82.9±6.20	P> 0.05	No significant differences	86.76±5.87/ 70.58±5.26	P< 0.05	Significant decrease
Normal/ Diabetic control	86.60±3.55/ 94.13±13.99	P> 0.05	No significant differences	86.76±5.87/ 96.5±7.66	P< 0.05	Significant increase
Diabetic/ Diabetic+GTE	94.13±13.99/ 83.16±7.37	P> 0.05	No significant differences	96.5±7.66/ 85±4.84	P< 0.05	Significant decrease

Significant decrease (P< 0.05) seen in total cholesterol level of “N+GTE” group as compared to “NC” group, while cholesterol level increased in “DC” group compared to “NC” group (P< 0.05) on 14th day. Significantly (P< 0.05) decrease observed in serum T.Chol levels of “D+GTE” group in comparison to diabetic control group on 14th day, while same results were not observed in on 7th day of experiment. No significant (P> 0.05) changes have been seen in “N+GTE” group compared with normal control group on 7th day. Similarly no significant differences have been seen between “NC” group and diabetic control on 7th day.

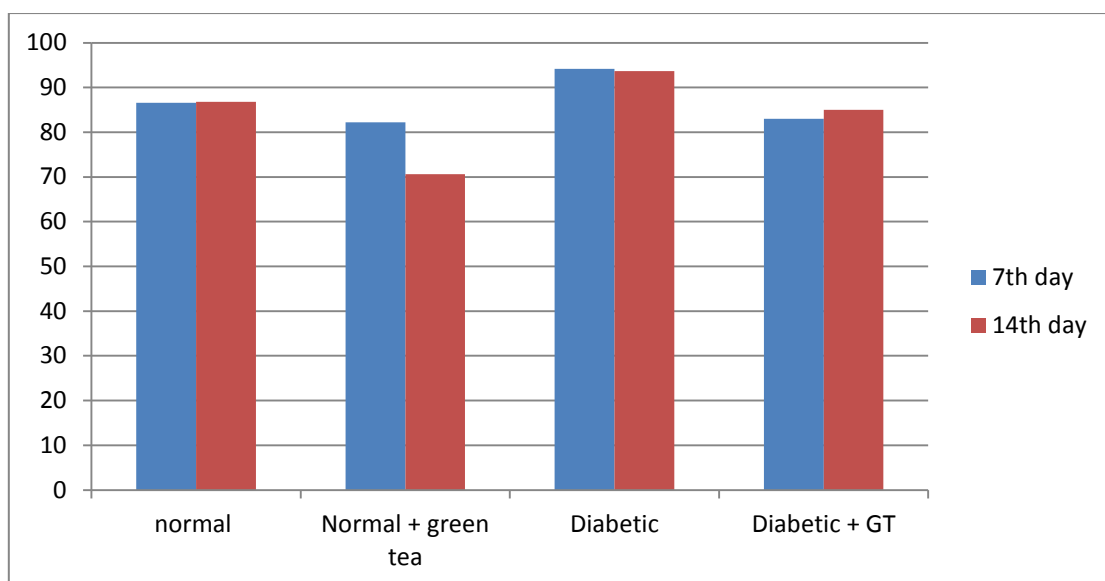


Fig 4-9: Serum total cholesterol levels in different groups on 7th and 14th days.

4.2.7. Serum bilirubin

Serum bilirubin was estimated on 7th and 14th days after diabetes induced. It was estimated by Diazo kit method of Pearlman and Lee (*Erba Mannheim Ltd.* Diagnostic assay kits). To exclude or differentiate the effect of other factors than effect of GTE and diabetes, we initially compared results of 7th and 14th days within each group (table 4-15, and then to know the effect of GTE and diabetes on serum bilirubin we compared results of 7th and 14th days among the different groups (4-16, figure 4-10).

Table 4-15: Comparison of serum total bilirubin levels of each group between 7th and 14th days

Groups	7 th day (mean±SD)	14 th day (mean±SD)	P-value	Remarks
Normal	0.76±0.19	0.81±0.28	P> 0.05	Insignificant increase
Normal + GTE	0.88±0.22	0.85±0.22	P> 0.05	Insignificant decrease
Diabetic control	1,07±0.26	0.99±0.27	P> 0.05	Insignificant decrease
Diabetic+ GTE	1±0.18	0.99±0.23	P> 0.05	Insignificant decrease

No group showed significant difference between 7th and 14th days of experiment. However, slight changes were present.

Table 4-16: Comparison of serum total bilirubin levels among experimental groups on 7th and 14th days

Days	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	0.76±0.19/ 0.88±0.22	P> 0.05	Insignificant Increase	0.81±0.28/ 0.85±0.22	P> 0.05	Insignificant difference
Normal/ Diabetic control	0.76±0.19/ 1.07±0.26	P< 0.05	Significant increase	0.81±0.28/ 0.99±0.27	P< 0.05	Significant increase
Diabetic/ Diabetic+GTE	1.07±0.26/ 1±0.18	P> 0.05	Insignificant decrease	0.99±0.27/ 0.99±0.23	P> 0.05	No significant differences

No significant differences in STB levels observed between “NC” and “N+GTE” groups on 7th and 14th days, while STB levels were significantly increased (P< 0.05) in diabetic control groups compared with normal control on 7th and 14th days. No significant differences considered between “DC” and “D+ GTE” groups on 7th and 14th days.

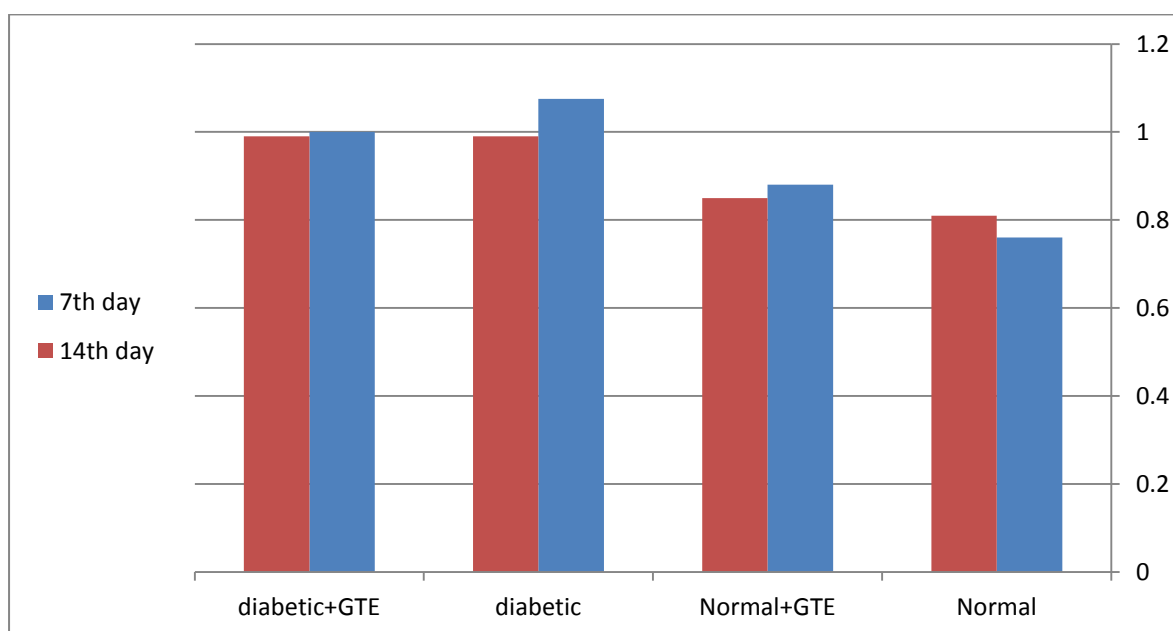


Fig 4-10: Serum total bilirubin levels in different groups on 7th and 14th day

As bilirubin conjugation is occurred in liver, so serum D.Bil level has strong connection with liver status. In our study D.Bil also showed similar differences to T.Bil. differences between 7th and 14th days of each group is shown in table 3-16.

Table 4-17: Comparison of serum direct bilirubin levels of each group between 7th and 14th days

Groups	7 th day (mean±SD)	14 th day (mean±SD)	P-value	Remarks
Normal	0.13±0.05	0.11±0.04	P> 0.05	No significant differences
Normal + GTE	0.13±0.04	0.11±0.04	P> 0.05	No significant differences
Diabetic control	0.15±0.05	0.18±0.07	P> 0.05	Insignificant increase
Diabetic + GTE	0.16±0.1	0.15±0.05	P> 0.05	Insignificant decrease

There were no significant differences ($P < 0.05$) between same group on 7th and 14th days, while slightly increase occurred in “DC” group and slightly decrease observed in diabetic + GTE group but not significantly. For observing the effect of diabetes and GTE on D.Bil level, values were compared among the groups (table 4-18, figure 4-11).

Table 4-18: Comparison of serum direct bilirubin levels among experimental groups after 7th and 14th days

Days	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	0.13±0.05/ 0.13±0.04	P> 0.05	No significant differences	0.11±0.04/ 0.11±0.04	P> 0.05	No significant differences
Normal/ Diabetic control	0.13±0.05/ 0.15±0.05	P> 0.05	No significant differences	0.11±0.04/ 0.18±0.07	P> 0.05	No significant differences
Diabetic/ Diabetic+GTE	0.15±0.05/ 0.16±0.1	P> 0.05	No significant differences	0.18±0.07/ 0.15±0.05	P> 0.05	No significant differences

No significant differences ($P > 0.05$) were observed among groups on 7th and 14th days of experiment in serum direct bilirubin levels, while serum direct bilirubin level was increased in diabetic and diabetic groups as compared to “NC” group, but not significantly ($P > 0.05$).

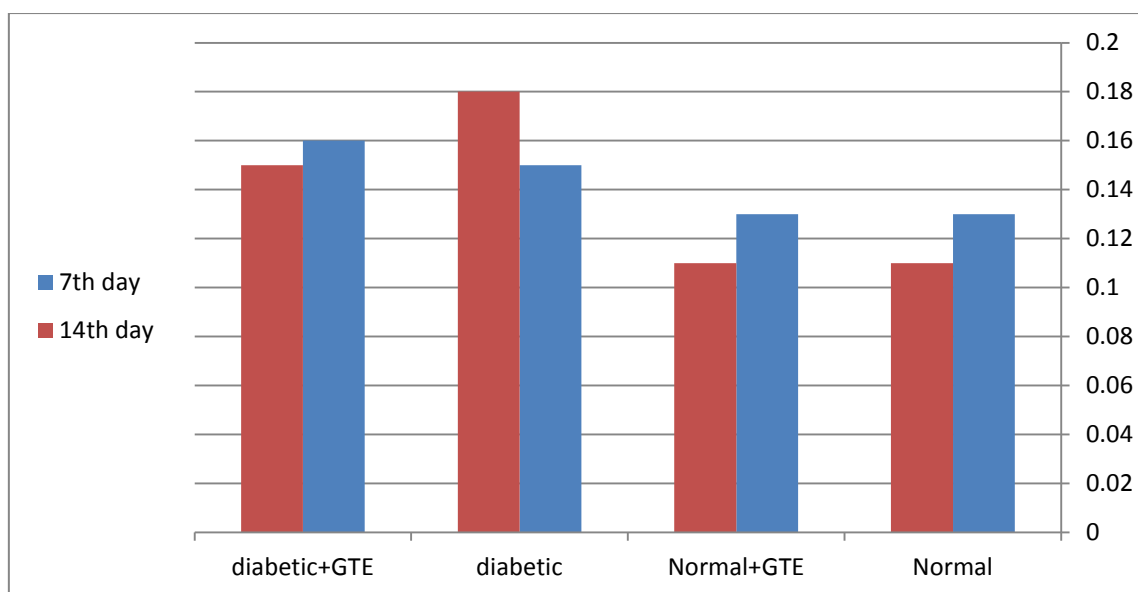


Fig 4-11: Serum direct bilirubin levels in different groups after 7th and 14th days of experiment

Indirect bilirubin was obtained from total bilirubin – direct bilirubin = indirect bilirubin.

Indirect bilirubin was only increased in diabetic groups in compare to normal control group on both 7th day and 14th day of experiment.

4.2.8. Serum Glutamate-Pyruvate Transaminase

SGPT enzyme was estimated after 7 and 14 days of experiment. It was estimated by IFCC kit method, kinetic (*Erba Mannheim Ltd.* Assay kit) by semi-autoanalyzer machine. To exclude or differentiate the effect of other factors than effect of GTE and diabetes, we initially compared results of 7th and 14th days within each group (table 4-19), and then to know the effect of GTE and diabetes on SGPT we compared results of 7th and 14th days among the different groups (4-20, figure 4-12).

Table 3-19: Comparison of SGPT levels of each group between 7th and 14th days

Groups	7 th day (mean±SD)	14 th day (mean±SD)	P-value	Remarks
Normal	22.66±3.55	22.83±2.78	P> 0.05	No significant differences
Normal + GTE	22.5±4.88	23±4.56	P> 0.05	No significant differences
Diabetic control	63.26±15.71	71.98±8.33	P< 0.05	Significant increase
Diabetic+ GTE	62.36±17.62	68.55±18	P> 0.05	Insignificant increase

Diabetic control group revealed significant ($P < 0.05$) increase in SGPT levels on 14th day as compared to 7th day, while this increase was not significant between 7th day and 14th day in diabetic + GTE group. 0.2% aqueous solution of GTE did not affect on SGPT level in healthy rats also. Data were compared among the groups on 7th and 14th days of GTE treatment to clarify the effect of diabetes and GTE supplementation on serum SGPT levels (table 4-20, figure 4-12).

Table 4-20: Comparison of SGPT levels among experimental groups on 7th and 14th days

Groups	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	22.66±3.55/ 22.5±4.88	P> 0.05	No significant differences	22.83±2.78/ 23±4.56	P> 0.05	No significant differences
Normal/ Diabetic control	22.66±3.55/ 63.26±15.71	P< 0.05	Significant increase	22.83±2.78/ 71.98±8.33	P< 0.05	Significant increase
Diabetic/ Diabetic+GTE	63.26±15.71/ 62.36±17.62	P> 0.05	No significant differences	71.98±8.33/ 68.55±18	P> 0.05	Insignificant decrease

SGPT level was increased significantly ($P < 0.05$) in diabetic control and “D+GTE” groups as compared to normal control group in both 7th and 14th days of experiment. Data showed that SGPT levels were decreased in diabetic + GTE on both 7th and 14th days but not significantly. No significant changes observed between “D+GTE” and “NC”.

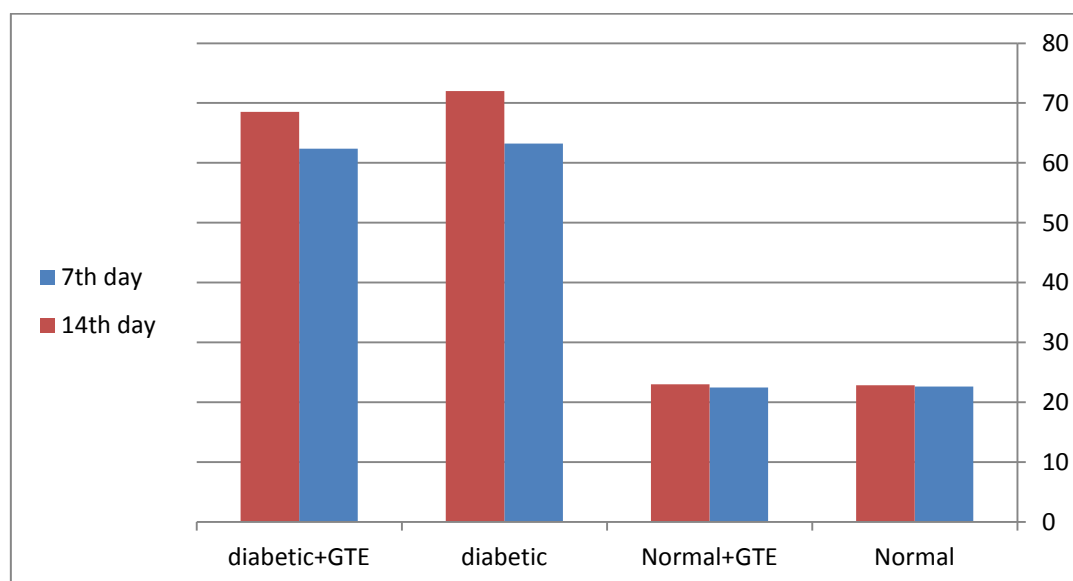


Fig 4-12: Serum GPT levels in different groups on 7th and 14th day of experiment

4.2.9. Serum Glutamate-Oxaloacetate Transaminase

Serum SGOT was estimated on 7th and 14th days of green tea treatment. It was estimated by IFCC kit method (*Erba Mannheim Ltd.* Diagnostic assay kits). For understanding whether there any other factor effecting affecting SGOT levels and to differentiate them, initially data of 7th and 14th days within each group were compared (table 4-21), and then to know the effect of GTE and diabetes on SGOT we compared results of 7th and 14th days among the different groups (4-22, figure 4-13).

Table 4-21: Comparison of SGOT levels of each group between 7th and 14th days

Groups	7 th day (mean±SD)	14 th day (mean±SD)	P-value	Remarks
Normal	29.729.7±7.83	28.26±5.21	P> 0.05	No significant differences
Normal + GTE	28.63±6.69	28±.695	P> 0.05	No significant differences
Diabetic control	106.66±24.68	94.1±10.21	P> 0.05	No significant differences
Diabetic + GTE	101.83±13.24	99.83±17.44	P> 0.05	No significant differences

No significant changes observed in subject groups between 7th and 14th days of experiment. To evaluate the effect of GTE on healthy animals, “N+GTE” group was compared with “NC” on 7th and 14th days, and for understanding the effect of diabetes on SGOT level, “DC” group was compared to “NC” group on both 7th and 14th days, and to evaluate the effect of GTE on SGOT level, “D+GTE” group was compared to “DC” group on 7th and 14th days of green tea treatment (table 4-22, figure 4-13).

Table 4-22: Comparison of SGOT levels among experimental groups on day 7th and 14th

Days	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	29.729.7±7.83/ 28.63±6.69	P> 0.05	No significant differences	28.26±5.21/ 28±.695	P> 0.05	No significant differences
Normal/ Diabetic control	29.729.7±7.83/ 106.66±24.68	P< 0.05	Significant increase	28.26±5.21/ 94.1±10.21	P< 0.05	Significant increase
Diabetic/ Diabetic+GTE	106.66±24.68/ 101.83±13.24	P> 0.05	No significant differences	94.1±10.21/ 99.83±17.44	P> 0.05	No significant differences

Level of SGOT increased in diabetic group significantly ($P < 0.05$) as compare to normal group on both 7th and 14th days, while there is no significant differences between “D+GTE” group and diabetic control group on both days. GTE did not affect SGOT level at this specific dose in 14 days.

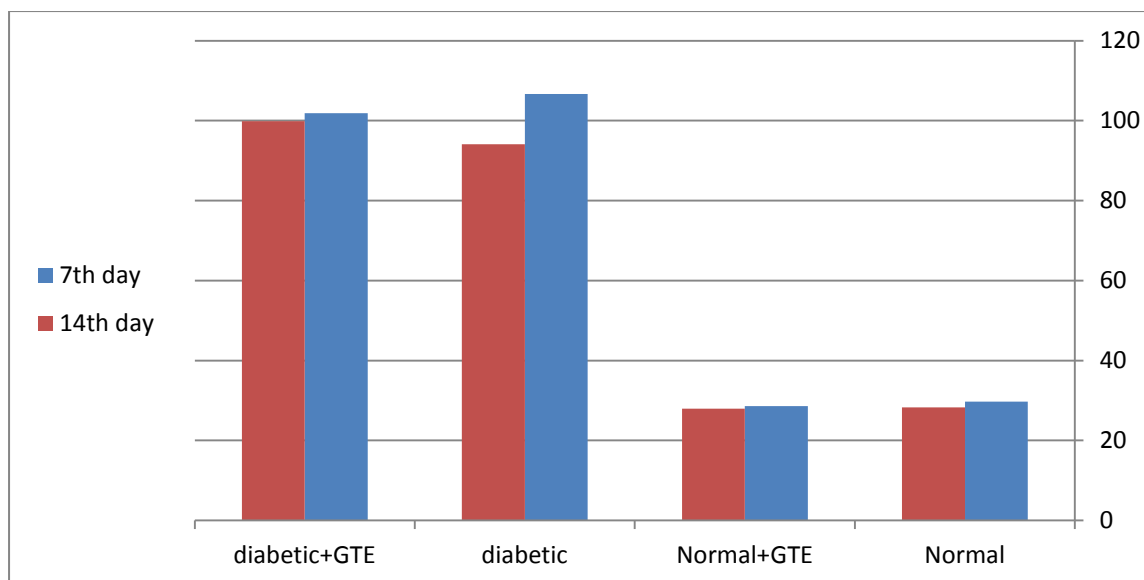


Fig 4-13: SGOT levels in different groups on 7th and 14th days of experiment.

4.2.10. Alkaline phosphatase

Serum alkaline phosphatase was estimated on 7th and 14nd days of GTE treatment by IFCC kit method, kinetic (*Erba Mannheim Ltd.* Diagnostic assay kits). To exclude or differentiate the effect of other factors than effect of GTE and diabetes, we initially compared results of 7th and 14th days within each group (table 4-23), and then to know the effect of GTE and diabetes on ALP, we compared results of 7th and 14th days among the different groups (table 4-24, figure 4-14).

Table 4-23: Comparison of ALP levels of each group between 7th and 14th days

Groups	7 th day (mean±SD)	14 th day (mean±SD)	P-value	Remarks
Normal	74.6±13.9	72±16.1	P> 0.05	No significant differences
Normal + GTE	76.8±13.7	74.3±13.7	P> 0.05	No significant differences
Diabetic control	175.26±31.4	170.86±45.3	P> 0.05	No significant differences
Diabetic + GTE	144.15±22.3	146.96±35.8	P> 0.05	No significant differences

No significant ($P > 0.05$) changes seen in each group between 7th and 14th days of experiment. It shows that there was no any other factor affecting ALP level during our experiment.

Data of both days (7th and 14th) were compared among the groups to understand the effect of diabetes and GTE on serum ALP level (table 4-24, figure 4-14).

Table 4-24: Comparison of ALP levels among experimental groups on day 7th and 14th

Days	7 th day	P-value	Remarks	14 th day	P-value	Remarks
Normal/ Normal+GTE	74.6±13.9/ 76.8±13.7	P> 0.05	No significant differences	72±16.1/ 74.3±13.7	P> 0.05	No significant differences
Normal/ Diabetic control	74.6±13.9/ 175.26±31.4	P< 0.05	significant differences	72±16.1/ 170.86±45.3	P< 0.05	significant differences
Diabetic/ Diabetic+GTE	175.26±31.4/ 144.15±22.3	P> 0.05	No significant differences	170.86±45.3/ 146.96±35.8	P> 0.05	No significant differences

Serum ALP level was increased in “DC” group as compared to “NC” group and according to the statistics the changes were significant at p< 0.05. Although, the level of ALP was lesser in “D+GTE” as compared to the diabetic control group, but it was not significant according to the statistic issues.

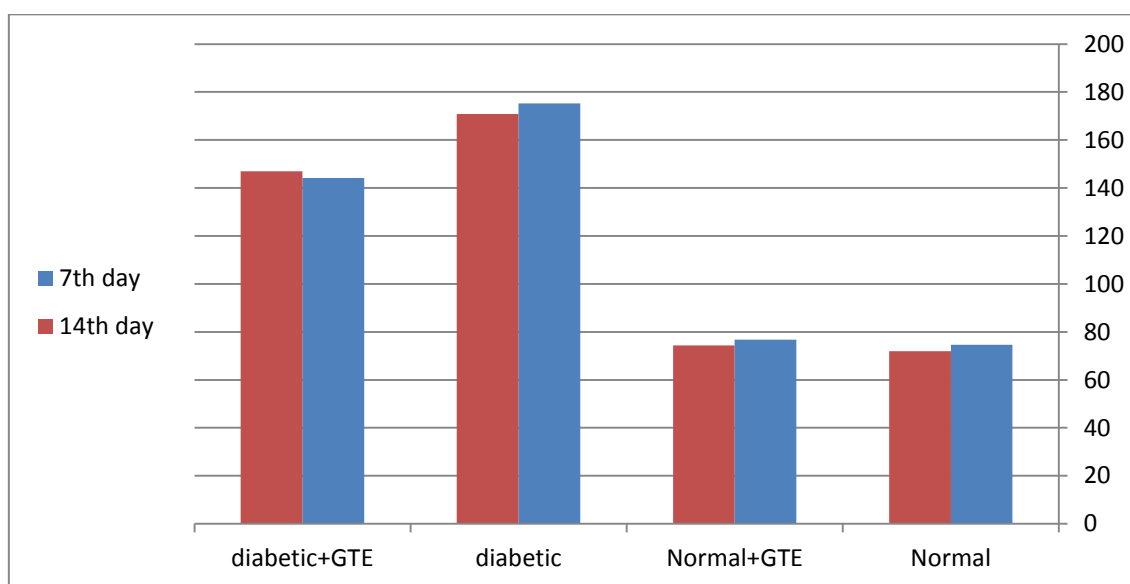


Fig 4-14: ALP levels in different groups on 7th and 14th days of experiment

4.2.11. Serum Total Antioxidant Capacity

DPPH was used as indicator of serum TAC. This test only applied after 14 days of green tea supplementation and the results of inhibition capacity were compared among the groups.

Table 4-25: TAC after 14th days of GTE supplementation

Groups	Inhibition capacity on 14 th day (mean±SD)
Normal	39.2±5.40
Normal + GTE	44.6±3.50
Diabetic control	21.2±3.63
Diabetic + GTE	26.4±4.39

After 14 days of GTE supplementation, DPPH inhibitory capability of all groups was compared that how much they could inhibit the DPPH. More inhibition of DPPH shows greater TAC (Table 4-26, figure 4-15).

Table 4-26: Comparison of TAC among experimental groups after 14 days of GTE treatment

Days	14 th day	P-value	Remarks
Normal/ Normal+GTE	39.2±5.40/ 44.6±3.50	P= 0.05	Significant increase
Normal/ Diabetic control	39.2±5.40/ 21.2±3.63	P< 0.05	Significant decrease
Normal/ diabetic + GTE	39.2±5.40/ 26.4±4.39	P< 0.05	Significant decrease
Diabetic/ Diabetic+GTE	21.2±3.63/ 26.4±4.39	P< 0.05	Significant increase

“N+GTE” group showed significant increase (P< 0.05) in TAC as compare to “NC” group on 14th day of experiment, while “diabetic control” group revealed significant decrease in TAC as compared to “NC” group. TAC of “D+GTE” group was significantly lesser than “NC” group but significantly higher than “DC” group.

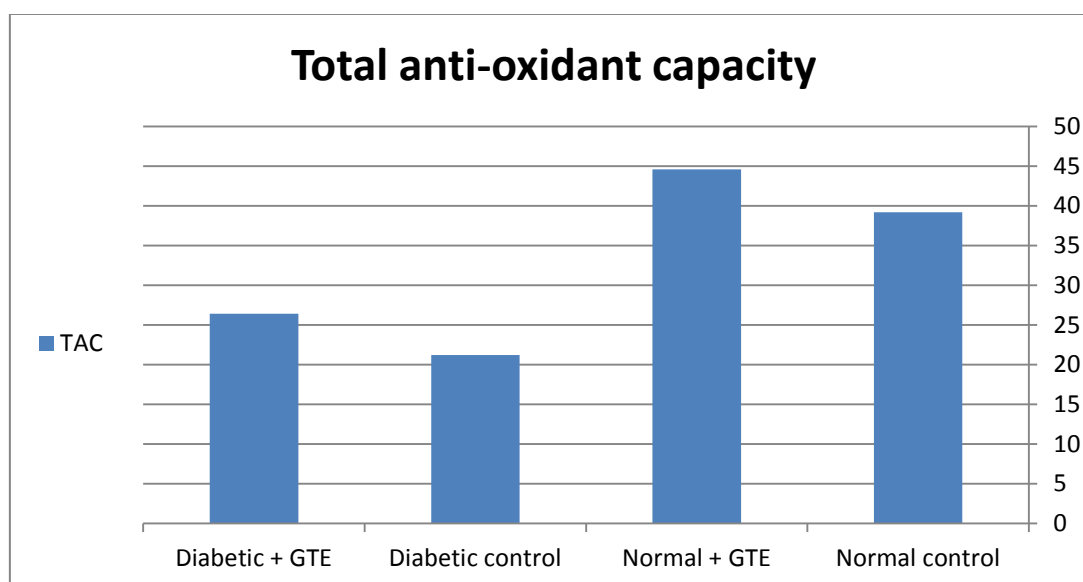


Fig 4-15: Total anti-oxidant capacity of experimental groups on 14th day

Table 4-27: Data of all estimated parameters on 7th and 14th days of experiment

Parameter	No. of animal per group	Normal			Normal + GTE			Diabetic control			Diabetic + GTE		
		7 th day	14 th day	P-value	7 th day	14 th day	P-value	7 th day	14 th day	P-value	7 th day	14 th day	P-value
Body weight	6	236±10	249±11.1	P<0.05	247±18	243±19	P>0.05	236±25	183±25	P<0.05	230±15	186±36	P<0.05
FBS	6	78.61±18.87	79.78 ± 16.5	P>0.05	84.16 ±13.3	77.65±14.06	P>0.05	151.07±15.10	154 ± 12.08	P>0.05	140.33 ± 5.5	122 ± 5.79	P<0.05
Serum Total protein	6	7.84 ± 0.09	6.52 ± 0.6	P<0.05	7.32 ± 0.46	6.45 ± 0.43	P<0.05	7.45 ± 0.57	6.71 ± 0.48	P<0.05	7.15 ± 0.91	5.6 ± 0.7	P<0.05
Serum albumin	6	5.1±0.45	4.34±0.18	P<0.05	4.7±0.22	4.2±0.15	P<0.05	4.8±0.49	3.5±0.2	P<0.05	4.8±0.29	3.31±0.17	P<0.05
Serum TG	6	49 ± 4	49.3±6	P>0.05	44±5	40.1±5	P>0.05	60.3±5.9	78.8±14	P<0.05	75.1±13.3	55.6±24.4	P<0.05
Serum Cholesterol	6	86.60±3.55	86.76 ±5.87	P>0.05	82.9±6.20	70.58±5.26	P<0.05	94.13 ±13.99	96.5±7.66	P>0.05	83.16±7.37	85±4.84	P>0.05
Serum total bilirubin	6	0.76±0.19	0.81±0.28	P>0.05	0.88±0.22	0.85±0.22	P>0.05	1.07±0.26	0.99±0.27	P>0.05	1±0.18	0.	P>0.05

Serum direct bilirubin	6	0.13±0.05	0.11±0.04	P>0.05	0.13±0.04	0.11±0.04	P>0.05	0.15±0.05	0.18±0.07	P>0.05	0.16±0.1	0.15±0.05	P>0.05
SGPT	6	22.66±3.55	22.83±2.78	P>0.05	22.5±4.88	23±4.56	P>0.05	63.26±15.71	71.98±8.33	P<0.05	62.36±17.62	68.55±18	P>0.05
SGOT	6	29.729.7±7.83	28.26±5.21	P>0.05	28.63±6.69	28±.695	P>0.05	106.66±24.68	94.1±10.21	P>0.05	101.83±13.24	99.83±17.44	P>0.05
ALP	6	74.6±13.9	72±16.1	P>0.05	76.8±13.7	74.3±13.7	P>0.05	175.26±31.4	170.86±45.3	P>0.05	144.15±22.3	146.96±35.8	P>0.05
TAC	6		39.2±5.4			44.6±3.5			21.2±3.6			26.4±4.39	

In above table only data of body weight under the title of 7th day belongs to first day of experiment (day of STZ injection).

4.1.3. Histopathological Analysis

After 14 days treatment with GT, one animal was dissected under diethyl-ether anesthesia from each group. Liver was immediately removed, washed by normal saline and the features were noticed. Tissue from “NC” group should normal liver histo-architecture. Number of cells were in proportional to the whole tissue, no considerable fatty vacuoles were present (figure 4-16). Liver tissue from “N+GTE” also showed normal tissue histo-architecture (figure 4-17). Histological appearance from “DC” showed macrovesicular fatty changes. Number of hepatocytes looks lesser in proportional to whole tissue as compared to “NC” (figure 4-18). Macrovesicular fatty changes are lesser in “D+GTE” group in compare to the “DC” group, while it has more fatty changes as compared to “normal control” group (figure 4-19).

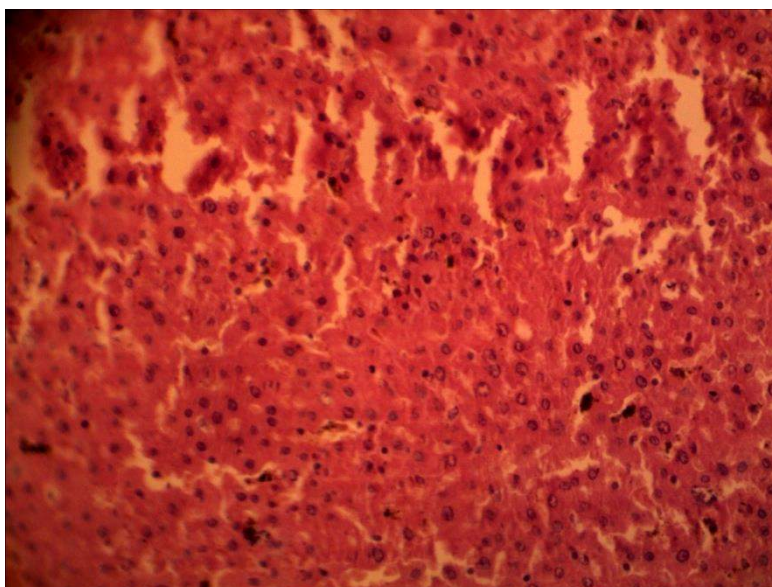


Fig 4-16: Microscopic appearance of liver tissue belongs to the “normal control” group (H&E stain, 40x).

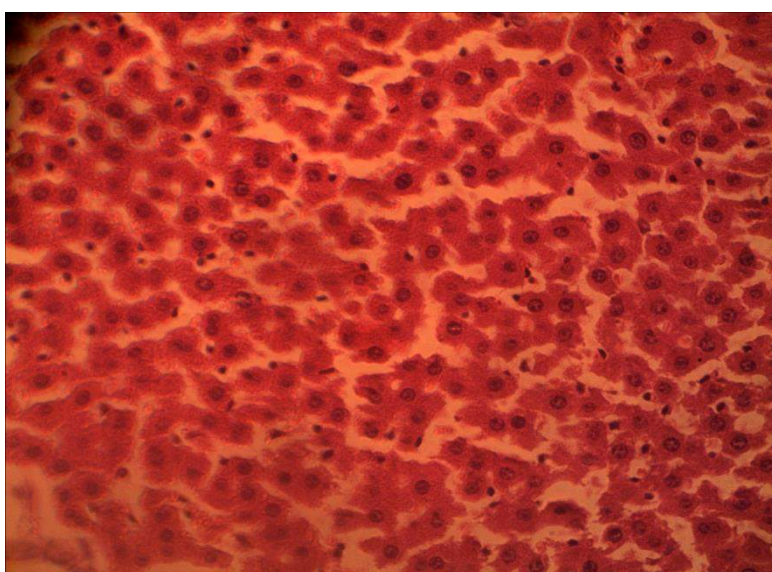


Fig 4-17: Microscopic appearance of liver tissue belongs to the “normal+GTE” group (H&E stain, 100x).

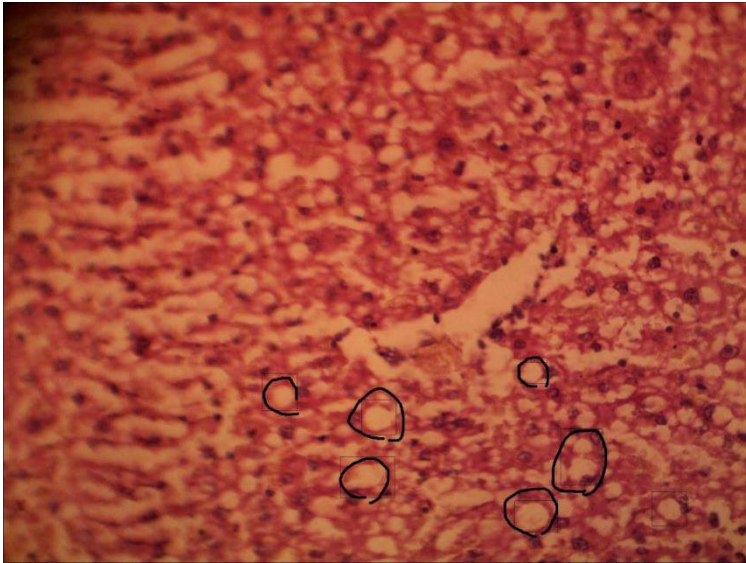


Fig 4-18: Microscopic appearance of liver tissue belongs to the “diabetic control” group showing fatty vacuolation of liver and mild inflammation (H&E stain, 40x).



Fig 4-19: Microscopic appearance of liver tissue belongs to the “diabetic+GTE” group showing mild fatty vacuolation (H&E stain, 40X).

Chapter V
Discussion, Summary and
Conclusion

Page 104- 118

Chapter-V

Discussion and Conclusion

5.1. Discussion

5.1.1. Effect of GTE on body weight

In this study, body weight in “NC” group was significantly ($P < 0.05$) increased in 14 days, while in “N+GTE” group instead of increase; it was decreased but not significantly ($P > 0.05$). This finding shows that GTE has anti-obesity effect. GTE prohibited the weight induction. This finding is in similarity with other reports. Wang *et al.*, (2010) had study on moderately overweight Chinese population and found that GTE had dose depending body weight lowering properties. While, Yanaga *et al.*, (2002) reported that EGCG (1%) treatment for 8 weeks did not significantly changed the body weight of experimental rats. We have found significant decrease in body weight of diabetic control group. Similar findings also reported by other papers (Sabu *et al.*, 2002). Gamal *et al.*, (2009) reported that orally treatment of 50 & 100 mg/kg BW given to rats for 28 consecutive days significantly modulated both the severe decrease and increase in body weight induced by alloxan and high cholesterol diet. Similar finding also reported by Sabu *et al.*, (2002). In our study we have not observed significant differences between “DC” and “D+GTE” groups which suggest that orally treating diabetic rats with 0.2% aqueous solution of GTE (having 90% catechins) as a drinking water for 14 days did not significantly change the body weight in diabetic subjects. Martha *et al.*, (2012) reported that as blood glucose levels were increased in “DC” rats, BW reduced as compared to “NC” rats during 8 weeks of experiment, and “D+GTE” rats have lowered blood glucose and reduced body weight lost as compared to the “DC” group.

5.1.2. Effect of GTE on serum glucose level

In present study “normal control” group was used as normal range provider to compare the influence of GTE on diabetic and non diabetic animals, and to exclude the effects of other factors than diabetes and GTE on the parameters. Daily fresh prepared 0.2% aqueous solution of GTE (having 90% catechins) was administrated instead of drinking water to “normal+GTE” and “diabetic+GTE” groups. No significant differences have been seen in FBG levels of “N+GTE” group compared to NC after 7 and 14 days, while diabetic group which were treated with GTE showed significant ($P < 0.05$) decrease in FBG after 14 days but not after 7 days as compared to the DC. Anti-diabetic effects of GT and GT-components have discussed by several researchers. Yoko Kobayashi, *et al.*, (2000) suggested that tea polyphenols especially EGCG and ECG inhibit

the sodium-dependent glucose transporter -1 (SGLT-1) in brush-border membrane vesicles of rabbit small intestine. GTE also have shown inhibitory effect on α -amylase (Yukihiko & Miwa, 1990; Matsumoto, *et al.*, 1993), sucrase and α -glucosidase (Matsumoto, *et al.*, 1993; Miwa & Yukihiko, 2014) in intestine. Marilyn (2002) found that GT increased the activity of insulin around 15-folds *in vitro* in an epididymal fat cell assay. Green tea extract decreased the level glucose after 6 h without changing the level of serum insulin (Hiroshi *et al.*, 2004). Md. Shahidul Islam and Haymie (2007) reported that lower dose of GT (0.5%) is insulinotropic while higher dose (2.0 %) is hyperglycemic. Ali Akbar *et al.*, (2012) have reported that GTE (1.5%) significantly reduced the blood glucose level in healthy normal rats and also caused significant hypoglycemic effect in diabetic rats. However, our finding showed no any significant changes in FBG level of “NC” group on 14th days which is supported by Fatemeh Haidari *et al.*, (2013) who suggested no changes in level of serum glucose in normal non-diabetic control group after administration of 100 and 200 mg/kg BW of GTE for 4 weeks. Several other mechanisms also suggested for anti-diabetic effect of GT such as decreasing food intake (Kao *et al.*, 2000; Hitoshi *et al.*, 2004), preventing destruction of RINm5F (an insulinoma cell line, Song *et al.*, 2003), decreasing adipose tissue weight without any change in other tissues, modulation of glucose uptake in skeletal muscles and adipose tissues (GT significantly decreased the glucose uptake along with decrease in translation of GLUT-4 in adipose tissue, while it oppositely increased glucose uptake by skeletal muscle cells through increasing GLUT-4 translation, Hitoshi *et al.*, 2004), reductive effect on the gene expression of rate-limiting gluconeogenic enzymes (Oyama, *et al.*, 2004). Yun *et al.*, (2006) reported that EGCG acts as pro-oxidant, not anti-oxidant, to pancreatic β -cells, and Takabayashi *et al.*, (2004) suggest that higher intake of GT may cause oxidative damage of pancreatic DNA. However, our finding suggests that GTE at lower doses it can act as anti-oxidant. Our finding also can be confirmed by Md. Shahidul Islam and Haymie, (2007) that lower intake of GT revealed insulin increasing as well as hypoglycemic activity while higher dietary intake had reverse effect in diabetic rats. Similarly Atef and Talal (2010) reported hyperglycemic effect of GT. A 5 years follow up study in Japan on non-diabetic population showed that consumption of GT is inversely related with the incidence of diabetic in normal individuals but not in diabetic people (Iso *et al.*, 2006). Similarly kim *et al.*, (2003) reported that pretreatment with epicatechin can protect islets cells of pancreas from STD-induced oxidative damage but does not have beneficial effect of developed diabetes. These findings are in contradictory to our finding in some points. We have found that GTE at lower doses at least for 14 days was beneficial for glucose level. As conclusion we suggest that consumption of lower doses of GT is beneficial while higher doses may be detrimental rather than beneficial for diabetic patients. In our study coefficient of variance for “DC” and “D+ GTE” were 7.83% and 4.74% respectively. This result shows that GTE had better control on variation of blood glucose among subjects.

5.1.3. Effect of GTE on serum total protein and albumin

Most of the serum and plasma proteins especially albumin are synthesized in liver. Low level of total protein in plasma provide information about status of nutrition or about some sever organ disease (protein losing state, liver disease). Raised level indicates presence of some paraprotien and patient should by further investigated. Albumin levels are more useful as indicators of nutritional status. This investigation is also important in a chronic liver disease and nephritic syndrome. In septicemia and general inflammatory disorders, hypoalbuminemia can occur due to leakage of albumin into interstitial compartment. Albumin along with total protein level gives indication of the globulin level (Saini and Kaur, 2001). In our study serum total protein level was within 7.20 and 7.84 in all experimental groups after 7 days while it was decreased to 6.21-6.71 after 14 days of experiment. Similarly serum albumin was within 4.74 and 5.11 in all groups after 7th day of experiment while it deceased to 3.31-4.38 on 14th day. These changes might be happened due to environmental factors and diet or kidney problems. In our study we did not consider any significant changes between all groups in results of serum total protein after 7 and 14 days. The only changes ($P < 0.05$) was occurred in level of albumin between “NC” and “DC” groups (decreased level in DC) after 14 days, while there was no significant changes between “DC” and “D+ GTE”. Similarly Hiroshi *et al.*, (2004) have reported treating GT brings no significant changes in serum total protein level. However, the fractions were significantly different between diabetic mice and wild-type mice. The peak intensities of proteins at m/z (mass/charge ratios) 4203, 4119, 4206, 4579, 18691, 9311 and 4211 were more than 3 times lower, while those at m/z 17406, 13075/ 17418, 17407, 17622, 26100 and 18431 were more than 3 times higher than the peak intensities of sera proteins of +m/+m mice. Gamal *et al.*, (2009) also reported there was no significant change in the serum total protein between alloxan-induced diabetic rats and diabetic rats treated with 50 and 100mg/kg BW GTE for 28 consecutive days. similarly they have reported no changes in serum albumin level between diabetic and diabetic + GTE groups, but there was significant differences between changes in serum total protein and albumin levels between normal control, and diabetic control and diabetic + GTE. In our observation decrease level of albumin in “DC” while no changes in total protein levels as compared to “NC” may shows STZ and hyperglycemia caused liver toxicity with inflammation inside the body.

5.1.4. Effect of GTE on serum TG and serum total cholesterol

In this study we have found that consumption of 0.2% aqueous solution of GTE—having 90% catechins, for 14 days did not affect serum TG level in non-diabetic group (normal+GTE group), while serum TG level was increased in diabetic group significantly. Dyslipidemia is one of the common characteristics of diabetes mellitus (Tina and Henry, 2006). Deng Zeyuan, *et al.*, (1998) reported that GT and BT and their aqueous extracts significantly decreased the blood glucose and blood serum TG. Thomposon and Yoon (1984); Hara and Honda (1990) suggested that polyphennols bind to proteins so decrease the activity of digestive enzymes and lead to reduced digestibility and/or absorption of lipids and glucose. Atef and Talal, (2010) reported significant increase in the level of FBG, TG, Chol, urea, creatinine, uric acid, SGPT and SGOT in STZ-induced diabetic mice fed with normal diet after 15 and 30 days, while insignificant alteration

have been observed in TG, chol, total protein, urea, creatinine, uric acid, SGOT and SGPT in mice treated with GT. Marked elevation can be seen in the level of TG-rich lipoproteins (chylomicrons and VLDL) in poorly controlled T1DM patients. The reason behind that suggested reduction in the activity of lipoprotein lipase (an insulin-regulated enzyme) in muscle and adipose tissues (Tina and Henry, 2006). In our study serum TG was significantly ($P < 0.05$) decreased in diabetic group which were administrated with 0.2% aqueous solution of GTE (having 90% catechins) instead of drinking water for 14 days. However, there were no significant differences between “DC” and “D+GTE” groups after 7 days of treating with GTE. Similarly, according to Anandh Babu *et al.*, (2006) report administration of GTE to diabetic rats significantly reduced TG, T.Chol, FFA and LDL-chol levels, and induced HDL-chol level in serum of diabetic rats. Usama El-Sayed Mostafa (2013) showed significant decrease occurs in TG and cholesterol levels after treating with GTE having higher amount of catechins. Our findings are supported by many other studies (Kao *et al.*, 2000; Hitoshi *et al.*, 2004; Ikeda I., 2008; Atef and Talal, 2010). In our study there was significant decrease ($P < 0.05$) in serum level of cholesterol between 7th and 14th days in “N+GTE” group, as well as there was significant ($P < 0.05$) decrease in cholesterol level of “N+GTE” groups as compared to “NC” group after 14 days. However, similar differences were not observed after 7 days between “N+GTE” and “NC” groups. Hitoshi *et al.*, (2004) similarly reported significant decrease in cholesterol level in normal animals treated with GT instead of drinking water for 3 weeks. In our study blood cholesterol level was increased but not significantly ($P > 0.05$) in “diabetic control” group. It was in contradictory to turan karaca *et al.*, (2010) findings. They have found reduction in cholesterol level in STZ-induced diabetic animal models after 6 weeks. While similar findings to our study reported by Atef and Talal, (2010) significant increase in blood cholesterol level in STZ-induced diabetic animals. TG-rich lipoproteins (chylomicrons and VLDL) are induced in patients with T1DM. Increased exchange of cholesteryl esters between HDL and LDL for TG in chylmicrons and VLDL may decrease HDL levels and generates LDL, mediated by cholesteryl ester transfer protein (CETP). Significant insulin deficiency may lead induction of LDL because LDL receptor expression is regulated partially by insulin (Tina and Henry, 2006). GTE significantly reduced serum cholesterol level in diabetic rats as compared to the “DC” group after 14 days in our study. These findings are in favor to Turan karaca *et al.*, (2010); Usama El-Sayed Mostafa, 2013 and others’ reports. Hasegawa *et al.*, (2003) suggested the hypocholesterolic properties of GT is due to inhibition of cholesterol synthesis in liver. In a review presented by Tina and Henry, (2006), showed that differences in serum TG level in diabetic groups in compare to normal groups are more than differences in cholesterol level. These observations are similar to our study.

5.1.5. Effect of GTE on serum bilirubin level

Bilirubin is formed from haemoglobin in the reticulo-endothelial system, enters to circulation and binds to albumin. This bilirubin is not water soluble so cannot excrete through urine or bile. Bilirubin conjugation occurs in liver which produce water soluble product to excrete through bile. Estimation of T.bil and D.Bil is routinely used as biomarker of liver (Harold Varley, 4th

Edition, 2002). In this study we have observed that total bilirubin and direct bilirubin levels were significantly ($P < 0.05$) increased in “DC” group as compared to “NC” group after 7 and 14 days. Increased total bilirubin level is common in diabetes; several studies have reported as such (Gamal *et al.*, 2009; Ali Akbar *et al.*, 2012). Total and direct bilirubin had no significant changes in “NC” and “N+GTE” groups after 7 and 14 days of our study. Similarly Jan Frank *et al.*, (2009) reported that daily consumption of GT does not affect total and direct bilirubin level in normal subjects. Total and direct bilirubin did not show significant differences in “D+ GTE” group compared to “DC” group after 7 and 14 days of our study. Similar finding also reported by Gamal *et al.*, (2009) who revealed GTE has no effect on serum bilirubin level of alloxan-induced diabetic rats. It is in contradictory to Ali Akbar *et al.*, (2012) who reported that serum bilirubin level significantly reduced in diabetic rats supplemented with 1.5% w/v GTE for 8 weeks. Perhaps the reason of these contradictory findings is less duration of our study; the level of bilirubin did not decrease significantly, however slightly decrease was observed between 7th and 14th days of experiment. Megan *et al.*, (2010) studied the beneficial effects of high T.Bil in DM. They showed that people with high level of T.Bil have lesser odds of diabetes prevalence (T1DM & T2DM). Endogenous and anti-inflammatory properties of bilirubin reported in several papers (Hammerman *et al.*, 1998; Arai *et al.*, 2001; Kapitulnik *et al.*, 2004; Pramil Cheriyaatha *et al.*, 2010). Stocker *et al.*, (1987) reported that bilirubin under 2% oxygen condition (physiologically relevant concentration) has more anti-oxidative property than α -tocopherol. It also has been postulated that the anti-oxidant properties of T.Bil may reverse the oxidative damage associated with hyperglycemic state (Ndisang *et al.*, 2009; Pramil Cheriyaatha *et al.*, 2010). Anti-oxidant and cardioprotective effects of bilirubin showed within high normal range (Novotny L, and Vitek L., 2003). A 50% reduction in T.Bil was associated with 47% induction in the odds of being in more severe coronary artery disease (CAD). So, serum bilirubin is an independent and inverse risk factor for CAD (Schwertner *et al.*, 1994). High level of serum bilirubin is toxic for some organs such as brain. The neurotoxic effects of bilirubin may be due to modulation of neuronal protein phosphorylation system (Thor Willy *et al.*, 1996). GTE has hepatoprotective effect also and has the ability to protect hepatocytes against oxidative stress, as 5% GTE reduced the elevated bilirubin in CCl₄-induced hepatic injury (Kyung *et al.*, 2007).

5.1.6. Effect of GTE on SGPT, SGOT and ALP

ALT (SGPT) is distributed in many tissues, with comparatively high concentrations in the liver. It is considered the more liver-specific enzyme of the transferases. Clinical applications of ALT assays are confined mainly to evaluation of hepatic disorders. Higher elevations are found in hepatocellular disorders than in extrahepatic or intrahepatic obstructive disorders. Cardiac tissue contains a small amount of ALT activity, but the serum level usually remains normal in AMI unless subsequently liver damage has occurred. AST (SGOT) is the other commonly estimated liver biomarkers widely distributed in human tissue. The highest concentrations are found in cardiac tissue, liver, and skeletal muscle, with smaller amount found in the kidney, pancreas, and erythrocytes. The clinical use of AST is limited mainly to the evaluation of hepatocellular

disorders and skeletal muscle involvement. Because wide tissue distribution, SGOT levels are not useful in the diagnosis of AMI (Bishop 6th Edit, 2010). In our study we have observed that use of 0.2% aqueous solution of GTE (90% catechins) instead of drinking water for 14 days had no significant effect on liver function and liver enzymes in normal animals. Significant increase ($P < 0.05$) has been seen in levels of SGPT and SGOT in “DC” group as compared to “NC” group after 7 and 14 days, as well as we did find any insignificant changes in levels of SGPT and SGOT in “D+GTE” group as compared to “DC” after 7 and 14 days. SGPT, SGOT and ALP increased level have been seen in diabetic animal models in several studies (Ali Akbar Abolfathi, *et al.*, 2012; Usama El-Sayed Mostafa, 2013) which is similar to our findings. While Ali Akbar Abolfathi, *et al.*, (2012) and Usama El-Sayed Mostafa, (2013) finding is in contradictory to our findings in the case of effect of GTE on SGPT and SGOT level in diabetic models. Treatment with GTE (dose dependent) significantly improves liver biomarkers (Usama El-Sayed Mostafa, 2013). Treatment with 1.5% of GTE for 8 weeks decreased the elevated liver MDA, serum AST, ALT, ALP, and bilirubin in STZ-induced diabetic rats compared to diabetic control group and increased the reduced serum albumin, SOD, GSH, GSH-Px, and CAT contents of liver (Ali Akbar Abolfathi, *et al.*, 2012). We did not consider association between liver enzymes and BMI which is in agreement with Prettu *et al.*, (2000).

Alkaline phosphatase belongs to a group of enzymes that catalyze the hydrolysis of various phospho-monoesters at an alkaline pH. Its activity is present on cell surfaces in most human tissues. The highest concentrations are found in the intestine, liver, bone, spleen, placenta, and kidneys. In the liver the enzyme is located on both sinusoidal and bile canalicular membranes. Elevations of ALP are of most diagnostic significance in the evaluation of hepatobiliary and bone disorders (Bishop, 6th Edit, 2010). In our study ALP levels in serum were not significantly changed among “NC” and “N+GTE” group after 7 and 14 days. This finding suggest that 0.2% aqueous GTE (90%) solution for 14 days was not toxic for liver or any other organs involved in induction of serum ALP levels. As many reports showed, higher and prolong consumption of GTE caused liver toxicity and increased liver biomarkers such as ALT, AST and ALP (Sabu *et al.*, 2002; Gloro *et al.*, 2005; Javaid and Bonkovsky, 2006; Jimenez and Martinez, 2006; Galati *et al.*, 2006; Bjornsson and Olsson, 2007; Gabriela *et al.*, 2009). In other side GTE showed the capability to decrease the level of AST, ALT and ALP in aged experimental animals (Shereen *et al.*, 2013) as well as in diabetic animals (Sabu *et al.*, 2002). In our study serum ALP levels were increased significantly ($P < 0.05$) in “DC” as compared to “NC” group after 7 and 14 days. Some other studies also showed induced level of ALP in diabetic animal models and patients (Stanely *et al.*, 2000; Ali Akbar Abolfathi, *et al.*, 2012) which in agreement to our finding. In our study, although the levels of ALP were decreased in “D+GTE” as compared to “DC” group after 7 and 14 days, but statistically this reduction was not significant. May be the reason behind it, the lesser time, and the concentration of GT we have used in our experiment.

5.1.7. Effect of GTE on TAC

Numerous in vivo and in vitro studies revealed elevation of oxidative stress in DM and it is thought to be the key mechanism of pathogenesis and complication of DM (Baynes JW. 1991; Wolff *et al.*, 1991; Giugliano *et al.*, 1996; Oberley 1998; Hinokio, *et al.*, 1999; Suzuki, *et al.*, 1999; Green *et al.*, 2004 Takeshi and Eiichi, 2007). All the mechanisms of diabetic oxidative damages are stimulated by mitochondrial ROS over formation (Ferdinando and Michael, 2010). In diabetic subjects TAC is decreased, MDA level is increased or both, and the GT and/or catechins are reducing oxidative stress by either increasing total plasma anti-oxidant activity or reducing MDA levels in tissues (Martha *et al.*, 2012).

In our study we have found that GTE significantly ($P= 0.05$) induced the TAC in “N+GTE” as compared to “NC” group. This finding is in similarity with previous works (Gow and Hui, 1995; Deng Zeyuan, *et al.*, 1998; Shengmin sang *et al.*, 2005, Jashue and Lambert, 2010; Sarah and Joshua, 2011; Ali Akbar *et al.*, 2012; Martha *et al.*, 2012). While Mahaboob S. Khan and Gurjot Kour, (2007) did not found any significant changes in the level of SOD, GPx, Cat and GST after treating of normal control group with 1.5% w/v GTE after 15 days. The total anti-oxidant capacity in “DC” group was significantly ($P< 0.05$) decreased as compared to “DC”. This finding is in agreement to other papers (C. Feillet *et al.*, 1999; Ali Akbar *et al.*, 2012; Martha *et al.*, 2012). Our findings showed that GTE significantly ($P< 0.05$) increased the TAC in “D+GTE” as compared to “DC” group which is in agreement with others findings (C. Feillet *et al.*, 1999; Ali Akbar *et al.*, 2012; Martha *et al.*, 2012). Deng *et al.*, 1998 reported that GT and GT water extract significantly increased the activity of superoxide dismutase and decreased the MDA. Fatemeh Haidari *et al.*, (2013) reported dose dependent anti-oxidant capacity of GTE. They have found that 100mg/kg BW and 200 mg/kg BW increased 3% and 14% respectively in the TAC of “N+GT” as compared to “NC” group, while “DC” group revealed significant decrease in TAC as compared to the “NC” group. They have reported 5.9% and 32.4% increase in TAC in “D+GTE” for 100 and 200 mg/kg BW respectively as compared to “DC” group. Their reports approve our findings.

However, these conflicting findings might be due to differences in doses, concentrations of green tea, time of exposure, type of green tea as well as the degree of hyperglycemia, type of animals, and the detection methods employed.

5.1.8. Histopathological analysis

Liver tissue sample from “NC” group did not show any histopathological changes. Cells were regularly arranged and no fatty degeneration was observed. Similarly liver tissue sample from “N+GTE” also did not show any histopathological changes and there was no fatty vacuolation in the tissue. Liver tissue of diabetic control animal showed fatty vacuolation and mild inflammation. This is in similarity with Ali Akbar *et al.*, (2012). In our study we have found that GTE could decrease the level of fatty vacuolation in diabetic subject. Similar finding also reported by Akbar *et al.*, (2012) that diabetic

group treated with 1.5% GTE did not show considerable fatty changes in liver tissue. These changes may be due to curative or preventive effect of GTE. As we had permission of dissection of only one animal in each group so we did not dissect any animal after 7 days GTE treatment that we would be able to guess whether this decrease in fatty degeneration in “D+GTE” group is due to preventing or curative effect of green tea.

5.2. Summary

Hyperglycemia is the word used for elevation of plasma glucose level above the normal range. Diabetes is a chronic metabolic disorder causing disturbances in carbohydrate, fat, and protein metabolism, characterized by hyperglycemia, polydipsia, polyphagia, and polyuria. Total diabetic population is reported 19.4 million and estimated to reach 60 million by the year 2025. DM is classified into two main groups, T1DM (insulin dependent DM) and T2DM (insulin independent DM), however, other types also exist. Both types of DM (type 1 & 2) are associated with induced risk of other comorbid conditions such as generalized atherosclerosis and other cardio-vascular diseases, peripheral vascular disease, hypertension, retinopathy, cerebro-vascular disease, nephropathy, and liver disease.

Green tea is widely consuming beverage after water globally. It possesses more than 4000 bioactive components. One third of its component is polyphenols which contains mainly catechins exhibiting strong antioxidative effects. Flavonoids, the largest class of polyphenols with low molecular weight, may be defined as a class of polyphenols possessing compounds that have 2 benzene rings linked by 3 carbon chain and one oxygen bridge. Flavonols and flavanols are the main classes of flavonoid found in tea.

Liver the largest organ of body which mainly performs the function of production and secretion of bile, to facilitate the digestion and absorption of fats from intestine, carbohydrate, lipid and protein metabolism, processing of drugs and hormones, excretion of bilirubin, synthesis of bile salts, storage, phagocytosis and activation of vitamin D. Different liver tests based on its functions can be done. Simple and routing tests for identification of liver status are T.Bil and D.bil, serum albumin, lipid profile, SGPT, SGOT and ALP.

The phrase of Reactive Oxygen Species (ROS) is used to describe a variety of molecules and free radicals derived from molecular oxygen such as superoxides, hydrogen peroxide and hydroxyl radical. Superoxide anion ($O_2^{\cdot-}$), the result of reduction of oxygen by one electron at a time and the initial product of the electron transport chain (ETC), is a mediator in oxidative chain reactions and the precursor of most ROS. Superoxide anion goes under dismutation, either spontaneously or by superoxide dismutase enzymes, and produces H_2O_2 . This H_2O_2 further may be fully reduced and produce water by the mean of catalase or glutathione peroxidase or partially reduced and produce hydroxyl radical (OH^{\cdot}), one of the strongest oxidants in nature, in presence of reduced transition metals. Superoxide anion may react with radical such as nitric oxide (NO^{\cdot}) and produces a very powerful oxidant (peroxynitrite). In vivo, $O_2^{\cdot-}$ production can be enzymatically or non-enzymatically. NADPH oxidase and CYP450-dependent oxygenases, and the conversion of xanthine dehydrogenase to xanthine oxidase are the enzymatic sources of $O_2^{\cdot-}$ and H_2O_2 formation, hence they also provide the source of OH^{\cdot} . The non-enzymatic production

of $O_2^{\cdot -}$ includes the direct transfer of a single electron to O_2 by a prosthetic groups (e.g., flavins or iron sulfur cluster), or reduced coenzymes, or by reduced xenobiotics (Julio, 2003).

Oxidative stress is the term used to describe various harmful processes due to excessive formation of ROS and/or RNS along with decreased or limited antioxidant defenses. Over production of ROS and RNS lead to more generation of free radicals which indiscriminately target DNA, proteins, polysaccharides and lipids.

Mitochondria is the main and primary source of ATP production, disturbance of its function may cause several disease such as Friedreich ataxia, Parkinson, pathophysiology of aging, diabetes and its complications, and Huntington disease. Studies showed that dysfunction of mitochondria in β -cells of pancreas damages secretion of insulin in response to induced glucose level. It is also has been reported that there is link between impaired mitochondrial oxidative phosphorylation in liver and muscle and diabetes type 2. Experiment on animal models revealed that induction DM altered mitochondrial respiration and disrupted energy production in liver, diaphragm, skeletal muscle and heart. Numerous in vivo and in vitro studies revealed elevation of oxidative stress in DM and it is thought to be the key mechanism of pathogenesis and complication of DM. Different mechanism contribute in development of oxidative stress in diabetes which are included non-enzymatic glycosylation (glycation), auto-oxidative glycosylation, metabolic stress (resulted from changes in energy metabolism), alterations of sorbitol pathway activity, alteration in the level of inflammatory mediators and antioxidant defense system, and ischemic reperfusion injury and hypoxia are caused localized tissue damage. Five major mechanisms are suggested for hyperglycemic-induced damage: 1). Induced flux of glucose and other sugars through the polyol pathway; 2). Induced intracellular formation of advanced glycation end products (AGEs); 3). Induced receptor expression of AGEs and its activating ligands; 4). Protein kinase C (PKC) isoforms activation; and 5). Increased activity of hexosamine pathway. All these mechanism are stimulated by mitochondrial ROS over formation.

Numerous studies showed that GTE have anti-diabetic or anti-hyperglycemic effects. The main mechanisms of anti-diabetic (anti-hyperglycemic) of GTE are suggested as following:

- decreasing the appetite,
- inhibiting α -amylase, sucrase and α -glucosidase in intestine,
- Reducing carbohydrate absorption from intestine by decreasing SGLTs,
- Reducing oxidative stress in liver, pancreas and other cells,
- Prevent β -cells of pancreas destruction and improve their damage,
- By affecting immune system can prevent β -cells from autoimmune type-1 diabetes
- Increasing insulin sensitivity, and insulin binding to adipocytes,
- Inducing IGF-1,
- Increasing glucose uptake by muscle cells by increasing GLUT-4,

- Down-regulating the levels of mRNA for gluconeogenic enzymes (PEPCK & G-6-Pase) in liver, and
- Decreasing adipose mass and resistin and blood lipids.

Some studies reported that anti-diabetic effect of GT is dose dependent. GT at lower doses possess insulin stimulatory and hypoglycemic effect while at higher doses it has insulin inhibitory and hyperglycemic effect.

As GTE shows anti-oxidant properties, it may also show pro-oxidant properties and may cause several side effect including liver toxicities. Mostly suspected hepatic reaction is attributed to the catechins, especially EGCG and its metabolites, and the possible mechanism suggested is generation of ROS. So, dietary supplements of GT with higher level of catechins should be utilized carefully. Rout of administration of green tea affects the bioavailability of catechins, likely it is lower after oral administration. However fasting condition and repeated administration of green tea may increase the bioavailability of catechins even up to the toxic doses. Catechins are capable to induce oxidative stress in the liver. Therefore, it is better to avoid consumption of GTE in fasting condition, as well as concentrated GTE, and also to avoid more repeated consumption of GTE. 4- 5 cups of GT is recommended per day as a safe and useful beverage, more than 9-10 cups can cause liver toxicity.

GT has lots of beneficial effect when taken in appropriate amounts. Bioactive component of GTE increased the activity of glutathione reductase, glutathione peroxidase and the contents of GSH, and reduced significantly in lipid hydroperoxides (LOOH), 4-HNE (4-hydroxynonenal) and MDA (malondialdehyde), in liver. Vit. A concentration also increased by 40%. All above parameters had minor changes in serum. But increase in total antioxidant status and decrease in MDA & 4-HNE strongly marked in blood serum. These anti-oxidant properties of GTE make it able to reduce liver toxicity induced by paracetamol, CCl₄, alloxan, streptozotocin, hyperglycemia, paraquat, glucose oxidase, Tamoxifen (anti-cancer medicine), lipopolysaccharid-1-D-galactosamine, azathioprine, ethanol, rubratoxin B, bromobenzene, iron and other transition metals.

GTE have shown both, inhibitory and stimulatory effect on CYP-450 enzymes. These contradictory effects depend on the dose of GT and type of enzyme. In same dose and same subject some enzymes were stimulated while some enzymes were inhibited by GTE.

Antioxidative properties of GTE are the main reason of widely usage of GT. Polyphenols are the important anti-oxidant component of GT. Polyphenols may exhibit their antioxidative activity by following mechanism (Lotito *et al.*, 2000; Skrzydlewska *et al.*, 2002; Anna, 2014):

- Direct scavenging of ROS lead to interruption of reactions which may lead to lipid peroxidation,

- Inhibition of enzymes involved in promoting ROS formation such as xanthine oxidase, cyclooxygenase I & II, ascorbic acid oxidase, protein kinase C, lipoxygenases, cAMP phosphodiesterase and Na⁺/K⁺ ATPase.
- Indirectly inducing endogenous antioxidant enzymes such as catalase, SOD, glutathione peroxidase and reductases.
- Scavenging of metal radical formation by chelating.
- Directly preventing decrease of endogenous antioxidants such as α -tocopherol & β -carotene.

Catechins also have the ability to indirectly induce the endogenous antioxidants to decrease the oxidative damage.

Catechins at low dose inhibit apoptosis while at higher doses stimulate apoptosis, as well as in cancer cells stimulate apoptosis while in normal cell inhibit apoptosis at proper doses.

Diabetes and liver diseases are highly associated. Patients with diabetes have high prevalence of liver disease and also patients with liver disease possess high risk of diabetes. Liver cirrhosis, fatty degeneration, liver cancer, chronic hepatitis and liver fibrosis are the most frequent liver diseases associated with diabetes. The prevalence of diabetes in cirrhotic patients has been reported almost from 12.3-57%.

Many papers have reported contradictory properties of different doses of GTE, antioxidant and pro-oxidant, hepatoprotective and hepatotoxic, hyperglycemic and hypoglycemic, anti-diabetic and diabetogenic. Hence, we have conducted study to evaluate effect of 0.2% aqueous solution of GTE (90% catechins) on liver function and histology of streptozotocin diabetic wistar rats, used instead of drinking water for 14 days. 24 rats were randomly divided into 4 groups each possesses 6 animals. First group was normal control fed with free access to food and water, second was normal animals administered with 0.2% aqueous solution of GTE (90% catechins) instead of drinking water for 14 days after diabetes induced in other groups. 3rd group was diabetic control induced by 45mg/kg BW single IP injection of STZ after overnight fasting condition and fed same as normal control group. 4th group was diabetic+GTE. FBG \geq 200 mg/dL was considered as diabetes. Biochemical parameters were compared within each group between 7th and 14th days of GTE treatment, and as well as among the groups on 7th and 14th days.

In this study, body weight in "NC" group was significantly (P< 0.05) increased in 14 days, while in "N+GTE" group instead of increase; it was decreased but not significantly (P> 0.05). This finding shows that GTE has anti-obesity effect. We have found significant decrease in body weight of diabetic control group, and we have not observed significant differences between "DC" and "D+GTE" groups.

In present study “normal control” group was used as normal range provider to compare the influence of GTE on diabetic and non diabetic animals, and to exclude the effects of other factors than diabetes and GTE on the parameters. No significant differences have been seen in FBG levels of “N+GTE” group compared to NC after 7 and 14 days, while diabetic group which were treated with GTE showed significant ($P < 0.05$) decrease in FBG after 14 days but not after 7 days as compared to the DC. We have found that GTE at lower doses at least for 14 days was beneficial for glucose level.

In our study serum total protein level was within 7.20 and 7.84 in all experimental groups after 7 days while it was decreased to 6.21-6.71 after 14 days of experiment. Similarly serum albumin was within 4.74 and 5.11 in all groups after 7th day of experiment while it decreased to 3.31-4.38 on 14th day. These changes might be happened due to environmental factors and diet or kidney problems. We did not consider any significant changes between all groups in results of serum total protein after 7th and 14th days. The only changes ($P < 0.05$) was occurred in level of albumin between “NC” and “DC” groups (decreased level in DC) after 14 days, while there was no significant changes between “DC” and “D+ GTE”.

In this study we have found that consumption of 0.2% aqueous solution of GTE—having 90% catechins, for 14 days did not affect serum TG level in non-diabetic group (normal+GTE group), while serum TG level was increased in diabetic group significantly. In our study serum TG was significantly ($P < 0.05$) decreased in diabetic group which were administrated with 0.2% aqueous solution of GTE (having 90% catechins) instead of drinking water for 14 days as compared to the DC group. But, there were no significant differences between “DC” and “D+GTE” groups after 7 days of treating with GTE. In our study there was significant decrease ($P < 0.05$) in serum level of cholesterol between 7th and 14th days in “N+GTE” group, as well as there was significant ($P < 0.05$) decrease in cholesterol level of “N+GTE” groups as compared to “NC” group after 14 days. However, similar differences were not observed after 7 days between “N+GTE” and “NC” groups. GTE significantly reduced serum cholesterol level in diabetic rats as compared to the “DC” group after 14 days in our study.

In this study we have observed that total bilirubin and direct bilirubin levels were significantly ($P < 0.05$) increased in “DC” group as compared to “NC” group after 7 and 14 days. Total and direct bilirubin had no significant changes in “NC” and “N+GTE” groups after 7 and 14 days of our study. Total and direct bilirubin did not show significant differences in “D+ GTE” group compared to “DC” group after 7 and 14 days of our study. Perhaps the reason of these contradictory findings is less duration of our study; the level of bilirubin did not decrease significantly, however slightly decrease was observed between 7th and 14th days of experiment. In our study we have observed that use of 0.2% aqueous solution of GTE (90% catechins) instead of drinking water for 14 days had no significant effect on liver function and liver enzymes in normal animals. Significant increase ($P < 0.05$) has been seen in levels of SGPT and SGOT in “DC” group as compared to “NC” group after 7 and 14 days, as well as we did find any

insignificant changes in levels of SGPT and SGOT in “D+GTE” group as compared to “DC” after 7 and 14 days. We did not consider association between liver enzymes and BMI.

In our study ALP levels in serum were not significantly changed among “NC” and “N+GTE” group after 7 and 14 days. This finding suggest that 0.2% aqueous GTE (90%) solution for 14 days was not toxic for liver or any other organs involved in induction of serum ALP levels. In our study serum ALP levels were increased significantly ($P < 0.05$) in “DC” as compared to “NC” group after 7 and 14 days. Although the levels of ALP were decreased in “D+GTE” as compared to “DC” group after 7 and 14 days, but statistically this reduction was not significant. May be the reason behind it, the lesser time, and the concentration of GT we have used in our experiment.

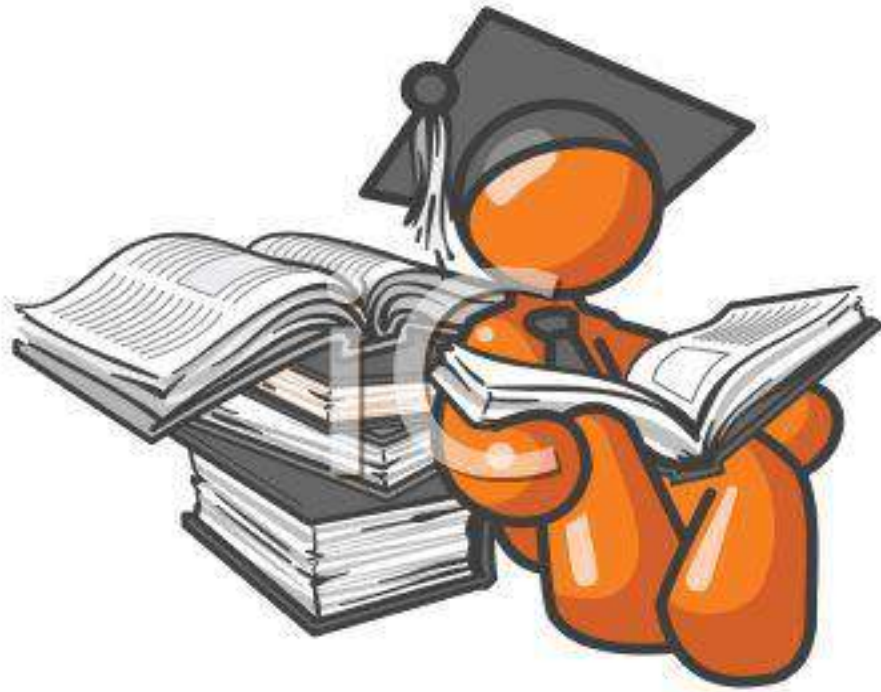
In our study we have found that GTE significantly ($P = 0.05$) induced the TAC in “N+GTE” as compared to “NC” group. Our findings showed that GTE significantly ($P < 0.05$) increased the TAC in “D+GTE” as compared to “DC” group. TAC was significantly reduced in “DC” group as compared to “NC” group.

However, these conflicting findings might be due to differences in doses, concentrations of green tea, time of exposure, type of green tea as well as the degree of hyperglycemia, type of animals, and the detection methods employed.

Liver tissue sample from “NC” group did not show any histopathological changes. Cells were regularly arranged and no fatty degeneration was observed. Similarly liver tissue sample from “N+GTE” also did not show any histopathological changes and there was no fatty vacuolation in the tissue. Liver tissue of diabetic control animal showed fatty vacuolation and mild inflammation. Fatty vacuolation was also observed in “D+GTE” but it was lesser in compare to “DC” group. As we had permission of dissection of only one animal in each group so we did not dissect any animal after 7 days of GTE treatment which we would be able to guess whether this decrease in fatty degeneration in “D+GTE” group is due to preventing or curative effect of green tea.

5.3. Conclusion

Diabetes mellitus is a metabolic disease increases day by day among the people. Hyperglycemia in DM may cause further complication such as neuropathy, retinopathy, nephropathy, liver damage and cirrhosis etc. The main reason behind their complications is the induction of oxidative stress. Consumption of antioxidants is one of the possible ways to reduce further complications. Natural antioxidants are the cheapest, available with lesser side effects. One of these natural antioxidants is polyphenolic compounds widely distributed among the some fruits and plants such as *Camellia sinensis* (green tea plant). Use of daily consumption of green tea can protect against DM and/ or can cure the DM complications due to its anti-oxidant, anti-apoptosis, anti-diabetic and anti-inflammatory effects. However, these effects are dose dependent; at lower doses it has anti-oxidant, anti-apoptosis, and anti-inflammatory, while at higher doses it showed pro-oxidant, pro-apoptotic properties. 0.2% aqueous solution of GTE (90% catechins) as drinking water for 14 days showed anti-daibetic (lowering hyperglycemia), anti-hypercholesterolemia, anti-triglyceridemia, and antioxidant effects, but couldn't modulate liver biomarkers.



Chapter- VI

References

Pages: 120- 139

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Chapter-VII

Appendages

Pages: 140-143

Poster presentation

Poster presentation from this article



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Antioxidative and Prooxidative Effects of Green Tea Extract Exhibit Hepatoprotective and Hepatotoxicity

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RTBS15-PPS6

Abstract

Green tea is widely consuming beverage after water globally. It possesses more than 8000 bioactive components. One third of its component is polyphenols which contains mostly catechins exhibiting strong antioxidant effects. Catechins scavenge the free radicals, reduce ROS-generation, and inhibit lipid peroxidation which are reported to be the possible mechanisms for hepatoprotection of green tea extract (GTE) against oxidative stress. GTE ameliorates oxidative damage of liver by modulation of biomarkers such as ALT, AST, ALP, GSH, glutathione peroxidase, catalase, superoxide dismutase, lipid hydroperoxide (LPOH), 4-TBN, 15-hydroxyprostaglandin, hydroxybutyric acid reductive activities (THARS) and MDA (malondialdehyde). Naturally derived or alternative antioxidants may be effective, but does not always guarantee safety when are taken in conventional dose. 100 ml of green tea contains 30-100 mg of polyphenols, which may show adverse effects including hepatic failure when taken frequently for prolonged period. Higher doses of GTE reported to elevate ALT, AST, ALP, GGT, bilirubin whereas reduce serum total protein, total antioxidant, and alter the ultra-architecture of hepatic tissue which finally lead to necrosis. The possible mechanism may be due to disturbance of mitochondrial metabolism, attenuated mitochondrial activity and formation of ROS by oxidation of GTE. Thus, GTE in conventional doses may act as antioxidant and protect hepatic cells against oxidative stress, but in higher doses may act as prooxidant which cause hepatotoxicity.

Keywords: Green tea, Antioxidant, Prooxidant, Catechins, Polyphenols, Hepatoprotection, Hepatotoxicity

Introduction

GTE, the infusion of *Camellia sinensis*, the most widely consuming beverage after water globally, with having more than 8000 bioactive components possesses complex chemistry such as polyphenols, alkaloids, amino acids, glycosides, proteins, volatile compounds, minerals and trace elements.

Dietary supplements containing GTE are used for different purposes such as anticancer, anti-diabetic, anti-oxidants, low weight reduction, EGCC, EGCG, ECG and EC are the most responsible for antioxidant and prooxidative effect of GTE.

They can scavenge OH⁻ groups, superoxide, 1,1-diphenyl-1-picrylhydrazyl radicals, peroxyl radicals, NO, nitrite oxygen, carbon-monoxide free radicals, lipid free radicals, and also scavenge peroxyl radicals by inhibiting the initiation of cyclooxygenase to protect hepatocytes against oxidative stress-induced damage.

They can inhibit different points of apoptosis chain such as caspase 3, glycogen synthase kinase 3 pathway, p38-AKP whereas proinflammatory cytokines, cytochrome c release, and also cell signaling through activation of the phosphatidylinositol-3-kinase/Akt pathway.

Polyphenols also may cause a mixture of salivome stress including hepatic failure when taken frequently for prolonged period through ROS induction. The possible mechanism is suggested through oxidative stress can cause hepatic damage.

Anti-oxidative Mechanism

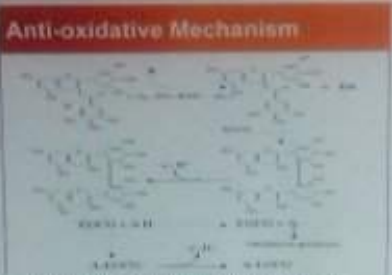


Figure 4: Proposed antioxidant mechanism of EGCG.

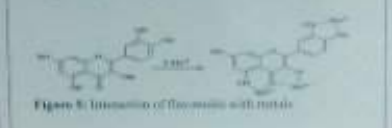


Figure 5: Interaction of flavonoids with metals.

↓ Oxidized (OH)[•] → Reduced (OH)⁻ H₂

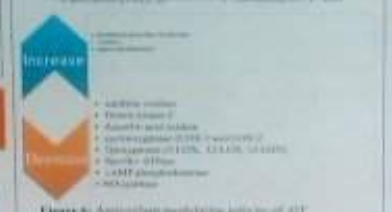


Figure 6: Antioxidant modulating activity of GTE.

Result & Discussion

- Catechins at low doses have antiapoptotic effect while at higher doses possess proapoptotic effect.
- Polyphenols at low doses possess antioxidant effect while at higher doses reveal prooxidative effects.
- GTE can protect the hepatocytes against several hepatocarcinomas through antioxidant activities and improve the liver biomarkers such as BSEP, BCL2, c-myc, Akt, p-ERK, cyclin D1 and p38, liver tumor and tumor anti-oxidants (superoxide and nitro-oxyl radicals).
- While at higher doses show prooxidative effect which may alter liver biomarkers such as ALT, AST, ALP, GGT, bilirubin, serum total protein, total antioxidant, and alter the ultra-architecture of hepatic tissue which finally lead to necrosis and hepatic failure.
- Flavonoids are used as antioxidants in hepatocarcinoma due to green tea.
- Green tea induced more hepatotoxicity when taken at fasting condition than feeding condition.
- Same dose of GTE when taken through IP may not lead to hepatotoxicity while through oral route may not lead to hepatotoxicity.
- Hepatotoxicity of green tea also depends on the dose of its administration.

ROS-formation and Oxidative Stress




Figure 2: Production of reactive oxygen species by Electron Transport Chain in mitochondria.

- ERH[•] - nitric oxide (NO), nitrogen dioxide (NO₂), and nitric acid (HNO₃).
- ↑ Organic radicals (O^{•-}) - steroids, lipids, carbohydrates and nucleic acids.
- ↑ GSH, Cat, SOD, HSP, P-7 and reduction.

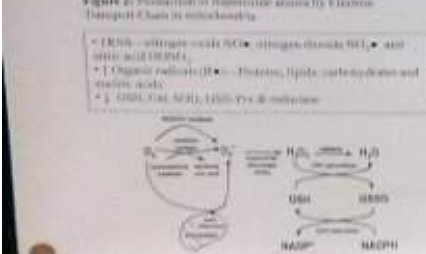


Figure 3: Role of natural anti-oxidant enzymes.

Pro-oxidative Mechanism

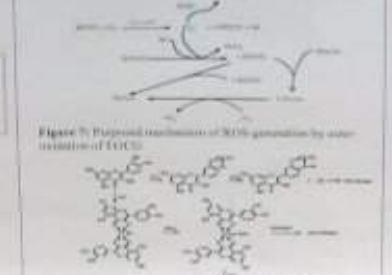


Figure 7: Proposed mechanism of ROS generation by auto-oxidation of EGCG.




Figure 8: The possible mechanisms of DNA damage induced by generation of super-oxides.

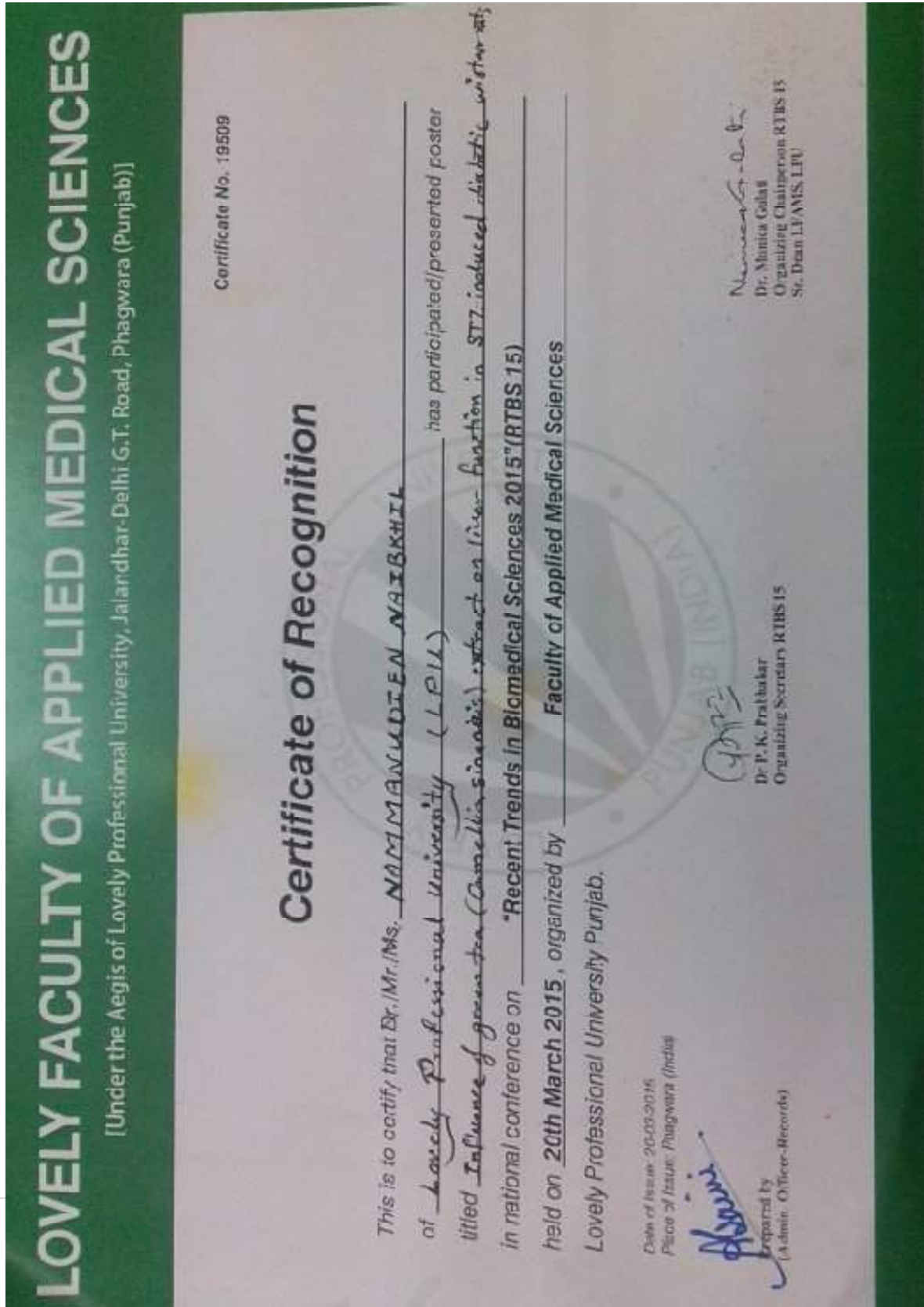
Conclusion

- GTE have both antioxidant and prooxidative effect but it depends on the amount, time and dosing/dosing condition of administration.
- GTE also have both anti-apoptotic and proapoptotic effect which depends on the amount of GTE taken.
- GTE may stimulate apoptosis in cancer cells while inhibit apoptosis in normal cells.
- GTE of conventional doses may protect liver against oxidative stress damage, while at higher doses may induce oxidative stress leads to hepatotoxicity.
- GTE may have inhibitory effects on some of CYP-450 enzymes while stimulatory effect on some others.
- 4-5 cups of GT is recommended for daily use, avoid drinking remaining of higher amount or conventional GTE.

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Abst: 34

ANTIOXIDATIVE AND PROOXIDATIVE EFFECTS OF GREEN TEA EXTRACT EXHIBITS HEPATOPROTECTIVE AND HEPATOTOXICITY

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Green tea is widely consuming beverage after water globally. It possesses more than 4000 bioactive components. One third of its component is polyphenols which contains mainly catechins exhibiting strong antioxidative effects. Catechins scavenge the free radicals, reduce NO-synthase, and inhibit lipid peroxidations which are reported to be the possible mechanisms for hepatoprotection of green tea extract (GTE) against oxidative stress. GTE ameliorate oxidative damage of liver by modulation of biomarkers such as ALT, AST, ALP, GSH, glutathione peroxidase, catalase, superoxide dismutase, lipid hydroperoxides (LOOH), 4-HNE (4-hydroxynonenal), thiobarbituric acid reactive substance (TBARS) and MDA (malondialdehyde). Naturally derived or alternative antioxidants may be effective, but does not always guarantee safety when not taken in recommended dose. 100 ml of green tea contains 50-100 mg of polyphenols, which may show adverse effects including hepatic failure when taken frequently for prolonged period. Higher doses of GTE reported to elevate ALT, AST, ALP, GGT, bilirubin whereas reduce serum total protein, total antioxidants, and alter the histo-architecture of hepatic tissue which finally lead to necrosis. The possible mechanism may be due to disturbances of carbohydrate metabolism attributed mitochondrial toxicity and formation of ROS by catechins of GTE. Thus, GTE in recommended doses may act as antioxidants and protect hepatic cells against oxidative stress, but in higher doses may act as prooxidants which cause hepatotoxicity.

Keywords: Green Tea, Antioxidant, Prooxidant, Catechins, Polyphenols, Hepatoprotective, Hepatotoxicity.