وزارة التعليم العالي والبحث العلمي

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قسم الكيمياء

تأثير المستخلص الايثانولي للعكبر المحلي على بعض مؤشرات مضادات الأكسدة لدى الجرذان المصابة بالفشل الكلوي نتيجة الإصابة بداء السكري

رسالة مقدمة الى
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وهي جزء من متطلبات نيل درجة ماجستير علوم في الكيمياء/كيمياء حياتية

من قبل

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الخلاصة

الهدف من هذه الدراسة هو التحقق من تأثير المستخلص الايثانولي للعكبر المحلي من خلال التجريع اليومي على مستوى الكلوكوز, النظام الدفاعي المضاد للأكسدة في الجسم و كذلك تأثيره على وظيفة الكلية في الجرذان المختبرية المصابة بالفشل الكلوي نتيجة داء السكري المحفز بواسطة الستربتوزوتوسين (٦٠ملغم/ كغم).

اجريت الدراسة على خمسة وسبعون جرذا (١٥٠- ١٦٠) غم قسمت الى خمس مجاميع: المجموعة الطبيعية , المجموعة المحفزة بداء السكري بعد ٣ اسابيع من بداية التجربة, مجموعه طبيعية تجرع بالمستخلص الايثانولي للعكبر المحلي يوميا مدة ٦ اسابيع, مجموعه تجرع بالمستخلص الايثانولي للعكبر المحلي لمدة ٣ أسابيع ثم يتم تحفيز داء السكري لمدة ٣ اسابيع اخرى و مجموعه طبيعية يتم تحفيز السكري بعد ٣ اسابيع من بداية التجربة ثم تجرع بالعكبر المحلي لمدة ٣ اسابيع بعد التحفيز.

في نهاية التجربة جمعت عينات مصل الدم من الجرذان تحت التخدير لتعيين التغيرات البايوكيميائية, وتم التضحية بالحيوانات لاستخراج الكلية وفحصها مجهريا في المجاميع المختلفة.

قيمت الحالة التأكسدية للمجاميع من خلال قياس فعالية سوبراوكساید دسمتيز, الكاتاليز, الكلوتاثيون بيروكسيديز , كلوتاثيون ترانسفيريز وتركيز الكلوتاثيون,ملونيل ثنائي الالديهايد, نايترك اوكساید, حامض اليوريك وكذلك تم تعيين مدى الإصابة بالفشل الكلوي من خلال قياس اليوريا, البروتين الكلي والكرياتينين في مصل الدم. لوحظ في نهاية الدراسة انخفاضا معنويا في وزن الجسم و فعالية النظام المضاد للأكسدة ومستوى البروتين في المصل, و زيادة معنوية في مستوى الكلوكوز في الدم وزيادة تركيز ملونيل ثنائي الالديهايد, نايترك اوكساید, يوريك اسيد, اليوريا و الكرياتنين في المصل ($p<٠,٠٥$), بالنسبة للمجموعة المصابة بالسكري.

كما بينت النتائج أن المستخلص الايثانولي للعكبر المحلي كان له تأثيرا معنويا في تقليل مستوى الكلوكوز , ملونيل ثنائي الالديهايد, نايترك اوكساید, يوريك اسيد, اليوريا و الكرياتنين, و حدوث زيادة معنوية في فعالية النظام المضاد للأكسدة وتركيز البروتين الكلي في مصل الدم ($p<٠,٠٥$) وتحسين البنيان النسيجي للكلية. يمكن الاستنتاج بأن المستخلص له فعالية مضادة للأكسدة و خفض مستوى السكر في الدم وبالتالي حماية الكلية من تأثير مضاعفاته كالفشل الكلوي.

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AGEs	Advanced Glycation End products
ATP	Adinosin Tri Phosphate
BGL	Blood Glucose Level
CAPE	Caffeic Acid Phenethyl Ester
CAT	Catalase
CDNB	1-Chloro-2,4-Dinitrobenzene
CKD	Chronic Kidney Disease
CKF	Chronic Kidney Failure
Conc.	Concentration
COx	Cyclo- oxygenases
CTGF	Connective Tissue Growth Factor
D.W	Distilled Water
DL	Deciliter
DM	Diabetes Milletus
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic Acid
DTNB	5-5-Dithiobis(2-nitrobenzoic acid)
ECD	Endothelial Cell Dysfunction
EEP	Ethanolic Extract of Propolis
FBG	Fasting Blood Glucose
FR	Free Radicals
GFR	Glomerular Filtrations Rat
GGT	ɤ-Glutanyl Transpeptidase
gm	Gram
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione-S-Transferase
H&E	Hematoxylin and Eosin stains
H_2O_2	Hydrogen Peroxide
HDL-C	High Density Lipoprotein Cholesterol
HOCl	Hypochlorous Acid
i.p.	Intraperitoneal
i.v.	Intraveins
IL-1	Interleukin 1
IL-6	Interleukin 6
iNOs	Inducible Nitric Oxide Synthase
Kg	Kilogram
L	Liter
LDL-C	Low Density Lipoprotein Cholesterol
LOx	lipoxygenase
MDA	Malondialdehyde
mg	Milligram
mM	Millimolar
MODY	Maturity-Onest Diabetes of Youth
mRNA	Messenger Ribonucleic Acid

NADP	Oxidized Nicotinamide Adnine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adnine Dinucleotide Phosphate
eNOS	Endothelial Nitric Oxide Synthase
nNOS	Nerve Nitric Oxide Synthase
NFK-β	Nuclear Factor Kappa-B
$NO^{\cdot}$	Nitric Oxide Radical
NOs	Nitric Oxide Synthase
$OH^{\cdot}$	Hydroxyl Radical
$ONOO^{-}$	Peroxynitrite
PKc	Protein Kinase c
PUFA	Poly Unsaturated Fatty Acids
RM	Reactive Metabolite
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
$RO^{\cdot}$	Alkoxy Radical
$ROO^{\cdot}$	peroxyl Radical
ROS	Reactive Oxygen Species
SD	Sprague-Dawley
SOD	Superoxide Dismutase
STZ	Streptozotocin
TBA	Thiobarbutric Acid
TC	Total Cholesterol
TCA	Trichloroacetic Acid
TG	Triglyceride
TGF-β	Transforming Growth Factor beta
TSP	Total Serum Protein
UA	Uric Acid
UV	Ultra Violet Radiation
VLDL-C	Very Low Density Lipoprotein Cholesterol
XO	Xanthine Oxidase
µg	Microgram
µl	Microliter
β-cells	Beta cells

1.1. Diabetes Mellitus (DM)

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. There are two major types of diabetes:

Type 1 diabetes (juvenile), β-cells destruction, usually leading to absolute insulin deficiency, Immune-mediated diabetes results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas.

Type 2 diabetes (adult onset), ranging from predominantly insulin resistance with relative insulin deficiency results from impaired insulin production by beta cells and impaired insulin action (Van Belle *et al*., 2011).

Other specific types of diabetes are associated with certain conditions (secondary) including genetic defects of β-cells function or insulin action, pancreatic disease, disease of endocrine origin, drug or chemical included insulin receptor abnormalities, and certain genetic syndromes. Maturity-onsets diabetes of youth (MODY) is a very rare form of diabetes that is inherited in an autosomal dominant fashion (Malchoff,1991).

Diabetic nephropathy (DN) is the most complication of the two kinds of diabetes mellitus (Valocikov, 2011). It is the major reason of end-stage renal disease (Ritz *et al*., 2011).

1.2. Nephropathy

Nephropathy is a common term referring to disease of kidneys. That result damages in the small vessels in kidneys, and causes kidneys lose their ability to filter the waste product from blood like toxins and protein (Levey *et al*., 2005).

Nephropathy can be classified to: (Hsu CY *et al*., 2008).

1. Chronic Nephropathy.
2. Acute Nephropathy.

1.2.1. Chronic Nephropathy(Chronic Kidney Disease) (CKD)

CKD is characterized by progressive deterioration of kidney function, which develops into a terminal stage of Chronic Kidney failure (CKF).Chronic disease can categorize CKD into five stages depending on

the glomerular filtrations rate (GFR) and presence of signs of kidney damage (Levey *et al*., 2005).

- Stage 1: GFR ≥ 90 ml/min that means kidney damage with normal GFR.
- Stage 2: GFR = 60-89 ml/min (kidney damage with mild GFR).
- Stage 3: GFR = 30-59 ml/min (moderate GFR).
- Stage 4: GFR = 15-29 ml/min (Sever GFR).
- Stage 5: GFR < 15 ml/min (kidney failure).

There are several factors lead to the serious complication and causes CKD:

1- Acid-base balance

Acid-base disorder is commonly observed in the CKD. The metabolic acidosis is noted in a majority of patient when GFR decrease to less than 20-25% of normal . In mild Chronic renal, metabolic acidosis is result because of reduce the ability to reabsorb bicarbonate to excrete ammonia. In a more severe renal insufficiency, Organic and other Conjugate anion of acids (nonvolatile acids) can't be exactly excreted , this disorder is called Uremic acidosis (Cibulka and Racek, 2007).

2- Protein metabolism

This disorder can be termed Uremic malnutrition. It is present nearly 20-50% of patients on dialysis and it can be distinguished by insidious loss of somatic protein stores and visceral protein (that explain increase serum creatinine and lean body mass) that causes decrease in serum total protein or albumin (Ikizler, 2004).

3- Carbohydrate metabolism

Disorder in metabolism of Carbohydrate is also frequent in CKD.

The important reason in this disorder is diabetes mellitus; it represents nearly 35% of patients on dialysis, so as insulin resistance (Alvestrano, 1997).

4-Lipid metabolism

In CKD the serum triglycerides are elevated , that result from several reason like dysfunction of TG degradation resulting from insufficient mitochondrial beta-oxidation of fatty acids , deficit of L-Carnitine and Hyperinsulinemia is the important factor that causes increasing the synthesis of TG and also directly decreasing the activity of lipoprotein lipase that hydrolyzes triglycerides in lipoproteins (Cibulka *et al*,2005).

1.2.2. Acute Kidney Injury

Acute kidney injury is a medical emergency characterized by a rapid (hours to days) fall in glomerular filtrations rate (Hsu Cy, 2008).

Acute kidney injury causes can be divided into three categories:

1. Pre-renal causes

This case occurs when the blood flow is reduced because the inability to maintain renal blood flow via auto regulation. It is the most common cause of acute kidney injury. The main causes of pre-renal injury are Hypovolaemia because of diarrhea, vomiting, diuretics, osmotic diuresis. From uncontrolled diabetes, decreased effective blood volume is the cause of heart failure or cirrhosis and may result from vasoregulation medicines (Hilton, 2006).

2. Intrinsic-renal causes

Intrinsic-renal causes include direct damage to nephrons. The common reason of this case is acute tubular necrosis or direct toxicity (hypotension, hemolysis or nephrotoxic medicine (Khalil *et al*., 2008).

3. Post-renal injury

Post-renal injury is caused by a blockage to the flow of urine resulting in a back pressure to the kidney, which causes damage the kidney. Most renal causes are urinary tract stones, prostatic hypertrophy and other pelvic malignancy (Hilton, 2006).

1.3.The mechanisms of DN Stimulating

Diabetic nephropathy is kidney damage that occurs as a result of diabetes. There are several mechanisms that cause development (DN) such

as increased formation of advanced glycation end products (AGEs), the activation of protein kinase C (PKC) isoforms (Brown *et al*., 2001). The activation of polyol pathway mechanism, and glucose auto oxidation, these mechanisms induced diabetic complication (Yan *et al*., 2008).

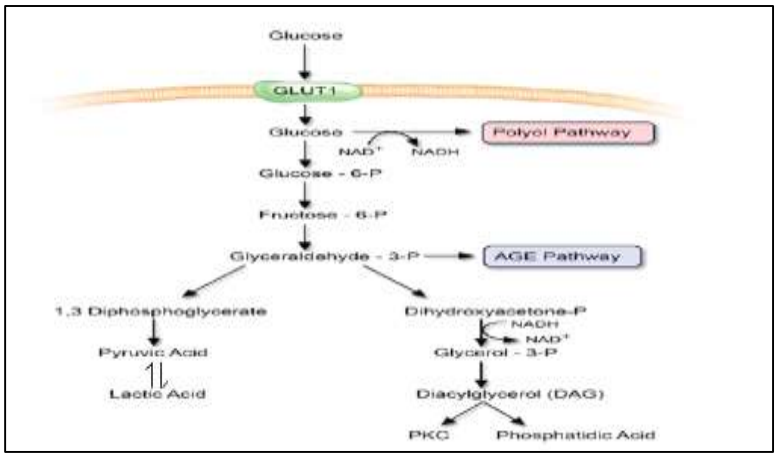


Fig (1-1): Hyperglycemia induced superoxide production prevents the normal conversion of glyceraldehyde-3-Phosphate into 1, 3 diphosphoglycerate. This diverts upstream metabolites into the polyol, and AGE pathways. Excess glyceraldehyde-3-Phosphate is converted into diacylglycerol (DAG), which subsequently activates protein kinase C (PKC) (David J. 2010).

1.3.1. Advanced Glycation End Products

(AGEs) compound are formed when reducing sugar react with amino acids in protein and other macromolecules in nonenzymatic reaction (Claudia *et al*., 2010). The first reaction between Glucose and the free amino acid of protein give a Schiff base. This step depends on glucose concentration and takes place within hours. When the concentration of glucose decreases the reaction is reversible, but when increases that cause forming Amadori products (within a period of days) (Ahmed, 2005). Fructose amine compound is considered early glycation adducts and then undergo rearrangement like (dehydration, condensation, fragmentation, oxidation and cyclization reaction) giving irreversible compound and so called advanced glycation End-products (AGEs) (Grillo and Colombatto., 2007).

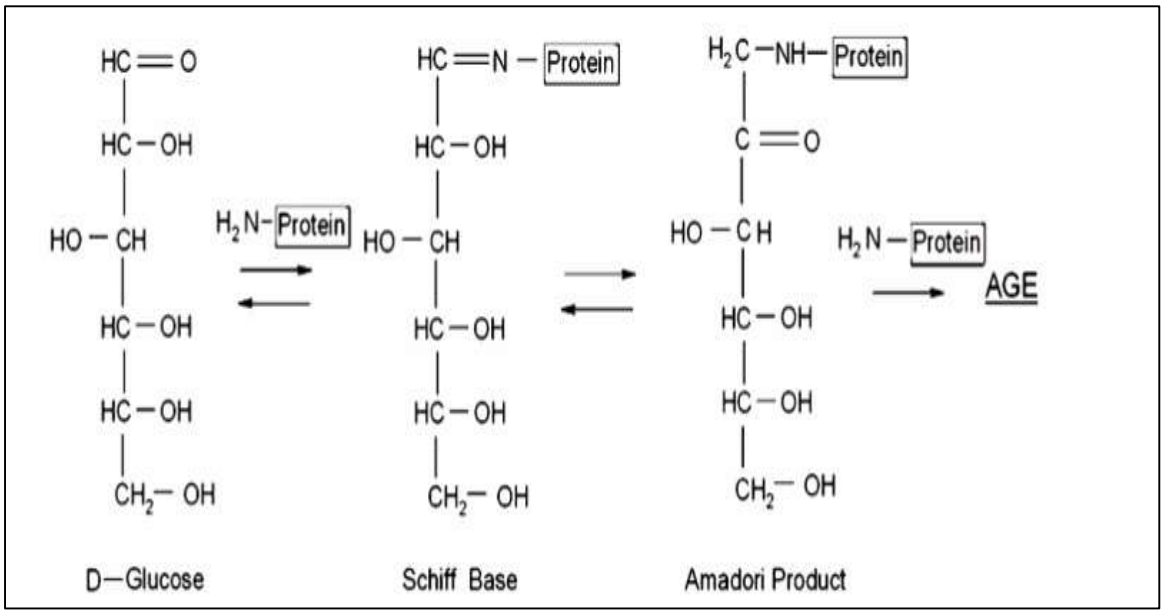


Figure (1-2): The non –enzymatic glycosylation(Grillo and Colombatto . 2007).

The AGEs are very stable and accumulate inside and outside the cells and interfere within protein function (Claudia *et al*., 2010). AGEs tend to accumulate on long-lived macromolecules in tissue-cross-linking AGE-protein with other macromolecules in tissue results in abnormalities of cell and tissue function, so, increased vascular structure binding to a specific macrophage receptor. This process includes the synthesis and secretion of cytokines such as TNF (tumor necrosis factor) that are produced in excess in inflammation and tissue destruction, which causes endothelial dysfunction and induced free radical generation (Brownlee *et al*., 1989).

1.3.2. Activation of Protein Kinase C

PK is a family of closely related enzymes that phosphorylate serine or threonine residues of various intracellular proteins and this is involved in a wide range of cellular functions (Kang *et al*., 1999). Hyperglycemia considers a key role in the pathogenesis of micro vascular diabetic complications, and causes activation of the protein kinase (PKC) system, that may represent an important mediator of glucotoxcity in diabetic nephropathy (Lee *et al*., 1989).

The hypothesis intracellular mechanism is the glucose induced *de novo* synthesis of diacylglycerol that is one of intracellular activators of PKC, when glucose inter into vascular and renal cells through GLUT1(Glucose transporter 1) which is a protein facilitates the transport of glucose across the plasma membranes of mammalian cells, it is phosphorylated and then converted to fructose-6-phosphate and glyceraldehyde-3-phosphate. By the action of various transferases and phosphatases, glyceraldehyde- 3-phosphate forms glycerol phosphate a precursor of DAG that is directly or indirectly activates PKC isoforms (Noh *et al*., 2007).

Therefor; activation of PKC may cause alteration in various vascular functions in diabetes because of a PKC dependent activation of NADPH oxidase that is the essential mechanism of increase ROS production in diabetic vascular tissues (Toyoshi *et al*., 2003).

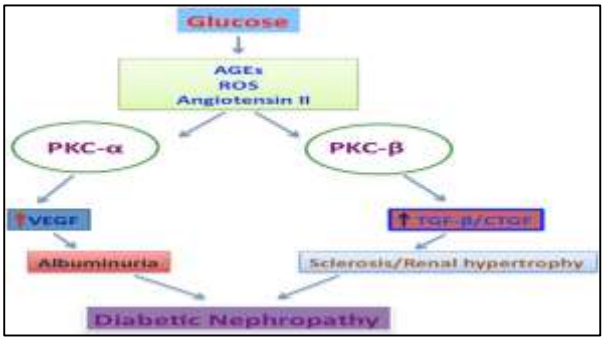


Figure (1-3): In diabetic nephropathy, hyperglycemia initiates various intracellular signaling pathways resulting in further downstream activation of different PKC isoforms. ROS, reactive oxygen species; CTGF, connective tissue growth factor (Thallas-Bonke, and Cooper, 2012).

1.3.3. The Activation of Polyol Pathway

The nonphosphorylated glucose reduced to sorbitol by the Aldose reductase and using NADPH, then the sorbitol converted to fructose by sorbitol dehydrogenase with the cofactor NAD^+, Aldose reductase possess

low affinity for glucose, when the level of glucose is high, there for a small amount of glucose being metabolized (Sheetz *et al*., 2002). Actually polyol pathway is active in kidney, because of increased sorbitol level in misangial cells of the kidney when there is high concentration of glucose. Besides, the changes in the metabolic that result because of the increased flux of the polyol pathway are consistent with the renal changes (Setter *et al*., 2003).

Reduction of glucose to sorbitol uses NADPH and oxidation of sorbitol increases NADH that causes rapid change in the cytoplasmic redox state and enhanced production of ROS (Dhruvk *et al*., 2011).

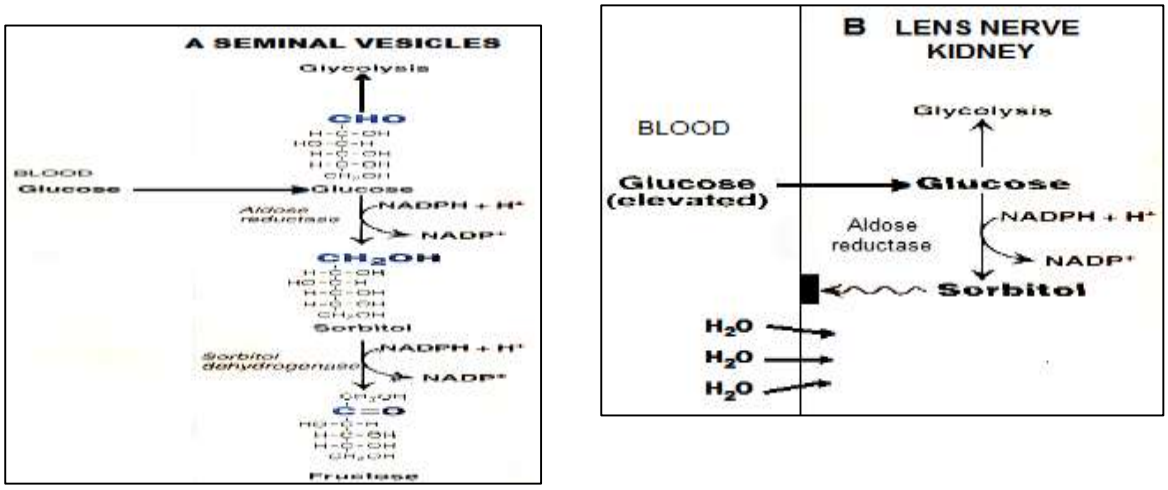


Figure (1-4): Sorbitol Metabolism

(Champe and Harvey, 2005)

1.3.4. Auto Oxidation

Glucose *in vivo* is in equilibrium with its enediol form, when there is a small amount, in the presence of transition metal like iron and copper, the enediols generate anion and themselves are oxidized to dicarbonyl keto aldehydes, this oxidation product reacts with amino group of protein in the presence of transition metal (Halliwell *et al*., 2007). That makes the transition metal have hazardous potential because of its ability to participate in the generation of powerful oxidant species, like $O^{-\bullet}_2$, H_2O_2, $HO^{\cdot}$ and dicarbonyls which can damage protein(Singh *et al.*,2009).

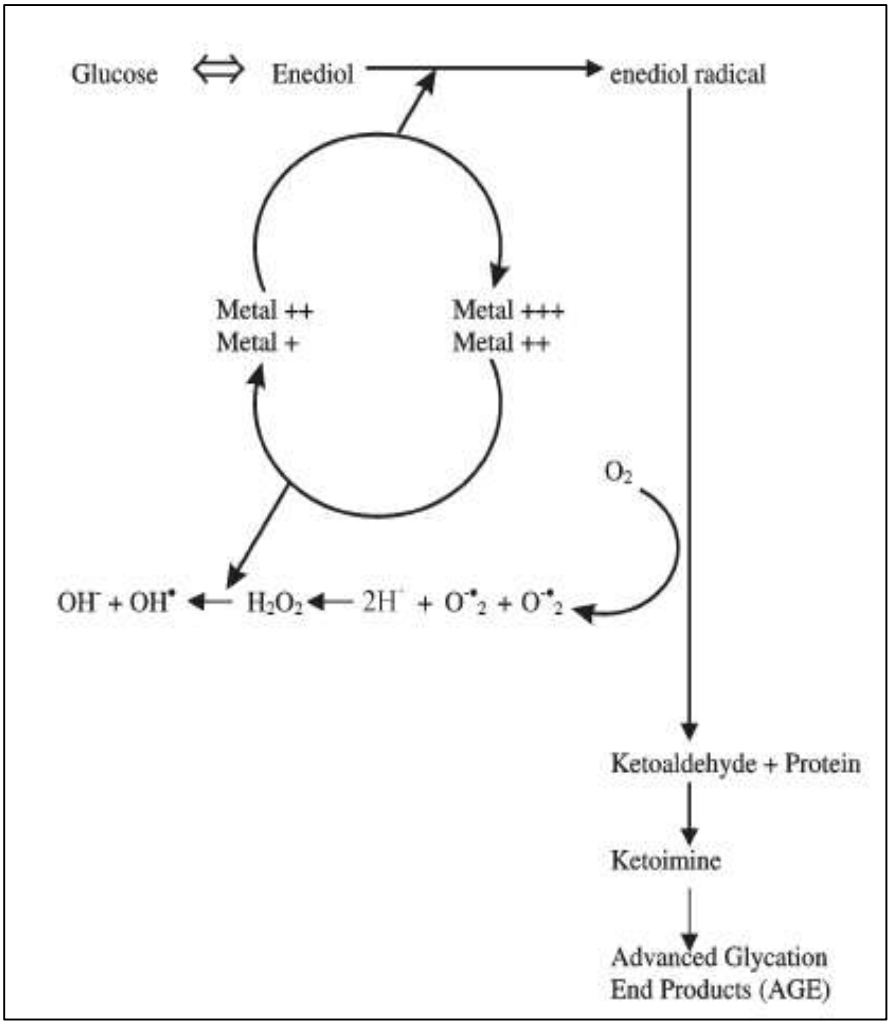


Figure (1-5): Auto oxidation of glucose resulting in oxidative stress through formation of superoxide anion ($O^{-\bullet}_2$) and free hydroxyl radical ($^{\bullet}OH$) (Wolff and Dean, 1987)

1.4. ROS (Reactive Oxygen Species):

The term of Reactive Oxygen Species refers to a group of oxidants, either free radical or molecular species have the capacity of generating free radicals. ROS mainly consists of super oxide radicals and nitric oxide (NO) radical. Both $O_{2.}^{-}$ and $NO^{\cdot}$ radicals are converted to powerful oxidizing radicals like hydroxyl radical ($OH^{\cdot}$), alkoxy radical ($RO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), singlet oxygen ($^1O_2$) by transformation reactions, some of these species can be converted into molecular oxidants like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{-}$), hypochlorous acid (HOCl) and other products(Winterbourn *et al*., 2008).

In the biological significance, the reactive oxygen species consist of free radicals (FR) and their reactive metabolites (RM). The association between free radical and their reactive metabolites can be summarized in figure (1-6).

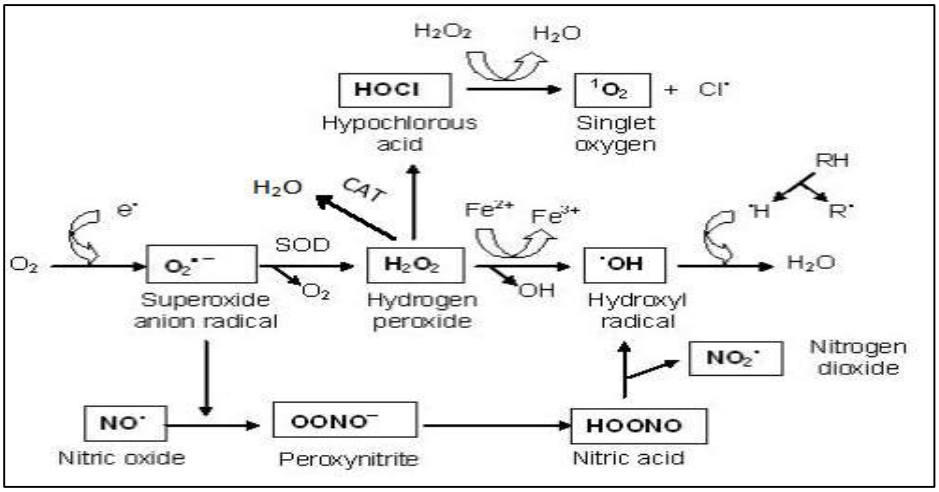


Figure (1-6) Mutual association between free radicals and their reactive metabolites (Ďuračová, 2010).

Under normal physiological conditions, nearly 2% of the oxygen consumed by the body is converted into $O_2^{-\cdot}$ through mitochondrial respiration (Winterbourn, 2008). The generation of ROS in mitochondria is not constant in different tissues and different condition. ROS generation is

not a simple byproduct of mitochondrial respiration as is frequently assumed (Barja, 2007).

1.4.1. Generation of ROS in Cells:

- Production of ROS in mitochondria

Electron transport chain produced superoxide anion in mitochondria by the reduction of molecular oxygen. ROS are generated by mitochondria, *via* the release of electrons from the electron transport chain and reduction of oxygen molecules into superoxide's ($O^{-\bullet}_2$) (Shafak, 2012)

- Production of ROS in endoplasmic reticulum

Cytochrome P_{450} complexes are used to detoxify the toxic hydrophobic chemical compound from the body, as a result of superoxide anions are formed. Enzyme cytochromeP_{450} reductase is used to detoxify into hydrophilic compound, NADPH and NADH donate electrons for reduction of cytochrome b_5 and p_{450} (Bedard and Krause, 2007).

- Phagocytosis

When the bacteria engulfed by the phagocytic cell results in the production of ROS. Bacteria and toxic cells are destroyed by respiratory burst produced by ROS (Shafak, 2012).

-Other sources

Apoptosis: Apoptosis is a process of programmed cell death, causes protein denaturation and phagocytosis of the cell (Kam and Ferch, 2000).

Autoxidation of small molecules: small molecules like dopamine, epinephrine , flavins and hydroquinons involve direct production of $O_2^{\cdot -}$ (Freeman and Crapo, 1982).

-Peroxisomes generating hydrogen peroxide:

Peroxisomes containing enzymes, glycolate oxidase, D-amino acid oxidase, urate oxidase and fatty acyl CoA oxidase are involved in generating H_2O_2. The Catalase involves varieties of per oxidative reactions (Tolbert and Essner, 1981).

-ROS Generation by Lysosomes: It carries electron transport chain which involves pumping of proton. This system promotes 3 electrons reduction to oxygen and from highly reactive $OH^{\cdot}$ (Kiebanoff., 2005).

1.4.2. Physiological Function of ROS

When the generation of ROS under the normal condition, it is necessary for physiological functions (Droge, 2002).

The important beneficial roles of ROS:

1. Generation of ATP (universal energy currency) from Alternative oxidases in the mitochondria of plant, oxidative phosphorylation.
2. Detoxification of Xenobiotic by Cytochrome P_{450} (oxidizing enzyme).
3. Apoptosis of effect or defective cells.
4. Killing of micro-organism and cancer cells by macrophage and cytotoxic lymphocytes.
5. Oxygenases (e.g. COX: cyclo-oxygenases, LOX: lipoxygenase) for the generation of prostaglandins and leukotriene, which have many regulatory function (Devasagayam *et al*., 2004).

1.5. Oxidative Stress

Oxidative stress is a common term that describes the effect of oxidation when the ROS level is abnormal, such as the free radical (like: Hydroxyl, nitric acid, superoxide) or the non-radicals (e.g. hydrogen peroxide, lipid peroxide), and the intensity of oxidative stress is detected by the balance between the rate which oxidative damage is induced and the rate at which it is efficiently repaired and removed. The balance provides certain steady-state ROS level (Lushchak, 2011).

ROS increases during infections, disease, exercise, exposure to pollutant, UV light, ionizing radiation, smoking...etc. that increase in ROS lead to the damage of the cells, lipids protein or DNA that results to inhibit the normal function, because of this oxidative stress has been implicated in growing list of disease (Pinar *et al*., 2012).

The relation between ROS and disease can be explaining by the concept of oxidative stress(Lushchak, 2011).

1.6.The Role of ROS in Pathogenesis of Diabetes Nephropathy

The pathophysiology of diabetic nephropathy can be viewed as a sequence of events evolving in a stepwise pattern, where it starts with endothelial cell dysfunction (ECD) and ends with end-stage renal failure. However, ECD is preceded by glomerular hyper fusion and hyper filtration (Mshelia, 2004)

The endothelium is a metabolically active system that maintains vascular homeostasis, by regulating the homeostatic, inflammatory, and reparative responses to local injuries mediated by NO by inhibiting the activation of an important nuclear transcription factor-Nuclear Factor kappa B (NFK-B) that binds to the promoter regions of genes which code for proinflammatory proteins.

Hence, NO production or availability can regulate diverse functions in endothelial cells. Therefore, any event that alters the function of NO will lead to endothelial cell dysfunction (Clines *et al*., 1998).Other study suggest that (NADPH) oxidase is the primary source of vascular and renal ROS production; however other possible source include Glucose-6-Phosphate dehydrogenase (Gao and Mann, 2009).

1.7. The Reasons which trigger ECD in Diabetes Mellitus

A causal relationship between oxidative stress, ECD and diabetic nephropathy has been established by observations that:

a) High glucose can directly cause ECD and increases oxidative stress in glomerular mesangial cells, a target cell of diabetic nephropathy.

b) Lipid peroxides and 8-hydroxydeoyguanosine, indices of oxidative tissue injury, were increased in the kidneys of diabetic with albuminuria.

c) Oxidative stress induces mRNA expression of NFk-β (nuclear factor kappa-light-chain-enhancer of activated β cells) genes which in turn promotes production of proinflammatory proteins TGF-β (transforming growth factor beta) , fibronection, laminin, elastin, IL-1, IL-6. Inhibition of oxidative stress ameliorates all the manifestation associated with ECD and diabetic nephropathy(Mshelia, 2004).

Other possible sources include nonenzymatic glycation of free amino groups on proteins and amino acids, flux of glucose through the sorbitol/polyol pathway increased *de novo* synthesis of diacylglycerol and subsequent activation of the protein kinase C pathway, these not only generate ROS but also attenuate antioxidant mechanisms creating a state of oxidative stress, these processes have been proven to account for numerous features of chronic diabetic complications (Forbes et al., 2008).

Sequence of events in the pathogenesis of diabetic nephropathy can illustrate by figure (1-7) (Mshelia, 2004).

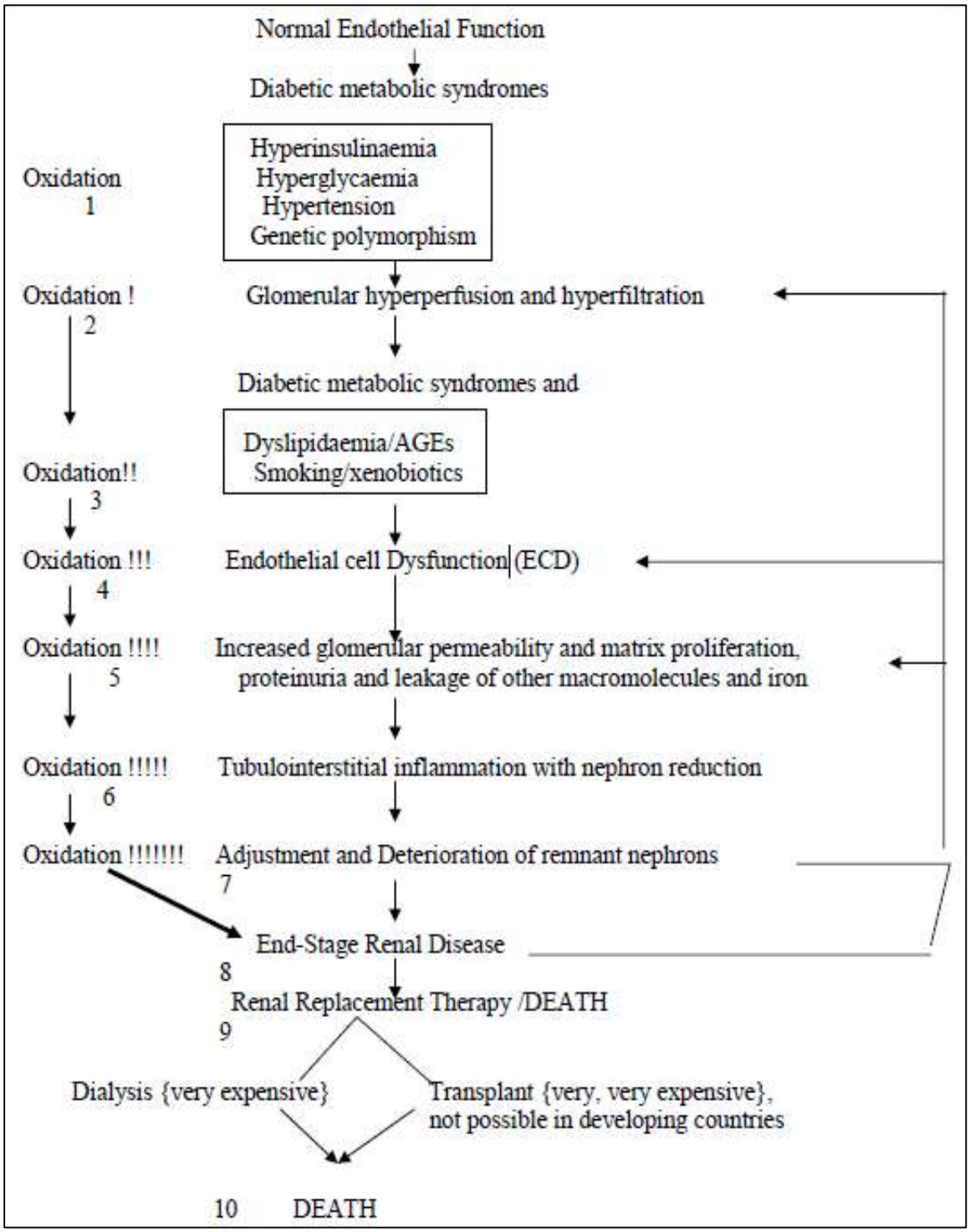


figure (1-7): Sequence of events in the pathogenesis of diabetic nephropathy (Mshelia, 2004).

1.8. The Biological Alterations in Oxidative Stress

1.8.1. Lipid Peroxidation

Malondialdehyde MDA, is a highly reactive three carbon di aldehyde produced, is one of the final products of lipid peroxidation, the levels of

various lipid peroxidation products have been used as an indicator and direct evidence of toxic processes caused by free radicals activity (Talas and Gulhan, 2009; Tatli Seven *et al*., 2009).

1.8.2. Nitric Oxide

Nitric oxide is an important messenger molecule brings both beneficial and deteriorating effects on the human body, NO synthesized by various NOS (Durackova,2010), during the conversion of arginine to citrulline catalyzed by nitric oxide synthase. It contains an unpaired electron and is classified along with other reactive oxygen species (ROS) (Dahiya *et al*., 2013).

The positive role of NO present at low concentration and produced by constitutive NOS either during vasodilating processes (eNOS) or during transmission of nerve impulses (nNOS). NO can cause damage to proteins, lipids and DNA when reaction with superoxide, leading to the formation of the very reactive peroxynitrite anion (nitro peroxide) $ONOO^-$ (Knott and Bossy, 2009).

1.8.3. Uric Acid

Uric acid (UA) is converted from xanthine, a degradation product of purine nucleotide (adenine and guanine) by the enzyme xanthine oxidase (XO), on the other hand when there is a small evaluation serum uric acid (a schema A) can be resulted from diet rich in purine or from the impaired renal excretion of uric acid decreased glomerular filtration. Because XO enzyme is not involved in this type of evolution of serum uric acid, no superoxide is produced. Any local tissue ischemia derived from any micro vascular disease may also lead to an increased UA synthesis due to an increased RNA and DNA breakdown, resulting in an increased purine concentration and an increased substrate concentration for enzyme XO.

Under this condition, superoxide is also produced by xanthine oxidase

(a schema B) that lead to increase oxidative stress and causes the inflammation induced (major cause of damage) (Lily and James, 2008).

1.9. The Biological Alternation in Kidney Function.

1.9.1 Total Protein

Serum protein is a mixture containing albumin and the immunoglobulin, liver responsible for formation of protein that can contain albumin and 30% serum globulins (Varley, 1985). A small visceral protein mainly synthesized in liver and catabolized in the kidney after glomerular filtrations, in diabetic nephropathy protein exertion by urine is strongly associated with structural and functional tubular kidney damage (Kamijo *et al.*, 2001).

1.9.2. Blood Urea

These substances are normal metabolic waste products that are excreted by the kidneys. Urea is a byproduct of protein breakdown, the test of blood urea is the simplest way to monitor kidney function. In kidney disease, these substances are not excreted normally, and so they accumulate in the body, this causes an increase in blood level (Molitoris, 2007).

1.9.3. Creatinine

Serum creatinine is primarily a metabolite of creatine, almost all of which is located in skeletal muscle. The normal levels of creatinine in female usually lower than male because they usually have less muscle mass.

The amount of creatine per unit of skeletal muscle mass is consistent and the breakdown rate of creatine is also consistent, thus, serum creatinine concentration is very stable and a direct reflection of skeletal muscle mass. Creatinine test is one of the best measure of kidney function (Martin and Sheaff, 2007).

1.10. Antioxidants

Antioxidants can be defined as the substances that neutralize free radicals or their action and have productive mechanism against any harmful effects of free radicals. These compounds contain mono hydroxyl polyhydroxy phenol and can donate hydrogen atom and therefore cannot initiate the second free radical and slow down the oxidation. A Cell has many anti-oxidant systems to prevent injury. Preventation of excessive ROS and repair of cellular damage is necessary for cell life. Antioxidants consist two main systems: Enzymatic antioxidant that is directly/indirectly contributes to defense against the ROS and Nonenzymatic antioxidants are actually the scavengers of ROS and RNS (Gutteridge, 1994).

1.10.1. Antioxidants Enzymes

1.10.1.1. Superoxide Dismutase (SOD)

Superoxide dismutase is an important enzyme in living family for maintaining normal physiological condition and for coping with stress (Olawale *et al*, 2008) it is spontaneously dismutase $O^{-\bullet}_2$ anion to form O_2 and H_2O_2. Superoxide dismutase mainly acts by quenching of superoxide $_{O^{-}}$ •2 (Vivek and Surendra, 2006).

$$2O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Superoxide dismutase exists in several forms, one form containing manganese is found in the mitochondrial matrix and other containing copper and zinc occurs in the cytoplasm. Cells are capable of increasing synthesis of SOD in response to hyper oxidant stress. Extracellular fluid contains a unique high-molecular weight SOD, the enzyme binds to external endothelial cell surfaces and may be important in the pathogenesis of free radical damage (Hisalkar *et al.*, 2012).

For each type, the mechanism of catalysis is thought to be the same, essentially involving a protein pocket bordered by positively charged amino acid residues that create an electrostatic sink for attracting the superoxide anion radicals to the active site. The transition metal is present

at the active site then carries out one electron transfer between two superoxide radicals and undergo alternative oxidation reduction reactions (Bowler *et al*., 1992).

1.10.1.2. Catalase (CAT)

Catalase is the class of enzymes, which catalyze the decomposition of hydrogen peroxide to oxygen and water. It is neutralized potentially lethal effects of hydrogen peroxide (Tetyane *et al*., 2005).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes by oxidase involved in B-oxidation of fatty acid. There are three isoenzyme localized separately in peroxisomes, cytosol and mitochondria (Scandalias, 1993).

1.10.1.3. Glutathione Peroxidase (GPx)

Glutathione peroxidases (GPxs) are members of the family of antioxidant enzymes that scavenge hydrogen peroxide in the presence of reduce glutathione, GPx is a selenium-dependent enzyme that contains a selenium atom incorporated within the selenocysteine residue (Sung *et al*.,2009).

GPx catalyzes the reaction of hydro peroxides with reduced glutathione to form glutathione disulfide (GSSG) and reduction product of the hydro peroxide (Shajeela *et al*.,2013).

This enzyme is important to detoxify not only H_2O_2 but also organic peroxide through the following reaction

$$ROOH + 2\,GSH \xrightarrow{GPx} GSSG + ROH + H_2O$$

These enzymes are located mainly within the cytosol of eukaryotic cells and may also occur intramitichondrially (Irshad and Chaudhary, 2002).

1.10.1.4. Glutathione-S-Transferase

Glutathione-S-transferase is another important enzyme known to catalyze antioxidant metabolism of thiol compounds, and this in turn protects cells from xenobiotics, free radical induced damage and oxidative stress. (Kemidi *et al*.,2013).

The transferase increases the nucleophilic properties of reduced glutathione and they catalyze formation of thioether bond by conjugated hydrophobic compound with GSH to form very stable compound, then kidney can break down glutathione conjugates to mercapturic acids, by the activity of ɤ-glutanyl transpeptidase (GGT), glutathione-Conjugate excretion are considered an important component of defense against xenobiotic (Dekant *et al*., 1990).

1.10.2.The Chain Breaking Antioxidant

Chain breaking antioxidant, are small molecules that can receive an electron from a radical or donor an electron to a radical with a formation stable by-products. In general, the charge becomes dissociated over the scavenger and the resulting product will not be ready accept an electron from or donate to another molecule, preventing the further propagation of the chain reduction (Young and Woodside., 2001).

Chain breaking antioxidant is divided into:

A-Aqueous phase: This antioxidant will directly scavenge radicals present in the aqueous compartment, antioxidant reacts with oxidants in the cell cytoplasm and the blood plasma. like:

Reduced glutathione (GSH), Glutathione is a tripeptide present in high concentration in most eukaryotic cells and reacts with free radicals in at least two ways. First, it may act as a reductant H_2O_2 directly to water with the formation of GSSG. Secondly, it may react directly with free

radicals such as $O_2^{-}\cdot$, $OH\cdot$ and $RO\cdot$ by a radical transfer process, yielding thiol radicals like $GSH\cdot$, $GS\cdot$ and eventually GSSG (Mohora *et al*., 2007) GSH protect cellular proteins against oxidation through the glutathione-redox cycle and directly detoxify reactive oxygen species (Selven and Rosamma, 2013).

Also Uric acid, Albumin, Thioredoxin, Sulphydryl groups, Vitamin C are considered aqueous phase of chain breaking antioxidant(Young and Woodside, 2001).

B- Lipid phase antioxidants: like Vitamin E (tocopherols and tocotrienols), the carotenoids, Flavonoid, Ubiquinol- 10 and alpha lipoic acid, these compounds may be synthesized in the body or obtained from the diet (Kibanova, 2009)..

The transition metal binding proteins, includes ferritin, tranferrin, lactoferrin and Caeruloplasmin act as an important component of the antioxidant defense system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical(Young and Woodside, 2001).

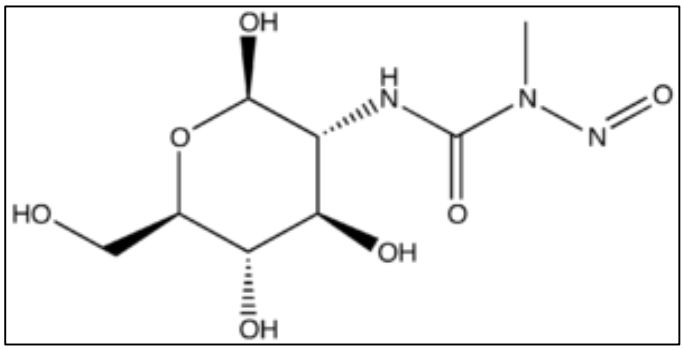
1.11. Streptozotocin

STZ, a glucosamine-nitrosourea compound obtained from Streptomyces achromogenes, is used as an experimental tool to develop animal models to study diabetes associated complication, it has been reported that STZ is capable of producing mild to severe types of diabetes according to the dosages used when it is given to animals by either single i.v. or i.p. injection (Standell , 1988).

1.11.1.Chemical properties of STZ

- A hydrophilic compound.
- An alkylating agent.
- Methylnitrosourea moiety considers the cytotoxic group that attached to the glucose.

- A beta cell toxic glucose analogue.
- Relatively stable at PH 4.7 and 37 C° (at least for up to one hour). (Poviski *et al*., 1993).



Figur (1-8): Streptozotocin

2-deoxy-2-({[methyl (nitroso) amino] carbonyl} amino)-β-D-glucopyranose

(Standell et al,.1988)

1.11.2. Biological Effect of Streptozotocin

Streptozotocin is the most prominent diabetogenic chemical in diabetes research. Streptozotocin is toxic glucose analogue that preferentially accumulate in pancreatic β-cells via the GLUT2 glucose transporter. Following its uptake into the β-cells, Streptozotocin is split into its glucose and Methylnitrosourea moiety. Owing to its alkylating properties, the latter modifies biological macromolecules, fragments DNA and destroys the β-cells, thereby impairing the function of β-cell mitochondrial metabolism causing a state of insulin-dependent diabetes, (Lenzen, 2008).

The hydrophilic properties of STZ reduces chance to enter cells without support, there for it prevents access to the brain by the blood brain barrier (Weiss, 1982).

STZ can be used in the treatment agent of human islet-cell carcinomas and tumors, because of the resistance of human beta cells to STZ toxicity, because there is very low level of constitutive glucose

transporter expression in the human β-cells, rather than the inability of human glucose transporter isoform to provide uptake STZ into the intracellular compartment that lead to high resistance of human against the diabetogenic action of STZ (Elsner *et al*., 2003).

The selective pancreatic β-cells toxicity of Streptozotocin in rodent can clarify, that the glucose moiety in the STZ structure which enables STZ to enter the β-cell via the low affinity glucose transporter in the plasma that caused enhanced expression of glucose transporter protein in the plasma membrane and occur greater toxicity of STZ (Elsner *et al*., 2000).

The importance of the glucose transporter of the toxic action of STZ is also proved by the noticing of STZ damage to other cells expressing this transporter such as hepatocytes and renal tubular cells, therefor any Streptozotocin treatment of animals leads not only to diabetes but can also cause liver and kidney damage of variable degree (Thorens *et al*., 1988), even before negative effect mitochondrial DNA and protein alkylation become evident. A STZ- induced depletion of NAD^+ may result in an inhibition of insulin biosynthesis and secretion (Stand ell *et al*., 1988).

Another factor considers important cause of the diabetogenic effect of STZ, STZ is potential to represent an intracellular nitric oxide ($NO^{\cdot}$) donor ,STZ contains a nitrous group, and can liberate nitric oxide, therefor the free radicals $NO^{\cdot}$ and nitrous radical like peroxynitrite may be an aggravating factor in the toxic action, so as the reactive oxygen species may be produced during uric acid generation as the final product of ATP degradation by xanthine oxidase from xanthine (Nukatsuka *et al*., 1990).

1.12. Propolis

1.12.1. General Description

Propolis called (bee glue) is an adhesive, balsam that smells like resin. It is collected from the buds, leaves and similar parts of trees and other plants like pine, oak, eucalyptus, poplar, chestnut and soon by bees and mixed with their wax (seven *et al*., 2010).

Propolis was being used to make the protective shield at the entrance of Beehive, also it is used to fill the cracks in the hive, to attach the corner of frames to the grooves in the hive, and also to polish the cells of the honeycomb. The bodies of dead lizards, snakes and mice that have entered hives are scaled into the wall with bee glue, thereby protecting the colonies against the unpleasant older and bacterial flora of the putrefying corpses (Ghisalberti *et al*., 1978).

1.12.2. Physical Properties of Propolis

A. Its color varies from yellow wish green to dark brown depending on its source and age (seven *et al*., 2010).

B. Consistency: it is hard and brittle when cold, but becomes soft and very sticky when warm (Vijay and Rameshwar, 2012).

C. The smell and taste: it has a pleasant flavor of popular buds, wax and vanilla but it can also have a bitter taste, when burnt, it exhibits a smell of aromatic of great value, the aroma are changed to the geographical zones (Arjun *et al*., 2002).

1.12.3. Chemical Composition of Propolis:

Propolis composed mainly by the plant resins and exudates that bees gather. Bees add wax, and also some secretions and pollen to it .The composition of propolis depend on its botanical and thus also on its geographical origin (Bank ova *et al*.,1984).

The propolis basically contains balsamic and non-balsamic component. It contains a main part of plant derived substances and minor part of bee and pollen derived substances. The balsam part of propolis originates from the collected glue, while the non-balsamic constituents are added by the bees (Cvek *et al*., 2008).

Simple fractionation of propolis to obtain compound is difficult due to its complex composition (Musa *et al.*, 2012), as that studies the chemical composition of Ethanolic Extract of Propolis attained that propolis have many effective compounds such as flavonoids (flavones and flavanones),

phenolic acid and ester in propolis extract, that it have positive physiological properties and non-toxicity (Talas and Gulhan, 2009).

Table (1-1) illustrated the composition of raw propolis (Qian *et al* ., 2008)

	Substances
BALSAM 40 - 70 % Ethanol soluble	*Phenolics* Phenols, phenolic acids, esters, flavanons, dihydroflavanons, flavons, flavonols, chalkones, phenolic glycerides ; *Others*: Aliphatics: acids, alcohols, esters, aldehydes, ketones, benzoic acid and esters
Essential oils 3-5 % ethanol soluble	Mono-, and sesquiterpenes
NON-BALSAM Wax: 20-35 % Ethanol insoluble	Beeswax components
others: ca. 5 % partly ethanol soluble	Mainly minerals average ash content 2.1 % Polysaccharides:2 % Proteins, amino acids, amines and amides: 0. 7 % Traces of carbohydrates, lactones, quinones, steroids, vitamins

Propolis also contains some minerals such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe as well as some vitamins like B_1, B_2, B_6, C and E, and number of fatty acids, in addition, it contains some enzymes such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase (Tikhonov and Mamontova,1987). Propolis contains copper 26.5 mg /kg, manganese 40 mg /kg and the ash residue contain iron, calcium, aluminum, vanadium, strontium and silicon (Moreira, 1986). 2,3- dihydroflavone derivative, 7- o- prenylstrobopinin, and 25 known diterpenes and phenolic compounds were identified from the n-butanol extract of Greek propolis (Melliou and Chinou, 2004). Iraqi propolis, a study by (Sulaiman *et al*., 2011) was performed on, propolis samples were collected from different geographical location in Iraq (Baghdad, Dahuk, Mosul and Salah al-din).

Concentrations of phenolic compounds (flavonoids, phenolic acid and their ester) in propolis were estimated, thirty eight different compounds were identified and thirty three of them were polyphenols. Other compounds were tentatively identified, diterpenoids. One compound considers unknown semi quantitative measurements showed that phenolic acid and their esters were the predominant constituents in propolis extracts.

1.12.4. The Biochemical Activity of Propolis

Propolis contains about 300 constituent (Turkez *et al*., 2010).

Recently, propolis has gained popularity in connection with oxidative stress (Tatli seven *et al*., 2008), and used widely in healthy drinks and foods to recuperate health and prevent disease such as inflammation, heart disease, diabetes and even cancer (Banskota *et al*., 2000). Because of the broad spectrum of biological properties and their different uses, there is a great interest in its biological activities, and showed that flavonoids concentrated in propolis are powerful antioxidants which are capable to scavenge free radicals, the amounts of total phenols and antioxidant activities depended on the collection site as well as on the harvesting season (Miguel et al., 2011).

Flavonoid of propolis is one of the most important compounds, thought to be responsible for many of its biological and pharmalogical activities including anti-cancer, anti-inflammatory, anti-microbial and antioxidant effect (Sforcin and Bank ova., 2011). Flavonoids from propolis play a protective role against the toxicity of the chemotherapeutic agents or radiation in mice, giving hope that they may have similar protective action in humans. The combination with an adjuvant antioxidant therapy may enhance the effectiveness of chemotherapy by ameliorating the side effect on leukocytes , liver and kidney are consequently enabling dose escalation (Orsolic, 2010).

Phenolic of different type of propolis are known to have a hepatoprotective function, which correlated to the antioxidants activity (Banskota *et al*., 2001).

Propolis counteracts hepatoxic effect of alcohol liver injury in mice (Lin *et al*., 1997), and also of paracetamol induced liver damage of mice (Conzalez, *et al* 1994) and carbon tetra chloride induced liver damage in rats (Conzalez, *et al* 1995; Bhadauria, 2011).

The anti-radiation effect of propolis has been reviewed by Orsolic, 2010, as an antioxidant propolis has a powerful effect to counteract radiation as tested in tumor cells or animal. Propolis acts also in apoptosis (cell death) of cancer cells thus improving the anti-cancer effect of radiation, as well as Orsolic , 2010 discussed the chemo preventive activity of propolis in animal models and cell cultures are likely to be the result of their ability to inhibit DNA synthesis in tumor cells ,their capability to induce apoptosis of tumor cells. And their property to active macrophages to produce factors capable of regulating the function of B-, T-, an NK-cells respectively.

The immunomodulating effect has been reviewed by Sforcin at 2007, that showed all propolis types have immunomodulating activity, in *vitro* and in *vivo* assays demonstrated the modulatory action of propolis on murine peritoneal macrophages increasing their microbicidal activity and stimulating the lytic activity of natural killer cells against tumor cells by enhancing antibody production, so the propolis has an immunomodulating effect by increasing antibody protection and by activating B and T lymphocytes, an adjuvant like activity of propolis.

Although many polyphenols have an anti-metastatic activity, caffeic acid phenethyl ester (CAPE) from propolis have identified as the most potent anti-tumor agents (Bank ova, 2009).

Regular consumption of propolis food supplements can have a preventive effect against mutation linked cancer in human (Ribeiro, 2003).

Propolis has the anti-inflammatory activity, by inhibitory effect on myeloperoxidase activity, NADPH-oxidase ornithine decarboxylase and this activity refers to the tyrosine-protein kinase from guinea pig mast cells, present of flavonoids, cinnamic acid derivatives and naringenin, the latter includes caffeic acid phenyl ester (CAPE) and caffeic acid (CA) (Almeida and Menezes, 2002).

CAPE and the galangin, both propolis constituents exhibited anti-inflammatory activity and significantly inhibited carrageenan edema, carrageenan pleurisy and adjuvant arthritis inflammation in rats (Borrelli, 2002).

As well as the propolis compounds like chrysin, quercetin and galangin have an anti-parasitic (Riou, 2011).

Propolis shows an antihypertensive effect in rats (Yoko *et al*., 2004).

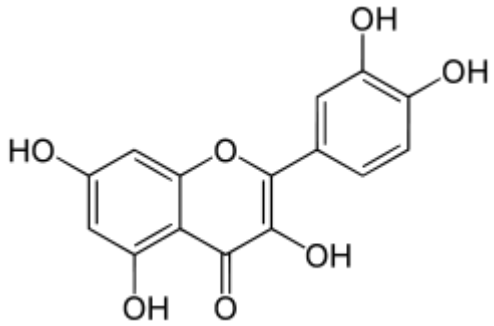
In diabetic rats, administration of bee propolis, extract led to decrease levels of Blood Glucose (FBG), Fructose Amin (FRU), Malondialdehyde (MDA), Nitric oxide (NO), Nitric Oxide Synthetase (NOS), Total Cholesterol (TC), Triglyceride (TG), Low Density Lipoprotein Cholesterol (LDL-C), Very Low Density Lipoprotein Cholesterol (VLDL-C) in Serum of fasting rats; and to increase serum level of High Density Lipoprotein Cholesterol (HDL-C) and Superoxide Dismutase (SOD). This suggest that propolis can control blood glucose and modulate the metabolism of glucose and blood lipid, leading to decrease output of lipid peroxidation and scavenge the free radicals in rats with diabetic mellitus (Fuliang *et al*., 2005).

This conclusion improve that the propolis is a powerful antioxidant.

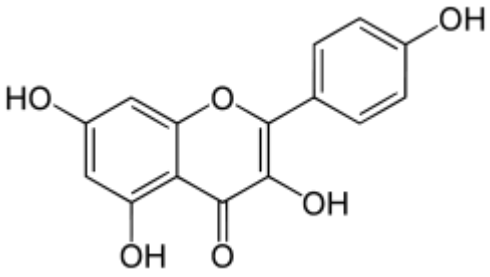
The antioxidants activity was four times higher than in vitamin in E and was 40-50 times greater than that of forest fruits and 25-50 greater than that of coffee and (Boganor., 2011).

The antioxidant activity of propolis is due to the high concentration of phenolics, flavonoids and other antioxidant compounds.

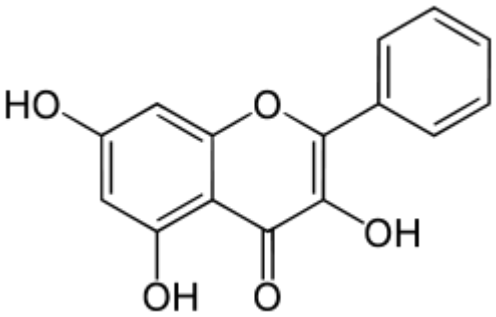
The most important flavonoids present in propolis are: Querecetin, kaempferol, galangin, fisetin and caffiec acid phenethyl ester (Martos *et al*., 2008).



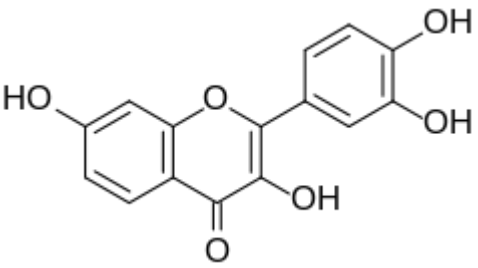
Quercetin



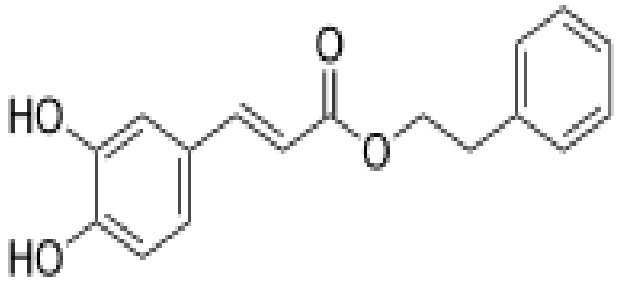
Kaempferol



Galangin



Fisetin



Caffeic acid phenethyl ester (CAPE)

Figure(1-9): The most important flavonoids present in propolis.

Aim of the study

The objective of this study was to investigate the treatment by ethanolic extract of local propolis (EEP) in Streptozotocin-induced diabetic rats, that may effective at onset of diabetes mellitus. The specific aims of experiment are:

1) This study provided evidence that these propolis samples exhibit interesting antioxidant properties.
2) To determine the anti-hyperglycemic effect of the EEP in the STZ-induced diabetes mellitus in rats.
3) To determine the regenerative effect of the effective extract in the STZ-induced diabetic rats on kidney function.

2.1. Chemicals

Table (2-1): Chemicals used in the present study.

Chemicals	Source
1-Chloro-2,4-Dinitrobenzene (CDNB)	Sigma. Aldrich. U. K
5-5-Dithiobis(2-nitrobenzoic acid) (DTNB)	Fluka. Company. Switzerland
Ammonia (98%)	Scharlau. Spain
Chloroform	BDH. England
Citric acid	BDH. England
Creatinine Kit	Syrbio . Company. Syrbio
Dipotassium hydrogen phosphate	BDH. England
Disodium hydrogen phosphate	Fluka. Company. Switzerland
Disodiumethylendiamintetraacetic acid	Fluka. Company. Switzerland
Epinphrine	Sigma. Aldrich. U. K
Ethanol(99%)	Scharlau. Spain
Ferric Chloride	BDH. England
Formalin	Scharlau. Spain
Glutathion	BDH. England
Hydrochloric acid (HCl, 38%)	BDH. England
Hydrogen peroxide	Scharlau. Spain
Iodine	Sigma. Aldrich. U. K.
Ketamine	Hameln gmbh. Germany
Lead acetate	Sigma. Aldrich. U. K.
Mercuric Chloride	BDH. England
N-(1-naphthyl)ethylenediaminedihydrochloride	Sigma. Aldrich. U. K
Phosphoric acid	Scharlau. Spain
Potassiumdihydrogen phosphate	BDH. England
Serum Total Protein Kit	Springer. Company. Spain
Serum Uric acid Kit	Syrbio . Company. Syrbio
Sodium Azide	Sigma. Aldrich. U. K
Sodium bicarbonate	BDH. England
Sodium Chloride	BDH. England
Sodium citrate	BDH. England
Sodium dihydrogen phosphate (NaH_2PO_4)	Kok. Germany
Sodium hydroxide (NaOH)	BDH. England
Sodium Nitrite	BDH. England
Streptozotocin	Sigma. Aldrich. U. K
Sulfosalicylic acid	BDH. England

Sulfuric acid	BDH. England
Sulfuric acid	Scharlau. Spain
Thiobarbutric acid(TBA)	Fluka. Company. Switzerland
Tri Chloroacetic acid	Merek. Germany
Urea Kit	Syrbio . Company. Syrbio

2.2. Equipment

Table (2-2): Equipment used in the present study.

Equipment	Company	Origin
Accu-chek Active meter and test strips for quantitative blood glucose	Roche Diagnostics Gmbh	Germany
Centrifuge	Gottlingen	Germany
Hot magnetic stirrer	IKAMAGRCT	India
High speed centrifuge	Heraens Biofugepico	England
Sensitive balance	Metler	USA
Refrigerated centrifugation	International Equipment. Co	England
Water bath	Techne Junior TE-8J	England
pH meter	Ino Lab 720	Germany
Microscope	Olympus	Japan
Oven	Memmert 8-1733	Germany
Hot plate	Lab-compinian Hp-3000	USA
Incubator	Memmert E 409-0963	Germany
Vortex Maxi mix III	Thermolyne 65800	USA
UV-VIS Spectrophotometer	Aple PD-303 UV	Japan
1-1000µl Micro pipette	Eppendrof Research	USA
10-100µl Micro pipette	Slamed	England
100-1000µl Micro pipette	Slamed	England
Surgical blade	JAI surgical limited	USA
Insulin injectors	Shanchuan	China
Eppendroff tubes(500µl)	Shanchuan	China
Syringe (5ml)	Xiang- Jiang	China
Hood	Bio Lasco	Thailand
Shaker incubator	Gallenkamp Orbital	England

2.3. Propolis collection

Propolis sample was obtained from different parts of Al-Qadisiya governorate (Diwaniya, Alseder, Saneya, and Shaffia). All propolis samples were collected by scrapping from walls of the beehives as solid samples.

The propolis samples were cleaned from debris and kept in dark and closed bottles and stored at room temperature until processing.

(Figure 2-1): Crude propolis

(Figure 2-2): Grinding crude propolis

2.4. Preparation of Local Ethanolic Extract of Propolis (EEP)

The samples of crude propolis were cut into small pieces by using medical mortar then, grinded by using of electrical grinder to a powder. The powdered crude propolis was sifted to ensure the proper practical size,

where in sieving the material is passed through a sieve of suitable mesh size giving two fractions. The fraction passing the sieve consists particles with a size smaller than or corresponding to the mesh size. The remaining fraction consists of coarser particles which are returned to the electrical grinder for further grinding.

By methods presented by (Yaghoubi *et al*,.2007). Fifty grams of crude powdered propolis macerated in (1000) ml of ethanol 70 % (was prepared from 95% ethanol by following the equation $C_1V_1 = C_2 V_2$), for (6) days with mixing and shaking by thermo magnetic stirrer (300 rpm at 25C°) (4hours/day).

The extract solution was stored over night at (4C°) to obtain crystallization of dissolved waxes. The resultant solution was filtered through a whatman filter paper no.4. Then the filtered solution was dried by using oven at (35- 45C°) till complete dryness, giving a resinous brown products.

Figure (2- 3): Extract Crude propolis.

Complete dryness of the samples was ascertained by the absence of weight loss during the last three continuous weighting, and was achieved about 15 days. The yield of extraction of propolis was determined from the proportion of dry weight of extracted propolis to that of crude propolis using the following formula:-

$$\text{The yield} = \frac{\text{Weight of crude propolis}}{\text{Weight of propolis}} \times 100$$

To prepare 2% milky solution of propolis extract, 2 grams of crude propolis was dissolved in 4 ml of absolute ethanol by using of vortex mixer, after complete dissolving, the volume was completed to 100 ml by adding distilled water.

Figure (2- 4): 2% (w/v milky solution of Local EEP)

The final concentration of ethanol in this milky solution didn't exceed 5% which had no side effect on *in vivo* and *in vitro* experiment according to what stated by (Nader, 2010).

2.5. Primary Chemical test of EEP

The ethanol extract was fractioned by using diluted HCl (Thamaraiselvi and Jayanthi, 2012).

2.5.1. Test for Flavonoids

One ml of a fraction of the extract was treated with concentrated H_2SO_4 and monitored for the formation of orange color , indicating the positive test for flavonoids (Thamaraiselvi and Jayanthi, 2012)

2.5.2. Test for Alkaloids

In this test, 0.5g of extract was stirred with 3ml of 1% aqueous hydrochloric acid, the extract was tested by the following methods:

1- Mayer's test: Mayer's reagent was prepared by dissolving 1.35g of mercuric chloride in 60ml of distilled water, then dissolving 2.5g of potassium iodide in 10ml of distilled water, then both solutions were mixed and completed the volume to 100ml by distilled water. To the extract, 2ml of Mayer's reagents was added, if alkaloids present, cream precipitate should appear.

2-Wagner test: Wagner reagent was prepared by dissolving Iodine (1.27g) and potassium iodide (2gm) in 5 ml of water and made up to 100ml with distilled water. To 2ml of filtrate, 2ml of Wagner's reagent was added, the formation of a reddish- brown precipitate confirms the test as positive (Seema and Parwez, 2011).

2.5.3. Test for Tannins

Few ml of a fraction of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish color solution, if Tannins is present(Thamaraiselvi and Jayanthi, 2012).

2.5.4. Test for Phenols

Few drops of 10% aqueous ferric chloride solution was added to 1 ml of a fraction of EEP. Appearance of greenish or yellow- greenish precipitation indicates positive result for the presence of phenols (Seema and Parwez, 2011).

2.5.5. Test for Terpenoids

5 ml of a fraction of EEP (1%) was heated with trichloroacetic acid. If a red to purple coloration was formed, indicating the presence of terpenoids (Seema and Parwez, 2011).

2.5.6. Test for Coumarins

To 1 ml of a fraction of extract, 1 ml of 10% sodium hydroxide was added . The presence of coumarins is indicated by the formation of yellow color (Harborne, 1973).

2.5.7. Test for Saponin

To 1ml of the extract, 5ml of water was added and the tube was shaken vigorously. Copious lather formation indicates the presence of Saponins (Seema and Parwez, 2011).

2.5.8. Test for Resins

50ml of ethanol (96%) was added to (5gm) of EEP. The mixture was filtered and heated over steam bath for 1 min. the mixture was filtered, then (100ml) of (4%) HCl was added to the filtered, presence of turbidity indicating presence of resins (Harborne, 1973).

2.6. Animals and Housing

Seventy-five Sprague- Dawley rats weighting between 150±10gm were used. Rats were reared at the animal house of the Veterinary medicine college in Al - Qadisiya University.

The animals were allowed to acclimatize for one week, before the experiment .The animals were housed in polypropylene cages inside a well-ventilated room. Each cage consists of not more than five rats. They were fed standard laboratory food (appendix1) and drinking water were freely available. They were maintained under standard laboratory condition of

temperature 22-25 c^{o}, and maintained on a regular feed and also they were exposed to clinical examination produced the beginning of experiment in order to ensure the good health status of all the rats.

2.7. Streptozotocin Preparation

STZ was used for induction diabetes by intraperitoneal injection of 60mg/kg bw. STZ must prepare in 0.1 M Citrate buffer (pH 4.5) (Sachin *et al*., 2009).

2.7.1. Preparation of 0.1 M Citrate Buffer

Citrate buffer (0.1M) has been prepared by mixing 10.5g of citric acid14.7g of Sodium Citrate 14.7g with 500ml deionized distilled water. The volume has been adjusted to 1000ml by adding distilled water while pH was adjusted to be 4.5 by adding drops of sodium hydroxide (0.1M) (Rajukar, 2011).

2.7.2. Preparation STZ solution

STZ was dissolved in cold 0.1M citrate buffer, pH 4.5 and always prepared freshly for immediate use within 5-20 minutes. The dose of STZ was determined according to the body weight of animals (Sachin *et al*., 2009). For this purpose, the final concentration of STZ in buffer 20mg/ml by dissolving 100mg of STZ in 5ml buffer (Noorafshan *et al*., 2005), on the other hand, all the doses of STZ were administered at volume not exceeding 1ml/100gm body weight of rats (Sachin, 2009) **.**

2.8. Induction of Diabetes:

Diabetes mellitus was induced in the overnight fasted rats by a single intraperitoneal injection of Streptozotocin (STZ, Sigma Aldrich) at a dose of 60 mg/ kg body weight**.** STZ was dissolved in citrate buffer (pH 4.5) and

freshly prepared before injection. Hyperglycemia in rats followed up for 72 hours, by using blood glucose test strips depends on the method by (Stedman, 2006), that used with Accu-Chek Active meter. Hyperglycemic rats were confirmed by the elevated glucose levels in blood on day 5 after injection. Male rats with blood glucose concentration more than 200 mg/ dl were considered as diabetic (Zhang *et al*., 2006), and used for evaluation the anti-hyperglycemic effect of the ethanolic extracts of propolis

2.9. Experimental Design

Seventy five (30 intact and 45 diabetic) rats were randomly assigned to 5 equal groups, (15rats for each group). Animals of all groups were treated as follow:

(G1) Control Rats: intact rats drenched orally with drinking water containing 4% ethyl alcohol *via* oral gavage at a dose of (10ml/kg. b. w) once a day for six weeks, including stopped for 5 days between every three weeks, after single injected of citrate buffer (3ml/kg. b. w).

(G2) Diabetic rats: Rats were drenched with drinking water containing 4% ethyl alcohol (10ml/kg. b. w) *via* oral gavage for three weeks, then after overnight fasting (deprived of food for 16 hours had been free access to water), diabetes were induced by i.p injection of STZ. After five days to ensure induction diabetes, rats were drenched with water containing 4% ethanol at a dose level (10ml/kg. b. w) daily for three weeks.

(G3) Treatment intact rats: Drenched with EEP at dose of (200mg/kg. b. w), in average of (10ml/kg. b. w) from freshly prepared milky solution for three weeks, then injected with single dose of citrate buffer (3ml/kilo. b. w). After stopped for 5 days, rats were continued in drenching EEP for other three weeks.

(G4) Diabetic with Pre- treated of EEP: Rats were pretreated with local EEP at a dose of (200mg/kg. B. w) *via* oral gavage for three weeks, after

that they were injected with STZ (60mg/kg) to induce diabetes , then after 5 days was continued drenched orally with distilled water containing 4% ethyl alcohol for three weeks.

(G5) Post-treated Diabetic rats: Rats were drenched with drinking water containing 4% ethyl alcohol at (10ml/ kg. b. w) for three weeks *via* oral gavage, after that post-treated with local EEP at dose of (200mg/kg. b. w) daily for other three weeks, starting after 5 days of STZ (60mg/kg. b. w) injection and diabetes induced.

Every three days body weights have been registered by electric balance, and blood glucose level of rats(groups 2, 4 and 5) were taken three days after induction diabetes, by using blood glucose monitoring instrument, type Accu-chek Active meter(blood was withdrawn from tail-vein).

After 49 days of the experiment, all rats were withheld for the following day and food for 12 hours before blood sample collection. All rats were anesthetized with xylazine and ketamine (10mg and 90mg/kg, i.p, respectively)(Lei *et al*., 2001). After sedation ,each rats was fixed on the rat dissecting table, and mid line thoracic incision was made to the lower abdominal posterior vein, which the greater vessels were easily exposed. After obtaining of blood, animals were sacrificed; samples from kidney in all groups have been quickly removed, and fixed in 10٪ neutral buffered formalin.

Figure (2-5): anesthetization rats

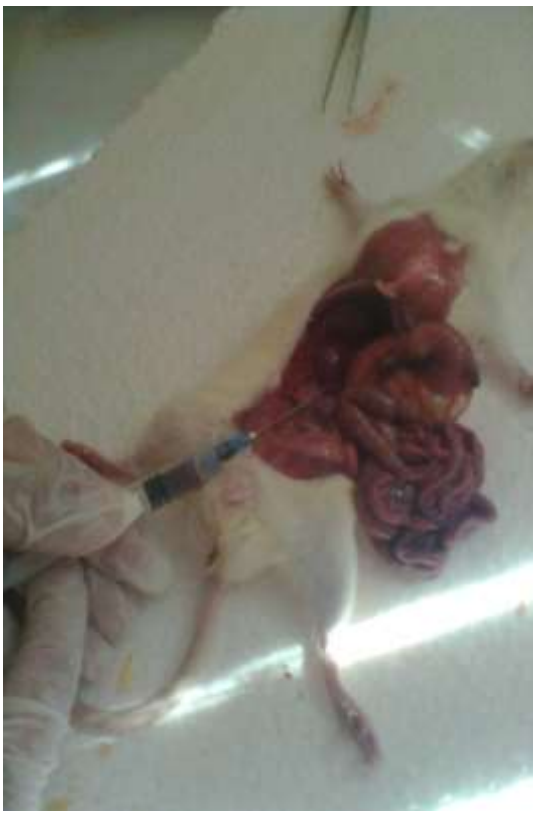


Figure (2-6): blood collection

2.10. Serum preparation

Blood was collected in test tubes with cap and allowed to clot (for 20 minutes), then serum was separated by centrifugation at (4000 rpm, 0.894xg) for 10 minutes (Laessig *et al*., 1976). The separated serum of each animal was subdivided nearly into (6) samples using appendroff tubes (0.5ml) and kept at deep freezer until using for assessment of the biochemical parameters.

2.11. Biochemical Test:

2.11.1. Determination of serum superoxide dismutase (Misra and Fridovich.1972)

A: Principle

The method is based on the SOD ability to inhibit the Epinephrine oxidation to adrenochrome.

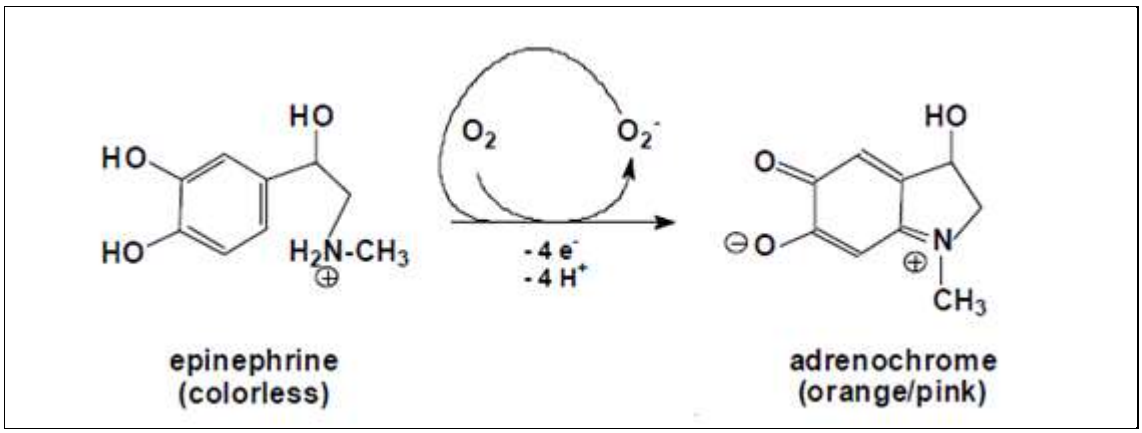


Figure (2- 7): Epinephrine oxidation to adrenochrome (Misra and Fridovich).

:

The $O_2^{-\cdot}$ Substrate for SOD is generated indirectly in the oxidation of epinephrine at alkaline pH by the action of oxygen on epinephrine. As $O_2^{-\cdot}$ Builds in the solution, the formation of adrenochrome accelerates because $O_2^{-\cdot}$ Also reacts with epinephrine to form adrenochrome.

SOD reacts with $O_2^{-\cdot}$ Formed during the epinephrine oxidation and therefor slows down the rate of formation of the adrenochrome as well as the amount that is formed. Because of this slowing process, SOD can inhibit the oxidation of epinephrine. One unit of SOD activity was defined as the concentration of the enzyme in the serum that caused 50 % reduction in the auto-oxidation of epinephrine (Jewett and Rockling, 1993).

B: Reagents

1-Working carbonate buffer (0.05M) pH 10.2:

This solution was prepared by dissolving 4.2gm of $NaHCO_3$ (M.W=84.01gm/mole) in about 800ml of distilled water. The pH was adjusted to 10.2 by added (3M) NaOH until reached to appropriate pH then the buffer solution was transferred to 1L volumetric flask and made up to the mark. The buffer solution was transferred to clean plastic storage bottle because silicates will be removed from the glass in the alkaline medium.

2-Na_2EDTA solution (1×10^{-4} M).

This solution was prepared by dissolving 0.039gm of $Na_2EDTA.2H_2O_2$ (M.W=372.2gm/mole) in about 250ml of distilled water, and then total volume was completed to 1L.

3- Epinephrine 0.01M.

This solution was prepared by dissolving 45.8mg of epinephrine (M.W=183.2gm/mole) into the clean dry 25ml volumetric flask, after that immediately added HCl (0.02M) to dissolve epinephrine and completed to the mark by distilled water.

C: Procedure

Three sets tubes were prepared as follow:

Reagent	Blank	Control	Sample
Serum	_	_	0.1ml
Carbonate buffer	1.8ml	1.8ml	1.8ml
Epinephrine	_	0.1ml	0.1ml
Na_2EDTA	1ml	1ml	1ml

Absorbance was determined at wave length 480nm immediately after addition of epinephrine and after 5 minutes, by using spectrophotometer Aple PD-303 UV.

D: Calculation

The percentage of inhibition was calculated as follow:

$$\text{Inhibition\%} = \frac{\Delta A\ control - \Delta A\ sample}{A\ control} \times 100$$

One unite of SOD was defined as the amount of enzyme that inhibits the oxidation 50% of epinephrine.

2.11.2 Determination of Serum Catalase (CAT)(Aebi, 1974).

A: Principle

Catalase (CAT) which catalyze the decomposition of Hydrogen peroxide, therefore its activity was determined by the decrease in absorbance due to H_2O_2 consumption (Mueller *et al.*, 1997)

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

B: Reagents

1. Phosphate buffer solution (50 mM, pH 7.0) was prepared by the following steps:

- Potassium dihydrogen phosphate (KH_2PO_4) (5 mM), was prepared by dissolving 6.81gm of KH_2PO_4 (136.09 gm /mole) in 1L of distilled water.
- Disodium hydrogen phosphate (Na_2HPO_4) (38.772 mM), was prepared by dissolving 6.9 gm of Na_2HPO_4 (141.96 gm/mole) in 1L of distilled water. Then 390 ml of (KH_2PO_4) solution was mixed with 610 ml of (Na_2HPO_4) solution , and the pH was adjusted to 7.0.

2. Hydrogen peroxide (H_2O_2) (30 mM).

The solution of hydrogen peroxide was prepared by dilution 0.34 ml of 30% H_2O_2 with phosphate buffer solution to 100 ml, this solution was prepared immediately before the test.

C: Procedure

1. 50 µl of serum was diluted with 5 ml of phosphate buffer solution (50 mM), pH 7.0 immediately before the assay.
2. Two sets of tubes were prepared as follow:

Reagents	Sample	Blank
Serum	2 ml	2 ml
Phosphate buffer solution (pH 7.0)	-	1ml
H_2O_2	1 ml	-

The reaction was started by adding hydrogen peroxide (freshly prepared), then all tubes were mixed immediately and the initial absorbance

after 15 seconds (t_1) and the final absorbance after (30) seconds (t_2) were read at 240 nm by using spectrophotometer Aple PD-303 UV.

D: Calculation

$$\text{Activity of CAT (K/ml)} = \frac{Vt}{Vs} \times \frac{2.3}{\Delta t} \times \log \frac{A1}{A2} \times 60$$

Where:

K: first order rate constant. It expresses the activity of catalase.

Δ_t: is (time $_2$ – time $_1$) and it is equal to 15 seconds.

A_1: is the initial absorbance at 15 seconds.

A_2: is the final absorbance at 30 seconds.

V_t : is total assay volume =3ml

V_s : is sample volume in the assay mixture = 2ml

K/ml = is specific activity

So the activity of CAT (K/ml) $= 13.8 \times \log \frac{A1}{A2}$

2.11.3. Determination of Serum Glutathione Peroxidase (Flohe and Gunzler, 1984)

A: Principle

The principle of this method is that the rate of glutathione oxidation by H_2O_2 as catalyzed by the GPx present in the supernatant is determined. The color that develops is read against a reagent blank at the range 420 nm on a spectrophotometer, one unit is the amount of GPx that produces 1μmole of GS-SG per minute (Rotruk *et al*., 1973).

$$2GSH + H_2O_2 \xrightarrow{GPX} GS\text{-}SG + 2H_2O$$

GPx activity is reported as units based on the definition :

1 Unit of GPx = the amount of enzyme necessary to catalyze the oxidation (by H_2O_2) of GSH to GSSG, per minute.

B: Reagents

1. Phosphate buffer (0.1M, pH7.4)

This solution was prepared as follow:

- 2.6gm KH_2PO_4
- 21.7gm Na_2HPO_4
- 8.71gmNaCl

All of these material were dissolved in 800 ml of distilled water ,adjusted pH to 7.4 (if necessary) ,then brought volume to 1L with distilled water.

2. 2mM Glutathione was prepared by dissolving 0.015gm of glutathione (M.W= 307.33) in small amount of distilled water, then total volume is completed to the mark of volumetric flask of 25 ml.

3. 10mM Sodium azide was prepared by dissolving 0.016gm of sodium azide (M.W=65.01) in small amount of distilled water ,then transferred the solution to volumetric flask of (25ml) and completed the volume to the mark.

4. 1mM Hydrogen Peroxide was prepared by adding 1µl of 30% hydrogen peroxide to 8.8ml of distilled water.

5. DTNB(5-5-dithiobis(2-nitrobenzoic acid) (M.W=396.3gm/mole) was prepared by dissolving 40mg of DTNB in a small amount of ethanol and completed the volume to the mark of volumetric flask of 25ml.

6. Trichloroacetic acid (TCA) 5%, this solution was prepared by dissolving 5gm of TCA in a small amount of distilled water, then completed to the final volume 100 ml.

C: Procedure

Two sets tube was prepared as follow:

Reagents	Blank	Sample
Serum	_	300µl
Phosphate buffer pH7.4	300µl	300µl
Glutathione	200µl	200µl
Sodium azide	100µl	100µl
Hydrogen peroxide	100µl	100µl

The sets of sample and blank tubes were put in water bath 37Cº for 15 minutes, after that added 300µl of 5% TCA.

Cooled sample tubes and centrifuge at 450X for 15 minutes, and the supernatant was taken out and prepared as follow:

Reagent	Blank	Sample
Supernatant	_	100µl
Distilled water	100µl	_
Phosphate buffer pH7.4	300µl	300µl
DTNB	700µl	700µl

All tubes were read at wave length 420nm by using spectrophotometer Aple PD-303 UV.

D: Calculation

$$\text{Glutathione Peroxidase Activity (U/L)} = \frac{(AT - AB) \times 1000000}{E \times L} \times D$$

Where: AT = Absorbance for test samples at 420nm.

AB = Absorbance for Blank at 420nm.

L = Cuvette length =1cm.

E = Extinction coefficient = $6220 M^{-1}.cm^{-1}$.

D= Dilution factor.

2.11.4. Determination of serum Glutathione-S- Transferase (Habig *et al*., 1974)

A:Principle

The activity of glutathione S-transferase (GST) was determined by the method of Habig and Jacoby. The conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), a hydrophilic substrate, was observed spectrophotometrically at 340 nm to measure the activity of GST. One unit of GST was defined as the amount of enzyme required to conjugate 1 µmole of CDNB with GSH per min.

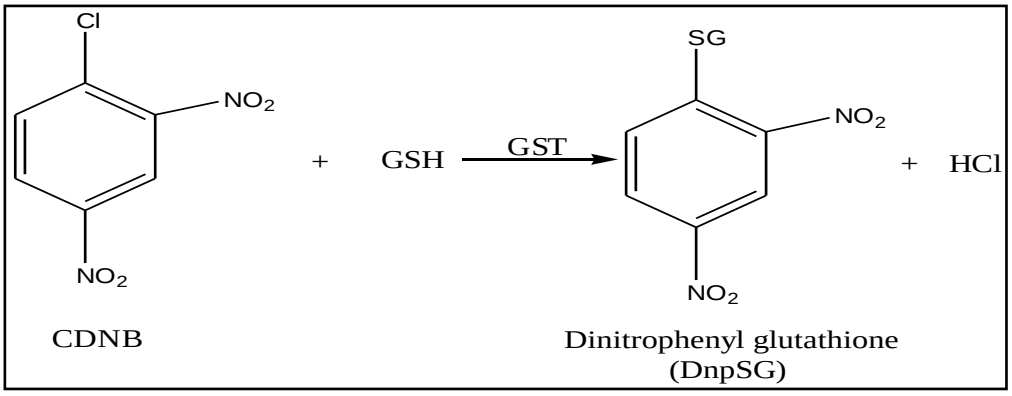


Figure (2-8): Reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione in the presence of glutathione S-transferase (GST) enzyme, (Yildiz & Kurman, 2004).

B: Reagents:

1. Glutathione solution (29.93 mM).

   This solution was prepared by dissolving 0.092 gm of glutathione (307.33gm/mole) in a small amount of distilled water, then total volume was completed to 10 ml.

2. 1-Chloro-2,4-Dinitrobenzene (CDNB) (22.463mM).

   This solution was prepared by dissolving 0.455gm of CDNB (M.W=202.55gm/mole) in small amount of ethanol (95%), then total volume was completed to 100ml with distilled water.

3. Phosphate buffer solution, pH 6.25.

   This solution was prepared by dissolving 0.4355gm of dipotassium hydrogen phosphate (K_2HPO_4) and 3.060gm of potassium dihydrogen phosphate in 250 ml of distilled water, and then the pH was adjusted to 6.25.

C: Procedure

Two sets of tubs were prepared as follow:

Reagents	**Sample**	**Blank**
Phosphate buffer pH 6.25	2.7 ml	2.7 ml
Serum	100 µl	-

Distilled water	-	100 µl
CDNB solution	100 µl	100 µl
After 3 minutes add GSH solution	100 µl	100 µl

Then, all tubes were mixed and the absorbance was firstly measured at 1minut and finally at 10 minutes was read at 340 nm.

D: Calculation

Enzyme activity was expressed as (U/L) is calculated according to the following equation:

$$\text{Activity of GST (U/L)} = \frac{\Delta A/10 \times V_t \times 1000}{\varepsilon \times V_s \times d}$$

Where:

ΔA: the difference between the first absorbance at 1st minute and the tenth one at 10th minute.

V_t: the total volume =3ml

V_s: the sample volume =0.1ml

ε: extinction coefficient (9.6 mM^{-1} cm^{-1}).

d: light bath = 1 cm

$$\text{Activity of GST (U/L)} = \frac{\Delta A/10 \times 3 \times 1000}{6.9 \times 0.1 \times 1}$$

2.11.5. Determination of Serum Glutathione Concentration (Burtis & Ashwood, 1999).

A: Principle

TNB (5,5'-Dithiobis(2-nitrobenzoic acid)), known as Ellman's Reagent, was developed for the detection of thiol compounds. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, the absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration, figure (2-11).

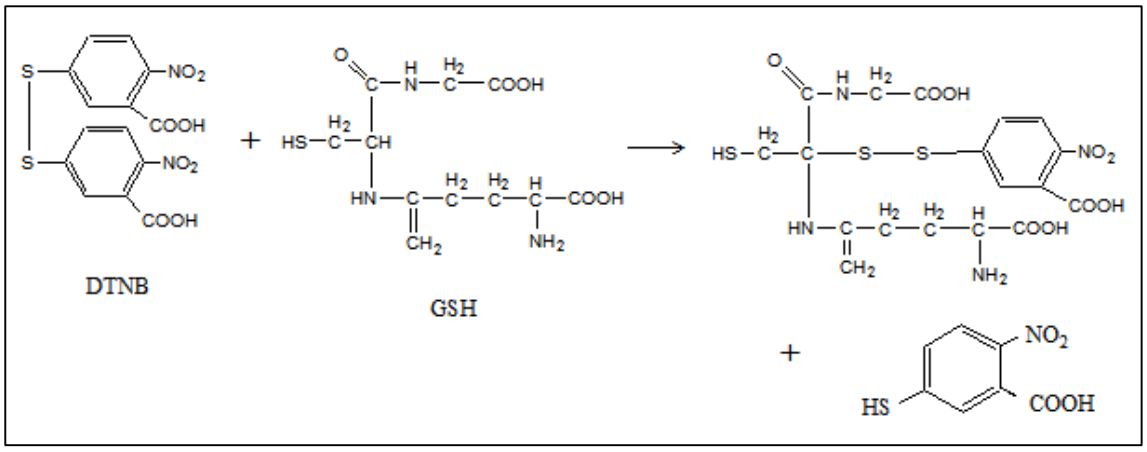


Figure (2-9): Reaction between glutathione (GSH) and 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) (Sedlak & Lindsay, 1968).

B: Reagents

1. 4% 5-sulfosalicylic acid.
2. Ellman's reagent 0.1mM was prepared from DTNB (5-5-dithiobis (2-nitrobenzoic acid).; M.W= 396.3 gm /mole) in phosphate buffer pH 8.0
3. Phosphate buffer pH 8 (this solution was prepared by a mixture of 0.6 M KH_2PO_4, and 0.8M Na_2HPO_4).

C: Procedure

Two sets of tubes were prepared as follow:

Reagent	**Sample**	**Blank**
Serum	150 µl	-
4% 5-sulfosalicylic acid	150 µl	150 µl
sample test tube was mixed, and centrifuged at 450 X g at 4°C for (5) minutes, then:		
Supernatant	150 µl	-
Ellman's Reagent	4.5 ml	4.5 ml

All tubes were mixed, and the absorbance of sample was read at 412 nm by using spectrophotometer instrument Aple PD- 303UV.

D:Calculation

The concentration of serum glutathione was measured by using the following equation:

$$\text{The concentration of GSH} = \frac{A_{\text{of sample}}}{\varepsilon \times L}$$

Where:

ε = Extinction coefficient (13600 M^{-1} cm^{-1})

L=Light path (1cm)

2.11.6. Determination of Serum Malondialdehyde Concentration (Guide and Shah, 1989)

A: Principle

The measuring MDA is based on the reaction with thiobarbituric acid (TBA), for assay by the Spectrophotometric method. MDA reacts with thiobarbituric acid under high temperature (90-100 ^{o}C) and acidic condition, The reaction yields a pink MDA-TBA.

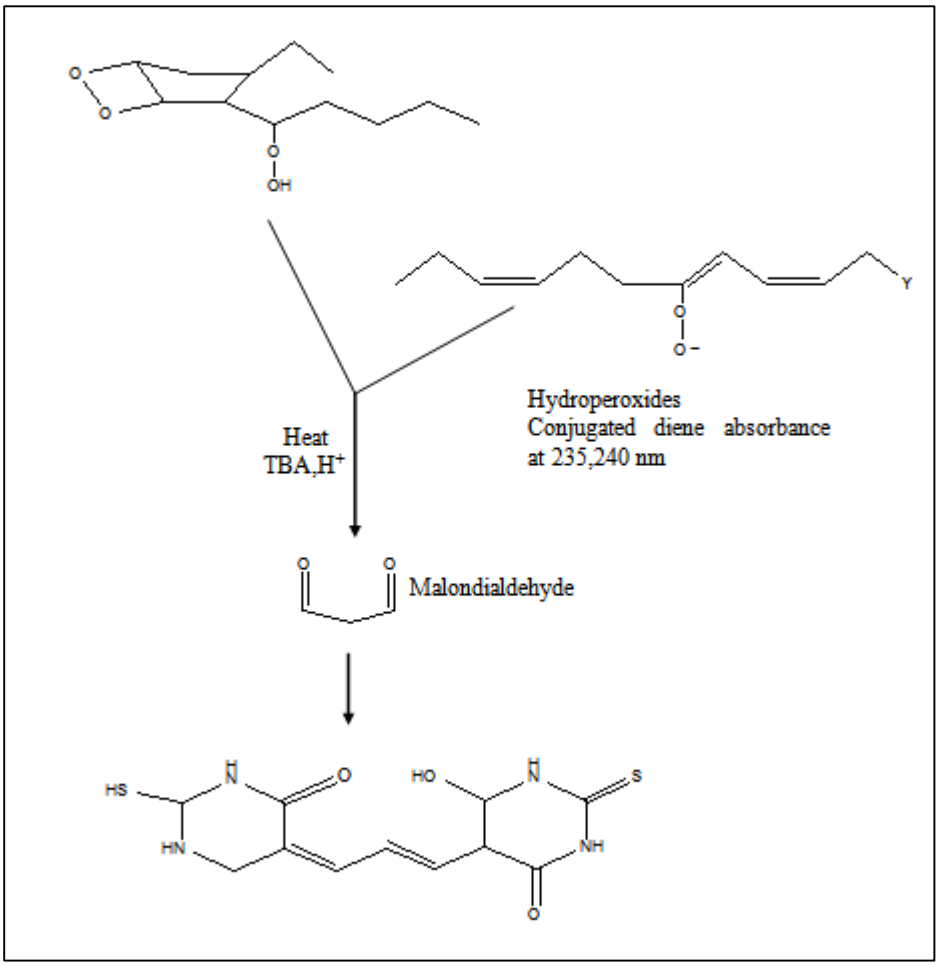


Figure (2-10): Schematic diagram for assessment lipid peroxidation via determination the byproduct; malodialdehyde (Lucene J., 1990)

B: Reagents

1- 0.6 % Thiobarbutric acid (TBA) reagent (0.6%) was prepared by dissolving 16mg of (TBA) in 10ml of distilled water using a water bath to ensure complete dissolving of (TBA).

2- 70 % Trichloroacetic acid, seventy grams of trichloroacetic acid (TCA) were taken and dissolved in final volume of distilled water.

3- 17.5% Trichloroacetic acid, five milliliter of 70%TCAwere taken and the volume was completed to 20ml with distilled water.

C: Procedure

1. Two set of tubes were prepared as follow:

Reagent	**Sample**	**Blank**
Serum	150 µl	-
TCA (17.5%)	1 ml	1 ml
TBA (0.6%)	1 ml	1 ml
All tubes were mixed well by vortex, incubated it in boiling water bath for (15) minutes, then allowed to cool		
TCA (70%)	1 ml	1 ml

2. The mixture was let to stand at room temperature for 20 minutes and centrifuged at 450X g for 15 minutes, and the supernatant was taken out to read the absorbent of sample at 532 nm by using spectrophotometer instrument type Aple PD- 303UV.

D: Calculation

$$\text{The conc. of MDA} = \frac{A_{\text{of sample at 532 mn}}}{L \times \varepsilon} \times D$$

where:

L = light path (1cm)

ε = Extinction coefficient ($1.56 \times 10^5 M^{-1} cm^{-1}$)

D = Dilution factor

$$D = \frac{\text{Volume used in reference}}{\text{Volume of the sample}} = \frac{1+1+1+0.15}{0.15} = 21$$

2.11.7. Determination of Serum Nitric Oxide Level (Hortelano *et al.*,1995).

A: Principle

A spectrophotometric determination using a reaction between nitrite, sulfanilamide and N-(1-naphthyl)ethylenediaminedihydrochloride to produce a pink colored complex which is measured by its absorbance at 548nm. Under acidic conditions, sulfanilic acid is converted by nitrite to a Diazonium salt, which readily couples with N-(1-naphthyl)-etheylenediamine to form a colored Azo dye that can be detected at 548nm.

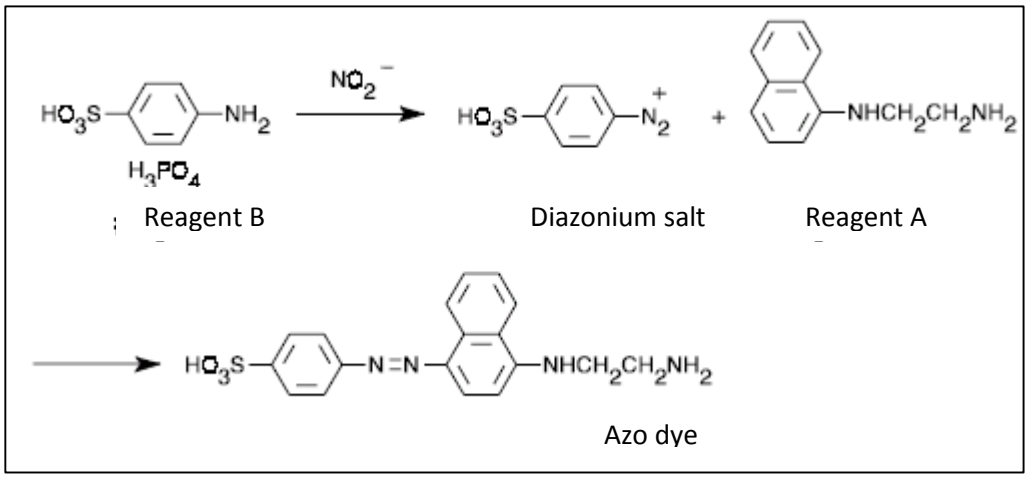


Figure (2- 11): Principle of nitrite quantitation using the Griss reaction (Green *et al.*,1982)

B: Reagents

1. Griess reagent containing:
   a) N-(1-naphthyl)ethylenediaminedihydrochloride (component A), 25ml of a 0.1% (1mg/ml) solution.
   b) Sulfanilic acid (Component B), 25ml of a 1%(10mg/ml) solution in 5% Phosphoric acid.

Equal volumes of components A and B were Mixed together to form the Griess Reagent, that must Prepared sufficient reagent for immediate experiments only.

2. Nitrite standard solution (Component C), 1ml of 1mM sodium nitrite in distilled water.

C: Procedure

Two sets of tubes were prepared as follow:

Reagent	Blank	Sample
Serum	_	300µl
Griess Reagent	100µl	100µl
Distilled water	2.9ml	2.6ml

After mixing reagents, let's incubate the mixture for 30 minutes at room temperature, then measured the absorbance of the nitrite containing a sample at 548nm relative to the reference sample.

Converted absorbance reading to nitrite concentration as described in the calibration of sodium nitrite.

D: Calibration

1mM Sodium nitrite standard was prepared by dissolving 0.69mg of Sodium Nitrite (M.W=69gm/mole) in a small amount of distilled water, then total volume is completed to 10 ml.

The calibration curve of Sodium Nitrite solution with concentration between 1-100µM by diluting the nitrite standard solution with distilled water, then the absorbance was read at 548nm for each concentration.

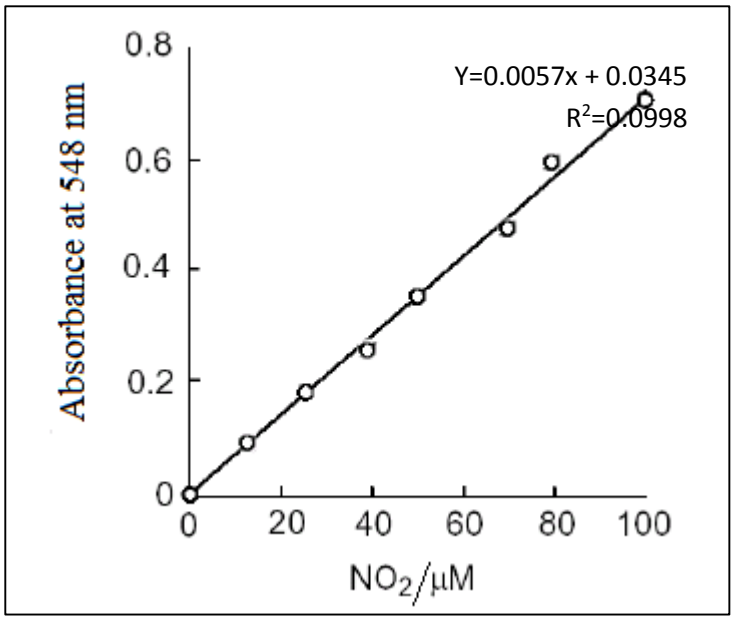


Figure (2- 12): Calibration curve of Nitrite Solution.

2.11.8. Determination of Serum Uric acid (Fossati etal.,1980)

A: Principle

This assay depends on the oxidation of Uric acid by Uricase to Allantoine and Hydrogen peroxide according to the following reaction:

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoine} + CO_2 + H_2O_2$$

$2H_2O_2$ + DHBS + 4-aminoantipyrine $\longrightarrow$ Red quinone $+H_2O$ +HCl (in presence of peroxidase).

B: Reagents

Reagent 1:

Phosphate buffer	150mmol/l

Reagent 2: It contains from:

Peroxidase	660U/l
4-Aminoantiyrine	1.0mmol/l
Uricase	90U/l

DHBS 2.0mmol/l

Reagent 3:

Standard Uric acid 6mg/dl

Preparation of working reagent:

Reagent 2 dissolved in contents of one bottle buffer R1 (125ml).

C- Procedure:

Three sets of tubes (samples, blank, Standard) were prepared as follow:

Reagents	Blank	Sample	Standard
Standard	_	_	20µl
Serum	_	20µl	_
Working reagent	1ml	1ml	1ml

Tubes were mixed and incubated for 15minutes at room temperature , then read the optical density (O.D) of the samples and standard against the blank at the wave length 510 nm, by using spectroscopy Aple PD-303UV.

D- Calculation:

The concentration of Uric acid was calculated by the following equation.

$$\text{Uric acid concentration (mg/dl)} = \frac{\text{O.D Sample}}{\text{O.D Standard}} \times \text{Standard concentration}$$

2.11.9. Determination of Serum Total protein (Burtis *et al.*, 1999)

A: Principle

The principle of this method, the proteins give an intensive violet-blue complex with copper salts in an alkaline medium. Iodide is included as an antioxidant.

The intensity of the color formed is proportional to the total protein concentration in the sample (Burtis *et al*., 1999). The absorbance of sample against blank was read at the wave length 540 nm .

B: Reagents:

R1: Biuret that contain from:

1. Sodium potassium tartrate 15mmol/L
2. Sodium iodide 100mmol/L
3. Potassium iodide 5mmol/L
4. Copper (II) sulfate 19 mmol/L

R2: T protein Cal (Bovine albumin) 7g/dL

C: Procedure

Three sets of tubes (sample, blank, standard) were prepared as follow:

Reagent	Blank	Standard	Sample
R1	1.0 ml	1.0 ml	1.0 ml
R2(Standard)	_	25µl	_
Serum	_	_	25µl

Tubes were mixed well ,incubated 10 min at room temperature, and the absorbance (A) of the samples and standard against the blank by using Aple PD-303 UV.

D: Calculation

The concentration of total protein was calculated utilizing the following equation :

$$\text{Total protein (g/dL)} = \frac{(A)\text{Sample}}{(A)\text{Standard}} \times 7(\text{Standard conc.})$$

2.11.10. Determination of Serum Urea Level (Chaney and Marbach, 1962).

A: Principle

This method is based on the fact that Urea is hydrolyzed by water and Urease into ammonia and Carbon dioxide .The ammonia produced is further acted with hypochlorite and salicylate to form a green complex.

$$Urea + H_2O \longrightarrow 2NH_3 + CO_2$$

The absorbance was done at 600 nm.

B: Reagents

R_1:

Phosphate buffer	100 mmol/L
Na Salicylate	62 mmol/L
Na Nitroprusside	5.0 mmol/L
EDTA	1.48 mmol/L

R_2:

Enzyme reagent (Urease) ≥	5 UI/ml

R_3:

Sodium Hypochloride	7.0 mmol/L
Sodium Hydroxide	150 mmol/L

R4:

Standard	50 mg/dL

Preparation of working reagent by dissolving the R_2 in contents of one bottle buffer R_1.Rinsing bottle 2 several times and waited for 15 minutes before use.

C- Procedure:

Three sets of tubes (sample, blank, standard) were prepared as follow:

Reagents	Blank	Standard	Sample
Standard (R4)	-	10 µl	-
Serum	-	-	10µl
Working reagent	1 ml	1ml	1ml

Mixed well incubate for 5 minutes at 37°C.

R3	1ml	1ml	1ml

Then, Mixed well incubate for 10 min at room temperature and then the absorbance of the samples and the standard were read against the blank by using spectrophotometer type Aple PD- 303 UV.

D: Calculation

The concentration of Urea was calculated by using the following equation:

$$\text{Serum Urea concentration (g/dl)} = \frac{\text{A sample}}{\text{A standard}} \times \text{standard concentration}$$

2.11.11. Determination of Serum Creatinine Concentration (Jaffe, 1886).

A: Principle:

It is based on the idea that Creatinine in alkaline picrate solution, forms a color complex . The rate of formation of complex is measured at 500 nm.

B: Reagents

R1: Sodium hydroxide 313 mmol/l

R2: Picric Acid 35 mmol/l

R3: Standard 2 mg/l

Perpetrated of working Reagent, by Mixed proportionally 1/1 the reagents R1 and R2.

C: Procedure

Reagents	Standard	Sample	Blank
Standard	100µl	-	-
Serum	-	100µl	-
Working reagent	1 ml	1 ml	1 ml

Mixed well, and after 30 second, read the optical density($O.D_1$) and exactly 1 minute after first reading ($O.D_2$).

D: Calculation

The concentration of creatinine in serum

$$= \frac{(O.D2 - O.D1)\ sample}{(O.D2 - O.D1)\ standard} \times \text{Standard concentration}$$

2.12. Histopathological examination

2.12.1. Preparation of Histological Sections (Lee, 1968)

The procedure includes the following steps:

1. Fixation: a complex series of chemical events that is coming up by reaction between the fixative (as neutral buffered formalin 10%, in order to keep everything as their in vivo relation to each other, and that can be accomplished by keeping kidney in neutral buffered formalin 10% for several days.
2. Tissue processing: Kidney samples were processes to embed them in a solid medium firm enough to support the kidney tissues and give it sufficient rigidity to enable thin section to be cut, and that was made in three stages:

a) Dehydration : To remove fixative and water from the tissues and replace them with dehydration fluid, the kidney samples were washed in water for three hours to remove the formalin residue, then the sample were passed through ascending grades of increasing ethanol concentration

(70%, 80%, 90%, 96% and 100%) for about 1- 2 hour for each concentration.

b) Cleaning: In order to replace the dehydration fluid with a fluid that is totally miscible with both the dehydration fluid and the embedding medium, kidney sample, were macerated in xylene for 3 hours.

c) Embedding : For providing sufficient external support during sectioning, kidney samples were macerated in a container contain liquid paraffin in 56- 58 Cº and leave it in room temperature to be solid rectangular blocks of paraffin which released from container to be kept in freezer.

d) Sectioning: By using of rotary microtome, blocks were cutting into sections each of 5µm thickness and the ribbons, were mounted on clean glass slide protracted with him film of Mayer's albumin fixative. The slides were put on a hot plate (50Cº) over night to day.

2.12.2. Staining of histological sections (Wood and Ellis, 1994)

It was accomplished as follows:

1. Slides were deparafinized by putting it in over (55- 70Cº) for 5 hours, and then it was macerated in xylene for 1 hour.
2. Slides were dried from xylene and the rehydrated by entering it in a series of ethanol concentration (100%, 95%, 70%) for 3minutes in each concentration.
3. Slides were rinsed in distilled water for (5) minutes.
4. Slides were stained in Hematoxylin stain for (6) minutes.
5. Then slides were rinsed well in running tap water for (20) minutes.
6. Slides were decolorized in acid alcohol few second, then rinsed well in tap water for (15) minutes.
7. Slides were immersed in lithium carbonate for (3) seconds and then rinsed well in tap water for (5) minutes.
8. Then slides were counterstained in Eosin stain for (15) seconds.

9. Slides were dehydrated by putting it in a graded series of ethanol concentration (95%, 95%, 100% and 100%), for (3)minutes in each concentration.
10. Slides then were cleaned in xylene for (5) minutes.
11. Finally mounting the slides were done by using of sticky material D.P.X. and cover slipping with cover slide and dried to be ready for microscopic examination.

Histopathological changes sections reading by helping pathologist in college of Medicine in Al-Qadisiya University.

2.13. Statistical analysis

All the groups data were statistically reading by SPSS program, Version 17 software (2010). Testing methods including one and two ways (ANOVA) for comparisons among groups followed by Least significant differences (LSD) test for comparison between groups. P values of $p < 0.05$ were considered to record statistical significance. All data were expressed as means ± standard error (SE).

3.1. The Propolis Extract and The Yield

In this study, the local EEP is obtained, after complete dryness was viscous and dark brown material. The yield of EEP according to different part of Al-Diwaniya province is range between 38%- 42%. This yield agreement with documented by previous study as(Cunha *et al*., 2004) they obtained 38.23%- 40.43% yield of EEP after extraction of Brazilian propolis with absolute alcohol by maceration, and also more than the yield of stated by (Al-Mohana,2004) who obtained 33% yield of EEP from propolis that obtained from different Iraqi provinces.

3.1.2. The Chemical Constituents of Local Propolis

The positive results for chemical constituent indicated that EEP contains a group of substances that have medical activity as well as exhibiting physiological activity.

The presence of flavonoids, tannins, resins, terpenoids, and saponin in propolis came in agreement with that reported by other researchers (Koo and Park,1997; Munzo *et al*.,2001).

The absence of Coumarines in EEP in this study is agreed with that stated by Al- Mohana (2004), but came in contrast with the result of Marcucci *et al* (2001).

The presence of alkaloids came in agreement with that reported by other researchers (Foket *et al*., 2010 and Robert *et al*., 2012), but it in disagreement with the result of Al- Mohana (2004) who noted the negative results for the presence of alkaloids. This contrast might be attributed to the variation in plant sources in areas of propolis as well as the difference in season of propolis collection.

3.2. Body Weights

All groups of rats was shown nearly the same gradual increase in their body weight until day 21,but after induction the diabetes, the differences in the body weight appeared as that shown in table (3-1). G1(control) and G3 (treated with EEP) rats shown gradual increase in body weight, while G2 (diabetic rats) showed marked and gradual significant reduction in body weight reaching 21% ($p<0.05$) compared to that of the G1 and G3 groups. In G4 (pre-treated with EEP) and G5(post-treated with EEP), the decrease in body weight was suppressed, and the reduction in the body weight recorded 7% and 14%) ($p<0.05$), respectively compared to

G1(control). Accordingly, with diabetes, the previous findings will have to match up with the breaking down in the muscles, when this happen rats will tend to lose weight. On the other hand, the glucose levels are high and body cells utilization of glucose was strongly depressed, because of this, glucose will tend to pass urine quit a few times, this will naturally result in dehydration. Because of this, the rats will tend to lose weight. However, male rats used in the present experiment were in the growing stage (they were in the early maturity). So, they didn't lose weight but gained little.

The mean body weight of control group rats and those given local EEP alone (G3) increased gradually throughout the experimental period, without significant difference between the gain in the body, that shown in figure (3-1),this result agrees with the research of (El-Nahrawy *et al*., 2012) but disagrees with the report of Denli *et al* (2005) whom shown increase in body weight when propolis was used in Coturnix bird. But in diabetic groups propolis treatment showed significant amelioration in body weight. As a strong antioxidant and free radical scavenging effect (Valadares *et al*.,2008), propolis may improve the disturbed metabolism associated with diabetes.

Table (3-1): Effect of EEP on body weight (g) in STZ- induced diabetic male rats.

Day	Control G1	Diabetic rats G2	Treatment with EEP. G3	Diabetic with Pre-treated of EEP. G4	Diabetic with post- treated of EEP. G5
1	154.38∓1.73 **Aa**	158.86∓0.98 **Aa**	156.73∓2.0 **Aa**	153.06∓1.2 **Aa**	158.53∓1.15 **Aa**
4	165.93∓2.4 **Bb**	170.46∓1.16 **Bb**	172.06∓1.22 **Bb**	174.86∓1.35 **Bb**	171.53∓2.09 **Bb**
7	176.5∓2.62 **Cc**	182.66∓1.24 **Cc**	185.40 ∓1.23 **Cc**	185.76∓2.07 **Cc**	181.06∓3.99 **Cc**
10	188.26∓2.51 **Cd**	195.8∓1.29 **Dd**	204.4∓4.5 **Dd**	195.53∓1.84 **Dd**	188.60 ∓4.1 **Ccd**
13	209.47∓2.91 **Ee**	209.33∓1.44 **De**	217.93∓1.79 **Fe**	214.40∓1.97 **Fe**	201.53∓2.08 **Ed**
16	219.76∓3.06 **Ff**	223.06∓1.82 **Gf**	231.92∓2.09 **Gf**	222.98∓1.49 **Gf**	218.13∓2.25 **Fe**

19	230. 8 ∓3.2 **Gg**	236.2∓2.09 **Gg**	241.68∓2.22 **Hg**	238.85∓1.35 **Gg**	232.46∓3.07 **Gf**
21	243.13∓4.21 **Hh**	246.66∓1.08 **Hh**	248.54∓1.5 **Hgh**	248.13∓2.26 **Hgh**	243.40 ∓1.98 **Hg**
24	255.7∓2.56 **Ii**	253.89∓2.11 **Ihi**	258.13∓3.14 **Ihi**	258.12∓1.79 **Ih**	255.89∓1.59 **Ih**
27	262.93∓2.4 **Jij**	260.78∓1.76 **Jij**	268.05∓1.22 **Ji**	263.86∓1.35 **Jhi**	261.53∓2.69 **Jhi**
30	271.66∓2.74 **Kjk**	266. 69 ∓1.21 **Jj**	285.80 ∓1.29 **Lj**	268.93∓1.69 **Jij**	272.07∓2.00 **Ki**
33	284.86∓2.48 **Lkl**	266.96∓2.23 **Jj**	296. 00 ∓2.49 **Mk**	274.13∓1.51 **Kjk**	279.09∓2.08 **Kij**
36	294. 04 ∓2.91 **Ml**	268. 59 ∓1.17 **Jjk**	313. 06 ∓1.82 **Nl**	277.93∓1.78 **Kjk**	286.46∓1.91 **Mjk**
39	306.35∓3.11 **Nm**	268.54∓2.34 **Jjk**	326. 2 ∓2.09 **Om**	282.26∓1.49 **Mkl**	294.73∓2.18 **Nkl**
42	318.53∓1.16 **On**	269.06∓1.83 **Jk**	337.66∓2.07 **Pn**	286.33∓1.45 **Mkl**	303.60∓2.48 **Nlm**
45	330.58∓3.08 **Po**	269.71∓2.45 **Jk**	344.99∓4.21 **Qno**	289.29∓1.84 **Ml**	311.31∓1.69 **Omn**
49	342.12∓3.46 **Qp**	270.06∓3.24 **Kk**	349. 42 ∓3.82 **Qop**	294.32∓2.19 **Nl**	319.35∓2.48 **Ono**

•G1= Control rats. •G2=Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre-treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different small letters represent significant difference in the same group($p<0.05$).

•Different capital letters represent significant difference between groups($p<0.05$).

•Values represent mean∓ standard error.

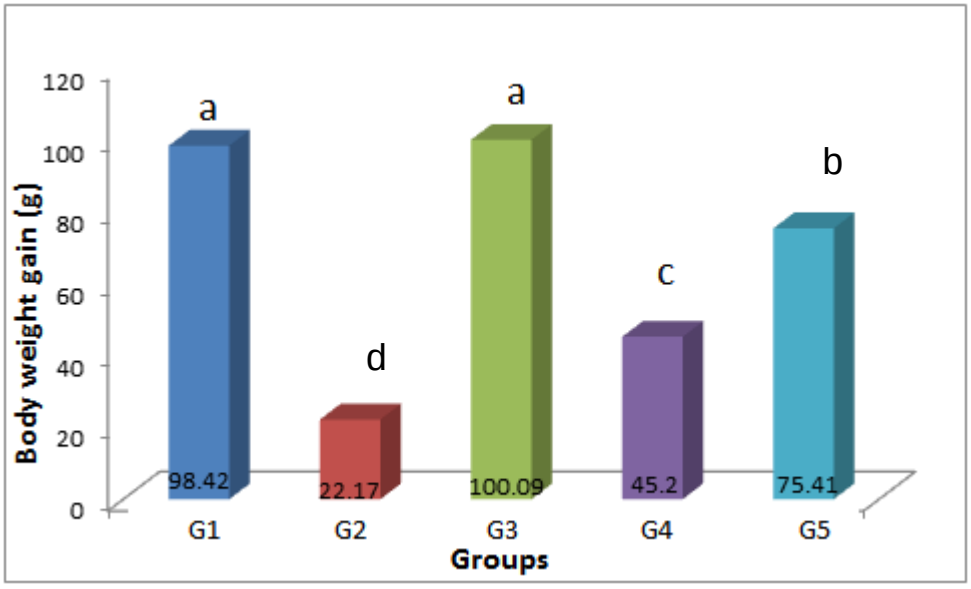


Figure (3-1): Effect of EEP on body weight gain (g) after day 21 when induction of diabetic by STZ in male rats.

•G1= Control rats. •G2=Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

3.3. Biochemical Analysis

3.3.1. Effect of EEP on Blood Glucose Level (BGL)

In STZ diabetic rats, there was a significant increase in blood glucose levels ($p<0.05$). The administration of local EEP(whether pretreated or post- treated) to the diabetic rats significantly reduced blood glucose level when compared with diabetic group, this reduction was not enough to reach that of normal rats(control and treatment), but it still significantly higher when compared with control group, as shown in figure (3-2). On the other hand , from table (3-2), it was clear that the level of blood glucose was decreased in rats post-treated with EEP significantly than pre-treated group, but it was significantly higher than control group($p<0.05$). The results agree with (Matsui *et al*.,2004) who demonstrated that administration of EEP in rats had a potent antihyperglycemic effect. The presence of flavonoids and polyphenolic components as main active ingredients having potent antioxidant activities, that may enhance the antioxidant defense systems in pancreatic tissue (Moreno *et al*.,2000; Hosnuter *et al.*,2004; El-Sayed *et al*., 2009),also Gary and his team (2000) proved that tannic acid worked to stimulate insulin secretion, and this component founds in local EEP.

Table (3-2): Effect of EEP on Blood Glucose level in STZ- induced diabetic rats.

Days	Control G1	Diabetic rats G2	Treatment with EEP. G3	Diabetic with Pre-treated of EEP. G4	Diabetic with post- treated of EEP. G5
21	93.73∓1.09 **a**	93.06∓1.66 **a**	92.66∓1.78 **a**	91∓1.78 **a**	91.26∓1.79 **a**

27	94.60∓1.29 **a**	485.13∓17.65 **b**	95.73∓1.25 **a**	289.12∓11.35 **c**	446.66∓16.17 **b**
30	96.43∓2.05 **a**	506.5∓15.58 **b**	94.2∓1.56 **a**	296 ∓ 10.01 **c**	395.61∓15.79 **d**
33	91.8 ∓ 1.55 **a**	508.14∓17.38 **b**	91.4 ∓ 1.74 **a**	301.86∓12.26 **d**	366.46∓13.89 **d**
36	92.3 ∓ 1.59 **a**	505.85∓17.12 **b**	87.94∓2.99 **a**	294.73∓10.74 **c**	354.26∓14.25 **d**
40	92.46∓1.60 **a**	512.56∓13.56 **b**	90.8 ∓ 2.01 **a**	302.2∓10.65 **d**	324.13∓14.28 **d**
43	96.1 ∓ 2.11 **a**	508.68∓14.98 **b**	91.81.68١ **a**	301.21∓10.65 **d**	299.86∓12.4 **c**
46	91.7 ∓ 1.27 **a**	514.16∓16.31 **b**	92.5 ∓ 1.69 **a**	302.71∓9.79 **d**	275.53∓9.44 **c**
49	91.3 ∓ 1.79 **a**	515.81∓16.33 **b**	91.2 ∓ 2.04 **a**	301.13 ∓ 11.4 **d**	256.66∓6.24 **c**

•G1= Control rats. •G2=Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre-treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

•Values represent mean∓standard error.

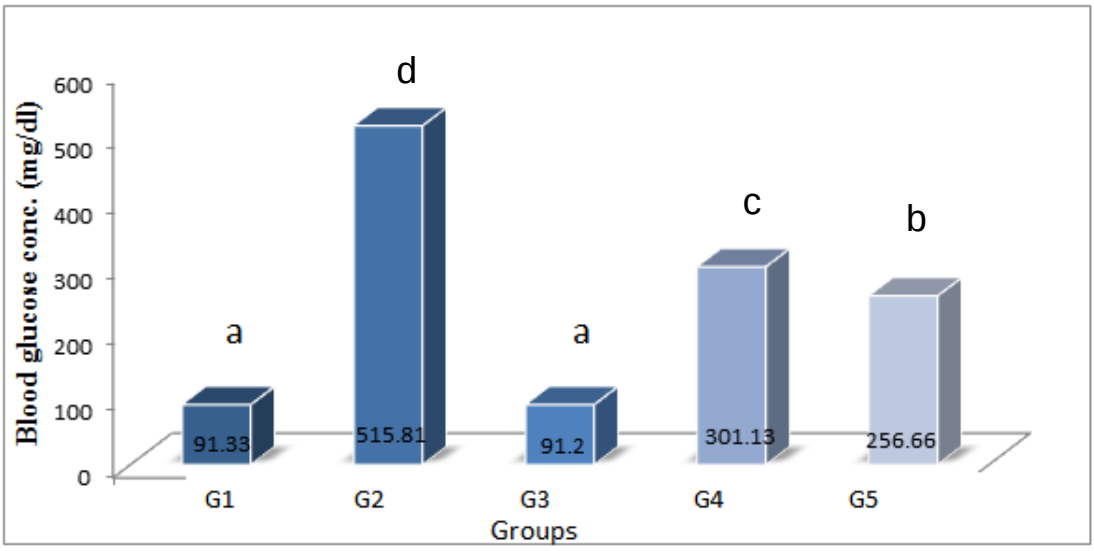


Figure (3-2): Effect of EEP on Blood Glucose Concentration in STZ- induced diabetic male rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

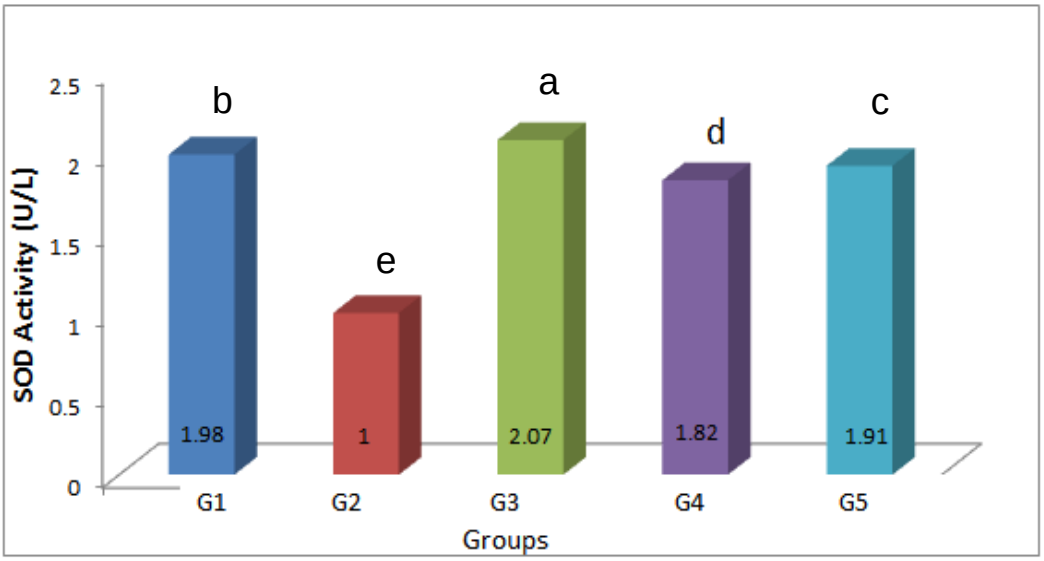
•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

3.3.2.Effect of EEP on Serum Superoxide Dismutase (SOD)

In the present study, activity of SOD enzyme was significantly decreased in non-treated diabetic rats (G2) by (50%) compared to control (G1) ($p<0.05$). Treatment group (G3) showed significant increased activity of SOD in ratio 104% ($p<0.05$). However, in pre-treated and post-treated groups(G4 and G5) there was significant increase and improvement in SOD Activity (91%, 96%) respectively compared to diabetic group G2($p<0.05$). Figure (3-3).

This study reveals a significant fall in SOD activity, which could be due to excessive oxidative stress. Decrease in SOD activity can result from several reasons, the decrease of SOD activity might be attributed to the hyperglycemia which could activates various biochemical pathways such as glucose autoxidation, nonenzymatic glycation of proteins (glycoxidation process of AGEs) and activation of protein kinase C, which, in turn, overproduce oxidants like superoxide and hydroxyl radicals as well as hydrogen peroxide. This is suggestive of the fact that increased autoxidative glycosylation of hemoglobin may also have led to enhanced generation of free radicals like the superoxide anion, thereby causing the depletion of SOD, which quenches it (Hisalkar *et al*., 2012), as well as loss of its two factors, Zn^{2+} and Cu^{2+}**,** this is in harmony with the finding that in diabetes, there is a close correlation between decreased SOD activity and loss of its two factors, Zn^{2+} and Cu^{2+} (Lin *et al*.,1996). There is substantial evidence that SOD activity in blood cells is reduced in the diabetic patients with DN as compared with those without diabetic complication (Hiroki *et al*.,2009).



Figure(3-3): Effect of EEP on Serum SOD Activity in STZ- induced diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

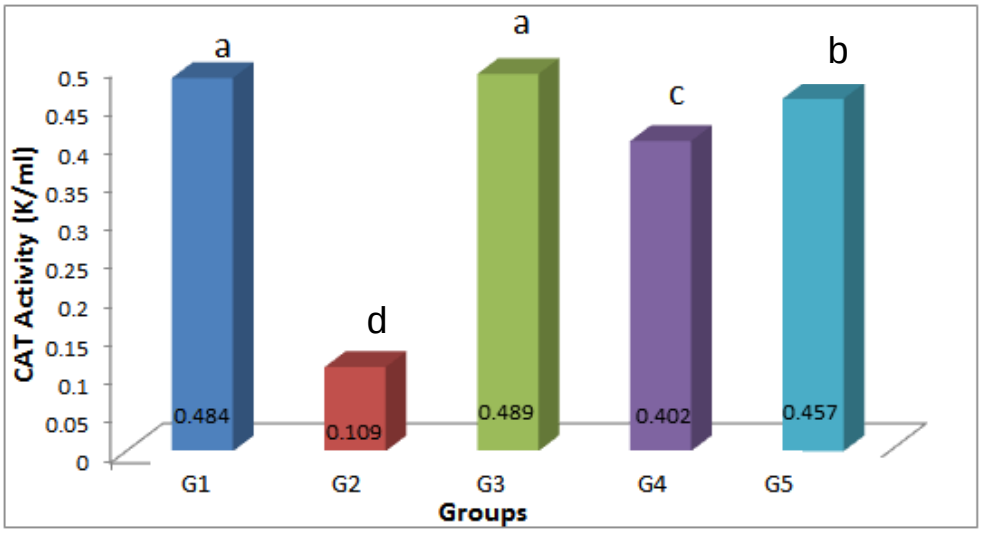
•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

Several studies have reported lower concentration of non-enzymatic antioxidants as well as enzymatic antioxidants in diabetes like SOD (Lodovici *et al.*, 2009; Likidlilid *et al.*, 2010; Bigagli *et al.*, 2012; El-Masry, 2012), but in some reports on rats, SOD activity was increased (Rauscher *et al.*,2000, Rauscher *et al.*,2001), the difference may be due to different doses of STZ. The effect of propolis was studied by Okutan *et al.*, (2005) and Mustafa *et al.*, (2008) when found that the diabetic group rats showed decrease the activity of SOD, but increased when treated with CAPE Caffeic acid phenethyl ester (is one of the major components of honeybee propolis) as CAPE possesses wide-ranging properties such as antioxidant, anti-inflammatory, that can be explained by its oxidative stress- reducing effect and by its scavenging activity on superoxide radicals. So, the diabetic rats treated with EEP had a higher activity of SOD as compared to untreated diabetic rats, which further indicates that the EEP possesses significant antioxidant activity (Osama *et al.*,2009. Wei *et al.*,2010).

3.3.3. Effect of EEP on Serum Catalase Activity (CAT)

As shown in figure(3-4), results of diabetic group (G2), showed a significant decline in CAT activity in serum (22%) compared with control ($p<0.05$). In treated intact group(G3) there is no significant differences in the activity of CAT compared with G1, but in pre-treated (G4) and post-treated (G5) showed a significant improvement in CAT activity reached to (83%, 94%) respectively($p<0.05$) in comparison with G2.



Figure(3-4): Effect of EEP on Serum CAT Activity in STZ- induced diabetic rats.
•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP rats.
•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.
•Different letters represent significant difference between groups($p<0.05$).

STZ-induced diabetes decrease the activity of CAT, that may be linked to exhaustion of enzyme as a result of oxidative stress. Furthermore, CAT activity was significantly lower in diabetes, exhaustion of catalase could also result from the accumulation of H_2O_2 on which the enzyme activity would be expended.

Mitochondrial reactive oxygen species (ROS) play an important role in diabetes complications, including diabetic nephropathy (DN). Plasma free fatty acids (FFAs) as well as glucose are increased in diabetes, and peroxisomes and mitochondria participate in FFA oxidation in an

interconnected fashion. Therefore, the deficiency of catalase, a major peroxisomal antioxidant, accelerates DN through peroxisomal dysfunction and abnormal renal FFA metabolism(Inah *et al*.,2012), or may be due to in activation of the enzyme CAT that may occure through glycation governed by prevailing glucose concentration. Thus, increased glycationin diabetes and subsequent reactions of proteins may effect amino acids close to the active site of the enzyme or disturb the stereochemical configuration and causes structural and functional changes in the in the molecules(Rahbani *et al*.,1999).

Oral administration of EEP may reactivated CAT, which might be via scavenging of free radicals or preventing its formation, that lead to decrease the overload on the antioxidation defence system and enhancement its activity which led to decrease in CAT exhaustion.

The present result agreed with that of Salem *et al*.,(2009) whom observed that treated diabetic rats with EEP, showed significant increase in SOD and CAT, as well as conformed with the report of Osama *et al*.,(2009) that showed the propolis normalized the activity of CAT at all tested doses, when treated the diabetic nephropathic rats with oral administration of propolis extract in doses of 100, 200 and 300 mg/ kg b.w, that may suggested a strong antioxidant effect of propolis which can ameliorate oxidative stress and delay the occurrence of diabetic nephropathy in rats with induced diabetes mellitus.

3.3.4.Effect of EEP on Serum Glutathione Peroxidase Activity (GPx)

This study observed that mean value of antioxidant enzyme GPx was significantly decreased in diabetic group(G2) compared with non-diabetic group (G1) reached to (43%) of the G1($P<0.05$), but in G4(pre-treated EEP) and G5(post- treated EEP), there is significant improved in GPx activity (79%), (88%) respectively, compared to G2(diabetic) ($P<0.05$), while there is significant increase in GPx activity compared to G2

(diabetic group). In G3(treatment) there is no difference in GPx activity compared to G1($P<0.05$), that shown in figure (3-5).

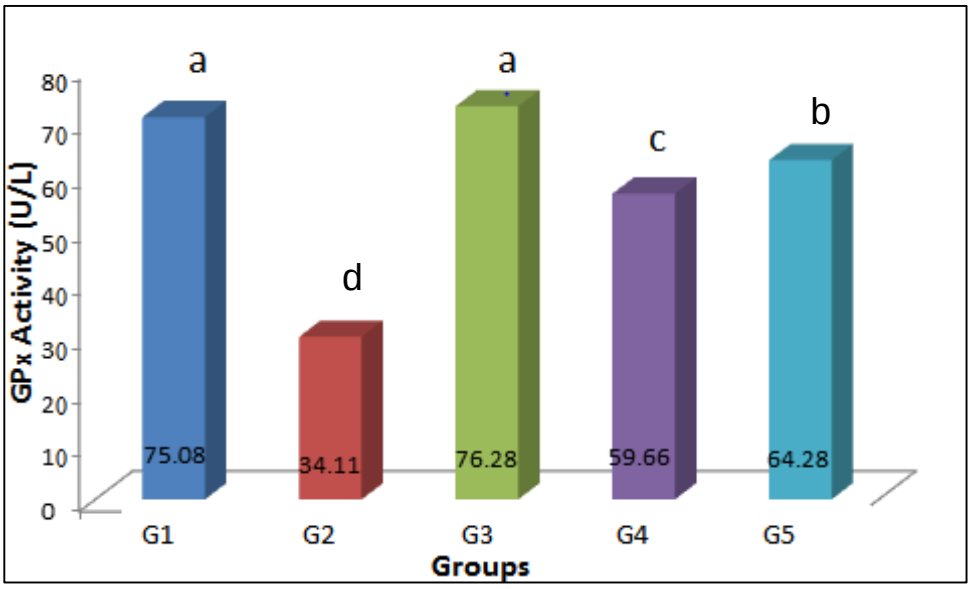


Figure (3-5): Effect of EEP on Serum GPx Activity in STZ- induced diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

•Similar letters represent insignificant difference between groups.

The observed mean value of antioxidant enzyme GPx was significantly decreased in diabetic rats groups compared with non-diabetics, these results are indicators of decrease in the protective antioxidant mechanism. This is in agreement with several studies, which have been carried out previously were reported by various authors like (Li et al,2010; Abo El-Soud et al.,2012; Al-Safar, 2013), but disagreement with the reports by (Rauscher *et al*.,2000, Rauscher *et al*.,2001).

The study by Okutan and his team (2005), investigated the effects of caffeic acid phenethyl ester (CAPE) which is a component of propolis on lipid peroxidation and antioxidant enzymes in diabetic rat. They found That GPx activity was increased in the CAPE-treated diabetic rats compared to those observed in untreated diabetic rats ,that concluded the protective role

of propolis might be related to its antioxidant effect, another researchers suggest that propolis might be considered to prevent oxidative stress in the broilers exposed to heat stress (Tatli Seven *et al*., 2009),and noticed the GPx activities in blood, liver and kidneys of heat stressed birds were significantly reduced in blood and some tissues, this may be explained by GPx inhibition at increased free radical levels. It can be concluded that dietary propolis regulated antioxidant enzymes activities.

3.3.5.Effect of EEP on Serum Glutathione-S-Transferase Activity (GST)

The result of measurement of GST activity of non-treated diabetic rats showed significant decreased in GST activity reached to (61%) from the activity of G2(diabetic group) ($P<0.05$), but in G4(pretreated EEP) and G5(post- treated EEP), there is a significant activation in GST activity reached to (80%), (92%) respectively, compared to G2 ($P<0.05$), while there is a significant increase in GST activity compared to G2 (diabetic group). In G3(treatment) there is a significant improvement in GST activity compared to G1($P<0.05$).

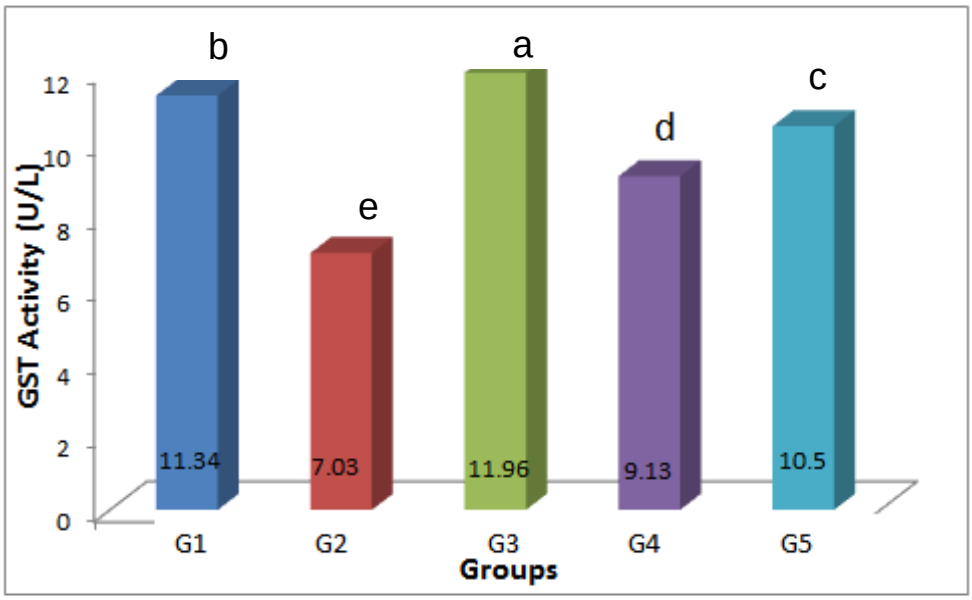


Figure (3-6): Effect of EEP on Serum GST Activity in STZ- diabetic rats. •G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP. •G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

•Similar letters represent insignificant difference between groups.

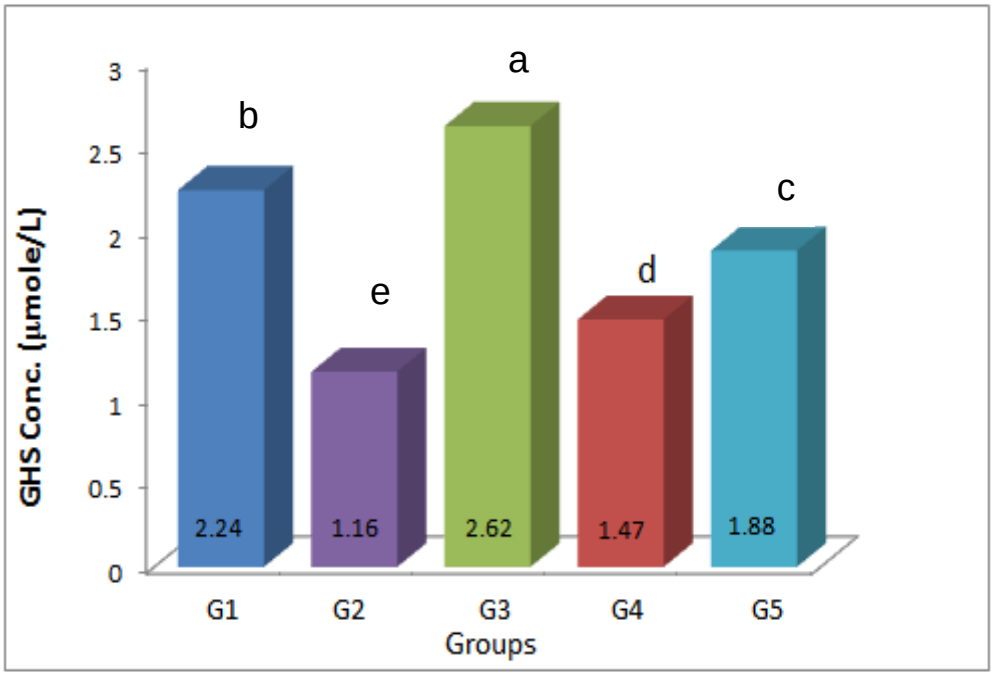
GST plays a crucial role in the detoxifying mechanisms of drugs and xenobiotics by preventing the binding of reactive metabolites to cellular proteins and modulating the by-products of oxidative stress by catalyzing the conjugation of electrophilic moieties to glutathione (Kumari and Mazumder, 2013). Decreased activity of glutathione-S-transferase, may due to the decreased availability of reduced glutathione (GSH), because GST works together with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides. Elevation of blood glucose level generates oxidative stress, which contribute to increase glutathione utilization and decrease GSH availability that lead to decrease GST activity that agree with many previous study such as the study by(Shajeela *et al*.,2013; Chin *et al*.,2008).

The kidney is an important target organ for many xenobiotic material and free oxygen radicals, the GST enzyme family prevents damage from oxidative stress, that decreases the activity of these antioxidant enzymes are a greater risk of oxidative damage (Akgul *et al*., 2012). In the present study, GST levels were decreased in DN animals but Treatment with local EEP significantly increased GST levels in the groups of DN treatment, by trapping and quenching the formed free radicals, or the induction of antioxidant system against toxicity metabolites of STZ-induced DN , where the EEP might encourage the induction of phases enzymes by its chemical ingredients as PPS (PhenylPropanoids) (Korkina,) and the report by (Ladli and Papiya, 2013) showed that the presence of alkaloid in Murraya Konigii Leaf, that is, also, present in local EEP, sprang on attenuating the progression of diabetic nephropathy in streptozotocin (STZ) induced DN.

3.3.6. Effect of EEP on Serum Reduced Glutathione Concentration (GSH)

In this study, the concentration of GSH significantly decreased in G2 (diabetic group) as compared to G1 (control group), reached to 51% ($P<0.05$), but in G4 (pretreated EEP) and G5 (post- treated EEP), there is a significance improved in GSH concentration reached to 66%, 84% respectively compared to G2 ($P<0.05$), while there is a significant increase in GSH concentration compared to G2 (diabetic group). In G3 (treatment) there is a significant increase in the GSH concentration, that show in figure (3-7).

Decreased levels of serum GSH in DN rats could represent an adaptive response to increased oxidative stress and free radicals generation that oxidized thiol group of GSH and decline GSH level, therefore, it is a good indicator to diabetic complications. (Moussa,2008) report maintained there is link between hyperglycemia and GSH, in hyperglycemia condition, glucose is preferentially used in polyol pathway, that consumes NADPH necessary for GSH regeneration by the glutathione reductase enzyme, lead to GSH depletion and increase oxidative stress.



Figure(3-7): Effect of EEP on Serum GSH Conc. in STZ- diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

This is in agreement with several studies, which have been reported about diabetic rats by various authors like (Dubey and Batra,2008; Osama *et al*.,2009 Shivani and Sunil,2013).

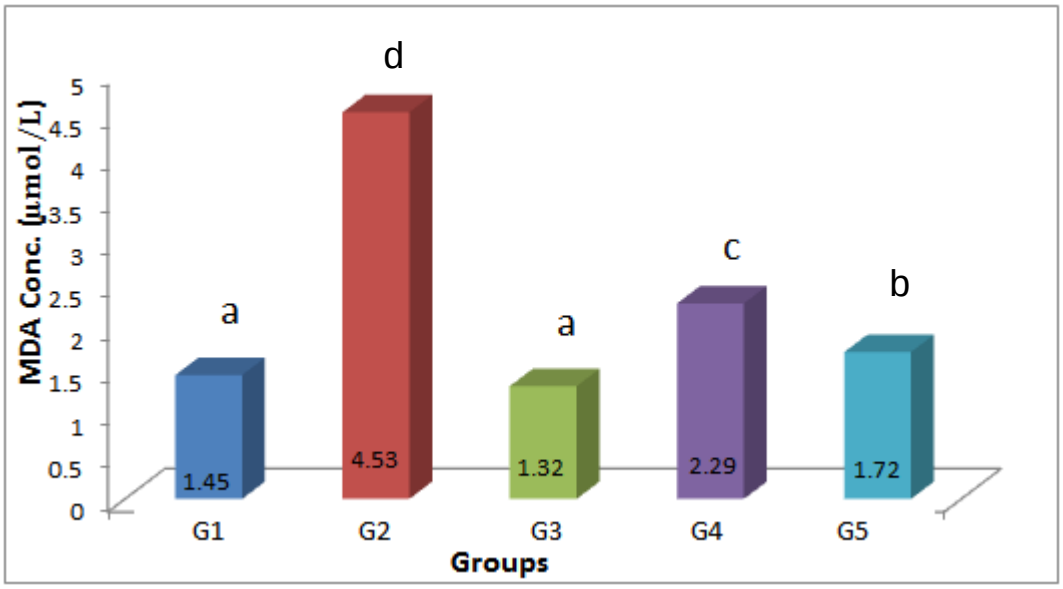
Propolis, owing to its characteristic chemical composition and biological activities, high total phenol amounts, caffeic acid; 3,4-dimethoxycinnamic kaempferol, chrysin, galangin, phenethyl caffeate, cinnamyl caffeate, tectochrysinthe main phenolic acids, ellagic acid, also, quercetin is one of the main constituents of the propolis flavonoids. This constituent could be responsible for the antioxidant activity(Ilhami *et al*.,2010), that can suggest a strong antioxidant effect of propolis which can ameliorate oxidative stress and delay the occurrence of diabetic nephropathy in diabetes mellitus.

3.3.7. Effect of EEP on Serum Malondialdehyde Concentration (MDA)

In this study, there is marked elevation in MDA concentration in diabetic rats group(G2), this significant increase reached to 300% compared to control group(G1) ($P<0.05$), but when treated with local EEP, that can moderate the effects of oxidative stress by decrease significantly the level of MDA reached to 157%, 118% respectively in pre-treated(G4) and post-treated(G5) ($P<0.05$), that means there is a significant decrease in MDA concentration in the treated groups compared with non-treated diabetic group, figure 3-8.

This result indicates that serum MDA levels are elevated in DN of rats, because of reduced antioxidant activity, so that shown in several studies that maintained that lipid peroxidation products, which increase in clinical and experimental diabetes, are important results of oxygen-derived

free radicals stress. These products may be important in the pathogenesis of vascular complications in DM (Mosaad and Abd-Allah,2004), as well as the elevation of MDA level is agreement with other study by (Al-Sa'aidi *et al*.,2012.; Wei *et al*.,2010.; Osama *et al*.,2009) in diabetic and diabetic nephropathy rats.



Figure(3-8):Effect of EEP on Serum MDA Conc. in STZ- diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP

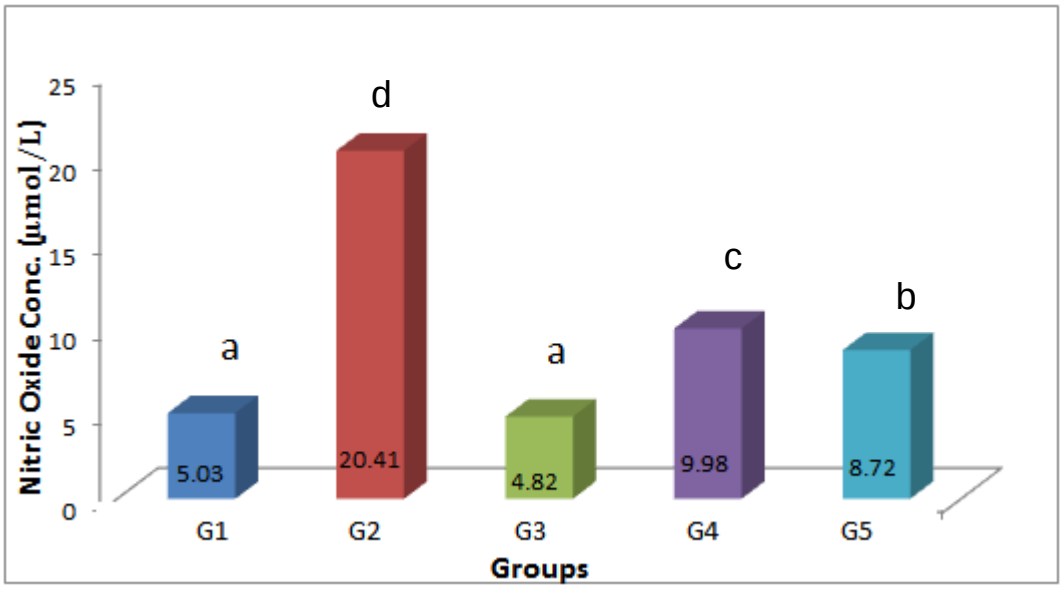
•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

The authors (Osama *et al*.,2009;Wei *et al*.,2010) found that might overcome the adverse effects of oxidative stress of STZ-induced DN by antioxidant effects of propolis are based on flavonoids and CAPE. It was reported that CAPE decreased MDA levels by blocking ROS production as an antioxidant (Seven *et al*., 2010).

3.3.8. Effect of EEP on Serum Nitric Oxide Level (NO).

In the present study, the levels of NO were found to be in a higher level in diabetic rats group (G2), compared with control group($P<0.05$), but in pre-treated (G4) and post-treated (G5) there is significant decrease in NO level compared to diabetic rats group(G2) , but still higher significantly than control group ($P<0.05$). There is no significant difference between G1and G3 in the level of NO, that shown in figure (3-9).



Figure(3-9): Effect of EEP on Serum NO conc. in STZ- induced diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

Excessive NO reacts with superoxide to form a strong toxicity oxidant Nitrite and Peroxynitrite which are linked to various diseases including diabetes, and associated with dysfunction of the endothelial cell. Scavenging of ROS by antioxidants helps to enhance NO bioavailability and alleviate tissue damage (Toda and Nakanishi, 2007). However, increased NO levels are found in diabetic patients and in the animal model therefor, over production of NO promotes the progression of diabetic retinopathy and early-stage DN(Prabhakar, 2004), several reports suggests

that enhanced generation or actions of nitric oxide of (NO) have been implicated in the pathogenesis of glomerular hyperfiltration and hyperfusion that occurs in early diabetes, like (Toda and Nakanishi, 2007; Prabhakar,2004), but there is some reports opposite these results like ((Sivitz and Yorek., 2010) that showed decrease NO level in DN.

The researches (Garcia- Mendiavilla *et al*.,2007) maintained that 30µg/ml of propolis inhibited NO production by 65% by decreasing iNOS gene expression and directly inhibiting its catalytic activity, whereas 30µM of chrysin,galagin, kaempferol or quercetin (constituents of propolis) displayed stronger reductions. Also the author (Luo, 2010) maintained propolis in concentrations ranging from 15µg/ml showed a high ability to scavenge peroxynitrite, a powerful oxidizing and nitrating molecule.

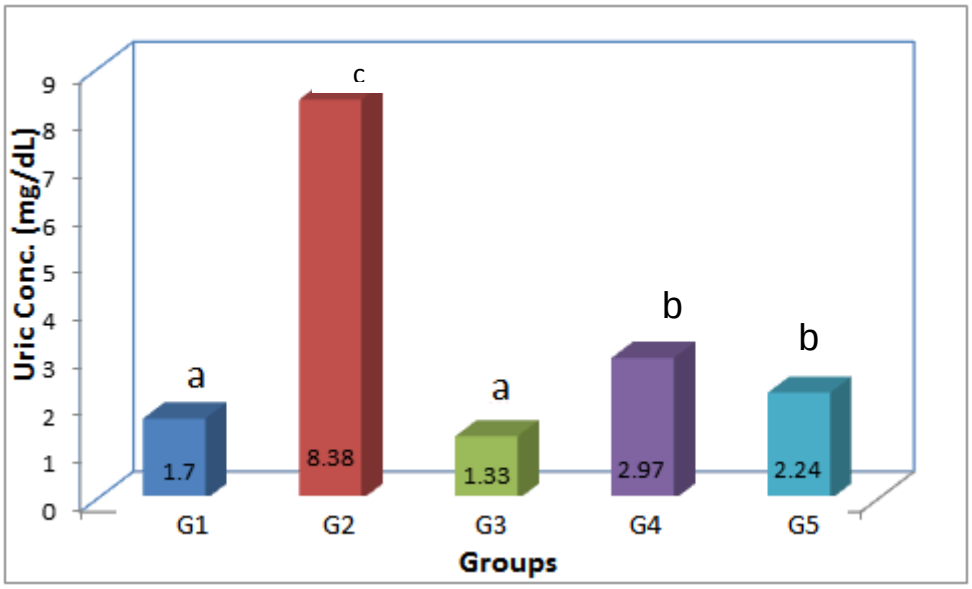
3.3.9. Effect EEP on Serum Uric Acid Concentration (UA)

This study revealed a significant increase in the level of Serum Uric acid in G2(Diabetic rats) compared to G1(control) ($P<0.05$), but oral administration of local EEP in G4(pre-treated) and G5(post-treated) improvement the level of serum uric acid by decreasing significantly compared to G2(diabetic rats) ($P<0.05$), but it is still more significantly than G1(control rats) ($P<0.05$). There is no significant difference between G1and G3 in the level of uric acid.

Because hyperuricemia can be induced not only by inflammatory risk factors, but is also associated with the generation of new acute and chronic inflammation, it should be noted that most damages found with hyperuricemia are not caused directly by UA but by superoxide free radical produced at the same time with UA by the enzyme XO (Wu and James, 2008).

Hyperuricemia has been known to be associated with renal dysfunction, as a result of reduced renal clearance of uric acid may involve a reduced GFR or dysfunctional handling of filtered uric acid by proximal

tubules. Furthermore, elevated serum uric acid itself may increase the risk for development of renal disease in patients with diabetes (Rosolowsky *et al*.,2008; Jerine *et al*., 2011). because hyperglycemia acutely reduces urinary uric acid excretion from the kidney, in type I diabetes, high serum uric acid may be the early sign of diabetes nephropathy before any significance change(Sapna *et al*., 2009).



Figure(3-10): Effect of EEP on level of serum uric acid in STZ- induced diabetic male rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

In experiments, numerous natural products have been found to inhibit xanthine oxidase *in-vitro* or in model animals (mice, rats). specific substance responsible for this inhibition has not been identified,. These include three flavonoids that occur kaempferol, myricetin, and quercetin and The natural product propolis inhibits xanthine oxidase in rats (Soni *et al*.,2011),the authors(Miguel *et al*.,2011) maintained that the samples of propolis, a relative high amount of flavonoids that lowest superoxide

levels, flavonoids attributed to the inhibition of xanthine oxidase, flavonoids are competitive inhibitors of xanthine oxidase, binding to the reactive site. With the inhibition of this enzyme, the oxidation of xanthine to uric acid yielded superoxide radical does not occur, preventing therefore oxidation and improved kidney function, there are many reports indicated the effect propolis on kidney function in diabetic nephropathy, like (Osama *et al*.,2009; Orsolic *et al*.,2012; Wei *et al*.,2011).

3.3.10. Effect of EEP on Total Serum Protein Conc. (TSP)

The results showed a significant decrease in the level of total Serum protein in G2(Diabetic rats) compared to G1(control) ($P<0.05$), but oral administration of local EEP in G4(pre-treated) and G5(post-treated) improvement the level of by increase significantly TSP level compared to G2(diabetic rats) ($P<0.05$), G4 is still less significantly than G1(control rats), but there is no significant difference between G1and G5 in the level of serum total protein($P<0.05$). That can be illustrated by the figure (3-11).

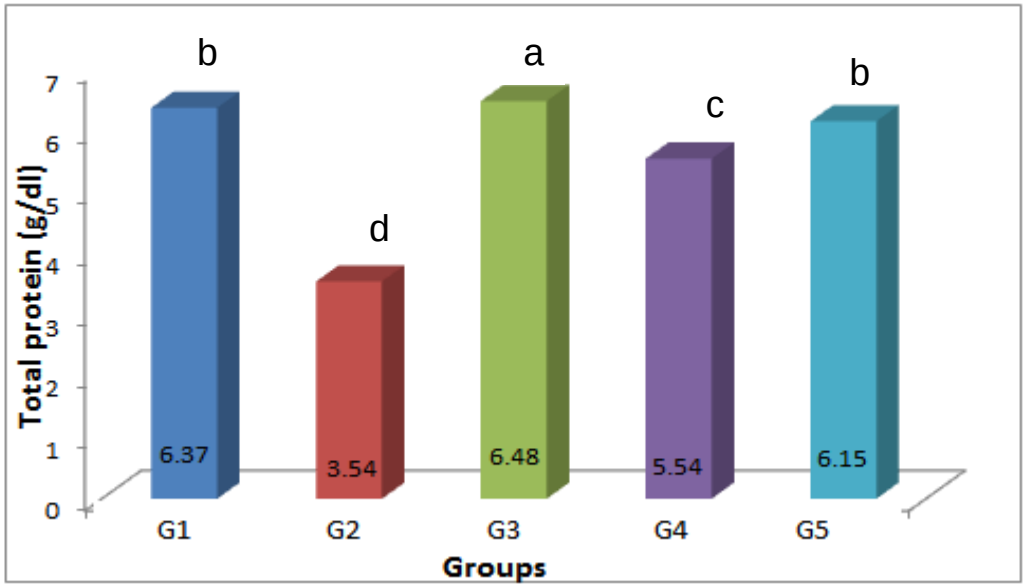


Figure (3-11): Effect of EEP on level of Total serum Protein(TSP) in STZ- induced diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

•Similar letters represent insignificant difference between groups.

Hypoproteinimia is a condition where there is an abnormally low level of protein in the blood. One common cause is due to excess protein in urine, which can be a medical sign of nephritic syndrome, development of lesions in the glomerular capillaries of the kidneys allows protein to escape because of changes in the basement membrane(Sapna *et al*.,2009)

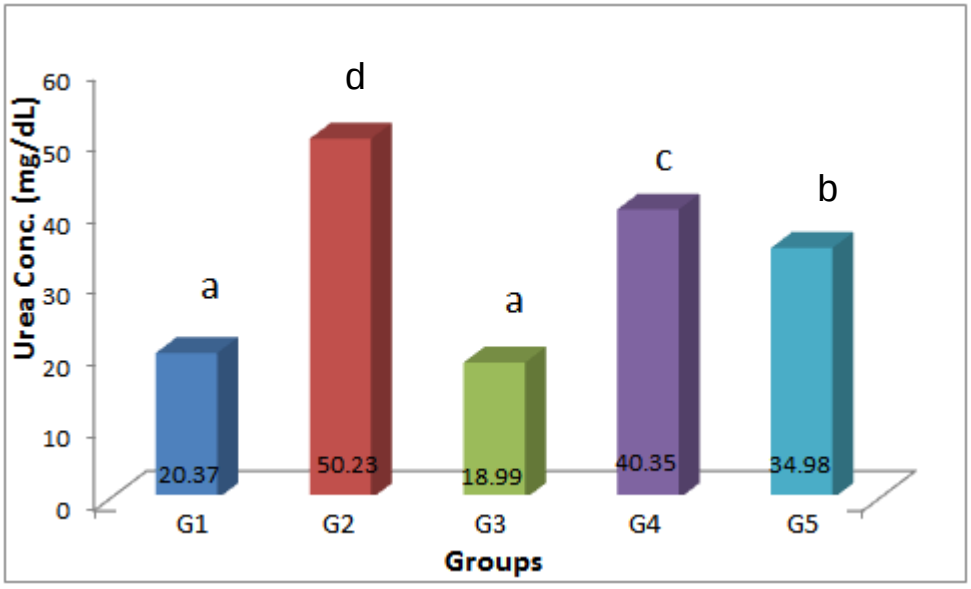
A decrease in serum protein contents with concomitant increase in urinary protein level of diabetic rats were observed because of several reasons like , Advanced oxidative protein products (AOPP), reactive oxygen species (ROS) and free radicals produce protein carbonyl products **(**PCO) and are considered as markers of oxygen-mediated protein damage, also indicating changes in glomerular filtration barrier that result in the increased permeability of the membrane (Rajnish *et al*.,2011),as well as, this decline may be due to the inhibited oxidative phosphorylation processes which leads to decrease in protein synthesis, increase in the catabolic process and reduction of protein absorption (Jerine *et al*., 2011). Similar result was also reported by (Yassin *et al*., 2007; Wanke and Wong,1991).

The report by(Giurgea *et al*.,1984) showed that daily administration of 20 mg/100 g b. w. standard propolis extract (SPE) to chicken for 15 days increased plasma total protein and gamma-globulin content. Interaction of purified propolis *in vitro* with serum albumin or human serum proteins caused conformational changes in the protein and increase ceruloplasmin activity (a major copper-carrying protein in blood), the increases in gamma globulins and proteins suggested that propolis had an anabolic effect, and that it stimulated the body's immune response.

The stimulatory effect of propolis on protein synthesis was also documented by (Ali, 1995) who reported a significant elevation in serum total protein after administration of a single dose of propolis to rats, and also Nassar and his group at 2012 reported that the using of Egyptian EEP in rabbits significantly increased values of TSP and it is can used as immune stimulant with human and animals vaccines.

3.3.11. Effect of EEP on Serum Urea Concentration

This study revealed a significant increase in the level of Serum Urea concentration in G2(Diabetic rats) compared to G1(control) ($P<0.05$), but oral administration of local EEP in G4(pre-treated) and G5(post-treated) improvement the level of serum urea by decrease significantly compared to G2(diabetic rats) ($P<0.05$), but it is still more significantly than G1(control rats) ($P<0.05$), there is no significant difference between G1and G3 in the level of urea. That is shown in figure (3-12).



Figure(3-12): Effect of EEP on level of serum Urea in STZ- induced diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP. •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

•Similar letters represent insignificant difference between groups.

The diabetic hyperglycemia induces degradation of protein and nucleic acid results in the formation of non-protein nitrogenous compound such as urea and creatinine, which are considered as significant makers of renal dysfunction, therefor elevated levels of serum urea in diabetic rats are indication of the development of diabetic nephropathy in rats (Punitha *et al*.,2006; Yasin *et al*.,2007). The presence of some toxic compounds might increase blood urea and decrease plasma protein (Ismael and Abd El Rahiem ,2007), so the STZ considers as a toxic compound.

This study revealed that coadministration of local EEP with STZ-induced diabetes to rats prevented the development of diabetic nephropathy by lowering the level of urea, this could be explained that there was increased clearance of blood urea by the kidney or that there where decreased protein degradation.

These results may indicate that propolis can attenuate renal damage in diabetic rats, agreement with these findings(Osama *et al*.,2009;Yamabe *et al*.,2006) reported that antioxidants and good control of diabetes led to improved renal functions . Moreover, caffeic acid phenethyl ester (CAPE), a biological active component of propolis was found to improve renal function tests in a rat model with lithium-induced renal tubular damage and oxidative stress that reported by(Oktem *et al*., 2005), as well as the researchs Premalatha and Parameswari at 2012, showed that coadministration of chrysin(a naturally present in propolis) with STZ to rats prevented the development of diabetic nephropathy by lowering blood urea.

3.3.12. Effect of EEP on Serum Creatinine Conc.

In the present investigation, there was a significant elevation in the levels of serum creatinine in STZ-induced diabetic rats compared to control

group(P<0.05), but when treated by local EEP, observed decreased significantly in the level of serum creatinine in both pre and post-treated groups (P<0.05). This could be explained that there was decreased clearance of creatinine in the kidney or that there were increased protein degradation in diabetic group (G2),the observed increased creatinine level in G2 (diabetic rats) indicates the development of diabetic nephropathy in STZ induced rats. Whereas the rats coadministered with local EEP to STZ-induced rats demonstrated reduced and decreased significantly(P<0.05) in the level of creatinine, whether pre-treated or post-treated groups.

Kidney failure is associated with many kinds of metabolic changes caused by the kidney disease and also dysregulation of metabolic pathways combine in the pathogenesis of these changes (Cibulka *et al.*, 2005). In the process of accumulation, decreased urinary excretion plays a crucial role and leads to retention of metabolites in the organism like creatinine and urea. Serum creatinine concentration is consider as a measure of the GFR and is used as an index of renal function (Cibulka and Racek, 2007).

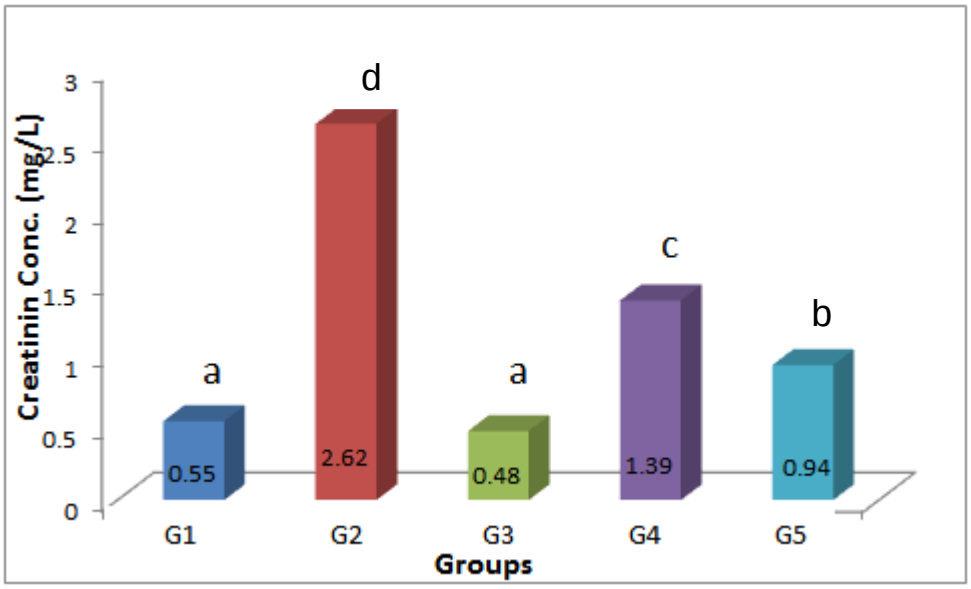


Figure (3-13): Effect of EEP on of serum Creatinin Conc. in STZ-induced diabetic rats.

•G1= Control rats. •G2= Diabetic rats. •G3= Treatment with EEP.

•G4= Diabetic with Pre- treated of EEP •G5= Diabetic with post- treated of EEP.

•Different letters represent significant difference between groups($p<0.05$).

Makino and Tanaka researchers at 2002, maintained that the increased of serum creatinine level in diabetic hyperglycemia induces in rats, are considered as significant makers of renal dysfunction and indication of the development of diabetic nephropathy in rats. The results in G4 and G5 demonstrated the protection effect of propolis on hepatorenal function in diabetic rats. This conclusion was consistent with several previous reports, which showed that propolis and its active constituent caffeic acid phenethyl ester (CAPE) have apparent therapeutic effects on liver and kidney lesions in animal models like(Nirala *et al*.,2008; Bhadauria *et al*.,2008; Ozen *et al*.,2004).

3.4. Histopathological Changes:

The results of biochemical alterations that ensured nephropathy due to diabetes in rats might be insured by microscopic examination of rats kidney, as well as the improvement in kidneys function by treatment with EEP. The destructive and degenerative changes that recorded after induced DN were including:

Microscopic lesion: there were sever vascular congestion and atrophy, with vascular wall destruction , figure (3-14) showing areas of red blood cells extravasation into the interstitium and amidst the spaces between the tubules, oedem changes (hydropic degeneration), with perivascular cells necrosis and mild to moderate hyaline degeneration.

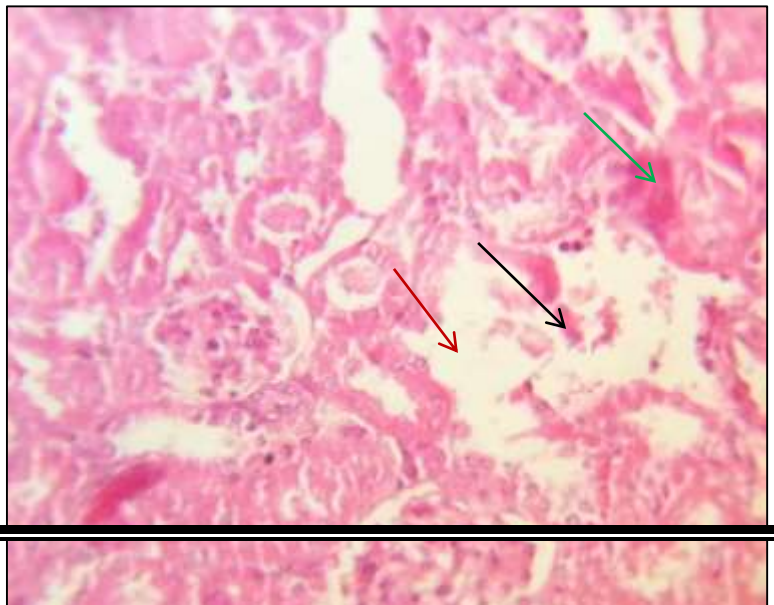


Figure (3-14): kidney of STZ-induced d iabetic in rats after 3 weeks showing sever vascular congestion and atrophy , with vascular wall destruction(black arrow), showing areas of red blood cells extravasation into the interstitium and amidst the spaces between the tubules(green arrow), oedem changes(hydropic degeneration) with inflammatory cells. with perivascular cells necrosis and mild to moderate hyaline degeneration(red arrow). 40X H&E.

The recorded pathological alterations were come in agreement with result of other researches (Muhammad *et al*.,2009; Jerine *et al*.,2011; Rexlin and Rajadurai,2012; Nishi *et al*,2013). They reported that STZ exhibits nephrotoxic and hepatotoxic activity, and severe hyperglycaemia induced by the streptozotocin caused the renal damage, Streptozotocin producing diabetes (hyperglycaemia) and hypoinsulinemia alters various metabolic and enzymatic functions of kidney resulting in various pathologic lesions. It may also be concluded that the diabetic complications in kidney are associated with alterations in enzyme levels.

The Histopathological lesions were observed in the kidney tissue of diabetic rats. Glomerular and tubular injury in untreated diabetic rats could be attributed to Transforming growth factor (TGF-β)stimulated expression of extracellular matrix proteins in glomerular mesangial cells, glomerular epithelial cells and tubular epithelial cells. (TGF-β) plays a pathogenic role in DN. TGF-β is up-regulated in renal fibrosis and its inhibition resulted in a potent reduction of fibrosis in DN. Several studies have shown that high glucose concentrations stimulate the expression of TGF-β in diabetic rat kidney (Abdel and Mason,2004; Chiarelli *et al*.,2009; Herman *et al*.,2011; Nishi *et al*.,2013), so that stimulate DN. ROS like hydrogen peroxide is

also reported to increase TGF-β and fibronectin production in mesangial cells(Ha and Lee ,2000). Moreover, (Buller *et al*., 2011) proved that increased renal insulin-like growth factor IGF-1 and increased nitrous oxide production during the early stages of STZ-induced diabetic nephropathy are associated with renal hypertrophy and hyperfiltration in diabetic rats.

Pre-treated of rats by local EEP as a protective material before injection of STZ-induced diabetes in rats successfully and partially mitigates the pathological changes that showed in microscopic changes. Microscopically, there is mild to moderate vascular congestion, with a small areas of red blood cell extravasation into the interstitium. Bowman's capsules are preserved, that shown in figure (3-15).

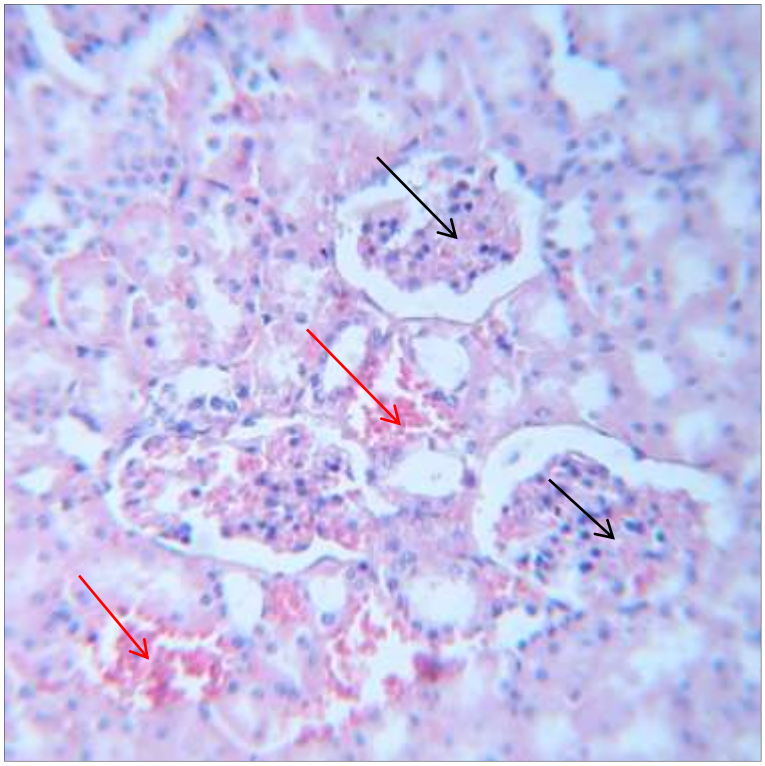


Figure (3-15): kidney, rats pretreated with EEP before injection of STZ-induced diabetes partially improvement of the histoarchitecture of kidney with mild to moderate vascular congestion(black arrow), with a small areas of red blood cell extravasation into the interstitium(red arrow). Bowman's capsules are preserved.40X H&E.

These results improved that EEP succeeded to reduce kidney damage which agreed with the finding of researches(Mesbah *et al*.,2010), they suggested that protective effects of an Algerian propolis extract against doxorubicin-induced oxidative stresses.

In this study, Streptozotocin represents toxic drugs similar to doxorubicin, stimulated oxidative stress and induced diabetic nephropathy in rats . Propolis restored the renal functions and clearly reduced the toxic effect of the drug, hypothesized that propolis effects could be due to a direct action on mitochondrial functions and protective the tissue from damage, polyphenols compounds had preventive properties against renal oxidative stress induced by toxic material.

The mechanism by which the natural product propolis extract may causes increasing rate of GSH or by induction of its synthesis or by the effects of flavonoids scavenger , instead of the toxic reactive metabolites bind to glutathione and consume, they will be captured by the flavonoids (naringenin, pinostrombin and galangin) (Mesbah *et al*.,2010).

Post-treated of local EEP as antioxidant after injection of STZ-induced diabetes in rats, significant successfully mitigates and improvement the pathological changes.

Microscopically, mild vascular congestion, and shows mild dilation of tubules and Bowman's capsules well preserved, showed near normal architecture of the renal tissue, that shown in figure (3-16).

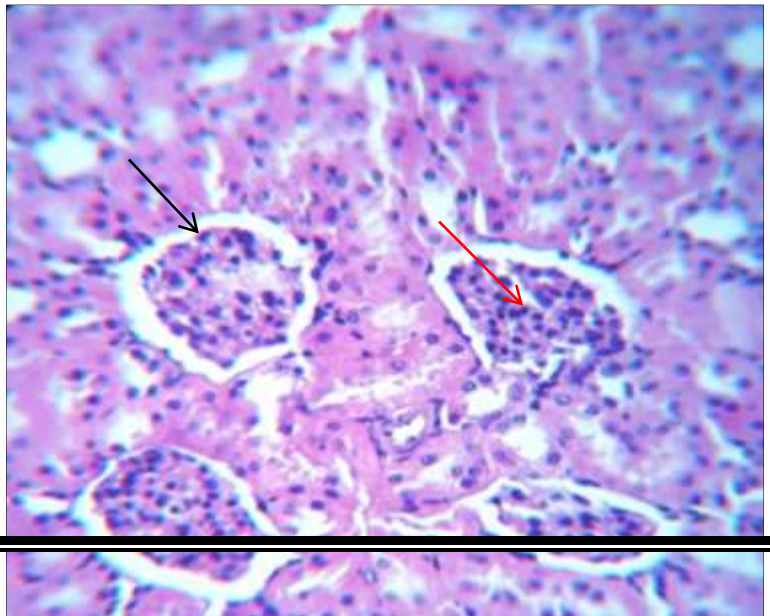


Figure (3-16): kidney, rats post-treated with EEP after injection of STZ-induced diabetes, showed , mild vascular congestion(red arrow), and shows mild dilation of tubules and Bowman's capsules well preserved(black arrow), showed near normal architecture of the renal tissue. 40X H&E.

In the present study, examination of propolis treated-diabetic rats reflected effective protection and improved histo-pathological findings. (Fuliang *el al* .,2005and El-Sayed *el al*., 2009) concluded that propolis can control blood glucose and modulate the metabolism of glucose and blood lipid, leading to decreased outputs of lipid peroxidation and scavenge the free radicals in rats with diabetes mellitus. The caffeic acid phenethyl ester, an active component of propolis, has several biological and pharmacological properties, including antioxidant, anti-inflammatory, anti-carcinogenic, antiviral, and immunomdulatory activities (Okutan *et al*., 2005) so as (Osama *et al*., 2009) maintained that the increase in renal antioxidant enzymes including glutathione, serum superoxide dismutase and catalase and the marked decrease in malonaldehyde (MDA) in diabetic rats under propolis administration,as well as(Zhu *et al*., 2011) showed the correlated between the decrease in nitric synthetase with decreased MDA, additionally, (Kang *et al*., 2010) proved that propolis inhibits the expression of Glucose-6-Phosphtase (G6Pase) is an enzyme that hydrolyzes glucose-6-phosphate resulting in the creation of a phosphate group and free glucose. In 2012, Li and his team proved that propolis can control blood glucose, modulate lipid metabolism, and improve the insulin sensitivity in diabetic rats, so the researches (El- Agawany *et al*., 2012)

showed that Propolis induced good glycemic control leading to a reduction in GFR reflecting reduction of hyperfiltration, so it delays onset of diabetic nephropathy and even correct it after it starts.

In control and treatment with local EEP groups, there is no microscopic changes were detected in both groups (figure 3-17 and 3-18) that indicating the local EEP has no nephrotoxic activity.

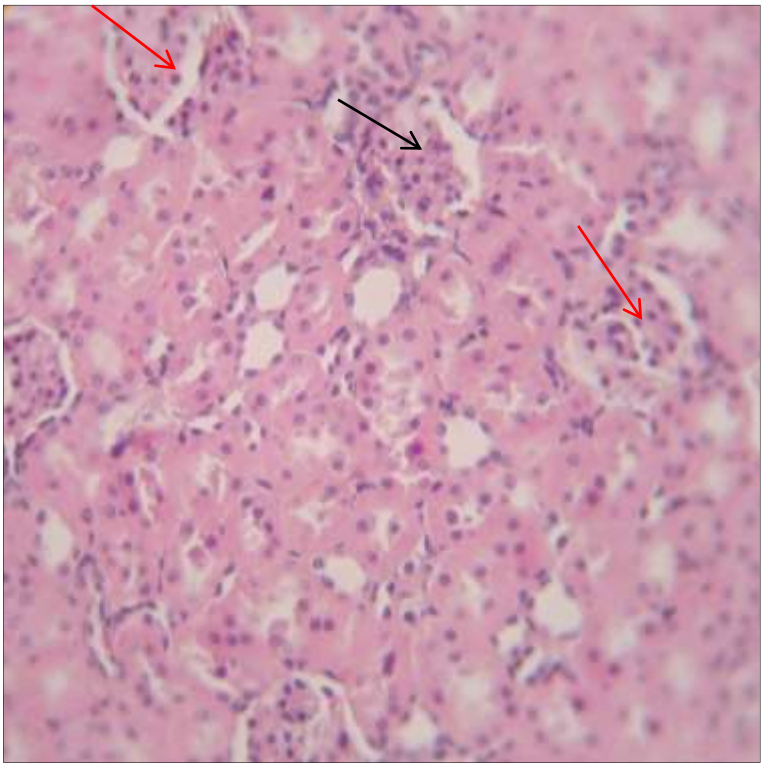


Figure (3-17): kidney of rat treated with local EEP, there is no pathological lesions, normal histoarchitecture of the renal tissue, shows intact tubules and glomeruli, well preserved(black arrow) normal structure of bowman capsules and un remarkable histopathology changes(red arrow). 40X H&E.

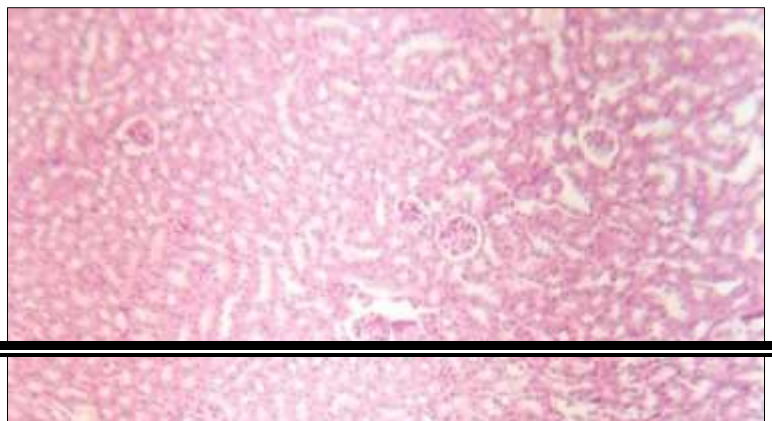


Figure (3-18): kidney of rats control group, normal histoarchitecture of the renal tissue, shows intact tubules and glomeruli . 10X H&E.

Conclusions

The results found in this study enable for concluding the following points:

1. Propolis has antioxidative and immunomodulatory properties. These properties of propolis are based on the presence, flavonoid, phenolic acid and terpenoid contents, which exhibited the antioxidant activity and enhancement of antioxidant status in rats.
2. Pre-treatment and post-treatment with EEP produced anti-hyperglycemic effect. Furthermore, EEP is capable of improving the impaired kidney functions in STZ- induced diabetic rats.
3. EEP has a moderate improvement on kidney function, which may evidence by the decrement of blood urea, serum creatinine, uric acid, and increased total protein. As well as the improvement of Histopathological changes in the kidney of diabetic rats and delays onset of diabetic nephropathy.

Recommendation

1. Further study are required about propolis and propolis extract, if the positive physiological properties and the non-toxicity of the propolis sample are proven, it could be used as antioxidant and preservative material to human.

2. Local propolis having the nephron protective and therapeutic activity in diabetic nephropathy rats in this study, further study can start to determine the positive effect on body organs other than kidney.
3. Further study must be done in order to isolate, identify, characterize and elucidate the structure of the other bioactive compounds that haven't yet identified.
4. Must be study the effect of propolis on the expression level of genes that are responsible for biosynthesis of insulin hormone, in pancreatic β-cell, and antioxidant enzymes and factors when used the local EEP.

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Summary

The present study aimed to investigate the ameliorating effect of daily oral administration ethanolic extract of local propolis (EEP) on blood glucose, antioxidant defense system and kidney function in Streptozotocin-induced diabetic rats.

Seventy five male rats (aged thirty eight days and weighted 150∓10 gm) were randomly assigned to five equal groups: intact rats (G1), diabetic rats without treatment (G2), intact rats with EEP (200mg /kg. b. w)for 6 weeks (G3), intact rats pre-treated with EEP for 3 weeks and then diabetes was induced and rats were monitored for other 3 weeks (G4), and 3 weeks diabetic rats post-treated with EEP for 3 weeks (G5). At the end of experiment blood samples were obtained from anesthetic fasting rats for determination of biochemical alterations, and kidneys were obtained and fixed with 10% formalin for Histopathological study. Antioxidant status has been evaluated by determination of Superoxide dismutase, Catalase, Glutathione peroxidase, and Glutathione-S-Transferase Activities as well as the assessment of Malondialdehyde, Reduced Glutathione, Nitric Oxide

and Uric acid contents. Kidney functions have been evaluated by measuring Serum Total Protein, Urea, and creatinine levels.

The results of non-treated diabetic group revealed significant decrease ($P<0.05$) in body weight, activities of Antioxidant enzymes and concentration of Reduced Glutathione and Total Protein, and significant increase ($P<0.05$) Blood Glucose, Malondialdehyde, Nitric Oxide, Uric acid, Urea, and Creatinine concentrations. Whereas intact and diabetic groups treated with EEP showed significant moderation of Blood Glucose Concentration and antioxidant defense system as well as significant decrease of Serum Malondialdehyde, Nitric Oxide, Uric acid and Creatinine Concentrations.

It can be concluded that 200mg/kg b. w of EEP has potent antioxidant, anti-hyperglycemic and Reno protective effects when used orally in adult rats.