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بسم الله الرحمن الرحيم

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صدق الله العلي العظيم

الآية (٥٩) من سورة الانعام

Dedication

I dedicate this work

To my dear Father

My beloved Mother

My Brothers

My Sisters

My wife

And

My children

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We certify that we have read this thesis "**Bio Chemical study and gene expression of *Glutathione-S-Transferase (GST)* in induced**

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List of Abbreviation

Abbreviation	
A	Absorbance
APX	Ascorbate peroxidases
Arg	Arginine
bp	Base pear
BSA	Bovine Serum Albumin
B.W	Body Weight
CAPE	Caffeic acid phenethyl ester
CAT	Catalase
CDNB	1-Chloro-2,4-dinitrobenzene
CT	Threshold Cycle number
DEPC	Diethylbyrocarbonate
DNA	Deoxyribo nucleic acid
DTNB	5,5-Dithiobis (2-Nitrobenzoic acid)
EDTA	Ethylene diamine tetra acetic acid
EEP	Ethanolic extract of Propolis
EPO	Eosinophil peroxidase
F	Forward
FAD	Flavin adnine dinucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glutathione reductase
GP	glutathione production
Glu-Cys-Gly	tripeptide

GSH	Glutathion reductase
GPX	Glutathion peroxidase
GSSG	Oxidized glutathione
GST	Glutathione –S-Transferase
GSTM1	Glutathione S-Transferase Mu 1
GSTP1	Glutathione S-Transferase pi
GSTT1	Glutathione S-Transferase (GST) theta 1
GST_S	Glutathione S-transferases (soluble)
HDM	House dust mite
IgE	Immunoglobulin E
iNOS	Inducible nitric oxide synthase
LPO	Lipid per oxidation
LSD	Least Significant Difference
MAP	Members of the mitogen – Activation Protein
MPO	Myeloperoxidase
mRNA	Messenger ribose nucleic acid
NADP	Primarily nicotinamaideadninedinucleotide phosphate
NAEPP	National asthma education and prevention program
NADPH	Reduced nicotinamaideadninedinucleotide phosphate
NHLBI	National Heart , Lung , and Blood Institute
NO	Nitric oxide
N.S.	Non Significant
MPO	Myeloperoxidase
OVA	Ovalbumin (Egg albumin)

PBS	Phosphate buffer solution
PCR	polymerase chain reaction
PPS	Phenyl propanoids
qPCR	Quantitative polymerase chain reaction
R	Reverse
RNA	Ribose nucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S.	Significant
S.C	Subcutaneous
SOD	Souper oxide dismutase
TNB	Thio nitro benzoic acid

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الخلاصة

تم فصل انزيم كلوتاثيون –S- ترانسفريز (GST) من كبد الجرذان عن طريق استخدام تقنية كروموتوغرافيا الترشيح الهلامي وباستخدام مادة سيفادكس G-75. كما تم استخدام ستون ذكر من الجرذان يوميا ومت عشوائيا الى اربع مجاميع و بواقع 15 جرذ في كل مجموعة .أعطيت المجموعة الأولى الماء المقطر يوميا و لمدة ثلاثة أسابيع بعدها حقنت بالمحلول الملحي بمقدار (0.5 مل) جرعة واحدة في اليوم الأول والثالث والخامس والتي اعتبرت مجموعة السيطرة. في حين جرعت المجموعة الثانية بالماء المقطر لمدة ثلاثة أسابيع بعدها حقنت (100 مايكرو غرام) من ألبومين البيض وبواقع جرعة واحدة في اليوم الأول والثالث والخامس . حقنت المجموعة الثالثة (100 مايكرو غرام) من ألبومين البيض جرعة واحدة في اليوم الأول و الثالث والخامس (200 ملغم/كيلو غرام)من وزن الجسم بعدها جرعت من المستخلص الايثانولي لثلاثة اسابيع .المجموعة الاخيرة جرعت (200ملغم/كيلوغرام) من المستخلص الايثانولي للعكبر المحلي لمدة ثلاثة اسابيع بعدها حقنت (100مايكرو غرام) من ألبومين البيض وبواقع جرعة واحدة في اليوم الأول والثالث والخامس . بعد 31 يوم من بدء التجربة تم قياس التغيرات البايوكيميائية وتشخيص التغيرات النسيجية للرئة . قيست حالة جهد التأكسد في الجرذان من خلال قياس الكلوتاثيون ، الكلوتاثيون -s- ترانسفيريز ، واوكسيد النايتريك .

أظهرت النتائج انخفاض كبير في فعالية انزيم GST في مصل الجرذان في المجموعة الثانيه بالمقارنة مع مجموعة السيطرة (G1)، كما اظهرت النتائج زيادة فعالية انزيم GST في مصل

الجرذان في (G3) بالمقارنة مع مجموعة (G2)، في حين اظهرت النتائج في (G4) تقريبا نفس فعالية إنزيم GST بالمقارنة مع (G1) حيث كانت نتائج فعالية GST (25 ، 12.5, 18.12, 24.96) U/L لـ G1, G2, G3, G4على التوالي.

كما تم حساب تركيز GSH وكانت القيم (2.15 , 1.04 , 1.47, 2.10) µmol / L وكذلك V_{max}و K_m حسب معادلة ميكاليس منتن وكانت قيمة K_m (12.96,11.03,13.2,11.73) mmol/L في حين كانت قيمV_{max} (4.351, 2.402 , 3.215 , 4.482) µmol/min لـ G1 , G2 , G3 , G4 على التوالي , كما تم حساب التعبير الجيني وكانت النتائج (1, 5, 2.9 , 0.97) لـ G1 , G2 , G3 , G4 على التوالي وتبين وجود زيادة في التعبير الجيني في (G2) بالمقارنة مع (G1) و(G3)، بينما في (G4) كانت نفس النتائج تقريبا بالمقارنة مع مجموعة السيطرة (G1).

توصلت هذه الدراسة الى ان الربو يزيد من الجذور الحرة والاجهاد التأكسدي وان EEP يمكن ان يستعمل كمضاد للأكسدة وللتقليل من تأثير الاجهاد التأكسدي وحماية الخلية من الجذور الحرة اثناء مرض الربو , بالإضافة الى ان الدراسة توصلت إلى الالتهاب الرئوي يؤدي الى زيادة في التعبير الجيني .

1.1 General Introduction of Asthma

A disease of the airways or branches of the lungs (bronchial tubes). Asthma narrows the airways, swells the lining airways and produces more mucus. As a result, the person will breathe with difficulty and feel that there is not enough air in the lungs [1]. This disease might appear in different ages but it usually starts in childhood. Important features of this disease are the recurrent attacks of breathiness and wheezing. These attacks may differ in frequency and severity from person to person. They may occur every hour, daily according to the conditions around the person [2].Asthma is a lung disease causes chronic inflammation of the airways in many types of cells, and in particular, mast cells, and lymphocytes in many individuals. This inflammation causes recurrent episodes of shortness of breath, chest tightness and coughing, particularly

at night or early in the morning [3]. Asthma not one case (heterogeneous), but a group of clinical phenomena which consists of a group of diseases, ranging from a bout of breath, and coughing and hoarseness spoke symptom-free periods on a regular basis with constant severe asthma [4]. Asthma is divided into two major types, these two types are: The first type is asthma exogenous (sensitivity), this disease often spreads between two groups of children and teenagers, with time this disease disappears, this will help us to avoid triggering factors and irritating him. And who experience in this type of asthma from excessive sensitivity towards irritants. Ameonocalobolin immune is immune system of the infected produce large quantities of antibodies (IgE) is the system which causes allergic symptoms. Pollen of some plants and cells physical are highlighted certain factors exciting allergens. These epithelial cells will accumulation tissues exposed to environmental factors such as the mucous membranes in the respiratory tract. The antibodies distinguish and alert mast cells will release histamine and chemical mediators if exposure infected factors excited again[5].

That affects bronchial tubes, leading to excessive secretion of mucus, such patients have increased concentrations of both total IgE and specific IgE against the allergen implicated, it occurs in children. The second type of asthma is endogenous asthma (not affected by allergies) often happens in adults. Virus or bacteria incidence most of the conditions which have no relation to hereditary or allergies [5]. The increase in the incidence and prevalence rates and deaths from asthma during the past few decades in many parts of the world, led to a review of this disease by researchers in basic and clinical science. There are many teams of experts in many countries and throughout the world, including the prevention program and teach the national asthma which is funded by the National Institute of

Heart, Lung and Blood in the United States, has been assembled to define the physiology pathogenesis of asthma and make treatment recommendations based on the severity of the disease (American Academy of Allergy and Immunology).

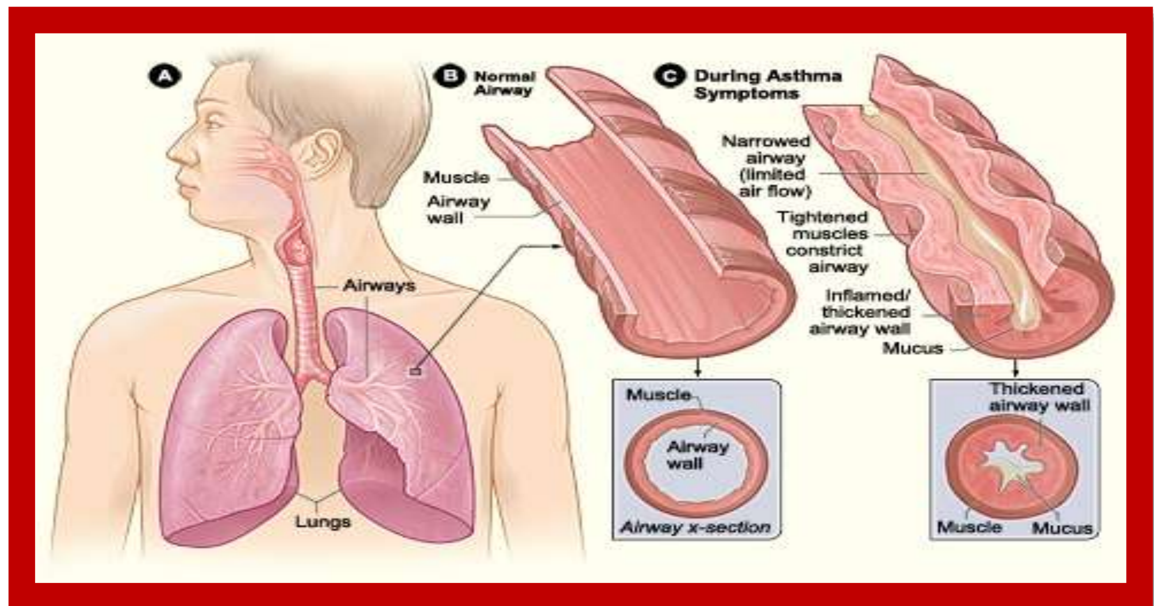


Figure 1-1 A: Shows the location of the lungs and airways in the body. B: Shows a cross-section of a normal airway. C: Shows a cross-section of an airway during asthma symptoms [6].

The National Asthma Education and Prevention Program (NAEPP) Expert Panel sponsored by the National Heart, Lung, and Blood Institute (NHLBI), improved the advice for the Diagnosis and Management of Asthma in 1991[7].The first recognition and treatment of asthma to prevent or weaken the loss of lung function over time was focused by the expert panels In 1997.The Prospective birth cohort studies have proved that for many patients, who has asthma have its roots from early child hood[8].The whistle in the first year of life is caused by the negative smoking sensitization and asthma has association with too much exposure to allergens in early ages. It is proved that there is little relation between exposures to allergens and wheezing is infants and kids. Males who suffer from asthma outnumber females around 2:51 in the first ten

years of life. It is also more severe than female especially in adults. To control symptoms aseravial in treating asthma it is necessary use the available drugs [9].

1.1.1 Mechanism and Cell Types Involved in Asthma

Asthma is a complex immunologic and inflammatory disease characterized by the presence of airway inflammation, asthma exists in three phases.

1.1.1.1Allergen Presentation

An allergen is a substance that can cause an allergic reaction. If an allergen is inhaled, an attack of asthma begins. The allergen which has binding sites on mast cells in the lungs bind to IgE antibodies. Binding triggers exocytosis the mast cells with the release of histamine and leukotrienes [10].

1.1.1.2 Early –Phase of Asthma

Early - phase of asthma means contracting the smooth muscle cells of the bronchi and leading to narrow the lumen of the bronchi which is caused by Histamine and leukotrienes [11].

1.1.1.3 Late – Phase of Asthma

Late – phase of asthma means attracting an accumulation of inflammatory cells (eosinophils, neutrophils, and macrophages) and the production of mucus [11]. As a result the lining of the bronchi becomes damaged with repeated attacks [12].

1.1.2 Pathophysiology of Asthma

The inflammation which is largely driven via immunoglobulin (IgE) is the characterized pattern of asthma according to dependent mechanisms. Several genes have now been identified, but the genetic factors have an important influence on whether atopy develops [13]. The genetic linkages in all allergic diseases are common in asthma. As it is proved that environmental factors may be more important in determining whether an atopic individual develops asthma. However, how severely the disease is expressed and amplification of inflammatory response may be influenced by genetic factors [14]. Asthma is a complex inflammatory disease that involves many inflammatory cells, over 100 different inflammatory mediators and multiple inflammatory effects including bronchi constriction, plasma exudation, mucus hyper secretion and sensory nerve activation. Mast cells play a key role in mediating acute asthma symptoms [15], whereas eosinophils, macrophages and T-helper 2 cells are involved in the chronic inflammation that underlies airway hyper responsiveness. There is increasing recognition that structural cells of the airways, including airway epithelial cells and airway smooth muscle cells become an important source of inflammatory mediators. Multiple inflammatory mediators are involved in asthma, including lipid and peptide mediators, chemokines, cytokines and growth factors. Chemokines play a critical role on the selective recruitment of inflammatory cells from the circulation, whereas cytokines orchestrate the chronic inflammation. This chronic inflammation may lead to structural changes in the airways, including sub epithelial fibrosis, airway smooth muscle hypertrophy/hyperplasia, angiogenesis and mucus hyperplasia Proinflammatory [16].

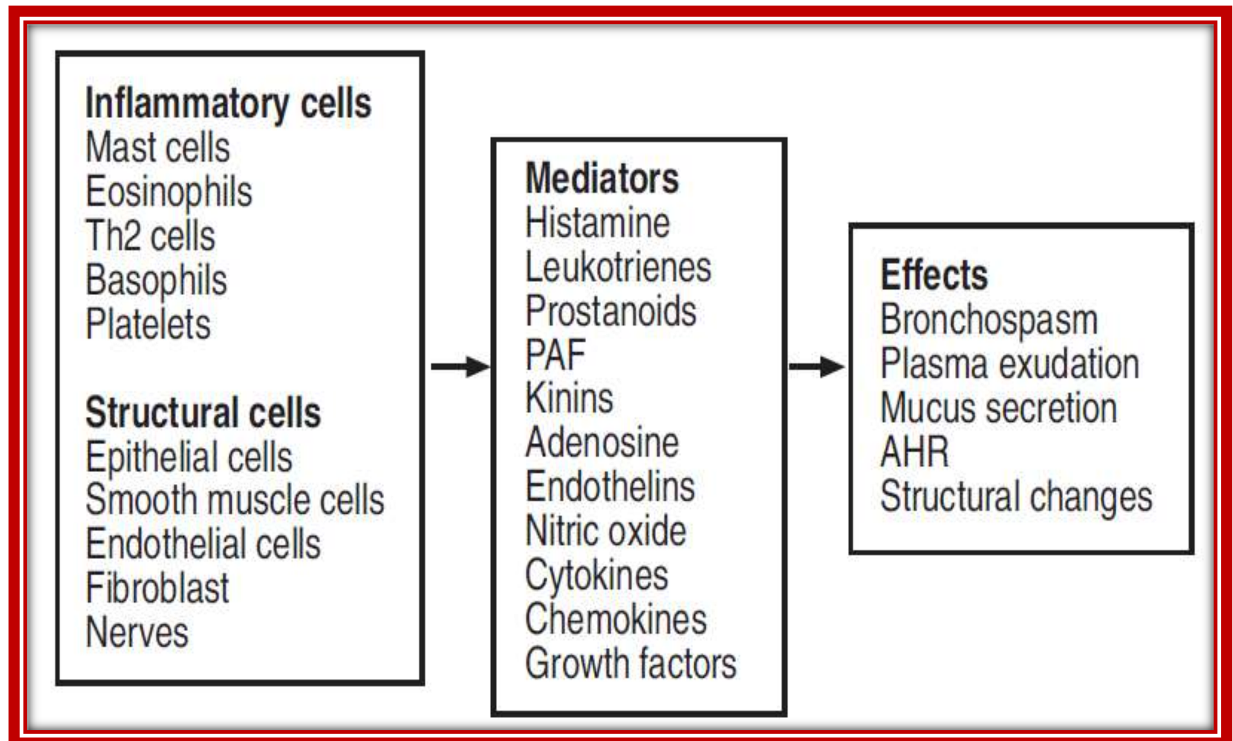


Figure 1-2 Many cells and mediators are involved in asthma and lead to several effects on the airways [17].

1.1.3. Effects of Inflammation

The acute and chronic allergic inflammatory responses have several effects on the target cells of the respiratory tract, resulting in the characteristic pathophysiological changes associated with asthma. Important advances have recently been made in understanding these changes, although their precise role in producing clinical symptoms is often not clear. There is considerable current interest in the structural changes that occur in the airways of patients with asthma that are loosely termed "remodeling it is believed that these changes underlie the irreversible changes in airway function that occur in some patients with asthma [18, 19].

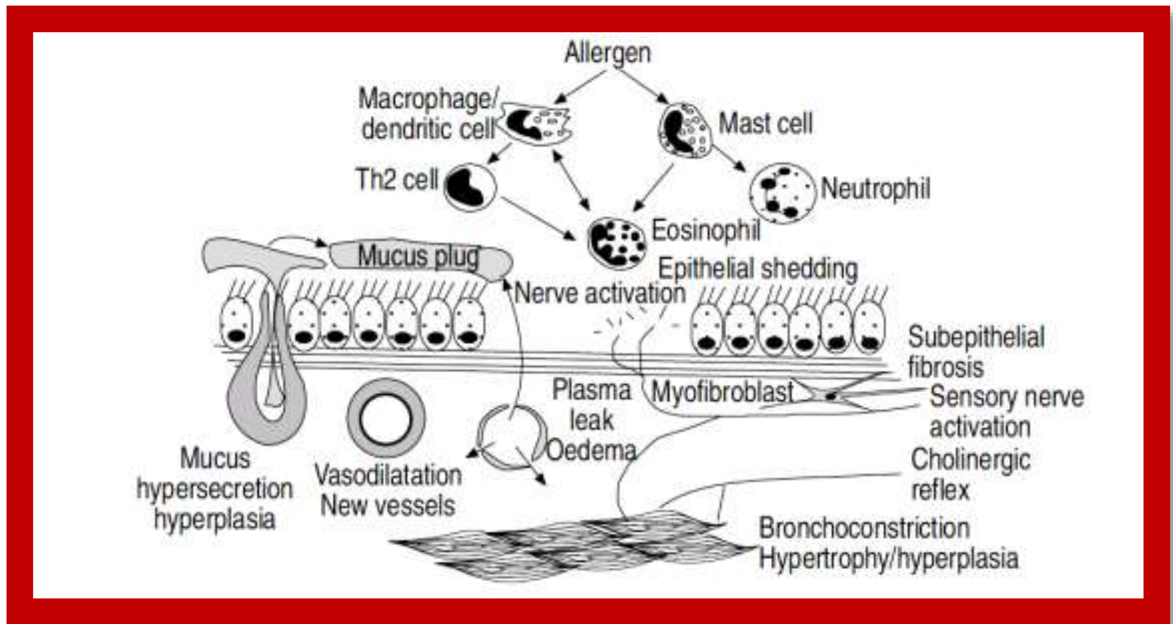


Figure 1-3 The pathophysiology of asthma is complex with participation of several interacting inflammatory cells which result in acute and chronic inflammatory effects on the airway [20].

1.1.4 Asthma Therapy

There is no permanent medicate for asthma however the trouble can be enough controlled with drugs. The optimal asthma control a valuation of severity is important before starting treatment and patient should be placed at the top of the class of seriousness asthma medication are divided into two groups: quick-relief medications and controller medications[21].

1.1.4.1Quick-Relief Medications

Quick-relief meds are to be taken as needed when asthma symptom exacerbations occur, they do not provide long-term asthma control, medications in this category are meant to be used to treat an asthma episode or attack – to relieve symptoms and open airways quickly [22]. They also may be used to pre-treat to prevent attacks, such as before

exercise. These falls into three categories: short-acting beta-agonists, anti-inflammatory drugs, and anticholinergics [23].

1.1.4.2 Controller Medication

Controller medication helps in prevent and reverse asthma attacks by decreasing the inflammation (swelling) in the airways. They actually treat the disease, not just the symptoms, but must be used every day for them to work effectively. Patients need to use a controller medication if they have persistent asthma. Persistent asthma occurs when the airways are swollen enough all the time that patient have asthma symptoms (cough, wheeze, shortness of breath) twice a week or more, or twice a month during sleep [24].

1.2. Asthma and Free Radical

Asthma is a chronic inflammatory disease of the respiratory while the major reason of asthma is not known, it is expected that a different of factors interacting with each other, in early age, result in the development of asthma. Inflammatory state is often associated with generations' increase of reactive oxygen species. Much clinical evidences have proved that there is a relationship between increased ROS and pathogenesis of bronchial asthma. Type of inflammatory cells, including, mast cells, eosinophils, neutrophils, and macrophages have an exceptional capability for generating reactive oxygen species (ROS), including, eosinophils, neutrophils, and macrophages [25]. Macrophages, neutrophils and neutrophils, release increased amount of ROS such as superoxide radical anions ($O_2^{\bullet-}$) and H_2O_2 via the membrane associated NADPH-dependent complex [26]. phagocytic cells can also release reactive oxygen species (ROS) such as HOCl in response to stimulation with various agents or to

phagocytosis. However, both species ($O_2^{\bullet-}$ and H_2O_2) result in formation cytotoxic radicals in biological systems through their reaction with other molecules. When metal ions such as iron and copper are found with $O_2^{\bullet-}$ and H_2O_2can release hydroxyl radical ($OH^{\bullet}$) that is the most reactive free radical. In the presence of eosinophil peroxidase (EPO) or myeloperoxidase (MPO) as a highly chaem enzymes, H_2O_2 can react with halides, e.g. Br^- or Cl^-, giving cytotoxic hypo halo use acids (HOCL, HOBr). monocytes or eosinophil peroxidase (EPO) from eosinophils (26, 27). ROS can be generated either endogenously by metabolic reactions, such as from mitochondrial electron transport during respiration or during activation of circulating inflammatory cells or phagocytes, and exogenously of air pollutants or cigarette smoke (28). As a result, increased levels of ROS have been proven to affect the extracellular environment affecting on a variety of physiological processes. In addition, ROS can start inflammatory responses in the lungs through the activation of redox-sensitive transcription factors (29).

1.2.1. Free Radical

A Free Radical is an atom, molecule, or ion that has unpaired electron and is important intermediates in natural processes. Radicals are very unstable molecules so; they must take the needed electron (30).To obtain stabilization by react quickly with stable compounds to form more unstable compounds the process results in the disruption of a living cell. In general, harmful effects of ROS on cell metabolism are often result to damage of DNA, oxidations of polyunsaturated fatty acids in lipids, oxidations of amino acids in proteins(31).

1.2.2. Generation and Sources of Free Radicals

Free radicals are *generated* from either endogenous or exogenous sources. *Free radicals may* be *formed through natural human physiological* processes. In addition, they may be *formed* from the environment. Free radicals are produced in a number of ways in biological systems:-

- The main *source of free radical* production is the electron transfer of the respiratory chain of mitochondria
- Electromagnetic radiation (X rays, gamma rays UV radiation)
- Metal-catalyzed reactions.
- Toxic chemicals and pesticides
- Organic matter burned during cooking , forest-fires, volcanic activities
- Environmental contaminants such as pollution and Cigarette smoke
- Macrophage of xenobiotics [32, 33].

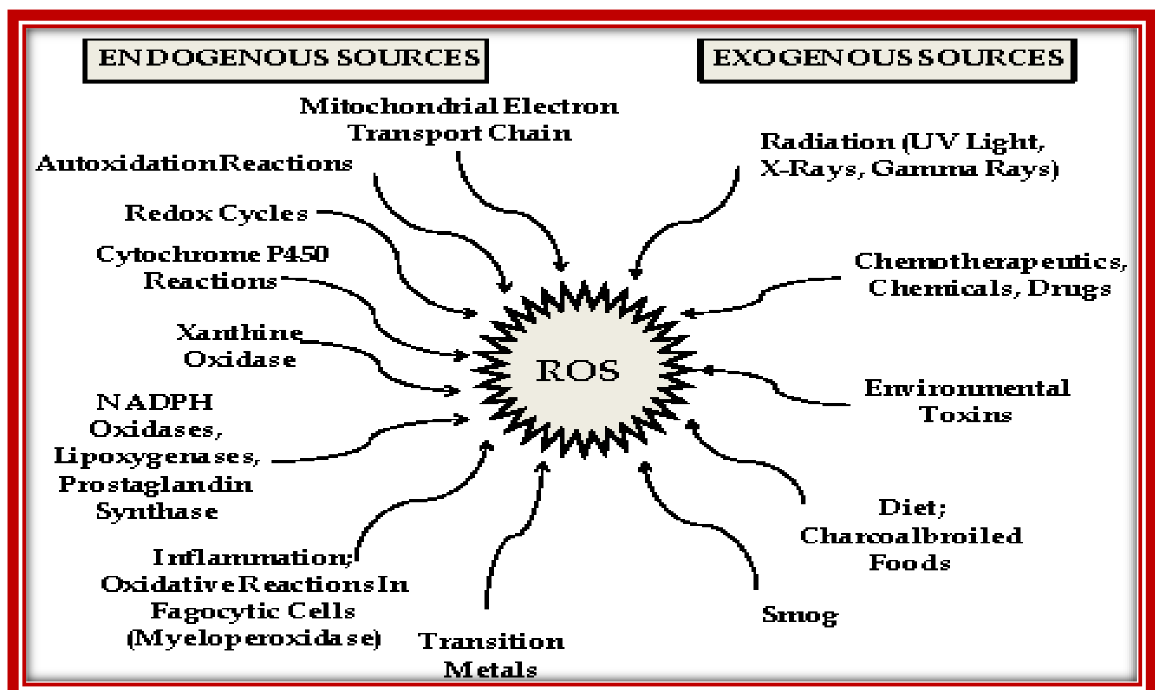


Figure 1-4 Sources of reactive oxygen species in the human body [34]

1.2.3 Free Radicals in the Physiological Control of Cell Function

There are many types of free radicals that can be generated within the body. Free radicals are not all bad, many of these play an essential role in a healthy human body by intracellular killing of bacteria .Free radicals would be produced to destroy enemies in human body [35]. High concentration of free radicals is dangerous because they attack all the major cellular constituents of the body. But at moderate concentrations, radicals have an important role in combustion, polymerization, plasma chemistry and many other chemical processes including human physiology. Free radicals may be involved in the physiological regulation of key changes in gene expression. ROS also acts a secondary messenger in intracellular signaling [36]. ROS are generated as undesirable side products of the oxidative energy metabolism. ROS- mediated responses regulate biomolecules and metabolism and product against microbial infection. Reactive nitrogen species *(RNS)* and ROS are *generated* by *tightly regulated enzymes*, such as *NO* synthase (*NOS*) and NADPH oxidase, respectively. *Abnormally* high levels of ROS may arise *either* less well –regulated sources such as mitochondrial electron-transport chain or from, *excessive stimulation* of excessive stimulation of NADPH oxidizes results in oxidative stress. Although these events that derived by different mechanisms result in cell die but this mechanism depends on different exogenous factors as well as the cell's capability to handle the oxidative stress. ROS attacking proteins and result in deification of proteins in different ways may be a loss function , a gain function, or a convert to several functions [37].

1.3. Oxidative Stress of Asthma

Asthma is a complex inflammatory disorder characterized by airflow obstruction of variable degrees, bronchial hyper-responsiveness, and airway inflammation[38], and chronic inflammation characterized by an influx and activation of inflammatory cells (macrophages, neutrophils, eosinophils, lymphocytes, and mast cells), generation of inflammatory mediators, and epithelial cell shedding. It has been shown that inflammation driven by increased oxidative stress occurs in the airways of patients with asthma, as reviewed by Dworski [39]**.** Inflammatory and immune cells in the airways, such as macrophages, neutrophils, and eosinophils, release increased amounts of ROS in asthmatic patients [40]. ROS can result in lung injury as a result of direct oxidative damage to epithelial cells and cell shedding [41, 42].

Oxidative damage plays an important role in the development of bronchial asthma. Increase production of ROS leads to mutagenic alternations resulting in many pathological processes and can be implicated in pathogenesis of asthma Protein oxidation, DNA modification and lipid peroxidation, all of these oxidative changes can be accumulated in airway and may participate to bronchial asthma persistence and lead to further release of mediators from epithelium resulting in further increase of oxidative damage which can again participate in asthma pathogenesis. Oxidative damage represents dynamic balance between a degree of oxidative damage and a degree of repair of

this damage. Changes are not happened only inconsequence of oxidative damage of biomolecules but also in consequence of damage of repair mechanism[43,44].

1.4. Antioxidant

Antioxidants are molecules which can protect the body from damage caused by harmful molecules called free radicals. Antioxidants interact with unstable radicals and prevent harm of free radicals. Antioxidants are essential and important for plants and animals' sustenance [45]. The oxidation processes in the human body damage cell and other structures including cellular lipids, proteins and DNA. Antioxidants can interact safely with free radicals and ending the chain reaction before vital molecules are damaged [46]. They are natural Antioxidants found in some foods that play a useful role in maintaining a healthy body and help neutralize free radicals in our bodies. Plant derived antioxidants, such as flavonoids and related phenolic compounds, have multiple biological effects, including antioxidant activity to reduce disease risk such as asthma, cancer and heart disease. One of the studies indicate that antioxidants can prevent oxidative damage caused by asthma [47].Antioxidants can effectively block oxidation process by neutralizing free radicals, while oxidized antioxidants work in two ways[48] **:**

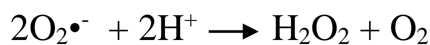
- **Chain-breaking** - where free radicals are created, they begin to react with a stable molecule, "taking" its electron, while the stable molecule loses its electron, converse to another free radical. This process continues to form more free radicals until termination and

stabilizing of free radical occur by a chain-breaking antioxidant molecules[48].

- **Preventive - antioxidant enzymes**: prevent oxidation process by stabilizing transition metal radicals such as copper and decrease the rate of chain initiation. They can also prevent oxidation by stabilizing transition metal radicals such as iron and copper. The human antioxidant defense system consists of both enzymatic and non-enzymatic antioxidant systems[49].

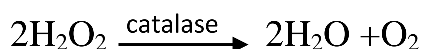
1.4.1. Enzymatic Antioxidants

Enzymatic system containing enzymes such as glutathione peroxidase (GPx) superoxide dismutase (SOD), catalase etc. SOD catalyses the dismutation of $O_2\bullet^-$ at a rate ten times higher than that for spontaneous dismutation at pH 7.4 [50].



$$2O_2\bullet^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Human cells have efficient protection against harmful effects of ROS by enzymatic and non-enzymatic antioxidant systems. Because the mitochondrial respiratory chain is a major site of formula generation in the cells, Mn-SOD plays an important role in maintaining cellular ROS balance. Catalase located in the peroxisomes can to convert H_2O_2 into H_2O and O_2. Another group of Se containing enzymes called glutathione peroxidase uses H_2O_2 as an oxidant to convert reduced glutathione (GSH) to oxidized glutathione (GSSG) [51].



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

$$2GSH + 2H_2O_2 \xrightarrow{\text{GSHPx}} GSSG + 2H_2O$$

Members of the enzymatic antioxidant defense system include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase, phenolic

peroxidases, such as guaiacol peroxidase (GPX), and the ascorbate/glutathione cycle that includes glutathione reductase (GR), Glutathione- S-Transferase (GST). The superoxide radical ($O_2^{\bullet}$-) is dissimulated to H_2O_2 by SOD, and CAT, APX and GPX metabolize H_2O_2 to H_2O. APX requires reduced ascorbate and GPX requires a phenolic compound like guaiacol to function. GR functions in the regeneration of reduced ascorbate after it is converted to mono dehydro ascorbate by APX(52, 53).

1.4.1.1. Glutathione-S-Transferase

Glutathione-S-transferases (EC.2.5.1.18 GSTs), represent a major groups of detoxification enzymes in living organisms, having a important role in cellular detoxification by protecting the cell through catalyse the conjugation of glutathione (GSH) to a wide group of exogenous and endogenous electrophilic compounds, Both exogenous and endogenous electrophiles compounds have ability to react with cellular components such as protein, lipids, DNA. Glutathione is a tripeptide (Glu-Cys-Gly) that is the specific substrate for glutathione *S*-Transferase. GST an antioxidant enzyme, GST family of detoxification enzymes includes three super families, cytosolic, microsomal and mitochondrial proteins (54).GSTs also have non-catalytic function; binding flavonoid natural products in the cytosol before their deposition. (GSTs) have functions in both common cellular metabolisms in addition the detoxification of a wide range of xenobiotic compounds.GST activity has been found in bacteria, plants, yeast, insects and in most mammalian tissues, mostly in the liver (55).In mammalian tissues , some GSTs can be purified to

homogeneity by ion –exchange chromatography , gel filtration and other traditional purification methods . (GST) uses 1-Chloro-2,4-dinitrobenzene (CDNB) which is suitable for the biggest possible range of GST isoenzymes when conjugation of the thiol group of glutathione to the CDNB substrate. Oxidative damage is exacerbated as a result decreasing in antioxidant enzymes activities such as catalase (CAT), (GST), and glutathione peroxidase (GPx) which acts as a trap of a free radical in conditions associated with oxidative stress. Last studies showed that GSTs catalyse glutathione-dependent isomerizations and decrease toxic organic hydro peroxide and also showed that high oxidative stress levels are associated with Glutathione S- transferases theta 1 (GSTT1) and Glutathione S- transferases Mu1 (GSTM1) which protects cellular DNA from oxidative damage [(56,57)].

Three mammalian glutathione-S- Transferase (GST) families, namely cytosolic, mitochondrial, and microsomal GST. Cytosolic or soluble GSTs represent the largest family of GSTs [(58,59)] and are ubiquitously distributed across living organisms, these are mainly recognized as detoxification enzymes involved in the cellular defense against chemically induced toxicity[(60,61)]. Two of cytosolic and mitochondrial GST_S comprise soluble enzyme that are only distantly related. Mammalian GST super-family consists of *GSTs*-alpha mu, omega, pi, sigma, theta and zeta based on their amino acid sequence similarity [(62,63)].

1.4.1.2. The Importance of Glutathione S-Transferase in Human Disease

GST family of detoxification enzymes catalyzing the conjugation of GSH with broad variation of endogenous and exogenous electrophilic compounds and is abundant during outmost of life forms. Human GSTs

are classified into two distinct super family members; The membrane bound cytosolic and microsomal family members. Microsomal GSTs plays very important role in the endogenous metabolism of leukotrienes and prostaglandins [64]. A lot of useful clinical medicine is also possible substrates for GST and development of medicine resistance can frequently be an essential element in treating failure not surprisingly, as a result , GSTs have been related, with the evolution, of resistance toward chemotherapy agents, herbicides , insecticides, and microbial antibiotics. In addition to the Transferase role, GSTs have been proved its ability to form protein: protein interaction with members of the mutagen -activated protein (MAP) kinase pathway thereby serving a regulatory function in the balance between cell apoptosis and survival [65, 66].

1.4.2. Non Enzymatic Antioxidants

Ascorbic acid (vitamin C), (vitamin E), glutathione (GSH), vitamin A, and β-Carotene are types of non-enzymatic antioxidant that act as a defense system in human body [67]. There is a balance between both the intracellular levels and action of these antioxidants which are necessary for the survival of organisms and their health1 [68 , 69]. It is known that the brain bears comparatively low antioxidant protection, and also contains high levels of polyunsaturated fatty acids which make it prone to increased lipid peroxidation [70] .

1.4.2.1.Glutathione (GSH)

GSH is most common non - protein, it is a water soluble tripeptide and which is very important for growth of cell. It contains amino acids, cysteine, glutamine, and glycine. GSH is a powerful reducing agent;

there for GSH is the most plentiful of intracellular small molecule thiol, to reach mill molar concentrations in several tissues. GSH plays an important role in a multitude of cellular processes such as antioxidant defense and detoxification of varied electrophilic compounds during catalysis by GPx and GST. The important function of GSH is clear in fungi, mammals, plants, and some prokaryotic organisms [71].In addition to the detoxification role , GSH plays important role in other cellular reactions, such as , glyoxalase system , protection against reactive oxygen and nitrogen species , nourishes the cells , preserves cell health , fights inflammation, and regulation of gene expression through thiol disulfide exchange reactions [72]. The accumulation of large amounts of reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet -}$) and peroxide (H_2O_2) lead to a process that is called to as 'oxidative stress , Hence, scavenging systems can eliminate them and control of the level free radical by formation of antioxidant in the cell . GSH works directly on removing ROS

during the action (GST) and glutathione peroxidase (GP_X) Reduced form of glutathione with GPx detoxifies peroxides act as an electron donor in the reduction reaction as show in Figure (1-5) , to produce the end product called Glutathione disulfide or oxidized glutathione (GSSG). GSSG is catalyzed by GSH reductase (GR) in a process that needs NADPH [73]. Glutathione reductase (GR) belongs to the wide family of flavoprotein disulfide oxidoreductase family that contain a single flavin adenine dinucleotide (FAD) or flavin mononucleotide which are is a redox cofactor . GSH also works as coenzyme of many enzyme systems such as glyoxalase and dehydrogenase. The highest concentration of glutathione is in the liver [74].

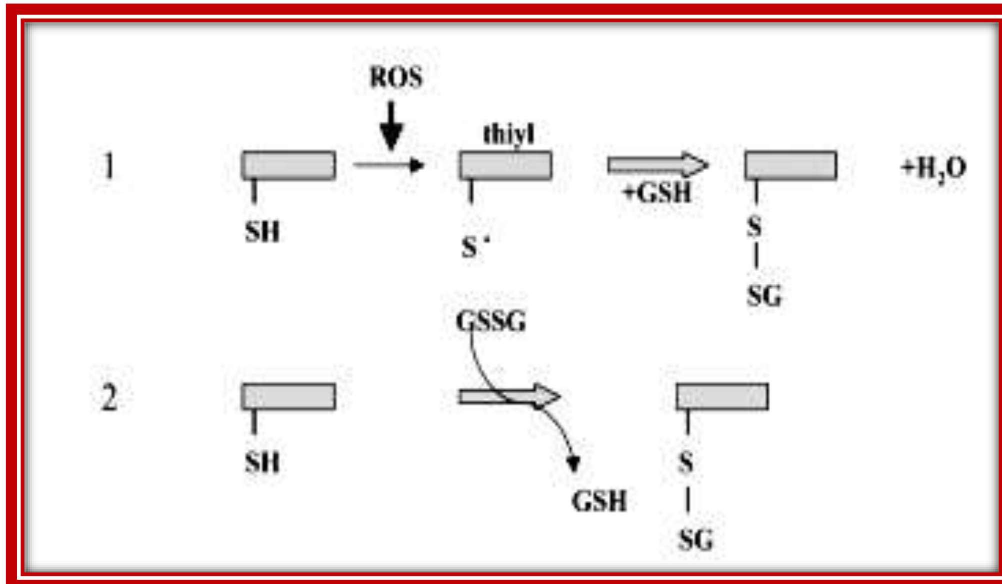


Figure 1-5 GSH redox cycle [74]

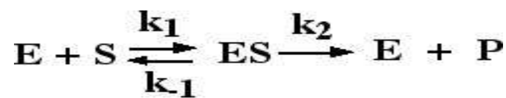
1.4.2.2. Nitric Oxide (NO)

NO has a multitude of physiological roles, including the ability to protect cell [75] .The cellular source of NO into the lung is unknown Nitric oxide radical NO• acts as a chain-breaking antioxidant in free radical-mediated lipid oxidation (LPO). In general chain breaking antioxidants act by reacting of NO• speedily with peroxyl radicals[76]. The combination of increased oxidative stress and NO may result in the formation of the strong radical peroxynitrite that may lead to nitrosylation of proteins in the airways. NO also possesses toxic effects such as pro-oxidant effects, genotoxicity and mutagenicity. Nitric oxide is synthesized from arginine and O_2 by nitric oxide (NO•) synthases [77]. These enzymes can convert arginine into citrulline, forming NO in the process. Nitric oxide (NO) also plays important role in synaptic transmission efficiency into nervous system [78].

1.5. Kinetic of Enzyme

1.5.1 Michaelis-Menten Equation

In biochemistry, Michaelis–Menten kinetics is one of the best-known models of enzyme kinetics . The model offers an explanation how the model offers an explanation how an enzyme can lead how the initial rate of this reaction, V_o, depends on the substrate concentration [S]. Michaelis-Menten kinetics is also called steady-state kinetics because it includes the steady-state assumption. Concept of Enzyme kinetics based on formation of a non-covalent complex (an ES complex when specific substrates binding occurs in a pocket on the enzyme called the active site (an ES complex).This chemical scheme is shown below [79].



Analysis of enzyme kinetic data requires the use of more than just comparisons of V_{max} and K_m values using the same error results of the parameters. The determination of enzyme kinetic parameters such as Km V_{max} is important in order to estimate of many biochemical reactions [80, 81].One of the most common mathematical function in use by biologists is the Michaelis-Menten equation which relates substrate concentration[S]to enzyme activity (v) [82].

$$V_0 = \frac{V_{Max} \quad [S]}{K_M + [S]} \quad \text{..} (1)$$

Where V_{max} and K_m are kinetic constants [83]. This equation is also employed in empirical models of a great variety of biological and physical processes[84, 85].

I.5.2. Conversions of the Michaelis-Menten Equation: The Double-Reciprocal Plot

The Michaelis-Menten equation is the rate equation for a one-substrate

Enzyme catalyzed reaction as follows

$$V_0 = \frac{V_{Max} \quad [S]}{K_M + [S]} \qquad \text{... (2)}$$

Double reciprocal plot is a graphical representation of the Line weaver–Burk equation of enzyme kinetics. It is difficult to determine V_{max} and accurately from a typical Michaelis-Menten. It is also difficult to determine an accurate value of V_{max} by estimating the limit of the hyperbola at infinity concentration of substrate. Accurate value of Vmax can be determined if Michaelis-Menten equation is transformed into equation that is more useful in experimental data and gives straight- line plot taking the reciprocal of both sides of equation 3 yields:

$$\frac{1}{V_0} = \frac{K_M + [S]}{V_{Max} \quad [S]} \qquad \text{... (3)}$$

Separation the components on the right side of equation (4) give:

$$\frac{1}{V_0} = \frac{K_M}{V_{Max} \quad [S]} + \frac{[S]}{V_{Max} \quad [S]} \qquad \text{..........................(4)}$$

This equation simplifies to

$$\frac{1}{V_0} = \frac{K_M}{V_{Max} \quad [S]} + \frac{1}{V_{Max}} \qquad \text{.............................. (5)}$$

This form of the Michaelis-Menten equation is called the Line weaver-Burk equation. For enzymes that obeying the Michaelis-Menten relationship, a plot of 1/Vo versus 1/[S] (the "double reciprocal" of the Vo versus [S] plot we have been using up to this point) yields a straight line [86], as show in Figure (1-6).

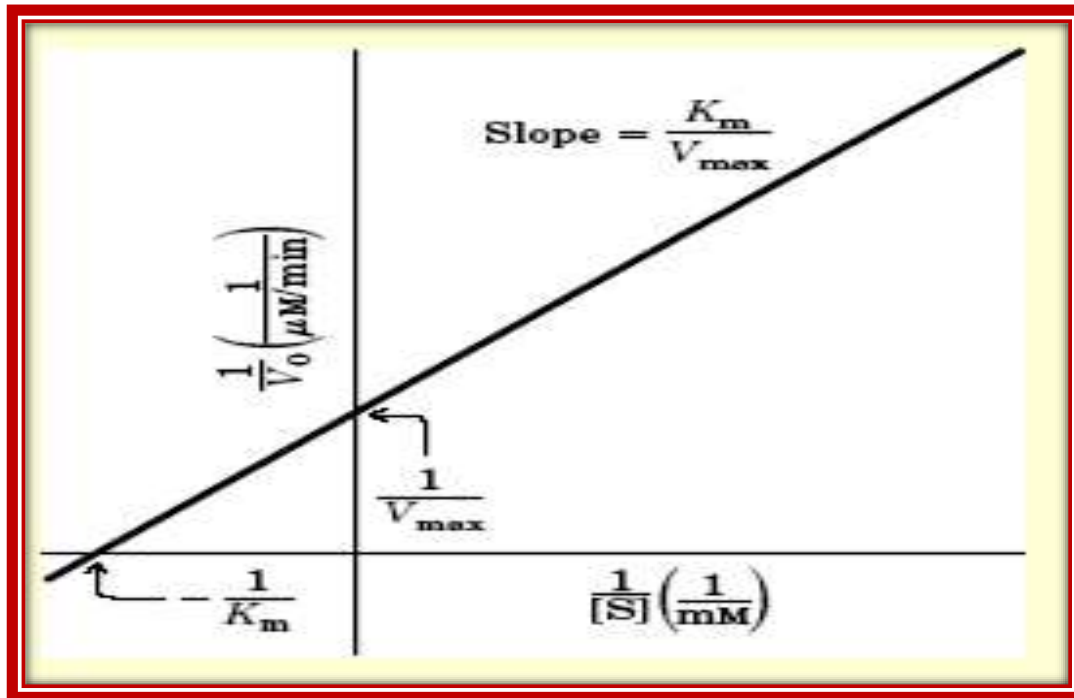


Figure 1-6: A double-reciprocal or Line weaver-Burk Plot [86]

1.6. Propolis

Propolis is a sticky substance collected by bees from some plants, and uses in the cell as building substance and defensive substance. In the last

years, it has been popular as a therapy in Europe but in this time, Propolis use in more than one recipe, such as for canker sores, infections caused by bacteria, strengthen the immune system [87].The obtained information about chemical composition of Propolis is phenolic, flavonoids, and various aromatic compounds. Propolis also contains several volatile oils, beeswax and terpenes, but these compounds are not thought to contribute to the large extent in chemical properties and implications of Propolis. The important characterization in Propolis is their significant biological activity. Propolis is a plant derived product, it form chemical elements which depends on the local flora at the site of Propolis is a plant derived product, it makes up of chemical elements which depends on the local flora at the site of collection, so it offers significant chemical diversity[88]. Now, a lot of discussion about the role of Propolis volatiles in identification of its plant origin.One of the reasons behind Propolis' popularity is that it is believed to have antiviral, antibacterial, antifungal, and anti-inflammatory properties. It is necessary also think about the contribution of volatiles and their constituents to the biological activity of Propolis[89]. Future perspectives are outline about the research on Propolis volatiles, Propolis extracts have also has been reported as important to prevention of toxic effects of some drugs or acting as radio protective. Propolis is a strong antioxidant. The free radical theory in human physiology confirms that the active free radicals are involved in all the cellular decomposition process and result to Oxidative stress that leading to cell death. Oxidative stress is believed to contribute to the development of chronic and degenerative diseases such as cancer autoimmune unrest aging, asthma, rheumatoid arthritis, asthma, cardiovascular and neurodegenerative diseases[90].

1.7. Ovalbumin and Asthma.

Ovalbumin is a glycoprotein which includes 54% of the total proteins of egg white. The function role of ovalbumin is unknown, despite it is supposed to be a storage protein. The properties of ovalbumin are discussed in relationship to their possible functional Importance. The Importance of ovalbumin as a model for the Serpin Family. Improvements in the functional properties of ovalbumin are of great importance in the food industry[91]. These include reasons for failure of ovalbumin to undergo a typical serpin conformational change involving the reactive centre loop, which explains why ovalbumin is not a protease inhibitor, and also the natural conversion of ovalbumin to the more stable form. Ovalbumin displays sequence and three-dimensional homology to the serpin super family[92] .Ovalbumin includes 3.5% carbohydrates and has four free sulphydrilic groups and a disulphide group. It cans decomposition by exposure heat, by surface absorption, in films, through agitation, or by the action of several denaturant agents [93]. Ovalbumin derived from chicken egg is a frequently used allergen that induces a robust, allergic pulmonary inflammation in laboratory rodents. A review of OVA challenge models has recently been published by Kumar et al[93]**.** Ovalbumin, however, is seldom implicated in human asthma, and other groups have used alternative allergens that may have greater clinical relevance, for example house dust mite (HDM)[94].

The Aims of The Study

1. To study the biological role of GST in asthma under oxidative stress conditions
2. To investigate the changes in level of GST (enzymatic antioxidant) and the level of GSH and NO (Non enzymatic antioxidants) related to oxidative stress
3. To evaluate the ability of EEP (Ethanolic extract of Propolis) to inhibit the free radicals and decrease the oxidative stress in asthmatic rats
4. To study kinetic of GST (calculate K_m and V_{max}) by Michaelis- menten equation
5. To estimate gene expressions of GSTM1 in the liver of asthmatic Rats, to study the biological role of GST in asthma under oxidative stress conditions
6-Investigation of histopathological change

3.1. The Ethanolic Extract of Propolis

The weight of final product was 18.631 g, for that the proportion of yield was:

18.631/ 50 *100 = 37.3 %

This result of EEP yield of local Propolis (37.3%) is similar to that obtained by (Meaad; 2004) [118] and (Paviani *et al*)[119] that presented a value of 38.34 ±2.05% and 39.45±1.20% , respectively of Iraqi and Brazilian Propolis .The difference in extraction yield and the concentration were probably related to characteristics of the raw Propolis

. This result also agrees with (AL-Mohana 2004) [99] who obtained 33% yield of EEP from Propolis collected from different Iraqi provinces.

3.2. The Primary Chemical Constituents

The primary chemical tests appeared positive results for flavonoids, resins, phenols, saponin and alkaloids .This positive result suggested that EEP contained substances that were known to show important medicinal activity as well as exhibiting physiological activity .

The presence of flavonoids, resins, phenols and saponin in Propolis came in a statement with that reported by other researchers [120, 121] . The test of alkaloids gave positive results, it agrees with (Fokt *et al*; 2010) [122]. But comes in contrast with the result of (Al-Mohana; 2004) who regarding the negative results for alkaloids in Iraqi EEP. This contrast regarded the chemical constituent of Propolis (presence of alkaloids as example) might be attributed to the variation in plant sources in areas of Propolis collection as well as the difference in season of Propolis collection .

3.3. Biochemical Analysis

3.3.1. Levels of Serum Nitric Oxide in Different Groups

The concentration of NO increased significantly in asthmatic rats (G2) as compared with control group (G1) ($P < 0.05$). This is a marked elevation of NO which was significantly reduced by pre administration of EEP in (G3) ($P < 0.05$). In addition ,there is almost the same concentration of NO showed in treated group with EEP and then induced asthma(G4)as

compared with control group(G1) as shown in figure (3-1) and table (3-1).

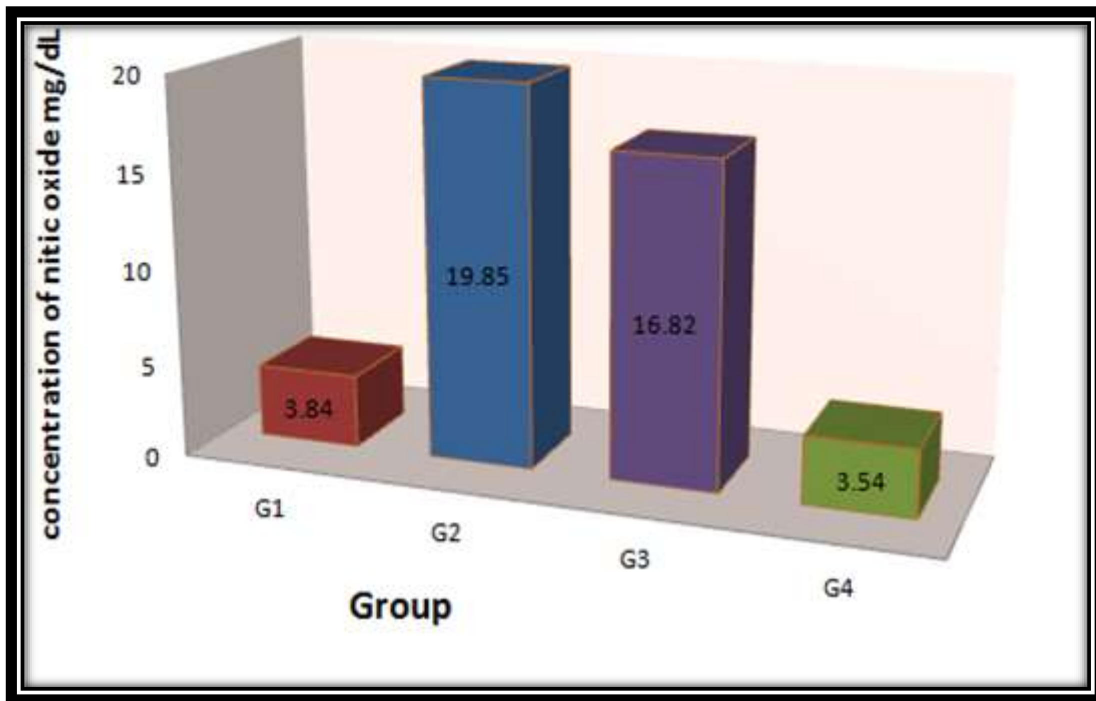


Figure 3-1 levels of nitric oxide in different groups
G1= control rats. , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

Table 3-1 Comparison between the control group (G1) and the three treatments on both alone to indicate any treatments more effect of nitric oxide

Groups of male Rats	Nitric oxide(mg/dL) M ± SD	Significant or Non Significant	P value

G1	3.84±1.1	-	-
G2	19.85±2.6	S.	$p < 0.05$
G3	16.82±1.9	S.	$p < 0.05$
G4	3.54±1.2	N.S.	-
LSD	5.8	Sign G2,G3	$p < 0.05$

S: Significant, N.S: Non Significant

G1= Intact health , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

NO play a critical role in the pathogenesis of airway inflammation in allergic asthma [(123)].The increased level of NO in (G2) may be due to ovalbumin which induced asthma. OVA induced airway inflammation, asthma and its greater expression of inducible nitric oxide synthase NOS (iNOS) in the lung tissue [(123)].airway inflammation is attributed to increased expression and activity of iNOS which increase NO production and consequently the numbers of eosinophils in the airway and airway inflammation [(124,125)].

The significant decrease in NO values in rats were pretreated with EEP before induce asthma might be attributed to the ability of EEP to inhibit different pathologic changes associating asthma or may be due to the effect of EEP to inhibited the OVA-induce and decrease level of IgE and iNOS in serum of asthmatic rats . This result agrees with (Meaad; 2004) [(118)].

Where CAPE a biologically active ingredient of Propolis , showed inhibitory effects on the inflammatory cells [(127)]and it may be a main regent of the decrease level of the iNOS and NO concentration in rats that received of EEP[(126)].

These finding suggest that EEP may be useful as an assistant therapy for patients with allergic airway inflammation and asthmatic patients in the future.

3.3.2. Levels of Serum GSH in Different Groups

The asthmatic rats (G2) showed significant decline in the mean of serum GSH level as compared with serum samples in control group(G1) ($P < 0.05$) .The oral administration of EEP and then induced asthma in (G3) caused a significant elevation in GSH as compared with results found in asthmatic group(G2) ($P < 0.05$) . Animal's administration with EEP before induced asthma in (G4) showed almost the same concentration in GSH level as compared with control group as shown in Figure (3-2) and table (3-2).

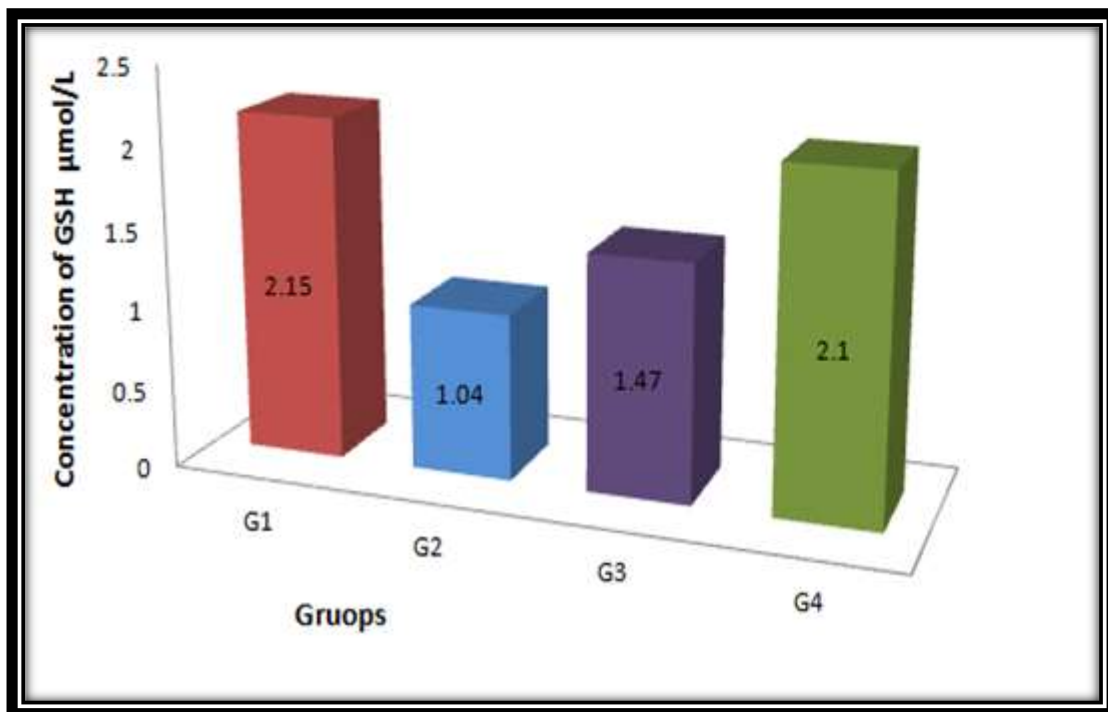


Figure 3-2 levels of GSH in different groups
G1= control rats. , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

Table 3-2 Comparison between the control group (G1) and the three treatments on both alone to indicate any treatments more effect of GSH

Groups of male Rats	GSH (µmol/L) M ± SD	Significant or Non Significant	P value
G1	2.15±0.4	-	-
G2	1.04±0.6	S.	$p < 0.05$
G3	1.47±0.5	S.	$p < 0.05$
G4	2.1±0.4	N.S.	-
LSD	0.9	Sign G2,G3	$p < 0.05$

G1= Intact health , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

Concentration plays a central role of antioxidant defense system, which detoxifies ROS [128,129]. The decreased level of serum GSH in asthmatic group (G2) may be returned to the increase in the oxidative stress, and so that GSH becomes oxidized to its diametric form (GSSG) as a result of its function as an antioxidant by acting as a sacrificial target for ROS and

other products of lipid peroxidation .This result agrees with (Nouf; 2010) [130] that showed the decrease in GSH level in Saudi Asthmatic patients.

The enhancement in the GSH level caused by EEP may be returned to its antioxidant components such as flavonoids. Flavonoids are ideal scavengers of peroxyl radicals due to their favorable reduction potentials relative to alkyl peroxyl radicals, and thus they are effective inhibitors of lipid peroxidation [131]. Therefore, this strong anti oxidative property of flavonoids has made them protective against airway diseases linked to oxidative stress [131]. On the other hand, the elevation of GSH level in this group (G 3) may return to increase GSH production ,where caffeic acid phenethyl ester (CAPE) ,one of the Propolis components ,is known to increase the expression of γ-glutamyl cysteine synthtase ,an important enzyme in synthesis of GSH resulted in production of glutathione [132]. The increased level of GSH in (G4) as compared with control group may be backed to that EEP increase the power and capacity of other endogenous antioxidant defense of the body and thus increase the steady state levels of GSH.

3.3.3. Levels of Serum GST in Different Groups

The changes in the serum Glutathione –S-Transferase activity of normal and experimental rats are illustrated in Figure (3-3) show significant reduction in the activity of GST in serum of rats in (G2) when compared with control group (G1), then simultaneous induced asthma and treatment with EEP (G 3) show increase activity of GST in serum of rats when compared with (G2), while in (G4) treatment with EEP before induced asthma was showed almost same activity of GST enzyme as compared with control group(G1),as shown in Figure (3-3) and table (3-3).

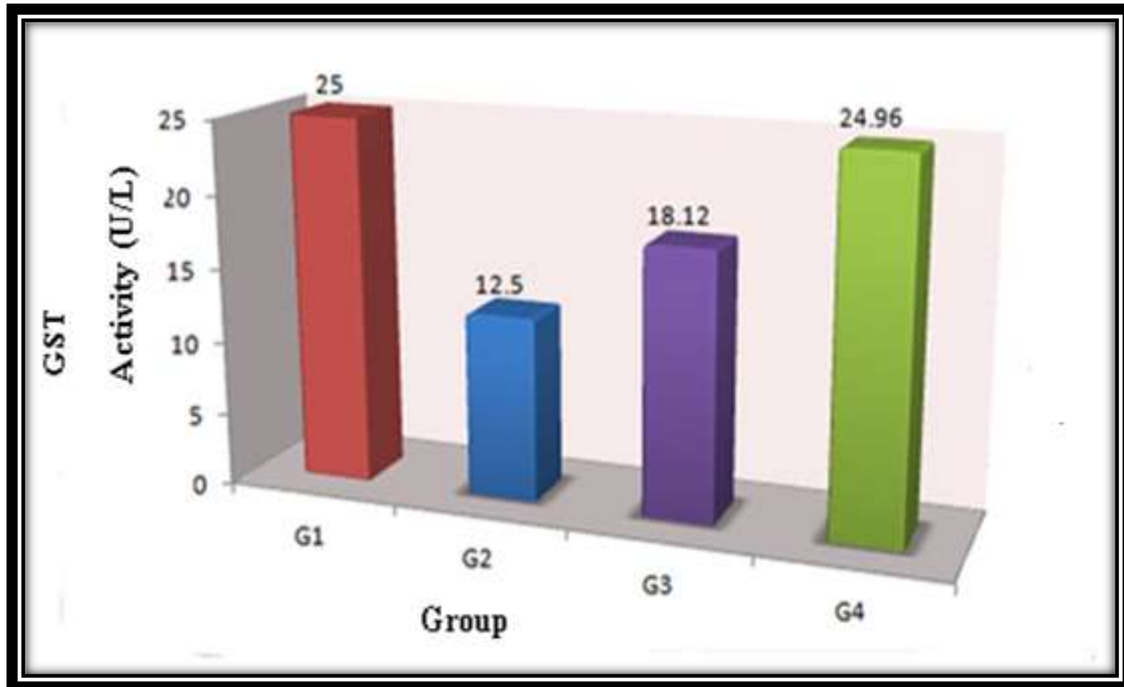


Figure 3-3 Levels of GST activity in different groups
G1= control rats. , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

Table 3-3 Comparison between the control group (G1) and the three treatments on both alone to indicate any treatments more effect of GST

Groups of male Rats	GST activity (U/L) M ± SD	Significant or Non Significant	Pvalue
G1	25 ±1.3	-	-
G2	12.5±1.6	S.	$p < 0.05$
G3	18.12±2.3	S.	$p < 0.05$
G4	24.96±1.1	N.S.	-
LSD	3.5	Sign G2,G3	$p < 0.05$

G1= Intact health , G2= Asthmatic rats.

G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

The body has an effective mechanism to prevent and neutralize the free radicals induced damage [133]. The oxidative stress plays an important role in the development and progression of bronchial asthma and chronic obstructive pulmonary disease [134].

The decrease activity of GST in asthmatic rats (G2) may return to the toxic effects of asthma they are largely due to its active metabolite, the free radicals [135] and that leads to increased oxidative stress. Or may be returned to the decrease availability of GSH in(G 2),the relation between GSH and GST its close, where it was well documented that GST enzyme catalyze the reaction via thiol (-SH) group of glutathione (GSH) by neutralizing and rendering the product more water soluble , this result agrees with (Cervello, et al :1992) [136]. The elevation in serum GST activity of rats received EEP in (G3) might be due to the decline of the production of free radicals and /or by trapping and inhibited the formed free radicals ,or may be the activation of antioxidant system against toxic metabolites of asthma . Where the EEP might inspire the induction of phases enzymes (GST) and the other antioxidant enzymes by its chemical ingredients as phenyl propanoids (PPS) [137].

3.4. Extraction of Enzyme Glutathione-S-Transferase (GST) and Purification from Rat Liver

3.4.1. Enzyme Extraction

Extraction process included cut liver of rats into small pieces and homogenized liver tissue with an equal volume of Tris – HCL (Tris hydroxy methyl amino methane) solution of 0.1 M and PH 7.5 for 15

minutes to get the extract crude enzymatic solution which reached its Specific activity 2.55,1.36,1.98 and 2.47 for G1,G2,G3 and G4 respectively shown as in tables (3-4) and then the homogenate was centrifuged at 1.1175 Xg for one hour as this step is very effective to form minutes colloidal which gives after centrifugal separation of the mixture into two layers surface of which was supernatant contain the enzyme .It was selected Tris-HCl as mentioned in the **(Hinberg)**[138] that Tris-HCl increases the efficiency of extraction and speed of GST reaction, and that the Tris-HCl solution content of amines containing hydroxyl group (- OH) that increase the speed of the catalyst interaction enzyme. As can be attributed to the fact that Tris-HCl achieves condition of good buffer, containing high resistance to change in pH as well as the Tris-HCl solution containing ions working to facilitate the solve of the enzyme and separated from the rest of the insoluble components, as well as to maintain the stability and effectiveness of the enzyme in the crude extract.

3.4.2. Enzyme Concentration

For the purpose of obtaining the concentration of the solution resulting from the enzymatic extraction it has been deposited with ammonium sulfate 50% by saturation as the precipitate process aimed at transforming the proteins present in the solution to the minutes insoluble and methods used in the precipitate of enzymes add salt neutral called salting-out and alcohols and organic solvents or change pH describes the salting-out term precipitate process using concentrations of salts, which works to achieve two important aims which are the concentration and purification of proteins. The mechanism of precipitate happen tendency of protein molecules accompanied by some because of the interference protein - protein become effective and preferred than the interactions protein -

solvent. As a result of the decline in hydrolysis of proteins work as neutral salt ions to withdraw layer surrounding protein molecules depends on the rate of protein charge is working to configure a few solubility leading to precipitation, note that the more groups charged on protein molecules was a high-protein hydrolysis [139]. The ammonium sulfate from more neutral salts commonly used to being cheap and high soluble as its non-toxic as well as it helps to install many of the enzymes [140], [141].Followed by the step precipitate and dissolution dialysis solution filtrate about the final solution versus 0.1M phosphate buffer and pH 6.25 for 72 hours with a change buffer solution has enabled this step of increase specific activity to 4.22, 2.31, 3.49 and 4.14 for G1,G2,G3 and G4 respectively shown as in tables (3-4).

3.4.3. Enzyme Purification

Enzyme purification were done by passing the solution of enzymatic product after dialysis process throughout purification column filled with purification sephadex G-75 dimension of 2 × 50 cm and has two balancing and recovery solution phosphate buffer 0.1M and pH 6.25, has enabled this step of Increase Specific activity to 6.19, 3.22, 4.84and 5.79for G1, G2, G3 and G4 respectively shown as in tables (3-4).

Table 3-4 Steps of partial purification of glutathione S-Transferase (GST) from rat liver for (G1, G2, G3 and G4) groups

Purification stage	Volume taken	Total protein	Activity U*/mL	Specific activity

	(mL)	mg/mL		U/mg Protein
G1				
Cytosol Fractions:	6	0.521	1.329	2.55
Precipitate by $(NH_4)_2SO_4$	5	0.446	1.885	4.22
Supernatant	3	0.211	0.40	1.89
Dialysis	2.5	0.346	2.146	6.19
Sephadex G-75 (Fractions)	0.5	0. 290	2.85	9.827
G2				
Cytosol Fractions:	6	0.491	0.671	1.36
Precipitate by $(NH_4)_2SO_4$	5	0.412	0.952	2.31
Supernatant	3	0.191	0.23	1.2
Dialysis	2.5	0.335	1.081	3.22
Sephadex G-75 (Fractions)	0.5	0.281	1.43	5.08
G3				
Cytosol Fractions:	6	0.489	0.97	1.98
Precipitate by $(NH_4)_2SO_4$	5	0.392	1.37	3.49
Supernatant	3	0.187	0.29	1.55
Dialysis	2.5	0.322	1.56	4.84
Sephadex G-75 (Fractions)	0.5	0.277	2.08	7.5

G4				
Cytosol Fractions:	6	0.513	1.27	2.47
Precipitate by $(NH_4)_2SO_4$	5	0.432	1.79	4.14
Supernatant	3	0.201	0.38	1.89
Dialysis	2.5	0.352	2.04	5.79
Sephadex G-75 (Fractions)	0.5	0.288	2.71	9.4

G1= Intact health , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

3.5. Estimate the kinetic Constants of the enzyme

Estimated kinetic constants of Michalis (K_m) and maximum velocity (V_{max}) for GST enzyme purified from rat liver using CDNB as the substrate by the method (the line weaver-Burk plot) used to draw a relationship between the reaction rate and the concentration of the substrate to determine constants(K_m) and (V_{max}) as shown in figures (3-4). The importance of hard estimate (K_m) and the known concentration of the substrate when the speed of the enzyme reaction is equal to half the maximum velocity , being the most important constants special enzyme and substrate, because it varies depending on the enzyme and the substrate used as well as to determine the value of this constant [142]**.** The results show that the value of K_m is (11.73, 13.2, 11.03 and12.96 mmol/liter), as well as show significant reduction in the V_{max} in (G2) when compared with control group (G1), then simultaneous induced asthma and treatment with EEP (G3) show increase V_{max} when compared with group (G2), while in (G4) treatment with EEP before induced

asthma show almost same V_{max} as compared with control group, As show in figure (3-4) and table (3-5).

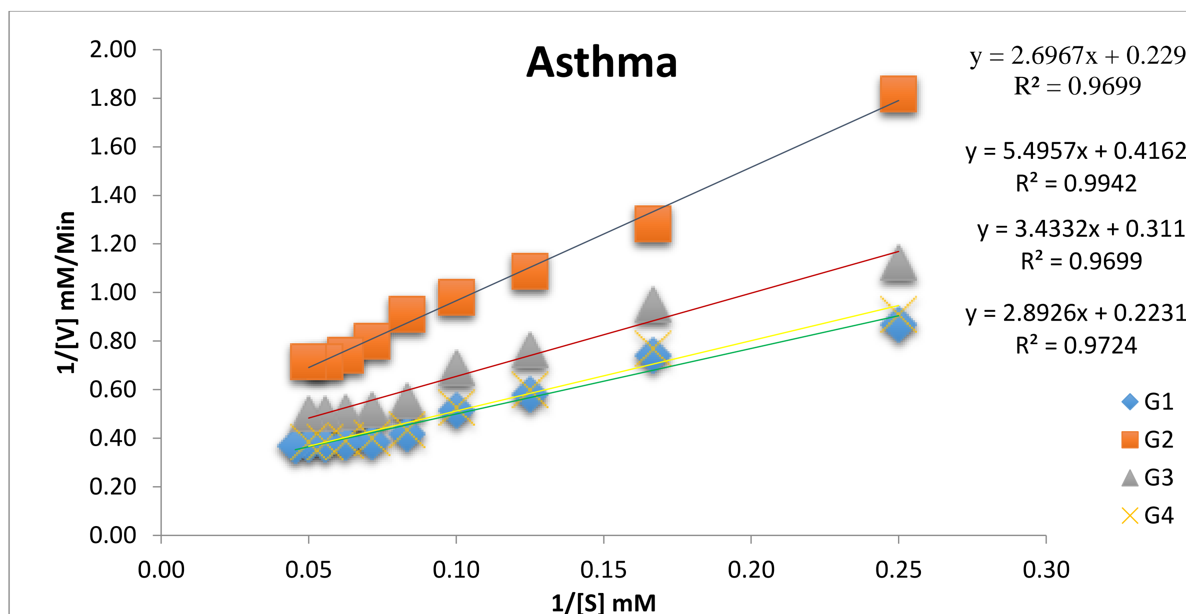


Figure 3-4 Line weaver-Burk plot of partially purified GST from rat's liver of G1, G2, G3 and G4

Table 3-5 show value of K_m and V_{max} for G1, G2, G3 and G4

Groups	V_{max}(µmol/min)	K_m(mmol/liter)
G1	4.35	11.73
G2	2.40	13.20
G3	3.21	11.03
G4	4.48	12.96

G1= Intact health , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

A result was obtained using a Line weaver-Burk plot by plotting the reciprocal of the inverted initial velocity versus the reciprocal of the inverted substrate concentration. A linear relationship was obtained as shown in Figure (3-4) giving a Km value of (11.73, 13.2, 11.03 and12.96 mmol/liter) and V_{max} (4.35, 2.40, 3.21 and4.48 µmol/min) For G1, G2, G3 and G4. The results showed that significant reduction of the V_{max} in (G2) when compared with control group (G1), then simultaneous induced

asthma and treatment with EEP (G3) show increase V_{max} when compared with group (G2), while in (G4) treatment with EEP before induced asthma show almost same V_{max} as compared with control group.

3.6. Gene Expression of Glutathione-S-Transferase (GST)

3.6.1. The concentrations and purity of total RNA

Total RNA concentrations (ng/µl) and purity was estimated using Nanodrop spectrophotometer in absorbance readings (260-280nm).All liver tissue samples that used in the present study gave high concentrations of total RNA and appeared quantitatively enough to proceed in quantitative reverse transcriptase real-time PCR as shown in table (3-6). The present results have shown that the ratio between optical densities at (260-280 nm) was within normal range (more than 1.8 and less than 2.0). The purity of total RNA samples of liver tissues that obtained from experimental adult male rats was *extracted* using TRIzol reagent.

Table 3-6 Effect asthma on RNA concentration (ng/µl) at 8 weeks in ova-albumin - induced asthma male rats.

Group	260/280R(purity)	RNA conc.(ng/ul)
G1	1.88	1425.0
G1	1.88	1339.7
G1	1.88	1376.3
G1	1.87	1484.6
G1	1.88	1165.6
G1	1.90	1557.5
G2	1.91	1696.7
G2	1.92	1508.0
G2	1.90	1351.8

G2	1.82	1926.9
G2	1.89	1227.7
G2	1.91	1350.8
G3	1.91	1305.4
G3	1.56	1372.4
G3	1.90	1737.4
G3	1.89	1643.0
G3	1.95	1197.6
G3	1.52	2053.8
G4	1.76	1858.0
G4	1.89	982.7
G4	1.12	1979.8
G4	1.87	1752.1
G4	1.93	998.7
G4	1.91	1490.4

G1= Intact health , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

3.6.2. Quantitative Reverse Transcriptase Real- Time PCR

Quantitative reveres transcription real-time PCR (RT-qPCR) was performed for measurement of relative quantification (gene expression analysis) for GSTM1 genes expression levels normalized by housekeeping gene expression.

RT-qPCR quantification method in real-time PCR system was dependent on the values threshold cycle numbers (CT) of amplification plot of target genes and housekeeping gene. Where the result of real-time PCR amplification plot of housekeeping gene make no difference in CT value, where the control group (CT=33) while the treatment groups which also

appeared (CT=33). Figure (3-5) show Amplification plot GAPDH gene of treated and control groups.

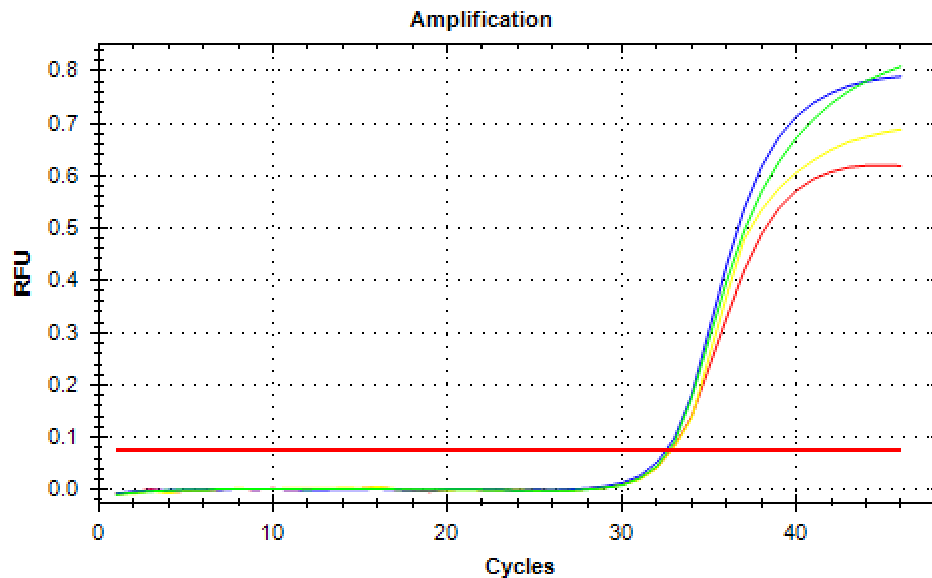


Figure 3-5 Amplification plot GAPDH gene of treated and control groups

Yellow: control rats (G1).
Red: Asthmatic rats (G2).
Green: induced asthma and Received EEP (G3).
Blue: Received EEP and induced asthma (G4).

The result of real-Time PCR amplification plot of target genes (GSTM1) were appeared difference in CT value between control and different groups. Figure (3-6) show amplification plot GSTM1 gene of different groups.

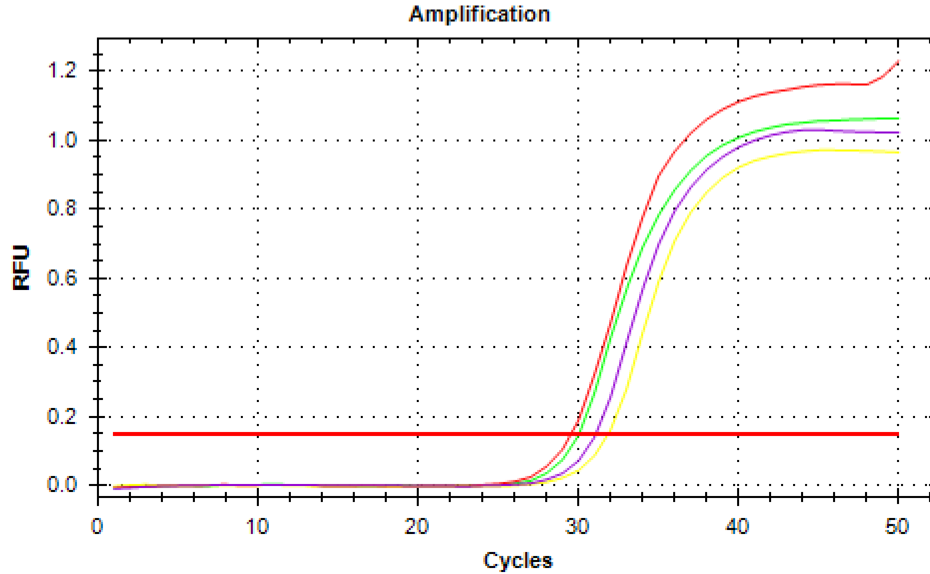


Figure 3-6 Amplification plot GSTM1 gene of different groups.
Yellow: control rats (G1).
Red: Asthmatic rats (G2).
Green: induced asthma and Received EEP (G3).
Blue: Received EEP and induced asthma (G4).

3.6.3. Relative Gene Expression

The relative expression of target genes (GSTM1) in rats liver tissue was calculated by using Livak Method ($2^{-\Delta\Delta CT}$) that dependent on normalization of RT-qPCR (CT values) of target genes with housekeeping gene (GAPDH) as reference gene in different groups.

The results of relative gene expression in GSTM1 gene which appeared clear difference in fold change of gene expression levels between different groups. Where, G2 was appeared up regulation at (5±1.3), G3 group was appeared up regulation at (2.9±1.6), G4 was appeared down regulation at (0.97±0.4), relative to control groups that is equal to 1 fold change of gene expression levels as shown in table (3-7) and table(3-8) and figure (3-7).

The statistical analysis of relative gene expression in GSTM1 gene was found significant differences in treatment groups compared with control groups at level ($P<0.05$) as shown in table (3-9).

Table 3-7 GSTM1 gene expression of G2, G3, and G4

Exp. Group	CT (Gstm1)	CT (GAPDH)	ΔCT (Test)	ΔCT (control)	ΔΔCT	Fold change (2^-ΔΔCT)	Mean
G2	29.43	31.23	-1.8	0.426666667	-2.2266667	4.680513013	**5.0032203**
G2	29.33	31.42	-2.08696638	0.426666667	-2.5136331	5.710563266	
G2	29.23	31.32	-2.08795676	0.426666667	-2.5146234	5.714484773	
G2	29.13	31.13	-1.99959849	0.426666667	-2.4262652	5.375001498	
G2	29.83	31.29	-1.46342122	0.426666667	-1.8900879	3.706578047	
G2	29.47	31.32	-1.84600797	0.426666667	-2.2726746	4.832181503	
G3	30.17	31.63	-1.46	0.426666667	-1.8866667	3.697798641	**2.9705300**
G3	31.24	31.44	-0.19696638	0.426666667	-0.6236331	1.540750281	
G3	30.34	32.02	-1.67795676	0.426666667	-2.1046234	4.300854809	
G3	30.56	31.38	-0.81959849	0.426666667	-1.2462652	2.372264964	
G3	30.14	31.35	-1.21342122	0.426666667	-1.6400879	3.116848193	
G3	30.49	31.55	-1.05600797	0.426666667	-1.4826746	2.794663623	
G4	32.42	31.49	0.925067068	0.426666667	0.4984004	0.707891226	**0.9768882**
G4	31.55	31.43	0.115711295	0.426666667	-0.3109554	1.240528923	
G4	32.18	31.52	0.656956172	0.426666667	0.2302895	0.852463811	
G4	31.43	31.44	-0.00770376	0.426666667	-0.4343704	1.351321006	
G4	32.13	31.44	0.686268633	0.426666667	0.25960197	0.835318349	
G4	32.05	31.43	0.621281027	0.426666667	0.19461436	0.873806438	
Mean G1	32.73	32.30	0.426666667	0.426666667	0	1	**1**

Table 3-8 GSTM1 gene expression of G 1

Exp. Group	CT (GSTM1)	CT (GAPDH)
G1	32.24	31.22
G1	32.89	31.25
G1	33.12	31.23
G1	32.34	31.37

G1	33.23	31.37
G1	32.54	31.29
Mean	32.72666667	31.29

Table 3-9 Comparison between the control group (G1) and the three treatments on both alone to indicate any treatments more effect GST of gene expression

Groups of male Rats	**GSTM1 gene expression (M ± SD)**	**Significant or Non Significant**	**Test result**
G1	1± 0.5	-	-
G2	5±1.3	S.	$p < 0.05$
G3	2.9±1.6	S.	$p < 0.05$
G4	0.97±0.4	N.S.	-
LSD	1.7	-	$p < 0.05$

G1= Intact health **, G2= Asthmatic rats.**
G3= induced asthma and Received EEP. **, G4= Received EEP and induced asthma**.

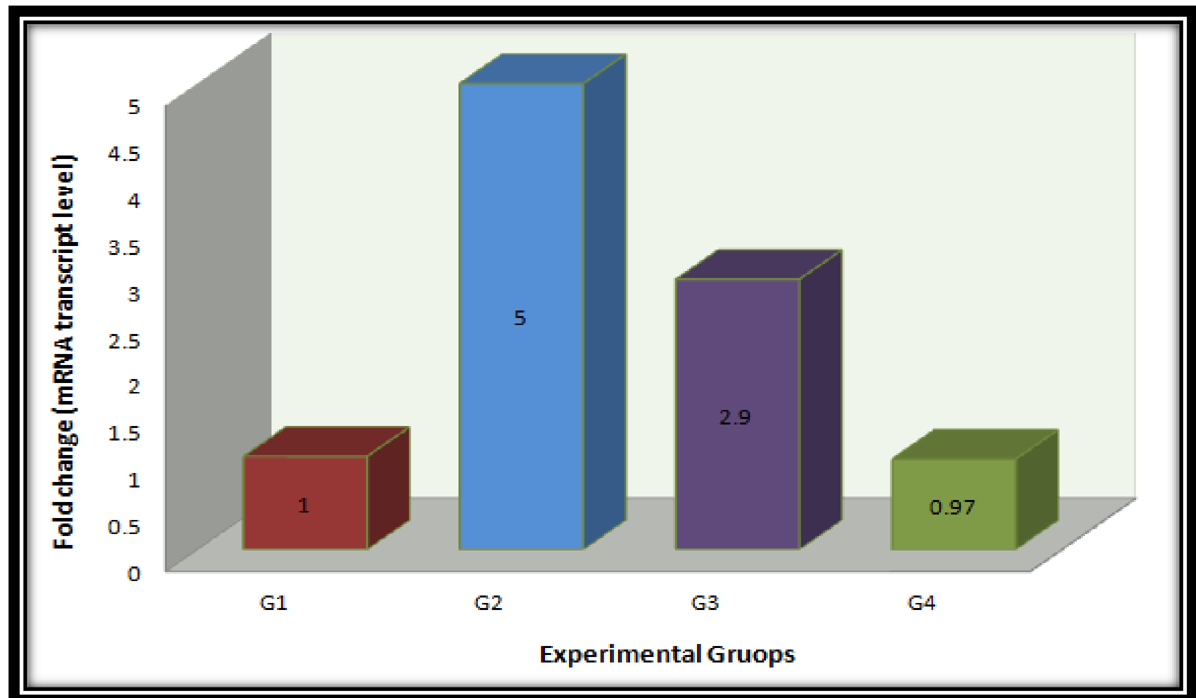


Figure 3-7 Relative gene expression

G1= control rats. , G2= Asthmatic rats.
G3= induced asthma and Received EEP. , G4= Received EEP and induced asthma.

Glutathione S-transferases (GST) include a number of subclasses, such as GST-P1 and GST-M1 that are expressed in the lungs and have been implicated in the pathogenesis of asthma. As well as calculated gene expression and show that result increase gene expression in (G2) when compared with (G1), while in (G 4) was showed almost same gene expression as compared with control group (G1).

3.7. Histopathological Changes.

The result of biochemical alterations was insured by microscopic examination of rat's lung, and to assess the anti-inflammatory or anti-modeling of Propolis. Lung tissue was collected (5days) after the last OVA challenge .OVA-challenge induced marked infiltration of inflammatory cells into the per bronchial and per vascular connective

tissue. The score of histological examination in OVA-challenged group was higher than in control group as show in Figure (3-8). This result was come in agreement with the result of (Meaad; 2004) [118].

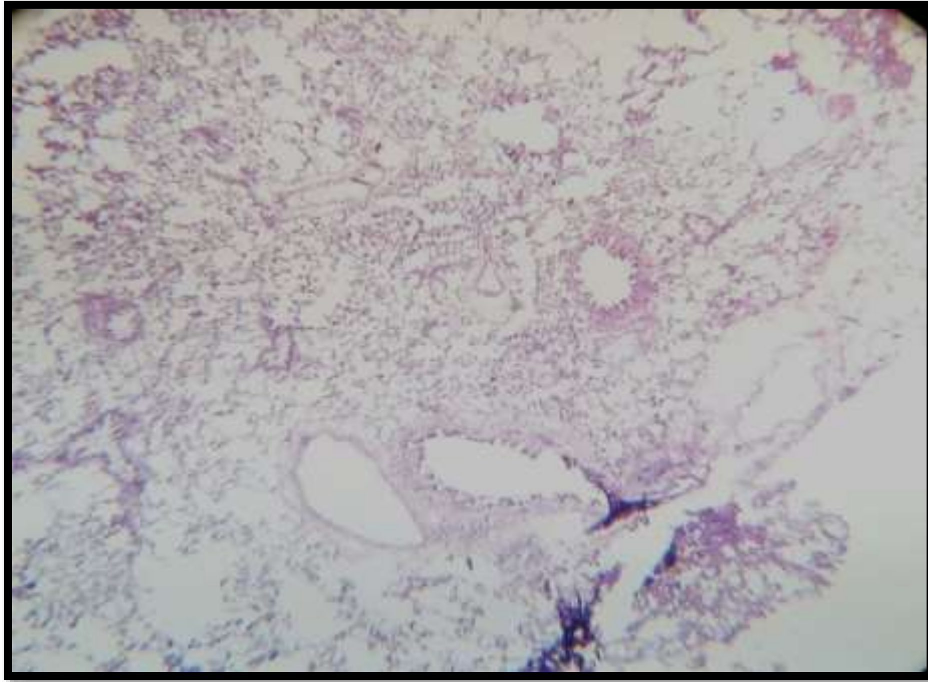


Figure 3-8 Normal lung (G1).

The majority of the infiltrated inflammatory cells were eosinophils and neutrophils. OVA-induced caused over production of mucus and goblet cell hyperplasia as show in Figure (3-9).

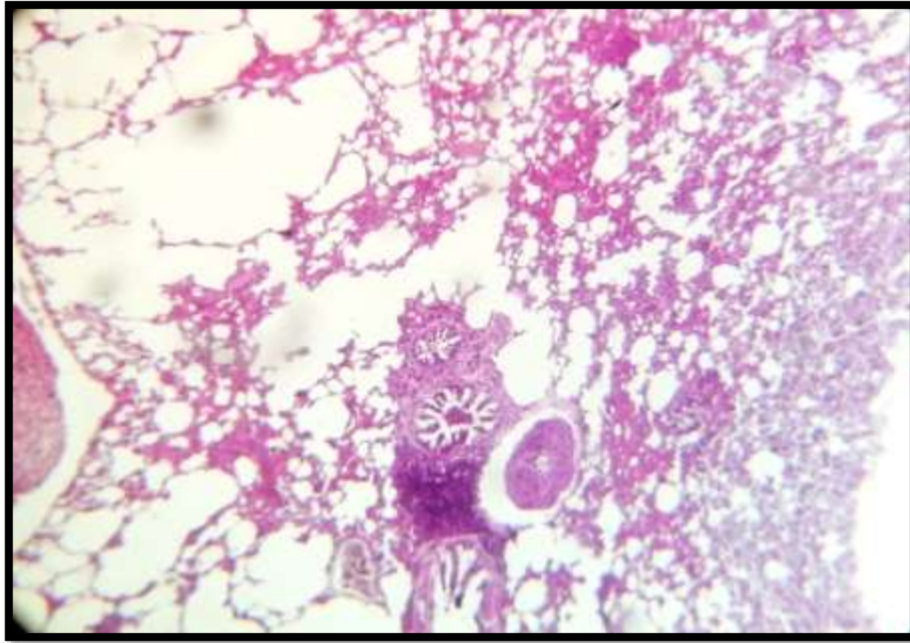


Figure 3-9 Lung rats induced asthma by OVA albumin only (G2) ,showed there were sever contraction of bronchi with sever inflammatory cell infiltration (eosinophils and neutrophils) with plug mucus that obstructed lumen .

Rats received EEP as antioxidant and anti-inflammatory before injection of OVA successfully and partially mitigates the above pathological changes which induced by OVA injection .EEP significantly attenuated eosinophil rich leukocyte infiltration compared with OVA –challenged rats (Figure 3-10),as well as blocked similarly changes in the structural cells of the airways , including increases in airway smooth muscle mass ,epithelial cell disruption and subepthelial fibrosis .

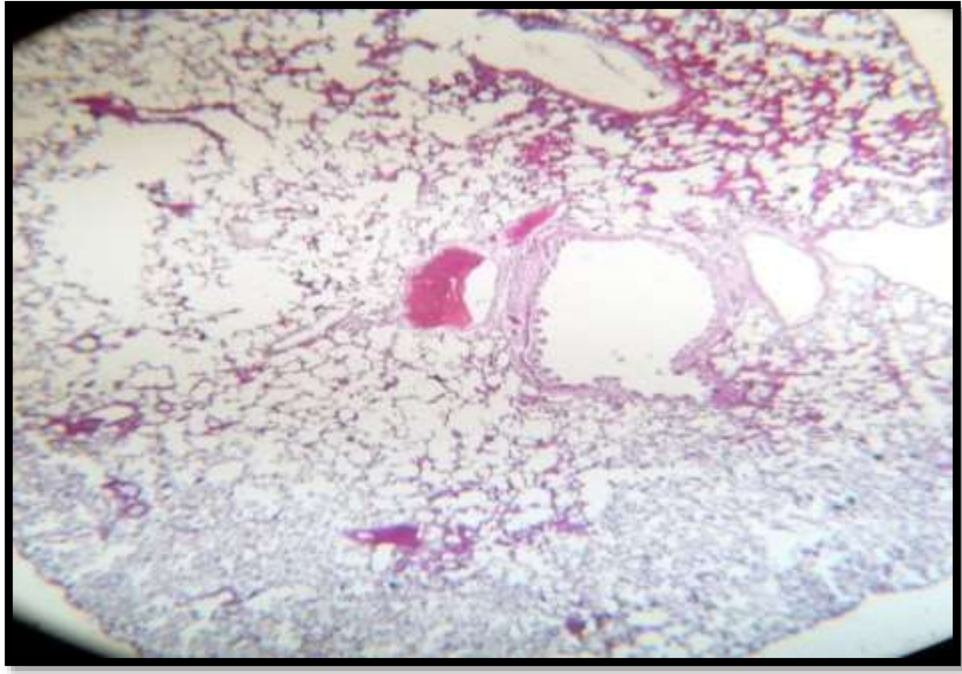


Figure (3-10) Lung rats induced asthma by OVA and then treated with EEP (G3), dilated bronchi without inflammatory.

The histopathological changes of treatment with EEP may be as a result of one or more of the followings:

First: due to its ability to inhibit eosnophils and neutrophils infiltration [143].

Second: by its ability to balance oxidant –antioxidant status via its effect as radical scavenger agent of various ROS [144].

Third: may be due to synergic effect and /or additive effect of various local Propolis compounds thereby to decreasing the inflammation observed [145].

Four : The extract are also capable of modulating the production of pro inflammatory and anti- inflammatory cytokines ,preventing amplification of the inflammatory process in the pulmonary site[146].

This result of experimental came in agreement with (Meaad; 2004) [118] . They demonstrated that treatment with Propolis inhibits pulmonary inflammation and decreases serum level of IgE.

No histopathological changes were detected in both G1 and G4 (Figure 3-11,

3-٨) indicating that EEP has not effect injury with lung tissue.

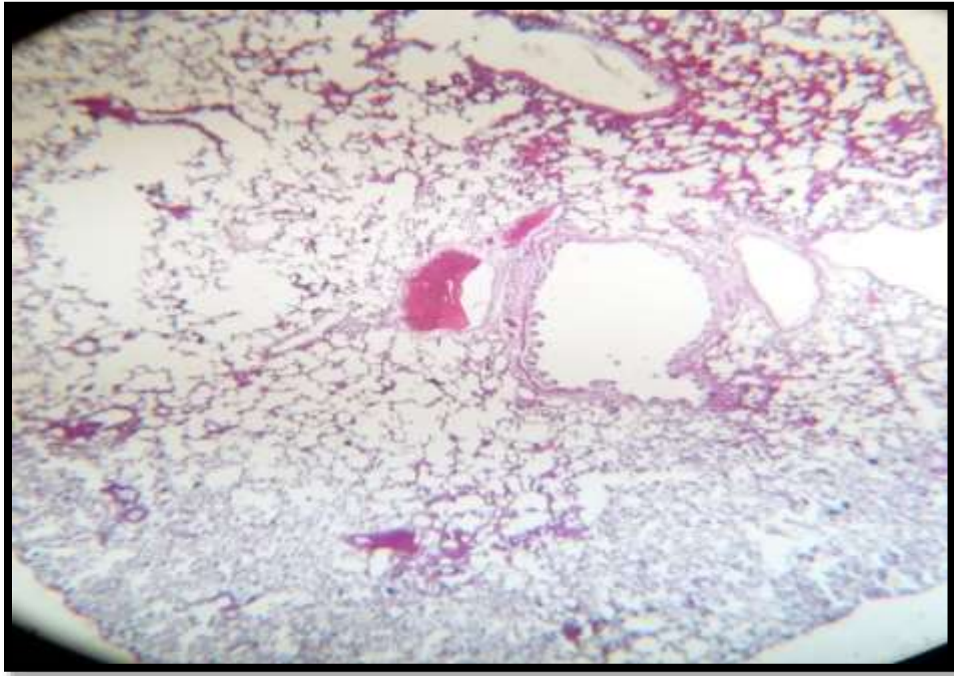


Figure 3-11 Lung rats treated with EEP and then induced asthma by OVA, there is no pathological change.

Conclusions

1. Asthma increases free radicals and oxidative stress.
2. Ethanolic extract of local Propolis can be used as antioxidant protective agent that decreases the oxidative stress in asthma disease.
3. The present study shows that the local EEP contains enough biological compound as flavonoids ,caffeic acid phenethyl ester (CAPE) and other antioxidant and anti inflammatory compounds which exhibited the antioxidant activity and enhancement of antioxidant status in asthmatic rats
4. The EEP has a strong potential to provide protection against asthma or asthma attacks that was confirmed improvement in both biochemical alteration and histological change induced by OVA.
5. The present study shows the local EEP can decrease the lipid peroxidation and protective the cells from free radicals
6. This study shows that chronic inflammation in asthma lead to increasing in gene expression.
7. This study shows, the activity of GST increased by increasing the substrate concentration till reaching the activity after which the activity of GST forming a plateau, this point called $V_{max.}$ Group which induced asthma and treatment with EEP shows increasing of $V_{max}$for CDNB substrates than control group.

Recommendations

1-Future studies should focus on possible interactions of the GST genes with environmental oxidative exposures and with other genes involved in the antioxidant pathway.

2-Because asthma can result from gene/environmental interactions, so studies should focus of the genetics of asthma have been performed, “the clinical implications of the genes and genetic variations associated with asthma phenotypes.

3-Future studies should focus on common polymorphisms of the GSTM1; GSTT1 and GSTP1 genes have been associated with asthma in children and adults.

4-More studies must be carried out to determine the importance of GSTs in drug resistance of asthma.

5-More studies must be carried out to assess actual levels of mRNA of — alpha (GSTA), mu (GSTM), and omega -class GSTs that is associated with asthma.

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Summary

Glutathione-S-transferases (GSTs) (EC: 2.5.1.18) are a family of multifunctional isoenzymes that was partially purified from rats' liver by using chromatography gel filtration using Sephadex G-75 technique. The experimental design consists of sixty male rats were used in this study divided into four groups. Every group contained 15 rats. First group (G1) received distilled water daily for three weeks then injected with (0.5 ml) one dose of normal saline on 1st, 3rd and 5th day as a control. Second group(G2) received distilled water for three weeks then was injected subcutaneous with three doses of 100µg of egg albumin on 1st,3rd and 5th day. Third group (G3) induced asthma by injected of 100µg of egg albumin on 1st, 3rd and 5th day and then received local Propolis for three weeks at doses 200mg /kg. The fourth group (G4) received local Propolis at doses 200 mg /kg for three weeks, then injected subcutaneous with three doses of 100µg of egg albumin on 1st, 3rd and 5th day.

Rats were leave to reach 31 days the total days after beginning the experiments to experience blood samples that were collected to determine biochemical parameters and examine microscopically of lung. Oxidative stress in rats were estimated by determining reduce glutathione, GST, and nitric oxide. GST was partially purified to estimate K_m and V_{max} by using line weaver-Burk plot equation. In addition estimated gene expression (fold change mRNA transcript level)

The results showed significant reduction in the activity of GST in serum of rats in (G2) when compared with control group (G1), and increase activity of GST in (G3) when compared with (G2), while in (G4) was showed almost same activity of GST enzyme as compared with control group (G1) and the results of the GST activity were (25, 12.5, 18.12 and 24.96) U/L and the concentration of GSH was also calculated. , and the results were (2.15 , 1.04 , 1.47 , 2.10) μmol / 1 , as well as calculated K_m and the V_{max} by using line weavr-burk plot equation and Km value of (11.73, 13.2, 11.03 and 12.96 mmol/liter) and V_{max} (4.351, 2.402, 3.215 and 4.482 μmol/min) for G1, G2, G3 and G4 respectively., as well as gene expression (fold change mRNA transcript level) was (1 , 5 , 2.9 , 0.97) for G1,G2,G3 and G4 respectively, and the result showed increase gene expression in (G2) when compared with G1 and G3 ,while in (G4) showed almost same gene expression as compared with (G1).

This study found that asthma increases free radical and oxidative stress and ethanolic extract of local Propolis (EEP) can be used as antioxidant protective agent to decrease the oxidative stress and protective the cell from free radical in asthma disease, as well as this study found that chronic inflammation in asthma leads to increase gene expression.

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Biochemical Study and Gene Expression of Glutathione-S-Transferase (GST) in Induced Asthma in Rats

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