Republic of Iraq
Ministry of Higher Education & Scientific Research
University of Babylon
College of science
Department of chemistry

Synthesis a New Glutathione Mimics Compound: Bioorganic Study to the Key Role of Glutathione Peroxidase in Regulating Antioxidant Status of Cigarette Smokers

A Thesis
Submitted to the Council of the College of science, University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry
by

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2012

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1443 A. H. **2021 A.D.**

صدق الله العلي العظيم

سورة المجادلة الآية 11

CERTIFICATION

We certify that this thesis entitled (**Synthesis a New Glutathione Mimics Compound: Bioorganic Study to the Key Role of Glutathione Peroxidase in Regulating Antioxidant Status of Cigarette Smokers)** was prepared under my supervision at the Department of chemistry/ College of Science / University of Babylon, in partial requirements for the Degree of Master of Science in Biochemistry and this work has never been published anywhere.

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We, the examination committee, after reading this thesis entitled "Bioorganic **Synthesis a New Glutathione Mimics Compound: Bioorganic Study to the Key Role of Glutathione Peroxidase in Regulating Antioxidant Status of Cigarette Smokers** " and examining the student **Ahmad Yasser Ahmad**, in its content, have found that it meets with "excellent" the standard and requirements as a thesis in fulfillment for the Degree of Master of Science.

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Dedications

Everything I have is from God, thank God for this blessing…

Forward the Hajj, peace be upon him;

The reason become today from his blessing, thank you for the support of my family and close friends, and thank God for every think

Ahmed

Acknowledgements

I thank God for giving me health and strength to facilitate my studies and completion. It gives me great pleasure to express my sincere appreciation and thanks to the supervisors of **Prof.Dr. Saadon Abdulla Aowda** and **Prof. Dr. Mahmoud Hussein Hadwan** for their assistance, support, supervision and encouragement, so is **Prof. Dr.Muhannad AL-Hijami**, **Dr. Ammar Abdel Hussein and Mr. Iyad Ali Dasher** to support me and provide materials and help me with some measurements I would like to thank the Deanship of the Faculty of Science, University of Babylon, for providing the necessary facilities.

My sincere thanks to the head and staff of the Department of Chemistry, Faculty of Science, University of Babylon for their assistance in this work.

Finally, I want to thank my friends who have helped me on this stage.

Ahmed

Summary

Cigarette smoke is linked to a number of major health issues, including mouth cancer, throat cancer, stroke, liver cancer, and bladder cancer. Cigarette smoking is a complicated cocktail of around 4,000 chemicals.

Two major types of glutathione peroxidase have been found. One type is distinguished by containing selenium in the form of covalently bound selenocysteine in its active site. The second type of glutathione peroxidase consists of proteins that do not depend on selenium for catalysis and have negligible activity with H_2O_2.

The selenium-dependent enzyme GPX1 is encoded by GPX1 and is found in epithelial tissues of the lung and other organs. By detoxifying hydrogen-and lipid peroxides, the enzyme is part of the enzymatic antioxidant defense that prevents oxidative damage to DNA, proteins, and lipids.

Glutathione Peroxidase Mimics: Oregano selenium compounds fulfill such requirements. In 1996, it was found that the organ selenium compound epsilon reacted in a rapid reaction with peroxy nitrite, the second-order rate constant being $2.0 \times 10^6\ M^{-1}\ s^{-1}$. With epsilon being known as a glutathione peroxidase mimic, interest turned to biologically occurring organoselenium compounds in proteins on one hand, and to other synthetic organoselenium and organotellurium compounds on the other.

A clinical study was performed in University of Babylon during 2021. Blood samples were drawn from the students of the University of Babylon. A total of 150 not smoking persons were enrolled and 150 subjects with Smokers, the current study was carried out on the following two groups:

1- Group I: 150 nonsmoker subjects .
2- Group II: 150 Cigarette Smokers.

study deals four parts: -

TOS and TAS levels of the participants were measured. The serum concentration of total oxidants species of cigarette smoke [15.211 (µmol/l)] were significantly higher than that of non-smokers [12.775 (µmol/l)]. While The serum concentration of total antioxidants capacity of

cigarette smoke [0.6850 (µmol/l)] were significantly lower than that of non-smokers [0.7429 (µmol/l)]. These results suggest evidence for oxidative stress and an impaired oxidant defense system of smokers.

Study Lipid peroxidation concentration in Sera of non-smokers **[**1.1265 (µmol/l)] and cigarette smokers **[**1.2207 (µmol/l)] Groups. Result of the current study refers to significant increase ($p < 0.05$) in Malondialdehyde concentration in group of study cigarette smokers, comparable to nonsmokers control group. Cigarette smoking has been reported to contain many free radicals and other highly reactive molecules. The increased concentration of these reactive molecules in tissues would induce lipid peroxidation with concomitant release of products such as MDA and It is one of the lipid oxidation products whose concentration was measured.

The glutathione peroxidase enzyme level was studied, and the results of the study indicated to significant decrease ($p < 0.05$) in glutathione peroxidase concentration in group of study cigarette smokers, comparison to nonsmokers control group [190.92 U/L], in cigarette smokers group decreased significantly to be [206.33 U/L].

From the results of this study, we conclude that smoking of all kinds is dangerous to health. Initially, we can use the provided approach to test glutathione peroxidase activity with high precision and accuracy in the presence of multiple types of biomolecules and low H_2O_2 concentration

An organic compound (COMPOUND A) with the same active group in the enzyme glutathione peroxidase was prepared and had an enzymatic activity similar to glutathione peroxidase.

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List of Abbreviation

Abbreviation	Details
CAD	Coronary artery disease
CAT	Catalase
CD	Cohn's disease
CKD	Chronic kidney disease
CNS	central nervous system
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CVD	Cardio vascular disease
CYP	Cytochrome p450 proteins
DETBA	1,3-diethyl-2-thiobarbituric acid
DPDS	Diphenyl Diselenide
EDTA	Ethylene diamine tetra acetic dihydrate
FRAP	ferric reducing antioxidant assay
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSNO	s- nitro so glutathione
GSSG	Glutathione disulfide
GST	Glutathione s- transferase
GSTMI	Glutathione s- transferase malignancies
Hb	Hemoglobin
IBDs	Inflammatory bowel disorders
LDL	Low-density lipoproteins
Leu	Leueine
LH	Luteinizing hormone
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
MDA	Malondialdehyde
NAT	N – acetyl transferase
NRF_2	The nuclear factor 2
NS	Non-smokers
O-dianisidine dihydrochloride	3,3′-Dimethoxybenzidine dihydrochloride
PLGSH-GX	Phospholipid hydroperoxides glutathione peroxidase
PMNs	Peripheral blood from non-smokers
PrO	Proline
Prx	Peroxiredoxin
Prx 4	Peroxiredoxin 4
RCF	Relative centrifugal force
RFLP	Restriction Fragment Length Polymorphism
$ROO^{\cdot}$	peroxy radical

rpm	**Revolutions per minute**
SDS	**Sodium dodecyl sulfate**
SECIS	**Selenocysteine insertion sequence**
Sepp	**Selenoprotein P**
SOD	**Super Oxide dismutase**
TAC	**Total antioxidant capacity**
TAs	**Total antioxidants**
TBA	**thiobarbituric acid**
TBARS	**Thiobarbituric acid-reacting**
TCA	**Trichloroacetic**
TOS	**Total oxidants status**
TRX	**Thioredoxin**
WHO	**World Health Organization**
X	**Xanthine**
ETS	**Environmental tobacco smoke**

CHAPTER ONE INTRODUCTION

1.Introduction

1.1. Cigarette smoking

In developed countries, cigarette smoking, also known as "smoking," is the leading cause of early mortality [1]. Males' smoking prevalence is higher internationally than it is in the United States, but women's smoking prevalence is often lower than that of men, though it has equaled or exceeded that of men in some northern European nations [2,3]. Smoking-related morbidity and death in poor nations have not yet overtaken those in industrialized countries due to the delayed health effects of smoking, but they are expected to do so in the next century [3,4].

Cigarettes are the most popular way to smoke, accounting for 65-85% of all tobacco consumption worldwide. It is estimated that one out of every three persons in the globe smokes. According to WHO estimates, there are around 1.1 billion smokers in the globe, with 80 percent of them living in low and middle-income nations. If current trends continue, the total number of smokers will be around 1.6 billion by 2025 [5]. Ammonia, nicotine, polycyclic aromatic hydrocarbons, acetaldehyde, polyphenols, nitrogen oxides, hydrogen cyanide, carbon monoxide, and trace metals are among the more than 4000 recognized components of cigarette smoke [6,7]. The components of a cigarette were depicted in figure: (1-1).

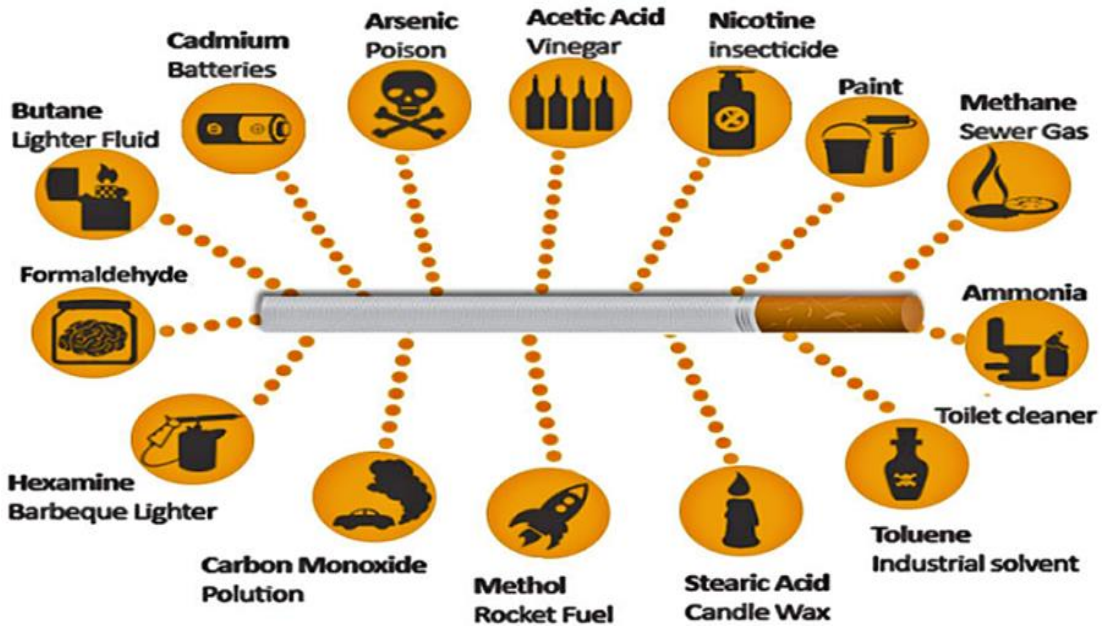


Figure (1-1) A Cigarette's Components Modified from [8]

Measures of exposure (i.e., nicotine, NNAL-Gluc1), carcinogen-macromolecular adducts, such as 4-amino biphenyl hemoglobin adducts), micronutrients (carotene), and genetic factors that may modify these factors or their effects have been the focus of biomarker research in smoking-related cancer [9].

1 .1.2. Smoking and Nicotine

Nicotine addiction has been identified as the psychopharmacologic process that keeps people smoking cigarettes [10]. Nicotine affects the mesolimbic dopaminergic reward pathway in the brain [11,12], causing dependency and physical and neurobiological withdrawal symptoms when abruptly stopped [13,14].

Nicotine modulates the release of neurotransmitters and ganglionic potentials by acting as an agonist for neuronal nicotinic acetylcholine receptors (nAChRs), which are pentameric ionotropic (Na^+ and Ca^{2+})

receptors found presynaptic ally throughout the central nervous system (CNS) and postsynaptic ally in the autonomic nervous system [15,16], The number of nAChRs is increasing, especially the 42 heteromer, which is the

most frequent nAChRs type in the mammalian brain [17,18]. To sustain nicotine levels, smokers of cigarettes increase their smoking intensity, rate, or inhalation, as evaluated by plasma nicotine levels in both ad libitum and laboratory smoking situations [19,20]. Individual smokers can achieve plasma nicotine levels of 20-50 ng/mL, according to nicotine levels measured in the arterial and venous circulation [20,21].

1.1.3. Mechanical and Structural Changes Caused by Smoking

The structural alterations in the respiratory region are caused by cigarette smoke and several of its components. Per bronchiolar inflammation and fibrosis, increased mucosal permeability, impaired mucociliary clearance, alterations in pathogen adhesion, and disruption of the respiratory epithelium are all examples of these changes [22]. These alterations are likely to increase the risk of upper and lower respiratory tract infections, which could exacerbate the lung inflammation caused by cigarette smoke.

Acrolein, acetaldehyde, formaldehyde, free radicals created by chemical reactions within the cigarette smoke, and nitric oxide are some of the components of cigarette smoke that may contribute to the structural changes in airway epithelial cells [23,24].

1.1.4. Immunologic Mechanisms

In humans, cigarette smoking has an impact on both cell-mediated and humoral-mediated immunological responses [25,26]. Peripheral blood cell counts and distribution average of the smokers have a 30 percent higher peripheral white blood cell count than nonsmokers [26,27]. Cigarette smoking has been found to alter the function of white blood cells in several investigations [28, 29]. When comparing PMNs from smokers' peripheral blood to PMNs from nonsmokers, smokers' PMNs have lower migration

and chemotaxis [29, 30]. Cigarette smoking is linked to a number of changes in the cellular and humoral immune systems. A decrease in circulating immunoglobulins, a depression of antibody responses to specific antigens, a drop in CD^{4+} lymphocyte counts, an increase in CD^{8+} lymphocyte counts, depressed phagocyte activity, and decreased production of proinflammatory cytokines are all examples of these changes [31].

1.1.5. Bacterial Infections

In patients with chronic obstructive pulmonary disease, cigarette smoking is a significant risk factor for pneumococcal pneumonia. Smoking, however, is a big risk factor for the chronic obstructive pulmonary disease [32].

1.1.6. Viral Infections

Colds are very common. Large epidemiologic research back up the link between smoking and the occurrence of colds and other lower respiratory illnesses. Blake et al., [33] studied a large group of US Army recruits (1230 soldiers) in a prospective cohort study and discovered that 22.7 % of smokers developed upper respiratory infection compared to 16% of nonsmokers. When 400 healthy volunteers were treated intranasally to a modest dosage of one of five respiratory viruses, Cohen et., [34] were linked smoking to an increased risk of symptoms leading to a clinical diagnosis among virologically confirmed sick people. This risk was unaffected by alcohol intake, as well as demographic, environmental, immunologic, and psychological factors among smokers.

1.1.7. Influenza

The link between cigarette smoking and the incidence of influenza infections has been proven in previous study [35]. Smokers have more

severe influenza infections, with greater cough, acute and chronic phlegm production, dyspnea, and wheezing [35].

1.1.8. Cigarette Smoking and Adverse Health Effects

Clinical data regarding lung cancer, the first disease definitely related to tobacco use, led to the discovery of a correlation between tobacco use and health. Over 7000 study articles on the topic of smoking and health were reviewed by the Office of the Surgeon General of the United States Health Service that covered the past 35 years. The involvement of smoking in different diseases, including lung cancer was officially recognized. Cigarette smoking is a key risk factor for lung cancer, which is the leading cause of cancer-related deaths in both men and women around the world [36]. The main health issues linked to cigarette smoking continue to be cancer, cardiovascular disease, and chronic obstructive pulmonary disease. Furthermore, smoking raises one's chances of dying from heart disease by four times. Tobacco is responsible for 24% of all male deaths and 7% of all female deaths worldwide; in some Central American Nations, these percentages reach to nearly 40% among men. Nonetheless, there is enough evidence to imply that cigarette smoke is a complex combination containing around 4800 distinct chemicals [37]. Approximately 100 of these substances have been identified as carcinogens, mutagens, or carcinogenic. In each mL of mainstream smoke, there are around 10^{10} particles of various sizes, as well as gases like ozone, formaldehyde, ammonia, carbon monoxide, toluene, and benzene. In addition, over the years, a variety of additional toxic, mutagenic, tumor promoter, and/or carcinogenic chemicals have been discovered in both mainstream and side stream cigarette smoke [38].

Figure (1-2): - The terms "health risk" and "smoking" are used interchangeably Modified from [39]

In industrial societies, cardiovascular illnesses, particularly atherosclerosis, are the main causes of death. Thermogenesis, which also causes atherosclerotic aortic and peripheral vascular disorders, is the most common underlying cause of coronary artery disease (CAD) [40]. Cigarette smoking contributes to the development and progression of atherosclerosis, both individually and in concert with other risk factors such as hypertension and hypercholesterolemia. Several studies have found that the risk of developing CAD rises with the quantity of cigarettes smoked per day, total smoking years, and age of initiation, showing a dose-related response. Smoking cessation, on the other hand, has been shown to lower atherosclerotic vascular disease mortality and morbidity [40]. The mechanisms by which smoking promotes the formation and progression of atherosclerosis are currently unknown, although new research suggests that smoking has a negative impact on endothelial and smooth muscle cell activities, as well as thrombotic disturbances [41]. Tobacco smoking has been shown to increase oxidative stress in the body through a variety of

processes, including the depletion of plasma antioxidants such vitamin C. At least two investigations have been conducted to assess the function of oxidative stress in increasing leukocyte-endothelial interactions, which occur before the onset of atherosclerosis in smokers. The adhesion of smokers' monocytes to endothelial cells was shown to be greatly reduced when they consumed a high dose of vitamin C [40]. This suggests that, at least in the early stages of smoking-induced leukocyte-endothelial interactions, NO levels are crucial. Passive smokers had higher amounts of 8-hydroxydeoxyguanosine, an oxidized DNA product, and F2-isoprostane, an oxidative arachidonic acid product [42,43]. The existence of increased levels of autoantibodies against oxidized LDL was shown to be raised in smokers, indicating that oxidation of low-density lipoprotein (LDL), a gold standard risk factor in the atherosclerotic process. It was also shown that dietary treatment with α-tocopherol, a lipid-soluble antioxidant, lowered plasma levels of oxidized LDL autoantibodies [44]. Similarly, consuming a cocktail of antioxidants was reported to increase smoker LDL's resistance to oxidative modification [45] and lower 8-hydroxydeoxyguanosine levels in passive smokers' blood [42]. However, animal studies are required to determine the significance of various processes in the progression of atherosclerosis and to develop effective therapies. Various epidemiological and experimental studies have not only confirmed the major role of tobacco smoke exposure in lung and bladder cancers, but have also reported on its association with cancers of various other sites, such as the oral cavity, esophagus, colon, pancreas, breast, larynx, and kidney [46]. It has also been linked to leukemia, particularly acute leukemia, in addition to the well-known involvement of cigarette smoking in lung cancer. It has also been linked to a variety of other chronic disorders, including chronic bronchitis and pulmonary emphysema [46]. The relevance of smoking in breast cancer is debatable because of its anti-

estrogenic protective effects. Recent research suggests, however, that both active and passive smoking may play a role in the development of breast cancer. One study reported a risk of breast cancer of 4.5 in women who were exposed to passive smoke before the age of 12 and a risk of 7.5 in active smokers [47,48]. When evaluating the risk of lung cancer in smokers of various types of cigarettes [49].

Thus, genes for various activating enzymes such as cytochrome P450 (CYP) proteins, and deactivating enzymes such as glutathione S-transferase (GST), N-acetyl transferase (NAT) and uridine diphosphate-glucose transferase have been the main target of many recent studies in the context of tobacco carcinogenesis. Because of the chemical complexity of tobacco smoke and the metabolic activation needs for many of its carcinogenic ingredients, genetic polymorphisms in biotransformation enzymes that metabolize tobacco smoke carcinogens have gotten a lot of interest. In tobacco-related carcinogenesis, pre-existing inherited mutations and/or mutation susceptibility of tumor suppressor genes such as p53, which are known to play a crucial role in predicting cancer susceptibility, have also been investigated [50,51]. In Japanese people, the CYP1A1 gene has been extensively investigated. Two polymorphic variations have been found that interact with smoking to modify lung cancer risk [52,53]. As a result, a homozygous minor allele paired with smoking was discovered to enhance the risk of lung cancer. The same gene has been studied in a number of different ways.

However, negative or inconsistent results have been recorded in western groups [54], despite the fact that an interaction of CYP1A1 variations with the GST genotype has been found to dramatically increase lung cancer risk in non-Japanese populations [55,56]. GSTs are another set of metabolic detoxification enzymes that has sparked a lot of research

in recent years due to their link to cancer risk [57]. These enzymes are classified into five groups based on their sequences. In the context of tobacco-related malignancies, three of these classes –GSTM1, GSTT1, and GSTPi–are relevant. The majority of populations surveyed have very high frequencies (20% to 50%) of homozygous GSTM1 and GSTT1 deletion carriers, according to extensive investigations on the link between these genes and cancer risks. GSTM1 and GSTT1 may have a role in cancer genesis at many sites. Furthermore, the risk to people with homozygous deletions is normally low, but it rises dramatically when combined with cigarette smoking [57].

1.1.9. Secondhand Smoke

Cigarette smoke has long been known to have harmful impacts on human health. It is a proven human carcinogen and the major etiological agent in chronic obstructive pulmonary disease and lung cancer. Evidence supporting the risk of involuntary exposure to environmental tobacco smoke (ETS) has been accumulated in recent years [58]. It is the most common way for nonsmokers to be exposed to toxicants through breathing. Despite recent laws, smoking is still prevalent in public places. In passive smokers, ETS is now recognized as a risk factor for lung cancer, cardiovascular illness, and impaired lung function [58].

Passive smoking has also been linked to an increase in atherosclerosis in people aged 15 to 65. Children who are exposed to ETS are more likely to have cardiovascular problems. In a large longitudinal atherosclerosis risk study of 10,914 people, quantitative risk estimations were generated by assessing the intimal-medial thickness of the carotid artery.

Current, ex- and passive smokers had increases of 50%,25%, and 20% above nonsmokers, respectively, demonstrating that all types of tobacco smoke exposure play a role in the advancement of atherosclerosis [59].

1.2. Antioxidants

The antioxidant defense system of aerobic organisms is well known for dealing with reactive oxygen species (ROS) formed as a result of aerobic respiration and substrate oxidation [60]. There is growing evidence that oxidative stress causes numerous biochemical changes and is a contributing factor in a number of human chronic diseases, including atherosclerosis, cardiovascular disease, mutagenesis, and cancer [61]. Various malignancies and leukemia have been linked to changes in the enzymatic and non-enzymatic antioxidant systems, as well as thiobarbituric acid (TBA) reactive products [62,63]. Intracellular superoxide dismutase (SOD) has been found to have a protective effect and could be a key factor in leukemic and cancer cell resistance to anticancer drugs and radiation [64]. While the absence of Mn-SOD activity has been identified as a phenotypic hallmark of tumor cells and has been shown to be useful in cancer therapy [65]. Superoxide dismutase (SOD), catalase (CAT), and peroxidase are the most essential antioxidant enzymes, with glutathione peroxidase (GPX) appearing to be the most important in mammalian cells [66]. Each of these enzymes comes in a variety of forms, and they are highly compartmentalized inside the cell [67]. Copper and zinc-containing SOD, for example, is mostly located in the cytoplasm, whereas manganese-containing SOD is mostly found in the mitochondria. Mn-SOD and CAT activity are almost always low in tumor cells, and Cu, Zn-SOD activity is usually low as well. The activity of Gpx varies [68].

Free radicals, particularly oxygen radicals, have recently been shown to play a significant role in the complicated process of multistep carcinogenesis, according to new data. The fact that antioxidants that directly scavenge free radicals or interfere with the formation of free

radical-mediated processes impede the neoplastic process [69]. Redox signaling mechanisms in cells are indicated in Figure (1-3).

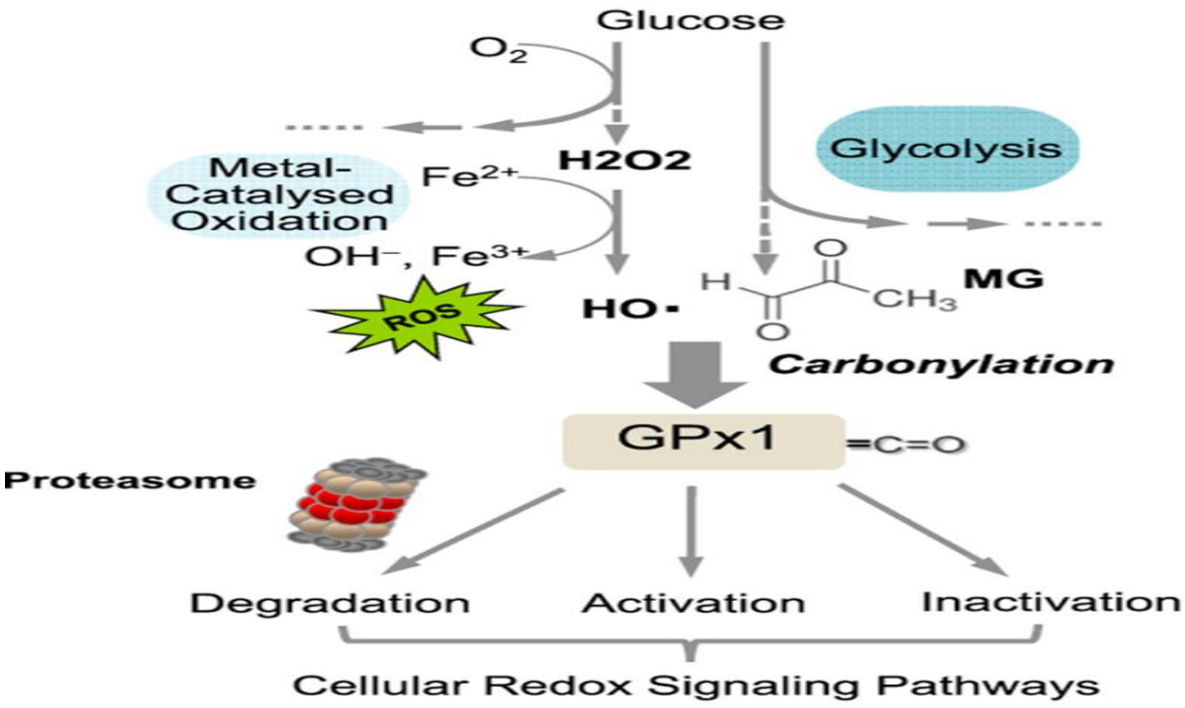


Figure (1-3) Redox Signaling Pathways in Cells Modified from [70]

Excessive production of reactive oxygen species (ROS) has severe implications, including damage to polyunsaturated fatty acids in membrane lipids, proteins, and DNA, as well as cell death [64]. Antioxidant species and mechanisms protect living organisms from the detrimental effects of reactive oxygen species (ROS). These include enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPX), as well as non-enzymatic antioxidants such as reduced glutathione (GSH), selenium compounds, vitamins A, E, and C, and thiol compounds.

1.3. Free Radicals

According to Hallowell and Guthridge's definition, a free radical is any species capable of independent existence that contains one or more unpaired electrons [71]. In a molecule, an unpaired electron occupies an orbital by itself [72]. Oxygen reduction requires 0.82 kcal/mole [73]. This

makes oxygen an excellent receiver of electrons from NADH and FADH in terms of cellular energy production [74].

The reduction of oxygen is accomplished as a series of partial reductions rather than in a single step. As a result, there's a chance that partially reduced oxygen molecules will escape the electron transport chain as superoxide ions. In addition to O_2, the mitochondria produce significant amounts of superoxide [75]. The production of reactive oxygen species (ROS) is a typical part of cellular metabolism. The enzymatically controlled reaction of oxygen with hydrogen in oxidative phosphorylation, which occurs within the mitochondria during oxidative metabolism, produces the majority of the body's energy. Free radicals are produced during the enzymatic reduction of oxygen to produce energy [76].

1.3.1. Free Radical-Reactions:

Because molecular oxygen possesses an electron pair on its outer orbital, it is being more stable. Excitation (singlet oxygen), reduction (superoxide free radical, hydrogen peroxide, hydroxyl free radical), molecular scission (oxygen atom), or oxidation (molecular oxygen ion) are all methods for producing free radicals [77].

Although H_2O_2 is not a free radical, it is nevertheless reactive; thus, it is classified as a reactive oxygen species (ROS), and it can produce even more reactive compounds, such as the hydroxyl free radical ($^{\bullet}OH$). Because the SOD process produces ROS from a free radical, which is a chemically less active molecule, both isoforms of SOD, cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD, are considered antioxidant enzymes. According to the Figure (1-4) hydroxyl radical has the shortest life-span of all free radical species since it is the most reactive. Cleavage reactions of H_2O_2,

such as the Haber-Weiss and Fenton reactions, produce hydroxyl radical. Figure (1-4) shows the oxidative stress in mitochondria [78].

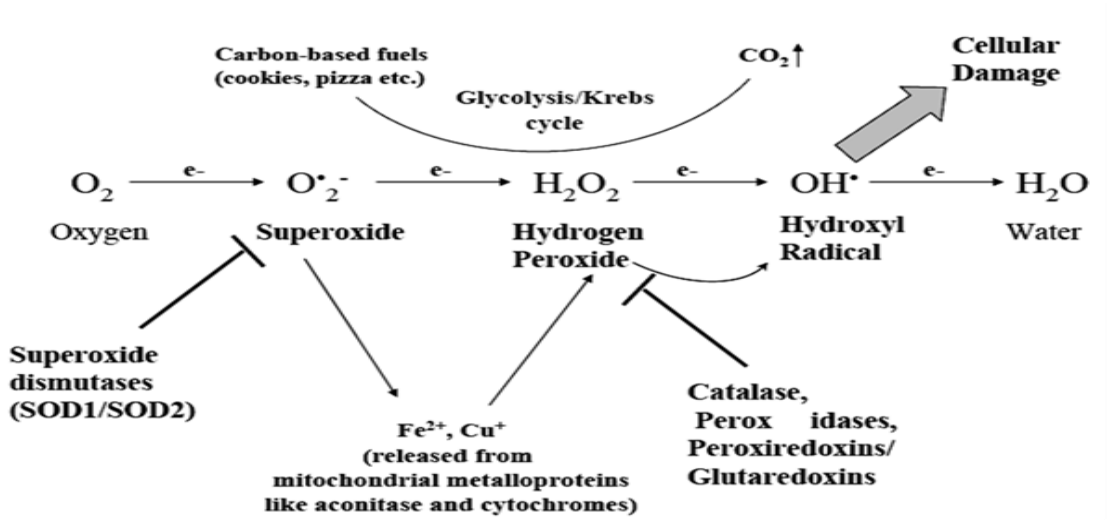


Figure (1-4): The Mitochondrial Respiratory Chain's ROS Sources [78]

1.3.2. Reactive Oxygen Species

Aerobic organisms have antioxidant defense enzymes that cope with the reactive oxygen species (ROS) that are created during aerobic respiration**.** Although reactive oxygen is linked to cell differentiation and growth arrest, larger levels of reactive oxygen may be damaging to cells and organisms [78]. Small differences from physiological values can have a big impact on a cell's resistance to oxidative damage of lipids, proteins, and DNA. As a result, poisonous oxygen plays a function in the aging process and a variety of human disorders [78]. In aerobic organisms, small amounts of reactive oxygen species (ROS) such as hydroxyl radicals (˙OH), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) are constantly produced in response to both external and internal stimuli [79]. Low quantities of ROS may be required for a variety of metabolic processes, such as intracellular massaging in cell differentiation and development or growth arrest, apoptosis [79,80], immunology [81], and antimicrobial resistance [82]. High levels and/or insufficient elimination of ROS, on the other hand,

create oxidative stress, which can lead to serious metabolic problems and damage to biological macromolecules. Lipid peroxidation prevention is a critical process in all aerobic organisms, as lipid peroxidation products can damage DNA. Increased lipid peroxidation and reduced antioxidant protection produce epoxides, which can spontaneously react with nucleophilic sites in the cell and bind to DNA, RNA, and proteins covalently [78]. Depending on the qualities of the epoxide in question, this reaction can cause cytotoxicity, allergies, mutagenicity, and/or carcinogenicity. Furthermore, oxidative processes may play a role in the mechanism of action of other lipids, and oxidizability may influence drug sensitivity in cells [83].

Superoxide dismutase (SOD), glutathione peroxidase (GPX), Catalase (CAT), glutathione (GSH), beta carotene, vitamin A, ascorbic acid (vitamin C), and α-tocopherol (vitamin E) are covered the enzymatic and non-enzymatic antioxidant defenses [84]. There is a link between these metabolites' activity and intracellular levels in terms of defending themselves from oxygen toxicity [85].

1.4. Glutathione peroxidase

The selenoprotein catalyzing the process is known as glutathione peroxidase (glutathione: H_2O_2 oxidoreductase, EC 1.11.1.9). [86]

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$$

It should not be used to other proteins with similar functions (e.g., glutathione S-transferases) [87]. There are two forms of glutathione peroxidase that have been discovered. One kind is identified by the presence of selenium in the active site in the form of covalently bound selenocysteine [88,89]. Gpx is a tetrameric protein that is active with both organic hydroperoxides and H_2O_2. Gpx is a selenium-dependent enzyme.

Its 3D structure was characterized using X-ray diffraction analysis [90], and its amino acid sequence was identified using traditional protein chemistry techniques [91]. The second type of glutathione peroxidase is made up of proteins that don't need selenium to catalyze and have very little action with H_2O_2[92]. Glutathione transferases [93] belong to this group. GSH conjugation with electrophilic chemicals such as aryl halides was initially characterized as a protein catalyzed reaction [93,94]. These enzymes are dimeric proteins that can be found in different forms in the same organ, with some isozymes having significantly higher glutathione peroxidase activity than others [95].

Despite the fact that the peroxidase activity of glutathione transferases is widely described [96], it is currently not thought to be their primary biological role. The activity of glutathione peroxidase has been found in all mammalian tissues studied [97]. The ratio of selenium-dependent to non-selenium-dependent activities can differ not only between animal species, but also between tissues within the same species [98]. When selenium deprivation occurs, the glutathione transferase's peroxidase activity becomes more important. Selenium-dependent glutathione peroxidase is largely found in the cytosol and the matrix of mitochondria in hepatocytes [98]. Antioxidants are molecules that prevent, minimize, or delay the oxidation of elements in living cells that are susceptible to oxidation, such as proteins, lipids, carbohydrates, and DNA. This is known as antioxidant defense. Enzymatic and non-enzymatic antioxidants are the two types of antioxidants [99]. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx) are enzyme-based antioxidants, while vitamin E, vitamin C, vitamin A, selenium (Se), transferrin, and lactoferrin are non-enzymatic antioxidants. Antioxidants are found both within and outside the cell [99].

Tumor cells may sequester antioxidants and sweep lipid peroxides, resulting in a decrease in circulating antioxidant [100].

The disadvantage of ROS generation is that it causes a variety of cancers, some of which are resistant to exogenous development [101]. For example, multidrug-resistantHL-60 is resistant to ROS development because the presence of endogenous antioxidants that are both detoxifying and ROS scavengers, such as leukemia [101, 102]. Several oncogene-induced cancer cells increase antioxidant activity while retaining the effect by activating nuclear factor erythroid 2-related factor 2 (NRF2) [102,103]. ROS levels allow pro-tumorigenic signaling pathways to be activated without causing cell death [102, 104]. Additionally, an increase in GSH levels, which play a function in protecting cells from cell death, appears to play a role in shielding cells from ROS-inducing therapy [102, 104]. The antioxidant defense system is a complex system of chemicals that eliminates free radicals and reduces the generation of reactive oxygen species (ROS) [105,106]. To compensate for ROS welded damage, endogenous antioxidant defense systems are present [100]. These systems work by using chelating to keep intracellular ROS activity and redox equilibrium in check [100, 107]. Glutathione peroxidase catalyzes a reaction involving the reduced form of glutathione (GSH) reacting with hydrogen peroxide or lipid peroxides while also supporting the detoxification of these molecules by forming a glutathione bridge with another glutathione molecule (GSSG) [108]. Catalase and glutathione peroxidase detoxify H_2O_2 [100]. The elimination of intracellular hydro peroxides is aided by the glutathione redox cycle. Because it binds four selenium atoms and provides glutathione peroxidase with catalytic activity, Gpx is classified as a selenocysteine molecule. As a co-substrate, glutathione is required [109]. The tripeptide glutathione is made up of

cysteine, glutamic acid, and glycine [110]. The -glutamic linkage and the sulfhydryl (-SH) group are two structural features of GSH. GSH is well-known for its numerous physiological roles as an antioxidant against reactive oxygen species (ROS) and free radicals in xenobiotic compound detoxification [110,111]. When the cell fails to protect GSH content no longer, certain cell death may be followed [111,112]. GSH, the most important antioxidant molecule in the intracellular environment [108], serves a variety of physiological functions, including xenobiotic detoxification, amino acid transport, maintaining sulfhydryl groups in proteins, and acting as a coenzyme in some enzymatic reactions not involving the antioxidant defense system [108, 113,114]. Glutathione, in its reduced form (GSH), converts to oxidized glutathione (GSSG) by forming a disulfide bond with another glutathione molecule while detoxifying these molecules by reacting with hydrogen peroxides or lipid peroxides, with the Gpx enzyme catalyzing the reaction [113]. GSSG must be converted back to its reduced form in order for free radical detoxification to continue in the cell. The GR enzyme converts GSSG to reduced glutathione via a process involving NADPH [108,113, 115]. Gpx is the most potent antioxidant against oxidative stress in erythrocytes and plays a role in phagocytic cells [116].

Gpx is an enzyme that removes the hydro peroxides that are generated in cells. It is assumed to be a selenoenzyme that protects cells from various insults since its subunits include a Se atom. Mills discovered this enzyme in mammalian erythrocytes for the first time in 1957. In endothelial cells, particularly in the lungs, it is the most efficient enzyme. The cytoplasm of eukaryotic cells contains 60–75% of enzyme activity, while mitochondria contain 25– 40%. In erythrocytes and the liver, the most common enzyme activity is understood [117]. Gpx is the most essential intracellular enzyme

that protects lipids from peroxidation. As a result, this enzyme, particularly in the cytosolic compartment of the cell, keeps the cell's structure and function under check. Phospholipid hydroperoxides glutathione peroxidase (PLGSH-Px), which converts membrane phospholipid hydroperoxides to alcohol, is also monomeric and contains the Se atom. In circumstances when vitamin E, a membrane-bound antioxidant, is deficient, PLGSH-Px protects the membrane against peroxidation [117,118].

Reduced glutathione catalyzes the detoxification of H_2O_2 and lipid peroxides by glutathione peroxidase. As a result, it shields membrane lipids and hemoglobin from peroxidase oxidation [119]. GSH-Px is also involved in xenobiotic detoxification. In mammalian cells, the antioxidant enzyme system is the most important defense against per oxidative damage to biological membranes. Glutathione peroxidase, catalase, and superoxide dismutase are three enzymes that work together to defend the cell from per oxidant compounds [119]. An increase in free oxygen radical levels can lead to mutagenicity, cytotoxicity, and alterations in gene expression, which can lead to the development of malignant tumors, and this mutagenicity can contribute to the transformation of benign development into malignant [120].

Finally, strong Gpx activity in the blood might be thought of as a factor that lowers cancer risk. When oxidative parameters were compared in Parkinson's disease and control groups, GSH-Px was shown to be considerably greater in the patient group [121]. Gpx activity levels were found to be lower in progressive hypothyroidism in the postnatal period [122]. Reduced Gpx activity, one of the body's most significant antioxidant defense systems, was either enhanced or lowered intracellularly in several tissues antioxidant enzymes [123,124]. The parotid glands of mice had increased Gpx activity in experimental colitis, while the submandibular

glands had decreased enzyme activity [125]. There were no statistically significant variations in Gpx activity in plasma between Crohn's disease (CD) patients and controls [126]. When plasma samples were analyzed, the results of Gpx and SOD activity measured in CD patients were found to be inconsistent [127].

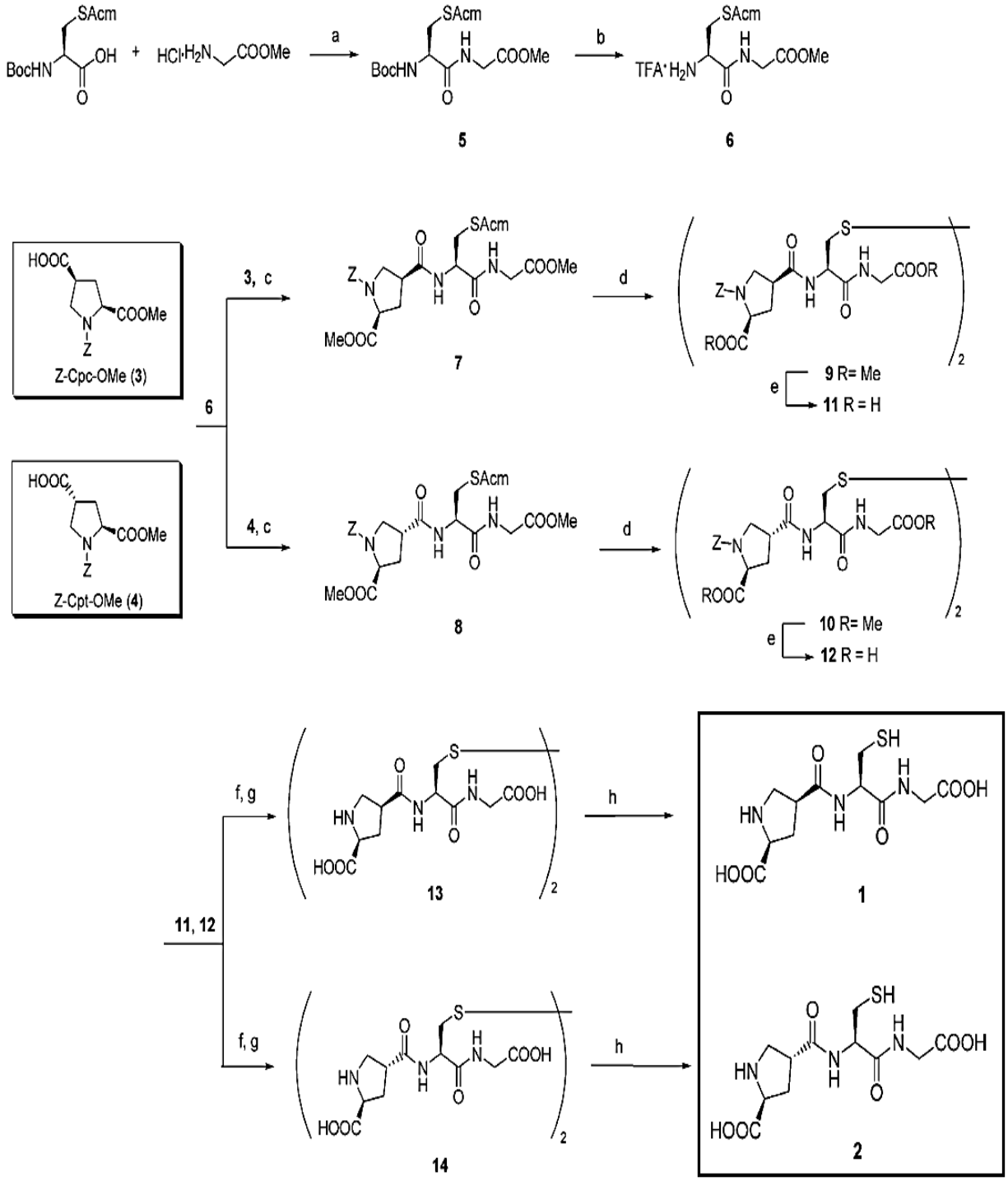
GPX1 SNP rs1050450, which frequently causes the C to T mutation, was studied in previous studies concentrating on the role of GPX1 single nucleotide polymorphisms (SNPs) [100]. The GPX1 SNP was discovered to affect r1010450's risk of lung cancer and bladder cancer [100]. The Leu allele of GPX1 SNPs affects the risk of arterial calcification and atherosclerosis [100, 128]. However, there is no indication that the GPX1 SNP rs1050450 has a role in the course of chronic kidney disease (CKD) or renal allograft failure [100, 129]. Individuals with reduced Gpx activity are thought to be more susceptible to intact antioxidant protection, resulting in oxidative damage to membrane fatty acids and functional proteins, and hence neurotoxic damage [130]. GPX1 loss was formerly thought to produce an increase in vascular oxidative stress, with endothelial dysfunction being directly involved by Forgone and colleagues [131]. The inhibition of Ferro ptosis by GPx4 provides neurodegenerative protection mechanisms. Furthermore, we believe that a decrease in Gpx activity causes selenium shortage to enhance vulnerability to ferroptotic processes and other programmed cell death pathways [132]. By regulating gene expression, protein function, and enzyme activity, GPX1 influences the effects of important variables involved in macro and micronutrient metabolism [133,134]. Some studies [135,136] emphasize the need of maintaining this selenoperoxidase's proper expression and activity in order to regulate redox balance, glucose, and lipid metabolism. In several populations, the GPX1 polymorphism has been linked to the risk of

diabetes and obesity [137,138]. The addition of SOD, glutathione peroxidase, and N-acetyl cysteine, which reduces intestinal tissue tumor necrosis factor-α concentrations and has anti-inflammatory and antioxidant characteristics, has been shown to prevent oxidative stress that induced intestinal injury [139, 140]. In ulcerative colitis (UC) patients in either active or remission stages, there was a considerable increase in Gpx activity in the inflamed mucosa. Other studies found that the UC and CD groups had considerably greater plasma Gpx levels than the control group [141]. When compared to control children, children with inflammatory bowel disorders (IBDs) showed higher Gpx activity and GSH levels [142]. Patients with susceptibility-related disorders had significantly lower levels of total glutathione, decreased/oxidized glutathione, and ubiquinone, whereas DNA fragmentation was much higher. Patients with susceptibility-related disorders had significantly lower levels of total glutathione, decreased/oxidized glutathione, and ubiquinone, whereas DNA fragmentation was much higher. These variations, however, were not linked to the GPx1 genetic background [143]. In postmenopausal women's erythrocytes, a linear link between estrogen and Gpx has been discovered [144]. In healthy women, Serviddio et al [145] found a favorable connection between Gpx activity and luteinizing hormone (LH) concentrations. They also discovered that estradiol and Gpx have a substantial positive connection. In postmenopausal women with abdominal obesity, there was a significant increase in Gpx activity [146]. Glutathione peroxidase (Gpx) is involved in the reduction of peroxides, which are thought to inactivate NO-mediated vasodilation, as well as the decomposition of S-nitrosoglutathione (GSNO), which is vital for vascular homeostasis [147].

1.4.1. Glutathione Peroxidase Mimics

The organ selenium compound ebselen was discovered to react rapidly with peroxy nitrite in 1996 [148], with a second-order rate constant of (2.0 x 10^6) M^{-1}. S^{-1} [149]. Because ebselen is a glutathione peroxidase mimic [150,151], researchers have been looking into physiologically occurring organ selenium compounds in proteins, as well as various synthetic organ selenium and organ tellurium compounds. The current forum article focuses on peroxynitrite interactions [152].

Mario Paglialunga and his coauthors have been synthesized a new two diastereoisomeric glutathione analogues by replacing the native g-glutamylic moiety with the conformational rigid skeleton of cis- or trans-4-carboxy-l-proline residue. Synthesis of the two conformation ally restricted GSH analogues 1 and 2 were performed according to Scheme (1).



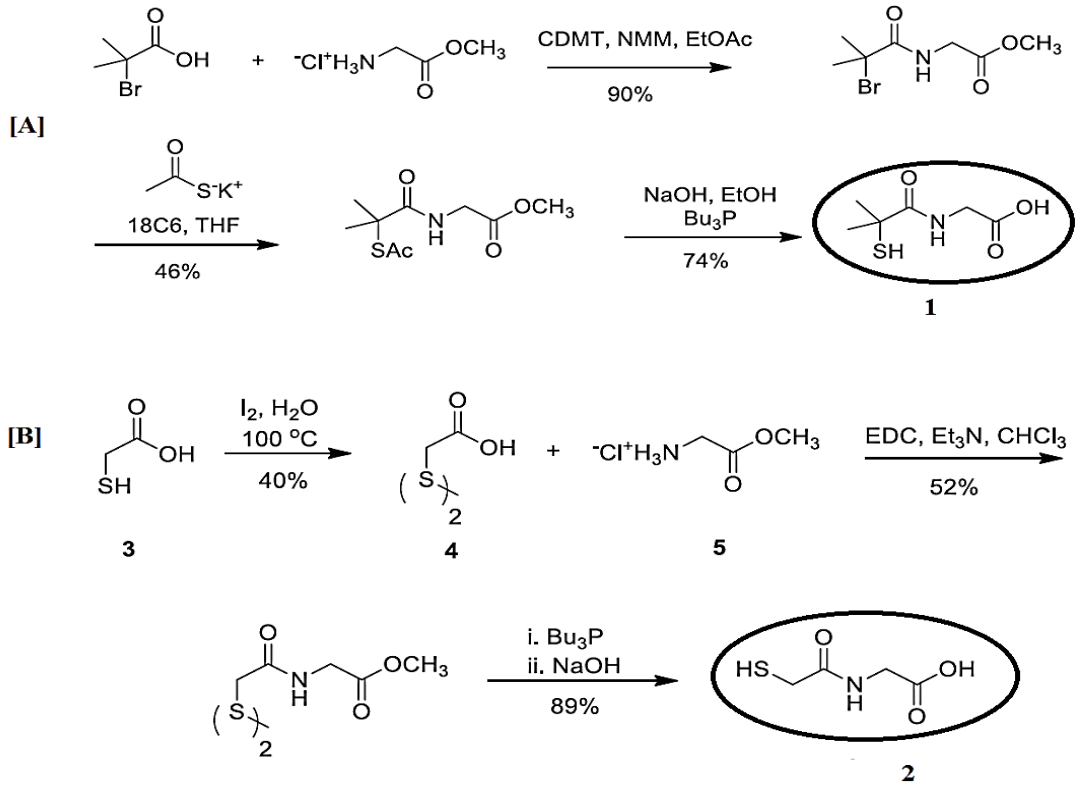
Scheme (1). Reagents and conditions: (a) DCCI, NMM, THF, 0 ˚C, 3 h then 5 ˚C, 16 h; (b) TFA, 1 h, rt; (c) isobutyl chloroformate, TEA, THF-15, ˚C, 15 min then 0 ˚C, 2h and 5 ˚C, 16 h; (d) I_2, MeOH, 4 h, rt; (e) NaOH 1N, THF, 0 ˚C, 20 min then 16 h, rt; (f) HBr/CH_3COOH, 5 h, rt; **(**g) 2N aq ammonia, rt, 20 min; (h) $(n\text{-But})_3P$, n-PrOH–H_2O, 3 h, rt.

1.4.2. Organo-Selenium and Sulfur Compounds

One of the most common Organo-selenium derivatives is Ebselen, as a glutathione peroxidase mimic, this chemical has been thoroughly investigated [152]. Ebselen has been shown to have anti-inflammatory properties in a variety of experimental paradigms [152,153]. As recently reviewed [154], the chemical has been developed for clinical usage (Phase III) [155,156]. The acute middle cerebral artery occlusion [157] was the indications addressed. Because these clinical disorders have an inflammatory component, it seems logical to presume that potential therapeutic benefits are linked to ebselen recognized anti-inflammatory activity.

The fact that Ebselen reacts with peroxynitrite, a powerful inflammatory mediator, was of particular interest [148,149]. In a first step, the molecule catalyzes the reduction of peroxynitrite to nitrite, with the reaction result being the matching selenoxide, which is then reduced back to Ebselen in two one-electron reduction steps via the selenodisulfide, using reducing equivalents in the form of glutathione.

Tiopronin is Another example for Organo-Suffer Compound, is a prescription thiol drug used primarily in the treatment of severe homozygous cystinuria. Matthew D. Hal and his co-authors have been tested the activity of new synthetic Tiopronin analogues and tested them for Inhibition of glutathione peroxidase as shown in scheme (2) [158].



Scheme (2): The synthetic routes of the Tiopronin analogues

1.4.3. Selenocysteine and Selenomethionine

Selenocysteine and Selenomethionine are the two primary selenium-containing amino acids in the body. A particular insertion machinery incorporates selenocysteine into a number of selenoprotein. In place of methionine, Selenomethionine is randomly integrated into proteins [159]. Selenomethionine has a substantially higher reactivity of peroxynitrite than methionine, as indicated by a larger second-order rate constant [160] and more effective protection against oxidation and nitration reactions [161]. Similarly, selenocysteine outperformed cysteine in protective tests [161]. The selenoxide generated during oxidation of selenocysteine residues or Selenomethionine in proteins would be reduced by reducing systems that could be selective or not. Glutathione, as well as dihydrolipoic acid and b-mercaptoethanol, has been shown to diminish the selenoxide of selenocysteine in proteins [162].

1.4.4. Gpx Mimics Catalytic Hydrogen Peroxide Scavengers

To detoxify peroxides, there are two basic design options. One model follows the catalase dismutation reaction and concentrates on molecules with redox-active metal centers, which are frequently manganese (Mn) or iron (Fe). Another technique uses selenium or tellurium active sites to model after the Gpx enzymes. At therapeutically effective quantities, an ideal mimic is stable and non-toxic. The mimetic's size and charge are frequently used to target cellular areas of oxidant generation, such as mitochondria, and increase their pharmacodynamics qualities [163]. Around the Gpx mechanism of H_2O_2 breakdown, a variety of selenium-containing compounds have been created (Fig :1-5)

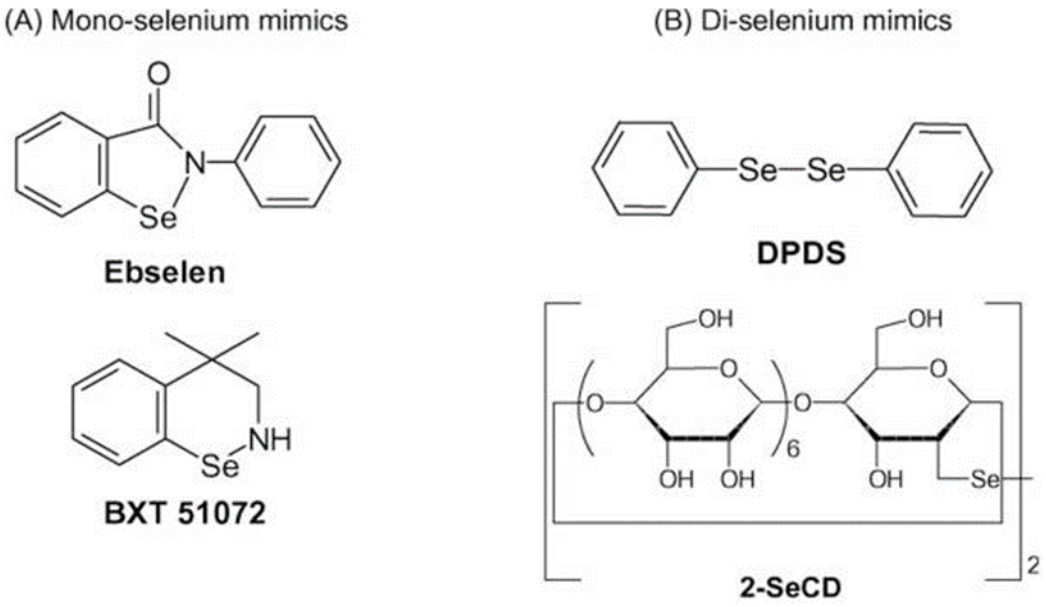


Figure. (1-5): - Mono-selenium mimics (A) and di-selenium mimics (B)are examples of glutathione peroxidase-like mimics' chemical structures. [163]

1.4.5. Diselenide and ditelluride compounds

A number of Diselenide and ditelluride-containing compounds have been shown to scavenge peroxides catalytically with stronger Gpx-like activity than ebselen [164]. Sulfur, selenium, and tellurium are all members

of Group VI of the periodic table and have chemical characteristics that are similar. Early chemicals like diphenyl Diselenide (DPDS) were electrophilic agents with cytotoxic, genotoxic, and mutagenic properties [165,166]. During the catalytic cycle, many previously described Diselenide compounds emit free selenium, which could pose a challenge in their development as therapeutic agents [167].

1.5. Glutathione peroxidase Gene Polymorphisms

The human GPx-1 gene is found on chromosome 3p21.3[168], where a cytosine-to-thymine (C > T) substitution polymorphism (rs1050450) occurs at codons 198 and 197, resulting in Pro198Leu and Pro197Leu variants. The Leu variation has been linked to a 40% reduction in GPx-1 activity and an increase in tumor susceptibility [169]. Given that growing evidence based on a small sample size and low statistical power suggested that GPx-1 could be a candidate gene for CVD risk, a meta-analysis of published data was conducted to investigate the connection of GPx-1 Pro 198 Leu and Pro 197 Leu polymorphisms with CVD risk [170]. The start and progression of chronic complex diseases such as CVD, neurological diseases, and malignancies are linked to redox homeostasis, which is regulated by reactive oxygen species producing enzymes and antioxidant enzymes [171]. In the cytoplasm and mitochondria, Gpx is an endogenous selenium-dependent antioxidant enzyme. GPx-1, which uses glutathione to detoxify hydrogen peroxide to water and lipid peroxide to alcohols, is a crucial antioxidant in oxidative stress defense [172,173]. GPx-1 activity in combination with homocysteine was found to predict cardiovascular risk in a prospective study [174]. Furthermore, the level of GPx-1 has been proposed as a useful marker for monitoring cardiovascular events [175]. Suggested mechanism indicated to the decrement of erythrocyte GPx-1 activity in multi-vascular atherosclerosis patients. Taken together, these

results suggested GPx-1 played an important role in the etiology of CVD. The risk of acute myocardial infarction was greater in patients with diminished GPx-1 activity, according to the study. Espinola-Klein et al. [176] examined the atherosclerotic load. According to the findings, erythrocyte GPx-1 activity was lower in individuals with multi-vascular atherosclerosis and was inversely connected to the event rate. These findings revealed that GPx-1 played a significant role in the genesis of CVD when taken together.

CVD is a complicated clinical condition caused by interactions between hereditary and environmental risk factors. CVD is the primary cause of death and disability in the United States, despite the introduction of tiered, preventative interventions [177]. For risk assessment and tailored treatment, the identification of genetic risk factors was contributed to CVD is critical. The glutathione peroxidase-1 (GPx-1) gene has been studied in the hunt for hereditary risk factors, however the results have been varied.

The antioxidant selenoenzymes glutathione peroxidases 1 (GPx1) and 4 (GPx4) detoxify peroxide radicals and lipid hydroperoxides, respectively [178]. Selenoprotein P (Sepp) has antioxidant and Se transport activities [179]. These three genes have a lot of functional polymorphisms in them. At codon 198 of the GPX1 gene, Rs1050450 causes an amino acid change from Pro to Leu [180], with the Leu variants being less active than the Pro variants [181]. Although the nature of the proteins impacted has not been determined, Rs713041 in GPX4 impacts protein binding to the 3′untranslated region (3′UTR) of the mRNA adjacent to an RNA structure (selenocysteine insertion sequence: SECIS) needed for selenoprotein synthesis [182, 183, 184]. Several functional polymorphisms in the human Sepp gene (SEPP1), such as rs3877899 (Ala234Thr) and rs7579 (a G/A

base change in the 3′UTR of SEPP1 mRNA), alter plasma and lymphocyte selenoprotein activity in vivo and the relative levels of selenoprotein.

Both SNPs have been linked to a higher proportion of plasma Sepp isoforms [185, 186]; additionally, both SNPs have been linked to a higher risk of colorectal and prostate cancer [187,188, 189]. Other selenoprotein genes [182] and non-selenoprotein genes, such as rs4880 in the SOD2 gene producing antioxidant manganese superoxide dismutase (MnSOD) [187, 188, 190], have reported a modest number of functional variants.

Until now, research on selenoprotein genetic variations and breast cancer (BC) risk has been restricted. Breast tumor DNA had a higher frequency of the Leu variation of rs1050450 (GPX1) than normal tissue, which was likely owing to tumor cells losing heterozygosity [181]. Two subsequent genetic association analyses [191,192] failed to find a link between rs1050450 and BC risk, however risk was raised in people who had both the Leu variant in GPX1 and the Ala variant in rs4880 (SOD2) [193]. Furthermore, carrying the Leu variation for rs1050450 in association with alcohol consumption was linked to a greater risk of BC [194]. As a result, whether rs1050450 (Gpx1) is a risk factor for BC is yet unknown [195]. A link between the rs713041 (GPX4) genotype and disease prognosis has also been discovered [190]. Although lymphocyte GPx4 mRNA expression was reported to be lower in breast cancer patients than controls [196], no genetic association research of genotype for rs713041 (GPX4) or other SNPs in SEPP1 with BC risk has been conducted to date.

Non-ductal breast cancer risk was found to be 1.9-fold higher in Leu carriers for rs1050450 (Pro198Leu) in GPX1. Furthermore, carriers of this variation were 2.6 times more likely to have a grade 3 ductal tumor than those with a grade 1 or 2 tumor. We propose that high GPx1 activity is required to counterbalance the levels of ROS and related damage that occur

during the initiation or progression of the disease [180], because enzymatic assays have previously shown that the Leu protein variant is less active than the Pro counterpart [180], [181].

1.6. A Validated Method to Assess Glutathione Peroxidase Enzyme Activity

Glutathione peroxidases (Gpx) act to reduce hydro peroxides (ROOH) by glutathione (GSH):

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$$

R may be an aliphatic, aromatic, or hydrogen-containing organic group. H_2O, alcohol (ROH) (or a second H_2O when H_2O_2 is the substrate), and glutathione disulphide (GSSG) are the products. The enzyme glutathione reductase is responsible for regenerating GSH from GSSG in the cell [197,198]. The glutathione peroxidase family (GPx1–8) catalyse the reduction of organic and inorganic peroxides by using reduced GSH. Proteins that contain selenocysteine make up five of the eight glutathione peroxidases (GPx1–4 and GPx6) [199]. The tendency of various Gpx to catalyse the degradation of hydro peroxides by thiols is their common denominator [200]. Gpx is a significant system to protect against endogenously and exogenously mediated lipid peroxidation that is present in many animal tissues. The enzyme is stoichiometric in selenium, and it reacts with several organic hydro peroxides as well as hydrogen peroxide [201].

Even though several protocols for assessing glutathione peroxidase activity have been established, only a few are still useful. To assess glutathione peroxidase activity in biological tissues, only two different test

systems have been used. The first system [197, 202] was based on measuring ROOH or GSH consumption at regular interval. The second system monitors GSSG production by coupling to the glutathione reductase-catalysed reaction. The decrease in NADPH concentration is continuously measured spectrophotometric ally or fluorometrically [202, 203].

Ellman's reagent (DTNB) is most commonly used in the first system to colour metrically evaluate glutathione consumption as a function of glutathione peroxidase activity [202]. Compared to other tests, the Gpx-DTNB assay is insensitive [204] and Ellman's reagent is relatively unstable [205]. Moreover, the process is time-consuming [206]. Flohé and Guzzler [207], on the other hand, showed another palaeographic method. It employed strong acid to stop enzyme-catalysed or spontaneous GSH-hydrogen peroxide reactions at a specified time (t). Polarography is then used to determine the GSH content.

Ugar et al. [208] recommended a microplate-based method that reduced the Cu(II)-neocuproine complex to highly coloured Cu(I)-neocuproine complex by using unreacted GSH. Catalase enzyme with high activity was used to stop the Gpx reaction. The absorbance decrement was correlated with Gpx activity. The method was suitable to assess Gpx activity in pure samples but not for assessing its activity in biological tissues because this does not take into account the interference arising from the presence of the catalase enzyme. Glutathione peroxidase and catalase act on hydrogen peroxide as a common substrate. All previous methods work to block interference with the catalase enzyme by adding sodium azide, which inhibits the catalase enzyme selectively. Fluorescent methods occupy an important part of the second

system of methods used to estimate the Gpx activity. Weiss et al. [209] documented a fluorometric method to measure Gpx activity in less than 100 µg of tissue. That assay depends on the fluorometric behaviour of $NADP^+$ that arises from the oxidised glutathione created by the Gpx reaction. Martinez et al. [210] developed a fluorometric procedure with high sensitivity that used the assay of oxidised glutathione with o-phthalaldehyde. Kamata et al. [211] developed a sensitive method to assess Gpx activity using the fluorometric reaction of oxidised glutathione with N-(9-acridinyl) maleimide. The procedure was used to evaluate human plasma samples and liver homogenates. Paglia and Valentino [203] were developed the most widely used protocol for measuring Gpx activity. It was based on the change of absorbance at 340 nm when NADPH is consumed by oxidised glutathione (GSSG). The protocol was modified by Lawrence and Burk [212] to study the activity of the Gpx enzyme in the liver supernatant of rats fed with a Se-deficient diet. The protocol was simple and selective, but the sensitivity was poor, and the enzyme and NADPH are costly [208]. Furthermore, NADPH + H^+ seems to be a potent Gpx inhibitor [206]. Since proteins and DNA absorb UV light, the method cannot have precise results when calculating Gpx activity in biological tissues. A simple method for determining Gpx enzyme activity is identified in this paper. Phosphate buffer was used to incubate the enzyme samples, which had appropriate concentrations of glutathione and peroxide as substrates. After appropriate incubation, the CUPRAC reagent ($Cu(Nc)_2^{2+}$) was added to stop the enzyme's reaction. Unreacted substrates reduced the Cu(II)-neocuproine ($Cu(Nc)_2^{2+}$) complex to highly coloured Cu(I)-neocuproine ($Cu(Nc)_2^{+}$) complex, which

has a maximum absorbance at 450 nm (CUPRAC method). The Gpx activity was correlated inversely with the decrease of absorbance of coloured Cu(I)-neocuproine ($Cu(Nc)_2^+$) complex.

The current protocol is precise, efficient, and trustworthy. The method is interference-free, simple to implement in laboratory experiments, and appropriate for clinical diagnosis.

1.7. The Aims of the Study

1- Synthesis and characteristics a new Glutathione Peroxidase Mimics compounds.

2- Study the defense role of glutathione peroxidase in smoking via

activity.

a. Assessment total antioxidant status and total oxidant concentration.
b. Measure the lipid peroxidation.
c. Study the glutathione peroxidase Gene Polymorphisms (Pro198Leu (rs1050450) gene polymorphisms).

3- Develop a novel precise method for assessment of glutathione peroxidase (Gpx1) activity and compared it with ordinary methods.

CHAPTER TWO MATERIALS AND METHODS

2. Materials

2.1. Chemicals:

All chemical and biochemical reagents were supplied of analytical grade and were purchased from standard chemical commercial providers, then consumed without any addition purification as shown in Table **(2.1):**

Table (2.1) The Chemicals Used in this Study.

Chemicals	Purity %	Supplied company
cysteine powder	99.0	BDH
Sodium nitrate	99.0	BDH
Methenol 10%	99.0	BDH
Iso propanol	99.0	BDH
$MgCL_2$	99.0	BDH
Primer		
ammonium sulfate	99.0	
Uric acid	99.0	BDH
Ammonium acetate.(NH_4Ac).	99	BDH
xylenol orange($C_{31}H_{32}N_2O_{13}S$)	99	BDH
Hydrochloric acid (HCl)	99	BDH
ascorbic acid	99	BDH
Ferrous ammonium sulfate $Fe(SO_4)(NH4)_2(SO_4)$	99.0	BDH
Glycerol ($C_3H_8O_3$)	99.0	BDH
Ethanol	96 %	BDH
glacial acetic acid	99	BDH
Sodium azide(NaN3)	99.0	BDH
Sodium chloride (NaCl)	99.0	BDH

Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$)	99.0	BDH
Sodium dodecyl sulfate. (SDS). $NaC_{12}H_{25}SO_4$	99.0	BDH
Sodium hydroxide (NaOH)	99.0	BDH
1,1,3,3- Tetra methoxypropane $C_7H_{16}O_4$	99	BDH
Sulfuric acid (H_2SO_4)	98	BDH
Trichloroacetatic acid. (TCA). $C_2HCl_3O_2$	99.0	BDH
Agarose	--------	Conda (Spain)
Ethylene diamine tetra acetic dihydrate (EDTA).2 H_2O	99.5	Fluka
Hydrogen peroxide (H_2O_2)	---------	Fluka
Tris base	-------	HI media (India)
PCR Master Mix Kit	---------	Promega Russian
Ethelium bromid	--------	Promega,USA
100 bp DNA ladder	-------	Russian (control)
Reduced glutathione	99	Sigma-Aldrich
2,9-dimethyl-1,10-phenanthroline (Neocuproine). $C_{14}H_{12}N_2$	98	Sigma-Aldrich
5,5'-dithio-bis-(2-nitrobenzoic acid). (DTNB). $C_{14}H_8N_2O_8S_2$	99.0	Sigma-Aldrich
solution of phthalic anhydride	99	Sigma-Aldrich
Copper (II) chloride $CuCL_2.2H_2O$	99.0	Sigma-Aldrich
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	99.0	Sigma-Aldrich
O-dianisidine dihydrochloride $C_{14}H_{16}N_2O_2.2HCl$	99.0	Sigma-Aldrich
Thio barbituric acid. (TBA). $C_4H_4N_2O_2S$	99.0	Sigma-Aldrich
Boric acid	---------	Thomas baker (India)

2.2. Instrument Analysis and Equipment:

Table (2-2): Instrument Used in this Study.

Instrument	Supplied company
Centrifuge	Heraeus (Germany)
Deep Freeze	GFL / Germany
Distillation device	Germany
Horizontal Gel electrophoresis unit	Cleaver scientific / UK
Magnetic stirrer	Gallin kamp (England)
Micro-centrifuge	Hettich / Germany
Micropipette 10M,100M and 1000M	Germany
Microplate reader	BioTech (USA)
Nano drop	Analytik jena
Oven	Hearson (England)
PG T80+ Spectrophotometer	Shimadzu / (USA)
pH meter	Jenway (Germany)
Sensitive balance	Stanton 461 AN (Germany)
Spectrophotometer UV -1601	Shimadzu / (USA)
Thrmocycler PCR(Biometra)	Germany
Vortex mixer	Karlkole (Germany)
Water bath	Karlkole (Germany)

2.3. Methodologies

This study was performed to the smoker and nonsmoker students in the University of Babylon, all the blood sample were collected between November 2020 and January 2021.

2.3.1. Collection of Blood and Serum Samples

When five ml of blood has been drawn into the needle was inserted, and the needle is withdrawn. This pad is firmly pressed onto until the bleeding stops. The needle is removed from the syringe and the blood slowly transferred to a Gel tube without anticoagulant. The blood permitted to clot for 15 minutes; the clot shrinks, and serum can be taken by centrifuging for approximately 10 minutes at a relative centrifugal force (RCF) of 1500 revolutions per minute (rpm) to 2000 rpm.

2.3.2. Samples: A clinical study was performed in University of Babylon during 2021. Blood samples were drawn from the students of the University of Babylon. A total of 300 male subjects ,150 subjects with non-smoking, 150 subjects with cigarette smoking.

The participants classified to:

- Group1: Non-smokers.
- Group2: Smokers of cigarette.

2.3.3. Exclusion Criteria

Diabetes mellitus, thyroid diseases, those under 18 years old, malignancies, those who had been on an antioxidant therapy during the past 2 months, and those with renal and liver failure were not included.

2.3.4. Collection of Data

The survey that questioned the sociodemographic characteristics of the participants (age and starting age of smoking) was filled through face to face interview.

2.4. Determination of Total Oxidant Status (TOS).

2.4.1. Principle

The TOS of serum was measured using a method, developed by Erel. **[213]** Oxidants existing in the serum oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is improved by glycerol molecules, which are abundantly found in the reaction medium. The ferric ion creates a colored complex with xylenol orange in an acidic medium. The color intensity, which can be determined spectrophotometric ally, is associated with the total amount of oxidant molecules existing in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micro molar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L).

2.4.2. Assay Reagents

Reagent 1: was prepared by dissolving 1.96 g of ferrous ammonium sulfate and 3.17 g of O-dianisidine hydrochloride in 1000 mL of H_2SO_4 solution, 25 mM. The final reagent was consisting of 5 mM ferrous ammonium sulfate and 10 mM O-dianisidine hydrochloride.

Reagent 2: was prepared by dissolving 114 mg of xylenol orange and 8.18 g of NaCl in 900 mL of 25 mM H_2SO_4 solution. One hundred milliliters of glycerol were added to the solution. The final reagent was composed of

150 µM xylenol orange, 140 mM NaCl and 1.35 M glycerol. The pH value of the reagent was 1.7. This reagent is stable for at least 6 months at 4°C.

Hydrogen Peroxide (STD): (100 µmol/L) was freshly diluted and standardized daily using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.

2.4.3 Procedure: shown in Table (2-3).

Table (2-3) Shows the Details of the Present Method.

	Blank	Standard	Sample
Distilled water	25 µl	-----	------
Serum	------	-----	25 µl
Hydrogen peroxide	------	25 µl	------
R1	1 ml	1 ml	1 ml
Test tubes was mixed by vortex, then add:			
R2	0.25 ml	0.25 ml	0.25 ml

Gently mix the content of every tube after addition, let to stand at room temperature for 3 minute, read spectrophotometrically at 560 nm [213].

2.4.4. Calculation

$$\text{Total oxidants status} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

2.5. Total Antioxidants Capacity (TAC) (Ferric-Ferro zine assay of total antioxidant capacity)

(ferric reducing antioxidant assay, Power)[214]

2.5.1. Principle

In this study, we measured TAC with the use of Ferric-Ferro zine reagent. The proposed assay depends on the reduction of a Ferric-Ferro zine reagent with antioxidants to the stable Ferrous-Ferro zine chelate in buffered medium. The concept of total antioxidant capacity of biological fluids is important because it determines the capacity of biological fluids to withstand oxidative stress. Most of the methods used for the

measurement of antioxidant activity are inhibition methods except for the ferric reducing antioxidant power (FRAP) and total serum reductive capacity, which measure the reductive capacity of antioxidants. The inhibition methods involve two phases, the first in which there is free radical generation with an endpoint indicating the presence of the free radical. In the second phase the antioxidant activity of the added sample inhibits this endpoint by scavenging the free radical.

The methods vary greatly with respect to the free radical that is generated, the reproducibility of the generation process and the end point used. The reductive methods do not generate free radical but measure serum ability to act against an oxidizing agent which was Fe^{3+} for the FRAP assay

2.5.2. Reagents

1- Iron (III) Ammonium sulfate ($NH_4Fe(SO_4)_2.12H_2O$) solution was prepared by dissolving 0.024 g of Iron (III) Ammonium sulfate and 1 ml of 1 M HCl was mixed with a separate solution containing 0.123 g Ferro zine in water. These two solutions were mixed and the mixture diluted to 25 mL with distilled water so as to make the final iron(III) concentration 2.0×10^{-3} M and Ferro zine concentration 1.0×10^{-2} M. This Ferric-Ferro zine complex solution, when kept in a stoppered, dark-colored bottle and protected from sunlight, was shown to be stable for a day.

2- Buffer solution 0.2 M (CH_3COOH/CH_3COONa) pH 5.5.

Steps:

- Prepare 400 mL of distilled water in a suitable container.
- Add 11.7 g of Sodium Acetate to the solution.
- Add 1.26 g of Acetic Acid to the solution.
- Adjust solution to final desired pH using HCl or NaOH.
- Add distilled water until volume is 0.5 L.

3- Uric acid (5.95 mM) was prepared by added 0.01g uric acid to (10 ml) Distilled water.

2.5.3. Procedure: shown in Table (2-4). [214,215]

To A (5) µl serum was added (45) µl EtOH (96%), then take 25 µl from it and 75 µl of Ferric-Ferro zine solution,100 µl of pH 5.5 buffer solution (0.2 M CH3COOH/CH3COONa), and 25 µl water so as to make the final volume 225 µl The absorbance against a reagent blank was measured at 562 nm after 30 min standing at room temperature.

Table (2-4) Shows the Details of the (FRAP) Method.

	Blank	Standard	Sample
Distilled water	5 µl	------	------
Serum	------	-----	5 µl
Uric acid	------	5 µl	----
EtOH (96%)	45 µl	45 µl	45 µl
Test tubes was mixed by vortex, then take 25 µl from each tube and			
ferric-ferrozine solution	75 µl	75 µl	75 µl
Buffer solution	100 µl	100 µl	100 µl
Distilled water	25 µl	25 µl	25 µl

After 30 min standing at room temperature was measured at 562 nm.

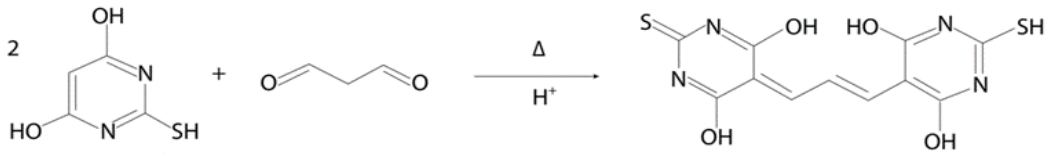
2.5.4. Calculation:

$$\text{Total antioxidants levels} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD \ (\mu mol/l)$$

2.6. Determination of Serum Lipid Peroxidation [216] (MDA method):

2.6.1 Principle

In the presence of heat and acid, MDA reacts with TBA to produce a colored end product that absorbs light at 530-540 nm. The intensity of the color at 532 nm corresponds to the level of lipid peroxidation in the sample.



Scheme 1: In the presence of acid and heat two molecules of 2-thiobarbituric acid (TBA) react with MDA to produce a colored end product that can be easily quantified

2.6.2 Reagents

Malondialdehyde (MDA) as the by-product of lipid peroxidation, reacted with thiobarbituric acid (TBA) to form TBARS. TBARS reagent was prepared by mixing 0.3 g of TBA, 12 g of trichloroacetic acid (TCA) and 1.04 mL of 70% per chloric acid ($HClO_4$) in 80 mL of double-distilled water.

2.6.3. Procedure: Shown in the Table (2-5)**.**

Sample (100 µL) was mixed with 1000µL of TBARS reagent and boiled at 90 °C for 20 min. After cooling on ice, the mixture was centrifuged at 309*g* for 10 min at 25 °C. Absorbance of the supernatant was read at 532 nm. The levels of lipid peroxidation were expressed as micromole MDA equivalents per liter of serum [217,218].

Table (2-5) Shows the Details of the Present (MDA method).

	Blank	Sample
Distilled water	100 µl	
Serum	------	100 µl
TBARS reagent	1 ml	1 ml
Boiled at 90 °C for 20 min. Then the mixture was centrifuged at 309*g* for 10 min at 25 °C		

Absorbance of the supernatant was read at 532 nm.

2.6.4. Calculation

$$serumMDA = \frac{Absorbance}{d\ X\ \zeta}\ X\ D.F$$

d = 1cm, ε =extinction coefficient = 1.56x 10^5 $M^{-1}cm^{-1}$

D. F = dilution factor

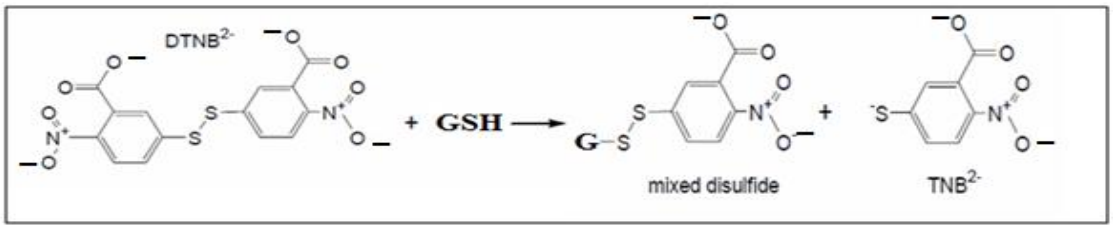
2.7. Assay of Se – Dependent Glutathione Peroxidase (Gpx) Activity:

2.7.1 Principle:

Glutathione peroxidase catalyzes the following reaction:

2GSH + LOOH —Gpx→ GSSG + LOOH + H2O

The decrement of reduced glutathione concentration can be monitored by Elman's reagent [5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB)].



2.7.2. Reagents:

1. Solution A: (0.4 M NaH_2PO_4) Dissolve 55.6 g of NaH_2PO_4 in 1L of water.
2. Solution B: (0.1 M Na_2HPO_4) Dissolve107.12 g of Na_2HPO_4 in 1L of water.
3. Sodium phosphate buffer (pH 7.0) (0.4 M): prepared by mixing 39 of solution A and 61 ml of solution B and dilute to 200 ml with D.W. which contain 0.0744 g EDTA.
4. Sodium azide (10mM): Dissolve 0.06501g of NaN_3 in 100ml of D.W.
5. Reduced glutathione (4 mM): prepared by dissolving 0.123 gm of a GSH in a final volume of 100 ml pbs
6. Tert- butylhydroperoxide (2.5mM)
7. Fresh H_2O_2 (2.5 mM) solutions were prepared by mixing 0.1134 ml of 30% H_2O_2 with 400 ml of phosphate buffer, and the solution was

adjusted to 2.5 mM using the molar extinction coefficient of H_2O_2 at 240 nm (43.6 M^{-1} cm^{-1}).

8. Na_2HPO_4 (0.4 M): Dissolve 5.68 g of Na_2HPO_4 in 100ml of D.W.
9. Sodium nitrate (0.1%)
10. DTNB {19.8 mg in 100 ml 0.1% sodium nitrate}

2.7.3. Procedure:

Table (2-6) Shows the Details of the Present Method.

Reagents	Test	Control	STD	Blank
Sodium phosphate buffer	1300µL	1500µL	1500µL	1500µL
Reduced glutathione	200 µL	-----	200 µL	------
D.W.	200 µL	400 µL	300 µL	500 µL
Sample	100 µL	-----	-------	------
The reaction was initiated by adding peroxide:				
Peroxide	200 µL	-----	-------	------
Mix by vortex and incubate for 10 minutes at 37°C, after that, the reaction was terminated with 0.5 ml of 10% TCA				
Sample	-----	100 µL	-----	-----
Mix well and centrifuge for 15 minutes at 3000 xg, then remove 1 ml of supernatant in a clean tube , and add				
Na_2HPO_4	3ml	3ml	3ml	3ml
DTNB	1ml	1ml	1ml	1ml

2. The color developed was read at 412 nm trough 3 min.

2.7.4. Calculation:

$$\text{The residue reduced GSH in test tube} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD$$

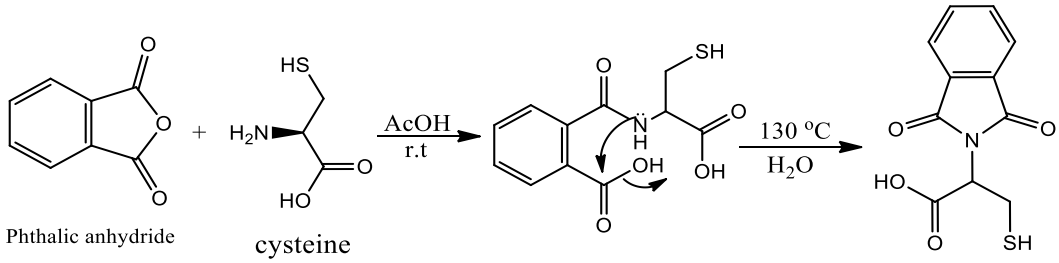
Se-dependent glutathione peroxidase activity (µmol of glutathione utilized/min) = Conc. of GSH in STD - Conc. of GSH in test * D.F.

$$\text{Se - GPX activity (µmol of GSH utilized/min)} = \frac{\text{Conc. of GSH in STD - Conc. of GSH in test}}{\text{time(3min)}} * D.F.$$

2.8. Synthesis of compound 2-(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid:

2.8.1. preparation [219]

cysteine (1.64 g, 13.5 mmol) was gradually added to solution of phthalic anhydride (2g, 13.5 mmol) in 100 mL of glacial acetic acid at room temperature with continuous stirring for 2 hours. Then the mixture was refluxed at 130°C for 4 hours until the whole white precipitate has dissolved and clear solution was formed. The solvent was removed using rotary evaporator and the remaining liquid was solidified to pale yellow crystals at 20 ˚C. The final product was recrystallized several times with cold mixture of diethyl ether and hexane (1:1) yield 70%.



Phthalic anhydride cysteine

2.8.2. Assessment of the glutathione peroxidase mimics

Reagents:

a. Sodium selenite $Na_2SeO_3.5H_2O$ (1mM): a 26.3014 milligrams of $Na_2SeO_3.5H_2O$ were dissolved in 100ml of D.W.
b. 2-(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid (1mM): a 27 milligrams of -(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid were dissolved in 100ml of 30% methanol.
c. Glutathione peroxidase mimics solution: the solution was prepared by mixing suitable volumes from (a and b) as shown in table (**2-7**).

Table 2 - 7: Glutathione Peroxidase Mimics Preparation

Solution	Volume of Solution a	Volume of Solution b
S_0	10ml	----
S1	9ml	1ml
S2	8ml	2ml
S3	7ml	3ml
S4	6ml	4ml
S5	5ml	5ml
S6	4ml	6ml
S7	3ml	7ml
S8	2ml	8ml
S9	1ml	9ml
S10	----	10ml

2.9. The Genetics Study

2.9.1. The solutions used for extraction and methods of preparation: [220]

(1) Preparation of the washing solution (90% Tris + 10% Methanol)
To prepare (100 ml) of Tris at a concentration of 20 mM
0.31 g of (Tris + HCl) Was dissolve in (90 ml) of D.W and adjust the pH to 7.5 and complete the volume to (100 ml) of D.W.

(2) To prepare the extraction solution (lyses)
(Tris - HCL) (0.3 g) + (0.06g) EDTA was taken and (0.2g) of SDS by dissolving volume of 90 ml of distilled water (DW). Adjust PH 8 and complete the volume to 100 ml.

(3) Solution EDTA to prepare the solution (50) ml EDTA by dissolving(0.03g) of EDTA in volume (40 ml) distilled water.
Adjust PH 8 and complete the volume to 50 ml distilled water.

(4) Sodium acetate solution to prepare (20) ml of the solution at a concentration of (3) M by dissolving (4.9g) of Sodium acetate in volume (15 ml) distilled water Adjust PH 4.5 and complete the volume to 20 ml distilled water.

(5) Buffer solution (TE) consists (Tris + EDTA) To prepare the solution (100) ml TE buffer by dissolving (0.157g) of Tris and (0.029g) of EDTA in volume (90 ml) distilled water Adjust PH 8 and complete the volume to 100 ml distilled water Put in or condensation at a temperature (121 C) and pressure (0.1) for a quarter of an hour.

2.9.2. Procedure Extraction [220]

1.Take 500 microliters of the sample and put it in a volume of one and a half ml Eppendorf, then add 1 ml of washing solution 10% Methanol + 90% Tris (18Mm) put on the Roar for 10 minutes, then Center fugue 10000 for 1 minute. The filtrate is discarded and the precipitate is taken.

2.The precipitate is suspended by the washing solution to reach full diffusion, then Center fuge 10,000 cycles for a minute. The filtrate is neglected.

3.The precipitate was suspended by 200 µl of 2 mM EDTA solution.

4. added 400 microliters of the extraction solution is and suspended well until complete dispersal, at 60 C for 15 minutes.

5. Then cool to room temperature, then add 100 µl of sodium acetate (4.5 ph M3) VORTEX for a minute.

6. Center fuge 10000 cycles for 10 minutes The sediment is neglected and the filter is taken and placed in the Eppendorf.

7. Center fusion 10000 cycles for 30 seconds.

8. Wash twice with 600 µl of 70% ethanol + 30% Tris (Ph 5Mm) (600 µl of column wash solution is added, then Centrifuge 10,000 cycles for 30 seconds and the process is repeated twice).

9.The column is dried after getting rid of the filter by placing it in Center fugue 10000 cycles for three minutes to get rid of the largest amount of washing solution.

10. The collection tube is discarded and the column is transferred to a sterile Abendrov, one or half ml volume.

11. 100 µl of Elution buffer solution is added to the center of the column and left for 5 minutes. After addition, it is confirmed that all the solution is absorbed from the column (and the solution is not left until complete absorption).

12. Then Center fuge10,000 cycles for a minute by taking the filter that represents pure DNA and neglecting the column.

Extracted DNA Quantity and Quality Assessment The extracted DNA was assessed by spectrophotometry method, (scan drop, analytical jena, Germany) sample quantity were ranged from 35-56 ng/µl and the quality (260/280 ratio) ranged from 1.7-1.85, each sample that retrieve a 260/280 ratio lesser than 1.7 would be re extracted, on the other hand the extracted DNA was checked by 1% agarose gel electrophoresis (figure: 2-1)

Fig: (2-1) The electrophoresis pattern of PCR product for Glutathione peroxidase gene.

2.10. Agarose Gel Electrophoresis

2.10.1. Preparation of TBE (10X) Stock Solution/Liter.

TBE (0.5X) 1000ml was prepared by adding 100ml of stock solution (TBE (5X) to a final volume of 900ml dH_2O.

2.10.2. Gel Electrophoresis Protocol.

1. The gel-casting tray was placed in plastic tray and checked that the teeth of the comb are 0.5mm above the gel bottom. Position the comb 1.5 cm from the edge of the gel.

2. TBE (0.5X) (80ml) was placed into a 250ml flask and 0.8g to deport DNA 1,6g to deport PCR of agarose was added and then melt the agarose by heating the solution on microwae for approximately 2 min. The agarose solution was swirled carefully to ensure that the agarose was completely dissolved and the solution became clear.

3. The agarose solution was cooled to approximately 60ºC and adds 2-3µl of ethidium bromide, and slowly pours the agarose into the gel-casting tray. If there are any air bubbles must be removed by tip.

4. Agarose was left solidify for about 20-30 minutes. After the agarose has been frozen the comb has been removed with the back and forth stirring, careful to prevent rupture of the gel.

5. The gel-casting tray was removed and placed the tray on the central supporting platform of the gel box.

6. Electrophoresis buffer was added to the buffer chamber until it reaches a level of 0.5-1 cm above the surface of the gel.

7. The samples were loaded in to the wells using white tips under the surface of the electrophoresis buffer just above the well. The sample was expelled slowly to allow it to sink to the bottom of the well.

8. Ladder molecular weight marker (5 µl) was loaded to one side of the gel (flanking and sample line) and 10µl of DNA specimen and 5micro of pcr product in the other well.

9. The lid was placed on the gel box and connected the electrodes. DNA will travel towards the positive (red) electrode positioned away from the well. Turn on the power supply.

10. Electrophoresis was continued until the tracking dye moves at least 10 cm of the gel length.

2.10.3. Photo Documentation.

Agarose gel was visualized in a UV trans illuminator provided with gel documentation unit, agarose gel was placed above the UV trans illuminator device, the gel was exposed to UV light and the photo was captured using canon digital camera.

2.10.4. Reconstituting and diluting primers.

Primers were commonly shipped in a lyophilized state. The units of a lyophilized primer were given as a mass, in pico-moles. In order to create a stock of primers, one would reconstitute the primer in sterile, nuclease free H_2O to be added to each primer to obtain a master stock that would be used again to obtain a working stock.

The following steps were followed for reconstituting and diluting the primers:

1. The tube was spied down before opening the cap.
2. The desired amount of water was added according to the oligos. Manufacturer to obtain a 100Pmoles/µl (Master Stock).
3. The primers were vortexes properly to re-suspend evenly.
4. A 10µl of the master stock was transferred to a 0.2ml Eppendorf tube that contained 90µl of sterile, nuclease-free H_2O (Working Stock).
5. The master stock and working stock were stored at -20 ºC.

6. The working stock was thawed on ice and vortex before using in PCR and then stored at -20 ºC.

Sequences of primers used for PCR amplification this study were shown in table (2-8):

Table (2-8): Sequences of primers used for PCR amplification of Glutathione Peroxidase Gene.

Primer gene name	Sequences
Glutathione Peroxidase Gene Rs 1050450	**F:5'-AATGACACAGGACATACACACAGTT-3′**
	R: 5'-CACCTGGTCTCCGGTGTGTC-3′

2.10.5. Amplification by Polymerase Chain Reaction (PCR)

Table (2-9): Optimization reaction mixture for (PCR)

No.	**Step**	**Temp.(Cº)**	Time **(min)**	No. of cycles
1	Initial denaturation	94	5 min	1
2	DNA Denaturation	94	30 sec	35
	Primer annealing	55-66	30 sec	
	Extension	72	30 sec	
3	final elongation	72	5 min	1

Table (2-10) The proportion of mixing materials are Shown below

	composition	Concentration	Volume
1	Master mix	100 ppm	8 µl
2	Forward primer	10 mol / ml	2 µl
3	Revers primer	10	2 µl
4	DNA sample	(30-55) ng / ml	2 µl
5	Nucleases free water		5.5 µl
6	Mgcl2	25 mM	0.5 µl
	Total volume		20 µl

2.11. Restriction Fragment Length Polymorphism (RFLP)for study the genotyping of Arachidonate 12Lipoxygenase

2.11.1. Principle:

RFLP is a distinction in homologous DNA sequences that can be identified by the nearness of fragments of various lengths after digestion of the DNA tests being referred in question with specific restriction endonucleases. RFLP, as a molecular marker, is explicit to a single clone/restriction enzyme combination [221]. Most RFLP markers are co-dominant (the two alleles in heterozygous example will be identified) and profoundly locus-specific. A RFLP probe is a marked DNA sequence that hybridizes with at least one fragments of the processed DNA test after they were isolated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single-or low-duplicate genomic DNA or cDNA clones are normally utilized as RFLP probes. The RFLP tests are much of the time utilized in genome mapping and in variety investigation (genotyping, crime scene investigation, paternity tests, inherited disease diagnostics, etc.) [222]

2.12. Programs:

The following programs and webs were used to validate the concentration of solutions that used in the current study:

1. SPSS 24 for Windows (SPSS Inc., Chicago, IL, USA).
2. Quickcals-graphpad.
   (https://www.graphpad.com/quickcalcs/Molarityform.cfm).
3. Buffer preparation
   (https://www.cusabio.com/m-296.html).
4. Molarity calculator
   (https://www.graphpad.com/quickcalcs/Molarityform.cfm).

2.13. A validated method to assess glutathione peroxidase enzyme activity

2.13.1. Materials and Methods

2.13.1.1. Chemicals and materials

Ammonium acetate, calcium chloride, copper(II) chloride, dipotassium phosphate (K_2HPO_4), hydrochloric acid, sodium dihydrogen phosphate dihydrate (NaH_2PO_4. $2H_2O$), hydrogen peroxide (30%), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), sodium azide, sodium hydroxide, sodium nitrate, and trichloroacetic acid were purchased from BDH.

Bovine serum albumin, glutathione, homocysteine, neocuproine (2,9-dimethyl-1,10-phenanthroline) and Tert-butyl hydro peroxide were purchased from Sigma-Aldrich.

2.13.1.2. Animals: The albino rats were obtained from Animal House, University of Babylon, Babylon governorate, Iraq. They were kept in well-

ventilated cages with monitored light and humidity, as well as free access to regular food and water. The current study was conducted in accordance with the WSAVA Animal Welfare Recommendations [223].

2.13.1.3. Reagents

1. The phosphate buffer solution (100 mM M, pH 7.0) consisted of 1:1.5 volumes of A and B solutions. Solution A contained 13.62 g of KH_2PO_4 dissolved in 1 L of distilled water (D.W.). Solution B contained 17.8 g of $Na_2HPO_4.2H_2O$ dissolved in 1 L of D.W. A 0.372 g EDTA and 0.6501 g of NaN_3 was added to the final solution and mixed gently.
2. Reduced glutathione (4 mM) was prepared by dissolving 0.1228 g of reduced glutathione in 100 ml of 100 mM phosphate buffer solution (pH 7.0).
3. Hydrogen peroxide H_2O_2 (2 mM) was prepared daily, in 100 mM phosphate buffer solution (pH 7.0). The final concentration was prepared by using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.
4. Copper(II) chloride (10^{-2} M) comprised 0.4262 g of $CuCl_2 \cdot 2H_2O$ dissolved in 250 ml of D.W.
5. Ammonium acetate buffer (NH_4Ac) (1.816M, pH 7.0) was composed of 35 g of NH_4Ac dissolved in 250 ml D.W.
6. Neocuproine (7.5 * 10^{-3}M OF 2,9-dimethyl-1,10-phenanthroline) (NC) was composed 0.039 g NC that was dissolved in 25 ml of 96% ethanol.
7. Fresh working reagent (CUPRAC reagent**)** was used for the experiment. The reagent was composed of Cu(II): Nc: NH_4Ac at a ratio of 1:1:1 (v/v/v).

2.13.2. Detailed of procedure

The details of the procedure are shown in table 2-10.

Table 2-11. The details of the protocol used to measure glutathione peroxidase activity.

Reagents	Test	Control	STD	Blank
Sodium phosphate buffer	1500µL	1900µL	1600µL	2000µL
Reduced glutathione	200 µL	----	200 µL	------
Sample	100 µL	100 µL	-------	------
The reaction was initiated by adding peroxide:				
Peroxide	200 µL	-----	200 µL	------
Mix by vortex and incubate for 10 minutes at 37°C, after that, the reaction was terminated with 0.5 ml of 8% TCA				
Mix well and centrifuge for 15 minutes at 3000 xg, then remove 1 ml of supernatant in a clean tube , and add:				
Working reagent	3ml	3ml	3ml	3ml

Absorbance was read against blank at 450 nm after 30 min.

2.13.3. Calculation

Unit definition: one unit of Gpx was defined as the amount of enzyme capable of oxidising 1.0 µmole GSH to GSSG per minute at 25 ºC, pH 7.0 [224, 225].

The residual glutathione concentration in test tube = $\left(\frac{A\ Test - A\ Control}{A\ STD}\right)$ × Conc. of STD

The concentration of standard glutathione was 400 µM.

Also, the residual glutathione concentration in the test tube could be calculated from the glutathione standard curve. Gpx activity equals the number of micromoles of consumed glutathione.

Glutathione peroxidase activity (U/L) =

$$\left(\frac{Conc.of\ GSH\ in\ STD - Conc.of\ residual\ GSH\ in\ test\ tube}{time\ (10\text{min})}\right) \times \left(\frac{Total\ volume\ (ml)}{\text{Volume of the sample (ml)}}\right) \times \text{D.f.}$$

2.13.4. Standard curve preparation

To create a standard curve for the assay, the stock solution of the standards (glutathione and peroxide) were diluted with the phosphate

buffer (0.1 M, pH 7.0) according to the layout in table 2. Once each standard tube was created and mixed, 1000 µL of each was added to the three ml of working solution according to the protocol listed in table 1. Absorbance was read against blank at 450 nm after 30 min (as shown in fig. 2-2).

Table 2 -12: Standard Curve Preparation

Standard	Volume of Glutathione 4mM	Final concentration of glutathione	Volume of peroxide 2mM	Final concentration of peroxide	Volume of phosphate buffer solution
S1	400µl	800µM	400µl	400µM	1200µl
S2	300µl	600µM	300µl	300µM	1400µl
S3	200µl	400µM	200µl	200µM	1600µl
S4	150µl	300µM	150µl	150µM	1700µl
S5	100µl	200µM	100µl	100µM	1800µl
S6	50µl	100µM	50µl	50µM	1900 µl
S7	25µl	50µM	25µl	25µM	1950 µl
S8	5µl	10µM	5µl	5µM	1990 µl
B	------	------	------	------	2000 µl

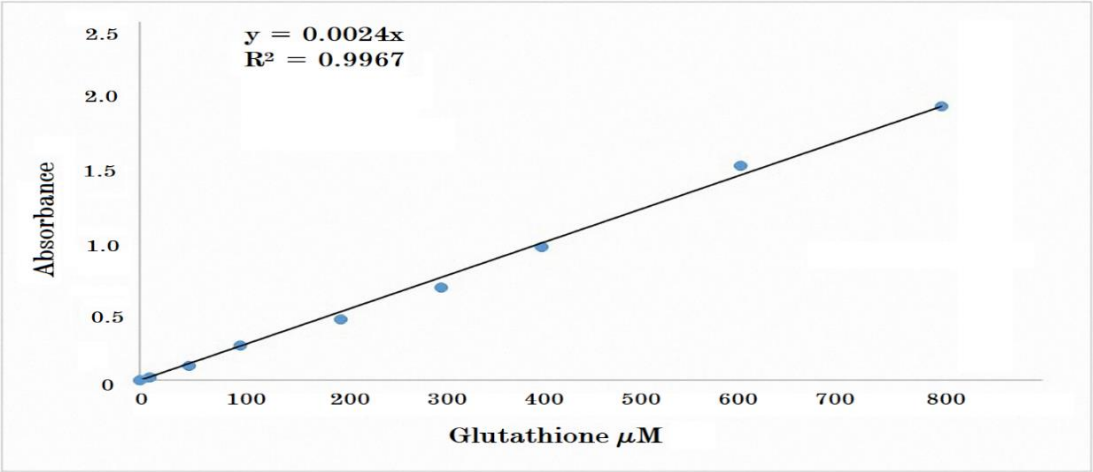


Figure 2-2. The standard curve of glutathione obtained by using the current protocol.

2.14. Statistical analysis

Data analysis was performed using SPSS 24for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean, SD, and range, and were

evaluated by one-way analysis of variance (ANOVA) followed by LSD test. The Kolmogorov Smirnov test was used to verify if data followed normal distribution. The statistical significance level was considered at $P < 0.05$.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Characteristics of the Study Subjects

This research was conducted on non-smokers and smokers at the University of Babylon, with all blood samples collected between November 2020 and June 2021. A total of 300 samples were taken from me The participants were placed into two groups: non-smokers (NS) and smokers (CS). There were no significant differences between nonsmokers and smokers in terms of age, height, or body mass (Table 3-1).

Table (3-1). Participants' characteristics non-smokers and cigarette smokers.

Group / Parameter	Nonsmokers (NS)	Cigarette Smokers (CS)
Number	**150**	**150**
Age (Years)	**20±2**	**20±2**
BMI (kg/m^2)	**22±1.5**	**21±2.2**

3.2. The Effect of Smoking on Oxidant/ Antioxidant Status:

3.2.1. Total Oxidant Status Concentrations in Sera of Non-smokers Groups and Cigarette smokers

Table 3-2 shows that the total oxidant status (TOS) concentrations found in sera of non-smokers (NS) were 12.775 µmol/l and cigarette smokers (CS) were 15.211 µmol/l. In addition, the conventional division in nonsmokers and cigarette smokers (4.478 and 5.853, respectively) µmol/l

Table 3-2: Total Oxidants Status Concentration in Sera (µmol/l) of Non-smokers Groups and Cigarette smokers.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	12.775	4.478	0.36566	12.0524	13.4975	0.001 *
CS	150	15.211	5.853	0.47791	14.2671	16.1558	

*. The mean difference is significant at the 0.05 level.

Smoking causes free radicals to activate inflammatory cells, resulting in elevated levels of reactive oxygen metabolites [226]. As a result, smokers are exposed to higher levels of oxidative stress, resulting in an imbalance between oxidants and antioxidants [227]. As a result, smoking behavior will alter indicators of oxidative stress, antioxidant, and redox state [228].

3.2.2 Total Antioxidant Status (µmol/l) in Sera of Nonsmokers and Cigarette Smokers

The total antioxidant status (TAS) was measured in smokers and non-smokers, as indicated in Table 3-3. Antioxidant levels were determined to be 0.7429 in nonsmokers and 0.6850 in cigarette smokers in the current study. In addition, the standard divisions for nonsmokers and cigarette smokers were and 0.19757 respectively.

Table (3-3): Total Antioxidant Concentration in Sera (µmol/l) of non-smokers Groups and cigarette smokers.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	0.7429	0.21927	0.01790	0.7075	0.7783	0.017*
CS	150	0.6850	0.19757	0.01613	0.6531	0.7168	

* The mean difference is significant at the 0.05 level.

As a result, smokers have fewer antioxidants than nonsmokers, which may contribute to their increased risk of vascular disease. Smokers have been shown to have lower levels of plasma vitamin C than nonsmokers [229,230].

The ratio of oxidized to reduced vitamin C has also been found to be greater in smokers [231]. Because of the medical and social problems that smoking addiction produces in our country, where about 16 million people regularly smoke, it is one of the most major causes of early and preventable deaths [232].

Increased quantities of reactive oxygen species cause oxidative stress, which damages cells, tissues, and organs. Oxidative stress arises when the body's antioxidant defense mechanism is overwhelmed by reactive oxygen species [233]. Smoking is one of the most important variables that causes oxidative stress. Cigarette smoking produces a significant number

of oxidants, which are thought to be responsible for many of smoking's negative consequences [234]. Oxidative stress is a condition in which cells, tissues, or organs are damaged by high quantities of reactive oxygen species.

TAS values were investigated in the current study to measure the entire effect of antioxidants, as well as TOS values. In the current study, there is a statistically significant difference between smokers and non-smokers in terms of mean TAS and TOS values (p0.05) [235]. The study that found that The TOS of smokers was much higher than that of nonsmokers, whereas the mean TAS of smokers was significantly lower than that of nonsmokers [236]. This result was attributed by to the existence of large levels of free radicals in cigarette smoke, which cause oxidative stress in the body. Furthermore, [237]. The effect of smoking on oxidative stress is depicted in Figure (3-1).

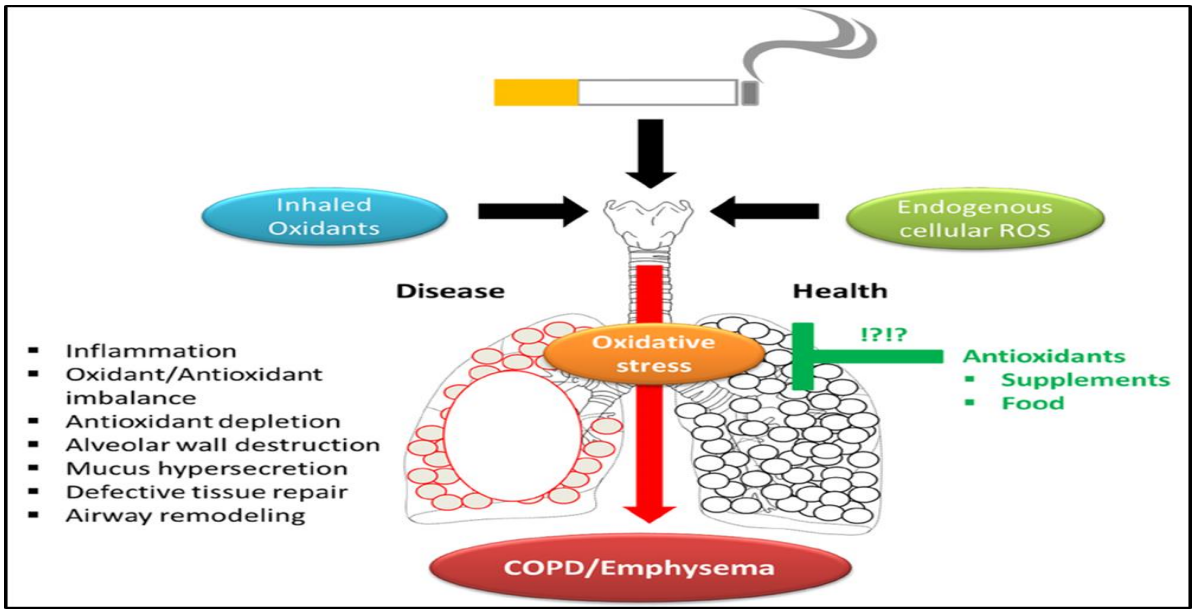


Figure (3-1). Smoking's Effect on Oxidative Stress [238].

3.3. Lipid Peroxidation in Sera of Nonsmokers and Cigarette Smokers:

The thiobarbituric acid-reactive compounds (TBARs) assay was used to assess the effect of smoking on lipid peroxidation. The results (Table3-4) revealed that the level of MDA in non-smokers' sera was 1.1265 and cigarette smokers' sera was 1.2207**.** Furthermore, the standard deviations for nonsmokers and cigarette smokers were 0.28165 and 0.47382 respectively.

Table (3-4): Lipid Peroxidation (MDA Concentration (µmol/l)) in Sera of Cigarette Smokers and Nonsmokers Groups.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	1.1265	0.28165	0.02300	1.0811	1.1719	0.034*
CS	150	1.2207	0.47382	0.03869	1.1443	1.2972	

*. The mean difference is significant at the 0.05 level.

3.4. Glutathione Peroxidase Activity (U/L) in Sera of Nonsmokers and Cigarette Smokers:

The results of Glutathione Peroxidase Activity (U/L) in smokers and non-smokers is shown in Table (3-5).

The average Glutathione Peroxidase Activity levels in nonsmokers were 206.33 (U/L), while cigarette smokers had

190.91 (U/L). In addition, the standard divisions for nonsmokers and cigarette smokers were (56.05190 and74.01852, respectively).

Table (3-5): Glutathione Peroxidase Activity (U/L) in Sera of Cigarette Smokers and Nonsmokers Groups.

Group	N	Mean	Std. Deviation (SD)	Std. Error	95% Confidence Interval for Mean		Sign.
					Lower Bound	Upper Bound	
NS	150	206.33	56.05190	4.57662	197.29	215.37	0.034*
CS	150	190.92	74.01852	6.04359	178.979	202.86	

*. The mean difference is significant at the 0.05 level.

Glutathione peroxidase participate directly and indirectly in decrement oxidative stress and xenobiotic levels in smokers as shown in figure (3-2).

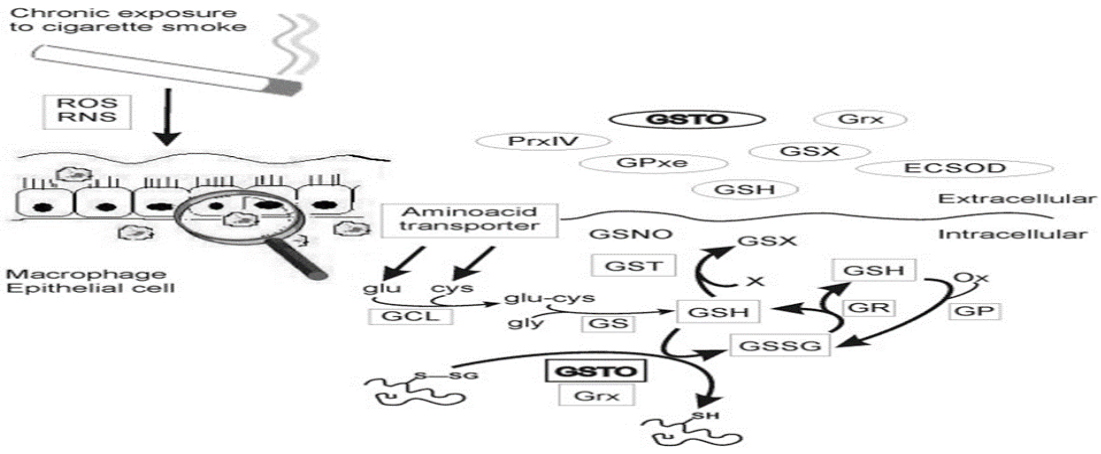
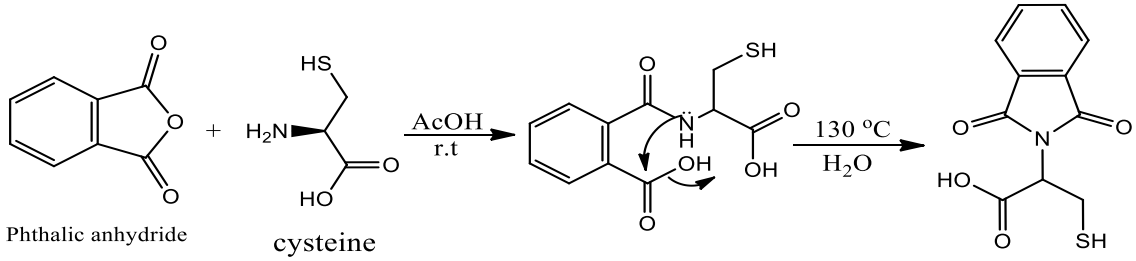


Figure (3-2) glutathione peroxidase play a role in oxidative damage caused by cigarette smoke. Protecting the lungs from oxidative damage caused by cigarette smoke requires enzymes that maintain GSH homeostasis. GSTO is a member of the Glutathione-S-transferase (GST) family, which uses GSH to detoxify hazardous substrates found in tobacco smoke Modified from **[**239**]**

3.5. Synthesis of compound 2-(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid [A]:

Compound [A] was prepared through direct addition-elimination mechanism by using equal amount of cysteine and phthalic anhydride in good yield [70 %] as yellowish white precipitate, mp. 20 °C.



Scheme 1: general equation of preparation 2-(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid

The prepared compound was characterized by FT-IR and ^{1}HNMR spectroscopies as following:

The FT-IR spectrum of prepared compound (figure 3.3) shows different bands as follow: 3523-2250 cm^{-1} (COOH), 3155 and 3030 cm^{-1} (=C-H, aromatic), 2950 and 2918 cm^{-1} (C-H, aliphatic), 2571 cm^{-1} (-SH), 1703 (C=O, phthaleimide), 1613-1457 cm^{-1} (C=C, Aromatic), 1387 (C-N).

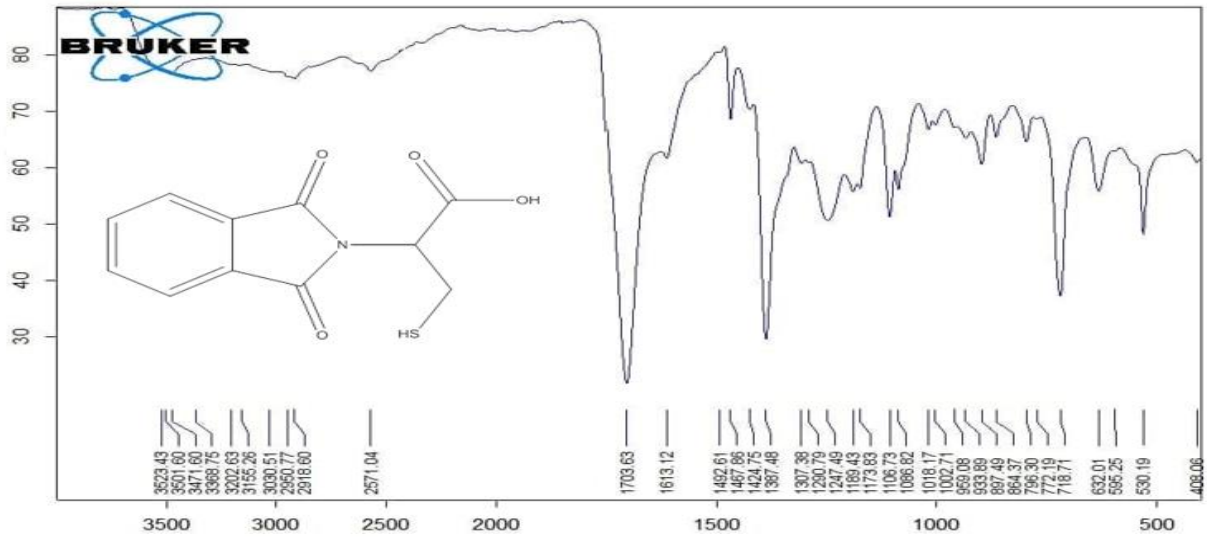


figure 3.3: FT-IR spectrum of new compound [-(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid]

The ^{1}HNMR spectrum of the prepared compound (Figure 3.4) shows differents chemical shifts for diferent protons as follow : 1.488 ppm (S,IH,-SH), 3.411 and 3.539 ppm (dd,2H, b protons, J =5.5 Hz), 4.862 and 4.883 ppm, (dd, IH,c proton, J= 4.5 Hz), 7.857 – 7.984 ppm (m, 4H, fused benzene ring), 11.948 ppm (s, IH, COOH).

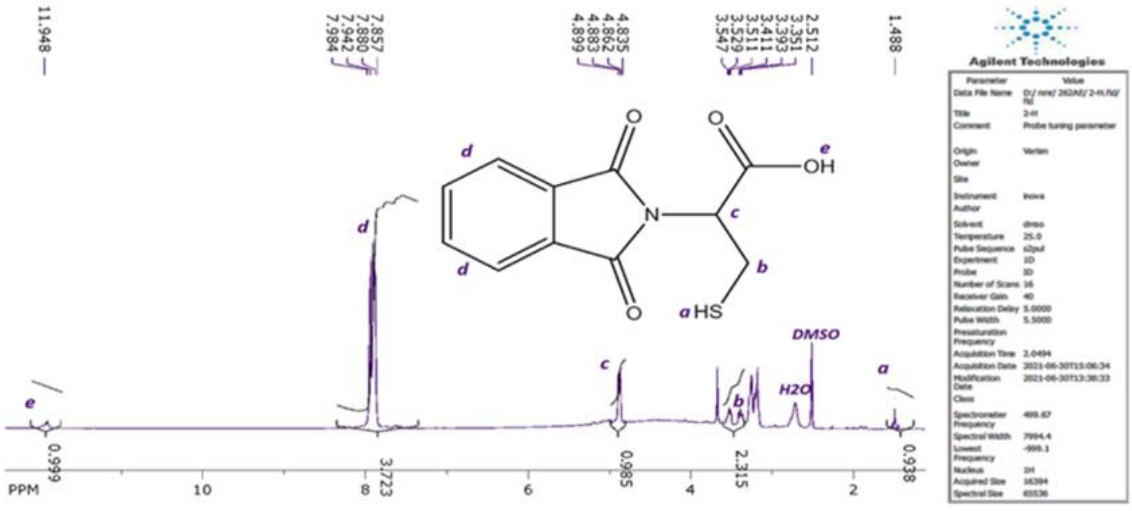


Figure 3.4 : ^{1}HNMR(500 MHz, DMSO,δPPm) of new compound [-(1,3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid]

3.6. Glutathione peroxidase mimics activity

Table (3-6) was shown the activity of glutathione peroxidase mimics activity

Table 3 -6: Glutathione Peroxidase Mimics Activity

Solution	Volume of Solution a	Volume of Solution b	Glutathione Peroxidase Mimics Activity U/L
S_0	10ml	----	---
S1	9ml	1ml	32
S2	8ml	2ml	68
S3	7ml	3ml	177
S4	6ml	4ml	201
S5	5ml	5ml	300
S6	4ml	6ml	223
S7	3ml	7ml	155
S8	2ml	8ml	76
S9	1ml	9ml	24
S10	----	10ml	----

3.7. Genotyping of Glutathione peroxidase:

The human GPx-1 gene is found on chromosome 3p21.3, where a polymorphism of the cytosine-to-thymine (C>T) substitution (rs1050450) occurs at codons 198 and197, resulting in Pro198Leu and Pro197Leu variatio The Leu variation has been linked to a 40% reduction in GPx-1 activity and an increase in tumor susceptibility [240].

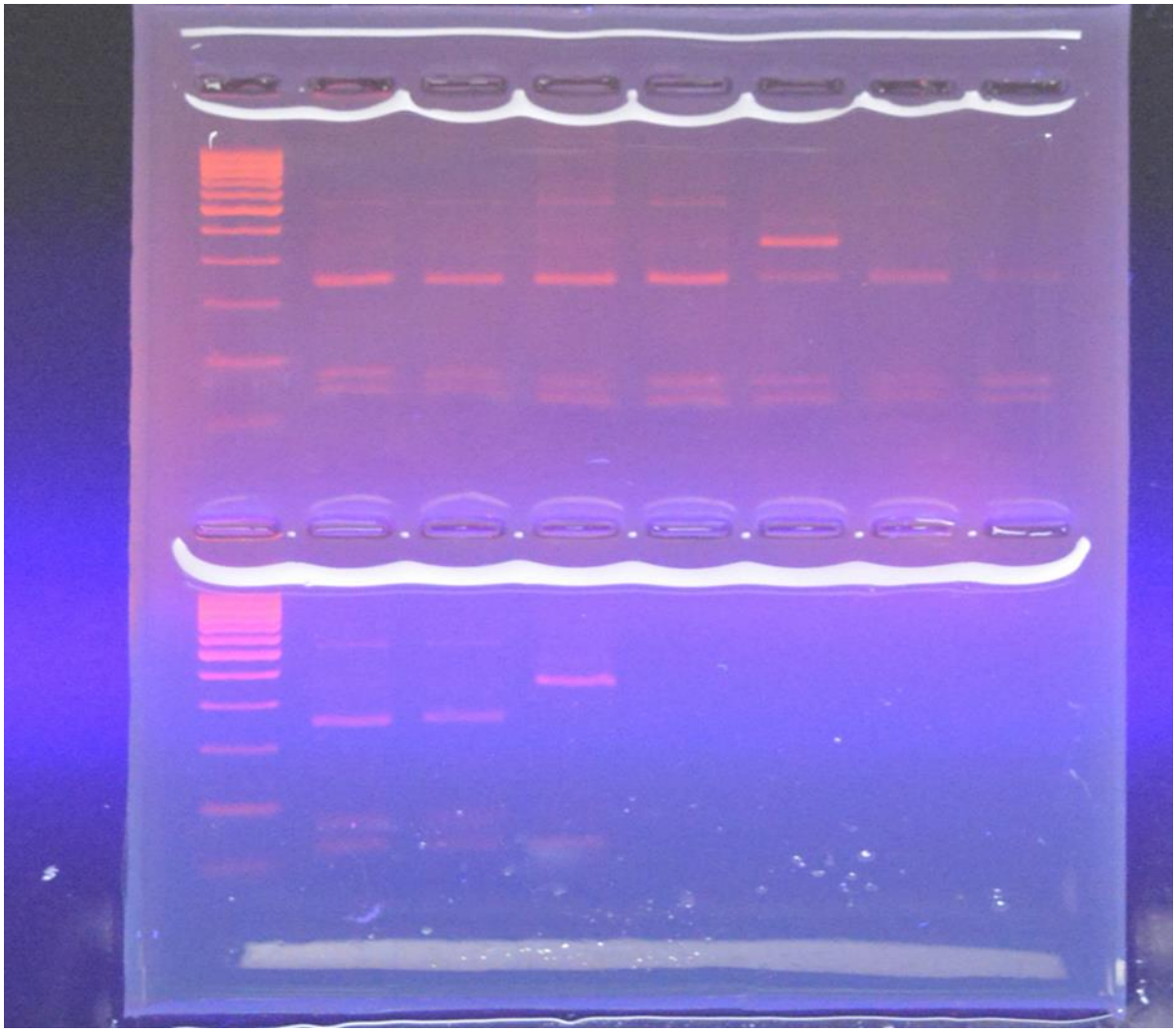


Figure (3-5): The electrophoresis pattern of PCR product for Glutathione peroxidase gene.

Extracted DNA Quantity and Quality Assessment The extracted DNA was assessed by spectrophotometry method, (scan drop, analytical jena, Germany) sample quantity were ranged from 35-56 ng/µl and the quality (260/280 ratio) ranged from 1.7-1.85, each sample that retrieve a 260/280 ratio lesser than 1.7 would be re extracted, on the other hand the extracted DNA was checked by 1% agarose gel electrophoresis

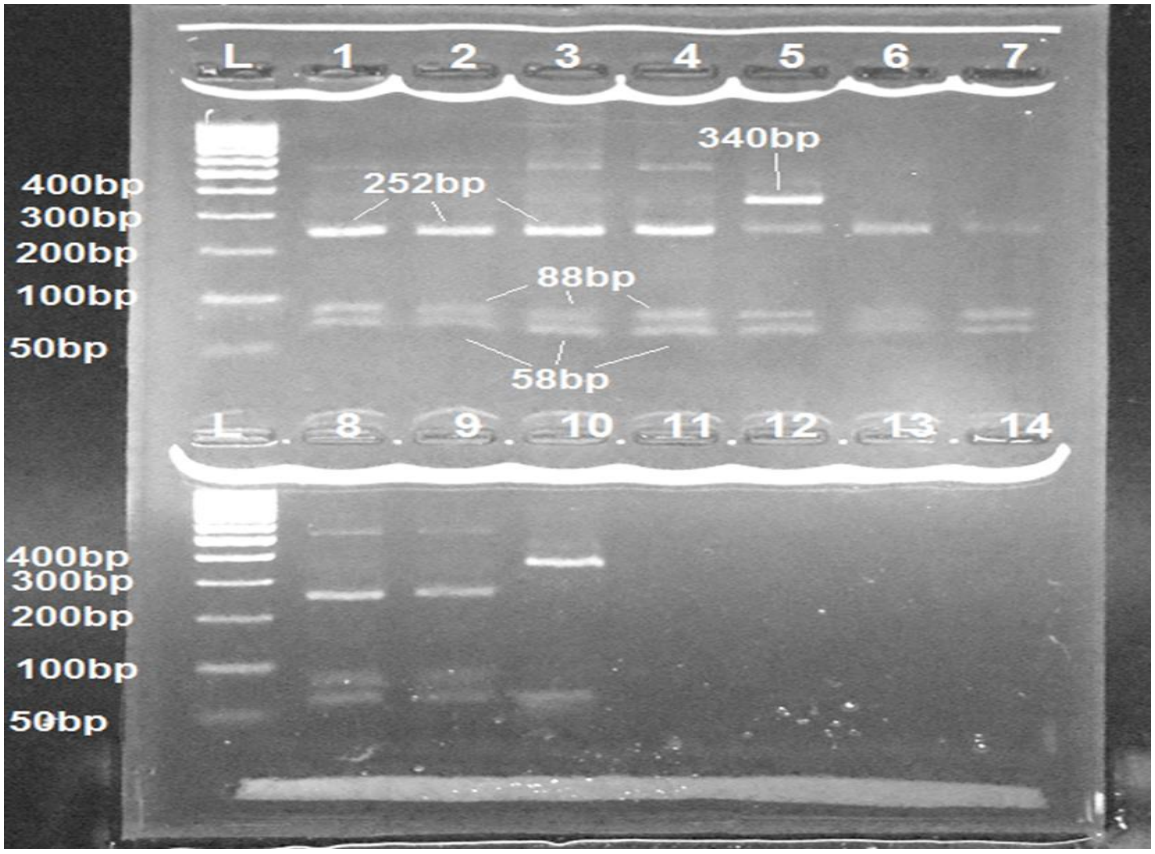


Figure (3-6): Glutathione peroxidase gene RFLP patterns electrophoresed on polyacrylamide gel electrophoresis.

As a positive check for restriction enzyme, the (58) and (88) bp length fragments were used. In diabetes patients and healthy controls, the genotype distribution and allele frequencies of the glutathione peroxidase rs1050450 (pro198leu) polymorphisms are presented in Table (3-7). For rs1050450 (pro198leu) polymorphisms, genotype frequencies were in HWE in both patients and healthy controls; there was a Hardy Weinberg equilibrium, which demonstrated the principle stating that genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors, such as mutations, which disrupt the equilibrium of allele frequencies by introducing new alleles

. **Table (3-7) Allelic frequency of rs1050450 (pro198leu) polymorphisms and their associations with risks of cigarette smokers and non-smokers Groups.**

	All subjects		non-smokers		cigarette smokers	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
A	114	0.19	60	0.2	54	0.18
G	486	0.81	240	0.8	246	0.82

The rs1050450 (pro198leu) polymorphisms showed a statistical difference (Table 3-8). (Table 3-8)

Table (3-8) Genotypic association of rs1050450 (pro198leu) polymorphisms in cigarette smokers and non-smokers Groups.

Model	Genotype	non-smokers	cigarette smokers	OR (95% CI)	p-value
Codominant	G / G	108 (72 %)	102 (68 %)	1.00	0.0033
	A / G	24 (16 %)	42 (28 %)	1.85(1.05-3.28)	0.0033
	A / A	18 (12 %)	6 (4 %)	0.35(0.13-0.92)	0.0033
Dominant	G / G	108 (72 %)	102 (68 %)	1.00	0.45
	A/G-A/A	42 (28 %)	48 (32 %)	1.21(0.74-1.98)	0.45
Recessive	G/G-A/G	132 (88%)	144 (96 %)	1.00	0.0091
	A/A	18 (12 %)	6 (4 %)	0.31(0.12-0.79)	0.0091
Over dominant	G/G –A/A	126 (84%)	108 (72 %)	1.00	0.012
	A/G	24 (16 %)	42 (28 %)	2.04(1.16-3.59)	0.012

Table (3-9) Hardy-Weinberg equilibrium test of rs1050450 (pro198leu) polymorphisms and their associations in cigarette smokers and non-smokers Groups.

	GG	AG	AA	G	A	P-value*
All subjects	210	66	24	486	114	< 0.0001
V2=1C	108	24	18	240	60	< 0.0001
V2=2S	102	42	6	246	54	0.58

Table (3-10) Variations of lipid profile, two tobacco biomarkers, glutathione peroxidase The rs1050450 (Pro198Leu) genotype frequencies according to smoking status.

	Smokers (n = 150)	**Nonsmokers (n = 150)**
Age (years)	**20 ± 2**	**20 ± 2**
BMI (kg/m2)	**21 ± 2.2**	**22 ± 1.5**
TAO (µmol/l)	**0.68902 ± 0.2019**	**0.72907 ± 0.205555**
ROS (µmol/l)	**14.9548 ± 5.671301**	**12.627 ± 4.335015**
Lp(a) (µmol/l)	**1.2207 ± 0.47382**	**1.1265 ± 0.28165**
GPX activity (IU/L)	**190.92 ± 74.01**	**206.33 ± 56.05**
GPX P198L		
pp, n (%)	**102 (190.765)**	**108 (213.419)**
pL, n (%)	**42 (195,64)**	**24 (195.635)**
LL, n (%)	**6 (160.54)**	**18 (178.084)**

Table (3-11) Effect of glutathione peroxidase rs1050450 (pro198leu) polymorphism activity on (Lipid Peroxidation, Total Antioxidant and Total Oxidant Status) concentration according to smoking status

Parameter	GPX P192L genotype	Smokers	Nonsmokers
GPX activity (IU/L)	PP	190.765 ± 72,63784	213.419 ± 55.05873
	PL	195.64 ± 76.67108	195.625 ± 56.18272
	LL	160.54 ± 75.6221	178.084 ± 48.55876
	P	0.033	0.021
TAO	PP	0.68902 ± 0.2019	0.72907 ± 0.205555
	PL	0.63429 ± 0.196458	0.67417 ± 0.2051
	LL	0.59167 ± 0.203805	0.76833 ± 0.270868
	P	0.01	0.17
ROS	PP	14.9548 ± 5.671301	12.627 ± 4.335015
	PL	15.059 ± 6,208677	12.9033 ± 4.754659
	LL	14.2867 ± 5.689651	12.1339 ± 4.646232
	P	0.15	0.6

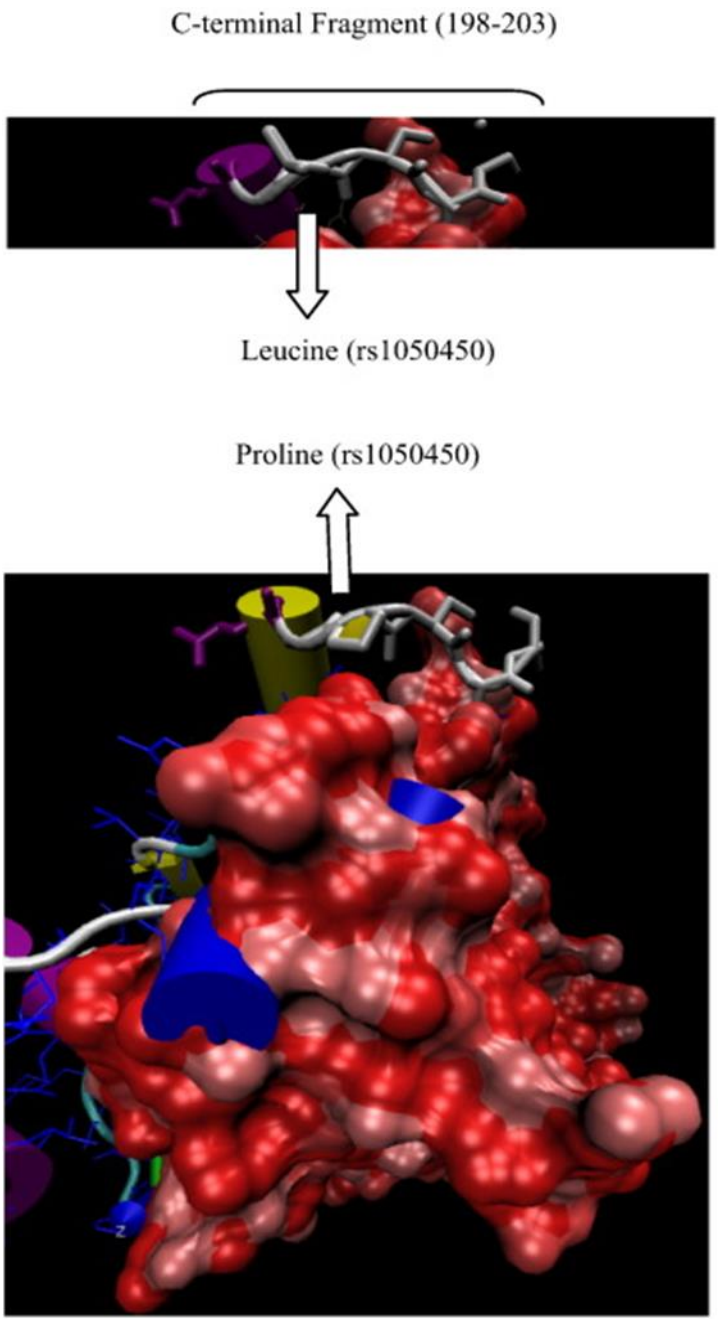


Figure. (3- 7) Gpx1 structure as predicted. [180]

(A) Leucine variation in the C-terminal segment. (B) Complete tertiary structure with conserved section (space-filling model, fragment 16–130aa) and C-terminal fragment (fragment 198–203 aa) including Proline variation [180]

Tobacco smoke contains a variety of carcinogens [241]. The great majority of lung cancer cases are caused by tobacco usage. Dietary variables and polymorphisms in genes involved in metabolism, ant oxidative defense, and DNA repair could help explain why some smokers acquire lung cancer and others do not. The selenium-dependent enzyme GPX1 is encoded by GPX1 and is found in epithelial tissues of the lung and other organs [242,243]. By detoxifying hydrogen-and lipid peroxides, the enzyme is part of the enzymatic antioxidant defense that prevents oxidative damage to DNA, proteins, and lipids [244]. In a recent research of male smokers, a polymorphism in the GPX1 gene (GPX1 Pro198Leu, rs1050450) was linked to lung cancer risk [245]. GPX1 activity is increased when selenium is consumed in the diet [246]. Reactive chemical exposure can set off a chain reaction of cellular oxidative stress reactions, including the activator of activated B cells, nuclear factor kappa-light-chain enhancer, and cytokine or chemokine-mediated signaling [247]. The oxidative damage to cellular components that results has been connected to aging [248], chronic diseases such as asthma, cardiovascular disease [254], and hypertension [250], among others. Inflammatory and oxidative stress responses are induced by pro-oxidant and electrophilic compounds in pollutant mixes from fossil fuel burning, according to toxicological studies [251]. Conjugated carbonyl chemicals, such as -unsaturated carbonyls, 1,2-dicarbonyls, and quinones, are toxicologically important because they can cause oxidative stress and cellular damage by covalently interacting with nucleophilic activities in proteins [252]. In contrast to carcinogenic PAHs, N-nitrosamines, and dioxins, reactive organic electrophilic chemicals do not require metabolic activation and can immediately react with proteins or produce mutagenic DNA adducts by

attaching covalently to nucleic acids [253]. The electrophilic, -unsaturated aldehyde acrolein, for example, is regularly identified in substantial amounts in the volatile part of diesel exhaust and cigarette combustion products [254].

Electrophilic chemicals are attracted to electrons and can use covalent bonding to inactivate the nucleophilic active sites of thiolate or selenocysteine enzymes like GPx-1. We recently discovered that diesel particles and ultrafine particles (less than 100 nm in diameter) collected in the Los Angeles area caused the thiol (cysteine) enzyme glyceraldehyde-3-phosphate dehydrogenase to become irreversibly inactive (GAPDH). [255]. Smoking is the primary source of acrolein in humans, as evidenced by a five-fold rise in urinary (3-hydroxypropyl) mercapturic acid (3-HPMA) levels in smokers compared to non-smokers and a 78 percent drop in median 3-HPMA levels following smoking cessation [256]. As a result, while the synthesis of these conjugates may be an important detoxifying mechanism, they may also disrupt the cellular redox equilibrium by causing GSH depletion via acrolein [257]. Acrolein has also been shown to generate two main DNA adduct isomers, -OH-Acr-dG and -OH-Acr-dG, respectively [258]. Both Acr-dG adducts are mutagenic and have been discovered in human lung tissues from smokers [253]. The antioxidant selenoenzyme GPx-1 protects cells from oxidative stress by catalyzing the reduction of hydrogen peroxide or organic hydro peroxides to water or the equivalent alcohols (R-OH) (Fig. 3-8). The selenol group in the enzyme's active site quickly ionizes at physiologic pH to the reactive selenolate, which is extremely vulnerable to electrophilic assault [255] due to its low pKa value. As a result, electrophile-derived covalent alterations of seleno enzymes implicated in the cytosolic defense against reactive oxidant and

nitrogen species may play a role in air pollutants' capacity to cause oxidative stress [255].

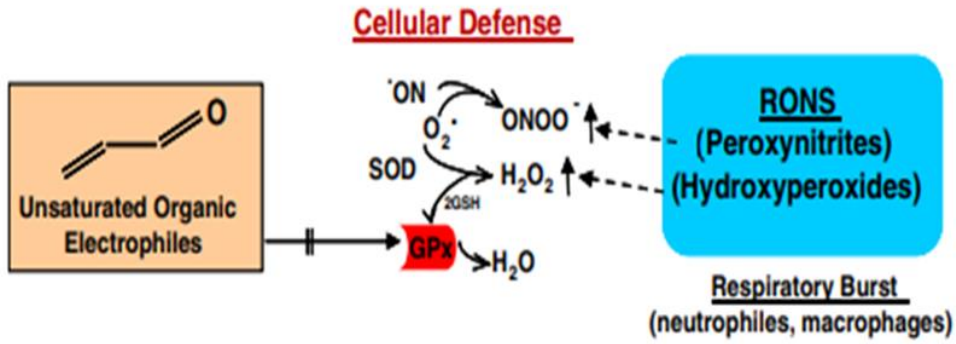


Figure. (3-8) In the cytosolic defense against RONS, GSH peroxidase (Gpx) and Cu/Zn-SOD are connected**.** The dismutation of superoxide to oxygen and hydrogen peroxide is catalyzed by Cu/Zn-SOD (H_2O_2). After that, the selenoenzyme Gpx reduces H_2O_2 and other hydro peroxides. Catalase, glutathione transferase, and reduced GSH are all part of a complex cellular antioxidant system that includes Gpx and Cu/Zn-SOD (not shown). Exposure to reactive electrophiles in the environment may increase the endogenous burden of oxidative stress by inactivating Gpx directly

3.8. A validated method to assess glutathione peroxidase enzyme activity

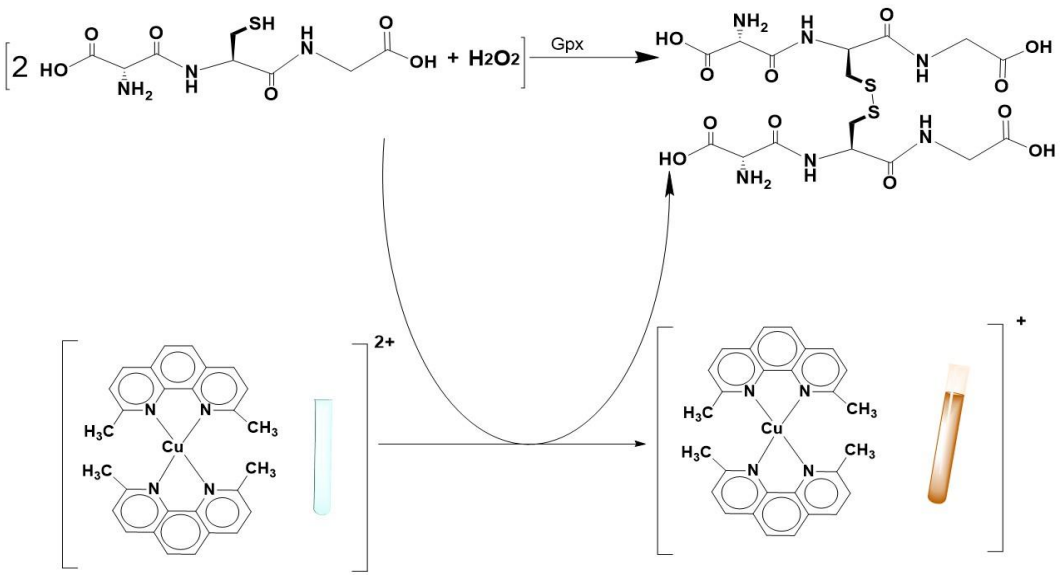
3.8.1. Results and Discussion

CUPRAC reagent as a suitable probe to measure Gpx activity [the Gpx-CUPRAC method]

By using the cupric neocuproine complex ($Cu(Nc)_2^{2+}$) as a suitable chromogenic oxidising probe, the present work explains a basic procedure to assay Gpx activity in biological samples (CUPRAC method). Apak et

al. [259] introduced the CUPRAC method to calculate antioxidant capacity. To establish Gpx activity, the enzyme samples were incubated with phosphate buffer, which contains suitable concentrations of the glutathione and peroxide as substrates. To stop the enzyme's reaction, the CUPRAC reagent ($Cu(Nc)_2^{2+}$) was added after a suitable incubation time.

Reducing the Cu(II)-neocuproine ($Cu(Nc)_2^{2+}$) complex to coloured Cu(I)-neocuproine ($Cu(Nc)_2^{+}$) complex by the unreacted substrates was quantified spectrophotometrically at 450 nm (CUPRAC method), as shown in Scheme 1. The decrease of absorbance of coloured Cu(I)-neocuproine ($Cu(Nc)_2^{+}$) complex correlated with the Gpx activity. The Gpx enzyme reaction formed the Cu(I)-neocuproine ($Cu(Nc)_2^{+}$) complex, which had a single peak at 450 nm. The absorbance was specifically associated with the unreacted substrates (glutathione and peroxide), as shown in Figure 3-9



Scheme 1: Glutathione peroxidase uses peroxide to convert reduced glutathione (GSH) to oxidised glutathione (GSSG). Cu(II)-neocuproine complex reacts with unreacted substrates (glutathione and peroxide) to produce yellow-orange Cu(I)-neocuproine stable complex.

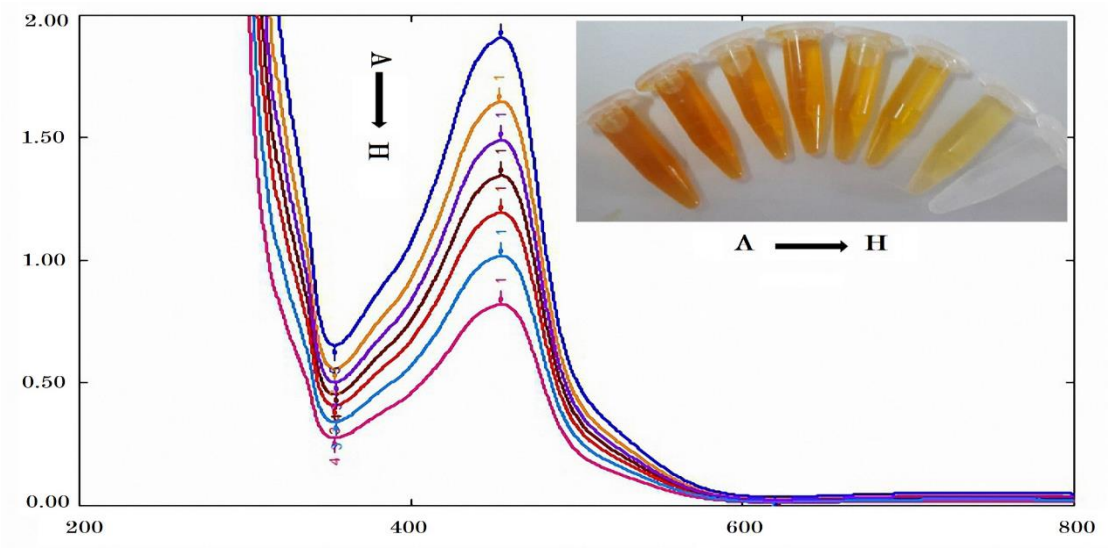


Figure. 3-9. Glutathione enzyme activity correlated inversely with the intensity of the formed Cu(I)-neocuproine ($Cu(Nc)_2^+$) complex.

Absorption spectra was achieved by reducing ($Cu(Nc)_2^{2+}$) to coloured Cu(I)-neocuproine complex ($Cu(Nc)_2^+$) as a result of adding 1 ml of the solution prepared by mixing a suitable concentration of glutathione (GSH) with peroxide (H_2O_2). (a) to (h) represent {(800/400), (700/350), (600/300), (550/275), (500/250), (450/225), (400/200)} μM of (GSH/H_2O_2).

3.8.2. Optimising the Gpx-CUPRAC assay

To achieve the optimum conditions, statistical methods were adapted with the Box–Behnken design (BBD) [260]. To optimise the Gpx-CUPRAC assay, BBD is an important measuring method with three central points to optimise glutathione, peroxide, and neocuproine concentrations to achieve optimal Gpx activity (see Table 3-12). The regression model for the Gpx-CUPRAC assay was determined using the analysis of variance (ANOVA) of the response surface methodology (RSM). The model's F-value (12.82) showed that it was significant, while the lack-of-fitness F-value (2.8405) showed that it was not significant as compared to the related p-value. The significance of model terms was proved by obtained p-value (p= 0.0019).

The adjusted response (Adjusted R^2 = 0.9918) was in acceptable agreement with the coefficient predicted response (Predicted R^2 = 0.9482). As a result, the Gpx-CUPRAC assay's ANOVA showed that the specific correlation between the independent variables of the proposed model was appropriate for description and highly significant.

To investigate the graphical results of the independent variables, contour diagrams and three dimensional (3D) of the BBD were used. When the third factor was constant, the creation of 2D and 3D graphs at the midpoint stage was based on a combination of two variables. In the response plot in Fig. 3-10 a–f, the relationships between the variables (glutathione, peroxide, and neocuproine concentrations) are depicted. All the figures exhibited good significant curvature. Gpx activity was optimum at 4 mmol L^{-1} glutathione, 2 mmol L^{-1} peroxide, and 7.5 mmol L^{-1} neocuproine concentrations. The actual activity level was 500 U/L. The actual value was consistent with the predicted value, indicating that the RSM investigation was reliable and well-suited to experimental conditions.

Table 3-12. ANOVA values for the Gpx-CUPRAC assay's experimental variables.

	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Regression	1.9322e+03	9	2.1469e+03	12.8227	0.0019
Residual	1.1720e+03	7	167.4265		
Lack-of-fit	1.0273e+03	5	205.4637	2.8405	0.2806
Pure error	144.6667	2	72.3333		
Total	2.0494e+04	16			
R^2	0.9428				
Explainable R^2	0.9918				

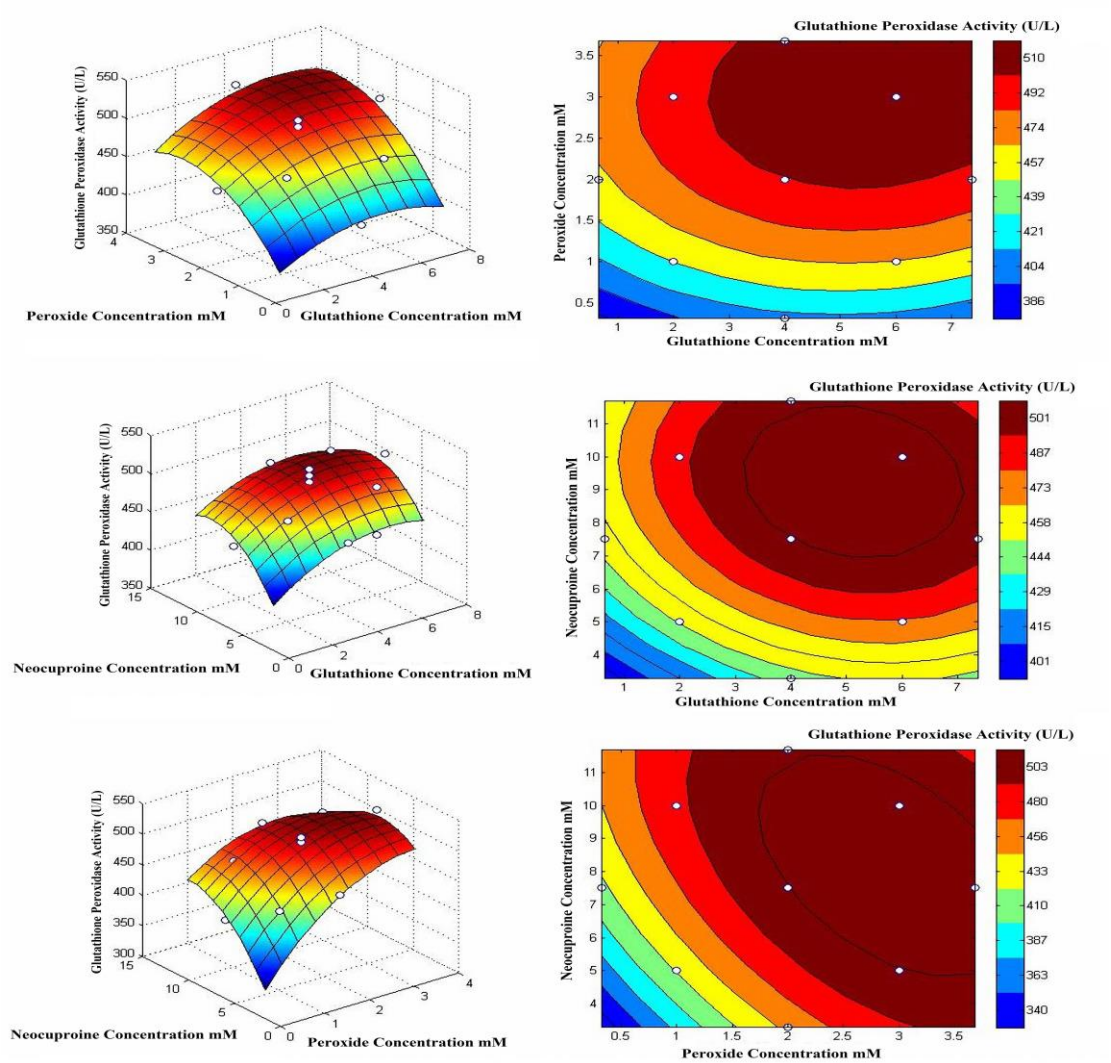


Figure. 3-10: 3D surface plot graphs and contours, demonstrating the interactions between glutathione concentration, peroxide concentration, and neocuproine concentration. The relationships between the variables (glutathione, peroxide, and neocuproine concentrations) are depicted in (a) through (f).

3.8.3. Signal stability

The coloured complex in the current study was remarkably stable at room temperature. At 25 °C, the CUPRAC complex's 450 nm absorbance remained remarkably stable for more than a week.

3.8.4. Linearity and sensitivity

According to the results shown in Fig (3-11), the Gpx-CUPRAC method was linear (y = 1.0006 x) within the range of 2–1000 $U.L^{-1}$ of Gpx activity

(Pearson's r = 0.999). The LOQ (1 $U.L^{-1}$) and LOD (3 $U.L^{-1}$) values demonstrated the high sensitivity of the Gpx-CUPRAC assay. The linearity of the new method was like that of the Gpx-DTNB assay.

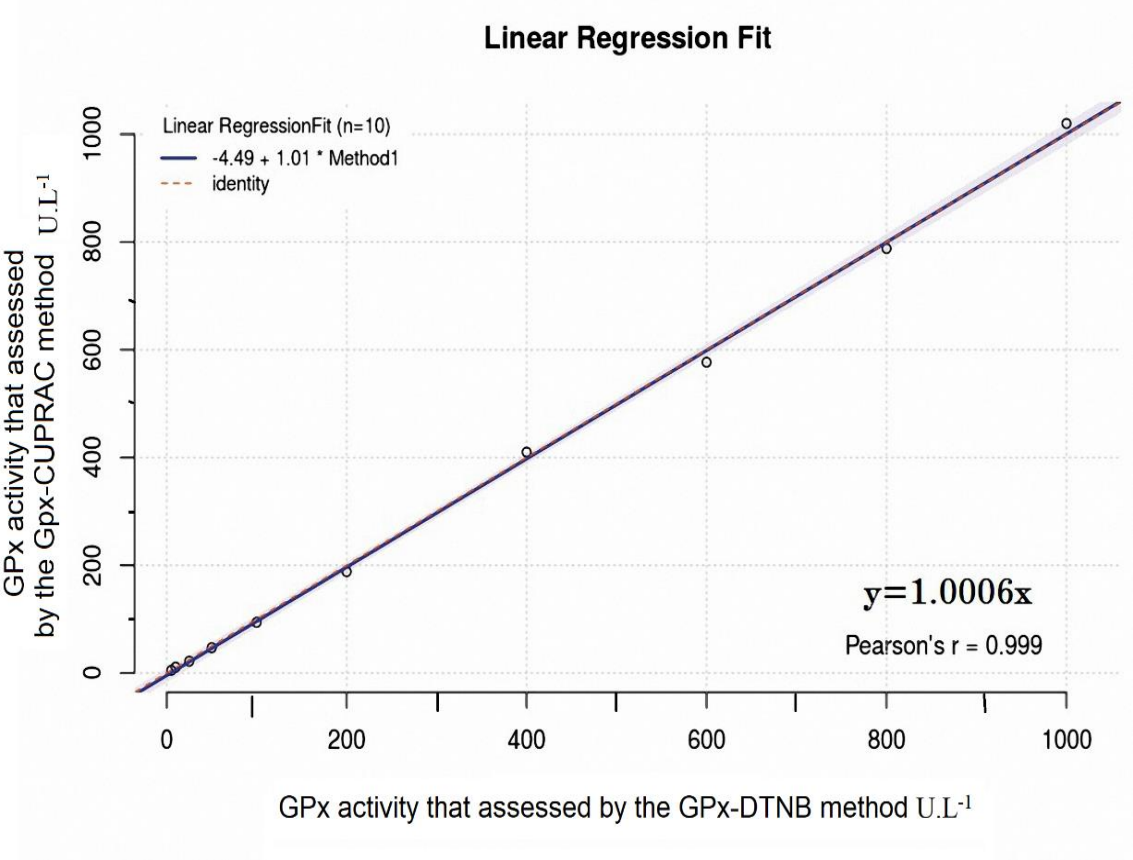


Figure (3-11): The Gpx activity of diluted tissue homogenates that were obtained by using the Gpx-CUPRAC method compared to the values achieved using the Gpx-DTNB method.

3.8.5. Selectivity, reproducibility, and accuracy of the Gpx-CUPRAC method

The findings in Table (3-13) showed that the analysed biomolecules cannot interact with the Gpx-CUPRAC assay. The current method differs from previous methods for determining Gpx activity because it does not interfere with biomolecules. The current assay employs a control test tube to eliminate interference caused by organic biomolecules in the sample containing Gpx enzyme activity. In the current procedure, the absorbance

of the test tube belongs to two kinds of compounds: unreacted substrates and sample interferences. The absorbance of the control test tube corresponds to interference compounds only. We excluded the interference of any compound that could reduce Cu(II)-neocuproine complex ($Cu(Nc)_2^{2+)}$ to coloured Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$) by subtracting the absorbance of the control test tube from the absorbance of the test tube. That means the remaining absorbance was exclusively for unreacted substrates.

Table 3-13: By using the Gpx-CUPRAC assay to assess Gpx activity, there is a relationship between relative percentage error and interfering biological interferences.

	Added Gpx U/l	Found Gpx U/l	Relative error (%)
Volumetric flask 1	300	300	0.00
Volumetric flask 2	300	302	0.66
Volumetric flask 3	300	303	1.0
Volumetric flask 4	300	305	1.66

The Gpx-CUPRAC assay was used to determine the Gpx activity of homogenates of liver tissue. The findings revealed that Gpx activity was elevated as predicted in liver tissue homogenates (Fig. 3-12). The Gpx-CUPRAC method demonstrated reasonable inter-day (RSD% = 2.2.8%–3.2%) and intra-day (RSD% = 2.7%–3.8%).

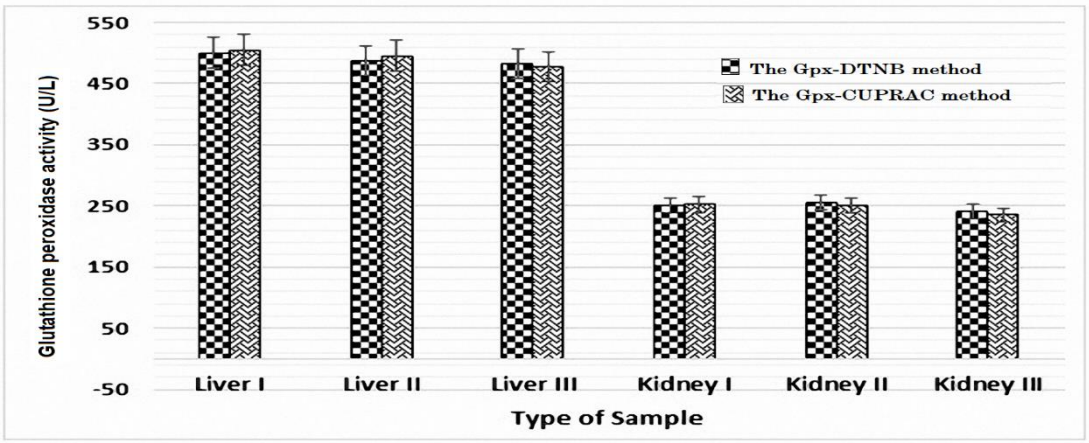


Figure. 3-12: Comparison of Gpx activity of diluted tissue homogenates that were obtained by the Gpx-CUPRAC method and the Gpx-DTNB method.

Gpx activity assessment is a useful parameter for evaluating the liver's ability to reduce the susceptibility to oxidative stress. Furthermore, several scientific experiments have focused on Gpx activity in the livers of some types of lab animals to assess the oxidative stress inclination [261, 262].

3.8.6. Validation

Using matched enzymatic samples, Bland–Altman plot analyses (QI Macros, 2016) were used to compare the Gpx activity assessed by the present method with the Gpx activity assessed by the Gpx-DTNB process [263]. Bland–Altman plot shows the relative differences between Gpx-CUPRAC and Gpx-DTNB methods, as well as the mean relative bias (Fig. 3-13). The correlation coefficient between the two protocols was 0.9994. This means that the new protocol is almost as accurate as the reference protocol. The comparison between the Gpx-CUPRAC method and the Gpx-DTNB method using the Passing–Bablok similarity analysis showed a good agreement correlation (Fig. 3-14).

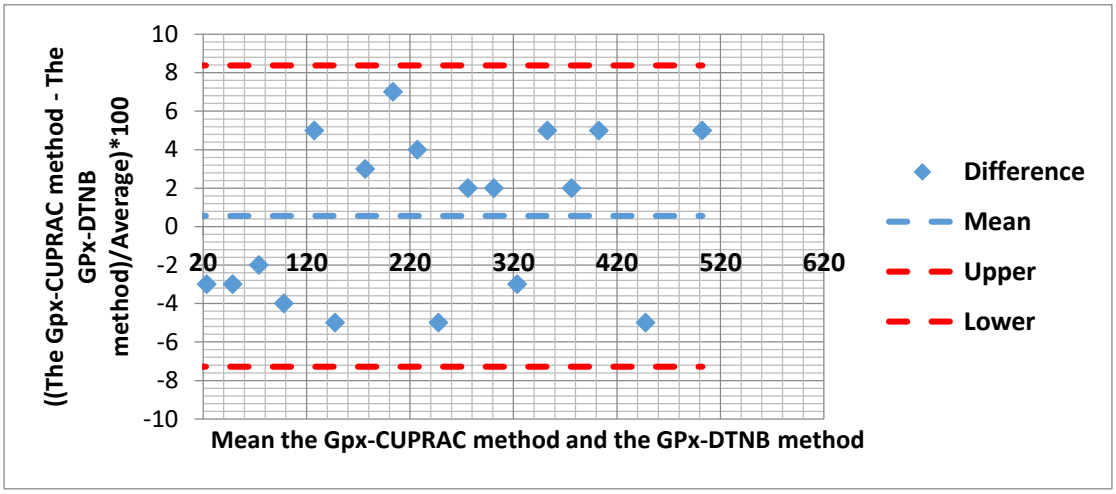


Figure. 3-13: Bland–Altman plot shows the relative differences between the Gpx-CUPRAC and GPx-DTNB protocols, as well as the mean relative bias.

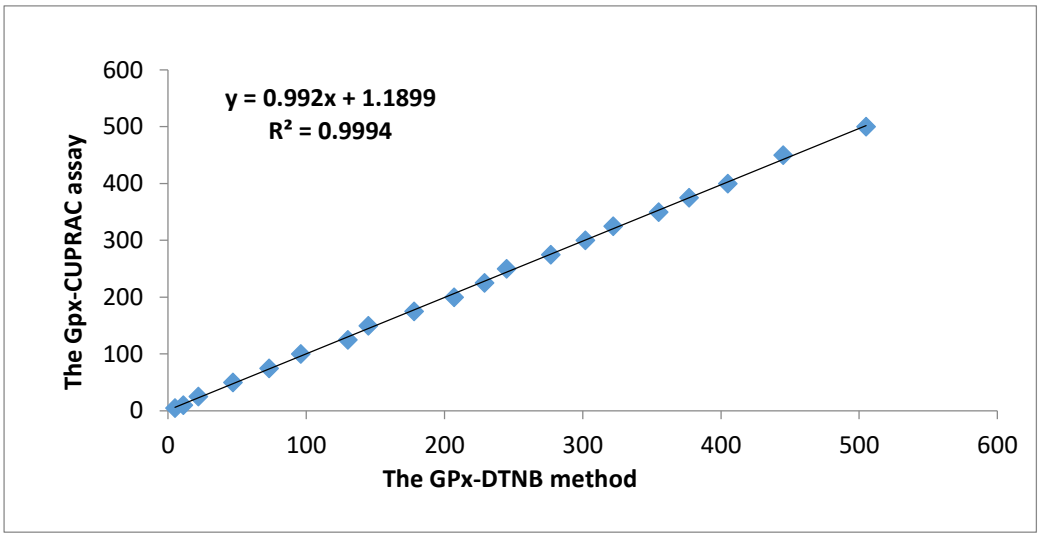


Figure. 3-14: Gpx activities were measured using the Gpx-CUPRAC and Gpx-DTNB methods over a series of Gpx dilutions.

The current method has numerous advantages over the method reported by Ugar et al. [264]. The current protocol is compatible with all previous methods in the use of sodium azide to inhibit and halt catalase enzymatic reaction completely; however, in the method reported by Ugar et al. [264], catalase and Gpx compete for the hydrogen peroxide substrate because sodium azide is not used to rule out catalase interference. Catalase certainly dominates because it has a considerably higher catalytic

efficiency than Gpx. Catalase has the highest turnover numbers of all enzymes. The Braunschweig Enzyme Database reports that one molecule of catalase can convert millions of hydrogen peroxide molecules into water and oxygen per second [265]. Catalase has a catalytic efficiency (k_{cat}/K_m) of 2.1×10^7 [266], whereas Gpx has a catalytic efficiency (k_{cat}/K_m) of 1.39×10^7 [267], i.e. catalase will overwhelm Gpx in breaking down the common substrate (hydrogen peroxide).

The trichloroacetic acid (TCA) precipitation of proteins is commonly used to concentrate protein samples or remove contaminants, including salts and detergents, prior to biochemical applications. TCA precipitation denatures proteins [268] to stop all enzymatic reactions. Raja lingam et al. [269] clearly demonstrated that TCA-induced protein precipitation is independent of the size and nature of proteins. The trichloro moiety is important for the protein precipitation capability of TCA. TCA-induced protein precipitation involves the reversible association of a stable partially structured intermediate [269].

The current protocol is well suited to quantify the Gpx-like activity of Gpx mimics. Some modifications must be made to ensure obtaining results with high reliability. The incubation time should be increased to 20 min, and the Gpx mimics should be dissolved in phosphate buffer (pH 7). The use of TCA is unnecessary because the enzymatic reaction solution does not contain protein compounds.

Conclusion

It is concluded that: -

1-An organic compound with the same properties and efficacy as glutathione peroxidase was prepared.

2-The described method for assessments of Glutathione peroxidase activity that show high precision and accuracy in the presence of high concentrations of several types of biomolecules, and at low H_2O_2 concentrations.

3-Smoking complications associated with decrement Glutathione peroxidase levels, Glutathione peroxidase activity and antioxidants concentration.

4- Smoking complications are associated with increment reactive oxygen species concentration and decrement total antioxidant levels.

5- Smoking complications are associated with inrement lipid peroxidation.

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الخلاصة

يرتبط دخان السكائر بعدد من المشكلات الصحية الرئيسية، بما في ذلك سرطان الفم وسرطان الحنجرة والسكتة الدماغية وسرطان الكبد وسرطان المثانة. دخان السكائر عباره عن مزيج معقد من حوالي 4000 ماده كيميائية.

يوجد نوعين رئيسيين من الكلوتاثيون بيروكسيديز يتميز احد الأنواع باحتوائه على السلينيوم في شكل سلينيوسستين المرتبط تساهميا في موقعه النشط، اما النوع الثاني من الكلوتاثيون بيروكسيديز يتكون من بروتينات لا تعتمد على السلينيوم في التحفيز ولها نشاط ضئيل مع (H_2O_2) .

رمز الانزيم المعتمد على السيلينيوم GPX1 ويوجد في الأنسجة الظهارية مثل الرئة والأعضاء الأخرى. ومن خلال ازاله السموم (بير وكسيدات الهيدروجين والدهون)، يعد الانزيم جزء من الدفاع الانزيمي المضاد للأكسدة الذي يمنع الضرر التأكسدي للحامض النووي والبروتينات والدهون. يمكن ان يؤدي التعرض للتفاعل الكيميائي الى إطلاق تفاعل متسلسل لتفاعلات الاجهاد التأكسدي الخلوي، بما في ذلك المنشط للخلايا B، المنشطة والمحسنة للعامل النووي، والاشارات الأخرى.

متشابهات الكلوتاثيون بيروكسيديز: وهي مركبات السلينيوم العضوية والتي لها نفس المجموعة الفعالة الموجودة في انزيم الكلوتاثيون بيروكسيديز.

تم اجراء الدراسة في جامعة بابل في عام 2021. تم سحب عينات الدم من طلاب الجامعة وأجريت الدراسة الحالية على مجموعتين: الأولى 150 شخصا من الغير مدخنين، الثانية 150 شخصا من المدخنين للسكائر.

تم قياس تركيز كل من (((TAS),(TOS)) للأشخاص المشاركين . كان تركيز المواد المؤكسدة لمدخني السكائر (15.211 ميكرو مول/لتر) اعلى بكثير من تركيز غير المدخنين (12.775 ميكرو مول/لتر)، بينما كان تركيز مضادات الأكسدة في مصل الدم لمدخني السكائر (0.6850 مكر ومول/لتر) اقل بكثير من تركيزها في غير المدخنين (0.7429 مكر ومول/لتر) تشير النتائج الى الاجهاد التأكسدي وضعف النظام الدفاعي للمدخنين.

قياس تركيز بيروكسيد الدهون في مصل الدم لدى غير المدخنين (1.1265 ميكرو مول/لتر) ومدخني السكائر (1.2207 ميكرو مول/لتر) تشير الدراسة الحالية الى الزيادة المعنوية في تركيز Malondialdehyde في مجموعة مدخني السكائر، مقارنة بمجموعة غير المدخنين. تم معرفة احتواء دخان

السكائر على العديد من الجذور الحرة وغيرها من الجزيئات شديدة التفاعل فسيؤدي التركيز المتزايد لهذه الجزيئات التفاعل في الأنسجة الى تحفيز بيروكسيد الدهون مع إطلاق ما يصاحب ذلك من منتجات مثل MDA وهو احد منتجات اكسدة الدهون التي تم قياس تركيزها.

تم قياس فعالية انزيم الكلوتاثيون بيروكسيديز وأشارت النتائج الى انخفاض في تركيز الكلوتاثيون بيروكسيديز في مجموعه مدخني السكائر (190.92 وحده/لتر) مقارنه بمجموعه غير المدخنين (206.33 وحده/لتر). نستنتج من هذه الدراسة ان التدخين بجميع انواعه يشكل خطورة على الصحة. يمكننا استخدام النهج المقدم لاختبار فعالية الكلوتاثيون بيروكسيديز بدقة عالية في وجود أنواع متعددة من الجزيئات الحيوية وتركيز منخفض من (H_2O_2).

تم تحضير مركب عضوي (مركب A) له نفس المجموعة الفعالة في انزيم الكلوتاثيون بيروكسيديز وكانت له فعالية مشابهه لأنزيم الكلوتاثيون بيروكسيديز.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية العلوم

قسم الكيمياء

تحضير مركب متشابه جديد للكلوتاثيون:

دراسة عضوية حيوية لدور الكلوتاثيون بيروكسيديز في تنظيم حالة مضادات الأكسدة لمدخني السجائر

رسالة

مقدمة إلى مجلس كلية العلوم /جامعة بابل كجزء من متطلبات نيل درجة الماجستير في علوم/ الكيمياء

من قبل

احمد ياسر احمد حسن

بكالوريوس علوم كيمياء –جامعة كربلاء

2012

بإشراف

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2021 م **1443هـ**