T. R.
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

DETERMINATION OF ANTIOXIDANT CAPACITIES OF SOME BENZOTHIOPHENE DERIVATIVES AND THEIR INHIBITION MODELS ON GSTP1-1 ISOZYME

M.Sc. THESIS

Prepared by: Ahmed Othman PIRDAWID
Supervisor: Asst. Prof. Dr. Can YILMAZ

VAN-2019

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ACCEPTANCE and APPROVAL PAGE

This thesis entitled "**DETERMINATION OF ANTIOXIDANT CAPACITIES OF SOME BENZOTHIOPHENE DERIVATIVES AND THEIR INHIBITION MODELS ON GSTP1-1 ISOZYME**" presented by Ahmed Othman PIRDAWID under supervision of Asst. Prof. Dr. CAN YILMAZ in the Department of Molecular Biology and Genetics has been accepted as a M.Sc. thesis according to Legislations of Graduate Higher Education on 25/10/2019 with unanimity / majority of votes members of jury.

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This thesis has been approved by the committee of The Institute of Natural and Applied Science on 01/11/2019 with decision number 2019/58-1

Signature

Director of Institute

THESIS STATEMENT

All information presented in the thesis obtained in the frame of ethical behavior and academic rules. In addition all kinds of information that does not belong to me have been cited appropriately in the thesis prepared by the thesis writing rules.

Signature

Ahmed Othman PIRDAWID

ABSTRACT

DETERMINATION OF ANTIOXIDANT CAPACITIES OF SOME BENZOTHIOPHENE DERIVATIVES AND THEIR INHIBITION MODELS ON GSTP1-1 ISOZYME

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With the formula C_4H_4S thiophene is located in the class which belongs to heterocyclic compounds that are composed of five member rings, with sulphur as heteroatom. This group of organic compounds has many pharmacologically active molecules. Benzothiophenes, as sulfur containing heterocyclic family, contains highly stable aromatic compounds that exhibit important physicochemical characteristics. They show broad range of biological/pharmacological activities such as anti-inflammatory, analgesic, anti-fungal, antidepressant etc. In this thesis project, two novel benzothiophene derivatives were investigated for their antioxidant capacities by the applications of four assays (DPPH, ABTS, galvenoxyl, and phosphomolybdenum) which were all based on different operating mechanisms. Derivative-1 and Derivative-2 demonstrated different levels of antioxidant capacities with respect to universal standards of trolox, ascorbic acid, and quercetin. Derivative-2 showed 2 times more antioxidant capacity than trolox in galvinoxyl assay. Derivative-1 was comparable with, especially quercetin, in phosphomolybdenum assay. Those two derivatives were subjected for their possible inhibitory effect on human GSTP1-1, which is one of the phase II drug metabolising enzymes performing the conjugation between glutathione and a various of substrates. According to kinetic calculations, Derivative-1 applied (full) uncompetitive inhibition, and Derivative-2 generated (partial) mix inhibition on this pure enzyme. As a conclusion, those novel benzothiophene derivatives have potential to be used as antioxidant agents, after detailed medical tests. On the other hand, some more derivatization is required for them to be better inhibitors of GSTP1-1.

Keywords: Antioxidant capacity, Benzothiophene, Glutathione S-transferase P1-1, Inhibition.

ÖZET

BAZI BENZOTİYOFEN TÜREVLERİNİN ANTİOKSİDAN KAPASİTELERİNİN BELİRLENMESİ VE GSTP1-1 İZOZİMİ ÜZERİNE İNHİBİSYON MODELLERİNİN TESPİTİ

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C4H4S formülü ile tiyofen, heteroatom olarak kükürt içeren beş üyeli halkalardan oluşan heterosiklik bileşiklere ait sınıfta bulunur. Bu organik bileşikler grubunun birçok farmakolojik olarak aktif molekülü vardır. Benzotiofenler, kükürt içeren heterosiklik bir aile olarak, önemli fizikokimyasal özellikler sergileyen oldukça kararlı aromatik bileşikler içerir. Antienflamatuar, analjezik, mantar önleyici, antidepresan vb. gibi çeşitli biyolojik / farmakolojik aktiviteler gösterirler. Bu tez projesinde, iki yeni benzotiyofen türevi, antioksidan kapasiteleri için hepsi farklı çalışma mekanizmalarına dayanan dört testin uygulanmasıyla araştırıldı (DPPH, ABTS, galvenoksil ve fosfomolibden). Türev-1 ve Türev-2, trolox, askorbik asit ve kuersetin gibi evrensel standartlarına göre farklı seviyelerde antioksidan kapasiteler gösterdi. Türev-2, galvinoksil analizinde, trolokstan 2 kat daha fazla antioksidan kapasite göstermiştir. Türev-1, fosformolibden denemesinde özellikle kuersetin ile karşılaştırılabilir seviyede antioksidan kapasiteye sahiptir. Bu iki türev, glutatyon ve çeşitli substratlar arasındaki konjugasyonu gerçekleştiren faz II ilaç metabolize edici enzimlerden biri olan insan GSTP1-1 üzerindeki olası inhibe edici etkileri için test edildi. Kinetik hesaplamalara göre, Türev-1 (tam) bağımlı inhibisyon uyguladı ve Türev-2, bu saf enzim üzerinde (kısmi) karışık inhibisyon oluşturdu. Sonuç olarak, bu yeni benzotiyofen türevleri, detaylı tıbbi testlerden sonra, antioksidan ajanlar olarak kullanılma potansiyeline sahiptir. Öte yandan, GSTP1-1'in daha iyi inhibitörleri olmaları için farklı kısımlarından türevlendirilmeleri gereklidir.

Anahtar kelimeler: Antioksidan kapasitesi, Benzotiyofen Glutatyon S-transferaz P1-1, İnhibisyon.

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2019
Ahmed Othman PIRDAWID

TABLE OF CONTENT

Pages

Pages

Pages

LIST OF TABLES

Tables **Pages**

LIST OF FIGURES

Figures **Pages**

Figures **Pages**

Figures **Pages**

SYMBOLS AND ABBREVIATIONS

Some symbols and abbreviations used in this study are presented below, along with Descriptions.

Abbreviations	Description
4NQO	4-Nitroquinolone 1-oxide
AA	Ascorbic acid
ABC	ATP-binding cassette
ABTS	2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
AD	Alzheimer's Disease
AED	Antiepileptic Drugs
ATP	Adenosine triphosphate
CDNB	1-Chloro- 2,4-dinitrobenzene
$CO_2^{\bullet}$	Carbon dioxide radical
$CO_3^{\bullet}$	Carbonate radical
COMT	Catechol O-methyl transferase
Cu	Copper
CYP 450	Cytochrome P450
DMEs	Drug metabolism enzymes
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EA	Ethacrynic acid
EC_{50}	Half maximal effective concentration
ELISA	Enzyme linked imunoabsorbent assay
Fe	Iron
FR	Free Radicals
GPO	Glutathione peroxidase
GPX	Glutathione peroxidas
GSH	Glutathione
GSTs	Glutathione S-transferases

Abbreviations	Description
GWAS	Genome wide association study
H_2O_2	Hydrogen peroxide
$^{\bullet}OH$	Hydroxyl radical
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
JNK	Junk N-terminal kinase
kDa	Kilodalton
K_i	Inhibition constant
K_m	Michealis Menten Constant
MAPEGs	Membrane associated proteins in eicosanoid and glutathione metabolism
MAPK	Mitogen activated protein kinase
MCF7	Michigan cancer foundation -7
MDR	Multidrug resistance
mM	Milimolar
MRP	Multidrug resistance associated protein
MRPs	Multidrug resistance proteins
MWT	Molecular weight
NATs	N-acetyltransferases
NBDHEX	6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol
NBDs	Nucleotide binding domains
NH	Ammonia
Nm	Nanomater
NO	Nitric oxide
NO_2	Nitrogen dioxide
O_3	Ozone
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide
$O2NOO^-$	Peroxynitrate ion
OATP2	Organic anion transporting polypeptide 2

Abbreviations	Description
ONOOH	Peroxynitrous acid
$ONOO^-$	Peroxynitrite ion
P-gp	P-glycoprotein
Pt	Ions platinum
Pt-SG	Platinum-GSH conjugates
RNS	Reactive nitrogen species
$RO_2^\bullet$	Peroxyl radical
RONS	Reactive oxygen nitrogen species
ROOH	Organic peroxides
ROS	Reactive oxygen species
SFN	Sulphoraphane
SOD	Superoxide dismutase
SODs	Superoxide dismutases
SULTs	Sulfotransferases
TMDs	Transmembrane domains
TPMT	Methyltransferases mainly thiopurine S-methyl transferase
UDP	Glucuronosyltransferases
USAN	United States Adopted Names
UV \VIS	Ultraviolet–visible spectrophotometry
V_{max}	Maximal rate
Zn	Zinc

Symbols	Description
% RSA	Radical scavenger activity
Z	Zeta
Ml	Microlitter
µM	Micromolar
π	Pi
ω	Omega
Ɵ	Theta
$_1O_2$	Singlet oxygen

1. INTRODUCTION

1.1. Composition of Thiophene

With the formula, C_4H_4S Thiophene is located in the class which belongs to heterocyclic compound that composed of five-member ring, with sulphur as heteroatom (Figure1.1). Thiophene as well as its derivatives present in petroleum or coal. Thiophene derived from the Greek word thion which means sulfur, and phaino which means shining. The structure of Thiophene can be found in many natural products, as well as combined in many pharmacologically active compounds (Mishra et al., 2011).

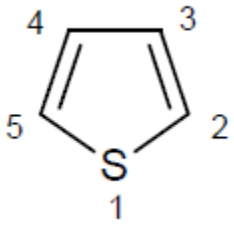


Figure 1.1. Thiophene structure.

Due to its usage in many therapeutic applications, Thiophene and its derivatives are becoming very well known in medicinal chemistry. Thiophene in its simple form is the stable liquid that very similar to benzene compounds in boiling point, and even in its odor (Meyer, 1883).

1.2. Benzothiophenes

Benzothiophene and its derivatives are at focus through sulfur-containing heterocyclic due to they have structural similarity to active compounds that can be developed to the new strong lead molecule in drug design (Keri et al., 2107). The Sulfur heterocyclic family contains highly stable aromatic compounds that exhibit physicochemical characteristics with relevance to design new materials, importantly those related to molecular conductors and magnets (Cava et al., 1975; Shishoo and Jain, 1992). Naturally, Benzothiophene exists as a component of oil-related deposits such as lignite tar (Boberg et al., 1992). As well as, they are present in some natural products

that possess beneficial biological activities for example bryoanthrathiophene, anti-angiogenic (Jeong et al., 2002). As benzothiophene derivatives have various applications in medicinal chemistry, thus they are in great interest in academia, and industry. They show broad range of biological/pharmacological activities for example analgesics, anti-inflammatory (Fakhr et al., 2009), anti-depressant (Berrade et al., 2011), anti-fungal (Jagtap and Agasimundin, 2015), estrogen receptor modulating (Qin et al., 2007), anti-mitotic (Romagnoli et al., 2007), anticancer (Martorana et al., 2015; Sweidan et al., 2015), enzyme inhibitors (Mourey et al., 2010). Some samples of the benzothiophene derivatives which including raloxifene, benocyclidine, sertaconazole, zileuton, and mobam (Figure 1.2) (Jordan, 2003).

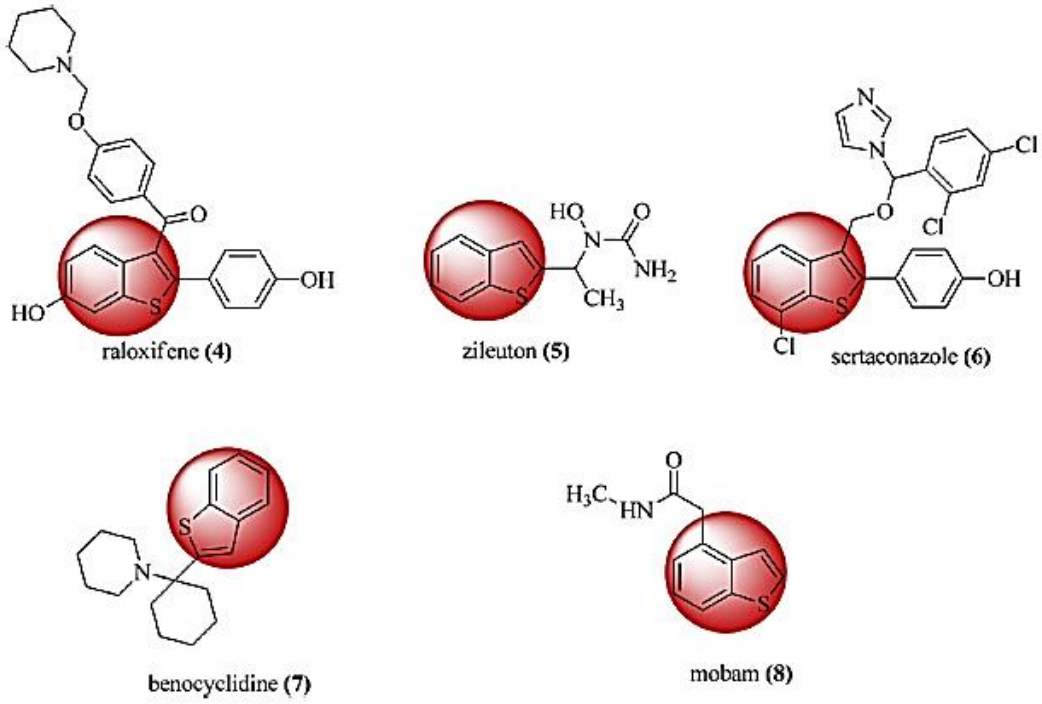


Figure 1.2. Drugs containing benzothiophene in their ring skeleton (Keri et al., 2107).

1.3. Lipinski's Rule

It is known as the 'rule of 5' which is based on the allocation of calculated characteristics between several thousand drugs. Subsequently, as mentioned from the definition, the number of drugs will not be located in the parameter cutoffs in the rule. However, solely a little number of therapeutic categories account for most of the USAN

drugs with characteristics lie outside parameter cutoffs. Those orally active therapeutic classes which lie outside the 'rule of 5' are antibiotics, antifungals, cardiac glycosides, and vitamins. It is believed that those few therapeutic class which consist of orally active drugs that don't join to the 'rule of 5' because their members consist of structural properties that allow them to act as substrates for natural transporters. As the 'rule of 5' has been modified to expel these few drug categories only a few exceptions can be found (Lipinski et al., 1997).

It known as the rule of five because the rules contain multiplication of five, the "rule of 5" states that: poor absorption or permeation is more likely when:

- There are more than 5 H-bond donors (expressed as the sum of OHs and NHs);
- The MWT is over 500 daltons;
- The Log P is over 5 (or MLogP is over 4.15);
- There are more than 10 H-bond acceptors (expressed as the sum of Ns and Os) (Lipinski et al., 1997).

Rules importance appear when new chemical compounds will synthesize (drug-likeness), then the rule of five will provide parameter to evaluate the compound faster, optimize the selection among them, and to know permeability and oral absorption capacity of compound, as well as its very useful for choosing chemical compounds (drug-likeness) from chemo libraries, while it should be used carefully and with criterion (Giménez et al., 2010).

1.4. Free Radicals

From normal cellular metabolism Free radicals could be produced. They can be defined as an atom or any molecule that has one or more unpaired electron from valence shell or outer orbit. The cause behind of unstableness of free radicals is a solo number of the electron(s), they live for a short time while extremely reactive. As they are very reactive so they will pick up electrons from other substances or compounds to get stability. As a result, the attacked molecule will lose its electron and change to free radical itself, then triggering chain reaction cascade which finally leads to damage to living cells (Pham-Huy et al,. 2008).

In the biological system Free radicals are manufactured as a result of normal aerobic cellular processes as well as can be generated by xenobiotics, and some disease processes (Sies, 2013). The compounds that have undergone an incompletely oxidizing process can generate Free Radicals (FR) which means they have undergone partial burning, and in their structure, they have oxygen groups that enable them to initiate the aggressive oxidation reaction from cell membrane surface or even inside of the cell. Free radicals resulted from both actions occurring in the body (energy production, incomplete catabolism, hepatic detoxification, etc.) and environment form outside (cigarette smoke, air pollution, foods, medicines, well/tap water, etc.) (Pickrell et al., 2009).

However, it sounds like around 10000-20000 free radicals assault every cell per day, while some of them are good for health that makes the human body defend against inflammation, kill bacteria, and controls those smooth muscles which work to regulate the proper functioning of internal organs and blood vessels. On the other side free radicals have an important role in the pathogenesis of various diseases for example diabetes mellitus, heart disease, Parkinson's disease, Alzheimer's disease, arthritis, aging, and cancer, etc… Both (ROS) and (RNS) together cover the free radicals on top of some other non-radical reactive species (Pala and Gürkan, 2008).

1.4.1. Reactive oxygen species

The Reactive oxygen species (ROS) is a mutual expression that is not only about oxygen radicals (superoxide and hydroxyl) but includes even some non-radical derivatives of molecular oxygen (O_2) such as hydrogen peroxide (Halliwell, 1999). Reactive oxygen species (ROS) are one of the natural by-products of cellular oxidative metabolism, it is very well known that they play fundamental roles in the differentiation, amendment of cell survival, inflammation-related factor production, cell death, and cell signaling (Touyz, 2005; Mueller, 2005).

Biologically important ROS elements contains free radicals, for example singlet hydroxyl ($HO^{\bullet}$), oxygen ($_1O_2$), hydroperoxyl ($_2HO^{\bullet}$), superoxide ($O_2^{\bullet-}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), carbonate ($CO_3^{\bullet}$), and carbon dioxide radical ($CO_2^{\bullet}$), and nonradicals, such as hydrogen peroxide (H_2O_2), hypobromous acid (HOBr), hypochlorous acid

(HOCl), ozone (O_3), organic peroxides (ROOH), peroxynitrite ($O2NOO^-$), peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH), nitric oxide (NO), hypochlorite (OCl^-), and peroxomonocarbonate ($HOOCO_2^-$) (Halliwell, 2006; Augusto and Miyamoto, 2011).

Always ROS produced in plants, through inescapable seepage of electrons onto O_2 which comes from the electron transport actions in plasma membranes, chloroplasts, and mitochondria, it is also possible that they can be manufactured as byproduct of different metabolic pathways that present in different cellular parts (Foyer, 1994; Heyno et al., 2011). Occasionally chilling, drought, salinity, UV-B radiation, and metal toxicity or another environmental status, and pathogen attack lead to facilitate the production of ROS in plants by disturbance homeostasis of the cell (Shah et al., 2001; Srivastava and Dubey, 2011). Common ROS has shown in (Figure 1.5.)

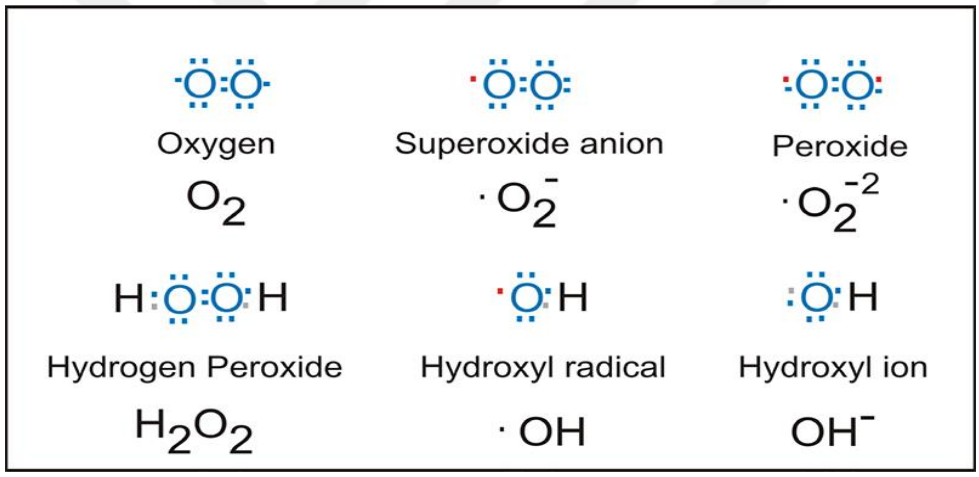


Figure 1.3. Electron structure of common reactive oxygen species. (Held, 2012).

O_2 can cause toxic effects on biological systems, for instance, deactivation of enzymes, breakdown (i.e., oxidation) of lipids, the introduction of the changes (i.e., mutations in the DNA, and crashing of the cell membrane) (De Groot, 1994; Toykuni , 1999). However, under the influence of environmental stress level of reactive oxygen species increased dramatically, in high concentration of ROS easily reacts with lipids, nucleic acids, proteins, and carbohydrates, it may causes injury to cell structure, as well as it may lead to certain case known as oxidative stress (Conner and Grisham, 1996; Brieger et al., 2012).

1.4.2. Reactive nitrogen species

Reactive nitrogen species (RNS) are those oxidants that contained nitrogen in their composition, such as peroxynitrite ($ONOO^-$), nitric oxide (NO^-), and nitrogen dioxide (NO_2) (klebanoff, 1980; Bedard and Krause, 2007). (RONS) reactive oxygen-nitrogen species, this term used for those radical compounds which contain reactive oxygen and nitrogen species together.

Table 1.1. List of ROS and RNS produced during metabolism (Kohen and Nyska, 2002; Halliwell, 2001; Mugoni and Santoro, 2013)

Free radical	Symbol	Half-life
Reactive oxygen species-ROS Radicals		
Superoxide	$O_2^{\bullet-}$	10^{-6} s
Hydroxyl	$OH^{\bullet}$	10^{-10} s
Alkoxyl radical	$RO^{\bullet}$	10^{-6}
Peroxyl Radical	$ROO^{\bullet}$	17 s
Non radicals		
Hydrogen peroxide	H_2O_2	Stable
Singlet oxygen	$^1O_2$	10^{-6} s
Ozone	O_3	s
Organic peroxide	ROOH	Stable
Hypochlorous acid	HOCl	Stable (min)
Hypobromous acid	HOBr	Stable (min)
Reactive nitrogen species-RNS Radicals		
Nitric oxide	$NO^{\bullet}$	s[a]
Nitrogen dioxide	$NO_2^{\bullet}$	s
Non radicals		
Peroxynitrite	$ONOO^-$	10^{-3} s
Nitrosyl cation	NO^+	s
Nitroxyl anion	NO^-	s
Dinitrogen trioxide	N_2O_3	s
Dinitrogen tetraoxide	N_2O_4	s
Nitrous acid	HNO_2	s
Peroxynitrous acid	ONOOH	Fairly stable
Nitryl chloride	NO_2Cl	S

a *The half life of some radicals depends on the environmental medium, for example the half life of NO• in an air saturated solution may be few minutes. S seconds, min minutes.*

They can be released from dendritic, macrophages, and neutrophils cells as defend against the inflammatory stimulus. Due to the absence of paired valence shell

electrons, on the other hand, there is no static bond therefor RONS are too much reactive, but their proper regulation is necessary for an effective immune response, as well as for limiting tissue damage (Salman and Ashraf, 2013).

1.4.3. Sources of free radicals

The sources of free radicals are three, internal sources, external source, and Physiological factors, for the first source which are enzymatic reactions, they serve as a source of free radicals, that contain those reactions involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and the cytochrome P450 system. There are many actions lead to generation of free radicals internally for example inside some organelles such as mitochondria free radicals will produce, while by the action of some enzymes, for instance, xanthine oxidase during phagocytes possess also free radicals may generated, as well as reactions involving iron and other transition metals results to production of free radicals. While the reaction of oxygen with organic compounds known as external sources. Free radicals may also come from those reactions who started by ionizing radiation. Some external sources are environmental pollutants, cigarette smoke, ozone, certain drugs, radiation, anesthetics pesticides, as well as industrial solvents. Finally emotion stress… etc. As well as disease situations located in Physiological Factors this includes mental status (Kumar, 2011).

Reactive species or free radicals include reactive oxygen and nitrogen species collectively and are called reactive oxygen-nitrogen species (RONS).

1.5. Antioxidants

Our first protective line versus free radical damage is antioxidants, and they are needed to preserve optimum health, as well as wellbeing. Antioxidants are widely used as a component in a dietary supplement to enhancing good health and banning diseases like cancer, cardiovascular disease (Atta et al., 2017).

Any substance if present in low concentration in comparison to oxidant substrate in the way that can lead to inhibit or delay oxidation of that substrate called Antioxidant (Halliwell and Gutteridge, 1995).

(Halliwell, 2007) defined antioxidants as any substance that able to inhibit or prevents oxidation destruction to a particular molecule. As antioxidants can stop of oxidation process even at comparatively low concentrations, thus they have various physiological roles in the body. It believes that antioxidants play significant roles in the body protection system versus ROS (ou et al., 2002; Yadav et al., 2016).

Human beings have enhanced a highly developed and complex antioxidant defense system. That contains widely different components, which in their origin they are exogenous and endogenous, in the way that they are working together to decline free radicals (Jacob, 1995). There are two classes of Antioxidants, synthetic antioxidants, and natural antioxidants.

Generally, antioxidants found in these forms:

- Antioxidants obtained from Nutrient such as tocopherol, ascorbic acid (vitamin C), carotenoids, tocotrienols (vitamin E), other compounds have low molecular weight, for instance, lipoic acid, and glutathione.

- Some enzymes are antioxidant enzymes, for example, glutathione peroxidase, superoxide dismutase, and glutathione reductase, that they capable of catalyzing free radical extinguish reactions.

- There are some proteins called metal-binding proteins, such as albumin, lactoferrin, ferritin, and ceruloplasmin that can isolate copper ions and free iron which can catalyze oxidative reactions.

- Finally, many antioxidants that present in various plant foods called phytonutrients (Kumar, 2014).

1.5.1. Enzymatic antioxidants

The typical members of these antioxidants are catalase, superoxide dismutase (SOD), glutathione peroxidase (GPO), and glutathione reductase, all enzymes of ascorbate glutathione cycle (Menshikova and Zenkov, 1993; Pradedova et al., 2011).

1.5.1.1. Glutathione peroxidases

It is known as selenium-containing peroxidase, which is one of the most remarkable examples among glutathione peroxidase (EC 1.11.1.19), that able to catalyze the reduction of a wide range of hydroperoxides (ROOH and H_2O_2) by using GSH, thus it results in saving mammalian cells from oxidative damage. At least there are five known GPX isoenzymes among mammals. While their expression is rife, but levels of expression for each isoform are different relying on tissue types (Matés et al., 1999).

1.5.1.2. Catalases

Hydrogen peroxide with the help of Catalase converted to oxygen and water, catalase which is an antioxidant enzyme, nullifies the effect of hydrogen peroxide that is presented intracellular. The precise amount of catalase present in the cytoplasm cannot be assessed because most of it is lost during tissue manipulation (Abdolsamadi et al., 2014).

1.5.1.3. Superoxide dismutases (SODs)

Their distribution has widely seen among those organisms which consuming oxygen, Superoxide dismutases its abbreviation is (SODs) (EC. 1.15.1.1), they are metalloenzymes. They have been seen among aerotolerant anaerobes, as well as some obligate anaerobic organisms. These enzymes have a vital role in protection mechanism versus oxygen toxicity, which they will generate during the biological reduction of dioxygen, which means they are acting as intermediary by the partially reduced oxygen intermediates (O_2^-, H_2O_2 and $^{\cdot}OH$). SODs have special substrates which are free radicals, as they are catalyzing the conversion of O_2- to H_2O_2, and O_2 (Hassan, 1988).

1.5.2. Non-enzymatic antioxidants

Non-enzymatic is represented by reduced glutathione (GSH), carotenoids, ascorbic acid, α-tocopherol, and flavonoids. All those compounds could neutralize ROS (Pradedova et al., 2011).

1.5.2.1. Glutathione

It is known that naturally reduced glutathione is protective. While in an oxidized composition is no preservative. Inside of the cell reduced glutathione serves to balance hydrogen peroxide that has been produced (Jeeva et al., 2015). the most tow significant enzymes which they have very important role in prevention of increasing levels of oxidative stress are glutathione reductase and Glutathione peroxidase (Townsend et al., 2003) as well as oxidation and reduction of glutathione by those enzymes couple that make GSH as a free radical scavenger (Maritim et al., 2003).

1.5.2.2. Vitamin C (ascorbic acid)

Ascorbic acid will be formed in liver from glucose in most mammalian species, it is six-carbon lactone in structure, while in order to human beings lack L-gulono-1, 4-lactone oxidoreductase (EC 1. 1. 3. 8) enzyme, which is necessary for buildup ascorbic acid immediate precursor 2-keto-l-gulonolactone, so they can't synthesize ascorbic acid from glucose, it is same situation for guinea pigs, and non-human primates. This is due to the piece of DNA which is responsible from the expression of L-gulono-1,4-lactone has faced substantial mutation, which is leads to an absence of functionalized enzyme (Nishikimi et al., 1994; Nishikimi and Yagi 1996).

So, if human beings could not get vitamin C from their nutrients, they will face deficiency states, which can be seen from the wide demonstration of medical guises. Clinically expression for vitamin C deficiency is scurvy which maybe causes death unless it will undergo treatment. That is why human beings need to ingest vitamin C for survival. Vitamin C is known as a reduced agent because it will donate the electrons. As long as the vitamin C donates its electrons, it prevents other compounds to be oxidized

and thus called vitamin C antioxidant. While on the other hand by nature of the reaction when vitamin C donates its electrons itself oxidized that means a change to free radical, but the species after losing electrons may form are ascorbyl radical or semidehydroascorbic acid. When comparing them to another free radicals ascorbyl radical is relatively stable that has 10^{-5} seconds half-life, which is fully unreactive (Padayatty et al., 2003).

2. LITERATURE REVIEW

2.1. Drug Metabolism and Its Phases

Mostly a great numbers of xenobiotics which have a contact with the human by exposing to them, this exposure mainly come from many ways such as the contamination of this area which human lives in, different types of ingredient that are used in food industry, different types of beautifying agents, some foods such as canned food and freezing food, chemicals that are used in agriculture, and different types of drugs. Generally, if there is no metabolism, they will not eliminate efficiently from the body due to they are lipophilic chemicals, it leads to toxicity as they pile in the body. Most of xenobiotics undergo one or multiple pathways of metabolism, which comprises phase I (phase one) and phase II (phase two) enzymatic systems as well as phase III (phase three) transporters (Meyer, 1996; Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

Generally, metabolism plays an important role to change those lipophilic compounds to more hydrophilic or less lipophilic compounds that can be easily eliminated from the body, through urine or bile. For cells to be available and to get their action sites, drugs are mostly have some physical properties that permit them to shift according to the concentration gradient and enter inside the cell. Hydrophobic properties of drugs make them easily enter cells through the lipid bilayer, where drugs interact with their target receptor or protein. As there are huge number of carriers on plasma membrane, this property provides easy way for entrance of lipophilic compounds into the cell, thus hydrophobicity will make it difficult to get rid of drugs, because, in the case of non-metabolism, they accumulate in the phospholipid bilayer and cell compartments (Morgan et al, 1998).

Small drug molecules are generally xenobiotics to the body (i.e., molecules that looks stranger to the normal biochemistry of body), ideally, the drug should be removed from the body when its pharmacological action is attained and its effect is not useful anymore. Several types of drugs are removed from the body and no changes in their structures happen. While most of the drugs require conversion to more hydrophilic compounds by a series of chemical modification, to be easily excreted through urine or

bile, those chemical modifications known as drug metabolism. Drug metabolism which is also known as detoxification function plays an important role to defend the human body from the xenobiotics which are present in the environment (Lohar et al., 2012).

The conversion process is called biotransformation. Biotransformation reactions of the drug are classified as functionalizing reactions from (phase one) phase I, and conjugation reactions from (phase two) phase II drug metabolism (Farombi, 2001; Lofholm and Katzung, 2001). Phase I reactions result in adding of functional groups to a parent compound (Mrozikiewicz et al., 2005), which leads to loss of their pharmacological activity. In some cases Phase I reactions convert pro-drugs to biologically active metabolites, usually it will happen in case of hydrolyzing the linkage of both an ester or amide. In rare cases, phase I metabolism is related to an altered pharmacological activity (Caira and Ionescu, 2006).

Phase II metabolism is coupling reactions, which creates a covalent bond between a functional group on the parent molecule (or on the phase I metabolites) with internally derived glucuronic acid, glutathione, sulfate, acetates or amino acids (Zamek-Gliszczynski et al., 2006). Thus those highly polar conjugates result in inactivating of drugs. So the normal effect of biotransformation could be called inactivation or detoxification. Biotransformation categorized into four categories: reduction, hydrolysis, oxidation, and conjugation. Phase I includes the first three, while phase II contains the last one (Caira and Ionescu, 2006).

2.1.1. Phase I drug metabolism

By the action of phase I reactions parent compounds will convert to more polar metabolites (Jancova et al., 2010). Reactions of Phase I include reduction, oxidation, and hydrolysis. These reactions will add one of these functional group to the original compound (–OH, $–NH_2$, –SH or –COOH), that results in increasing of hydrophilicity (Parkinson, A. 2001). CYP450s are enzymes that involve in the phase I drug metabolism that is present in high amounts in some organs and systems such as lung, digestive system, and kidneys. Liver is triggering both types of metabolism which are known as oxidation and reduction, for many chemicals and substances that exist in the human body (Tseng et al., 2005; Xu et al., 2005). There are some idea that in humans

there are five gene families, which are named cytochromeP1, cytochromeP2, cytochromeP3, cytochromeP4 and cytochromeP7, they have an important role in the elimination of drugs and xenobiotics, as well as they play critical roles in hepatic and extra-hepatic metabolism. About %70 of clinically most used drugs metabolized by CYP1A2, CYP2C9, CYP2D6 and CYP3A4/5(Xu et al., 2005; Saad et al., 2017) as shown in (Figure 2.1). However, the main responsible enzyme families for metabolizing xenobiotics are CYP 1-3 families (Guengerich, 2008; Saad et al., 2016).

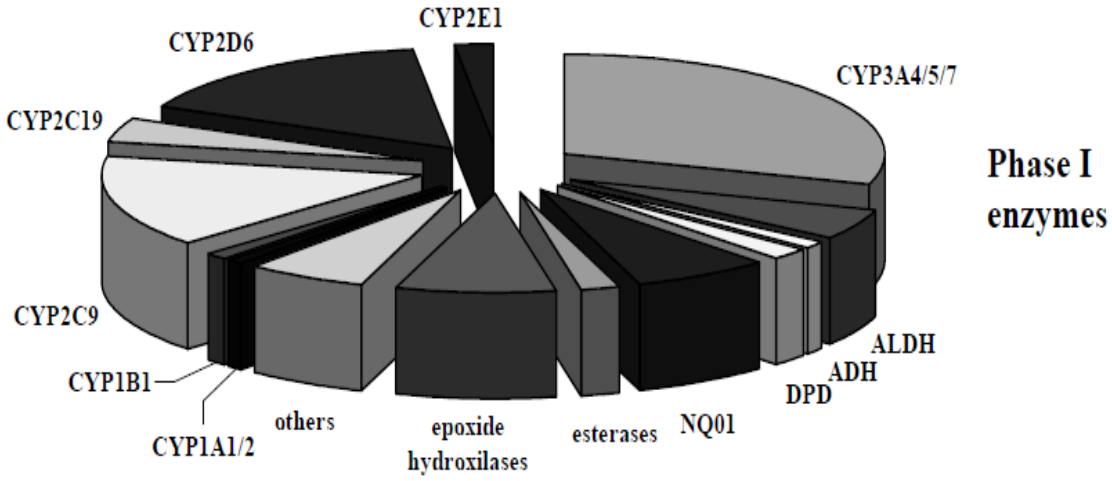


Figure 2.1. Phase I drug metabolism enzymes (Caira and Ionescu, 2006).

Table 2.1. General pathways of xenobiotic biotransformation and their major subcellular location (Parkinson, 2001)

REACTION	ENZYME	LOCALIZATION
	Phase I	
	Esterase	Microsomes, cytosol, lysosomes, blood
Hydrolysis	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
	Azo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
Reduction	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Reductive dehalogenation	Cytosol, microsomes
	Reductive dehalogenation	Microsomes
	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
Oxidation	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostaglandin H synthase	Microsomes
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes

2.1.2. Phase II drug metabolism

Enzymes of Phase II (phase two) metabolism have an important role in the conversion of xenobiotics as well as endogenous compounds to readily retractable forms and results in reducing the pharmacological activity of the parent compound. The aim of phase II (phase two) metabolism is to carry out conjugation reactions. Which contain glucuronidation, sulfation, methylation, acetylation, glutathione, and conjugation of the amino acids (Parkinson, 2001). As mentioned in (Table 2.2). Many different Gene types of biotransformation enzymes that are available will perform many different pathophysiological processes (Jančová and Šiller, 2012). These reactions are known to perform detoxification, while in certain cases also cause toxication (Lofholm and Katzung, 2001). The new compounds that are formed in metabolism known as metabolites and generally they are more water-loving than the original compounds (Jancova et al., 2010).

Table 2.2. General enzyme types in phase II drug metabolism (Parkinson, 2001)

ENZYME	LOCALIZATION
Phase II	
Glucuronide conjugation	Microsomes
Sulfate conjugation	Cytosol
Glutathione conjugation	Cytosol, microsomes
Amino acid conjugation	Mitochondria, microsomes
Acylation	Mitochondria, cytosol
Methylation	Cytosol, microsomes, blood

The new compounds that are formed in metabolism known as metabolites and generally they are more water-loving than the original compounds ((Jancova et al., 2010).

Another important point of drug metabolism enzymes (DMEs) is that they are differentiated by their capability to stick xenobiotics to a molecule that is an organic donor and have small molecular weight for example acetyl-A, glutathione, or glucuronic acid UDP. The conjugated metabolites may become a substrate for specific transport enzymes, which results in easy excretion from the body (Hines and McCarver, 2002). Usually, conjugated metabolites have no more pharmacological activity, as well as they are highly water-soluble (Lofholm and Katzung, 2001). Mostly transferases located in Phase II drug metabolism such as UDP glucuronosyltransferases (UGTs), sulfotransferases, N-acetyltransferases glutathione S-transferases and various methyltransferases (mainly (TPMT) and (COMT)) as shown in (Figure 2.2) (Jancova et al., 2010).

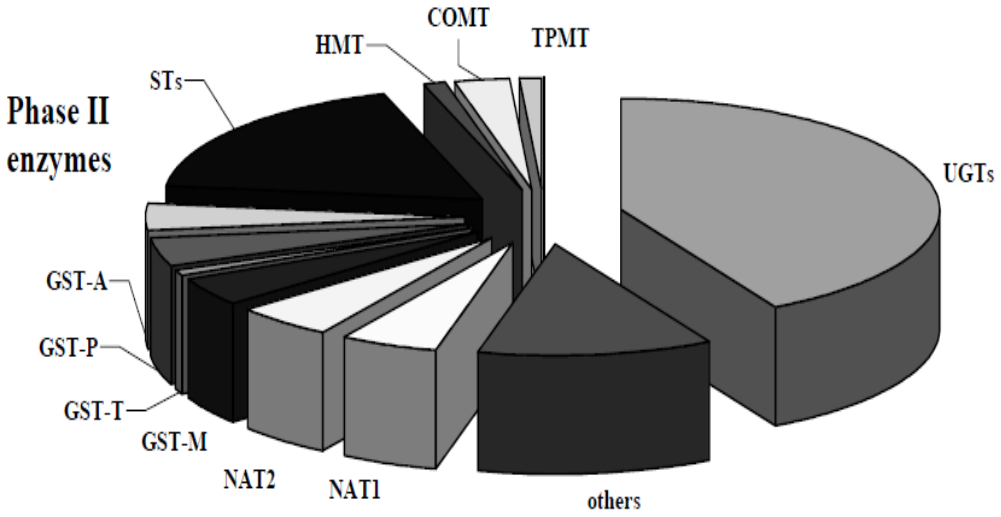


Figure 2.2. Phase II drug metabolism enzymes (Lofholm and Katzung, 2001).

2.1.3. Phase III drug metabolism

In the third phase drug metabolism, contains several transporters such as, (P-gp) which is the abbreviation of P-glycoprotein (Brinkmann and Eichelbaum, 2001), (MRP) which is the abbreviation of multidrug resistance associated protein (Kerb et al., 2001), and(OATP2) which is the abbreviation of organic anion transporting polypeptide 2 (Tirona and Kim, 2002), they can be seen in many organs and tissues in human body for example: liver which filter blood that comes from digestive tract, intestine which includes both (large and small intestine), kidney which removes the waste product through urine, and the last one is brain, all these organs can serve as a massive barrier for drug penetration and have an important function in drug pharmacokinetic which means the movement of drug throughout the body such as absorption, distribution, and excretion. (Brinkmann and Eichelbaum, 2001; Kim, 2003; Mizuno et al., 2003; Staudinger et al., 2003). It is reported that in chemotherapy P-gp is related with multidrug resistance (MDR). Both P-gp and MRP use the energy that produced by the hydrolysis of ATP to the substrate for transport via the cell membrane, called ATP cassette binding carriers (ABC) (Mizuno et al., 2003). ATP cassette binding carrier transporters included in one of the greatest super families of proteins, and a wide number of substrates that include amino acids, ions, sugars, lipids, xenobiotics, and many therapeutic drugs are either import or export by these proteins. (Dean et al., 2001; Kerb et al., 2001). In the eukaryotes, there are only proteins that can export the substrate

or in other words, only exporters are present. About forty six different types of ABC transporters protein have been founded in the human body (Dean et al., 2001; Mizuno et al., 2003). Two (NBDs) and two (TMDs) are the constituents of the ABC transporters. The hallmark feature of this transporter family is that The NBD is also called an ABC. The role of TMD is to recognize and help the transport of substrates through the membranes of the cell (Dean et al., 2001; Kerb et al., 2001).

2.2. Glutathione S-transferase Enzyme Superfamily

2.2.1. General properties of glutathione S-transferases

Conjugation between glutathione (GSH) and hydrophobic electrophile molecules which can be seen in (Figure 2.3) are accelerated and catalyzed by Glutathione S-transferase (GSTs, EC 2.5.1.18) enzymes. Substrates are endogenous compounds which means they are originated within the system or organism such as (prostaglandin endoperoxides or epoxides) (Burger et al., 1989), or xenobiotic which can be defined as a substance that is not present in the organism naturally. Such as weed killers or insecticides which are substances that can kill insects (Voss and van Bladeren, 1990), and mutagens can be defined as substances that change the genetic materials for example DNA, or carcinogens substance that promotes the formation of cancer. (Ketterer, 1988). Glutathione S-transferase composed of different types, shapes and multifunctional enzymes, which present as homodimers and heterodimers. These enzymes have a great potency and responsibility in regulating susceptibility to cancer, (Hayes and Pulford, 1995; Armstrong, 1997). The Glutathione S-transferases (GSTs) are important enzymes in the second phase of biotransformation which they have a great activity in cellular detoxification, macromolecules protection from been attacked by reactive electrophiles (RE), and carcinogens agents that are present in environment, and chemicals that are used in some therapies that known as chemotherapeutic agents , and ROS (Strange RC, 2000). In phase two metabolism the main detoxifying enzymes are GSTs enzymes which are widely distributed in nature and are available in both prokaryotes and eukaryotes (Hayes et al., 2005). There are different types of GSTs isoenzymes that are found throughout nature, they could be present in organisms such as microbes, plants, fish, insects, birds, and mammals (Hayes and Pulford, 1995). GSTs

first time had been found in the1960s as long as they had an important role in both drug detoxification and metabolism (Mozer et al., 1983). In the 1960s GST has been founded when its action has been noticed in cytosolic fraction of the liver of rat, after noticing its activity it became studied well (Combes & Stakelum, 1961). After finding the GST in the 1960s, the important goal of GST studies was to investigate of their crucial role in detoxification (Mannervik, 2012). Also, it was founded that cytosolic GSTs from the liver of the rat can bind to some compounds or substances such as steroids, bilirubin, and organic anions by non – covalent bonds. (Litwack et al., 1971).

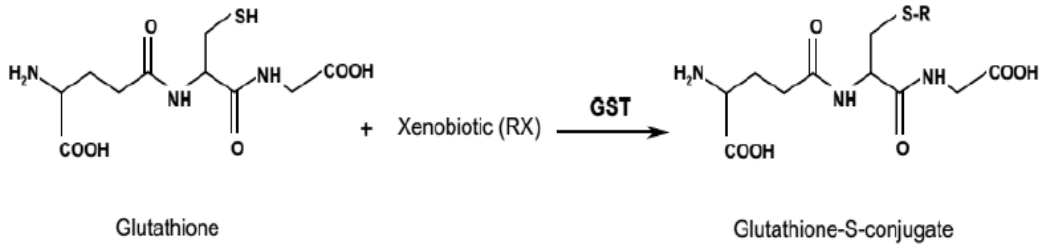


Figure 2.3. Formation of glutathione conjugation (Jancova et al., 2010).

2.2.2. Classification of glutathione S-transferases

After a detailed examination of Phylogenetic, it shows that all isoenzymes of GSTs have appeared from an old progenitor gene. There are many classes of The GSTs of mammals that are known as theta, zeta, phi, and tau classes, both types of GSTs, Zeta and theta can be seen in both animals and plants, but other types of GSTs enzymes such as tau and phi classes are only present in plant it means that they are plant-specific (McGonigle et al., 2000). And a prokaryote-specific beta class of GST can be found in bacteria. (Figure 2.4) (Rossjohn et al., 1998).

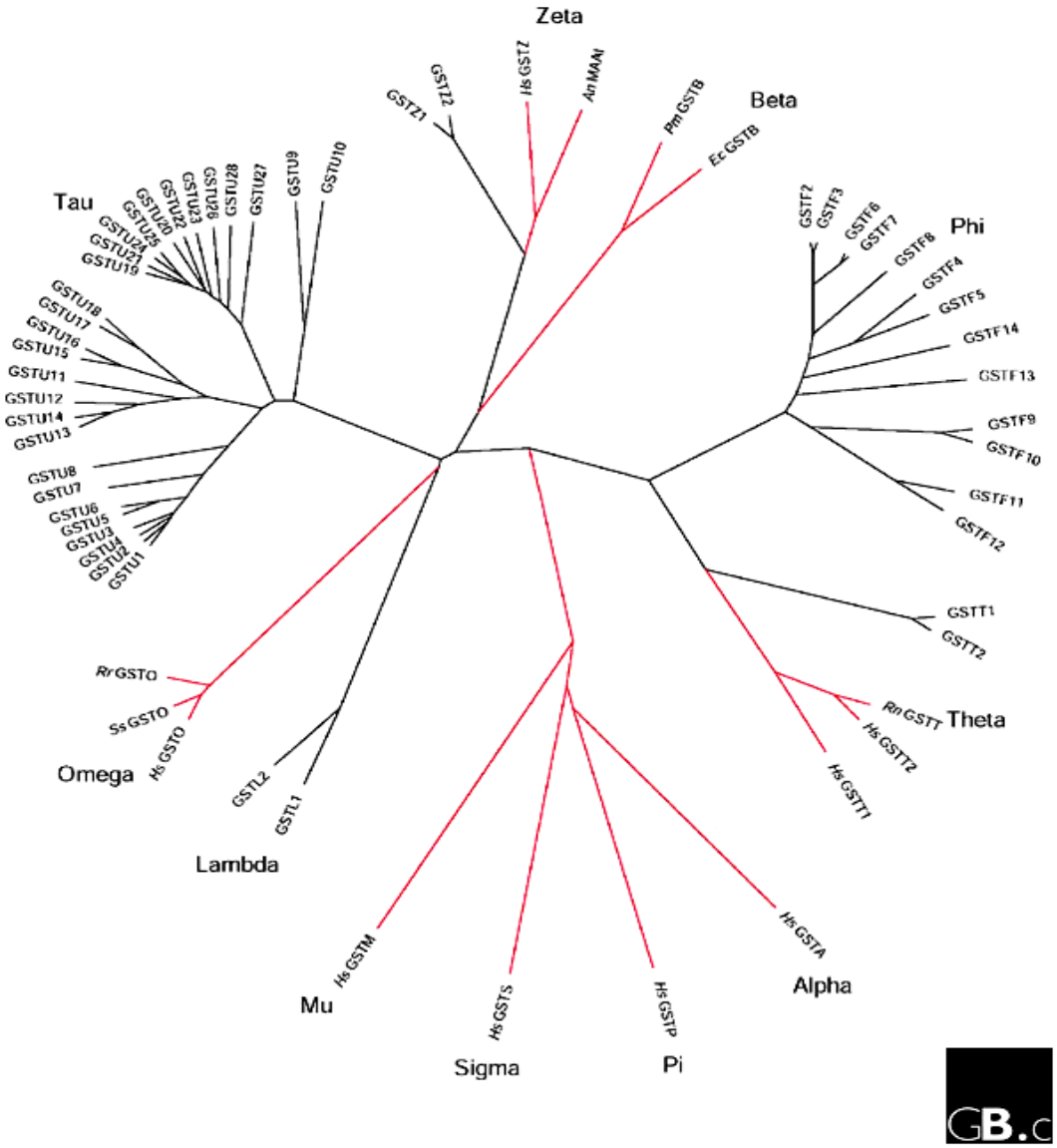


Figure 2.4. Phylogenetic tree illustrating the diversity of GSTs and the relationships between classes. (Rossjohn et al., 1998).

2.2.3. Human glutathione S-transferases

There are three major families of Human GSTs are known which are: cytosolic, mitochondrial and membrane-bound microsomal. The mitochondrial and cytosolic GSTs are known as soluble enzymes, and they have similar three- dimensional fold. There is an idea that with subunits of 23–30 kilodalton (kDa) all the soluble GSTs are active as dimers, and have 199–244 amino acid subunits which can be mentioned as its length. (homodimers or different, identical, heterodimers) subunits, for any dimer the independent gene will be encoded (Ji et al., 1995). Microsomal GSTs which are stated

as (MAPEGs) stands for membrane associated proteins in eicosanoid and glutathione metabolism there structures are differ from cytosolic GSTs but their activity or function are the same in that they have the capability to accelerate the conjugation of GSH to electrophilic compounds by catalyzing the reaction. Each group is coded via its various genes which can be found on different chromosomes. The GSTs of the cytosolic family are the most complicated group as long as they are divided into seven classes and they are designated as Zeta, Omega, Pi, Alpha, Sigma, Theta and Mu. Their classification based on several properities such as physical structure of the genes, immunological cross-reactivity and amino-acid sequence similarities, (i.e., intron number and position). (Hayes et al., 2005).

2.2.3.1. Genetic polymorphisms of human glutathione S-transferases

Availability of different types of drug-metabolizing enzyme genes make several subgroups within the population, which have different capability in transforming the drug; in another way it means that the polymorphism of the gene within those enzymes can enhance or decrease activity (Pawlak-Adamska et al., 2014). The predictive genotype of genes that metabolizing the drug can help to produce the drug with less toxicity, accurate doses of the drug for each patient, and less expensive drugs (Cai et al., 2014).

2.2.3.2. Human glutathione S-transferase alpha (α) class

There are five genes of alpha (α) GST, in which they have been classified as GSTA1, GSTA2, GSTA3, GSTA4, and GSTA5, that they were found on sixth chromosome (Sá et al., 2014), by using both methods gene-specific probes as well as real-time PCR it could demonstrate that GSTA1, GSTA2, and GSTA4 mostly their apportionment and localization found in all tissues, while GSTA3 was not common, on the other hand GSTA5 could not be detected (Dasari et al., 2018). There are some northern blot studies that illustrate that GSTA4 transcripts were found in all tissues (Dasari et al., 2018). GSTA1 is responsible for mechanisms inside the cells that protect the organism from those metabolites which generated during lipid peroxidation. The

length of GSTA1 protein is composed of 222 amino acids long, it has the ability to accelerate the conjugation of themyelosupressive agents, for example, the conjugation of busulfan with GSH, but in condition of polymorphism it could detect that there is variation in base of GSTA1 alleles, which consequently results in lower expression and changed biotransformation (Bredschneider et al., 2002), while there is an idea that (haeme and bilirubin) transportation is GSTA2 responsibility. (Morel et al., 2004) has found the GSTA3, and mentioned that GSTA3 is uncommon subclass of the alpha (α) GST, as well as it has two important functions which they are double-bond isomerization, and (steroids) biosynthesis (Johansson and Mannervik, 2001).

2.2.3.3. Human glutathione S-transferase mu (μ) class

There are five known mu (μ) GST genes, which they are GSTM1, GSTM2, GSTM3 GSTM4, and GSTM5. It is known that those five genes located on chromosome number 1 (Pearson et al., 1993). Both GSTM1 and GSTM2 are known to the members that have the most activity amongst all GSTM subclasses. It is believed that GSTM1 null allele is in relationship with head and neck carcinomas (Lohmueller et al., 2003), by GWAS it could study statistical association which is between GSTM1 expression and SNP rs366631 (Huang et al,. 2008). There is little knowledge about GSTM2, GSTM3, GSTM4 and GSTM5 functions in comparison to the function of GSTM1. A dimeric enzyme which is GSTM2 can be seen in the tissues of the liver and muscle while large numbers of GSTM3 can be seen in the brain (Laisney et al., 1984). It was believed that the (T2517C) C-T polymorphism within the sixth intron of the GSTM4 gene is responsible for causing lung cancer (Liloglou et al., 2002). Takahashi et al. found the GSTM5 by examination of the cDNA library human frontal cortex with cDNA rat that cross-hybridized with other human and rodent GST cDNAs. GSTM5 isoenzyme usually located in tissues of the lung and brain this is showed by Northern blot analysis and believes that it's nearly composed of 217amino acids (Takahashi et al., 1993).

2.2.3.4. Human glutathione S-transferase theta (Θ) class

The class of Theta (Θ) GST generally divided into two isoenzymes, they are GSTT1, GSTT2 and by in-situ hybridization studies it could find out that their location is on chromosome 22 (Webb et al., 1996). (Pemble et al.,1994) cDNA clones of theta (Θ) GST realized that the length of GSTT1 is composed of 239 amino acids. (Chen et al., 1996) 244 amino acids are the building block of the GSTT2 and it has about fifty-five percent similarity with the GSTT1 sequence. Studies of the frequency of the GSTT1 null genotype in myelodysplastic syndromes and 46% of myelodysplastic syndrome, patients with this syndrome have null GSTT1than 16% of controls (Tan et al., 1995). This is a gene (−537 G→A, −277T→C and−158G→A) and it has SNPs and these SNPs in the promoter region may allow the higher risk of colon and rectal cancer (Jang et al., 2007).

2.2.3.5. Human glutathione S-transferase omega (ω) class

Omega (ω) is class of a GSTs which are composed of two members, known as GSTO1 and GSTO2, these are located on the tenth chromosome (chromosome 10), according to the structure and function omega (ω) GST are different from all other GSTs. by using (EST) database stands for expressed sequence tag, GSTO1 cDNA was founded. Which is composed of 241 amino acids, GSTO1 shares many properties with zeta (ζ) and that's why it is closely related to zeta (ζ) GST which is abbreviated to (GSTZ1). In both regions (coding and noncoding) of the GSTO genes Number of polymorphisms have been found (Whitbread et al., 2003). A genetic relationship has been noticed between both (AD) and the chromosome 10q, where both GSTO1 and GSTo2 genes located, that leads to hypothesis that GST omega genes maybe a risk factor for (AD) Alzheimer's Disease, the GSTO1 locus has several functions and activity, and it shows positive relation with Alzheimer's disease (AD) (Piacentini, et al., 2012).

2.2.3.6. Human glutathione S-transferase zeta (ζ) class

By application of sequence alignment and analysis of phylogenetics, the zeta has been found, the position of genes of this class was on chromosome 14 (Blackburn et al., 1998). A great number of are present in liver, kidney tubules such as proximal convoluted tubules, they can accelerate the GSH-dependent reaction on electrophilic alpha-halogenated acids. It believes that in the catabolic pathway of the tyrosine and phenylalanine, Zeta GST has similar activity as maleylacetoacetate isomerase; any decrement in the catalytic property of(ζ) GST leads to some metabolic diseases which type I phenylketonuria and tyrosinemia can be evolved (Fernández-Cañón et al., 1998).

2.2.3.7. Human glutathione S-transferase pi (π) class

Pi class GSTP members are not widely known except one member, and its position is on chromosome 11, can be found in all tissues which red blood corpuscles included while small amount found in liver (Hussey et al,. 1986) This protein has a length which is measured as 209 amino acids and it is composed of seven exons which expand to nearly 2.8kb (Morrow et al., 1989). In many tumors, the level of (π) GST is increased (Dasari et al., 2018). Nowadays there is more attention on GSTP polymorphism which means various phenotype and types of GSTP, there is a great relationship between the level of GST-π in endothelial cells and glial cells/astrocyte and many different medical conditions, for example, the medical intractable epilepsy, AED which stands for antiepileptic drugs treatment cannot affect on GST-π because it is resistance this treatment. (Shang et al., 2008). Three-dimensional shapes of those isoenzymes are presented in (Figure 2.5).

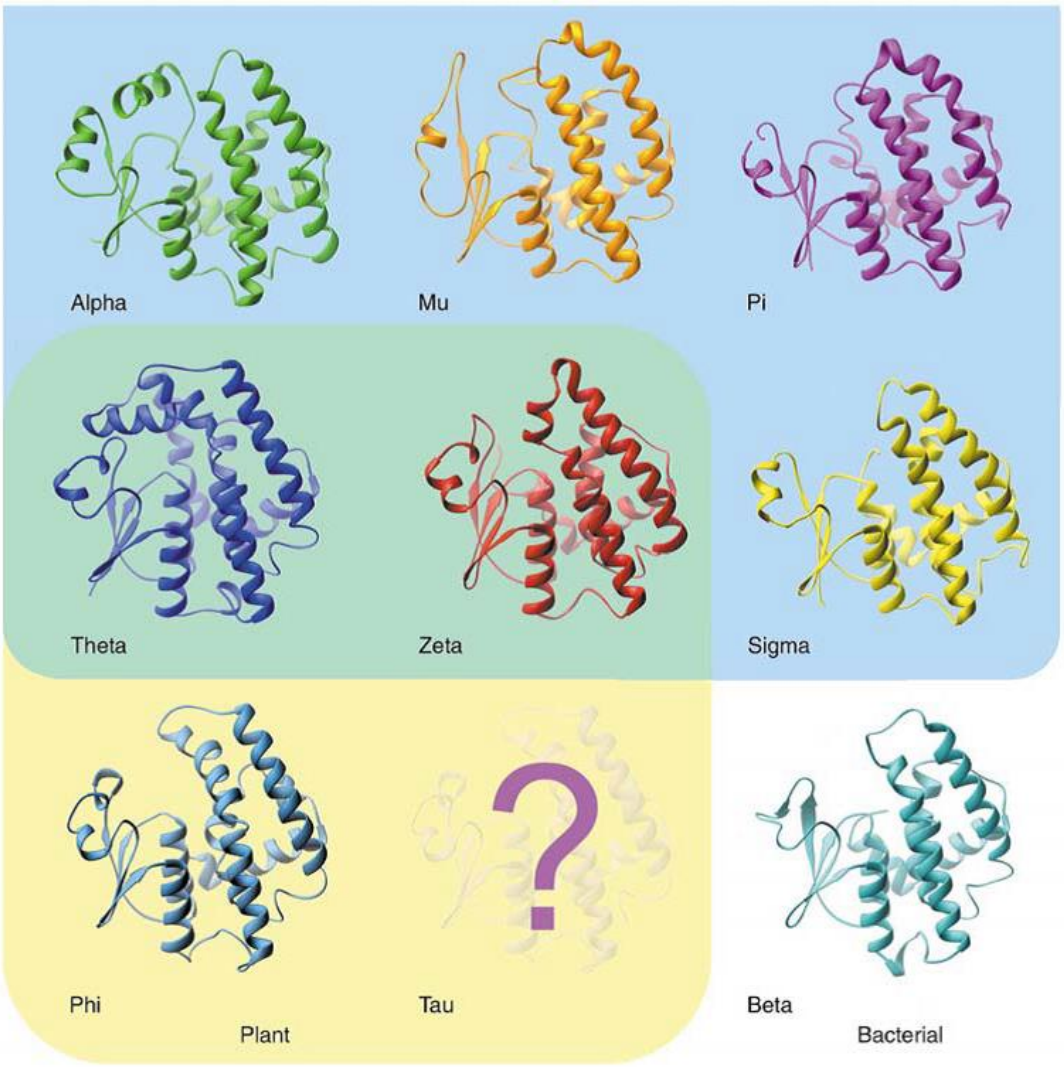


Figure 2.5. Ribbon representations of the structures of GST subunits.

The mammals GSTs known as (alpha, mu, pi and sigma) their background color is blue; while the plant GSTs are known as (phi) and bacteria GSTs known as (beta) their background color is yellow and white, respectively; GSTs (theta and zeta) that included in both animals and plants have backgrounds of green color. Even between different enzyme classes still there is a little similarity between them. There is significant conservation in overall structure (Fischer et al., 2006).

2.2.3.7.1. Properities of human glutathione S-transferase pi (π) class

A family of enzymes which is known as Glutathione S-transferase π composed of a group of enzymes that are associated with Phase II detoxification or metabolism

and it can be seen in numerous eukaryotes and prokaryotes (Townsend and Tew, 2003). The P of GSTP1 is standing for the human placenta because the human placenta was the first place where the GSTP1 was found which it about %75 of total GSTs (Kano et al., 1987). Conjunction between GSH and its electrophilic substrates is accelerated by GSTπ catalysis during contact with free radicals that are deteriorating. As well as its detoxifying activity, GSTπ also shows another prosperity which is ligand-binding properties that can initiate programmed death of the cells in case enhanced by cellular stress (Tew and Townsend, 2011).

This protein is a product that is composed only of a single gene, in which the coding is done by seven exons; it has 7.0 pI value and having a mass of 24 kDa. The Pi (π) gene is regulated by four elements which contain the following (two enhancers and one of each of AP-1 site and GC box) (Aliya et al., 2003). About two decades ago, the presence of unusual GSTπ has a relationship with tumor resistance to chemotherapy medicine, which means that this enzyme play a role in metabolism (Dong et al., 2018). The position of GSTP1 is on chromosome 11q13. It is also subject to polymorphic variation (Hayes and Pulford, 1995). The Codon number 105 residues from the active site of the GSTP1 is for binding of hydrophobic electrophiles and the catalytic activity of the substrate and stability of the heat of encoded protein can be affected by Ile-Val substitution protein (Parl, 2005). Human GSTP1 can act as a catalyst in the isomerization of 13-cisretioic acid to all-transretioic acid (Fukai et al., 1992). That is why, the many studies have been done about the GSTP role in the susceptibility of acute myeloid leukemia (Allan et al., 2001).

2.2.3.7.2. Toxicological role of human glutathione S-transferase pi (π) class in conjugation of compounds

GSTP1 has a number of functions but its main function just like other GSTs is the protection of cells, the structures of the cells such as (DNA) have been protected from the collapse by some substances which reactive compounds are an example of these substances. (MRPs) which stands for Multidrug resistance proteins are can be defined as (ABC) which is an abbreviation of ATP-binding cassette transporter proteins, they have important role in flowing out different types of exogenous and endogenous compounds, contains organic anions that can form a conjugation with many

compounds and substances such as, sulfate glucuronide and GSH (Cole & Deeley, 2006).

These two enzymes GSTP1 and MRP1 (gene symbol ABCC1) are overexpressed in the MCF7 cells, they are functioning synergistically to detoxify ethacrynic acid (Morrow et al., 1998). GSTP1 also can conjugate sulphorophane to GSH, and indeed, the studies described herein demonstrate that the GSH conjugate of SFN is transported by MRP1 (Sibhatu et al., 2008). Arsenic trioxide which is catalyzed by GSTP1 is a chemotherapeutic drug, resulting in the conjugation of GSH and arsenite forming arsenic triglutathione [As(GS)3] complex that is transport by MRP1 (Leslie et al., 2004). With this evidence, it could be said that GSTP1 catalyzes the formation of As(GS)3 from GSH and AsIII . 4-Nitroquinoline 1-oxide (4NQO) which is genotoxics, GSTP1 catalyzes the formation of a 4NQO-GS, and it depends on MRP1 for excluding them from the cell (Sibhatu et al., 2008).

One of unsaturated aldehyde product is Acrolein will lead to the cellular damage by forming adducts with proteins and it comes from cigarette smoke, Acrolein is also one of the GSTP1 substrates. Some studies have suggested that the GSTP1 family potentially saves aortal endothelial cells from acrolein, because it catalyzes the GSH conjugation reaction to form substrate (Conklin et al., 2009).

Other studies whow the GSTP1 has different binding sites and that each site is catalyzing different substrates, for example, benzyl isothiocyanate (BITC) is GSTP1 substrate and its required binding sites are Cys48 and Tyr104. On the other hand 1-chloro- 2,4-dinitrobenzene (CDNB) which is another GSTP1 substrates it requires GSTP1-Tyr103Ser and GSTP1-Tyr103Phe sites to bind (Ralat and Colman, 2004).

Many more researches have been done and realized that the GSTπ is present in large number in tumor cells and it has a relationship with the formation of cancers from different parts of our body, production of tumors and chemotherapy resistance. (Sawers et al., 2014; Sato et al., 1989) the more researches done about drug-resistant cell lines the more it realized that the GSTπ expression is increased (Su et al., 1993; Qin et al., 2002).

It realized that the GSTπ present at highr level In multidrug-resistant HL60/VCR acute myelogenous leukemia cells, if compared to the levels of GSTπ in HL60 (Zhu et al., 2002). GSTπ in tumor cells is responsible for tumor resistance and

apoptosis suppression by two major ways. First, the effect of chemotherapy drugs will be decreased GSTπ weakens the efficacy of by forcing them outside. Second, GSTπ prevents the apoptosis of the tumor cells by working as an MAPK-pathway inhibitor (Dong et al., 2018).

2.2.3.7.3. Human glutathione S-transferase pi (π) class expression in tumor cells

Generally, GSTP 1-1 is present in many different cancer cells, and the GSTP 1-1 is the most predominant isoenzymes which can be seen in many different sensitive and resistant cells of cancer (Hayes and Pulford 1995; Tew et al., 1996). The level of this isoenzyme in cells usually associated with both cell reproduction and de-differentiation. GSTP 1-1 has a property that can inhibit the effect of reactive oxygen species (ROS) on cell division and that is why GSTP 1-1 has an important role in the proliferation of the cell (McCaughan et al., 1994). In some types of cancer cells which are resistance to (DOX) abbreviation of doxorubicin, the overexpression of GSTP 1-1 is preferred, because it enhances cellular (redox) oxidation-reduction reaction status and by prevention of DOX change to semiquin and producing of superoxide anion radicals, and peroxides. So the GSTP 1-1 overexpression is the main cause in cancer cells that leads to the formation of drug resistance, is because of owing to enhance of anticancer drugs detoxification, as well as suppression ROS which can cause apoptosis in many cases. (Aliya et al., 2003).

GST-Pi is available at a high level in patients who suffering from leukemia and it has high relapse risk which means deterioration after a temporary improvement from a disease. GST-P is present in a high amount in response to insulin in many tumors (Hatayama et al., 1991). It suggests that in immunohistochemistry retrospective study that the presence of GST-Pi is a sign for some tumor progression such as astrocytomas (Aliya al., 2003). And during breast tumor researches (estrogen receptor (ER)-poor) which were to the detection of sensitivity or resistance of some anticancer drugs, results showed that there was no detection of GST-alpha in both tissues (tumoral and peritumoral). Furthermore, study proves that in tumors GST-Pi level is higher compared to that of peritumoral tissues (Albin et al., 1993).

Studies that were done for GST-Pi on (esophageal squamous cell carcinoma) could understand that the GST-Pi can be sign for being exposure to carcinogens, while it can't be used for diagnosis of developed cancer in human esophagus (Sasano et al., 1993).

2.2.3.7.4. Human glutathione S-transferase pi (π) class and drug resistance

It believes that the GSTP1-1 may have an important role in causing drug resistance. And if there is a huge number of the GST detoxification enzyme, drug resistance may occur. Three major classes of GSTs have been known which are classified as following alpha, mu, and pi, in the cancer cells, GST-pi is the most predominant form. When there are high level of GST-pi, resistance to alkylating agents will occur, examples for alkalizing agents include cyclophosphamide melphalan, and, chlorambucil. In lung cancers, GST-Pi will not show its main action when there is chemo and radio resistance, (Miyara et al., 1996) and brain tumors as GST-Pi, was found only in 38% of patients (Mousseau et a1., 1993). Oxygen radicals will be formed as a result of radiation and may kill the cells, oxygen radicals are known to be a reactive oxygen atoms that may cause damage to the DNA of tumor cells. GSTPi lowers the ROS and making them inactive and this leads to the formation of tumors radiation-resistant (Aliya et al., 2003). In many experimental systems elevation of (GST) P1-1 has been linked with cisplatin-based chemotherapy resistance and with cellular resistance to cisplatin cytotoxicity (Ban et al., 1996; Saburi et al., 1989). After finding out that the GSTP1-1 can increase the rate of (Pt-SG) formation, which stands for platinum-GSH conjugates, it suggests that a potential catalytic mechanism for GSTP1-1-associated cisplatin resistance (Goto et al., 1999). Many studies suggest that exposure to anticancer drugs have may have the ability for induction and expression of gene products that can save the cells. Tumoral overexpression of GSTs have been implicated in the resistance formation against chemotherapy, antibiotics to kill microbes, and insecticides (substance that can kill insects), herbicides use to destroy unwanted vegetation, (McLellan and Wolf, 1999).

2.2.3.7.5. Human glutathione S-transferase pi (π) class inhibitors

It suggests that GSTs Inhibitors will increase the sensitivity of cancer cells to the drugs that are used as an antitumor and that is why they may serve several therapeutic applications (Mahajan and Atkins, 2005). So for this purpose, many inhibitors for GSTS and peer to GSH have created with less toxic as well as more specific properties (Tew and Gaté, 2001; Wu and Batist 2013). Then, some different natural inhibitors seen in plants were also investigated (Singh, 2015; Harshbarger et al., 2017).

2.2.3.7.5.1. Ethacrynic acid and analogues

Ethacrynic acid, which is an α,β-unsaturated ketone, is used as a diuretic drug. It is, also, a common substrate/inhibitor of some GSTs (Figure 2.6) (Sau et al., 2010; Townsend and Tew, 2003).

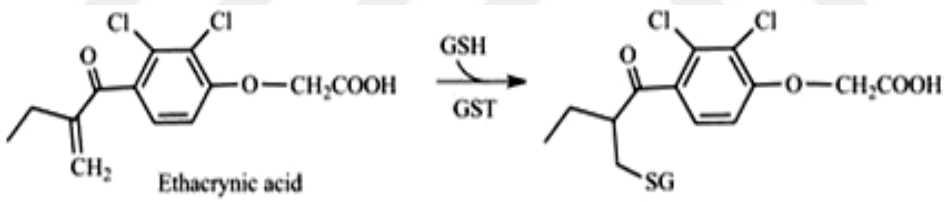


Figure 2.6. GST-catalyzed conjugation of ethacrynic acid (Sau et al., 2010).

Ethacrynic acid can cause inhibition to GSTP1-1, due to it has inhibitory properties so it has great activity against cells of human tumor, in the way that GSTP1-1 will covalently bind to the GSH-EA complex. While, this molecule has great pharmacological property on some cancers types, but still it has problems to be used in the clinic as long as it has strong diuretic properties. That is why some laboratories have created Ethacrynic acid analogues with less problematic properties. For example, when conjugation happened between ethacrynic acid and 2-amino-2-deoxy-D-glucose (EAG), it resulted in high anticancer activity just like EA but it may lead to some undesired problems such as diuretic activity (Punganuru et al., 2016). EAG and Ethacrynic acid

are structurally similar, its conjugation is done by GSH and adduct inhibits GSTP1-1 (Figure 2.7).

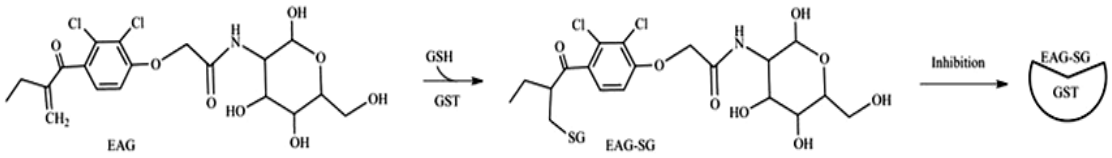


Figure 2.7. A conjugate of ethacrynic acid and glucosamine (EAG) reacts with GSH and inhibits GSTs (Punganuru et al., 2016).

More recently, the test has been done about the anticancer activity of new EA derivate about antiproliferative assays on two various tumoral cell lines (Mignani et al., 2016). Efficient anti-proliferative activities have been shown by the new molecules against human cancer cells emphasizing the potential of them as novel anticancer agents (Allocati et al., 2018).

2.2.3.7.5.2. Ethacraplatin

A bifunctional drug which known as Ethacraplatin is produced to fight against GST mediated cisplatin resistance. Ethacraplatin – prodrug of a dual-threat platinum (IV) – is a cisplatin molecule binds to two ethacrynic acid ligands which has a great ability to irreversibly inhibit GSTP1-1 as a consequence of binding enzymatic activity being reduced and cleaved (Figure 2.8) (Johnstone et al., 2016; Parker et al., 2011). So consequently it will lead to increase or enhance to Pt (platinum) ion diffusion as well as it can result in resistant of platinum drugs (Parker et al., 2011). Ethacraplatin also can revert cisplatin resistance in microsomal GST1-1 overexpressing cells (Johansson et al., 2011). Recently by encapsulating of ethacraplatin by nanoscale micelles, could reach to great drug preparation, in which results in a remarkable increase of cisplatin ability to form an accumulation in tumoral cells, and elevates the effectiveness in cells which are resistant to cicplatin, as well as decreases the toxicity (Li et al., 2017).

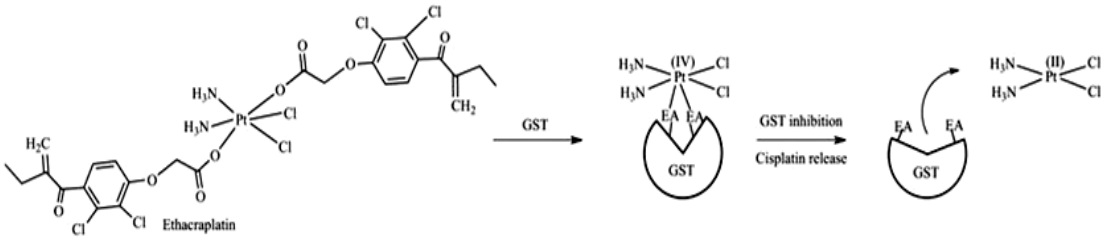


Figure 2.8. Ethacraplatin is a Pt(IV)-complex compound which contains two ethacrynic acid moieties. When exposed to GSTs, this compound inhibits the enzyme and liberates cisplatin (Johnstone et al., 2016).

2.2.3.7.5.3. NBDHEX

NBDHEX is an abbreviation that refers to 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol, which is of both GSTP1-1 and other GSTs are a highly and efficiently inhibited by (NBDHEX) and which in several cancer cells it stimulates apoptosis (Tentori et al., 2011; Graziani et al., 2015). It is mentioned that GSTP1-1 in some cancers could be overexpressed. H-site from GSTP1-1 will receipt NBDHEX and with GSH leads to the formation of a complex compound which results in inhibition of the enzyme (Figure 2.9) (Federici et al., 2009). Another important point is that it's also known that NBDHEX can separate GSTP1-1 from GSTP1-1 TRAF2 complexes that result in the activation of both TRAF2 and (c-Jun N-terminal kinase) JNK (Sau et al., 2012). On cisplatin-resistant human osteosarcoma cells, the NBDHEX is significantly active and it is shown by Drug combination studies (Pasello et al., 2008). There are some problems in the clinical use of NBDHEX and the most important one its lack of specificity for GSTP1-1. For example, this drug has a high affinity for GSTM2-2 but not for GSTP1-1(Federici et al., 2009). That is why, some novel analogues of NBDHEX have been synthesized and characterized, with better selectivity toward GSTP1-1, to provide new therapeutic options that can be used in the treatment of tumors that are drug-resistant including human melanoma (De Luca et al., 2015; Luisi et al., 2016). The MC3181 derivative was found later to be highly effective in preventing cancer growth and metastasis in a xenograft mouse model of vemurafenib resistant melanoma (De Luca et al., 2017).

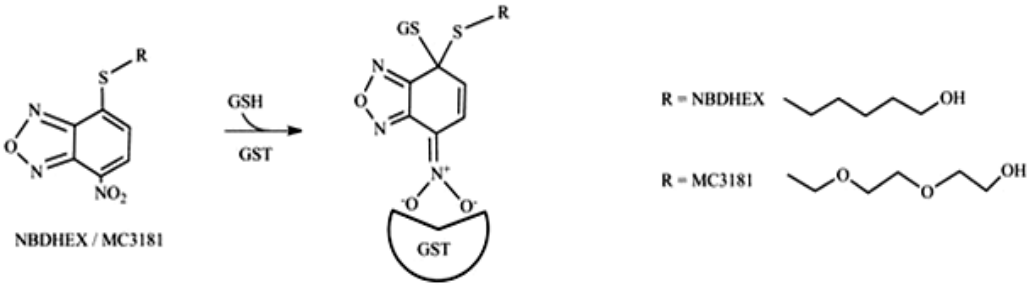


Figure 2.9. NBDHEX and its MC3181 derivative are GST inhibitors that bind the enzyme H-site and are conjugated by GSH leading to the formation of a stable σ complex (De Luca et al., 2017).

2.2.3.7.5.4. Auranofin

It is an anti-arthritic compound, that containing gold phosphine in its composition and it shows an anticancer activity which is the same as cisplatin. Auranofin is similar to other antiarthritic drugs which contain gold in some properties, while it is different from cisplatin, one of these differences is that auranofin exerts its activity through enzyme inhibition while others exert their activity through DNA damage. The latest researches illustrate that there are some enzymes which inhibited through auranofin and one of these enzymes is known as GSTP1-1 (Figure 2.10). So with cysteine mutants and wild type enzyme, the same kind of inhibitions could be seen, this is a recommendation that there is no need to thiol conjugation for inhibiting GST through auranofin, opposite to other mentioned targets (De Luca et al., 2013). Therefore, effective researches will be done to show us the exact inhibition mechanism on GSTs by auranofin. Finally, it is of notable interest that there will be a relationship between antiplasmodial effects of auranofin and GST inhibition.

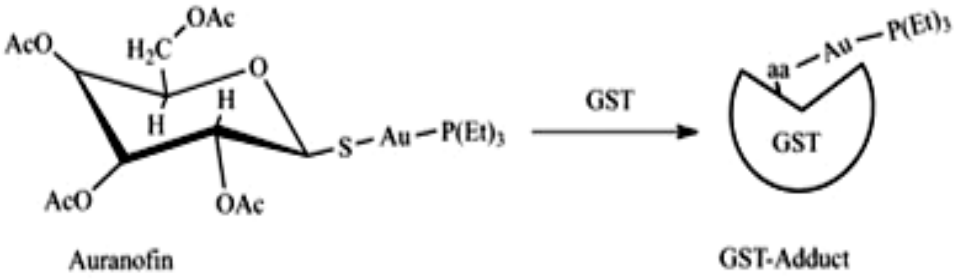


Figure 2.10. Auranofin structure and its inhibitory effect on GSTs (De Luca et al., 2013).

2.3. Kinetics of Enzyme Inhibition

The enzyme can be defined as a protein molecule which is acts as a catalyst to accelerate the enzymatic reaction. Prevention of enzymes from their activity is a science of enzyme-substrate reaction that can occur when there is any organic chemical or inorganic metal or biosynthetic compound due to their interaction with the active site of an enzyme either by a covalent or non-covalent bond. It is well known that all these inhibitors follow the same rule to interplay in enzyme reaction (Sharma, 2012).

Some substances can act on enzymes and inhibit their action and usually known as inhibitors of enzymes, they are playing an important role in pharmaceutical filed such as in human and veterinary medicine besides herbicides and pesticides. The study of Enzyme inhibition always a greatly, deep and detailed study and greatly analyzed due to its great benefit in both studies of enzyme mechanisms (Doehlert, 1987), and pharmacological studies (Sandler and Smith, 1989), while the science of activation of enzymes is not as extensive and detailed as enzyme inhibition (Saboury, 2009).

The substances that inhibit the enzymes (enzyme inhibitors) are usually chemical compounds. That has Low molecular weight. They lower the enzyme's catalytic action or they inhibit its action completely either temporary or permanently. The enzyme Inhibitor will change only one amino acid or some side chain(s) which are responsible for the catalytic action of an enzyme. For safety testing of inhibitors to value any drug type, as well as protecting the enzyme from denaturation, and protecting ligand binds that have specific side chain in an enzyme, therefore chemical changing could be done (Sharma, 2012). In the discovery of drugs, the number of drug-likeness will choose or created, which can causes inhibition to a specific enzyme. In the same

situation for testing antitoxins, to lower toxic effect, or detoxification they will undergo an examination of their inhibition activity on the specific enzymes. So, it is very important to study structure-function relationship as well as study aforesaid enzyme kinetic to learn what means by enzyme inhibition kinetics, this point by its role is very important in the industry of pharmaceuticals which have a modern design (Sami and Shakoori, 2011).

There are two types of apoenzyme inhibitors;

i) Firs type is reversible inhibitors; which their mode of action is non-permanent due to their confederation with the enzymes are temporary.

ii) Second type is irreversible inhibitors; in which they make permanent change by covalent bound of an essential residue of enzyme.

K_m and V_{max} will be affected by Apoenzyme. Metabolic antagonists other name for reversible inhibitors, which they divided for four groups: 1- competitive, 2- uncompetitive 3- noncompetitive finally 4- mixed type (Sharma, 2012).

2.3.1. Irreversible inhibitors

They composed of both nonspecific proteins denaturing agents and Specific inhibitors, example for nonspecific protein denaturing agents are acids and alkalis, while specific agents, which attack a specific area of the system. There are several groups of Specific inhibitors which are known as : (a) coenzyme inhibitors; (b) inhibitors of specific ion cofactor; (c) prosthetic group inhibitors; (d) apoenzyme inhibitors; and the last one is (e) physiological modulators of the reaction, which contain the pH and temperature that catalytic site of the enzyme will be denatured by both of them. Usually, the great numbers of irreversible inhibitors are either inhibiting the activity or destroy the enzyme, by interacting or binding with functional groups on the enzyme through a covalent bond. In the reaction mechanisms science, the irreversible inhibitors are very beneficial (Mohan et al., 2013). There are no structural relationships between the substrate and the irreversible apoenzyme inhibitors which bind covalently. The irreversible apoenzyme inhibitors also bind by a non-covalent bonds with the active site of the enzyme or break down an important functional group that is in the active site. So, irreversible inhibitors play an important role to detect

functional groups in the active sites of an enzyme at which location they bind (Sharma, 2012).

2.3.2. Reversible inhibition

Inhibitors can be defined as reversible inhibitors when they bind reversibly to an enzyme. A molecule can bind to the active site of an enzyme and act as a competitive inhibitor when it has the same structure as the normal substrate. For example, malonate and succinate dehydrogenase, malonate is a competitive inhibitor and succinate dehydrogenase is the enzyme, it has the ability to binding to the active site of the enzyme due to its close structure of malonate to the natural substrate of the enzyme succinate (Figure 2.11). Malonate can fill the succinate dehydrogenase's active site it inhibits the succinate which is a natural substrate, from binding to the enzymes active site this inhibition can cause slowing down the rate of oxidation of succinate to fumarate (i.e. inhibiting the reaction) (Robinson, 2015).

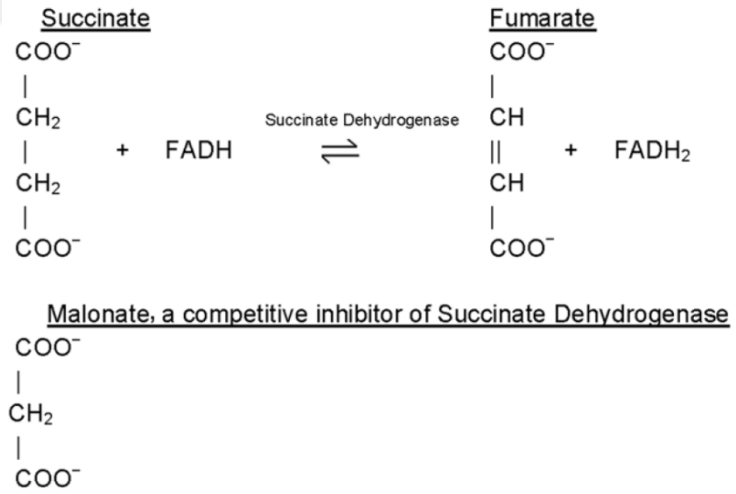


Figure 2.11. Reversible inhibition (Robinson, 2015).

According to the particular substrate, reversible inhibitors can be classified into several types such as competitive, noncompetitive, or uncompetitive inhibitors relative. Products that produced as a result of enzymatic reactions can act as reversible inhibitors of the enzymes. The decrement in the enzyme activity rate caused by the accumulation of its product plays an important role in the balance and least expensive usage of

metabolic pathways. For example, the accumulation of glucose 6-phosphate can inhibit hexokinase activity (Sharma, 2012).

They bind to an enzyme with several types of noncovalent interactions, which include hydrogen bonds, ionic bonds, and hydrophobic interactions (Figure 2.12) (Mohan et al., 2013).

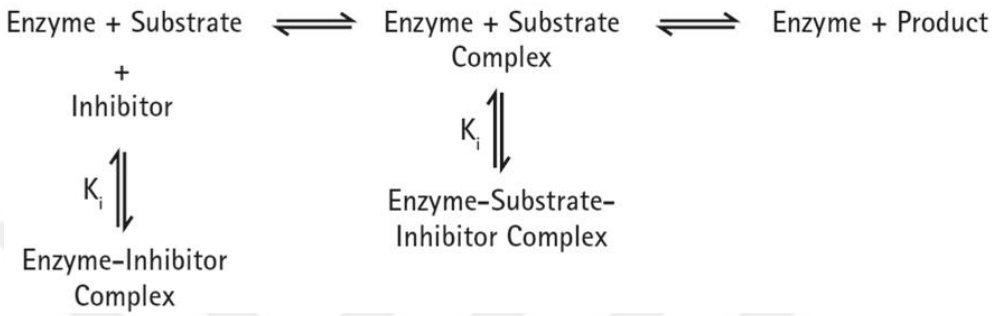


Figure 2.12. A general scheme of reversible enzyme inhibition (Mohan et al., 2013).

2.3.2.1. Competitive inhibitors

In this type of inhibition, there is often a structural likeness between natural substrate and inhibitor, so as a result inhibitor compound will compete against the natural substrate to reach the enzyme's active site (Figure 2.13). As a condition, if the inhibitor compound more strongly binds to the active site that of the natural substrate it will be a strong competitive inhibitor. On the other hand, if the inhibitor molecule weakly binds to enzymes active sites they will know as a poor inhibitor. In the case of competitive inhibition, there is the only free site of enzyme which inhibitor allowed to be attached, which means inhibitor could not bind to the enzyme-substrate complex. Consequently, it suggesting that if the amount of substrate was increased through mixture then the inhibition process could be controlled. In the case if competition was absence against substrate, then it could still reach to the V_{max}, while if inhibitor was present it needs to elevate the substrate volume in order to reach to get V_{max}, consequently it will result in increasing the value of K_m (Mohan, et al., 2013; Sharma, 2012).

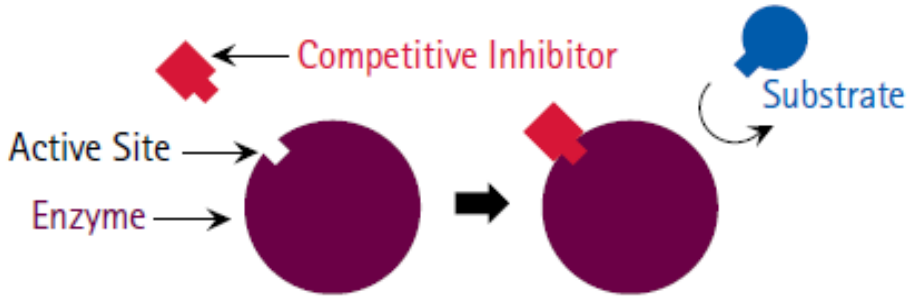


Figure 2.13. A simplified model of competitive inhibition (Mohan et al., 2013).

For competitive inhibition, we can find inhibition constant (Ki), the inhibition constant, that roles as separation constant for the enzyme-inhibitor complex. When K_i value decreased, the amount of inhibitor which is necessary for lowering the rate of reaction could decrease also. (Figure 2.14) illustrates that relation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$+$$

$$I$$

$$K_i \Updownarrow$$

$$EI$$

$$K_i = \frac{[E][I]}{[EI]}$$

Figure 2.14. The result of competitive inhibition can be presented in a Lineweaver-Burk plot.

During competitive inhibition, K_m rises up when inhibitor present then X-intercept will become closer to the origin. When the volume of inhibitor elevates, that results in rising of K_m furthermore, as well as X-intercept, goes closer to origin. Due to competitive inhibitor will not change V_{max}, so all lines will pass the same Y-intercept (Mohan et al., 2013).

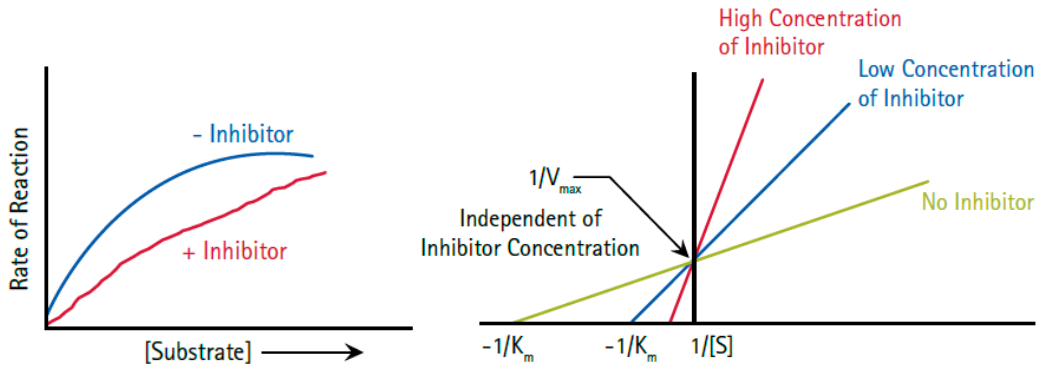


Figure 2.15. Kinetics of competitive inhibition (Mohan et al., 2013).

An example of competitive inhibitor would be anticancer agent methotrexate. That performing the inhibition for enzyme dihydrofolate reeducates, which participates in the synthesis of tetrahydrofolate. Another example would be sildenafil citrate drug, in which its structure is similar to cyclic- GMP (cGMP), that performing inhibition competitively to phosphodiesterase V enzyme, that leads to accumulation of cGMP and results in relaxation of smooth muscles (Mohan et al., 2013).

2.3.2.2. Uncompetitive inhibitors

This type of inhibition, the inhibitors will bind only to enzyme-substrate complex. The difference between uncompetitive inhibition and noncompetitive inhibition is that these inhibitors do not bind to the active site of the enzyme, as well as it is not conditioned for inhibitor to be similar to the substrate. During uncompetitive inhibition, V_{max} will decrease as long as activated enzyme-substrate complex removed. It is known that ESI complex volume depends on inhibitor concentration. K_m will decrease as a consequence of ES complex removal. Simultaneously both K_m and V_{max} will decrease in uncompetitive inhibition at the same rate, which means V_{max}/ K_m is unchanged. (Figure 2.16) shows that with uncompetitive inhibitor, $1/V_{max}$ is increased. Consequently, the Y-intercept moves up. Inhibition also rises $1/K_m$ to a degree that maintains the ratio of Km/V_{max}, which is the slope of the curve. That is why, in uncompetitive inhibition, the weaver-Burk plot lines are parallel to each other, even in the case of inhibitor existence and in the absence of inhibitor. Relatively uncompetitive inhibition is rare, however, it could happen in those systems that contain a metameric

enzyme, this is an important evolutionary point due to uncompetitive inhibition in the metabolic process has a larger influence on the concentration of intermediates during metabolism, than competitive inhibition, as well as may result in elevating of toxicity. Oxalate is an example of an uncompetitive inhibitor that results in lactate dehydrogenase inhibition (Mohan et al., 2013).

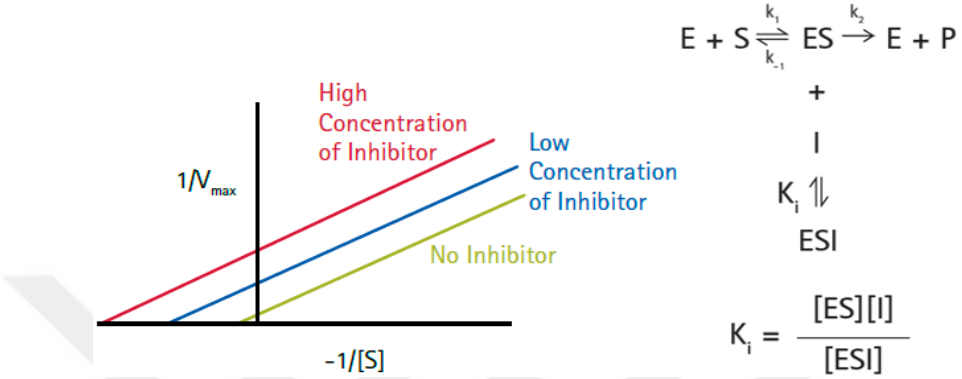


Figure 2.16. Kinetics of uncompetitive inhibition (Mohan et al., 2013).

2.3.2.3. Mixed type inhibitors

There are some situations, which inhibitor binds to the free enzyme which means enzyme without binding to the substrate (with a dissociation constant Ki) and also enzyme-substrate [ES] complex which means enzyme binding to the substrate (with dissociation constant Ki'). However, they have a different affinity, hence, Ki ≠ Ki'. Here, the binding of the inhibitor will become lower by the addition of more substrate, in the competitive inhibition will be overcome totally but in mixed-type inhibitor inhibition cannot be overcome. It is believed that mixed-type inhibitors will intervene with binding beside of that it will lower the effectiveness of turnover. Mostly these inhibitors are allosteric in nature, it means the inhibitor will tie up to any site of the enzyme rather than active site, which results in a conformational change in enzyme structure, this is by its turn reduces substrate association with the active site. That is why V_{max} is decreased, while Km increased (Figure 2.17) (Mohan et al., 2013). An example of mixed-type inhibition is that of xanthine oxidase by palladium ions.

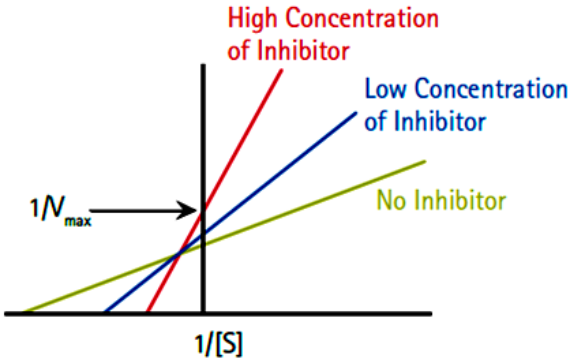


Figure 2.17. Kinetics of mixed type inhibition (Mohan et al., 2013).

2.3.2.3.1. Noncompetitive inhibitors

Noncompetitive inhibitors reduce enzyme activity by binding to them, and it does not effect on substrate binding. (Figure 2.18). it means that the inhibition level only dependent on the inhibitor concentration. These inhibitors (non-competitive inhibitors) bind to the sites of the enzyme by non-covalent bond but it is not competing with the substrate to the substrate- binding site. Inhibitor binding does not influence the availability of the binding site for the substrate. Hence, there is no relationship between the binding of the substrate and the, and inhibition cannot be reduced by substrate concentration increment. Noncompetitive inhibitors affinity for the enzyme are same affinity of an inhibitor to enzyme-substrate complex; therefore, Ki = Ki'. Hence, V_{max} is reduced, but K_m remains constant. V_{max} cannot be attained in the presence of a noncompetitive inhibitor (Mohan et al., 2013). Equilibrium for noncompetitive inhibition can be simplified as follows:

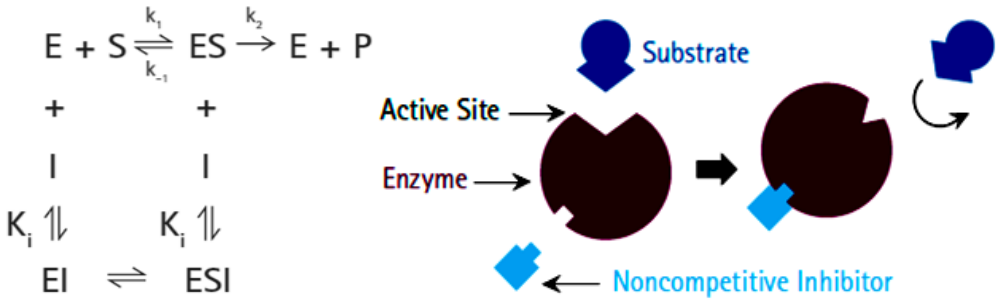


Figure 2.18. A simplified model of noncompetitive inhibition (Mohan et al., 2013).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Experimantal equipments

Brown glass bottle, spectrophotometer, 2ml Eppendorf tube, sonication machine, vortex, 500 µl & 1000 µl pipette, blue and yellow pipette tips, incubator, refrigerator, steel Micro Spatula Medicine Spoons.

3.1.2. Benzothiophene derivatives

In this study, two benzothiophene derivatives, whose antioxidant capacities and inhibitory/activatory effects will be tested, were synthesized by Prof. Dr. Arif KIVRAK and his group in the Chemistry Department of Van Yüzüncü Yıl University.

3.1.3. Other chemicals

Ascorbic acid, DPPH 2,2-diphenyl-1-picrylhydrazyl, ammonium molybdate, galvinoxyl, ABTS 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), quercetin, trolox, pure ethanol, trisodium phosphate, human Glutathione S-transferase pi 1 (GSTP1) (60.45 units/mg protein, 2.51 mg/mL stock), 1-Chloro-2,4-dinitrobenzene (CDNB), benzothiophene derivative one, benzhothiophene derivative two.

3.2. Methods

3.2.1. Synthesis and NMR analysis results of derivative-1

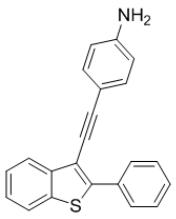


Figure 3.1. (derivative-1) 4-((2-phenylbenzo[*b*]thiopen-3-yl)ethynyl)aniline.

3-iodo-2-phenyl benzo[b]thiophene (0.7mmol, 235mg) was poured into 50ml balloon flask; and 7 ml of DMF and 3 ml of Et_3N was dissolved in it, under argon gas pressure. Then, $Pd(PPh_3)CI_2$ (0.035mmol, 24,5 mg), para-etinilanilin (0.75mmol, 87.8 mg) and CuI (0.035mmol, 6.7 mg) were added. The mixture was stirred overnight by argon flushing. The organic phase was washed by ethyl acetate and salted water; and, it was dried by Mg_2SO_4. Under low pressure applied by rotary evaporator, the solvent was vaporized and the dried organic phase was handled by n-hexane in 58% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.57-3.95 (brs, 2H), 6.50-6.74 (m, 2H), 7.30-7.68 (m, 6H), 7.72-7.93 (m,1H), 8.00-8.43 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 82.14, 95.87, 112.47, 114.27, 114.82, 122.13, 123.47, 124.90, 125.27, 128.38, 128.64, 128.71, 132.98, 134.14, 137.57, 141.21, 144.73, 146.97.

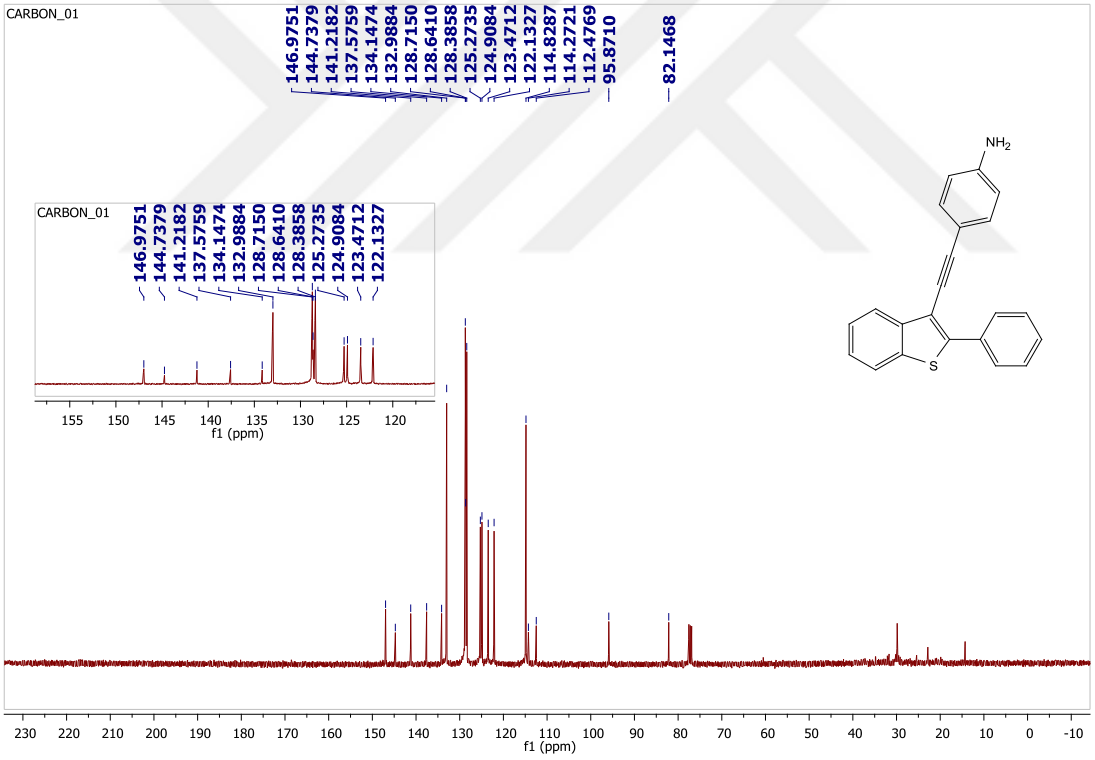


Figure 3.2. ^{1}H NMR spectrum of derivative-1.

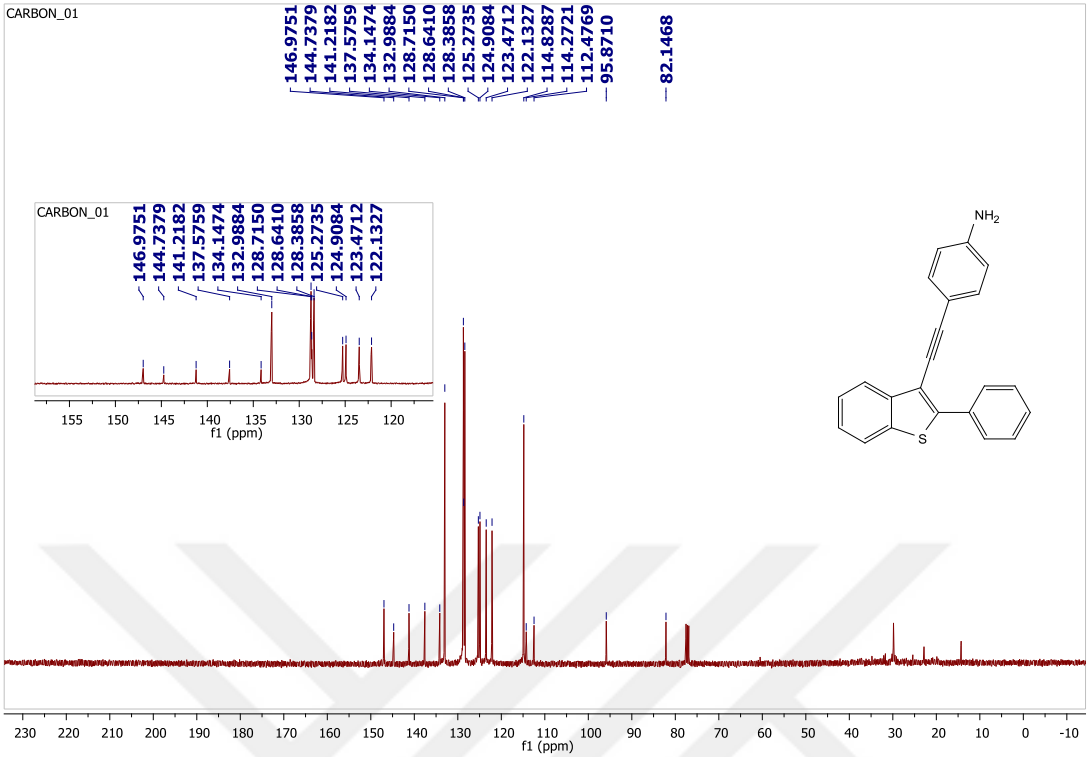


Figure 3.3. ^{13}C NMR spectrum of derivative-1.

3.2.2. Synthesis and NMR analysis results of derivative-2

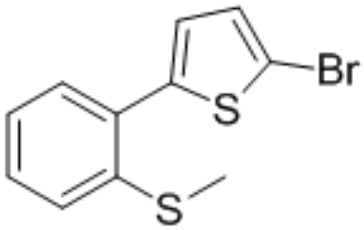


Figure 3.4. (derivative-2) 2-bromo-5-(2-(methylthio)phenyl)thiophene.

2-(2-(metylthio(phenyl)thiophene (0.05 mmol,103.2 mg) was dissolved by 20ml of chloroform, under argon; and, the temperature was decreased to 0˚C by ice bathing. N-bromosuccinamide (100 mg, 0,56 mmol) was added bit by bit. The mixture was stirred in the ice bath for 2 hours and extraction was performed by adding 10 ml of double- distilled water. The organic phase was dried by Mg_2SO_4 and filtered. The filtrate was dried by evaporator under low pressure and the targetted product was handled 99% yield. ^1H NMR (400 MHz, $CDCl_3$) δ 2.34 (s, 3H), 7.02 (d, J = 3.8 Hz, 1H), 7.06 (d, J = 3.8 Hz, 1H), 7.19 (td, J = 37.2-1.6 Hz, 1H), 7.28-7.38 (m, 3H). ^{13}C

NMR (100 MHz, $CDCl_3$) δ 16.27, 112.63, 124.98, 126.09, 127.83, 128.76, 129.84, 130.63, 132.47, 137.80, 142.84.

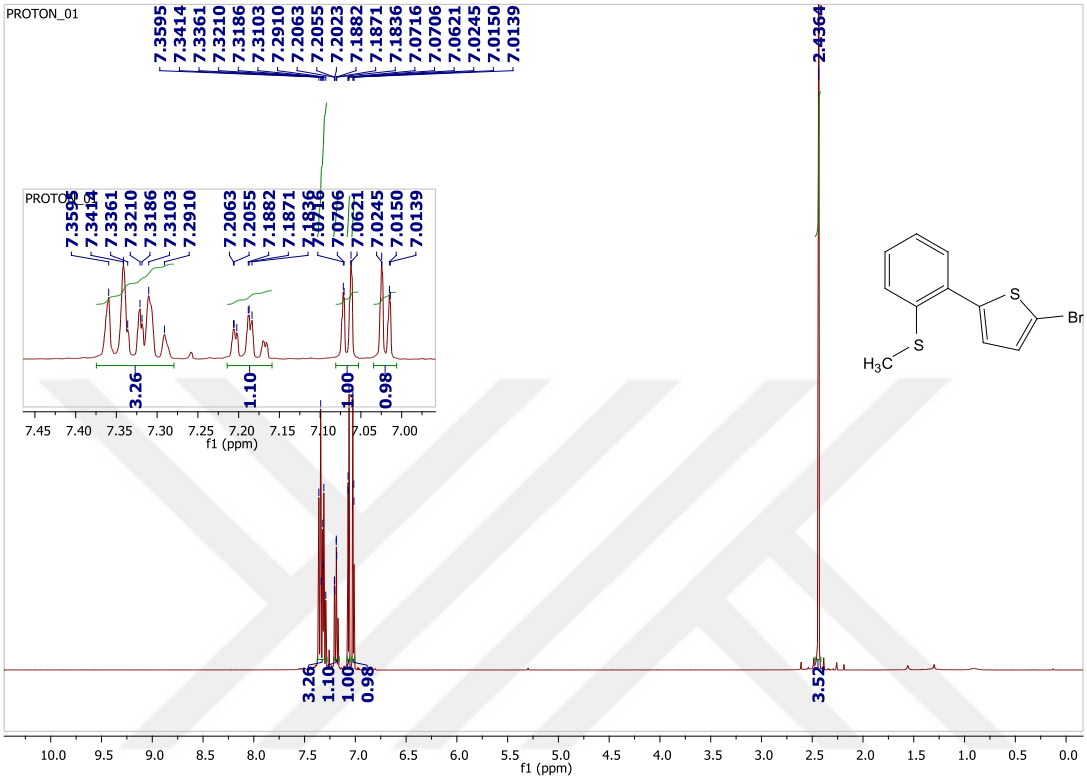


Figure 3.5. ^{1}H NMR spectrum of derivative-2.

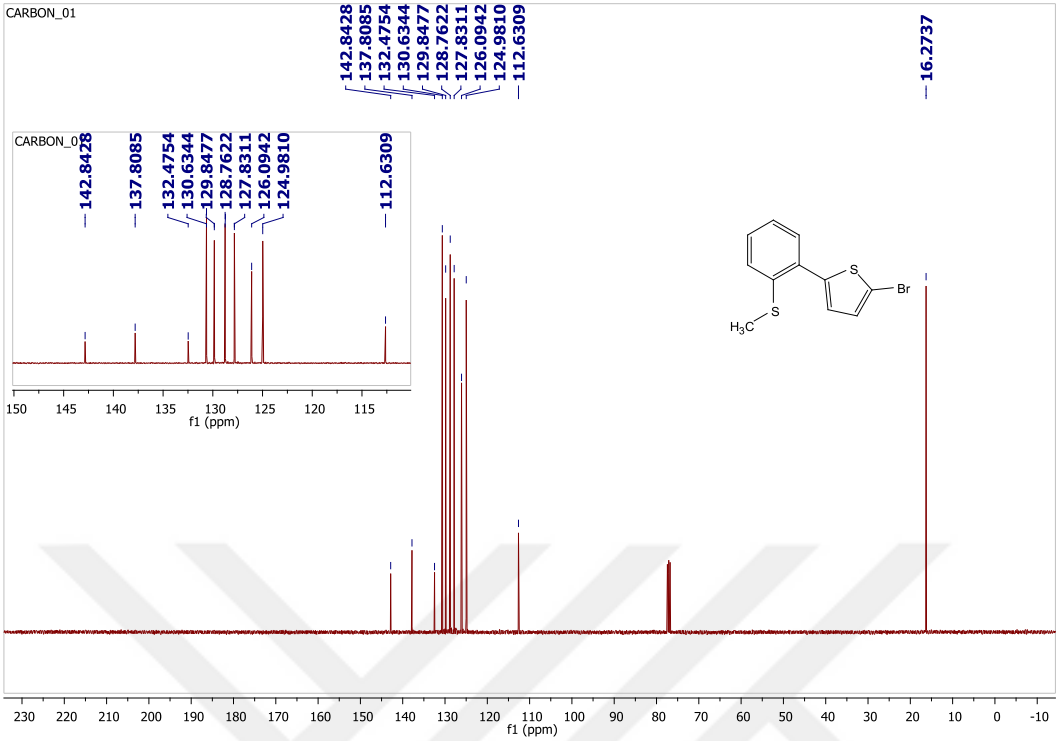


Figure 3.6. ^{13}C NMR spectrum of derivative-2.

3.2.3. GST enzyme activity assays and inhibition modelling

By method which had illustrated by Habig et al. The specific activity of GST enzyme has been measured, and then adapted to the ELISA Plate Reader System (Yilmaz *et al*). 100 mM phosphate buffer (pH: 7.0) in the control groups were used, 1mM GSH and 3 different CDNB substrate concentrations (0.5mM, 1mM and 1.5 mM) were used, and in the 96-well microplates had prepared to test different concentrations of benzothiophene derivatives. Each assay was prepared in triplicate: 3 wells had the same reaction medium and a specific blank was prepared. In the case measurements of derivatives, 7 different concentrations were taken between 0 micromolar and 250 micromolar, thus this procedure had been repeated for 3 different final CDNB concentrations. Activity measurements were started as the enzyme had added and recorded for 10 minutes at 30sec intervals and the results which gained were solved by authorized software, Sigma Plot 13 with Enzyme Kinetics Module, and the optimal inhibition model was found.

It is important to mention that, for testing antioxidant capacity of derivative-1 and derivative-2, four methods for this purpose have used, because the reaction behave

of these two derivatives are unknown, as well as behave of reaction medium to these derivatives unknown, maybe some unwanted reaction between derivatives and medium will happen to cause in showing low antioxidant activity or maybe opposite, so it is tried to testing both of them in four different assays for sure, and to get correct result.

3.2.4. Antioxidant assays

3.2.4.1. DPPH method

Blois's (1958) DPPH method was modified to determine the radical scavenging capacity of derivative-1 and derivative-2.

DPPH was dissolved in clean vials with a concentration of 400 µM in brown vials. DPPH put in ultrasonicator for complete dissolving for 30 minutes. For dissolving both Derivative-1 and Derivative-2, the same amount of pure ethanol was used which was 4ml, trolox, and ascorbic acid solutions used as standards, 0.001g of both, as trolox and ascorbic acid were dissolved in 10ml of ethanol. All dilutions were made with pure ethanol. A concentration range for ascorbic acid (5.323µM- 85.168 µM), for trolox (3.75 µM- 60 µM), for derivative-1 (57.609 µM- 921.75 µM), for derivative-2 (65.737 µM- 1051.8 µM) had established. Five separate concentrations were generated in the dilutions. Three samples were prepared for each test as blank. For each sample, 900µl of substance (ethanol for the blanks) and 300 µl of DPPH had been added. The vials were incubated at room temperature for 30 minutes after the caps of vials were closed. After incubation, the UV / VIS spectrophotometer was read against the reference (1200 µl of ethanol) at 517 nm. Ascorbic acid and Trolox were used as standard. Whole measurements have done in triplicate. Radical scavenger activity (% RSA) was calculated by the Equation (1), mentioned below and compared with trolox and ascorbic acid.

$$\%RSA = \frac{(ABS_{Blank} - ABS_{Sample/\,S\tan dart})}{ABS_{Blank}} x100 \qquad (1)$$

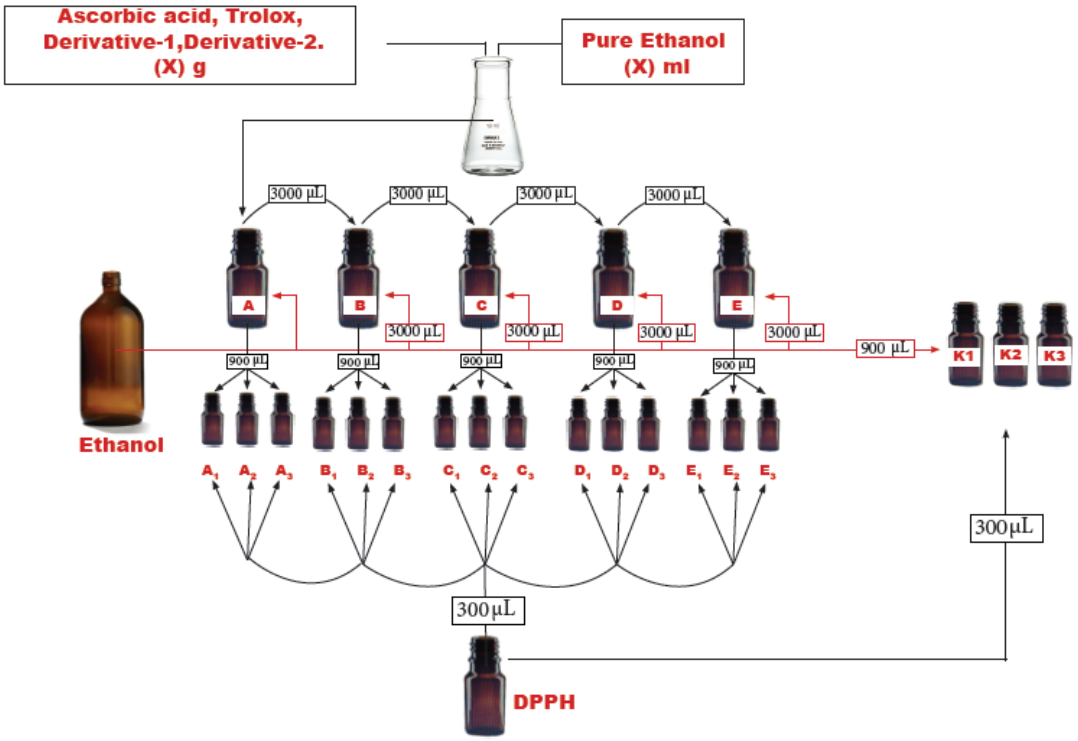


Figure 3.7. DPPH assay procedure.

3.2.4.2. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) method

Depending on the modified method via (rea et al., 1999), antioxidant capacities of synthesized chemicals could determine, the used antioxidant standards for this procedure were ascorbic acid, trolox, and quercetin. ABTS (7 mM) and potassium persulphate (2,45 mM), it has prepared by dissolving them in distilled water and incubated for 12-16 h at room temperature. Both derivatives one and two have prepared in ethanol, as well as standards prepared in pure ethanol, Dilutions were made by using pure ethanol at five different concentrations. Concentration range for ascorbic acid (0.887µM- 14.194 µM), for trolox (0.625 µM- 10 µM), for quercetin (0.129 µM- 2.067 µM), for derivative-1(3.840 µM- 122.9 µM), and for derivative-2 (43.825 µM- 701.2 µM) had established. Three samples were prepared for each test as blanks. Before the experiment, an intermediate stock was prepared from the ABTS stock solution with ethanol at an absorbance of 0,700-0,710 at 734 nm. 600 µl of substance (ethanol for the blanks) and 600 µl of diluted ABTS were then added for each sample. The vials were left at room temperature for 30 minutes after capping. After incubation, the UV / VIS

spectrophotometer was read against the reference (1200 µl of ethanol) at 734 nm. Ascorbic acid, Trolox, and quercetin were used as standards. whole measurements were done in triplicate. Radical scavenging activity (% RSA) for both derivatives one and two was calculated as indicated in equation (1), from the top and compared with ascorbic acid, trolox, and quercetin standards.

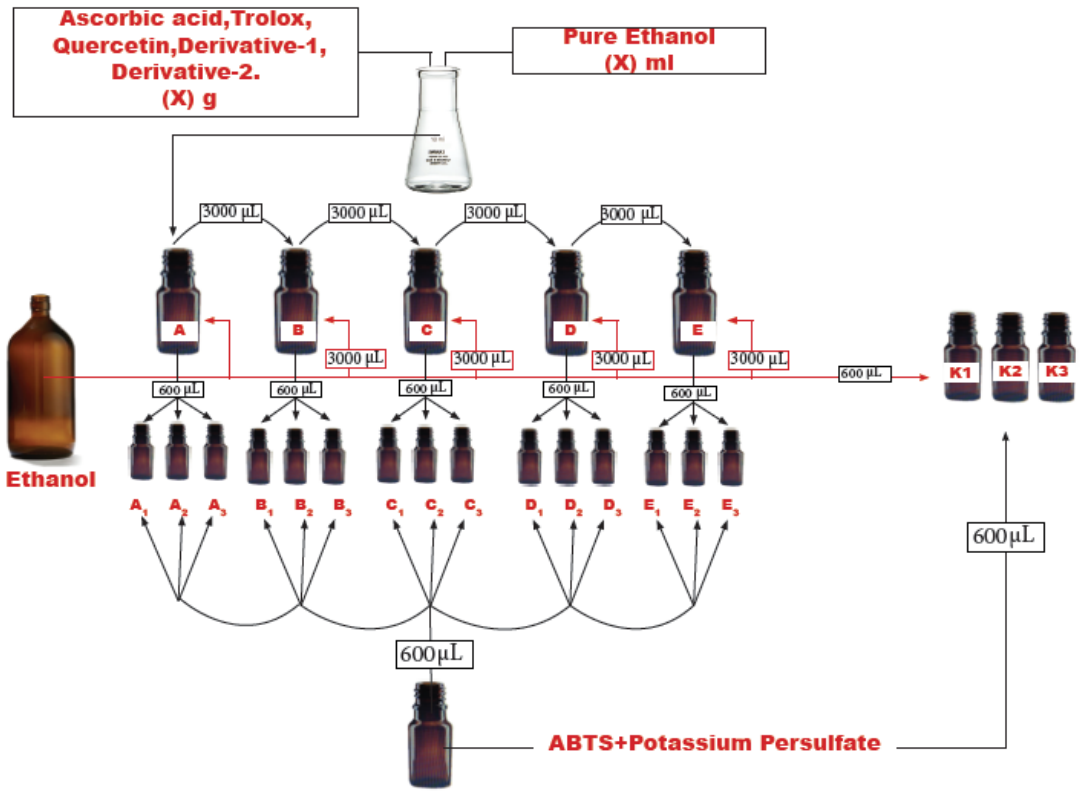


Figure 3.8. ABTS assay procedure.

3.2.4.3. Galvinoxyl method

Shi and Niki (1998) were applied by optimizing the galvinoxil method. In this method, the 160 µM galvinoxil solution was prepared with ethanol. The substances tested derivative-1, derivative-2, trolox and quercetin were dissolved in pure ethanol. dilutions were made with pure ethanol to five different concentrations.

A concentration range of 14.630 µM - 234.095 µM for derivative-1, 3.339 µM - 53.424 µM for derivative-2, 4.761 µM - 76.190 µM for trolox, and 0.196 µM - 3.151 µM for quercetin were established. Three samples were prepared for each test as blanks.

1000 µl of substance and 50 µl of galvinoxyl solution prepared at different concentrations were added to each tube. The tubes were incubated for 30 minutes in the dark and the spectrophotometer was read at 428 nm against the reference (1050 µl ethanol). For the blanks, 1000 µl ethanol and 50 µl galvinoxyl solution were added to the dark tubes to read against the reference. whole measurements had done in triplicate. Trolox and quercetin were used as standard. (% Radical scavenging activity) was calculated by the equation (1), mentioned above, and antioxidant potentials of the samples were compared with trolox and quercetin.

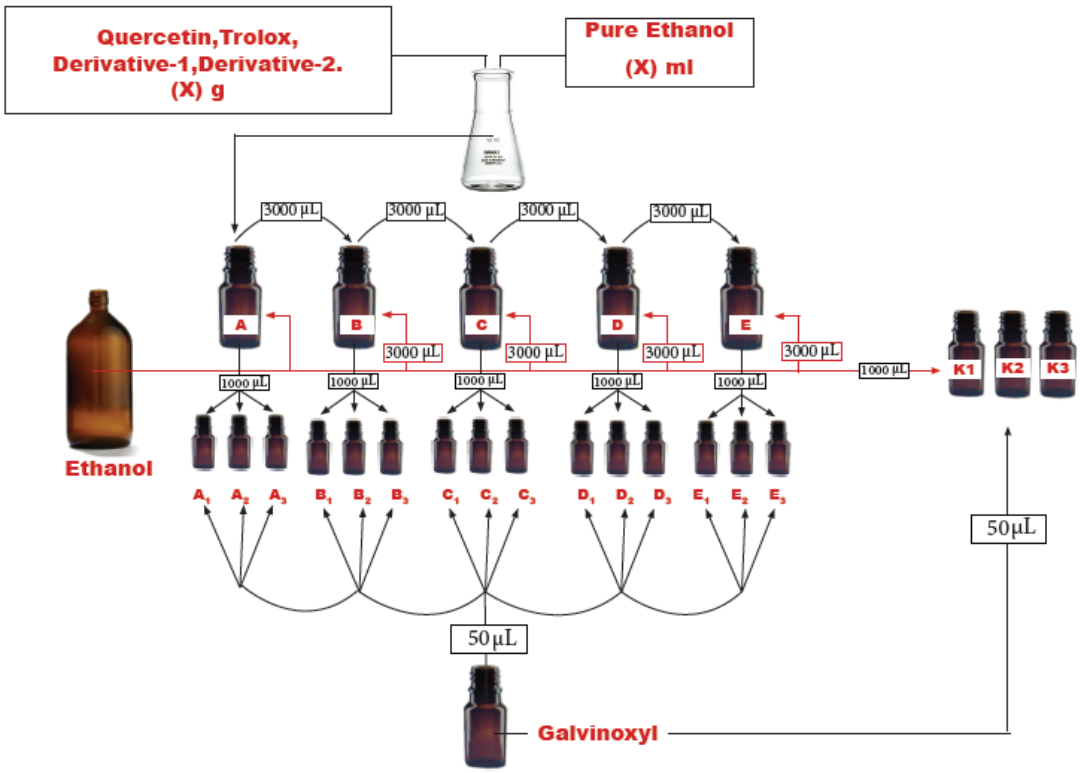


Figure 3.9. Galvinoxyl assay procedure.

3.2.4.4. Phosphomolybdenum method

The main principle of this method is converting Mo (VI) to Mo (V) by the aid of antioxidant compound which leads to the production of green phosphate Mo (V) compound in acidic medium. This method was modified by Prieto et al. (1999). The radical mixture solution required for this method was prepared with distilled water (dH_2O) containing 28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M

sulfuric acid. Derivative one, derivative two, trolox, quercetin and ascorbic acid were dissolved in ethanol. Dilution with pure ethanol was performed to five different concentrations.

7.681 µM - 1229 µM for derivative-1, 87.65 µM -1402.4 µM for derivative-2, 0.5 µM -8 µM for trolox, 2.067 µM - 33.086 µM for quercetin, and 3.548 µM - 56.779 µM for ascorbic acid were established. Three samples were prepared for each test as blank.

To each Eppendorf tube 900 µl radical mixture solution and 100 µl of different concentrations of diluted derivative one and two were added, then incubated for 90min at 95^{o}C in hot block, After incubation, the samples were allowed to stand and cooled for 25-30 minutes at room temperature and their absorbance was read on a spectrophotometer against a blind tube at 695 nm wavelength. All measurements were performed in triplicate. The values obtained were expressed as ascorbic acid equivalent, Trolox equivalent; Quercetin equivalent, standard curves prepared at different concentrations. Trolox, quercetin, and ascorbic acid were used as standards.

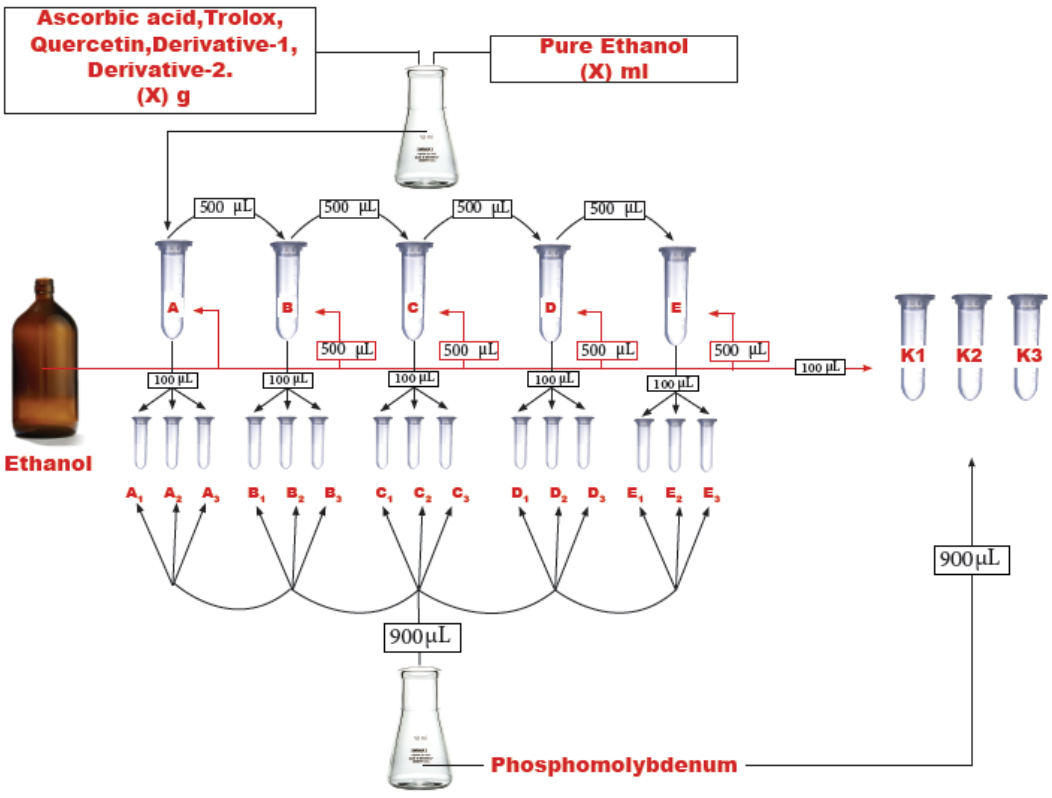


Figure 3.10. Phosphomolybdenum assay procedure.

4. RESULTS AND DISCUSSION

4.1. DPPH Assay Results

During this assay, electrons were transferred and caused the formation of a violet solution in ethanol, DPPH (2,2-diphenyl-1- picryl-hydrazyl-hydrate) free radical, it is stable at room temperature, it can be reduced with the existence of an antioxidant molecule, change its color from violet to colorless in the ethanol solution. Thus DPPH assay was used because it was an easy and rapid method that gives a clear evaluation of antioxidants by spectrophotometry at 517 nm (Huang., 2005).

This method is based on the principle that the amount of radical scavenged increases as the antioxidant substance increases, thus increasing the RSA%. This is seen in the following ascorbic acid and trolox standard curves (Figure 4.1) and (Figure 4.2).

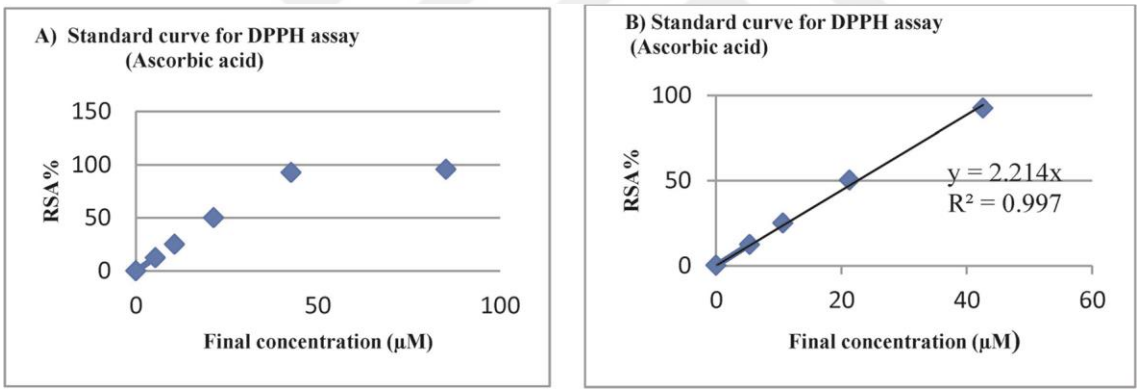


Figure 4.1.Standard curve of Ascorbic acid for DPPH assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}= 22.583 Mm.

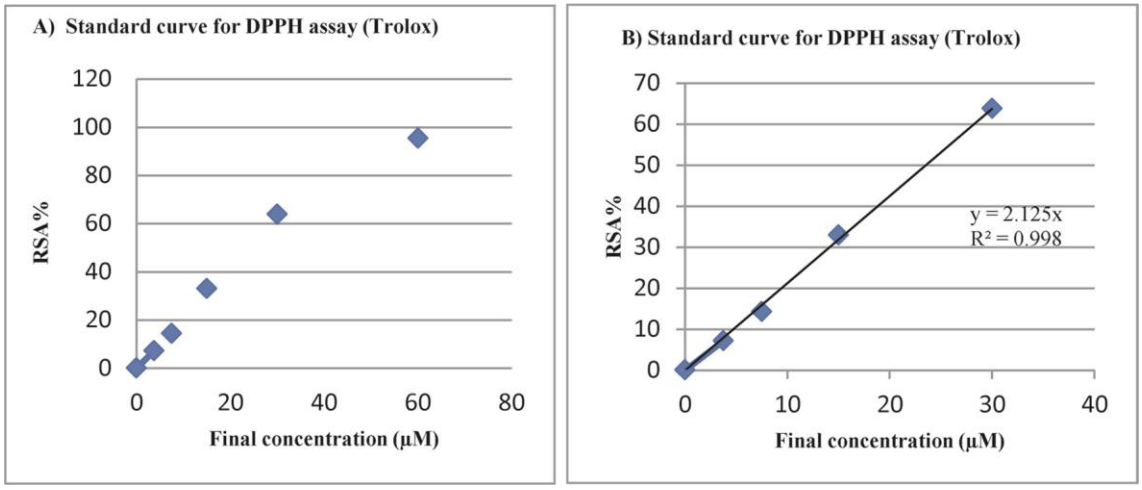


Figure 4.2.Standard curve of Trolox for DPPH assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B) EC_{50}=23.529 µM.

When specifying the radical scavenging activity of the antioxidant substance, the EC_{50} value is used to express the amount of antioxidant that sweeps 50% of the radical present in the medium. A low EC_{50} value indicates high antioxidant activity. The EC_{50} values of ascorbic acid and trolox used as standards, they were found to be 22.583 µM and 23.529 µM from the graphs (Figure 4.1, Figure 4.2), respectively.

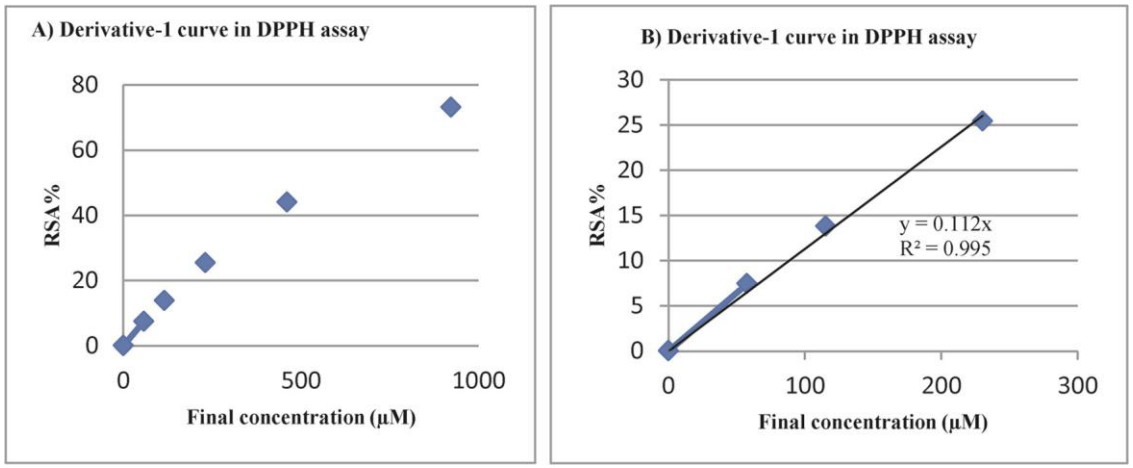


Figure 4.3.Derivative-1 curve for DPPH assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50} = 446.428 µM.

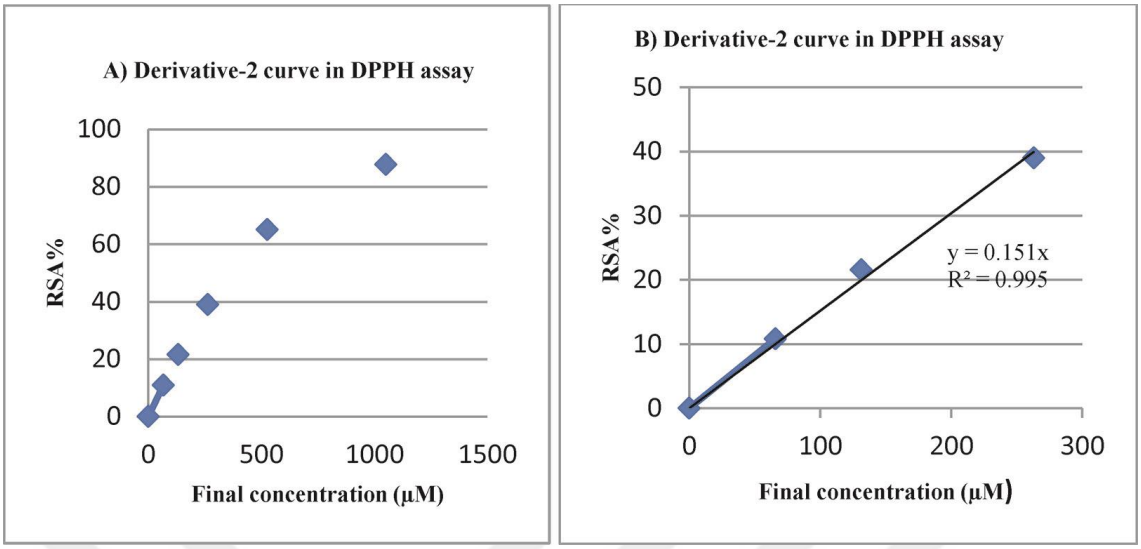


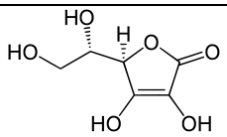
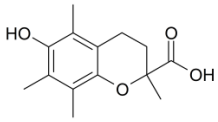
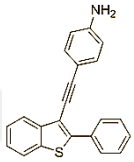
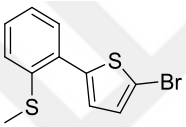
Figure 4.4. Derivative-2 curve for DPPH assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation EC_{50} value (B). EC_{50}= 331.125 µM.

The EC_{50} values stated that ascorbic acid had higher antioxidant capacity than trolox, as well as from both derivatives. It means that standards were comparable to each other, as well as the reaction medium and standards worked properly. In other words, assay was proper for testing both derivatives, then, for comparing them with standards.

Both derivatives showed very low antioxidant activity through DPPH assay. Their EC_{50} values were very high. They were not comparable to standards for total antioxidant capacities. Derivative-2 showed higher antioxidant activity in DPPH assay according to its EC_{50} value which was lower than that of Derivative-1. It might be because of its being a better proton acceptor/donor than Derivative-2.

DPPH radical does not react with aromatic acids that have single hydroxyl group in the benzene ring and with flavonoids that do not contain any hydroxyl group (Selçuk, 2012). Both of the benzothiophene derivatives tested don't contain strong electron donator/acceptor sites, and this might be the reason why they did not show high antioxidant activity in this assay.

Table 4.1. The comparison of the antioxidant capacities of standards and derivatives in DPPH assay

Standards and Derivatives	Structure	Molecular Weight	EC_{50} value	"RSA% / Highest Applied Concentration" Value
Ascorbic acid		176.12 g/mol	22.583 μM	-
Trolox		250.29 g/mol	23.529 μM	-
Derivative-1		325.426 g/mol	446.428 μM	-
Derivative-2		285.223 g/mol	331.125 μM	-

The overall ranking of increasing antioxidant capacities of standards and tested compounds were found to be Ascorbic acid > Trolox > Derivative-2 > Derivative-1, by DPPH assay. DPPH assay didn't worke for quercetin standard.

4.2. ABTS Assay Results

This method originally relies on the activation of metmyoglobin, which acts as a hyperoxidase in the existence of H_2O_2 with the production of ferryl myoglobin root which then causes the oxidation of phenothiazine ABTS, and thus $ABTS^{\bullet+}$ radical cation formation is performed (Miller et al., 1993:Rice et al., 1994).

ABTS cation root ($ABTS^{\bullet+)}$ (Marc et al., 2004), which is seen as bluish-green, absorbs light at 743 nm. In the presence of trolox (or other antioxidants that donate hydrogen), the nitrogen atom in the structure of ABTS works to irrigate the hydrogen atom, which removes the color solution.

ABTS is oxidized by potassium sulfate (Pellegrini., 2003), resulting in the appearance of the radical of the ABTS cation ($ABTS^{\bullet+}$), and it is noted that in the case of trolox defined as a standard antioxidant, its absorption decreases at 743 nm (Pellegrini et al., 2003). The idea of this test is that antioxidants can inhibit radical cation absorption ($ABTS^{\bullet+}$) indicating maximum absorption at 415 nm, and second

maximum absorption at 660, 734, 820 nm. This method is based on the principle of increasing the amount of radical scavenged as the antioxidant capacity increases. This is clearly seen in the standard curves of ascorbic acid (Figure 4.5), quercetin (Figure 4.6), and trolox (Figure 4.7) below.

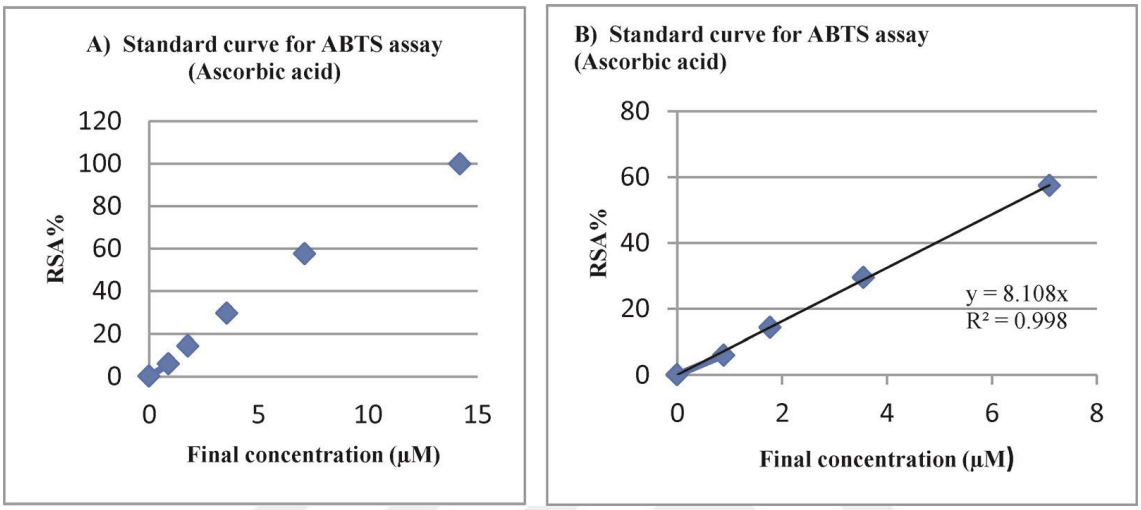


Figure 4.5. Standard curve of Ascorbic acid for ABTS assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}=6.166 µM.

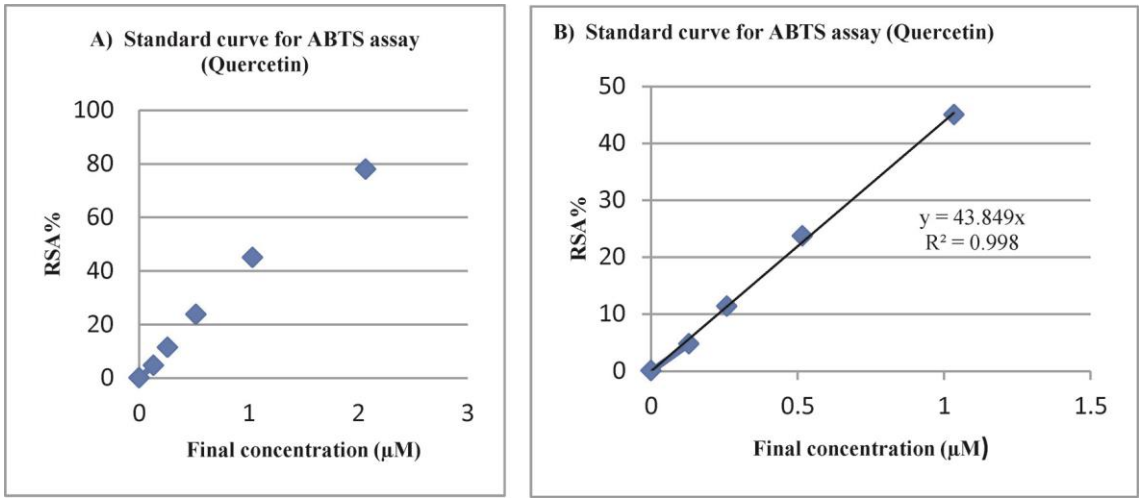


Figure 4.6. Standard curve of Quercetin for ABTS assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}=1.140µM.

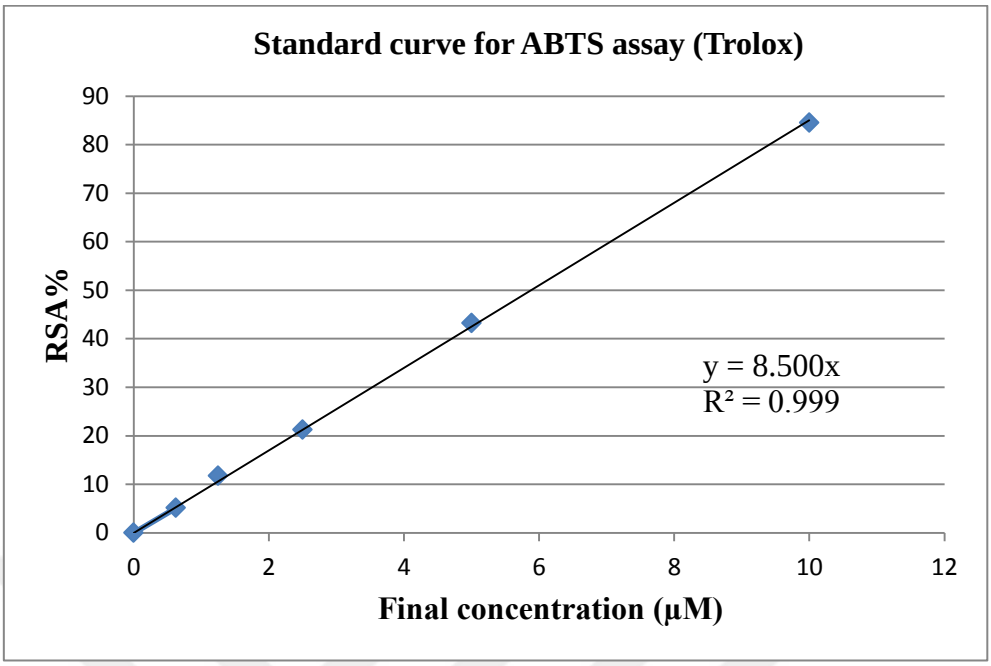


Figure 4.7. Standard curve of Trolox for ABTS assay. The plot was used in calculation of EC_{50} value. EC_{50}=5.882 µM.

The EC_{50} value is used to evaluate the antioxidant capacity. The low EC_{50} value indicates that the antioxidant activity is high. The EC_{50} values of ascorbic acid, quercetin, and trolox used as standards were calculated from the standard curves (Figure 4.5, Figure 4.6, and 4.7). The EC_{50} values were 6.166 µM, 1.140 µM, and 5.882 µM respectively, which means quercetin, had the highest response to ABTS assay.

EC_{50} values could not be calculated, as in the trolox, ascorbic acid, and quercetin standards, for Derivative-1 (Figure 4.8) due to the fact that there was an early saturation below 50% of RSA value. This might be the result of an unwanted reaction between Derivative-1 and any component in reaction medium. Because of this very low levels of %RSA, instead of EC_{50} value, another calculation was performed for Derivative-1: %RSA/ highest applied concentration were calculated to demonstrate the level of capacity for it. EC_{50} value of Derivative-2 was very high (Figure 4.9); but still, Derivative-2 showed higher antioxidant activity than that of derivative-1, in response to ABTS assay.

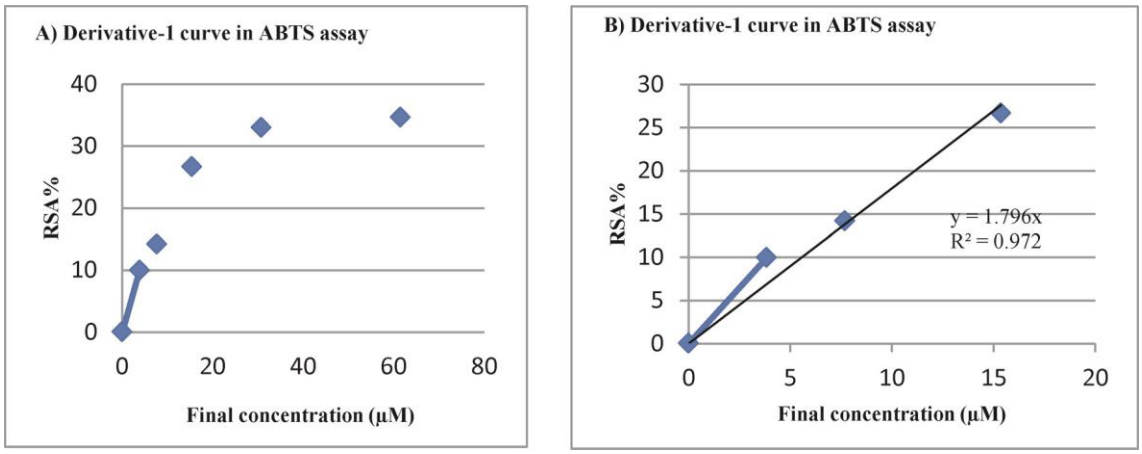


Figure 4.8. Derivative-1 curve for ABTS assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of RSA % / highest applied concentration value (B). RSA % / highest applied concentration = 0.563 $\mu M^{-1.}$

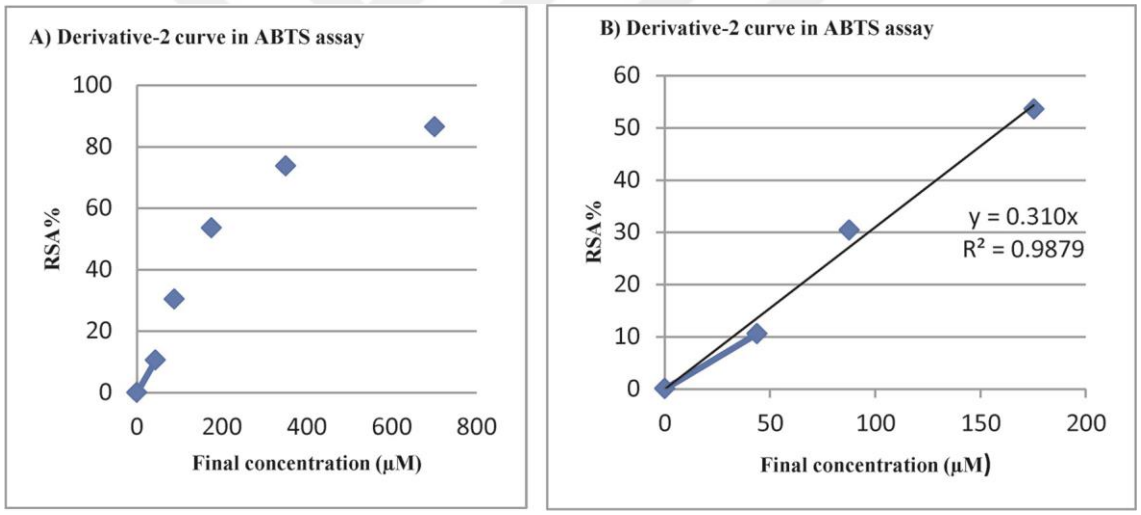
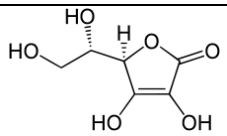
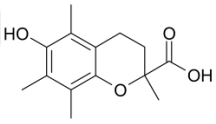
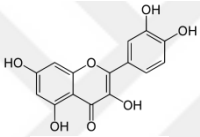
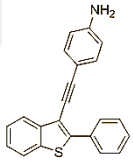
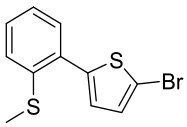


Figure 4.9. Derivative-2 curve for ABTS assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}= 161.290 µM.

All standard compounds used in the ABTS method (trolox, ascorbic acid, and quercetin) have hydroxyl groups in their structures. However, there is no such groups in the structure of the compounds that have been tested, as seen in Figures (3.1 and 3.4). The hydroxyl group makes hydrogen transfer easier and the ABTS method is based on hydrogen transfer. The low capacity levels of the tested derivatives might be due to the absence of such hydrogen-prone groups, such as the hydroxyl group. In the structure of Derivative-1, a thiophene ring is connected to the other benzyl rings (see Figure 3.1 and

Table 3.4) which were not such good bonding sites for hydrogen transfer. Table 4.2 shows the comparison between both derivatives and standards.

Table 4.2. The comparison of the antioxidant capacities of standards and derivatives in ABTS assay

Standards and Derivatives	Structure	Molecular Weight	EC_{50} value	"RSA% / Highest Applied Concentration" Value
Ascorbic acid		176.12 g/mol	6.166 μM	-
Trolox		250.29 g/mol	5.882 μM	-
Quercetin		302.236 g/mol	1.140 μM	-
Derivative-1		325.426 g/mol	-	0.563 μM^{-1}
Derivative-2		285.223 g/mol	161.290 μM	-

The resulting ranking of increasing antioxidant capacities of standards and tested compounds were found to be Quercetin > Trolox > Ascorbic acid > Derivative-2 > Derivative-1, by ABTS assay.

4.3. Galvinoxyl Assay Results

Depending on its composition, galvinoxyl is a fairly stabilized molecule. Due to the strange electron, in ethanol at 428 nm, galvinoxyl displays strong absorption; at low concentration, the galvanoxyl solution appears yellow. Once the electron becomes coupled, it results in the disappearing of absorption, and this discoloration is equal to the number of electrons that has been taken. Taking advantage of the color variation of galvinoxyl in the presence of an antioxidant, the dynamics of the antioxidant activity, hydrogen-donated activity, can be measured based on its structure (Shi et al., 2001).

This method is based on the principle that the amount of radical scavenged increases as the antioxidant substance increases in the environment and thus the % RSA increases. This is clearly seen in the plots drawn for quercetin (Figure 4.10) trolox (Figure 4.11) below.

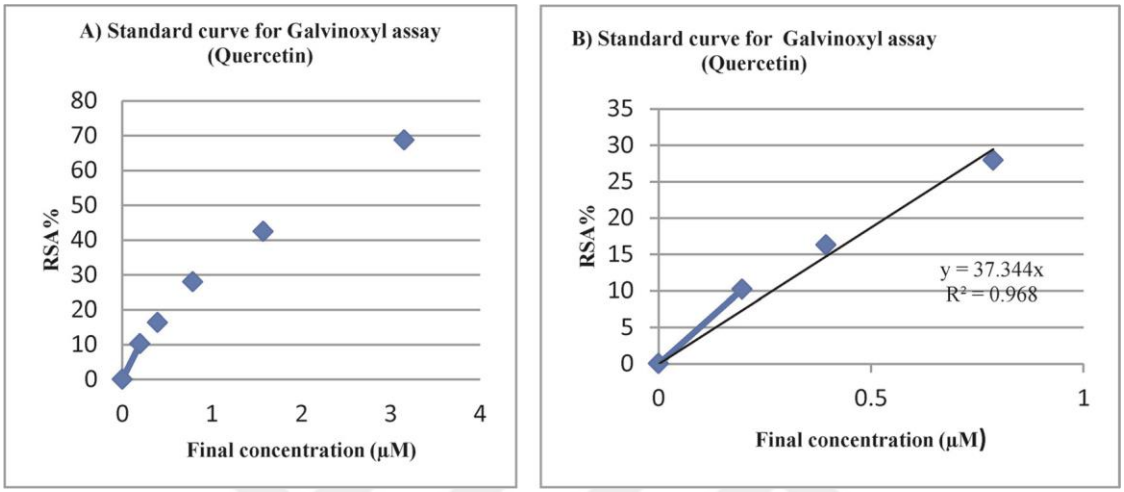


Figure 4.10. Standard curve of Quercetin for Galvinoxyl assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}= 1.338µM.

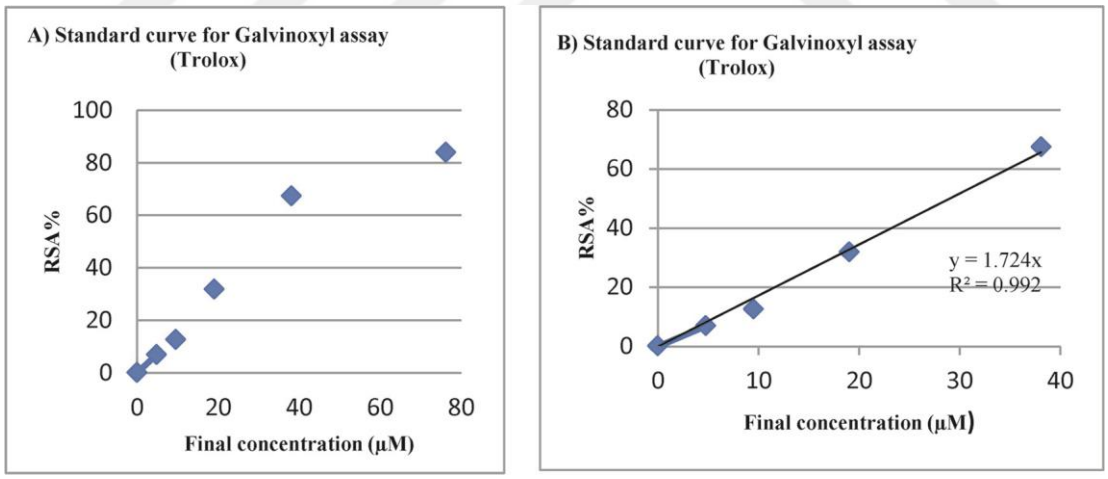


Figure 4.11. Standard curve of Trolox for Galvinoxyl assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}= 29.002µM.

The EC_{50} values of the quercetin and trolox, used as the standards in galvinoxil method, were calculated as 1.338 µM and 29.002 µM, respectively (Figure 4.10 and Figure 4.11).

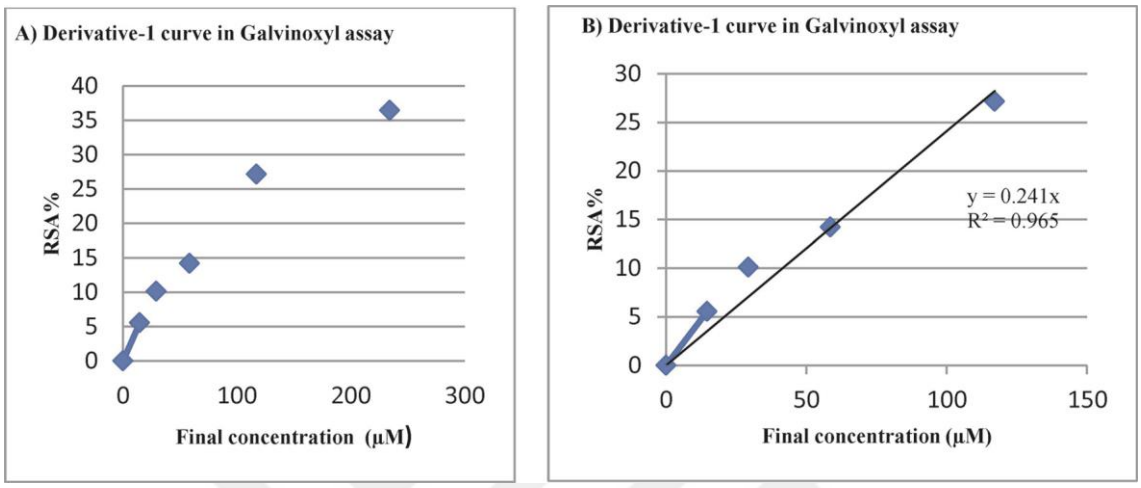


Figure 4.12. Derivative-1 curve for Galvinoxyl assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of RSA % / highest applied concentration value (B). RSA % / highest applied concentration = 0.155μM^{-1}.

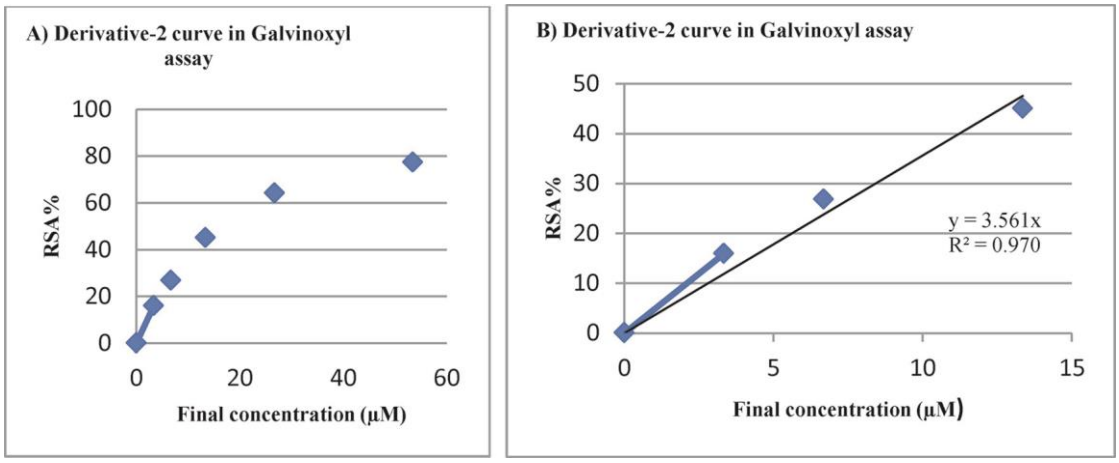
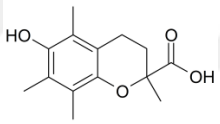
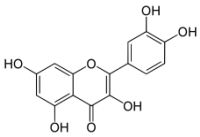
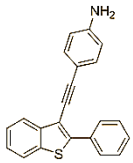
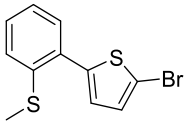


Figure 4.13. Derivative-2 curve for Galvinoxyl assay. The first plot demonstrates the saturation of reaction (A); and, second plot was used in calculation of EC_{50} value (B). EC_{50}= 14.040 µM.

When the concentration of Derivative-1 was increased up to 245.8 µM, there was no change in color, which didn't give sign that it reacts in this method. Its EC_{50} value was incomparable to standards; it was very high that indicated this compound has

low antioxidant activity in galvinoxyl method (Figure 4.12). This situation might be due to the nature of Derivative-1: it might undergo some unwanted reactions with galvinoxyl compound that lead to early saturation. On the other hand, Derivative-2 showed activity and its EC_{50} value (Figure 4.13) was comparable to standards, which was 14.040 μM. This relatively low value was smaller than that of trolox standard (Figure 4.11), which means this compound has higher antioxidant level of a vitamin E derivative in galvinoxyl assay. The structure of Derivative-2 lets it to transport electrons in a way that provide higher reducing capacity in a reaction medium contaning galvioxyl. Table 4.3 summarize the comparison of tested compounds in galvinoxyl assay.

Table 4.3. The comparison of the antioxidant capacities of standards and derivatives in galvinoxyl assay

Standards and Derivatives	Structure	Molecular Weight	EC_{50} value	"RSA% / Highest Applied Concentration" Value
Trolox		250.29 g/mol	29.002μM	-
Quarcetin		302.236 g/mol	1.338 μM	-
Derivative-1		325.426 g/mol	-	$0.155\mu M^{-1}$
Derivative-2		285.223 g/mol	14.040 μM	-

The overall ranking of increasing antioxidant capacities of standards and tested compounds were found to be Quercetin > Derivative-2 > Trolox > Derivative-1, by galvinoxyl assay. galvinoxyl assay didn't worked for Ascorbic acid and Derivative-1.

4.4. Phosphomolybdenum Assay Results

The test depending on the reduction of Mo (VI) to Mo (V) by sample analysis and the subsequent formation of the phosphate compound Mo (V) at acidic pH. The total antioxidant capacity can be calculated in the manner described before (Prieto et al., 1999).

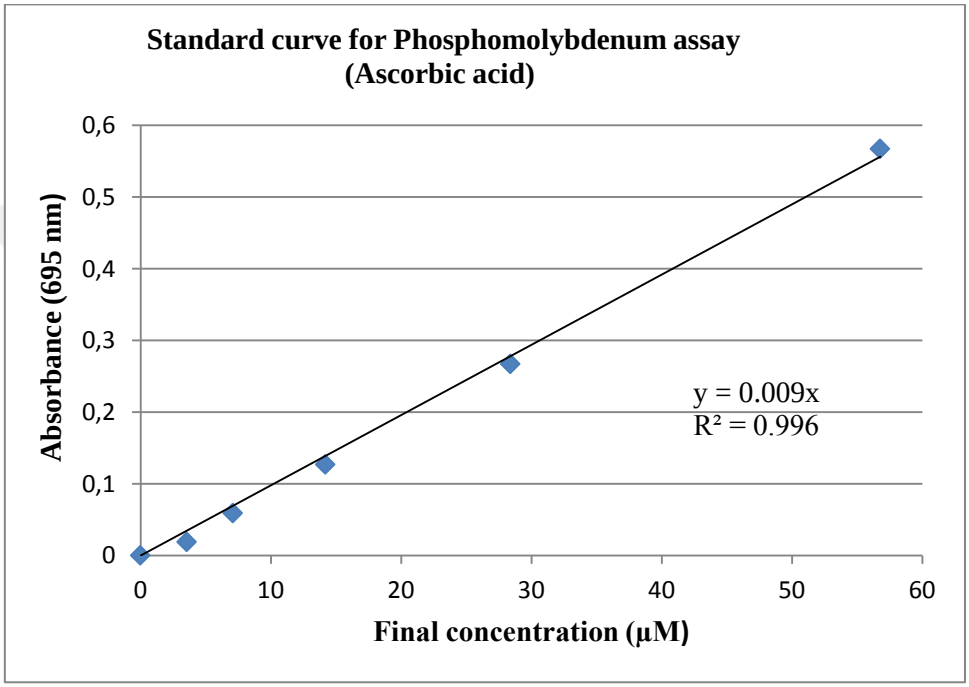


Figure 4.14. Standard curve of ascorbic acid at 695 nm in the phosphomolybdenum assay.

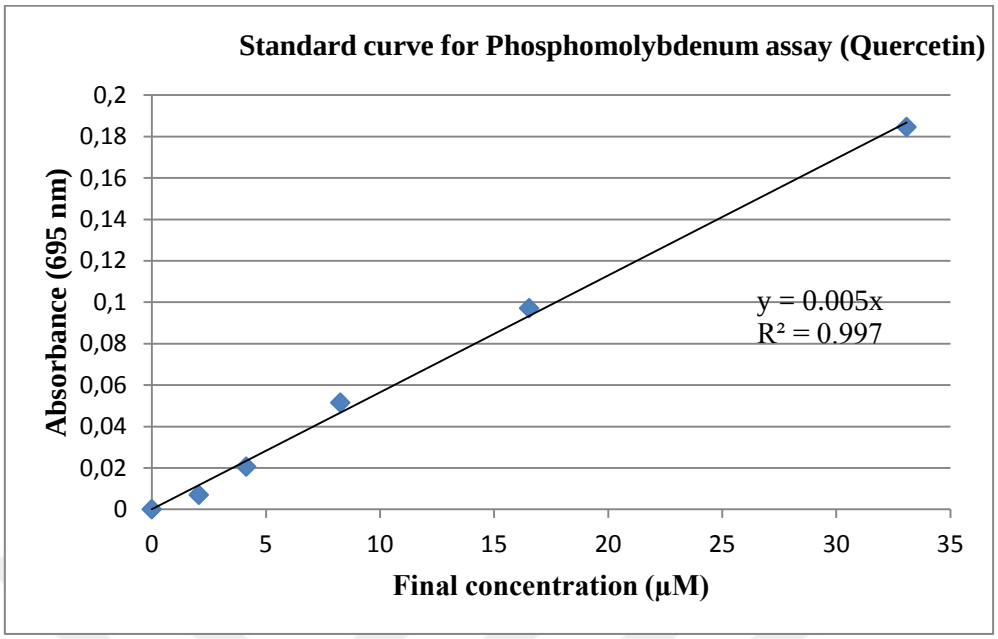


Figure 4.15. Standard curve of quercetin at 695 nm in phosphomolybdenum assay.

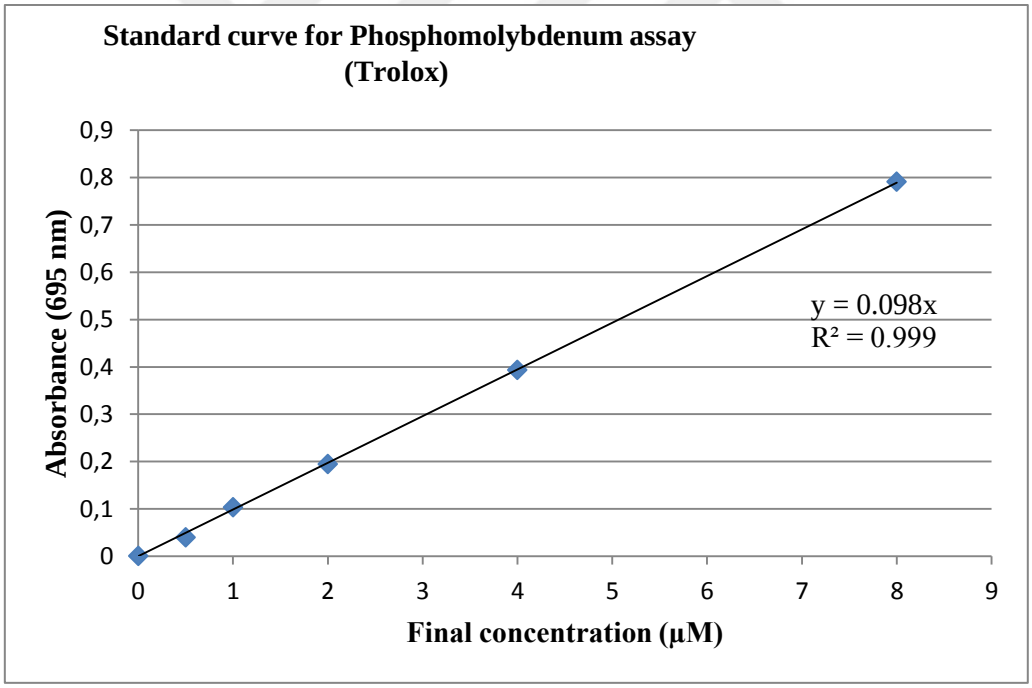


Figure 4.16. Standard curve of trolox at 695 nm in phosphomolybdenum method.

As it is shown in (Figure 4.14, Figure 4.15, and Figure 4.16) there were linear increasing of antioxidant capacity for ascorbic acid, quercetin, and trolox, in response to phosphomolybdenum assay with respect to their concentrations, 56.779 µM, 33.083 µM, and 8 µM, respectively.

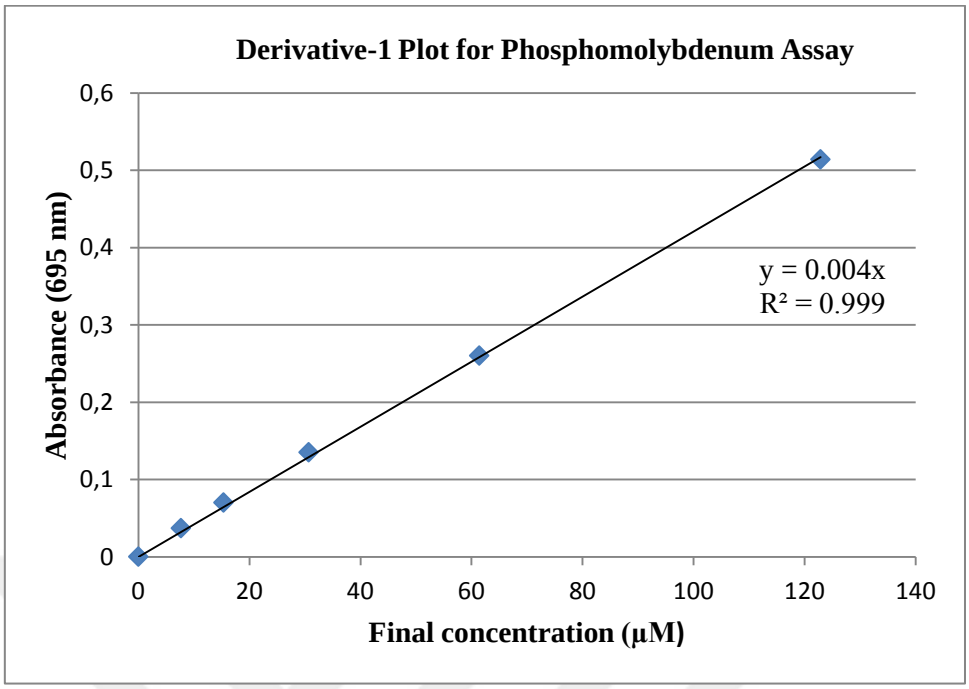


Figure 4.17. The plot of Derivative-1 for the phosphomolybdenum method.

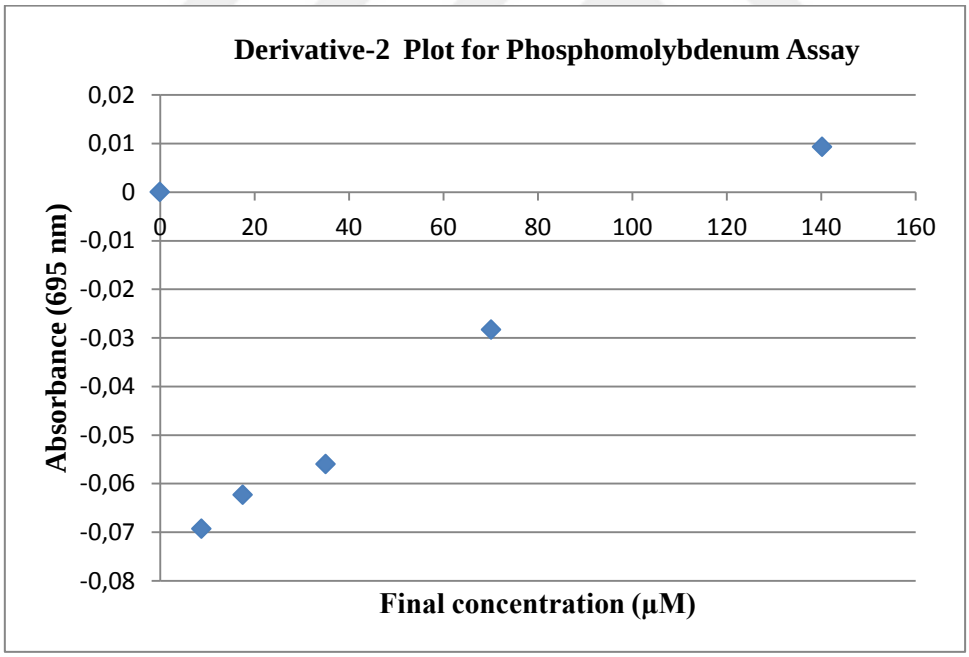


Figure 4.18. The plot of Derivative-2 for the phosphomolybdenum method.

Amongst standard curves trolox shows the highest antioxidant activity, according to its slope which is 0.098, which is about 10 times higher than that of quercetin and ascorbic acid.

The slopes of plots for both derivatives were compared to that of standard. Derivative-1 (Figure 4.17) was evaluated to be statistically comparable with those standards; it might indicate that this compound could work very well even in acidic medium, or it coldn't be affected by acidity. On the other hand, Derivative-2 (Figure 4.18) did not give any signs to have antioxidant capacity with phosphomolybdenum assay. In the way that plots showed no response to this assay even with increasing concentration from 56.096 μM to 1402.4 μM; that might be due to acidic medium which caused denaturation or damage to Derivative-2.

The ranking of all compounds for their reducing activity in phosphomolydenum assay as: Trolox > Ascorbic acid > Quercetin > Derivative-1 > Derivative-2.

4.5. Inhibition Models of Benzothiophene Derivatives on Human GSTP1-1

Inhibition effect on GST P1-1 isoenzyme tested at 0.0, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 250.0 μM concentrations for both substances Derivative-1 and Derivative-2, at three different concentration (0.5, 1.0 and 1.50 mM) of CDNB for inhibition examination, with the help of SigmaPlot software.

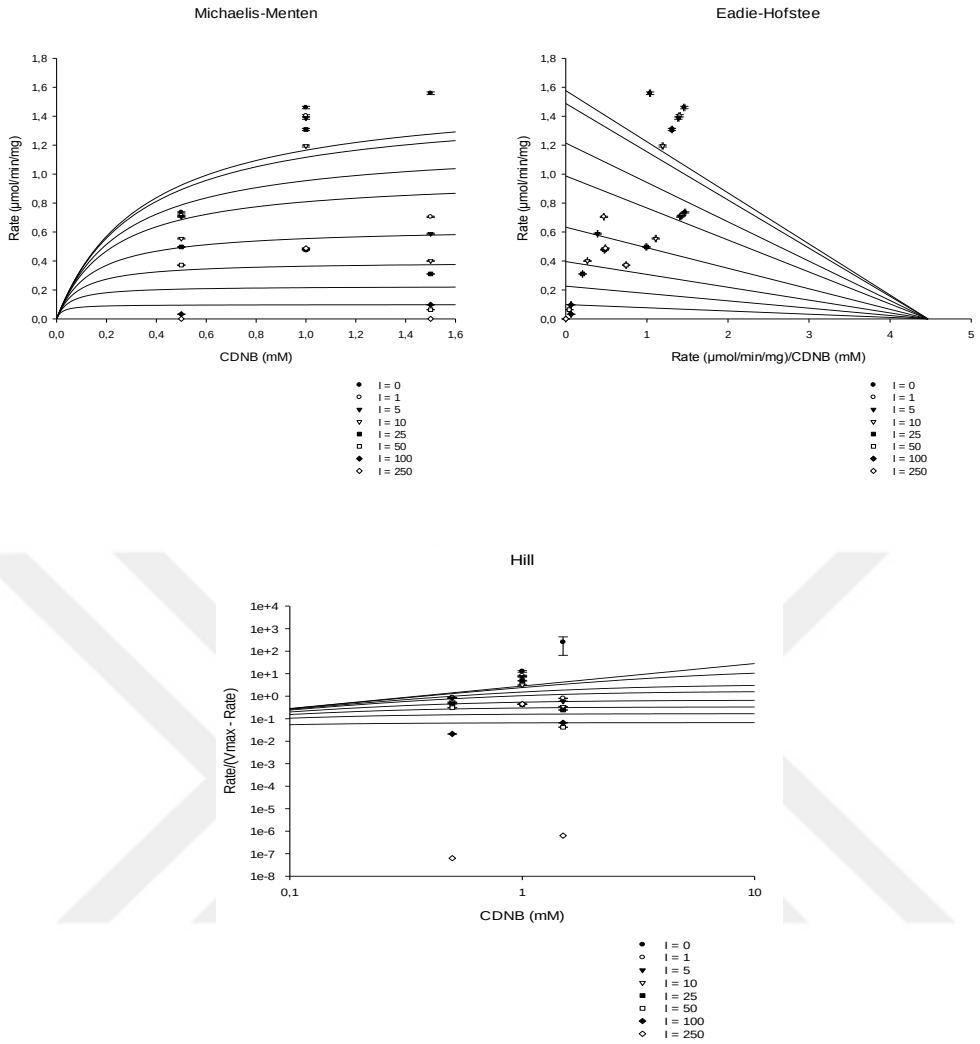


Figure 4.19. Michaelis-Menten, Eadie-Hofstee plot and Hill plot of derivative-1which were generated by Sigma Plot® 13 Enzyme Kinetics Module.

As it mentioned in (Table 4.4) three different concentrations of CDNB (0.50, 1.00 and 1.50 mM) respectively were used then for each concentration of CDNB eight different concentrations were used for Derivative-1 (0.0, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 250.0 µM). Then, by using of software, Vmax was calculated as 1.5770 µmol / min / mg protein, while Km and Ki were 0.3530 and 16.7760 µM, respectively. Then program stated that Derivative-1 caused (full) uncompetitive inhibition.

Table 4.4. The summary of the values of kinetic parameters for GSTP1-1 by inhibition of derivative-1

CDNB concentration mM	Derivative-1 concetntration µM	Vmax Mmol / min / mg protein	Km µM	Ki µM	AICc	R^2
0.50	0.0	1.5770	0.3530	16.7760	-157.713	0.574
1.00	1.0					
1.50	5.0					
	10.0					
	25.0					
	50.0					
	100.0					
	250.0					

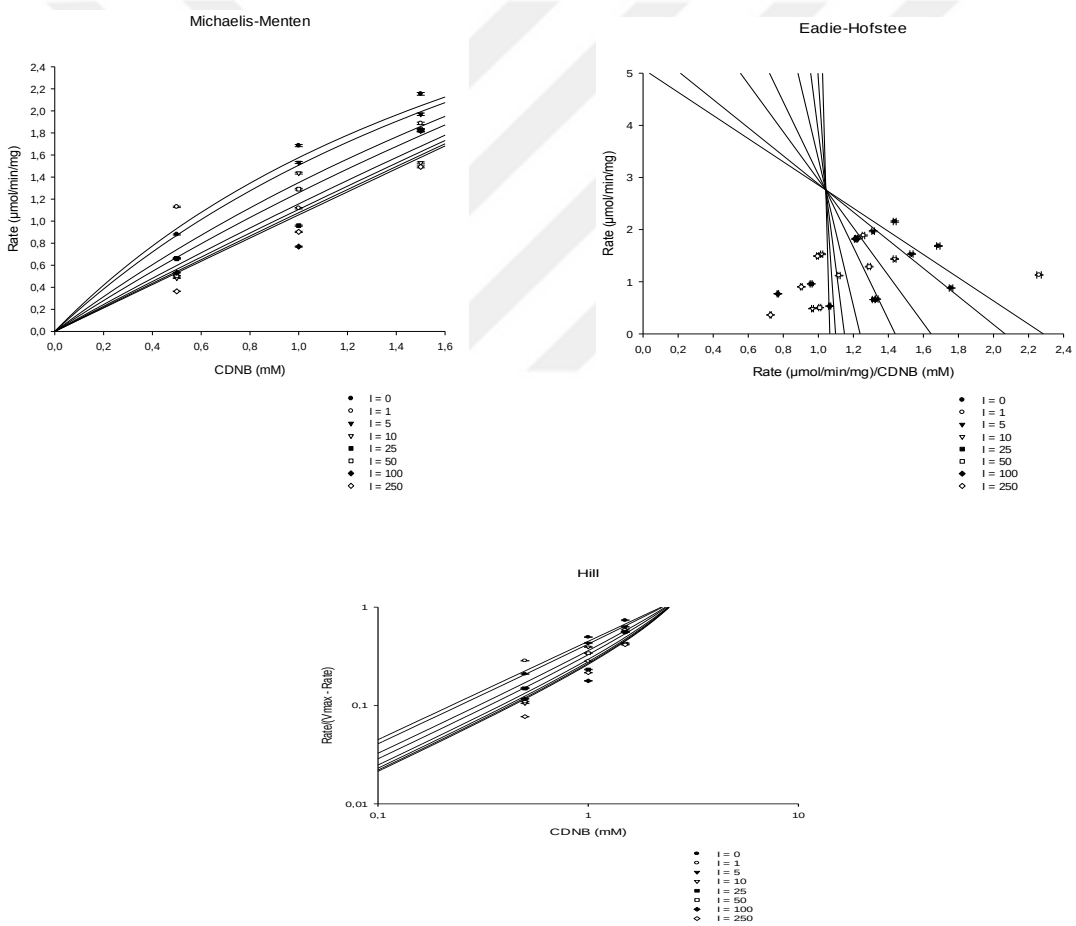


Figure 4.20. Michaelis-Menten, Eadie-Hofstee plot and Hill plot of Derivative-2 which were generated by Sigma Plot® 13 Enzyme Kinetics Module.

As it shown in (Table 4.5.) three deferent concentration of CDNB (0.50, 1.00 and 1.50 mM) were used; and, for each of those concentrations, seven different concentrations of Derivative-2 was used (0.0, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 250.0

μM). V_{max} was determined as 5.0798 μmol / min / mg protein, while Km and Ki were 2.2234 and 4.6897 μM respectively. The software stated Derivative-2 as a (partial) mixed inhibitor of GST P1-1 isoenzyme.

Table 4.5. The summary of the values of kinetic parameters for GSTP1-1 by inhibition of derivative-2

CDNB concentration mM	Derivative-2 concentration μM	Vmax μmol/min/mg protein	Km μM	Ki μM	AICc	R^2
1.00	0.0	5.0798	2.2234	4.6897	-233.394	0.887
0.50	1.0					
1.50	5.0					
	10.0					
	25.0					
	50.0					
	100.0					
	250.0					

5. CONCLUSION

The main aim of our study was to investigate the antioxidant capacitis of two novel benzothiophene derivatives by using four different methods, as well as the inhibitory characteristics of them on human GSTP1-1 isozyme. On the basis of acquired results, it was realized that these derivatives showed considerable antioxidant capacities in some methods, while they did not show results for some others. It indicated that they should be evaluated by further studies due to the fact that they had some potential. On the other hand, they were not good inhibitors of GSTP1-1 isozyme.

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EXTENDED TURKISH SUMMARY (GENİŞLETİLMİŞ TÜRKÇE ÖZET)

ÖZET

BAZI BENZOTİYOFEN TÜREVLERİNİN ANTİOKSİDAN KAPASİTELERİNİN BELİRLENMESİ VE GSTP1-1 İZOZİMİ ÜZERİNE İNHİBİSYON MODELLERİNİN TESPİTİ

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C_4H_4S formülü ile tiyofen, heteroatom olarak kükürt içeren beş üyeli halkalardan oluşan heterosiklik bileşiklere ait sınıfta bulunur. Bu organik bileşikler grubunun birçok farmakolojik olarak aktif molekülü vardır. Benzotiofenler, kükürt içeren heterosiklik bir aile olarak, önemli fizikokimyasal özellikler sergileyen oldukça kararlı aromatik bileşikler içerir. Antienflamatuar, analjezik, mantar önleyici, antidepresan vb. gibi çeşitli biyolojik / farmakolojik aktiviteler gösterirler. Bu tez projesinde, iki yeni benzotiyofen türevi, antioksidan kapasiteleri için hepsi farklı çalışma mekanizmalarına dayanan dört testin uygulanmasıyla araştırıldı (DPPH, ABTS, galvenoksil ve fosfomolibden). Türev-1 ve Türev-2, trolox, askorbik asit ve kuersetin gibi evrensel standartlarına göre farklı seviyelerde antioksidan kapasiteler gösterdi. Türev-2, galvinoksil analizinde, trolokstan 2 kat daha fazla antioksidan kapasite göstermiştir. Türev-1, fosformolibden denemesinde özellikle kuersetin ile karşılaştırılabilir seviyede antioksidan kapasiteye sahiptir. Bu iki türev, glutatyon ve çeşitli substratlar arasındaki konjugasyonu gerçekleştiren faz II ilaç metabolize edici enzimlerden biri olan insan GSTP1-1 üzerindeki olası inhibe edici etkileri için test edildi. Kinetik hesaplamalara göre, Türev-1 (tam) bağımlı inhibisyon uyguladı ve Türev-2, bu saf enzim üzerinde (kısmi) karışık inhibisyon oluşturdu. Sonuç olarak, bu yeni benzotiyofen türevleri, detaylı tıbbi testlerden sonra, antioksidan ajanlar olarak kullanılma potansiyeline sahiptir. Öte yandan, GSTP1-1'in daha iyi inhibitörleri olmaları için farklı kısımlarından türevlendirilmeleri gereklidir.

Anahtar kelimeler: Antioksidan kapasitesi, Benzotiyofen Glutatyon S-transferaz P1-1, İnhibisyon.

1.GİRİŞ

C_4H_4S formülü ile tiyofen, heteroatom olarak kükürt içeren beş üyeli halkadan oluşan heterosiklik bileşiklere ait sınıfta bulunur. Pek çok terapötik uygulamada kullanılmasından dolayı tiyofen ve türevleri, tıbbi kimyada çok iyi bilinmektedir. Doğal olarak benzotiofen, linyit katranı gibi yağ ile ilişkili birikintilerin bir bileşeni olarak bulunur ve faydalı biyolojik etkilere sahip bazı doğal ürünlerde bulunur. Bunlar, örneğin, analjezikler, anti-enflamatuar, antidepresan, mantar önleyici, östrojen reseptörü modüle edici, anti-mitotik, antikanser, enzim inhibitörleri gibi çeşitli biyolojik / farmakolojik aktiviteler gösterir. Hesaplanan özelliklerin tahsis edilmesine dayanan "5 kuralı", binlerce ilaç dizayn çalışmasının temelinde yer almaktadır. '5 kuralı'nın dışında kalan oral olarak aktif terapötik sınıflar şunlardır: antibiyotikler, antifungaller, kardiyak glikozitler ve vitaminler. Yeni kimyasal bileşikler sentezlendiğinde (ilaç benzerliği) kuralların önemi ortaya çıkar, daha sonra 5 kuralı, bileşiği daha hızlı değerlendirmek, aralarındaki seçimi optimize etmek ve bileşiğin geçirgenliğini ve oral emilim kapasitesini bilmek gibi parametrelerin tahminini sağlar. Kemo-kütüphanelerden kimyasal bileşiklerin (ilaçların benzerliği kriterleri esas olmak üzere) seçilmesinde yararlıdır, bununla birlikte dikkatli bir şekilde kullanılmalıdır. Bu tez çalışmasında 2 yeni benzotiyofen türevi, antioksidan kapasiteleri ve GSTP1-1 üzerinde potansiyel inhibitör / aktivatör etkileri üzerine kapsamlı çalışmalara tabi tutulmuştur. 4-((2-phenylbenzo[*b*]thiopen-3-yl)ethynyl)aniline Türev-1 olarak ve 2-bromo-5-(2-(methylthio)phenyl)thiophene Türev-2 olarak.

2. KAYNAK BİLDİRİŞLERİ

Antioksidanlar, sağlığı artırmak ve kanser ve kardiyovasküler problemler gibi hastalıkları önlemek için diyet takviyesinde bir bileşen olarak yaygın şekilde kullanılmaktadır. İnsanlar oldukça gelişmiş ve karmaşık bir antioksidan savunma sistemine sahiptir. Bu, serbest radikalleri ortadan kaldırmak için birlikte çalışan, kökeni itibariyle, eksojen ve endojen bileşenler içerir. Bu antioksidanların tipik üyeleri, askorbat glutatyon döngüsünün enzimleri, katalaz, süperoksit dismutaz (SOD), glutatyon peroksidaz (GPO) ve glutatyon redüktazdır. Enzimatik olmayanlar ise

indirgenmiş glutatyon (GSH), karotenoidler, askorbik asit, a-tokoferol ve flavonoidler ile temsil edilir. Bütün bu bileşikler ROS'u nötrleştirebilir. Vücutta veya biyokimyasal stres koşulları altında herhangi bir antioksidan kıtlığı durumunda, fazladan antioksidan desteği tüm metabolizmayı doğru bir şekilde devam ettirebilir. Bu noktada, ilaç aktif bileşenleri olarak yeni antioksidan alternatifleri hastalar için çok faydalı olabilir.

Çoğunlukla, gıda endüstrisinde kullanılan farklı türdeki süspansiyon türleri, farklı türlerde güzelleştirici maddeler, konserve ve dondurucu gıdalar, tarımda kullanılan kimyasallar ve farklı olarak birçok kaynaktan gelen çok sayıda ksenobiyotik ilaç türleri, insanla temas halindedir. Genel olarak, eğer metabolizma olmasaydı, lipofilik kimyasallar vücuttan etkin bir şekilde yok edilemezler, vücutta biriktikçe toksisiteye neden olurlardı. Ksenobiyotiklerin çoğu, Faz I ve Faz II enzimatik sistemlerin yanı sıra Faz III taşıyıcıları içeren bir veya birden fazla metabolizma yolundan geçerler. Dönüşüm sürecine biyotransformasyon denir. Bazı durumlarda Faz I reaksiyonları, ilaçları biyolojik olarak aktif metabolitlere dönüştürür, genellikle hem ester hem de amidin bağının hidrolize edilmesi durumunda ortaya çıkar. Nadir durumlarda, Faz I metabolizması değişmiş bir farmakolojik aktivite ile ilişkilidir. Bu gerçeklerin bir sonucu olarak, ilaç adaylarının bu yolların bileşenleri üzerindeki etkilerini kontrol etmek, ilaç tasarımı konularının bir gerekliliği olacaktır

Glutatyon S-transferazlar (GST'ler), biyotransformasyonun ikinci aşamasında, hücresel detoksifikasyonda büyük bir aktiviteye sahip olan, çevrede bulunan kanserojen maddelerin, kullanılan reaktif elektrofillerin (RE) taşınmasında ve böylece makromoleküllerin korunmasında önemli bir enzimdir. Sitosolik, mitokondriyal ve zara bağlı mikrozomal olmak üzere insan GST'lerin bilinen üç ana ailesi vardır. Mitokondrial ve sitozolik GST'ler çözünür enzimler olarak bilinir ve aralarında benzer üç boyutlu bir katlanma vardır. Pi sınıfı GST üyeleri, bir üye dışında yaygın olarak bilinmemektedir ve konumu, kromozom 11 üzerindedir; karaciğerde az miktarda bulunurken, kırmızı kan korpüsküllerinin dahil olduğu tüm dokularda bulunabilir. Günümüzde GSTP'ye ilgi artmıştır; endotel hücrelerinde GST-π düzeyi ile glial hücreler / astrosit ve birçok farklı tıbbi durum arasında büyük bir ilişki vardır. GSH ve elektrofilik substratları arasındaki bağlantı, deforme edici olan serbest radikallere temas sırasında GSTP katalizi ile hızlandırılır. Detoksifikasyon aktivitesinin yanı sıra, GSTπ, hücresel stresin artması durumunda hücrelerin programlanmış ölümünü başlatabilen ligand bağlama özellikleri

olan başka bir uygunluk da gösterir. Aşırı miktarda GSTπ'nin varlığı, kemoterapi ilaçlarına karşı tümör direnci ile ilişkilidir; bu da enzimin metabolizmada başka bir rol oynadığı anlamına gelir. Bazı araştırmacılar, GSTπ ekspresyonunun ilaca dirençli kanser hücre hatlarında arttığını bulmuşlardır. Bu nedenle, bu tez projesinde, yeni benzotiyofen türevlerinin GSTP1-1'in iyi inhibitörleri olarak potansiyellerinin değerlendirilmesi de amaçlanmıştır. Yeni GSTP1-1 inhibitörleri kemoterapi uygulamasının etkinliğini arttırmada kullanılabilir.

3. MATERYAL VE YÖNTEM

Türev-1 ve Türev-2 olarak kodlanan benzotiyofen türevleri Prof. Dr. Arif KIVRAK ve laboratuvarı tarafından sentezlenmiş ve yapı ve saflık testleri gerçekleştirilmiştir.

Türev-1'in sentezinde, ilk aşamada, 3-iyodo-2-fenil benzo[b]tiyofen DMF ve Et_3N ile argon gazı altında çözdürüldü. Daha sonra sırasıyla $Pd(PPh_3)CI_2$, para-etinilanilin, CuI eklendi. Karışım argon gazı geçirilerek oda sıcaklığında bir gece karıştırıldı. Etil asetat ve tuzlu su ile organik faz yıkandı ve Mg_2SO_4 ile kurutulup süzüldü. Elde edilen süzüntüdeki kuru organik fazdan, düşük basınç altında evaporatör yardımıyla, çözücü uzaklaştırıldı. Sadece n- hegzan kullanılarak %58 verim ile hedeflenen ürün elde edildi.

Türev-2'nin sentezinde ise ilk olarak, 2-(2-(metiltiyo)fenil)tiyofen kloroform içerisinde argon altında çözdürüldü ve sonrasında sıcaklık buz banyosu yardımıyla 0°C ye düşürüldü. N-bromosüksinamid eklendikten sonra buz banyosunda karıştırıldı ve daha sonra saf su eklenerek ekstraksiyon yapıldı . Organik faz Mg_2SO_4 ile kurutuldu ve süzüldü. Elde edilen süzüntüdeki kuru organik fazdan, düşük basınç altında evaporatör yardımıyla, çözücü uzaklaştırıldı. Sadece kloroform-hekzan kullanılarak %99 verim ile hedeflenen ürün elde edildi.

En az beş farklı konsantrasyonda hazırlanan maddeler öncelikle antioksidan kapasite testlerine tabi tutuldu. Üç farklı zamanda ve her bir konsantrasyonu da üç tekrarlı olmak üzere, birbirlerinden farklı çalışma esaslarına sahip dört farklı metot ile antioksidan kapasiteleri ölçüldü. Böylece, test edilen maddeler ile reaksiyon ortamındaki kimyasallar arasında olabilecek ve ölçümü engelleyecek/yanıltacak

istenmeyen muhtemel reaksiyonlar için B planı işlenmiştir. Kısaca, eğer denenen metotlardan biri müsbet sonuç veremese dahi, diğer testlerden alınan sonuçlarla antioksidan kapasite ortaya çıkarılmıştır. Bu amaçala ABTS, DPPH, Galvinoksil ve Fosfomolibden metotları kullanılmıştır. Metotların laboratuvar koşullarında çalıştığını göstermek ve teste edilen benzotiyofen türevleri için birer standart olarak kullanılmak üzere troloks ve askorbik asit antioksidanlarının kapasite testleri gerçekleştirildi.

4. BULGULAR VE TARTIŞMA

DPPH metodunda troloks için EK_{50} değeri 23.529 μM ve askorbik asit için EK_{50} değeri ise 22.583 μM olarak tespit edildi. Türev-1 ve Türev-2 için EK_{50} değerleri hesaplanabildi; ancak, standartlara oranla bu değerler çok yüksekti: Türev-1 için 446.428 μM ve Türev-2 için 331.125 μM. Türev-2 diğerinden daha yüksek antioksidan kapasiteye sahiptir.

ABTS metoduyla gerçekleştirilen ölçümlerde troloks için EK_{50} değeri 5.882 μM, askorbik asit için 6.166 μM ve Türev 2 için EK_{50} değeri 161.290 μM olarak hesaplanmıştır. Ancak benzer bir hesaplama Türev-1 için gerçekleştirilememiştir; Türev-1, nispeten yüksek konsantrasyonlarda uygulanmasına rağmen, tüm radikalin sönümlenmesini gerçekleştirebilecek antioksidan kapasiteyi sergileyememiştir. Bu durumda, diğer testlerdeki benzer durumlarda da yapıldığı üzere, "%RSA/uygulanan konsantrasyon" değeri hesaplanarak, bu değerin kullanılmasıyla Türev-1'in antioksidan kapasitesi hesaplanmıştır. Türev-1 için bu değer 0.563 μM^{-1} olarak hesaplanmıştır. "%RSA/uygulanan konsantrasyon" değeri ne kadar yüksekse, antioksidan kapasite de o derece yüksektir.

Galvinoksil metoduyla gerçekleştirilen ölçümlerde askorbik asit için sonuç elde edilememiştir. Ancak troloks için EK_{50} değeri 29.002 μM ve Türev-2 için 14.040 μM olarak hesaplanabilmiştir. Türev-1 için bu metotla elde edilen "%RSA/uygulanan konsantrasyon" değeri 0.155 μM^{-1} tir. Türev-2, DPPH metodunda elde edilen sonuca paralel olarak, daha yüksek antioksidan kapasiteye sahiptir.

Uygulanan son test olan fosfomolibden metodunda her iki türevinde eğimleri standartlarla karşılaştırıldı. Türev-1'in standartlarla istatistiksel olarak

karşılaştırılabildiği ve aktivitesinin oldukça iyi olduğu görüldü. Ancak Türev 2'nin antioksidan özelliğiyle ilgili herhangi bir aktivite gözlenemedi.

Saf, insan GSTP1-1 ile gerçekleştirilen enzim aktivite ölçümlerinde her iki maddenin 7 farklı konsantrasyonu (1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM ve 250 μM) reaksiyon ortamına eklenerek kontrol ölçümleriyle birlikte aynı anda okuma gerçekleştirilmiştir. Denemeler 3 kuyucukta yani üçlü gerçekleştirilmiştir. Ayrıca tüm ölçümler, CDNB'nin üç farklı konsantrasyonu için (0.5 μM, 1.0 μM ve 1.5 μM) bütünüyle tekrarlanmıştır. Her üç CDNB konsantrasyonunda da hem Türev-1 hem de Türev-2 için artan konsantrasyonlara da bağlı olmak üzere inhibisyon gözlemlenmiştir. Türev-1 için izlenen inhibisyon etkisi, Türev-2'ye göre daha belirgindir. Tüm spesifik aktivite değerleri hesaplandıktan sonra Sigma Plot Enzyme Module ile işlenmiştir. AICc ve R^2 değerleri göz önüne alınarak yazılımın istatistiksel testlerini geçen modellerden en uygunu inhibisyon modeli olarak kabul edilmiştir (en küçük AICc ve en yüksek R^2 değerine göre). Bu kinetik modelleme çalışması sonucunda Türev-1 için kabul edilen inhibisyon modeli tam bağımlı modeldir. Bu model de inhibitör etkinin gösterilebilmesi için öncelikle substratın enzime bağlanması gereklidir. Hesaplanan K_m 0.3530, V_{max} 1.5770 ve K_i değeri ise 16.7760'dır. Türev-2 için aynı kinetik testler uygulanmıştır ve kabul edilen inhibisyon modeli kısmi karışık modeldir. Bu modelde inhibitör değişen oranlarda olmak üzere hem enzim-substrat kompleksine hem de serbest enzim moleküllerine bağlanabilmektedir. Hesaplanan K_m 2.2234, V_{max} 5.0798 ve K_i değeri ise 4.6897'dır.

5. SONUÇ

Bu tez çalışması sonunda, ilaç olma potansiyeline sahip iki yeni benzotiyofen türevinin hem antioksidan kapasiteleri hem de ilaç metabolize eden enzimler içerisinde önemli bir yeri olan GSTP1-1 üzerindeki inhibisyon modelleri belirlenmiştir. Mevcut antioksidanlardan bazıları kadar yüksek kapasiteye sahip olabildikleri ancak ilaç olarak kullanılmakta olan GST inhibitörleri kadar önemli bir etkiye sahip olmadıkları belirlenmiştir. Ancak bu maddelerin *in vivo* testleri ile de etkilerinin gözlemlenmesinin önemli olduğu düşünülmektedir.

CURRICULUM VITAE

He is from Iraq, he is 25 years old; He has got Bachelors degree in biology, form university of Duhok, Science Collage, Biology Department in 2015-2016. As well as, he is marrid, his bachelor research was about Cholera disease, and he has worked in many laboratories as lab technician such as: Media Diagnostic Center, and central laboratory of birth hospital. He has studied M.Sc. in Van Yuzuncu Yil University between 2017-2019.

T.C
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Tez Başlığı / Konusu: **Bazı benzotiyofen türevlerinin antioksidan kapasitelerinin belirlenmesi ve GSTP1-1 izozimi üzerine inhibisyon modellerinin tespiti**

Yukarıda başlığı/konusu belirlenen tez çalışmamın Kapak sayfası, Giriş, Ana bölümler ve Sonuç bölümlerinden oluşan toplam **17** sayfalık kısmına ilişkin, **31/10/2019** tarihinde ~~şahsım~~/tez danışmanım tarafından **iThenticate** intihal tespit programından aşağıda belirtilen filtreleme uygulanarak alınmış olan orijinallik raporuna göre, tezimin benzerlik oranı **% 8 (sekiz)** dır.

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