SCREENING FOR ANTIOXIDANT ACTIVITIES OF SEVERAL MEDICINAL PLANT EXTRACTS AND THEIR EFFECTS ON GLUTATHIONE-S-TRANSFERASE ACTIVITY

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ABSTRACT

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The consumption of fresh fruits, vegetables, and medicinal plants are known to be associated with a long life span and low incidence of oxidative stress related diseases such as Alzheimer's, Parkinson's, cancer, aging and cardiovascular diseases. Fitotherapeutic effects of medicinal plants is virtually attributable to their phenolic compounds with low cytotoxicity.

In this study, plants used in Anatolian folk medicine for their effects such as antiinflammatory, antiulcer, antipyretic, fertility, analgesic and aphrodisiac, namely *Aesculus hippocastanum* L., *Papaver bracteatum* L., *Urtica urens* L., *Gundelia tournefortii* L., *Prangos ferulacea* L., *Chaerophyllum macropodum* Boiss., *Heracleum persicum* Desf., *Allium vineale* L., *Aconitum cochleare* Woroschin, *Rheum ribes* L., *Ferula rigidula* DC., *Rosa heckeliana* Tratt, were screened for their antioxidative effects. Antioxidant characteristics of the specified plants were studied using lipid peroxidation inhibiton and DPPH radical scavenging methods.

Total phenolics content and their effects on glutathione-S-transferase activity of the plants were further investigated. *Rheum ribes* L, *Ferula rigidula* DC, *Rosa heckeliana* Tratt., *Prangos ferulacea* L. were found to be very effective antioxidants and also effective inhibitors for glutathione-S-transferase activities among the plants.

Rosa heckeliana Tratt. root extracts exhibited very high total phenolics content (0.7 mg/mg of extract) and antioxidant activity with IC_{50} values of 11.2 µg/mL and 5.1 µg/mL for DPPH scavenging and lipid peroxidation inhibition, respectively. *Ferula rigidula* DC was identified as the most potent inhibitor for glutathione-S-transferase activity, with IC_{50} values of 49 µg/mL.

Keywords: Antioxidant, DPPH, peroxidation, GST, phenolic

ÇEŞİTLİ BİTKİ ÖZÜTLERİNİN ANTİOKSİDAN AKTİVİTELERİNİN VE GLUTATYON-S-TRANSFERAZ AKTİVİTESİ ÜZERİNE ETKİLERİNİN TARANMASI

ÖZ

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Taze meyve, sebze ve tıbbi bitkierin tüketimi, uzun bir yaşam süresi ve Alzheimer, Parkinson, kanser, yaşlanma ve kardiyovasküler hastalıklar gibi oksidatif strese bağlı hastalıkların görülme sıklığında düşüş ile ilintili olduğu bilinmektedir. Tıbbi bitkilerin fitoterapötik etkileri çoğunlukla içerdikleri toksisitesi düşük fenolik maddelere bağlanmaktadır.

Bu çalışmada, Anadolu'da halk arasında, antienflamatuar, antiülser, ateş düşürücü, kısırlığa karşı, ağrı kesici ve afrodizyak etkileri için kullanılan bitkilerden *Aesculus hippocastanum* L. (atkestanesi), *Papaver bracteatum* L. (haşhaş), *Urtica urens* L. (küçük ısırgan), *Gundelia tournefortii* L. (kenger), *Prangos ferulacea* L. (heliz), *Chaerophyllum macropodum* Boiss. (mendi), *Heracleum persicum* Desf. (sow), *Allium vineale* L.(sirmo), *Aconitum cochleare* Woroschin, *Rheum ribes* L (ışkın), *Ferula rigidula* DC. (siyabo), *Rosa heckeliana* Tratt. (şilan kökü)'ların antioksidan etkileri bakımından taranmıştır. İlgili bitkilerin antioksidan karakteristikleri, lipit peroksidasyon engelleme ve DPPH radikal yakalama metodları kullanılarak çalışılmıştır.

Bitkiler, toplam fenolik içerikleri ve glutatyon-S-transferaz aktivitesi üzerine etkileri bakımından da incelenmiştir. Bitkiler arasında *Rheum ribes* L, *Ferula rigidula* DC, *Rosa heckeliana* Tratt., *Prangos ferulacea* L. en etkili antioksidanlar ve glutatyon-S-transferaz aktivitesi inhibitörleri olarak belirlenmiştir.

Yüksek miktarda fenolik madde içeren *Rosa heckeliana* Tratt. kök ekstreleri (0.7 mg/mg ekstre) aynı zamanda, sırasıyla IC_{50} değerleri 11.2 µg/mL and 5.1 µg/mL olan DPPH radikal tutucu ve lipit peroksidasyon inhibisyonu özellikleriyle yüksek antioksidan aktivite göstermiştir.

Ferula rigidula DC ise IC_{50} değeri 49 µg/mL ile en etkin glutatyon-S-transferaz inhibitörü olarak belirlenmiştir.

Anahtar kelimeler: Antioksidan, DPPH, peroksidasyon, GST, fenolik

To my family…

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NOMENCLATURE

BSA	: Bovine serum albumin
CAT	: Catalase
CDNB	: 1-chloro-2,4-dinitrobenzene
DMEs	: Drug metabolizing enzymes
DMSO	: Dimethyl sulfoxide
DPPH	: 2,2-diphenyl-1-picrylhydrazyl radical
EDTA	: Ethylene diamine tetraacetic acid
FA-OOH	: Fatty acid hydroperoxides
GAE	: Gallic Acid Equivalents
GPx	: Glutathione peroxidase
GR	: Glutathione reductase
GSH	: Reduced glutathione
GSSG	: Oxidized glutathione
GST	: Glutathione-S-transferase enzyme
LPO	: Lipid peroxidation
MDA	: Malonedialdehyde
MDA	: Malonedialdehyde
PL-OOH	: Phospholipid hydroperoxides
ROS	: Reactive oxygen species
SOD	: Superoxide dismutase
TBA	: Thiobarbituric acid
TLC	: Thin Layer Chromatography
TRIS	: Hydroxy methyl aminomethane

CHAPTER 1

INTRODUCTION

All living organisms, except for anaerobic microorganisms, require molecular oxygen as an electron acceptor for efficient production of energy. However, oxygen is a strong oxidant which can cause deleterious consequences (Sorg, 2004). It is evident that oxidative stress plays a central role in the pathogenesis of many diseases including atherosclerosis, Ischemic heart disease, cancer, Alzheimer's, Parkinson's, even in the aging process (Ceconi *et. al.*, 2003).

It is generally agreed that a diet rich in fruits and vegetables is associated with a low incidence of several degenerative diseases and contributes to the maintenance of health, by decreasing both oxidative damage and cancer incidences (Stahl, 2000; Halliwell, 2002). Such plant foods contain certain naturally occurring phenolic compounds that have antioxidative properties, many endogenously produced compounds also exhibit antioxidant functions, and often act synergistically with antioxidants of dietary origin, such as reactive oxygen species (ROS) scavenging, electrophile scavenging, metal cheletion and inhibition of ROS generation systems. (Diplock, 1994; Ames, 1998; Stahl, 2000)).

Most naturally occurring phenolic compounds in foods are flavonoids, but others include chlorogenic acid, caffeic acid, ferulic acid, catechins and diallylheptanoids such as curcumin. Besides their antioxidative activities, they have been reported to be antimutagenic and/or anticarcinogenic and to possess several other biological activities and have limited cytotoxicity (Aruoma, 1999; Lee, 2003).

1.1. Free Radicals

Reactive oxygen species (ROS) such as superoxide, $O_2^{-\bullet}$, hydrogen peroxide, H_2O_2, and hydroxyl radical $OH^{-\bullet}$ are free radicals which are generated mainly as the end products of cellular metabolism, primarily in the mitochondria during respiration and are highly toxic to cells when their cellular production overwhelms the cellular antioxidant capacity. A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge, 1989) .

Molecular O_2 in the atmosphere is also classified as a free radical because it has two unpaired electrons with parallel spin in different p-antibonding orbitals. The formation of reactive oxygen species from oxygen is shown in **Figure 1.1** This spin restriction accounts for its relative stability and paramagnetic properties. O_2 is capable of accepting electrons to its antibonding orbitals, becoming reduced in the process therefore, functions as a strong oxidizing agent and major promoter of radical reactions in living cells (Thannical and Fanburg, 2000; Halliwell and Gutteridge, 1989)

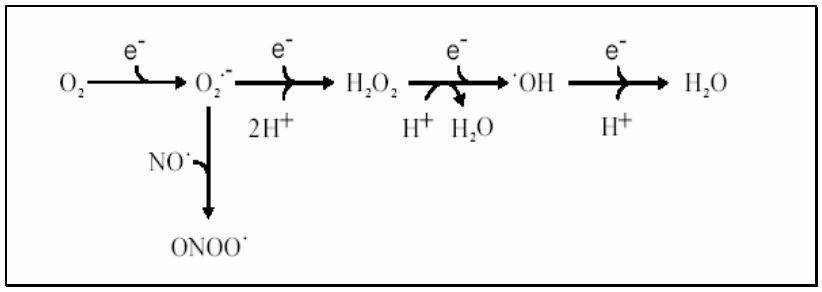


Figure 1.1. The formation of reactive oxygen species.

The most important ROS are the superoxide anion $O_2^{\bullet-}$, hydroxyl radical $OH^{\bullet}$, nitric oxide $NO^{\bullet}$, and hydrogen peroxide H_2O_2 (**Table 1.1**). The primary ROS formed *in vivo* are superoxide and hydrogen peroxide. H_2O_2 is generated through nonenzymatic or enzymatic dismutation of superoxide. However, the most reactive and harmful ROS is the hydroxyl radical, which can be formed from H_2O_2 and $O_2^{\bullet-}$,

as well as the reaction of superoxide with NO to produce peroxynitrite OONO- that consecutively decomposes to form NO_2 and OH (Halliwell and Gutteridge, 1989).

Table 1.1 Examples of molecules mediating oxidative stress (Sorg, 2004)

ROS	NAME	STRUCTURE	MAIN REACTIONS
Radicals	Superoxide	•O–O$^-$	Catalysis of Haber–Weis reaction by recycling Fe^{2+} and Cu^+ ions; formation of hydrogen peroxide or peroxynitrite
	Hydroxyl	•OH	Hydrogen abstraction; production of free radicals and lipid peroxides; oxidation of thiols
	Alkoxyl	L-O• R–O •	Hydrogen abstraction; formation of radicals; decomposition of lipids and other biomolecules
	Peroxyl	L-O-O• R–O–O •	
	Nitric oxide	•N=O	Formation of peroxynitrite; reaction with other radicals
Non radicals	Peroxynitrite	O=N–O–O$^-$	Formation of hydroxyl radical; oxidation of thiols and aromatic groups; conversion of xanthine dehydrogenase to xanthine oxidase; oxidation of biomolecules
	Hypochlorite	ClO$^-$	Oxidation of amino and sulphur-containing groups; formation of chlorine
	Hydrogen peroxide	HO–OH	Formation of hydroxyl radical; enzyme inactivation; oxidation of biomolecules
	Ozone	$^-$O–O $^+$=O	Oxidation of all kinds of biomolecules, especially those containing double bonds; formation of ozonides and cytotoxic aldehydes
	Singlet oxygen	O=O	Reaction with double bonds, formation of peroxides; decomposition of amino acids and nucleotides
	Hydro peroxide	R–O–OH	Oxidation of biomolecules; disruption of biological membranes
	Copper ions Iron ions	Cu^{2+}, Fe^{3+}	Formation of hydroxyl radical by Fenton and Haber–Weis reactions

1.1.1 Sources of Free Radicals in Biological Systems

Radicals can be produced in the cells and tissues of our bodies by various processes and reactions. The impact of radiation such as high energy radiation, ionizing radiation, visible light with photosensitizers and thermal degredation of organic materials can produce homolytic fission, by the loss of a single electron from a non-radical, or by the gain of a single electron by a non-radical, where the electron pair of a covalent bond is shared between the two atoms resulting in each having an odd electron (Halliwell and Gutteridge, 1989; Başağa, 1990).

The most important sources of $O_2^{\bullet -}$ in vivo in most aerobic cells are the electron transport chains of mitochondria and endoplasmic reticulum. During the production of ATP in the electron transport chain, approximately 1-5% of all oxygen used in metabolism escapes as free radical intermediates.

Ubisemiquinone species generated in the course of electron transport reactions in the respiratory chain donate electrons to oxygen and provide a constant source of superoxide (Raha and Robinson, 2000).

Oxidative reactions are very important in other biological reactions and many of these also have the potential for generating free radicals under physiological conditions. Cytochrome P450 and cyclooxygenases as well as certain lipoxygenases, dehydrogenases and peroxidases generate free radical intermediates.

The various Fe-S proteins and NADH dehydrogenase are possible sites of superoxide and hydrogen peroxide formation (Turrens et al., 1982). A well known active oxygen producing enzyme is xanthine oxidase, which produces superoxide anions during oxidation of xanthine to uric acid. Molecular oxygen is a potential oxidizing substrate for xanthine oxidase as well as aldehyde oxidase. They are both flavoproteins containing FAD, iron-sulfur centers and molybdenum that can undergo redox cycling (Fisher, 1987).

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{XO}} \text{Uric acid} + H_2O_2 + O_2^{\bullet -}$$

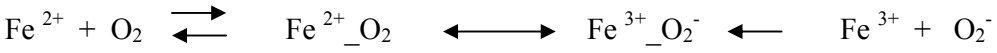
Another enzyme is NAD(P)H oxidase in the plasma membrane of neutrophiles which produces superoxide anion within the plasma membrane or on its outer surface (Başağa, 1990). However, this enzyme is present only in phagocytes and therefore does not contribute to the intracellular oxidant load in most cells (Fisher, 1987). Consequently, the distribution and the activity of the enzymatic pathways varies with different cell types depending on their specialized function.

$$2O_2 + \text{NAD(P)H} \xrightarrow{\text{NAD(P)H Oxidase}} \text{NAD(P)}^+ + 2O_2^{-\bullet} + H^+$$

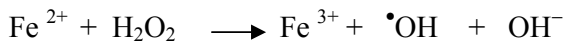
The main sources of ROS in the lung include neutrophils, eosinophils and alveolar macrophages but also alveolar epithelial cells, bronchial epithelial cells and endothelial cells are capable of generating superoxide and/or H_2O_2. The generation of ROS in the lungs is further enhanced after exposure to numerous exogenous chemical and physical agents, which include mineral dusts, ozone, nitrogen oxides, ultraviolet and ionizing radiation, and tobacco smoke.

Exposure of living organisms to background levels of ionizing radiation leads to homolytic fission of oxygen-hydrogen bonds in water to produce hydroxyl radical. Hydroxyl radical can also be generated when H_2O_2 comes into contact with certain transition metal ion chelates, especially those of iron and copper. In general, the reduced forms of these metal ions (Fe^{2+}, Cu^{+}) produce $OH^{\bullet}$ at a faster rate upon reaction with H_2O_2 than the oxidized forms (Fe^{3+} , Cu^{2+}) and so reducing agents such as $O_2^{\bullet -}$ and ascorbic acid can often accelerate $OH^{\bullet}$ generation by metal ion/H_20_2 mixtures. However, both Cu^{2+} and certain Fe^{3+} complexes do generate some $OH^{\bullet}$ upon reaction with H_2O_2 (Halliwell and Aruoma, 1991).

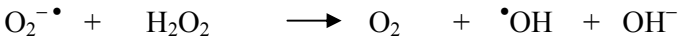
All the transition metals have variable valency which allows them undergo changes in oxidation state involving one electron. In solution, in the presence of air, the Iron (III) state is the most stable whereas Iron (II) salts are weakly reducing and ferryl compounds are powerful oxidizing agents.



In 1894, Fenton observed that a mixture of hydrogen peroxide and an iron (II) salt reacts with many organic molecules where the reactivity is due to the formation of the hydroxyl radical. If H_2O_2 can accumulate, $O_2^{-\bullet}$ and H_2O_2 can react according to the Haber-Weiss reaction (Vuillaume, 1987).

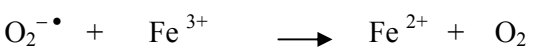
Fenton Reaction : 

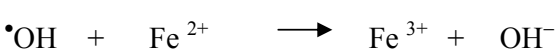
Haber-WeissReaction:



Fe^{3+} can react further with H_2O_2:

Even other reactions can occur:





$O_2^{-\bullet}$ can also be chemically generated by photoreduction of flavins, auto-oxidation of quinones and hydroquinones and by NADH oxidation by phenazine methosulphate (Greenwald, 1985).

Hydrogen peroxide can cross biological membranes and because of their limited reactivity, both H_2O_2 and $O_2^{-\bullet}$ may diffuse some distance from their sites of production. The much more reactive $OH^{\bullet}$ and singlet O_2 are consumed nearer their sites of production and their intracellular half-lives have been estimated to be 10^{-9} seconds and 10^{-6} seconds, respectively (Fisher, 1987).

Although the production and diffusion of free radicals in the cells persisits, the concentration of oxidants is effected by antioxidant status of the cell, mainly by the antioxidant enzymes. Dismutation of $O_2^{-\bullet}$ by superoxide dismutase (SOD) in mitochondria and cytosol, removal of H_2O_2 by catalase (CAT) in peroxisomes and by glutathione peroxidase (GPx) in cytosol are all enzymatic processes that modifies the oxidant pool of the cell.

1.1.2 Reactivity of Free Radicals and Oxidative Damage

The earliest idea that suggested the relation of oxygen toxicity with free radicals was proposed by Gershman and Gilbert in 1954 (Halliwell, 1987). Since then, numerous research conducted in this field has revealed that free radicals are responsible for many degenerative effects in biological systems and oxidative stress arises when reactive oxygen species are not adequately removed, leading to peroxidation of membrane lipids, depletion of nicotinamide nucleotides, increase in intracellular Ca^{2+} ions, cytoskeleton disruption and DNA damage (Vuillaume, 1987). The presence of highly oxidized biomolecules in the pathological tissues has been regarded as a strong evidence for the involvement of ROS in disease progression.

Free Radicals can react with biological molecules in a number of ways (Slater, 1984). Hydroxyl radical reacts with extremely high rate constants with almost every type of molecule found in living cells: sugars, amino acids, phospholipids such as phosphatidylcholine, nucleotides and organic acids. It is one of the most reactive chemical species known. Altered biomolecules play a critical role in the development of degenerative diseases such as atherosclerosis, Alzheimer's, Parkinson's and cancer (**Figure 1.2**).

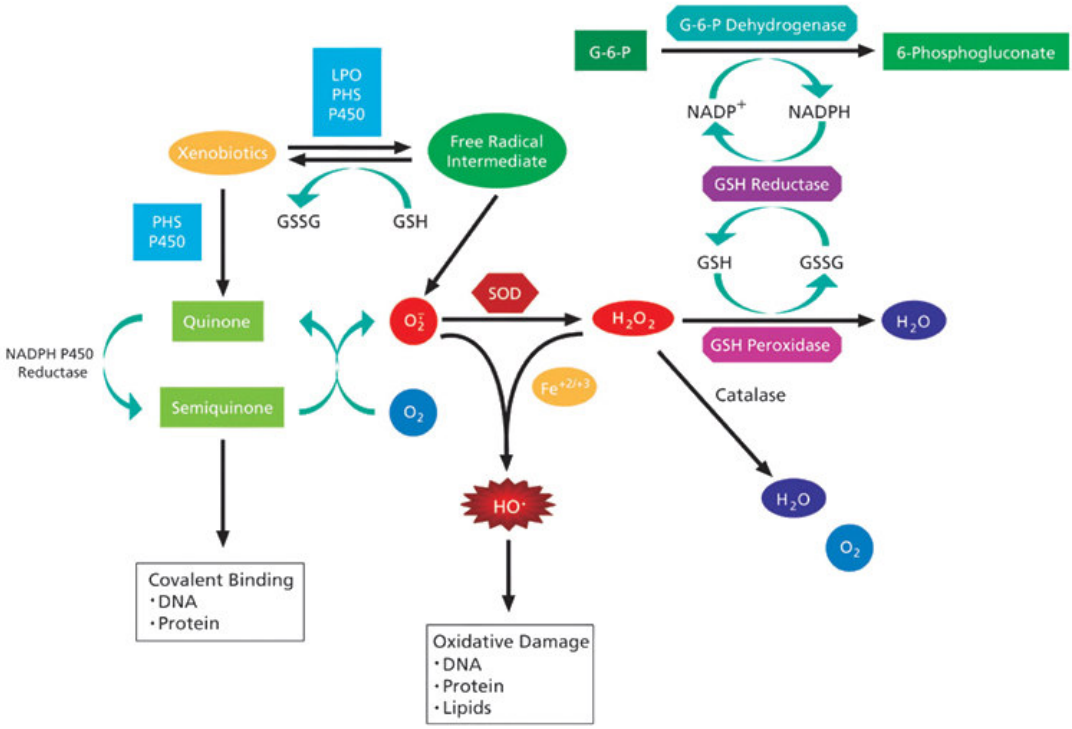


Figure 1.2. Free radical induced damage

Reactions of $OH^{\bullet}$ with biomolecules can occur as hydrogen abstraction, addition and electron transfer reactions. The reaction of $OH^{\bullet}$ with aromatic ring structures can proceed by addition and similar reactions occur with the purine and pyrimidine bases present in DNA and RNA. Addition of $OH^{\bullet}$ to guanine in DNA, addition of $OH^{\bullet}$ across a double bond in the pyrimidine base thymine, reduction of

O_2 to $O_2^{-\bullet}$ by paraquoat, oxidation of ascorbic acid and the hydrogen atom transfer in the reaction of alpha-tocopherol are some of the common reactions of free radicals in biological systems (Halliwell, 1995,1998). Free radicals can also react with non radicals to give radicals.

A substantial portion of H_2O_2 lethality involves DNA damage from iron mediated Fenton reactions (Henle and Linn, 1997). Several examples of DNA base damage induced by hydroxyl radicals have been identified recently. Some of these lesions have already been shown to be mutagenic. The sugar moiety is also a target for $OH^{\bullet}$ radical attack in DNA, although to a lesser extent than the bases. The predominant center of attack is at C-4 and the carbon centered radical produced by hydrogen abstraction undergoes rearrangements which eventually lead to base loss and strand break with two types of termini, phosphoryl and phosphoglycolate (Meneghini et.al., 1993)

Free radical reactions play an important role in the widely accepted oncogene theory of carcinogenesis, which is believed to be a multistep process. Oncogenic activators acting as initiators and promoters act by free radical mechanisms (Başağa, 1990).

The demonstration that oxidatively modified forms of proteins accumulate during aging, oxidative stress and in many pathological conditions has focused attention on physiological and non-physiological mechanisms for the generation of ROS and on the modification of biological molecules (Berlett and Stadtman, 1997; Beckman and Ames, 1997; Halliwell, 2002).

ROS can alter protein structure and function by modifying critical amino acid residues, inducing protein dimerization, and interacting with Fe-S moieties or other metal complexes. Oxidative modifications of critical amino acids within the functional domain of proteins may occur in several ways. Biological aging represents a fundamental process that results in a progressive decline in cell metabolism over time and represents the major risk factor with respect to the

development of cancer, neurodegenerative and cardiovascular diseases (Squier and Bigelow, 2000). Oxidized proteins increase in an exponential fashion with age (Tabatabaie and Floyd, 1996) and this age-related increase in carbonyl content of proteins isolated from many tissues, have been identified and estimated that 20-50% of the total protein is oxidized in aged humans. Consequently, age-dependent alterations in the calcium homeostatis also takes place (Squier and Bigelow, 2000).

Despite of its non-radical nature, peroxynitrite is more reactive than its parent molecules, where it initiates lipid peroxidation, causes DNA breakage and reacts with thiols while exerting protein modification especially by oxidation on methionine, cysteine, tryptophane or tyrosine residues and nitration of tyrosine or tryptophane residues (Virag, *et.al.*, 2003)

Several other primary targets of the oxidants have been defined by many researchers including cellular membranes as for lipid peroxidation, glyceraldehyde-3-phosphate dehydrogenase, Ca^{+2} reservoirs, and mitochondria (Cochrane, 1987).

Most biomolecules within the cells are nonradical in nature and they can become radical molecules when they react with free radicals. Consequtively, the reaction of free radicals with nonradicals in biological systems is usually proceeding as chain reactions. Lipid peroxidation is the most studied biologically relevant free radical chain reaction which has many consequences in pathobiology of several diseases (Sağdıçoğlu, 2000).

1.1.3 Lipid peroxidation

In 1940's, Farmer and coworkers have established the mechanism where unsaturated lipids can react with molecular oxygen to undergo autooxidation or peroxidation (Gutteridge and Halliwell, 1990). Later it was revealed that a free radical that has sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid is able to initiate a chain reaction in bulk lipid. The resulting carbon-centered radical reacts rapidly with molecular oxygen to form

a peroxy radical, which itself can abstract a hydrogen atom from an unsaturated fatty acid, leaving a carbon centered radical and a lipid hydroperoxide (Gutteridge, 1987).

Hydroxyl radical can initiate the first chain of a peroxidation sequence in a membrane or polyunsaturated fatty acids. By contrast, $O_2^{\bullet -}$ is insufficiently reactive to abstract hydrogen from lipids; in any case, it would not be expected to enter the hydrophobic interior of membranes because of its charged nature (Halliwell, 1989). Furthermore, peroxyl radicals can combine with eachother or they can attack membrane proteins, but they are also capable of abstracting hydrogen from adjacent fatty acid side chains in a membrane and so propagating the chain reaction of lipid peroxidation (Gutteridge and Halliwell, 1990) (**Table 1.2**).

Table 1.2. Chain reaction mechanism of lipid peoxidation

Initiation	$LH + R^{\bullet} \longrightarrow L^{\bullet} + RH$
Propagation	$L^{\bullet} + O_2 \longrightarrow LO_2^{\bullet}$ $LH + LO_2^{\bullet} \longrightarrow LOOH + L^{\bullet}$
Termination	$L^{\bullet} + L^{\bullet} \longrightarrow LL$ $LO_2^{\bullet} + LO_2^{\bullet} \longrightarrow LOOL + O_2$ $LO_2^{\bullet} + L^{\bullet} \longrightarrow LOOL$

Although a number of compounds initiate lipid peroxidation through direct production of oxidizing radicals or iron-oxygen species, further peroxidation occurs by secondary processes involving enzyme activation in which unsaturated lipids serve as substrates. Proposed mechanism for formation of lipid hydroperoxides and cyclic peroxides from arachidonic acid is shown in **Figure 1.3.**

Among the best examples are lipoxygenases that are activated by hydrogen peroxide and lipid hydroperoxides and particularly in response to disrupted membrane structure or composition (Sevanian and Ursini, 2000).

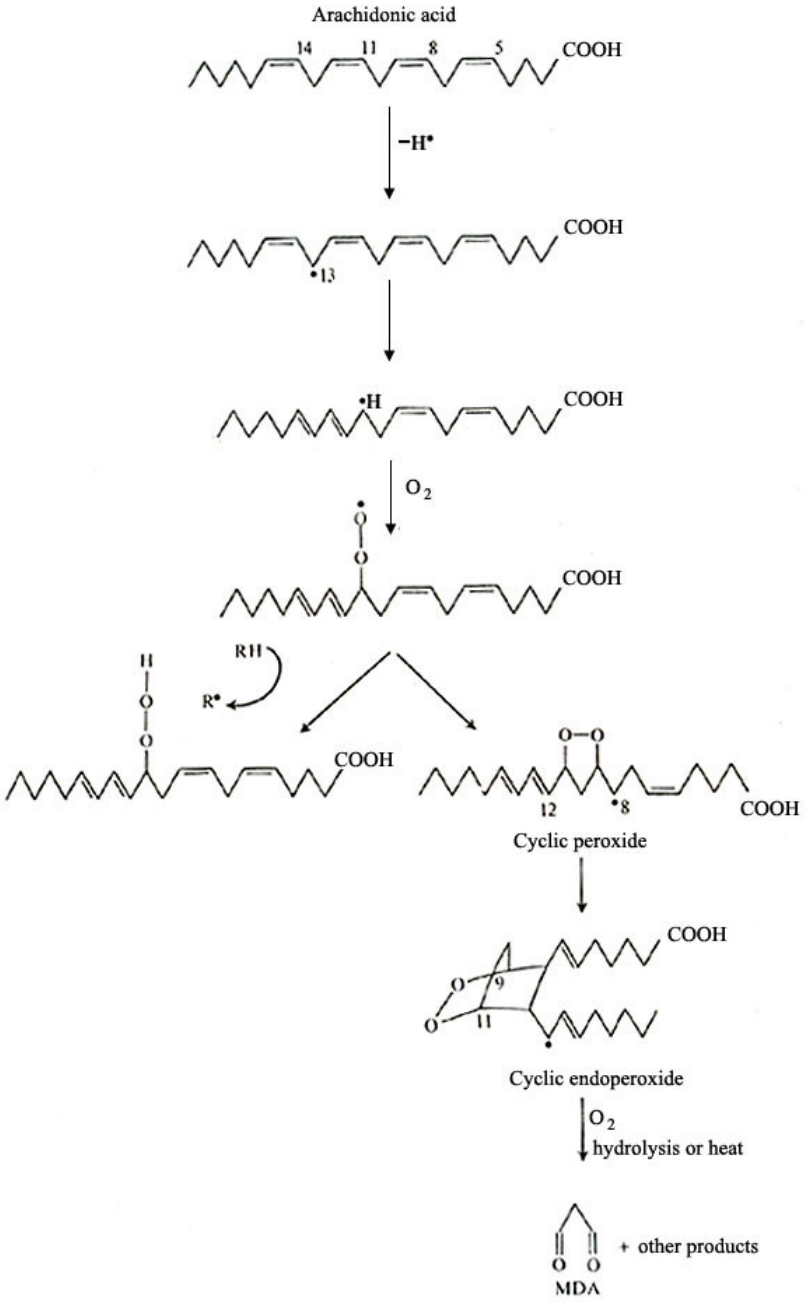


Figure 1.3. Proposed mechanism for formation of lipid hydroperoxides and cyclic peroxides from arachidonic acid (Halliwell and Gutteridge, 1989).

Iron (II) ions are themselves free radicals and they can take part in electron transfer reactions with molecular oxygen. Thus the addition of an iron (II) salt to a peroxide-free unsaturated lipid should bring about a first-chain initiation of lipid peroxidation, by H abstraction of hydroxyl radical. Iron plays a second important role in lipid peroxidation. Pure lipid peroxides are fairly stable at physiological temperatures but in the presence of transition metal complexes, including iron and copper salts, their decomposition is greatly accelerated. Thus a reduced iron complex can react with lipid peroxide in a similar way to its reaction with H_2O_2. An iron (III) complex can form both peroxy and alkoxy radicals, according to the overall equation:

$$R\text{-}OOH + Fe^{+3} - \text{complex} \rightarrow RO_2^{\bullet} + H^{+} + Fe^{+2} - \text{complex}$$

The reaction of iron and copper complexes with lipid peroxides generates a huge range of products including aldehydes, epoxides, unsaturated fatty acid aldehydes, alkanes, epoxy fatty acids, hydroxy fatty acids and hydrocarbon gases (Halliwell and Gutteridge, 1989; Başağa, 1990). Consequently, lipid peroxidation is considered in many cases including xenobiotics metabolism, inflammatory processes, ischemia and reperfusion injury, and chronic diseases such as atherosclerosis and cancer as an evidence for cellular oxidative stress (Sevanian and Ursini, 2000).

Many of the carbonyl groups produced by metal-ion dependent decomposition of lipid peroxides are aldehydes. An aldehyde isolated from peroxidizing membranes is the unsaturated aldehyde 4-hydroxy-2,3-trans-nonenal. Malonedialdehyde is also formed in small amounts in most tissues. Larger amounts of malonedialdehyde are formed during the peroxidation of other cell membrane fractions.

Dialdehydes such as MDA can attack amino groups on protein molecules to form both intramolecular cross links and also cross links between different protein molecules. MDA is a potentially important contributor to DNA damage and mutation that is produced endogenously via lipid peroxidation and prostaglandin

biosynthesis. Mutagenicity and carcinogenicity of MDA has been shown in bacterial and mammalian cell assays (Sharma, 2001; Laura et al., 2003).

The biological effects of lipid peroxidation produce extensive changes in polyunsaturated fatty acids that occur during peroxidation of biomembranes and involve the highly unsaturated fatty acids arachidonate and decosahexaenote. Therefore, lipid peroxidation decreases the relative content of these two fatty acids, leading the destruction of anatomical integrity of membranes.

Formation of lipid hydroperoxides may inhibit or stimulate various enzymes associated with biomembranes. A lipid peroxide diminishes membrane fluidity, increases nonspesific permeability to ions such as Ca^{2+}, inactivate membrane bound enzymes by oxidizing various thiol groups that are required for the activities of enzymes in the membrane (Halliwell and Gutteridge, 1989).

1.1.4 Cell Signaling

Although elevated levels are associated with the pathogenesis of various diseases including cancer, ROS are also important second messengers generated in response to many types of environmental stress. In this setting, changes in intracellular ROS can activate signal transduction pathways that influence how cells react to their environment (Victor *et al.*, 2004).

ROS can regulate the cellular signaling pathways by regulating the gene expression (Ryter and Tyrrel, 1989 Thannickal and Fanburg, 2000). Several cytokines, growth factors, hormones, and neurotransmitters use ROS as secondary messengers in the intracellular signal transduction (Nordberg and Arner, 2001) Recent studies have also implicated ROS that are generated by specialized plasma membrane oxidases in normal physiological signaling by growth factors and cytokines. (Thannickal and Fanburg, 2000)

The immune system highly relies on accurate cell-cell communications for its function, and any damage to the signaling systems results in an impaired immune responsiveness. Since the phagocyte cells produce ROS as part of the defense against infection, adequate amounts of neutralizing antioxidants are required to prevent damage to the immune cells themselves (Victor et al., 2004)

Reactive oxygen species are generated as by-products of cellular metabolism and are known to be very important for cellular signaling netwoks, however, when cellular production of ROS overwhelms its antioxidant capacity, damage to cellular macromolecules such as lipids, protein, and DNA may appear. Such a state of oxidative stress is thought to contribute to the pathogenesis of a number of human diseases including atherosclerosis, Alzheimer, Parkinson, cancer and aging. Therefore, the control of cellular oxidant-antioxidant status is very important (Thannickal and Fanburg, 2000).

In addition, expression of glutathione-S-transferase is inducible by several pro-oxidants and regulated by the cellular redox status working as a sensor for transmitting the redox variations to apoptosis machinery through modulating the kinases pathway. Therefore these enzymes are also play important roles in the signaling cascades (Hayes and McLellan, 1999; Adler et.al, 1999; Pastore *et al*, 2003).

1.2 Antioxidant Protection Mechanisms Against Free Radical Damage

In the nature, there are some enzymes and small molecular weight molecules having antioxidant properties which are protecting tissues and cells from the adverse effects of free radicals. The aerobic organisms constitute a defence system either independently, cooperatively or even syneristically.

An antioxidant can be defined as a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly prevents or delays a pro-oxidant initiated oxidation of the substrate (Prior and Cao, 1999).

Antioxidants can show their antioxidative property either by decreasing localized O_2 concentration, preventing first-chain initiation by scavenging initiating radicals such as $OH^{\bullet}$, binding metal ions in forms that will not generate such initiating species as $OH^{\bullet}$, ferryl or Fe^{+2}, Fe^{+3}, O_2 and/or will not decompose lipid peroxides to peroxy or alkoxy radicals, decomposing peroxides by converting them to non-radical products such as alcohols, or by chain breaking by scavenging intermediate radicals, such as peroxy and alkoxy radicals, to prevent continued hydrogen abstraction (Halliwell and Gutteridge, 1989).

According to Niki, the antioxidants acting in the defence systems are classified into 3 categories depending on their functions as preventive antioxidants, radical-scavenging antioxidants or repair and *de novo* antioxidants (**Figure 1.4)** (Niki, 1993)**.**

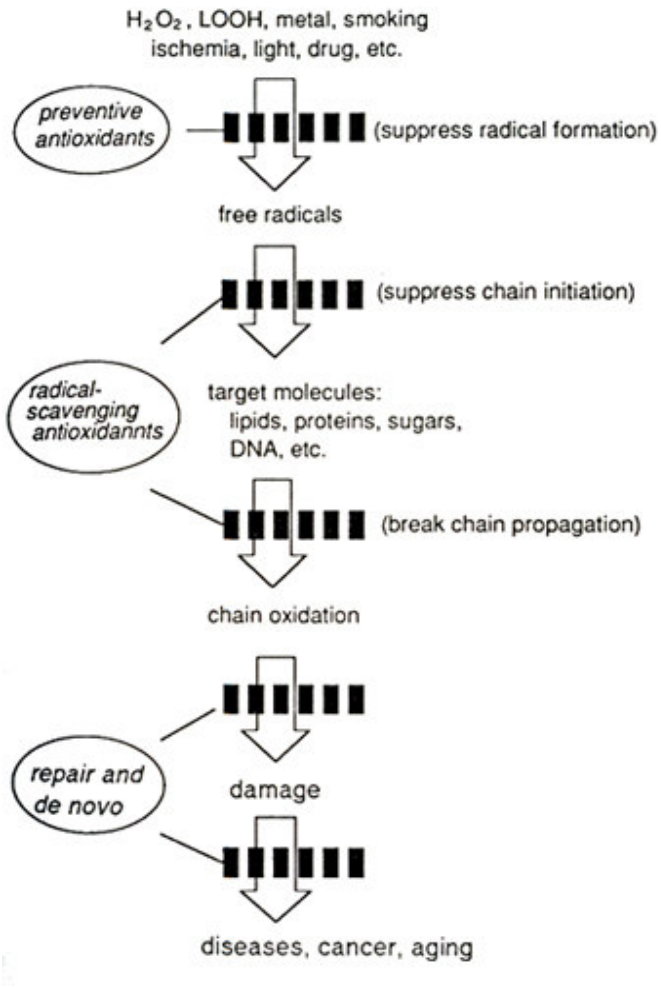


Figure 1.4. Antioxidants in the defence system against free radical induced oxidative damage (Niki, 1993)

The first line of defence are preventive antioxidants which supress the formation of free radicals by decreasing O_2 concentration, preventing first-chain initiation by scavenging radicals and binding metal ions. Catalytic removal by antioxidant enzymes Superoxide dismutase, catalase, and peroxidases constitute also a supportive team of defense against ROS (**Table 1.3**).

The enzymes Glutathione peroxidase, glutathione-s-transferase, phospholipid hydroperoxide glutathione peroxidase and peroxidase which decompose lipid hydroperoxides to corresponding alcohol are also important preventive antioxidant enzymes (Niki, 1993; Meneghini et al., 1993).

Table 1.3. Endogenous antioxidants **(Sorg, 2004)**

Antioxidant	**Phase**	**Action**
Superoxide dismutases (SOD)	Hydrophilic	Dismutation of O_2- into H_2O_2 and O_2
Catalase	Hydrophilic	Dismutation of H_2O_2 into H_2O and O_2
Glutathione peroxidases (GPx)	Hydrophilic or lipophilic	Reduction of R–OOH into R–OH
Glutathione reductase (GR)	Hydrophilic	Reduction of oxidised glutathione
Glutathione-S-transferases (GST)	Hydrophilic	Conjugation of R–OOH to GSH (GS–OR)
Metallothioneins	Hydrophilic	Binding to transition metals (= neutralisation)
Thioredoxins	Hydrophilic	Reduction of R–S–S–R into R–SH
Glutathione	Hydrophilic	Reduction of R–S–S–R into R–SH Free radical scavenger Cofactor of GPx and GST
Ubiquinol	Lipophilic	Free radical scavenger
(Dihydro)lipoic acid	Amphiphilic	ROS scavenger Increases antioxidant and phase II enzymes
Ascorbic acid (vitamin C)	Hydrophilic	Free radical scavenger Recycles tocopherols Maintains enzymes in their reduced state
Retinoids (vit. A) and carotenoids	Lipophilic	Free radical scavengers
Tocopherols (vitamin E)	Lipophilic	Singlet oxygen ($^1O_2$) quencher
Selenium	Amphiphilic	Free radical scavenger (prevents LPO) Increases selenium absorption Constituent of GPx and thioredoxins

The first enzyme involved in the antioxidant defense is the superoxide dismutase: a metalloprotein found in both prokaryotic and eukaryotic cells (Fridovich, 1983, 1986). There are three of them in mammalian systems: a cytosolic CuZn superoxide dismutase (SOD1), an intramitochondrial manganese superoxide dismutase, (SOD2) and an extracellular CuZn superoxide dismutase (SOD3) (Fridovich, 1995).

It is a specific catalyst that lowers $O_2^{\bullet-}$ by converting it to H_2O_2 (Bandyopadhyay et al., 1999).

$$O_2^{\bullet-} + O_2^{\bullet-} \xrightarrow[\text{SOD}]{2\ H+} H_2O_2 + O_2$$

Superoxide can be converted to hydrogen peroxide by manganese superoxide dismutase (Mn-SOD) in the mitochondrial matrix and copper-zinc superoxide dismutase (Cu/Zn-SOD) largely in the cytosol, consequently converted to oxygen and water by catalase or glutathione peroxidase (Brand *et.al.*, 2004)

Catalase is present in almost all the mammalian cells and localized in the peroxisomes or the microperoxisomes (Chance et.al, 1979). It is a hemoprotein which catalyses the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage (Deisseroth and Dounce, 1970).

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

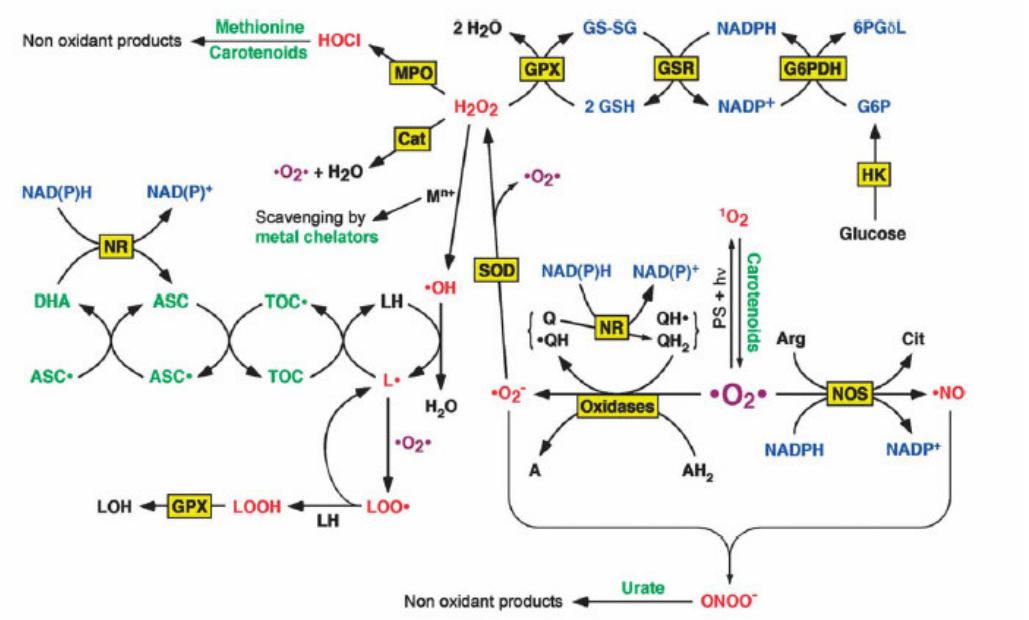


Figure 1.5. Oxidants and antioxidants in the cell (Sorg, 2004).

Peroxidases also lowers the stady-state kevels of H_2O_2 (Bandyopadhyay et al., 1999). The most important H_2O_2 removing enzymes in human cells are glutathione peroxidases. They reduce H_2O_2 to water using two molecules of reduced glutathione (GSH). The oxidized glutathione (GSSG) is subsequently reduced by glutathione reductase (GR) under consumption of NADPH (Nordberg and Arner, 2001). Oxidants and antioxidants in the cell are shown in **Figure 1.5.**

Glutathione peroxidases require selenium as selenocysteine at their active sites (Halliwell, 1991). Various GPx isoforms exist, which are specific for hydrophilic or lipophilic phases (Sorg, 2004).

Glutathione also reduces glutaredoxins which in turn reduce various substrates. Specific for glutaredoxins is the reduction of glutathione mixed disulfides such as glutathionylated proteins (**Figure 1.6**). Since GSTs catalyze the conjugation of glutathione with other molecules, they function as an intermediate step in the detoxification of miscellaneous toxic substances (Nordberg and Arner, 2001)

Glutathione reductase is an FAD-containing enzyme that regenerates GSH from GSSG with NADPH as a source of reducing power.

Glutathione (L-g-glutamyl-L-cysteinylglycine) is the principal nonprotein thiol involved in the antioxidant cellular defence against ROS. It plays an important role both in protecting cells against cytotoxins and in regulating the cellular response to stress (Kauvar, 1998). GSH reacts directly with radicals in nonenzymatic reactions. The redox status of the cell depends on the relative amounts of the reduced and oxidized forms of glutathione (GSH/GSSG) and is a very critical determinant in cell. Glutathione functions in the detoxification of hydrogen peroxide, other peroxides and free radicals. It also plays a role in the detoxification of a variety of xenobiotics, which interact with glutathione (Dringen, 2000; Pastore, 2003).

Glutathione can also prevent oxidative stress caused by redox-cycling of quinines (Cnubben et. al., 2001).

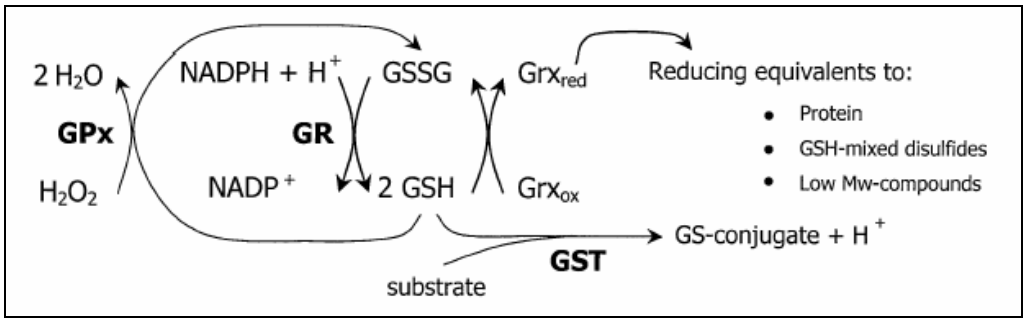


Figure 1.6. Schematic summary of the major glutathione-associated antioxidant systems (Nordberg and Arner, 2001**).**

Metallothioneins are small proteins with several cysteine residues which bind transition metal ions: this can both detoxify metals and avoid them catalysing the Haber–Weiss and Fenton reactions that lead to the production of the hydroxyl radical (Sorg, 2004).

The second line of defence is the antioxidants which scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions. Vitamin C, uric acid, bilirubin, albumin and thiols are the hydrophilic while α-tocopherol (vitamine E), ubiquinol, retinoic acid and carotene are lipophilic radical scavenging antioxidants. (Burton and Ingold, 1984; Meneghini et al., 1993). Lipid-soluble antioxidants are important in preventing membrane polyunsaturated fatty acids from undergoing lipid peroxidation, which leads to loss of membrane integrity (Victor, 2004).

Chain–breaking antioxidants are often phenols or aromatic amines (Halliwell and Gutteridge, 1989).

Major antioxidant action of alpha-tocopherol in biological membranes under most conditions is to react with lipid peroxy and alkoxy radicals, donating labile hydrogen to them and so terminating the chain reaction of peroxidation by scavenging chain propagating radicals (**Figure 1.7**):

$$RO_2^{\bullet} + TH \rightarrow ROO_2 + T^{\bullet}$$

$$RO^{\bullet} + TH \rightarrow ROH + T^{\bullet}$$

The tocopheryl radical T is insufficiently reactive to to abstract H from membrane lipids because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure so increasing its stability and its reactivity with organic peroxyl radicals is associated with the redox properties of the chromane ring (Halliwell et al., 1995; Stahl, 2000).

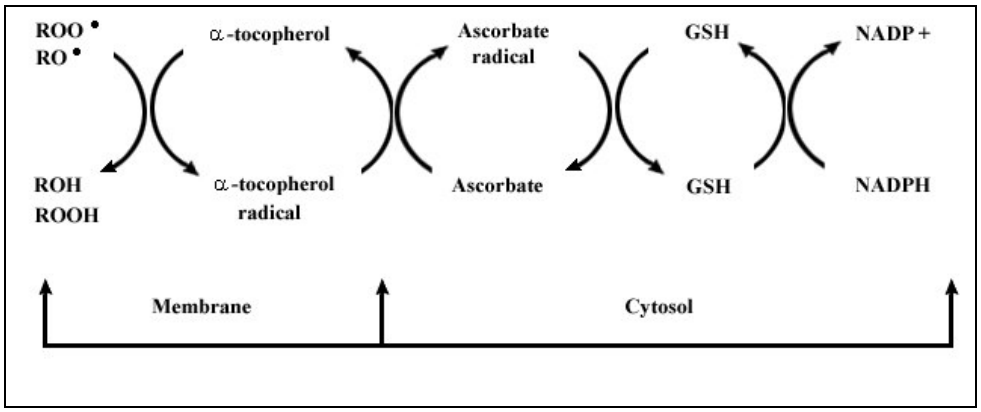


Figure 1.7. Mechanism of free radical scavenging action of cellular low molecular weight antioxidants, α-tocopherol, ascorbate and reduced glutathione (GSH), through NADPH-glutathione reductase system (Bandyopadhyay et al., 1999).

Vitamin C, the prominent water-soluble antioxidant, has a number of well-defined biological functions, including collagen, catecholamine, and carnitine biosynthesis. It very effectively scavenges a wide array of ROS and free radicals, forming the first line of antioxidant defense in plasma against many different types of ROS and radicals and, surprisingly, also protects LDL more effectively against oxidation than vitamin E (Frei, 1994).

Numerous metal-binding plasma proteins function as valuable antioxidants together with their transport roles including apotransferrin, lactoferrin and caeruloplasmin. Albumin is also effective via its oxidizable thiol group, which permits radical scavenging, and the binding of reactive transition metal ions (Victor, 2004)

Other important factors which determine the overall antioxidant activities are the fate of the radical derived from the antioxidant and the site of radical formation. In general, the radical scavenging antioxidant donate hydrogen to active radicals and new radical is formed from the antoxidant.

Various phospholipases repair the oxidatively damaged phospholipids where they are classified in the repair and *de novo* antioxidants group. The proteolytic enzmyes, proteinases, proteases and peptidases present recognize, degrade and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins (Davies, 1988).

There is also a fourth line of defence which is the adaptation mechanism where the signal for the production and reactions of free radicals induce formation and transport of the appropriate antioxidant to the right site (Niki, 1993).

1.3 Methodology in Free Radical Research and Antioxidant Activity

The role of ROS in chronic degenerative diseases such as autoimmune, inflammatory, cardiovascular, neurodegenerative diseases, cancer and the outcome in the oxidative reactions occuring in foods, pharmaceuticals and cosmetics are currently under investigation. Free radicals and antioxidants are considered as an emerging research area of science where the new findigs open promising horizons in health and disease management. To prevent the damaging effects of free radicals, a number of investigations are working to reveal especially the natural antioxidants using various methodologies (Sanchez-Moreno, 2002; Aruoma, 2003; Gulcin *et. al.*, 2004).

Dietary compunds have long been suggested to reduce the risk of some cancers and are even used for the treatment of certain diseases. Consequently, food and plant extracts have been the subject of study for many researchers to reveal the potential antioxidants and other bioactive components such as vitamins and flavonoids (Ogawa *et.al.*, 1999; Nuutila *et.al.*, 2003; Gülçin et.al., 2004). Nevertheless, there are difficulties associated with methods used in evaluating the antioxidant activity both *in vivo* and *in vitro.* This fact brings out the necessity to use a mix of possible tools for the evaluation of antioxidant activity (Aruoma, 2003).

The main methods to measure the antioxidant activity comprise superoxide radicals scavenging; hydrogen peroxide scavenging; hypochlorous acid scavenging; hydroxyl radical scavenging; peroxyl radical scavenging, among them are the methods that use azo-compounds to generate peroxyl radicals, such as the 'TRAP' method (Total Radical-Trapping Antioxidant Parameter) and the 'ORAC' method (Oxygen-Radical Absorbance Capacity); the scavenging of radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) or the ABTS or the 'TEAC' method (Trolox Equivalent Antioxidant Capacity); the scavenging of stable radical 2,2-diphenyl-1-picrylhydrazyl or DPPH radical method and the scavenging of radical cation N,N-dimethyl-p-phenylenediamine or DMPD method (Sanchez-Moreno, 2002**).**

Each assay used to evaluate the antioxidant activity measures a different criteria therefore none of them is the method of choice alone (Halliwell and Gutteridge, 1989).

Certain enzymes such as lipoxygenases, cycloxygenases and xanthine oxidase produce reactive oxygen species during specific reactions. The inhibitors of these enzymes are also considered as the targets for decreasing reactive oxygen species in biological systems (Vaya and Aviram, 2001).

1.4 Plants As Natural Sources of Antioxidants

It is generally agreed that a diet rich in fruits and vegetables containing various micronutrients is important for the prevention of several degenerative diseases and contributes to the maintenance of health, by decreasing both oxidative damage and cancer incidences (Stahl, 2000; Halliwell, 2002).

Some vitamins and other micronutrients are among the most important non-enzymatic antioxidants, but many endogenously produced compounds also exhibit antioxidant functions, and often act synergistically with antioxidants of dietary

origin. An appropriate selection of nutrients contributes to the prevention of various disorders and reduces risk factors (Diplock, 1994; Ames, 1998; Stahl, 2000)

There is a growing interest to use natural antibacterial compounds, like plant extracts of herbs and spices for the preservation of foods, as these possess a characteristic flavour and sometimes show antioxidant activity as well as antimicrobial activity. Consumption of foods containing significant amounts of polyunsaturated fatty acids has increased the importance and use of the antioxidants to prevent oxidation. The addition of antioxidants is a method of increasing the shelf life, especially of lipids and lipid containing foods. Synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytolune, have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic. Therefore, the importance of search for natural antioxidants, especially of plant origin, has greatly increased in recent years (Habsah, 2000; Aruoma, 2003; Xu et al., 2005).

Plants contain numerous different compounds which have various pharmacological actions. Besides the compounds of primary metabolism such as carbohydrates, lipids and amino acids, they have very important bioactive constituents such as phenolic acids, coumarins, lignans, tannins, anthocyanins, sesquiterpenes, iridoids, cardiac glycosides, caretenoids and saponins (Bruneton, 1995).

Among natural antioxidants, polyphenolic compounds, which contain multiple phenol rings within their structures, are reported to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical. Also, phenolic compounds of plant materials have been shown to neutralize free radicals in various model systems. The antioxidant effect of polyphenols has been demonstrated in both *in vitro* studies (human low density lipoprotein, liposomes) and in normal human subjects (Shon et al., 2003). Consistantly, chain–breaking, natural antioxidants are usually pointed out to be phenols, polyphenols or aromatic amines (Halliwell and Gutteridge, 1989; Velioglu, 1998)

Dietary antioxidants also confer significant protection to gut epithelial cells from pro-apoptotic oxidant stress. The phytochemical mixtures found in the teas, cat's claw and green tea were evaluated to be more effective than vitamin C, where the benefits of antioxidant-rich diets are in part related to a reduction in signals leading to apoptosis and cell activation. (Miller, 2001).

Flavonoids and polyphenols are recognized to be suitable antioxidants for the prevention of diseases associated especially with the process of lipid peroxidation (Biesalski, 1997; Diplock, 1998)

Researchers have been commonly working on the antioxidant activities of plant extracts since plants are natural sources for antioxidant (Erdemoglu and Sener, 2000; Aruoma, 2003; Cheung *et. al.*, 2003; Shon *et. al.*, 2004; Xu *et. al*., 2005; Skerget, 2005). Consequently, many natural antioxidants have already been isolated from different kinds of plant materials, such as oilseeds, cereal crops, vegetables, leaves, roots, spices, and herbs (Yesilada, 2002; Ersoz *et. al.*, 2003; Cakir *et. al.*, 2003; Taubert *et. al.*, 2003; Tan, Bay and Zhu, 2004). It is also obvious that the structural diversity provided by the natural products is greater than that provided by most of the synthetic techniques (Harvey, 1999)

Phenolic phytochemicals are mainly expressed in three different groups according to their chemical structures and their synthesis pathways as flavonoids, non-flavonoid ployphenols and phenolic acids (**Figure 1.8**).

Among phenolic compounds, flavonoids attracted growing global interest during the last decade, therefore, the number of reported flavnonoids has increased dramatically (Rauha, 2001). The existence of 6500 different known flavonoids was stated in 1999 (Harborne and Baxter, 1999) Flavonoids are further divided into groups of flavones, flavonols, isoflavones, flavanones and anthocyanins.

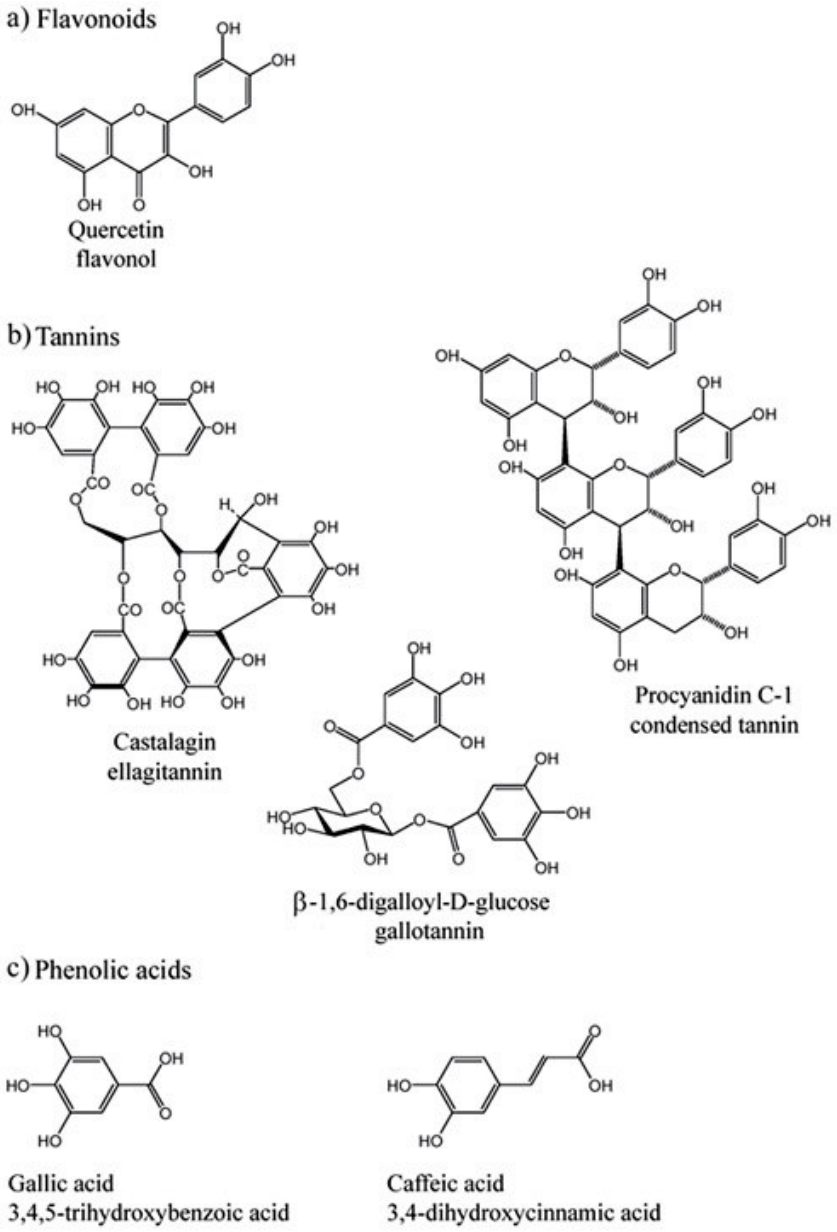


Figure 1.8. Typical structures of different groups of plant phenolics: a) Flavonoids, b)Tannins, c) Phenolic acids (Rauha, 2001)

Major types of phenolics which are found in commonly used medicinal plants and their parts are given in **Table 1.4.**

Table 1.4. Major types of phenolic compounds found in some medicinal plants (Cai, *et. al*., 2004 ; Rauha, 2001)

Scientific name	Medicinally used parts	Major types of phenolics
Acacia catechu (L.) Willd.	Branch, stem	Hydrolyzable and condensed tannins, Flavan-3-ols ((+)-catechin, epicatechin)
Salvia miltiorrhiza Bge.	Root	Phenanthroquinones, flavonoids (baicalin), phenolic acids
Glycyrrhiza uralensis Fisch.	Root	Flavanones , lycopyranacoumarins
Punica granatum L.	Peel	Tannins (ellagitannins), flavonols (quercetin), phenolic acids (gallic acid, chlorogenic acid)
Rosa chinensis Jacq.	Flower	Flavonoids (quercetin, catechin, anthocyanins), phenolic acids (gallic acid), tannins
Gardenia jasminoides Ellis	Fruit	Phenolic acids (chlorogenic acids), flavones (gardenins)
Citrus aurantium L.	Immature fruit	Flavanones (hesperidin, naringenin, naringin)
Camellia sinensis (L.) Kuntze	Young leaf or flush	Flavanols (cathecin, epicathecin), flavonols (kaempferol and quercetin glycosides), condensed tannins
Zingiber officinale	Rhizome	Phenolic volatile oils (gingerols)
Rheum officinale Baill.	Root	Anthraquinones (emodin, chrysophanol, rhein, physcion and their glycosides), phenolic acids (gallic acid), hydrolysable tannins
Magnolia officinalis Rehd at. Wils.	Bark	Neolignans (magnolol, isomagnolol), tannins
Thymus vulgaris	Plant	Flavones, flavonols, flavanones
Allium cepa L.	Bulb	Flavonols
Solanum tuberosum L.	Tuber	Flavonols, flavanones, anthocyanins, flavans

Medicinal preparations phytopharmaceutials which are obtained after prosecing the medicinal plant by extraction, distillation, fractionation, purification, condensation and fermentation. They can be prepared as extracts, teas, volatile oils, non-volatile oils or powders. The amount of bioactive compounds found in the

plants are variable depending on the collection time, place, drying conditions, percolation and extraction. Consequently, standardization of the product should be defined from one kind of bioactive chemicals known to exist in that plant.

A number of randomised, placebo-controlled plant originated antioxidant supplementation trials are under way since many years (Guillaume and Padioleau, 1994; Salonen, 1999; Leach, 2004)

1.5 Drug Metabolizing Enzymes

The drug metabolizing enzymes (DMEs) are a diverse group of proteins that are responsible for metabolizing a vast array of xenobiotic compounds including drugs, environmental pollutants, endogenous compounds such as steroids and prostaglandins and many other phytochemicals found in food and plants. They are largely found in the liver as microsomal enzymes and in many other tissues including the lung and GI tract.

Drug metabolizing enzymes are divided into two groups: Phase I enzyme systems, the oxidative drug metabolizing enzymes, which include Cytochrome P450s (CYPs) and flavin monoxygenases (FMOs), catalyze the introduction of an oxygen atom into substrate molecules, generally resulting in hydroxylation or demethylation. Phase II, the conjugative enzyme families including the UDP-glycosyltransferases, glutathione transferases, sulfotransferases, and N-acetyltransferases. The second group catalyze the coupling of endogenous small molecules to xenobiotics that usually results in the formation of soluble compounds that are more readily excreted (Guengerich, 1995; Parkinson, 1996).

DMEs play a very important role in chemoprevention as well as chemotherapy since they catalyze the detoxification reactions. They are induced in the body when they encounter xenobiotics, as a defence system of the body. In several animal studies it was reported that cancer incidence was reduced while the

activity of phase II enzymes, in particular glutathione S-transferase, was increased (Percival, 1997)

However, in some case, a non-toxic compound can be transformed into a toxic substance by Phase I enzymes, as in the case of cigarette smoke. In such a situation, the activity of Phase II enzymes becomes very important to effectively eliminate biotransformed intermediates. One important mechanism by which natural compounds found in food may exert their in vivo chemopreventive effects is through their inhibition of phase I metabolizing enzymes, such as cytochrome CYP1A1 and CYP2E1, which metabolically activates procarcinogens to reactive intermediates that trigger carcinogenesis (Ren et al., 2003)

Flavonoids galangin and resveratrol have been shown to inhibit CYP1A1 thereby preventing the metabolism and activation of polycyclic aromatic hydrocarbons (Wattenberg, 1992).

On the other hand, tumors develop drug resistance where the activity of GSTs increase, which is the major obstacles of cancer chemotherapy. The development of drug resistance and the severe side effects of chemotherapy also damage normal tissues. Therefore, the fact that activity of the human glutathione S-transferase enzymes can be influenced by multiple natural compounds (Haaften *et. al.,* 2003), is regarded as a promising strategy.

1.5.1 Glutathione-S-Transferases

GSTs (EC 2.5.1.18) are a multigene family of phase II drug-metabolizing enzymes found in all eukaryotic and prokaryotic systems, located mainly in the cytoplasm, in the microsomes and in mitochondria. (Morgenstein and DePierre, 1988; Landi, 2000; Cnubben *et. al.*, 2001). In humans, about 3–5% of total soluble protein of liver consists of GST protein (Awasthi *et. al.*, 2004)

1.5.1.1 Structure and Classification

Nine different classes of mammalian GSTs have been identified regarding to the substrate and inhibitor specificity, chemical affinity, structure, amino acid sequence, antibody cross reactivity and kinetic behavior of the enzyme. Eight classes namely, alpha (A), mu (M), pi (P), kappa (K), sigma (S), zeta (Z), omega (O) and theta (T) of the gene families encode the cytosolic GSTs and the ninth encodes a membrane-bound form (Hubatsch and Mannervik, 2001; Gyamfi e*t. al.,* 2004) .

Subunits of GSTs are classified on the basis of a general nomenclature proposed by Mannervik *et. al.* and is applicable to all vertebrate GSTs and extendable to prokaryotes and other organisms (Mannervik, 1985; 1992). A lower case letter is used for denoting the species, an upper case letter for the class, and an Arabic number for the subfamily for the mammalian subunit GSTs. When the enzyme rather than the subunit are named, repeated number of subunits is written, to express the homodimeric structures (Landi, 2000).

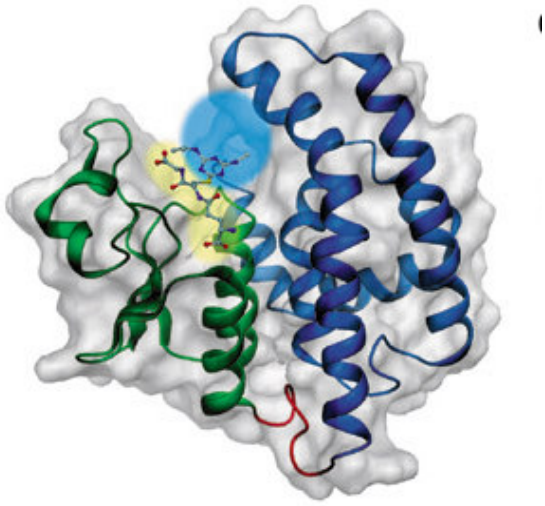


Figure 1.9. Overview of GST dimer structure and substrate binding. A ribbon/surface representation of a typical GST subunit, with the amino-terminal domain in green, the linker region in red, the carboxy-terminal domain in blue and the protein surface in gray (Dixon et.al, 2002).

The soluble GSTs exist as homodimers or heterodimers of approximately 25 kDa, having kinetically independent active sites and a common ancestry evolutionarily (Landi, 2000). Each of the subunits is comprised of a glutathione binding site (G site) and an adjacent relatively hydrophobic site (H site) for the binding of the electrophilic substrate. Besides, in various isozymes, at least in the alpha and pi class, a non-substrate binding site was reported, possibly functioning as a transport site or a regulatory site (Van Bladeren and Van Ommen, 2000; Cnubben et. al, 2001; Zanden *et. al.,* 2003). Structure of GSTs are shown in **Figure 1.9.** Membrane-bound microsomal GSTs present in human tissues are trimers of about 17 kDa subunits (Awasthi *et. al.*, 2004)

1.5.1.2 Reactions

GSTs catalyze the general reaction:

$$GSH + R\text{-}X \rightarrow GSR + HX$$

These superfamily of isoenzymes catalyse the conjugation of electrophilic compounds with the –SH group of glutathione (**Figure 1.10**) thereby neutralizing their electrophilic sites and rendering the new products more water-soluble. (Habig *et. al.*, 1974; Cnubben *et. al.*, 2001)

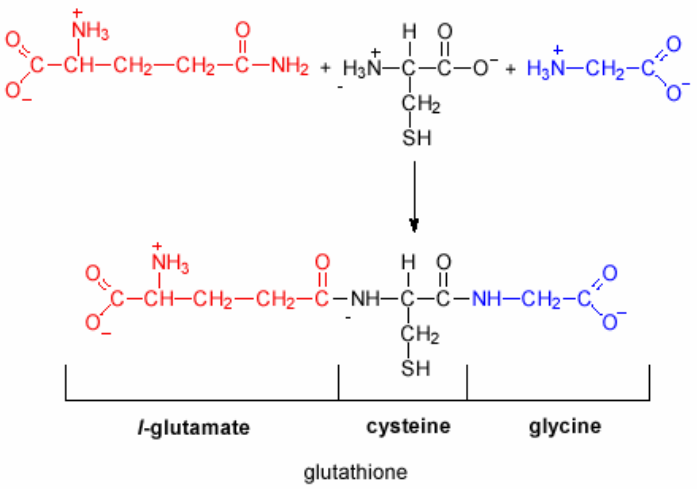


Figure 1.10. Chemical structure of glutathione

They catalyze the conjugation of various xenobiotics, such as herbicides, insecticides, carcinogens and anticancer agents (Mannervik, 1985; Board et al., 1990) therefore playing an important role in the major defense system of cells against electrophilic compounds. (Zanden *et. al.,* 2003). Some of the substrates for GSTs are shown in **Figures 1.11, 1.12 and 1.13.**

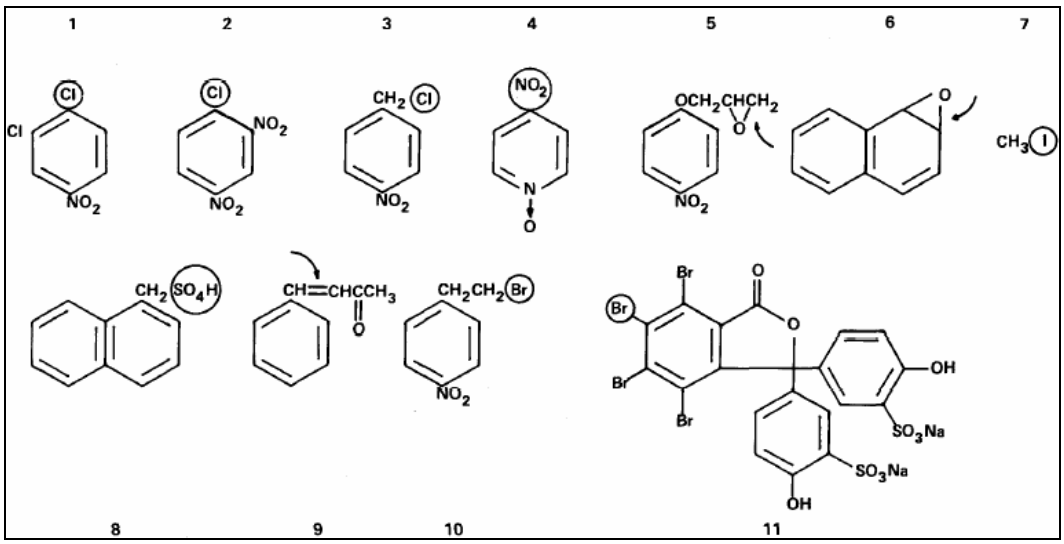


Figure 1.**11.** Substrates for glutathione transferases. 1) 1,2-dichloro&nitrobenzene; 2) 1-chloro-2,4-dinitrobenzene; 3) p-nitrobenzyl chloride; 4) 4-nitropyridine-N-oxide; 5) 1,2-epoxy-3-(p-nitrophenoxy)propane; 6) 1,2-naphthalene oxide; 7) iodomethane; 8) 1-menaphthyl sulfate; 9) trams-4-phenyl-3-buten-2-one; 10) p-nitrophenethyl bromide; 11) bromosulfophthalein. Leaving groups are circled, whereas arrows are indicating the addition site of GSH to alkenes and epoxides (Habig *et. al.*, 1974).

Especially chemotherapeutic agents such as cyclophosphamide and chlorambucil, are metabolised by GSTs. Examples of GSTs substrates from different compound categories are given in **Table 1.5.**

Table 1.5. Examples of GSTs Substrates from Different Compound Categories (Hayes and Pulford, 1995).

Compound Type	**Substrates**
Metabolites of Carcinogens	Aflatoxin B_1-8,9-epoxide Benzo[*a*]pyrene-7,8-diol-9,10-oxide 5-hydroxymethylchrysene sulfate 7-hydroxymethylbenz[*a*]anthracene sulfate 4-nitroquinoline *N*-oxide
Pesticides	Alachlor Atrazine Dichlorodiphenyltrichloroethane (DDT) Lindane Methyl parathion
Oxidative-damage products	Acrolein Base propenals Cholesterol α-oxide Fatty acid hydroperoxides 4-hydroxynonenal
Anticancer drugs	1,3-*bis*(2-chloroethyl)-1-nitrosourea Chlorambucil Cyclophosphamide Melphalan Thiotepa Fosfomycin

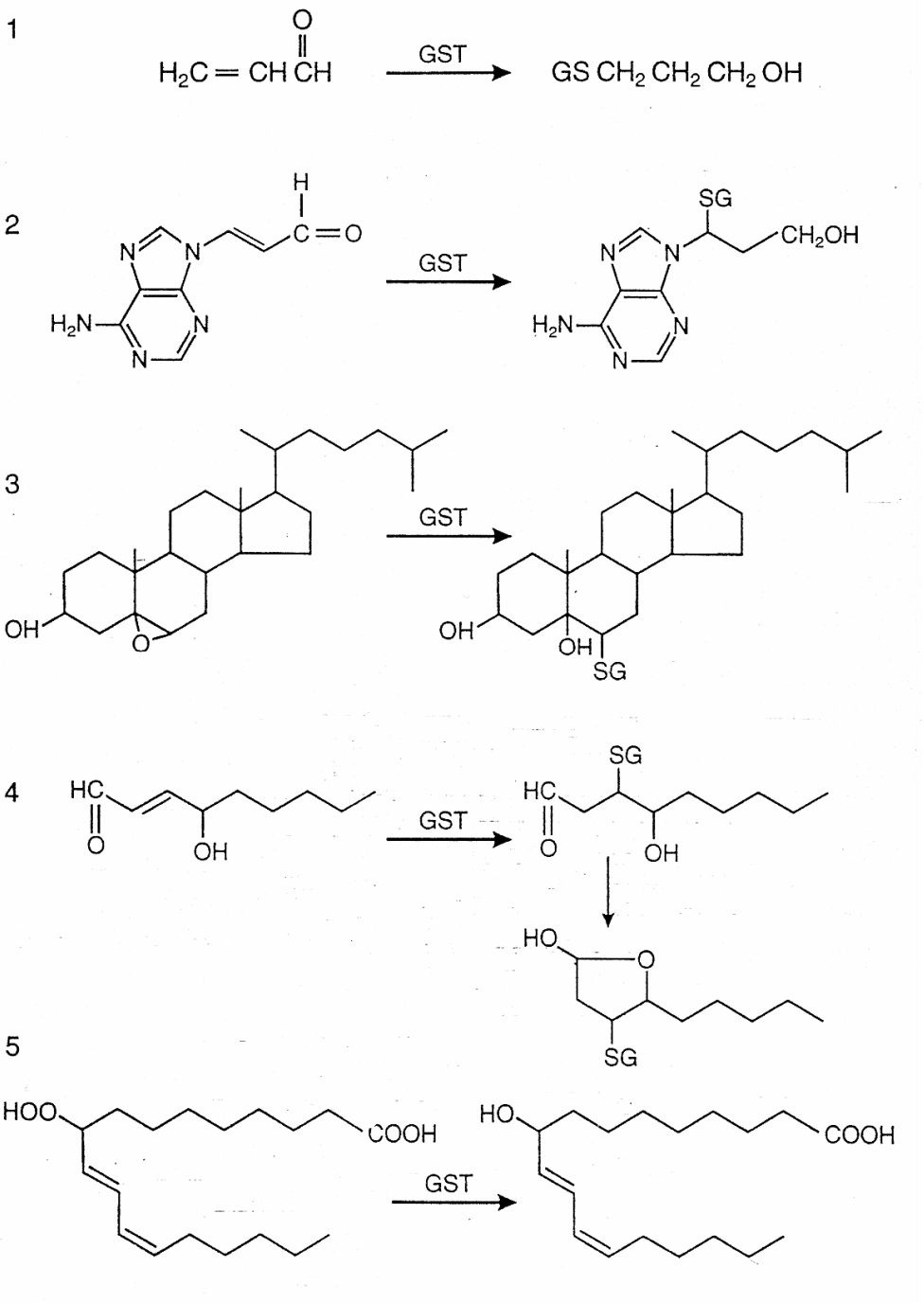


Figure 1.12. Examples of GST substrates that are produced by oxidative stress: (1) acrolein; (2) adenine propenal; (3) cholesterol-5,6-oxide; (4) 4-hydroxynon-2-enal (5) 9-hydroperoxy-linoleic acid (Hayes and Pulford, 1995).

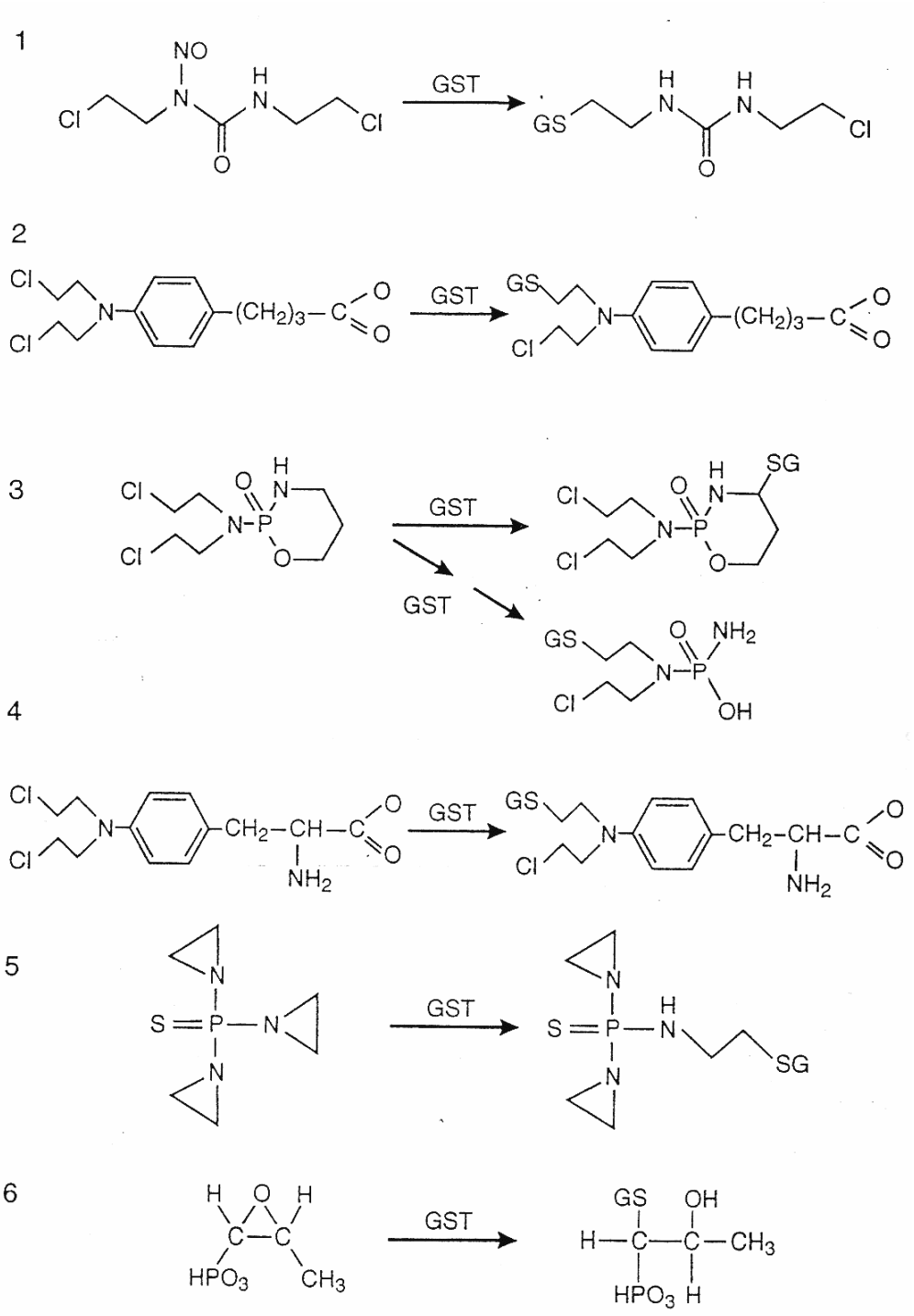
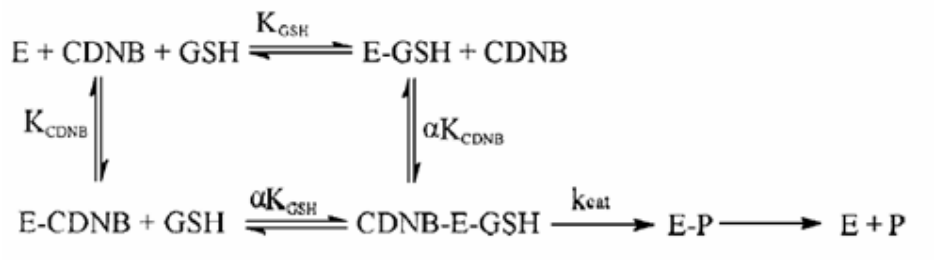


Figure 1.13: Examples of chemotherapeutic agents that are GST substrates: (1) BCNU; (2) chlorambucil; (3) cyclophosphamide; (4) melphalan; (5) thiotepa; (6) fosfomycin (Hayes and Pulford, 1995).

1.5.1.3 Reaction Kinetics

The catalysis of nucleophilic aromatic substitution reactions can be divided into steps involving substrate binding to the enzyme active site, activation of GSH by deprotonation of the thiol to form the nucleophilic thiolate anion and nucleophilic attack by the thiolate at the substrate electrophilic centre. The enzymic mechanism involves tyrosine- or serine- mediated deprotonation of the thiol group of the bound GSH. Important questions with regard to the determinants of the reaction pathway that influence k_{cat} and K_m still need to be addressed.

Kinetic data reported in many studies suggest that the GST I-catalysed reaction between CDNB and GSH follows a rapid equilibrium, random sequential Bi Bi kinetic mechanism. The kinetic mechanism of the GST-catalysed conjugation reaction is quite complex and isoenzyme-dependent. For example, the p class GST follows a rapid equilibrium random Bi Bi kinetic mechanism, whereas a steady-state random Bi Bi kinetic mechanism was proposed for rat GSTs M1-1, M1-2 and A3-3 and octopus hepatopancreatic GST. In the case of h class, for the rat GST T2-2 a hysteric reaction mechanism was suggested based on presteady-state and steady-state kinetics, whereas for the *Lucilia cuprina* enzyme, a steady-state random Bi Bi mechanism was proposed to explain the non-Michaelian substrate rate curves (Labrou, 2001). Kinetic data were fitted to the equation for the rapid equilibrium, random sequential Bi Bi model according to scheme (Lo Bello, 1997):



1.5.1.4 Distribution

Cytosolic glutathione-S-transferases are differentially expressed in the different organs (Mannervik, 1985; Prabhu, 2001)

In mammalian tissues, alpha, mu and pi classes account for the majority of GST activity (Mannervik et al., 1985), in human liver, adrenal gland, kidney and testis class alpha and mu isozymes, (Casalino et. Al., 2004) whereas in lung, muscle, brain, placenta, pancreas, erythrocytes, and skin, the Pi class isozyme is predominant (Awasthi *et. al.*, 2004)

The pi class isoenzyme is the most abundant and widely distributed GST isozyme found in many tissues (Awasthi et al., 1994, Board et al., 1990), and it is expressed mostly in tumour cells (Ouwerkerk-Mahadevan and Mulder, 1998). Particularly, GSTP1-1 isozymes are present at high levels in a preponderance of tumor types (Kauvar, 1998).

There are relatively few studies on membrane GSTs, which have an ideal location to participate in the detoxification of lipid peroxidation products as well as lipophilic drugs and other xenobiotics. (Prabhu, 2001; Tahir, 1988)

GST M1 is expressed in only 60% of human individuals and people lacking the GST M1 have a higher risk of developing lung cancer (Van Bladeren and Van Ommen, 1991, Board et al., 1990). GSTM1, GSTM3 and GSTT1 exhibit genetic polymorphisms (Iyer and Ratain, 1998)

1.5.1.5 Antioxidant Activity

GSTs are important enzymes for the organisms since they account for the defence against oxidative stress. They can detoxify endogenous harmful compounds such as hydroxyalkenals and base propenals that are the breakdown products of lipid peroxidation, or DNA hydroperoxides, as well as electrophilic xenobiotics and/or

reactive intermediates formed during their biotransformation like epoxides and quinones (Tew, 1994; Hayes and Pulford, 1995; Cnubben *et. al.*, 2001).

In mammalian tissues, especially the Alpha class GSTs, particularly the isozymes GSTA1-1 and GSTA2-2, can effectively reduce lipid hydroperoxides to their corresponding alcohols through their glutathione peroxidase activity with oxidized glutathione (GSSG) and water as by-products (**Figure 1.14**) (Awasthi *et. al.*, 2004).

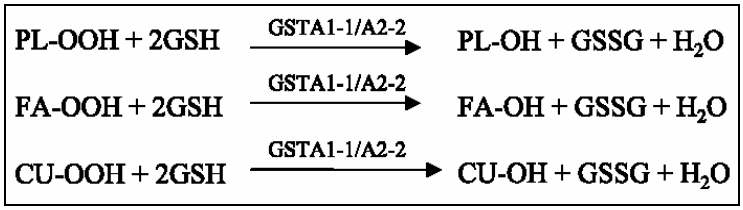


Figure 1.14. Glutathione peroxidase activity of GSTs. PL-OOH, phospholipid hydroperoxides; FA-OOH, fatty acid hydroperoxides; CU-OOH, cumene hydroperoxide. (Awasthi *et. al.*, 2004)

4-HNE is the major 4-hydroxyalkenal formed during peroxidation of arachidonic and linoleic acids, which has shown to be genotoxic and cytotoxic (Prabhu, 2001). The major pathway for its disposition is believed to be GSH conjugation catalyzed by a group of GSTs (Cnubben *et. al.*, 2001), particularly by the alpha classes (Awasthi *et. al.*, 2004).

Class theta GSTs are also reported to possess GPx activity (Cnubben *et. al.*, 2001).

Pi form of GST, particularly GSTP1-1 is shown to deactivate products of lipid peroxidation such as oxidative DNA-bases, lipid hydroperoxides and their derivatives such as hydroxyalkenals, malondialdehydes and base propenals (Ketterer and Christodoulides, 1994; Berhane et al., 1994).

It can even directly react with ROS via a sensitive SH-group, and cause inactivation by disulphide formation, pointing out the specific role of this enzyme in oxidative stress. (Cnubben *et. al.*, 2001)

GSTs also have Se-independent GPx activity and can catalyze GSH-dependent reduction of hydroperoxides. Physiological significance of GPx activity is suggested by the high abundance of Alpha class GSTs in primary metabolic organs such as liver, which are constantly exposed to reactive oxygen species (ROS) due to high metabolic activity. (Awasthi *et. al.*, 2004)

Selenium is a limiting component in glutathione peroxidase structure and in its function. This is one of the major reasons why Se has antioxidative and anticancer effects. Depletion of GSH can diminish the body's ability to defend against lipid peroxidation since GSH is a cofactor for glutathione peroxidase activity (Beasley, 1999).

1.5.1.6 Importance of GSTs in Drug Resistance

A major obstacle in human cancer therapy is ability of tumor cells to develop cross-resistance to chemotherapeutic agents. This resistance of cancer cells acquired upon exposure to a single antitumor drug, usually extends over a range of other drugs even of a completely different chemical nature (Guadiano, 2000).

One of the proposed mechanisms for multidrug resistance is the ability of resistant tumor cells to efficiently promote glutathione-S-transferase catalyzed GSH conjugation of the pharmacologically active compounds such as antitumor drug (Guadiano, 2000; Zanden *et. al,* 2004). Therefore, increasing glutathione S-transferase activity is regarded as an indicator for the resistance to chemotherapy (Gyamfi e*t. al.,* 2004).

This correlation of drug resistance with the overexpression of GST isozymes has been demonstrated in a variety of cells, including several human tumor cell lines (Morgan *et. al*, 1996; Iscan, 1998; Burg, 2002). Consequently, elevated plasma GST levels are regarded as a potential marker for certain cancers (Akçay, 2005) and can contribute to the detoxifcation of the DNA-alkylating cytotoxic metabolites of chemotherapeutics such as cyclophosphamide (Iyer and Ratain, 1998).

It was demonstrated that GSH conjugation activities of all isoenzymes in purified forms, in human liver cytosol (GST M and GST A) and lysate of human erythrocytes (GST P) were inhibited, in a concentration dependent way, by RRR-a-tocopherol (Rachel, *et. al.,* 2003).

Among all GSTs, GSTP1-1 was reported as the most important enzyme in multidrug resistance since it is over expressed in a large number of solid tumors which makes it a valuable target for inhibition studies. Therefore, development of GSTP1-1 inhibitors has been considered a promising strategy to increase chemotherapeutic efficiency. Many natural constituents, including plant polyphenols like flavonoids were highly promising candidates. Inhibition mechanism of GSTP1-1 by quercetin in vitro was demonstrated as well (Zanden *et. al.,* 2003, 2004).

In addition, several researchers have reported that drug sensitivity of tumor cells in culture are increased by treatment with inhibitors of GST such as ethacrynic acid, piriprost, indomethacin, and gossypol, leading a significant retardation of tumor growth (Morgan *et. al*, 1996). Deficiency in certain GST isoenzymes, GSTM1 or GSTT1, were also reported to have a positive influence in cancer chemotherapy (Iyer and Ratain, 1998)

The correlation of inhibition of GST P1-1 as the mechanism of enhancement of drug toxicity is important since it is indicating the potential for selective inhibition of specific GST isozymes elevated in tumors as an effective method for modulating drug resistance in tumor cells (Morgan *et. al*, 1996).

In this respect, inhibitory effects of novel antioxidants from the medicinal herbs are continuously investigated by researchers (Gyamfi e*t. al.,* 2004).

Continuous research for natural compounds is underway, where they can be used as adjuvant in cancer therapy (Burg et al., 2002). The inhibitors of GST that have been discovered thus far have shown little effect on GST P1-1 (Ouwerkerk Mahadevan and Mulder, 1998; Haaften, *et. al.,* 2003)

1.6. Effects of Phytochemicals on Drug Metabolizing Enzymes

Several compounds present in plant foods that help support detoxification of toxic substances are strongly associated with the reduced risk of cancer (Percival, 1997). Polyphenols are usually pointed out to be responsible for this protective effects of fruits and vegetables against cardiovascular diseases and certain forms of cancer (Zanden *et. al.,* 2003).

The effects of polyphenols on drug metabolizing enzymes have different consequences when considered *in vivo* and *in vitro*. The mechanism of chemopreventive activity of flavonoids is observed by their induction of phase II metabolizing enzymes *in vivo*. However, the activity of these drug metabolizing enzymes has to be controlled during chemotherapy to overcome multidrug resistancy. The activity of drug metabolizing enzymes can efficiently be modulated by flavonoids, for achieving a successful therapy.

In vivo, an important mechanism responsible for the chemopreventive activity of flavonoids is the induction of GSTs, NAD(P)H:quinine oxidoreductases and UDP-glucuronyltransferases, therefore readily helping detoxification and elimination of carcinogens from the body. For instance, green and black tea extracts, which contain several flavonoids and other dietary phenolics, were demonstrated to inhibit neoplastic transformations, and DNA adduct formation while efficiently inducing GSTs. (Galati and O'Brian, 2004). Alpha-tocopherol, phenobarbital and

other anticarcinogenic drugs can also increase the levels of GSTs, while coumarin is one of the most potent inducers (Landi, 2000).

Garlic and onion, which also contain flavonoid compounds, quercetin, in particular, the monoterpenes from the oil of citrus peel such as D-limonene, and curcumin which is a natural constituent of the spice turmeric support xenobiotic detoxification. Several compounds present in cruciferous vegetables such as broccoli, cauliflower, brussels sprouts, watercress and cabbage have been shown to be inducers of Phase II detoxification enzymes including glutathione S-transferase (Percival, 1997)

On the other hand, polyphenols may also exert their chemopreventive effects through inhibition of phase I metabolizing enzymes, such as cytochrome P450, which metabolically activates procarcinogens to reactive intermediates that trigger carcinogenesis (Galati and O'Brian, 2004).

They can even bind to receptors and stimulate xenobiotic metabolism, modulate protein kinase activities and influence the transcription (Moskaug *et. al*., 2004).

Terpenoids such as geraniol, menthol, carvone and limonene which occur in high concentrations in grapefruit and orange juice limonin and nomilin being the principal ones in citrus, possess the ability to inhibit tumor formation by stimulating glutathione S-transferase (Lam, et.al., 1994; Elson and Yu, 1994)

Phthalides in celery seed, the sulfides in garlic and onions, the dithiolthiones and isothiocyanates in broccoli and other cruciferous vegetables and the curcumins in ginger and turmeric are also among the substances considered as inhibitors of cancer regarding to their activity of stimulating GST *in vivo*, since they stimulate xenobiotic metabolism (Steinmetz, 1991; Lam, et.al., 1994; Zheng, 1994*)*.

The strategy for achieving effective chemotherapy brings about the idea of inhibiting GSTs *in vitro*. The inhibitory effects of naturally occurring plant polyphenols such as tannic acid, ellagic acid, ferrulic acid, caffeic acid, silybin, quercetin, curcumin and chlorogenic acid against GST have long been reported by many researchers in this respect (Gyamfi e*t. al.,* 2004).

Quinines are also well-known examples of covalent inhibitors of GST enzymes (Zanden *et. al.,* 2003).

The inhibitory effects of thonningianin A, a novel antioxidant isolated from the medicinal herb, *Thonningia sanguinea* on uncharacterized rat liver GST and human GST P1-Th was demonstrated and reported as a potent inhibitor of the conjugation of CDNB by crude cytosolic GSTs with IC50 of 1.1 lM (Gyamfi e*t. al.,* 2004)

The glutathione S-transferase activity of the cancer cells was also reported to be inhibited by the polyphenols (Zhang and Wong, 1997).

1.7. Importance of Bioinformatics in Drug Discovery from Natural Resources and Approaches in Biotechnology

In the last century, there has ben a great increase in the data, together with the development of technology. The improvement in the field of computational and laboratory techniques and rational drug design, has also provided important progress in drug development by refining the molecular structures to give a greater drug activity with fewer side effects. New computational techniques such as Quantitative Structure Activity Relationships (QSAR) are commonly used to detect the functional groups in bioactive compounds. Therefore, the time and money spent for the drug discovery is reduced and it is possible to screen more sources by the help of computer applications.

The second approach after the detection of bioactive compounds is their isolation in appropriate amounts from suitable sources. Biotechnology is the main solution, when the engineering site of research is considered. In this respect, genetic engineering approaches are used to manipulate the production of bioactive compounds in different plant parts. For instance, plants can be genetically engineered to improve the production of potentially profitable compounds. Biotechnological approaches to plant breeding are promising , because they offer the opportunity to design different qualities of compounds of interest. All plants contain numerous phytochemical compounds, usually in minute concentrations. Besides plant breeding efforts to increase the content of desired phytochemicals in crops, their isolation and the fortification of special foods with these compounds is already in progress and the first of such products have entered the marketplace. When the bioactivity of a specific compound in a medicinal plant is identified that plant can efficiently be multiplied at a commercial scale at plant tissue-culture laboratories.

1.8. Scope of the Study

The aim of this study was to investigate the antioxidant properties of medicinal plants which have been used in Anatolian folk medicine against inflammation, ulcer, fewer, infertility, and also as expectorant, vasodilator, analgesic, aphrodisiacs. *Aesculus hippocastanum* L, *Papaver bracteatum* L., *Urtica urens* L., *Gundelia tournefortii* L, *Prangos ferulacea* L., *Chaerophyllum macropodum* Boiss., *Heracleum persicum* Desf, *Allium vineale* L, *Aconitum cochleare, Rheum ribes* L, *Ferula rigidula* DC, and *Rosa heckeliana* Tratt. were selected among the medicinal plants commonly used in Anatolia.

Antioxidant activities of the plants were evaluated by determining their DPPH radical scavenging effects, lipid peroxidation inhibiton capacities and their total phenolic contents. Selected plants were further investigated for their influences on activity of glutathione-S-transferase enzyme which plays an important role in cellular detoxification mechanisms, as well as multi-drug resistancy.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1 Chemicals

Potassium chloride (KCl), ethylene diamine tetraacetic acid (EDTA), glycerol, bovine serum albumin (BSA), copper sulfate ($CuSO_4$), sodium potassium tartrate, sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), hydroxy methyl aminomethane (Tris), hydrochloric acid (HCl), iron salt ($Fe_2SO_{4.7}H_2O$), glycerol, thiobarbituric acid (TBA), ortho phosphoric acid (H_3PO_4) alpha-tocopherol, β-aescin 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), gallic acid, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), alpha-tocopherol, quercetin, Sephadex LH-20 were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

Preparative chromatography grade methanol, ethanol and butanol was used throughout the experiments. Dimethyl sulfoxide (DMSO), Ciocalteu`s Folin phenol reagent, Silica Gel 60 (0.063-0.200mm) for column chromatography and Silica Gel 60 F-254 Thin Layer Chromatography (TLC) aluminium sheets were purchased from E.Merck, Darmstadt, Germany.

All other chemicals were analytical grade and were obtained from commercial sources at the highest purity available.

2.1.2 Plant Materials

Flowers, leaves, seeds and bark samples from *Aesculus hippocastanum L.* were collected in consecutive seasons during 2003 on the campus of Middle East Technical University, Ankara and deposited in the Department of Chemistry with the voucher specimens numbers 4-2002-AhFG, 3-2002-AhLN, 1-2002-AhSG, 2-2002-AhBE respectively. All the other plant samples of the study were collected in various periods in 2003, from Van, Turkey. The plant specimens with their localities and the necessary field records were written and enumerated. They were pressed, dried according to herbarium techniques and identified by Flora of Turkey (Davis 1965-1988) and kept at the herbarium of Yüzüncü Yıl University. .

Aesculus hippocastanum L.
Family: Hippocastanacea
Common names: Horse chestnut, buckeye
Turkish name: At kestanesi
Collected from: METU campus forestry, Ankara.

Papaver bracteatum Lindl.
Voucher number: F 11 172
Family: Papaveraceae
Common names: Great scarlet poppy, scarlet poppy
Turkish name: Haşhaş
Collected from: Pirreşit Mountain, Van.

Urtica urens L.
Voucher number: F 11 173.
Family: Urticaceae
Common names: Burning nettle, dog nettle**,** dwarf nettle**,** small nettle
Turkish name: Küçük Isırgan
Collected from: Bahçesaray, Van.

Gundelia tournefortii L. var. ***armata*** Freyn. & Sint.

Voucher number: F 11 174

Family: Asteraceae (Compositae)

Common names: Tournefort's gundelia

Turkish name: Kenger

Collected from: Alacabük Mountain, Van.

Prangos ferulacea (L.) Lindl.

Voucher number: F 11 175

Family: Apiaceae (Umbelliferae)

Turkish name:Heliz

Collected from: Alacabük Mountain, Van.

Chaerophyllum macropodum Boiss.

Voucher number: F 11 176

Family: Apiaceae (Umbelliferae)

Turkish name: Mendi

Collected from: Alacabük Mountain, Van.

Heracleum persicum Desf.

Voucher number: F 11 177

Family: Apiaceae (Umbelliferae)

Common names: Sow

Collected from: Alacabük Mountain, Van.

Allium vineale L.

Voucher number: F 11 178

Family: Alliaceae, also placed in Liliaceae

Common names: Crow garlic, false garlic, field garlic, wild garlic, wild onion

Turkish name: Sirmo

Collected from: Alacabük Mountain, Van.

Aconitum cochleare Woroschin

Voucher number: F 10 947

Family: Ranunculaceae

Collected from: Hoşab, Güzeldere Passage,Van.

Rheum ribes L.

Voucher number: F 11589

Family: Polygonaceae

Turkish names: Uçkun, Işkın, Rıbes

Collected from: Alacabük Mountain, Van.

Ferula rigidula DC.

Voucher number: F 11 030.

Family: Apiaceae (Umbelliferae)

Turkish names: Siyabo, çakşır

Collected from: Alacabük Mountain, Van

Rosa heckeliana Tratt.

Voucher number: F 5474.

Family: Rosaceae

Turkish names: Kuşburnu, Şilan kökü

Collected from: Sağmalı village, Van

2.2 Methods

2.2.1 Preparation of Plant Extracts

The collected plant specimen were air dried and ground with Waring type stainless steel commercial Blender.

Matured seeds from *Aesculus hippocastanum* L. trees were collected for isolation of aescin and 100 g of dry seeds were extracted in ethanol, water, 80%,

50% and 20% ethanol-water solutions in Heidolph Laborota 4000 rotary evaporator at 50°C for 24 hours using a seed to solvent ratio of 1: 6.

The extracts from *Aesculus hippocastanum* L. flowers, leaves, seeds and bark were prepared by using 5 g of each of samples and extracted in pure ethanol at 50°C for 24 hours with a sample to solvent ratio of 1:10.

10 g of each of all the other plant samples were extracted with 100 mL of methanol at 50°C for 24 hours using a sample to solvent ratio of 1:10 for overnight in Heidolph Laborota 4000 rotary evaporator at 50^0C.

The extracts were filtered through double cheesecloth to remove any artifacts and were dried under vacuum at 50^0C. The resulting crude extracts were weighed and dissolved in suitable solvents for further investigations. All the prepared extracts were kept at $0 – 4^0$C during the experiments.

2.2.2. Isolation of Sheep Liver Microsomes and Cytosol

For the isolation of sheep liver microsomes, fresh livers were obtained from Mısırdalı slaughterhouse, Sincan, Ankara and were placed in plastic bags containing crushed ice. In the laboratory, all the subsequent steps were carried out at 0-4 °C. Livers were washed with cold distilled water to remove excess blood and cut into small pieces with scissors. Then the liver pieces were washed with 1.15 % (w/v) KCl containing 1mM EDTA solution.

Connective and fatty tissues were removed and the resulting 30 g of minced tissue was homogenised in a teflon glass homogeniser (Black and Decker Teflon glass homogeniser) in 120 mL of 20 mM Tris-HCl, pH 7.4 containing 1.15 % KCl (w/v) and 1mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). Homogenisation was achieved by using a Potter-Elvehjem homogenizer coupled with a motor (Black and Decker, V850, multi-speed drill)-driven Teflon pestle with 6 strokes at at 2400 rpm speed for 20-second intervals.

The homogenate was centrifuged at 10,800 x g for 25 minutes in Sorvall RC 5C Plus Centrifuge by using SS-34 rotor, in order to remove unbroken cells, nuclei and mitochondria. After centrifugation, supernatant solution was filtered through double layers of cheesecloth and to sediment the microsomes, ultracentrifugation was carried out in Sorvall Combi Plus Ultracentrifuge at 133,573 x g for 50 minutes. The resulting pellet was resuspended in the same homogenisation solution by using teflon glass homogeniser. The microsomes were resedimented by Sorvall Combi Plus Ultracentrifuge at 133,573 x g for 50 minutes. The supernatant solution was discarded and the microsomal pellets were suspended in 15 mL of 25% (v/v) glycerol containing 1 mM EDTA solution by using teflon glass homogeniser. The microsomal suspension containing approximately 25-30 mg protein / mL was then aliquoted and stored in -80°C in deepfreeze not longer than three months.

For the isolation of sheep liver cytosol, the supernatant after the first ultracentrifuge step is taken and kept at -80^0C until used.

2.2.3 Protein Content Determination by Lowry Method

The protein content of microsomal suspension was quantitatively determined by Lowry Method. (Lowry *et al.,* 1951.) Crystalline Bovine Serum Albumin (BSA) was used as standard protein. 0.5 mL of each standard aliquot containing 0.05 mg BSA/mL, 0.1 mg BSA/mL, 0.15 mg BSA/mL and 0.2 mg BSA/mL, respectively was poured into four seperate test tubes with duplicates. 1: 100 diluted microsomes were poured into three separate test tubes with duplicates at 0.5 mL, 0.25 mL and 0.1 mL respectively. The tubes containing 0.25 mL and 0.1 mL of unknown were completed to final volume of 0.5 mL with distilled water. For the blank tubes, 0.5 mL of distilled water containing tubes were prepared in duplicate (**Appendix A**). Then the tubes were mixed with 2.5 mL of alkaline copper reagent (ACR), which was obtained by mixing 2% copper sulfate, 2% sodium potassium tartrate and 1 N NaOH containing 2% sodium carbonate, with the ratio of 1:1: 100, respectively. All

the tubes were vortexed and allowed to stand for 10 minutes at room temperature (25^0C). Finally, 0.25 mL of Folin-Phenol reagent was added to test tubes and wortexed rapidly. The tubes were incubated at room temperature for 30 minutes and the colour development was measured at 660 nm. BSA calibration curve was used to calculate the protein content of microsomal suspension. Microsomal protein content was found to be approximately 25-30 mg/mL of protein.

2.2.4 Thin Layer and Column Chromatography

Thin Layer chromatography was used both for partial characterisation of some bioactive compounds in the extracts and also for solvent optimisation for elution used in silica gel column chromatography. Methanol, chloroform, ethanol, water and n-butanol were used in various proportions as the mobile phase in thin layer chromatography, using F-254 TLC aluminium sheets. Finally, 1:1:2 ratios of [ethanol:water:butanol] were chosen as the optimum elution solvents which is applied on column chromatography.

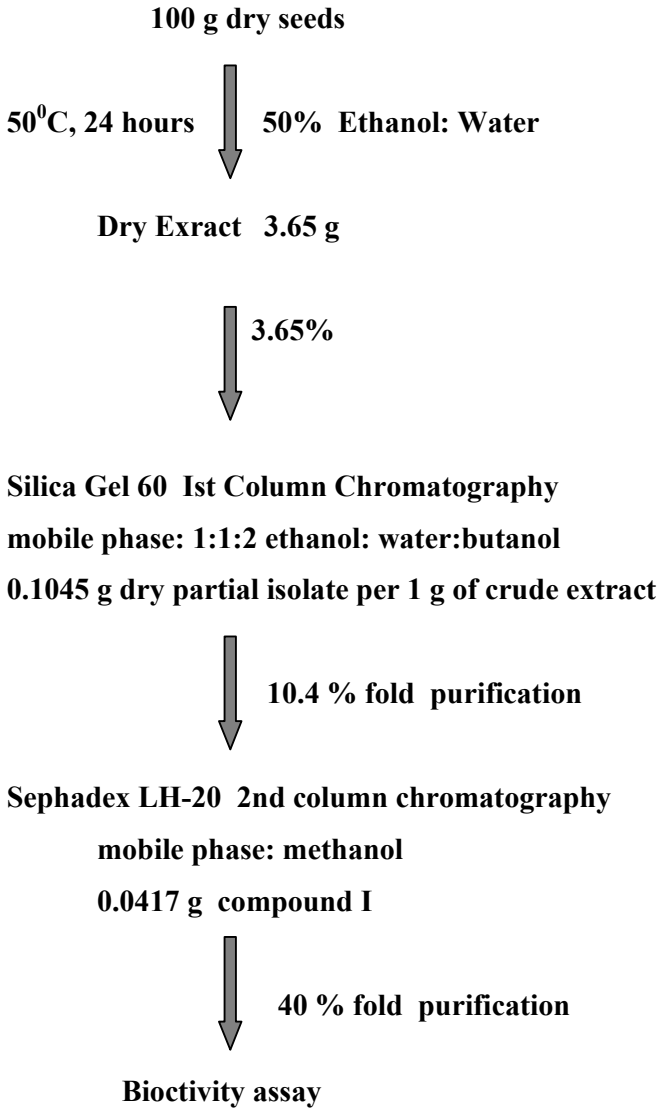
Column chromatography was applied using two separate supporting materials; silica gel 60 and lipophilic sephadex. Silica and sephadex columns were connected to peristaltic pump (Pharmacia Fine Chemicals P-3) for the continuous flow of eluents. Eluted fractions were collected as 1.5 mL volumes by using fraction collector (BIO-RAD 2110).

Silica Column

Silica Gel 60 in the mesh size of 0.063-0.200 mm was prepared by soaking 60 g in 100 mL of distilled water for couple of hours. Silica gel was poured into a glass column in the dimensions of 3 x 30 cm. 1.0 g of crude extract was applied after dissolving in 2.0 mL of water and the column was washed with previously optimised solvent system [1:1:2] of ethanol:water: butanol with 2.5 mL/min flow rate.

Lipophilic sephadex Column

Lipophilic sephadex was prepared by soaking dry sephadex in methanol for 24 hours. Wet sephadex was poured into 2.5 x 120 cm glass column and equilibrated with methanol . The extract was applied on the column after dissolving in 1 mL of methanol and eluted with methanol with 2.5 mL/min of flow rate. Isolation scheme is given in **Scheme 2.1**.



Scheme 2.1. Flow-chart for isolation of aescin from *Aesculus hippocastanum* seeds

2.2.5 Measurement of Lipid Peroxidation by Thiobarbituric Acid Test

There are various methods to detect and measure biological lipid peroxidation such as measuring the the loss of unsaturated fatty acids, the uptake of oxygen, cytotoxic aldehydes, antibody techniques. (Gutteridge and Halliwell, 1990). The TBA test is an old method used for measuring the peroxidation of fatty acids, membranes and food products (Ohkawa, 1979). The material to be tested is merely heated with thiobarbituric acid under acidic conditions. The product of lipid peroxidation , namely malonedialdehyde (MDA), reacts with TBA to generate a coloured product, $[TBA]_2$-malonedialdehyde adduct (**Figure 2.1**).

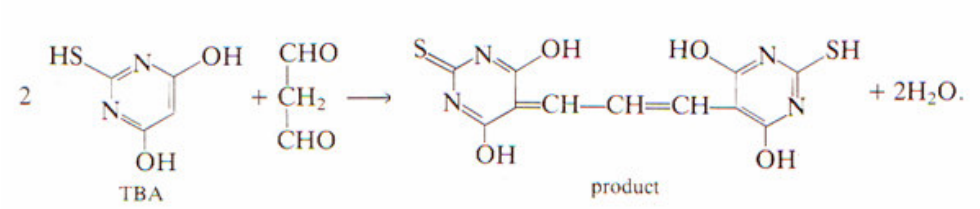


Figure 2.1. Reaction of MDA with TBA

In acid solution, the product absorbs light at 532 nm and fluoresces at 553 nm and it is readily extractable into organic solvents such as butan-1-ol. As the TBA test is calibrated by MDA, the results are expressed in terms of the amount of MDA produced in a given time. The molar extinction coefficient of the MDA -TBA adduct is 1.56 x 10^5 M^{-1} cm^{-1} at 532 nm which is used to calculate the amount of MDA formed (Heath, Packer, 1968).

Microsomal lipid peroxidation end products were measured by Thiobarbituric Acid (TBA) Test using the method of Ohkawa et al., 1979., with some modifications (Sagdicoglu, 2000). Lipid peroxidation is induced by incubating 1mg protein/mL of microsomes with 100µL iron salt (Fe_2SO_4.7H2O) of 100µM final concentration, in 0.2M Tris-HCl buffer, at pH 7.4.

All the tubes were containig 500µL of buffer, 100µl of microsomal suspension, 100µL of iron solution and 100µL of extracts. The suspensions were completed to 1mL with distilled water. DMSO, ethanol or distilled water was used as the blank. The endproducts of lipid peroxidation were quantified by determining the MDA contents of microsomal lipid suspensions in the absence and presence of the antioxidants. The tubes containing antioxidants were incubated for 30 minutes at room temperature after the antioxidants were added. Alpha-tocopherol was used as the standard for antioxidant.

The prepared tubes were vortexed and incubated in shaking water bath at 37^0C for 30 minutes. After incubation, they were put in ice for 10 minutes to stop the formation of lipid peroxides. Then, 1mL of 1% (v/v) ortho-phosphoric acid solution was added to the tubes to make the medium acidic, and 1mL 0.67% of thiobarbituric acid (0.1g TBA dissolved in 0.33 mL 2 N NaOH, completed to 20 mL with dH_2O) were added into the tubes and the tubes were again vortexed. Then they were incubated in boiling water for 45 minutes for the reaction of MDA and TBA to take place. After incubation, the tubes were again put in ice for 10 minutes. After cooling the tubes, 3mL of n-butanol was added on each tube and the tubes were wortexed and centrifuged for 10 minutes at 4500 rpm in clinical centrifuge. 1 mL of supernatant was put into tubes to read the absorbance at 532 nm with Unicam UV/VIS spectrophotometer.

The MDA values were calculated by using the extinction coefficient $1.56x10^5$ M^{-1} cm^{-1} and expressed as nmoles/mg of protein/minute. Microsomal lipid peroxidation inhibition activity is expressed as IC_{50}, which is defined as the concentration of isolated compound required to inhibit microsomal lipid peroxidation by 50 percent.

2.2.6 DPPH Radical Scavenging Effect

DPPH $^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical) is a stable free radical that can accept an electron or hydrogen radical and become a stable diamagnetic molecule (**Figure 2.2).** In its radical form, DPPH is stable, with a purple color in ethanol solution and is reduced into the yellow colored diphenylpicryl hydrazine upon abstracting an electron or hydrogen radical from an antioxidant or a radical species. The reduction in the concentration of DPPH $^{\bullet}$ is dedicated as the measure of antioxidant activity which is followed by monitoring the decrease in its absorbance at 517 nm wavelength during the reaction (Blois, 1958).

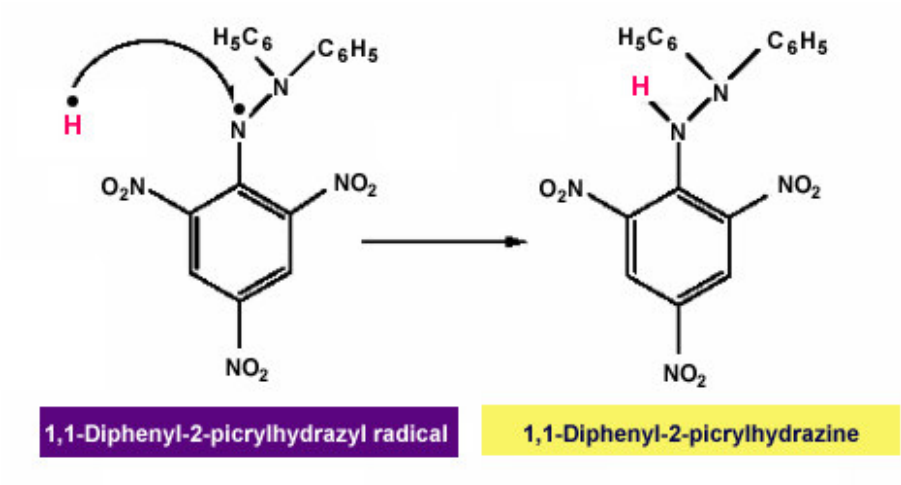


Figure 2.2 DPPH radical scavenging reaction.

1.4 mL of 0.05 mg/mL DPPH solution in ethanol and 0.1 mL of extract were mixed and the reaction mixture was shaken vigorously. The absorbace was determined at 517 nm after 5 minute. 0.1 mL of the solvent of the extract was used as control. The radical scavenging activity was obtained from the following equation:

Radical scavenging activity (%) = {(ODcontrol _ODsample) / ODcontrol}x100.

The antioxidant activity is expressed as the amount of antioxidant necessary to decrease the initial DPPH $^{\bullet}$ concentration by 50% (IC_{50}), which were calculated after constructing the percent inhibition versus log extract concentration curve. The lower the IC_{50}, the more efficient the antioxidant action.

2.2.7 Determination of Total Phenolic Content

Chemically, phenolic compounds are defined as substances possessing an aromatic ring that bears one or more hydroxy substituents, including their functional derivatives (Shahidi and Naczk, 1995). Plant phenolic compounds are a very important group of plant secondary metabolites, which have numerous medicinal applications (Shetty, 2004). Total content of phenolic compounds in the plant extracts were determined by using the method of Singleton and Rossi, 1965, with some modifications. A calibration curve was constructed using gallic acid, a well-known phenolic acid, as the standard and then the phenolic content in the test samples were evaluated using this curve. Results were expressed as milligram per gram of gallic acid equivalents (GAE).

The concentration of phenolic compounds in the plant extracts was measured using the method of Sato et. al., 1996 and calculated using gallic acid (0.05 – 0.3 mg/mL) to construct a standard curve. 0.1 mL of each of the extracts and the standards were mixed with 2 mL of 2% Na_2CO_3 and shaken vigorously. After 3 minutes, 0.1 mL of 50% Folin- ciocalteu phenol reagent was added to the mixture and the tubes were vortexed. After 30 minutes of incubation at room temperature, the absorbance was measured at 750 nm using a spectrophotometer. Results were expressed as milligram per gram of gallic acid equivalents (GAE).

2.2.8 Determination of Sheep Liver Glutathione-S-Transferase Activity toward CDNB

The single most important substrate used for the demonstration of multiple forms of GSTs in various biological species is 1-chloro-2,4-dinitrobenzene (CDNB). When CDNB is conjugated with GSH, it gives (2,4-dinitrophenyl)glutathione, a compound possessing an absorbance spectrum sufficiently different from that of CDNB to allow a simple spectrophotometric assay at 340 nm (Habig, 1974). The enzymatic reaction of CDNB with GSH is illustrated in **Figure 2.3.**

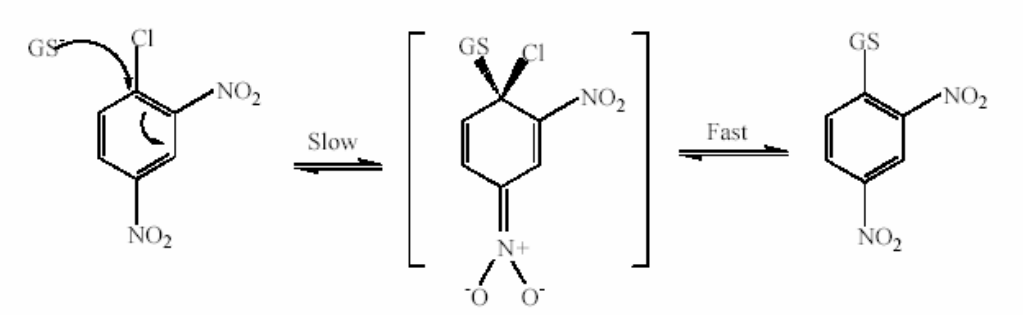


Figure 2.3. Structure of 1-chloro-2, 4-dinitrobenzene (CDNB) and its reaction with GSH (Armstrong, 1991).

GSTs activities were determined spectrophotometrically by monitoring the thioether formation at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate (Habig *et. al.*, 1974). Sheep liver cytosolic fractions were prepared used as the enzyme source to measure GST activity torard CDNB. 0.2 M potassium phosphate buffer and final 1 mM GSH was used throughout the experiments. Combination buffer and the substrate solutions were prepared fresh (**Appendix B)**. Enzyme activities were carried out at 25^{o}C. 21mg/mL sheep liver microsomes were used after 1/100 dilution with 10 mM potassium phosphate, buffer, pH 6.0. Concentrations ranging between 0.2 – 2 mM CDNB was used as substrate The reactions were started by the addition of the enzyme. Incubation mixtures without the enzyme source were used as blanks (non-enzymatic reactions). Enzyme activities with 10 µl of DMSO were regarded as control. The enzyme activities were

expressed in terms of percent specific activity. One unit of GSTs activity is defined as the amount of enzyme producing µmole thioether formed per minute.

The reactions were started by the addition of the enzyme. Incubation mixtures without the enzyme source were used as blanks (non-enzymatic reactions). Enzyme activities with 10 µl of DMSO were regarded as control. The enzyme activities were expressed in terms of percent specific activity. One unit of GSTs activity is defined as the amount of enzyme producing µmole thioether formed per minute. The kinetic constants Km and Vmax were determined for GST activity after Lineweaver-Burk Plots were constructed. The enzyme activities were expressed in terms of percent specific activity. The extinction coefficient for CDNB conjugate at 340 nm is 0.0096 $\mu M^{-1}cm^{-1}$ (Habig *et. al,* 1974)

2.2.9 Determination of Kinetic Constants for Glutathione-S-Transferase Activity

Lineweaver-Burk Plots were constructed for GST Activity toward CDNB in the absence and presence of 50 µg/mL plant extracts. The kinetic constants Km and Vmax were determined for GST activity to be able to observe any shift on the values. GST activities were determined at different substrate concentrations ranging between 0.2 – 2 mM CDNB concentrations. 0.2 M potassium phosphate buffer at pH 7.5 and final 1 mM GSH was used throughout the experiments. Enzyme activities with 10 µl of DMSO were regarded as control. The enzyme activities were expressed in terms of percent specific activity. The extinction coefficient for CDNB conjugate at 340 nm is 0.0096 $\mu M^{-1}cm^{-1}$ (Habig *et. al,* 1974)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Antioxidant Activities of *Aesculus hippocastanum* L. Seed Extracts and Isolation of Aescin

Ethanol extracts of A*esculus. hippocastanum* L. seeds were prepared and their antioxidant capacities were evaluated by using three different methods, inhibition of sheep liver microsomal lipid peroxidation, DPPH radical scavenging and the total phenolics content.

3.1.1 Determination of Antioxidant Capacity of the Crude Extracts and Partial Characterisation

100g of matured dry seeds from *Aesculus hippocastanum* L. trees were extracted in pure ethanol, water, 80%, 50% and 20% ethanol-water solutions in Heidolph Laborota 4000 rotary evaporator at 50°C for 24 hours using a seed to solvent ratio of 1: 6. Extracts were dried under vacuum, weighed and kept in dark at $+4^{o}C$ until used.

Thin layer chromatography method was used for partial characterisation and for achieving the optimum conditions in isolation of bioactive compounds from *Aesculus hippocastanum* L. extracts. Extracts were dissolved in ethanol and 5 µL from 1mg/mL extracts were applied on F-254 TLC plates (**Figure 3.1**). Among all the trials to obtain optimum mobile phase for the seperation of extracts, 1:1:2 ethanol:water:butanol solution mixture was found to be the best. After the extracts are applied on TLC plates, the plates were dried and were sprayed with 1:1 mixture of 1% vanilin-ethanol and 5% H_2SO_4-ethanol solution and heated at $120^{o}C$ for 5 minutes for visualisation.

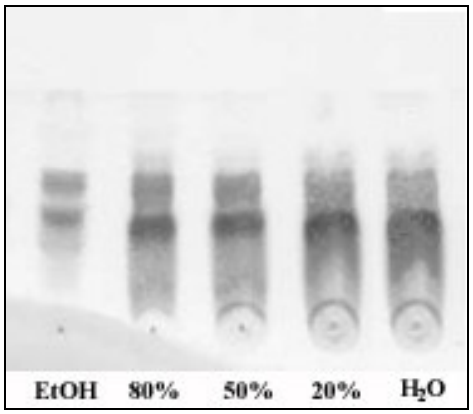


Figure 3.1. Optimization of horse chesnut seed extracts with silica thin layer using solvents as ethanol, 80%, 50%, 20% ethanol and water solutions respectively, mobile phase is 1:1: 2 (ethanol: water: butanol) with 5 µl loading of each samples

Antioxidant efficiencies of the extracts were investigated by using lipid peroxidation inhibition method.

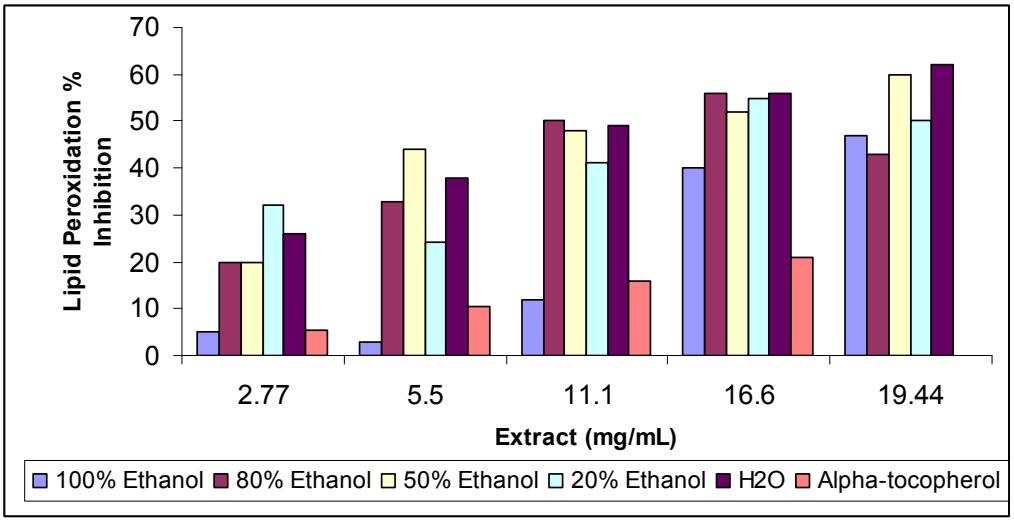


Figure 3.2. Lipid peroxidation percent inhibition capacities of different concentrations of *Aesculus hippocastanum* L. seed extracts prepared in varying solvent ratios.

The antioxidant capacity of the crude extracts were measured as the inhibition of iron induced microsomal lipid peroxidation on sheep liver. 1mg/mL microsomal suspension was used as the lipid source and 100 µM Iron (II) was used

for the induction of lipid peroxidation. Control measurements were obtained in the absence of extracts. Alpha-tocopherol was used as positive control.

Extraction which was carried out with 50% ethanol-water has led to the highest amount of substance and also high antioxidant activity as given in **Figure 3.2**. As a result, 50% ethanol-water crude extraction has been used for the isolation of bioactive compounds from *Aesculus hippocastanum* L. seeds. 3.65 g of dry extract was obtained from 100 g dry seeds with 50% ethanol-water solution in 1:6 seed to solvent ratio.

3.1.1.1 UV-VIS Characterisation of Crude Extract

UV-VIS absorbance spectrum of the 50% ethanol-water crude extract is shown in **Figure 3.3**, which gives a general idea about possible existance of different compounds. In the absorbance spectrum, the peaks that are observed at 200 – 210 nm, and 345 – 355 nm may be result of the π-π*, and n-π* transitions, respectively. Mainly, π-π* transitions are due to the C=C double bond structures, and n-π* transitions are due to the carboxyl groups with the transitions from nonbonding oxygen n orbitals to π* antibonding orbitals. The absorption peak at 266 nm is mostly due to the phenolic structures and ring systems.

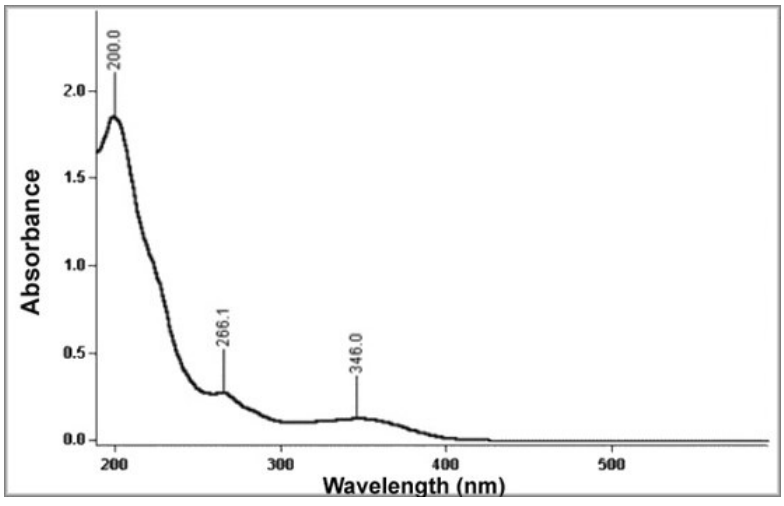


Figure 3.3. UV-VIS absorbance spectra of 50% ethanol-water crude seed extract

3.1.2 Isolation

3.1.2.1. Silica Gel Column Chromatography

Isolation has been carried out using simple column chromatography techniques to reveal individual active components in the crude extract. Silica gel 60 column (3x30cm) was prepared as given in methods. 1.0 g of 50% ethanol:water extract dissolved in 2.0 mL of water was applied to the silica gel column and eluted by using [1:1:2] ethanol:water:butanol solution system. The elutions from the column were collected as 2 mL for each fraction. Consecutive fractions were loaded on the TLC plates as much as 5 microliters (**Figure 3.4**). Every fraction was scanned for the UV-VIS spectrum. Depending on the TLC and UV-VIS data, similar fractions were pooled and evaporated to concentrate.

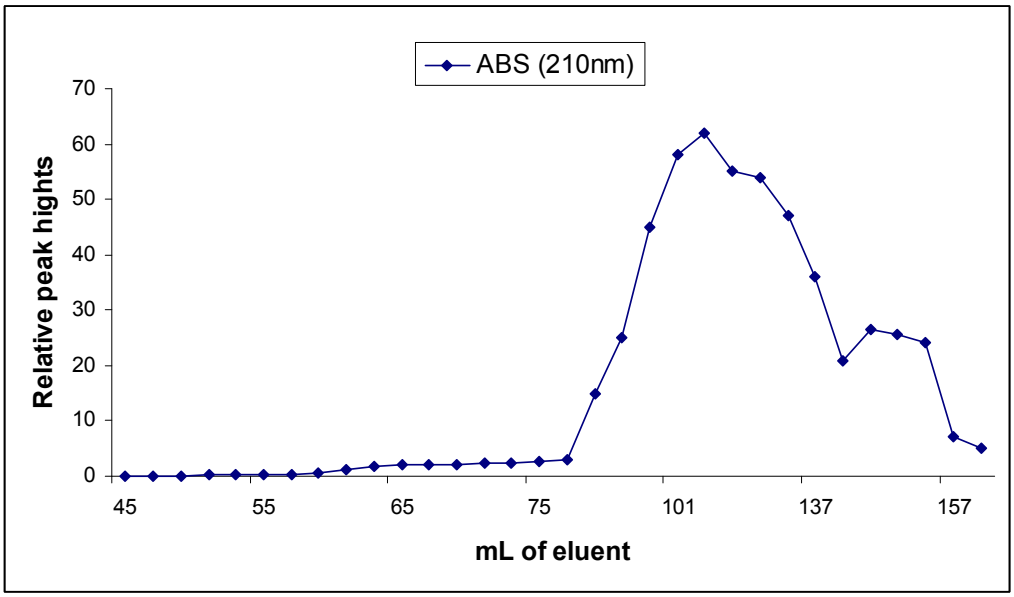


Figure 3.4. Elution profile of Silica Gel 60 column chromatography, eluted with [1:1:2] Ethanol:Water:Butanol mixture, at 2.5 mL/min flow rate.

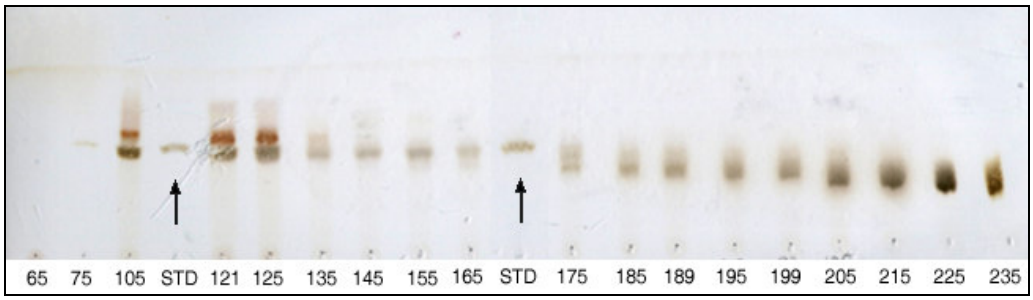


Figure 3.5. TLC data for Silica Gel 60 column chromatography elutions and applied on TLC plate as 5µl samples. Ethanol:Water:Butanol [1:1:2:] solvent system was used as mobile phase. The lanes are numbered as mL of eluted volume. Plates were dried sprayed with 1:1 mixture of 1% vanilin-ethanol and 5% H_2SO_4-ethanol solution and heated at 120^oC for 5 minutes for visualisation.

Eluted fractions between 80 to 157 mL were pooled as seen in **Figure 3.4 and 3.5,** were pooled, and evaporated under vacuum until dryness and 0.1044 g of partially purified component was obtained. From 1g of extract. 10.4 % yield was obtained. All of the partially purified isolate (0.1044 g) was applied on lipophilic sephadex by dissolving in 1 mL of methanol.

3.1.2.2. Isolation by Sephadex LH-20

Further purification was carried out through lipophilic sephadex (LH-20) at 2.5x120 cm diamentions, and the elution solvent was methanol as the mobile phase. All the fractions were scanned for their UV-VIS spectra and the elution profile of LH-20 sephadex column is given in **Figure 3.6,** at specific wavelengths**.**

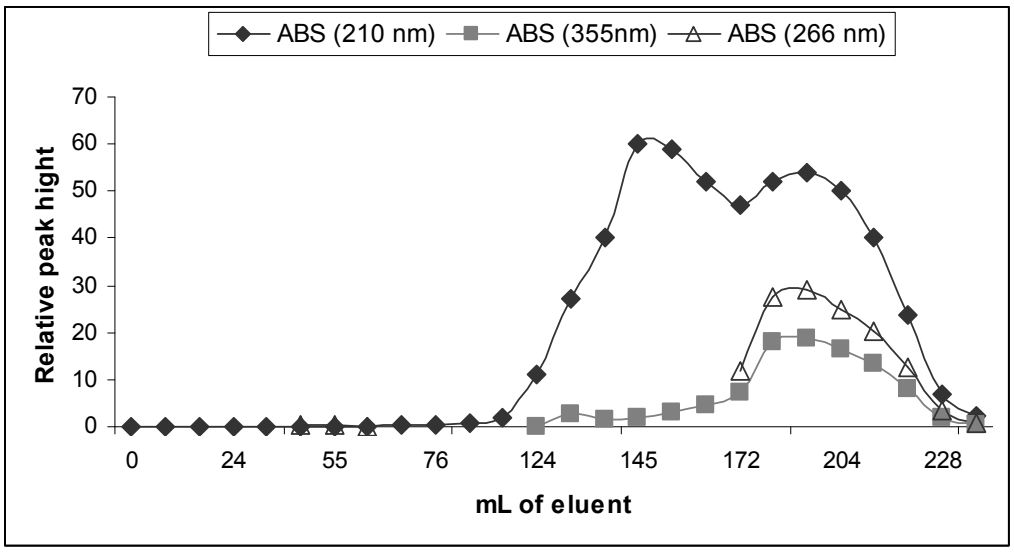


Figure 3.6. Elution profile of Sephadex LH-20 column chromatography, eluted with methanol. Each fractions were collected as 1.5 mL, at a 2.5mL/min flow rate.

3.1.2.3. Characterisation of Isolated Compounds

The eluton profile was contstructed for each fraction by taking the absorption not only at a single wavelength but scanning from 200nm through 600 nm given in **Figure 3.6,** which points out semi-resolved twin peaks. Selected fractions from the Sephadex LH-20 column elutions were applied on the TLC slica F254 plates with 5 µL of sample loading using 1:1:2 ethanol: water:butanol mixture as mobile phase are given in **Figure 3.7.**

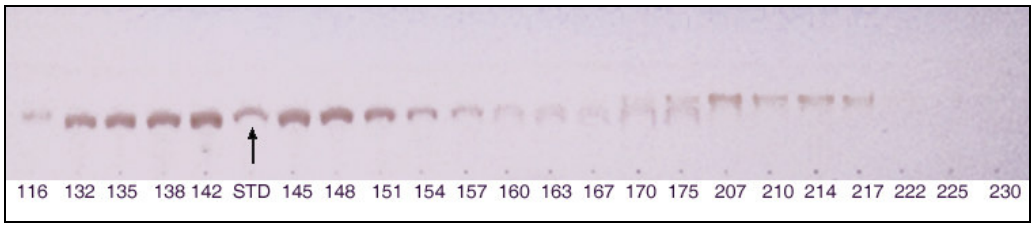


Figure 3.7. TLC slica F254 plate data from the elution fractions of LH-20 column chromatography. 5µl of sample application from selected fractions. Mobile phase of 1:1:2 .ethanol: water:butanol. Sprayed with 1:1 mixture of 1% vanilin-ethanol and 5% H_2SO_4-ethanol solution and heated at 120 ^oC for 5 minutes for visualisation.

In **Figure 3.6,** first one of the unresolved twin peaks starting from 116 mL through 160 mL volume of eluent absorbs only at 210nm wavelength, which indeed indicates a single compound in the elution, and its UV-VIS absorption spectrum confirms that as given in **Figure 3.8.** The elution fractions between 116th to 160th mL were collected and pooled yielding 0.0417 g of dry sample, names as compound I.. On the other hand the second part of this unresolved twin peak starting from the 165 mL through 225 mL volume of eluent gives an absorbtion spectrum with three peaks including 210, 266 and 355 nm, **Figure 3.9**, indicating the presence of at least one other compound. When it is compared with the **Figure 3.7,** it is obvious that there is another compound with some interference from the first part of 210 nm absorption peak.

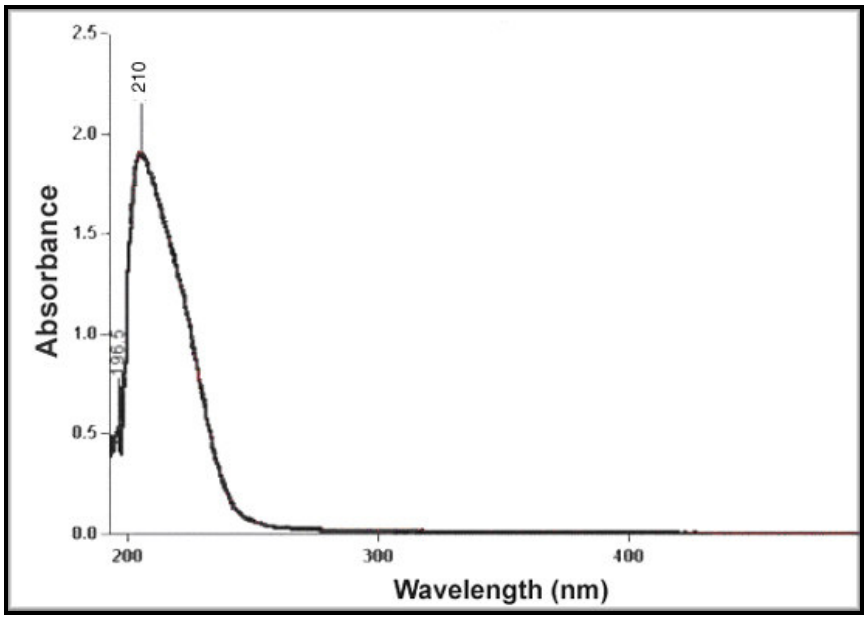


Figure 3.8. UV-VIS Spectrum of 145th fraction containing isolated compound I, aescin from Sephadex LH-20 column.

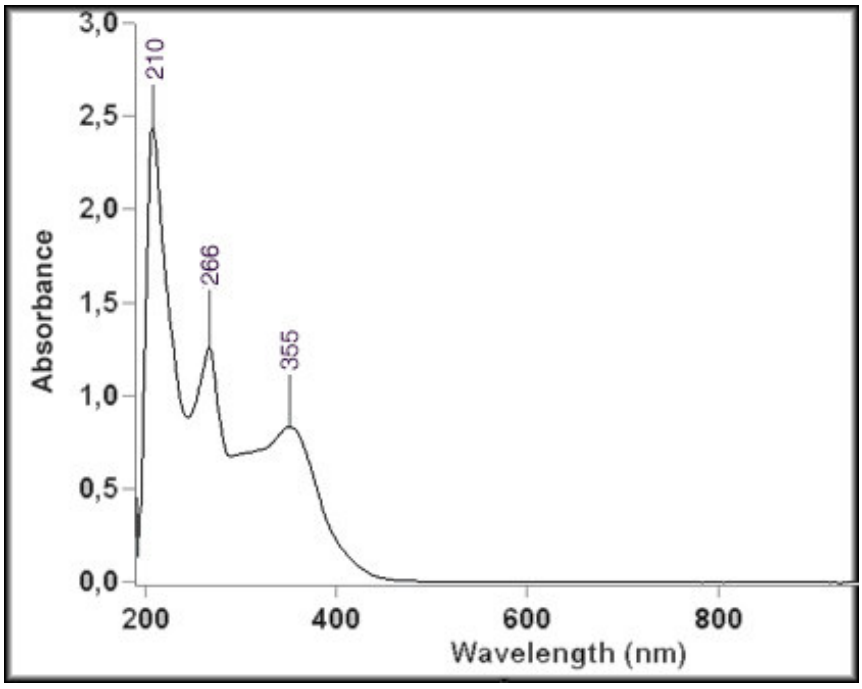


Figure 3.9. UV-VIS Spectrum of Compound II (180th fraction) from Sephadex LH-20 column chromatography.

Peaks eluted from the sephadex LH-20 column, namely compound I and compound II, were analysed for their antioxidant activities, and only compound I was found to be active. Since the first peak was a single compound, and it was carried on for further characterisation.

Infrared and UV-VIS spectroscopy data of the isolated compound I was compared with certain standard references from the spectral database and it has been found to match significantly with aescin (**Figures 3.8, 3.10**).

Aesculus hippocastanum L and isolation of aescin were studied by Kockar *et. al.*, 1990.

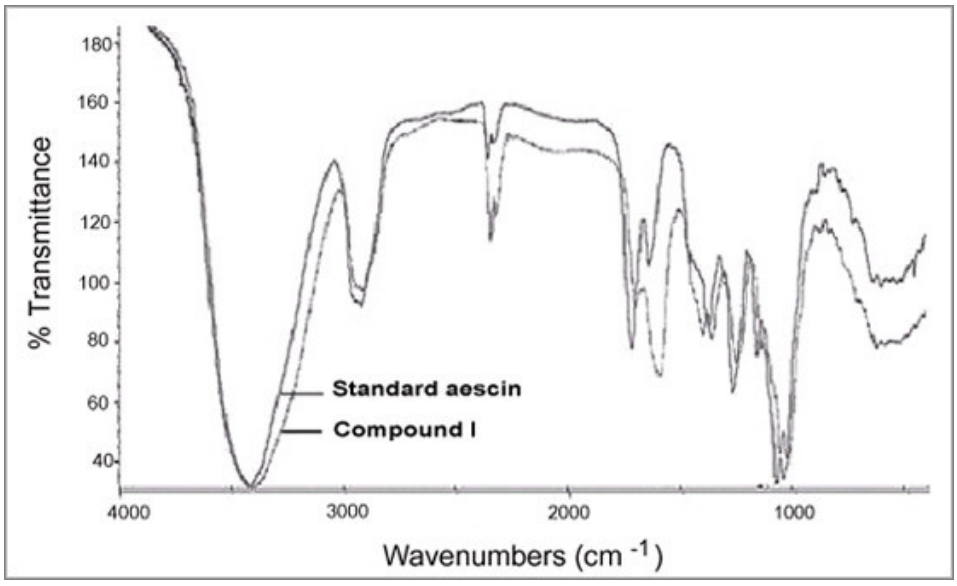


Figure 3.10. IR Spectra of isolated compound I and the standard aescin.

In this study, 100 g of *Aesculus hippocastanum* L. dry seeds were extracted in 50% ethanol: water solution . Total of 3.65 g crude extract was obtained and 1g of it was applied on the first column (Silica Gel 60), 0.1045 g of partially purified isolate was obtained. That was applied on the second column (Sephadex LH-20) for further purification and 0.0417 g of pure isolate Compound I (Aescin) was obtained. The isolation flow-chart is given in **Scheme 1.1**. In every step isolation, per cent recovery is calculated as shown in figure. At the last step of isolation, 40% fold purification was obtained.

Many other products have also been isolated from the chestnut seeds, i.e. bioflavonoids such as: quercetin, kaempherol and their diglycosyl derivatives, as well as antioxidants, such as proanthocianidin A2 and the coumarins esculin and fraxin, by using HPLC (Bombardelli *et. al.*, 1996).

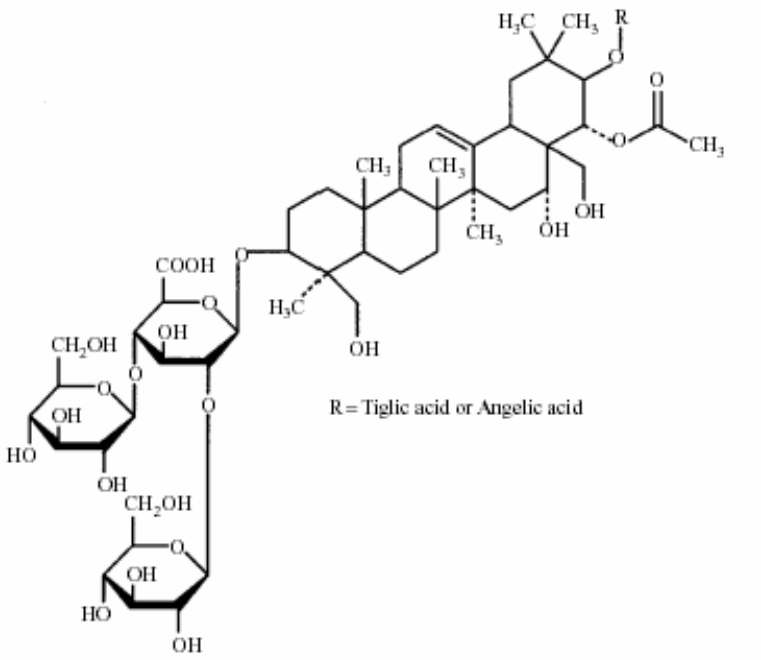


Figure 3.11. Chemical sructure of aescin (Bombardelli *et. al.*, 1996).

3.1.2.4 Determination of Antioxidant Activity of Isolates

Antioxidant capacity studies were carried out to determine the lipid peroxidation inhibitory effects of the 50% ethanol-water seed extract, isolated compound I, namely aescin and standard compounds. Results are presented in **Table 3.1** from which it is clear that the compound I has the same inhibitory effect on the lipid peroxidation as the standard aescin was purchased from the chemical company Sigma. In the literature, commercially available horse chesnut extract was specified to contain 70% aescin, had also shown inhibition on lipid peroxidation (Guillaume and Padioleau, 1994)

Table 3.1. Effect of 50% ethanol crude extracts from *Aesculus hippocastanum* L. seeds and its isolates on microsomal lipid peroxidation inhibition.

Tested material	IC_{50} (mg/mL)
Crude seed extract	0.50
Compound I	0.46
Compound II	None
Standard Aescin	0.44
Alpha-tocopherol	0.23

Average of 6 determinations

In comparison of the antioxidant activities of the ethanolic seed extract and isolates, the most potential antioxidant component in the seed extract should be attributed to the isolated compound I, which turned out to be identical to aescin, as confirmed by TLC, NMR, IR and UV-VIS spectral data. Especially aescin, as the principle compound residing in the seeds, has quite similar IC_{50} values with alpha-tocopherol when compared in molar concentrations, however the difference in their molecular weights is making up the variation in IC_{50} values in mg/mL concentrations.

The fruits of *Aesculus hippocastanum L.,* where the aescin content is highest, have been used for the treatment of mammary indurations and cancer (Hartwell, 1982). Potential antitumor agents showing significant in vitro cytotoxicity were isolated and characterized (Konoshima and Lee, 1986).

Various methods for the isolation and determination of aescin in pharmaceuticals were developed (Lehtola and Huhtikangas, 1990; Koçkar *et. al.*, 1994; Constantini, 1999). In this study, aescin has been isolated using simple column chromatography and TLC techniques.

Pharmacological properties of *Aesculus hippocastanum* L., containing 70% aescin, the main active component of commercial product Veinotonyl 57®, were investigated to elucidate its efficacy in chronic venous insufficiency. Antiradical action of *Aesculus hippocastanum* L. extract was investigated by in vitro non-

enzymatic and enzymatic lipid peroxidation and in vivo. *Aesculus hippocastanum* L. has inhibited lipid peroxdation in a concentration-dependent manner. IC_{50} values for *Aesculus hippocastanum* L. were reported as $1.4x10^{-4}$ mol/L and $1.7x10^{-5}$ mol/L in the non-enzymatic and enzymatic lipid peroxidation inhibition tests, respectively (Guillaume and Padioleau, 1994). IC_{50} values detemined in this study are consistent with the results of Guillaume and Padioleau when we express the results in terms of mg/mL concentrations

3.2. Phenolics Content and Antioxidant Capacity of *Aesculus hippocastanum* L.: Flowers, Leaves, Seeds and Bark

Crude seed extracts of *Aesculus hippocastanum* L. has shown substantial amount of antioxidant activity, which attracted our inters to investigate the antioxidant activity for the other parts of *Aesculus hippocastanum* L trees.

Air dried flowers, leaves, seeds and bark were ground and 5 g of each sample were extracted overnight at 50°C with ethanol using a sample to solvent ratio of 1:10. Extracts were filtered through double cheese cloth and were dried under vacuum. The extraction yields for flowers, leaves, seeds and bark of *Aesculus hippocastanum* L. are given below in **Table 3.2**.

Table 3.2. Extract yields obtained from ethanol extraction of *Aesculus hippocastanum* L.

Parts used	**Yield of extract (mg) - (%)**
Bark	51.75 ± 14.11 - 1.04
Seeds	103.8 ± 4.67 - 2.08
Leaves	143.5 ± 31.11 - 2.87
Flowers	312.5 ± 10.68 - 6.25

3.2.1 Lipid peroxidation inhibition activity of *A. hippocastanum* Flowers, Leaves, Seeds and Bark extracts

Iron induced microsomal lipid peroxidation inhibition was used to evaluate antioxidant capacity efficiency of the *A. hippocastanum* extracts. Stock solutions of flowers, leaves, seeds and bark extract were prepared by redissolving the 15 mg of the dried extracts in 1mL ethanol. The stock solution was then used for the preperation of the final concentrations, in the range of 0.05 mg/mL to 10 mg/mL, for the lipid peroxidation inhibition experiments. Alpha-tocopherol which is a well-known antioxidant, was used as the standard. During the measurements, absorbance of each sample was corrected for the ethanol background. Lipid peroxidation inhibition capacities of each ethanol extract were calculated as the percent inhibiton and the results are shown in **Figure 3.12**.

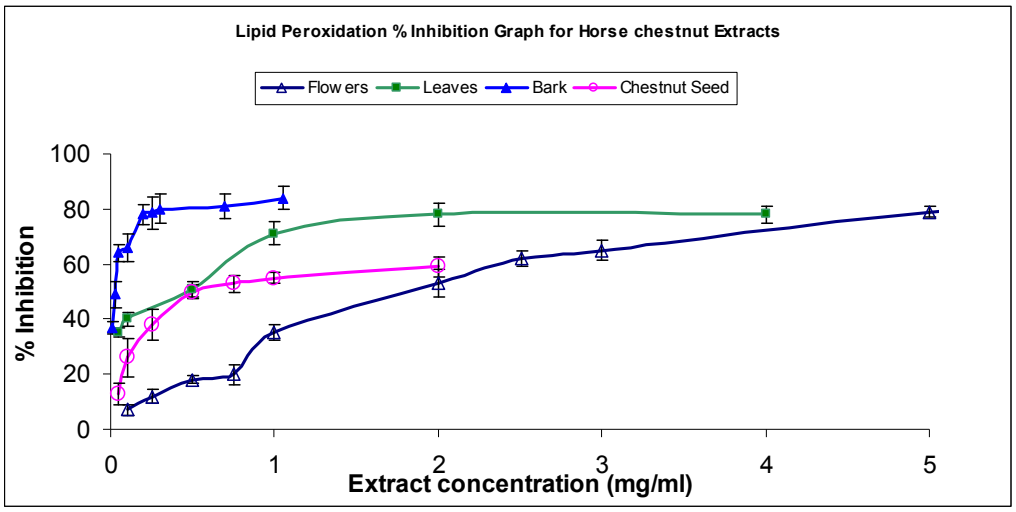


Figure 3.12. Lipid Peroxidation % Inhibition for *A. hippocastanum* flowers, leaves, seeds and bark extracts. 1mg/mL microsomal suspension was used as the lipid source. Each data were obtained by collecting 6-9 independent measurements.

50 Percent inhibition of lipid peroxidation was calculated for each of the extract in mg/mL to compare their antioxidant efficiencies, and the results are given in **Table 3.3.** The results indicated that bark extract was the most effective inhibitor

of lipid peroxidation with the lowest concentration of 0.025 mg/mL at 50 percent inhibition (IC_{50}). All the other extracts need at least 10 times higher concentrations to show the same effect as that of bark extract. In the experiments, alpha-tocopherol was used as the reference had an IC_{50} value of 0.23 mg/mL.

3.2.2 DPPH radical scavenging activity of *A. hippocastanum* extracts

Free radical scavenging activity of ethanol extracts from flowers, leaves, seeds and bark were evaluated by using DPPH radical scavenging method. Dry extract concentration range of 0.1 mg/mL – 15 mg/mL were dissolved in ethanol. DPPH radical scavenging activity of the extracts were expressed as % radical scavenging by measuring the decrease in the intensity of purple color due to DPPH radical. Quercetin being a well known antioxidant standard was used as a reference for comparison. The results were plotted in **Figure 3.13** as percent scavenging activity of each extract. 50 percent of the scavenging activities calculated and given in **Table 3.3**. The highest free radical scavenging activity was obtained with bark extract with a value of 0.014 mg/mL and next to that was the seed extract with an IC_{50} of 0.082 mg/mL. Extracts from leaves and flowers were less effective as radical scavengers with IC_{50} of 0.17 mg/mL and 0.6 mg/mL, respectively. In comparison of the extracts with quercetin, which has an IC_{50} of 0.085 mg/mL, bark extract is still the most effective one as a free radical scavenger.

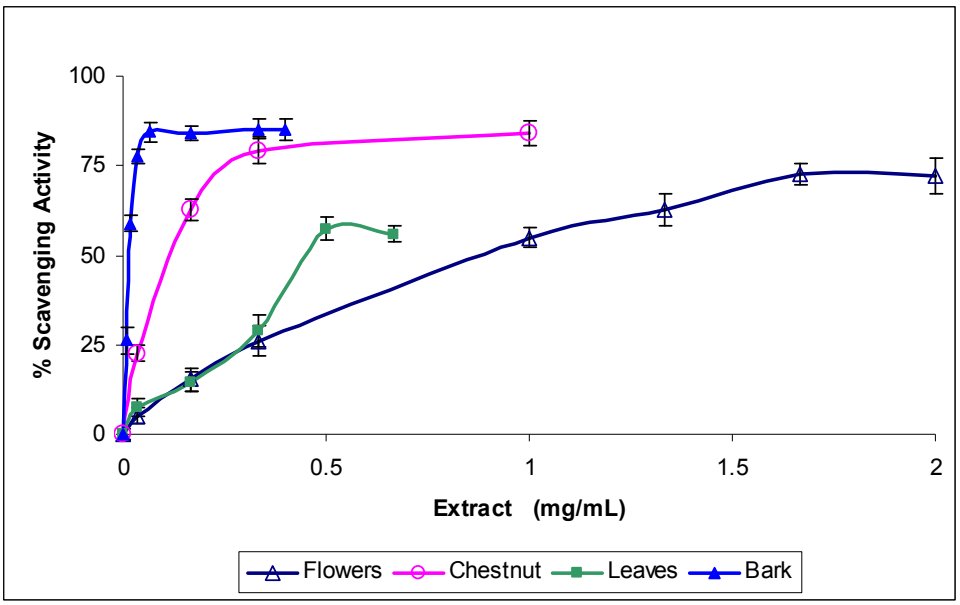


Figure 3.13. Free radical % scavenging activity of *A. hippocastanum* extracts Each data were obtained by collecting 6-9 independent measurements.

As given in **Table 3.3,** there is a considerable correlation between DPPH radical scavenging and lipid peroxidation inhibition capacities of extracts, with the highest activities for bark extracts and the lowest for that of the flower extracts. Bark extract has even revealed better antioxidant activities than the well-known references, α-tocopherol and quercetin in lipid peroxidation inhibition and DPPH radical scavenging capacity measurements.

Concerning the lipid peroxidation inhibition only, seed and leaves extracts were also good but not any better that α-tocopherol. In case of DPPH radical scavenging, however, seed extracts were much better than leaves extracts, in fact, as good as the reference quercetin. This might have been related to the high saponin content found in the seeds, since saponins are know to be good free radical sinks. Flower extracts were the least effective antioxidants in both methods.

3.2.3 Total phenolics content of *A. hippocastanum* extracts

The total phenolics content of the extracts from flowers, leaves, seeds and bark were investigated by using the method of Singleton and Rossi, 1965, and the results are expressed in **Table 3.3** as milligram per miligram of gallic acid equivalents (GAE).

The total content of phenolics was significantly higher in the leaves and bark extracts with values of 0.0747 and 0.0607 mg / mg extract GAE repectively. Bark extract, is expected to have quite high phenolics content, since it is known to contain the polyphenolic compounds like esculetin that is an important antioxidant (Gastaldo, *et al*., 1996). Seed extract however, contains the lowest amount of total phenolics with a value of 0.0207 mg per mg extract, as expected. Aescin which is saponin in structure, is the major antioxidant in the seeds (Sağdıçoğlu, 2000; Koçkar *et al*., 1994). This fact reflects itself in low phenolic content of the seeds.

Table 3.3. Comparison of the antioxidant activity and the total phenolics content of the flowers, leaves, bark and seeds *of Aesculus hippocastanum* L. ethanolic extracts

Parts of *Aesculus hippocastanum* L.	**Lipid peroxidation IC_{50} (mg/mL)**	**DPPH radical scavenging IC_{50} (mg/mL)**	**Total Phenolic Content (mg / mg extract) GAE**
Bark	0.025	0.014	0.0607 ± 0.0131
Seeds	0.51	0.082	0.0207 ± 0.0042
Leaves	0.19	0.17	0.0747 ± 0.0167
Flowers	1.58	0.60	0.045 ± 0.008
Alpha-tocopherol	0.23	**ND**	**NA**
Quercetin	**ND**	0.085	**NA**

Each data were obtained by collecting 6-9 independent measurements.

In spite of the low antioxidant activity, there was a considerable phenolics content in the flower extracts. Many aromatic compounds, found in plants, contain phenolic hydroxyl groups, carbonyl groups or both of them. The arrangements of these hydroxyl groups and substitution of the hydroxyl groups by glycosylation decreases the antioxidant capacity of these polyphenols (Robinson, 1963). This might explain the low antioxidant potential exerted by the flower extracts, despite their high total phenolic content.

Leaves extract has one of the highest total phenolic content besides the bark. Therefore, leaves extract is also expected to show much higher antioxidant activity.

Importance of phenolic constituents, mainly the flavonoids, have been studied extensively as contributers to the antioxidant activity of plants (Lo'pez et al., 2003; Skerget et al., 2005; Rice-Evans et al., 1997; Aruoma, 2003) . The tendency of polyphenols for metal chelation, especially for iron and copper, is very important since they inhibit the formation of metal-catalysed free radical chain reactions (Rice -Evans et al., 1997, Gheldof et al., 2002). Therefore, polyphenols are also important antioxidants for their cancer preventive characteristics. In the literature, there are many articles expressing results about the positive correlation between total phenolics content and the antioxidant activity of the studied plant extracts (Cai et al., 2004; Yen and Duh, 1993; Kaur and Kapoor, 2002; Velioğlu, et al., 1998). However, there are also a number of studies that has shown no correlation at all (Kaur and Kapoor, 2002; Mau et. al, 2004; Heinonen et. al, 1998; Cuvelier et. al, 1992).

The fact that *Aesculus hippocastanum L*. extracts have beneficial effects on certain disesases points out its antioxidant potential, where epidemiological data suggests positive effects of natural antioxidants againts many diseases including cancer. Consequently, a good correlation between the total phenolic content and the antioxidant activity could be expected for all the *Aesculus hippocastanum L*. extracts.

Aesculus hippocastanum L. extracts were shown to have significant superoxide anion-scavenging effects among 65 medicinal plants tested, therefore, the activity of other active-oxygen scavengers such as hydroxyl radicals, singlet oxygens and lipid peroxides in the *Aesculus hippocastanum* L. extracts was further examined in detail by both ESR spin-trapping and malondialdehyde generation. In addition, *Aesculus hippocastanum* L. was found to have strong active-oxygen scavenging activity and protective activity against cell damage induced by active oxygen, on a murine dermal fibroblast culture system. *Aesculus hippocastanum L*. extract is proposed as potent plant extract with potential application as anti-aging or anti-wrinkle material for the skin (Masaki, *et.al.,* 1995).

As a conclusion, all the ethanol extracts *of Aesculus hippocastanum L*. have shown significant antioxidant capacity. However, flower extracts have shown poor antioxidant activity, despite its high phenolics content.

Leaves extract could be considered as good antioxidant as it is reflected by both methods, besides it has the highest phenolics content. Seed extracts also have significant activity despite its lowest phenolics content.

Bark extract, especially, was very effective as lipid peroxidation inhibitor. It was quite rich in phenolic content and was remarkable in DPPH radical scavenging activity. The extract can even be considered as a better antioxidant than α-tocopherol and quercetin

3.3. Screening of Plants from East Anatolian Region for Their Antioxidant Activities, Phenolic Contents and Effects on Glutathione-S-Transferase Activity

In the flora of East Anatolian region, *Papaver bracteatum* L., *Urtica urens* L., *Gundelia tournefortii* L, *Prangos ferulacea* L., *Chaerophyllum macropodum* Boiss., *Heracleum persicum* Desf, *Allium vineale* L, *Aconitum cochleare, Rheum*

ribes L, *Ferula rigidula* DC, *Rosa heckeliana* Tratt have been selected to study their phenolics contents, antioxidant activities and their effect on GST since they are the most commonly used plants as food sources and as folk medicine in that region. Antioxidant activity of the specified plants were studied both with DPPH radical scavenging and lipid peroxidation inhibiton methods, total phenolic contents were determined and their effects on cytosolic GST activities toward CDNB were investigated.

3.3.1. Antioxidant activity of *Allium vineale* L. (*Liliaceae*) Sirmo

The word "allium" is an ancient Latin word for garlic. The name is now applied to all the plants closely related to garlic. It is native to Northern Africa, Western Asia, Turkey, Armenia; Azerbaijan and Europe. Today alliums are used for their flavor, aroma and taste, and being prepared domestically or forming raw material for a variety of food manufacturing processes (dehydration, freezing, canning and pickling). (Stajner, 2003)

Allium species are added into to a special type of cheese in the Van region for its aromatic and flavouring property (Baser, 1997).

***3.3.1.1. Extraction yield*:** 10 g air dried samples from aerial parts of *Allium vineale* L. were extracted with 100 mL of methanol for overnight at 50^0C. The amount of resulting crude extract was 675.50 ± 79.9 mg, which is 6.75 % by its dry weight.

3.3.1.2 DPPH percent scavenging activity of Allium vineale L.

DPPH percent scavenging activity of *Allium vineale* L. crude extract was measured in concentrations ranging between 0.01 and 1.4 mg/mL. The results are shown in **Figure 3.14.**

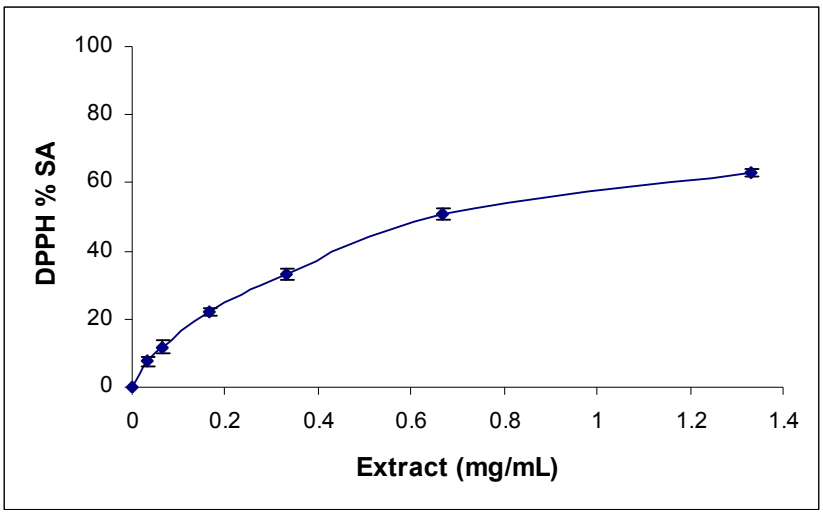


Figure 3.14. DPPH % scavenging activity of *Allium vineale* L.

IC_{50} value for DPPH percent scavenging activity were calculated from the percent inhibition versus log extract concentration curve and was found to be 0.790 mg/mL for *Allium vineale* L. extract.

3.3.1.3 Lipid peroxidation inhibition capacity of Allium vineale L.

Lipid peroxidation inhibition capacity of *Allium vineale* L. extract was evaluated with thiobarbituric acid test using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations varying between 0.01 – 1 mg/mL. Lipid peroxidation percent inhibition capacity of *Allium vineale* L. crude extract is shown in **Figure 3.15.**

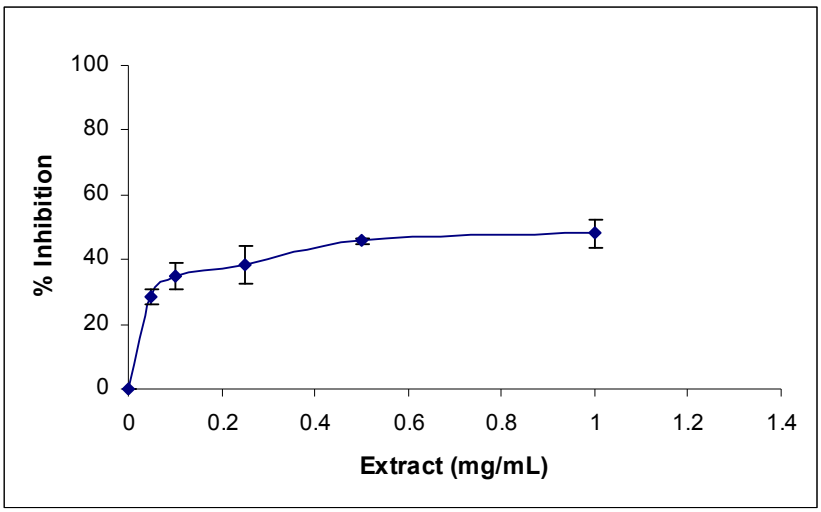


Figure 3.15. Microsomal lipid peroxidation percent inhibition capacity of *Allium vineale* L.

IC_{50}, the concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, was calculated as 1.097 mg/mL for *Allium vineale* L. crude extract.

3.3.1.4. Total phenolics content in Allium vineale L.

Total phenolics content of *Allium vineale* L. was measured as 0.0649 ± 0.0047 mg in 1 mg of methanolic extract using gallic acid as the standard to constructing calibration curve.

When the antioxidant activities of *Allium vineale* L..are compared, it is observed that, the extacts are not very active in scavenging free radicals such as superoxide and hydorxyl radicals as can be deriven from relatively high IC_{50} for DPPH percent scavenging activity (0.79 mg/mL), especially when compared to the IC_{50} for the standard, quercetin which is 0.085 mg/mL . The same situation has been observed for lipid peroxidation inhibition capacity (IC_{50}=1.097 mg/mL), when compared with α-tocopherol (IC_{50} =0.23 mg/mL). In fact, the total pheolics content

of *Allium vineale* L.. extract was also quite low with the amount of 0.065 mg of GAE in mg of extract, which could be correlated to the low antioxidant activity.

In the literature, Stajner reported the results related to flavonoid and, vitamin C content of the leaves for different *Allium* species (Stajner, 2003).

Garlic and onion, which also contain flavonoid compounds, quercetin, in particular, the monoterpenes from the oil of citrus peel such as D-limonene, and curcumin which is a natural constituent of the spice turmeric may act as blocking agents and may support xenobiotic detoxification (Percival, 1997).

3.3.2. *Chaerophyllum macropodum* Boiss. (*Apiaceae*)

Chaerophyllum macropodum is a common plant used for making cheese with herb, before its flowering period. It is mainly used in Van, Bitlis, Siirt, Hakkari, Ağrı and Muş in Turkey.

3.3.2.1 Extraction yield: 10 g air dried samples from aerial parts of *Chaerophyllum macropodum* Boiss. were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50 ^{0}C. The amount of resulting crude extracts was 338.50 ± 85.6 mg that is 3.38 % of the dry extract.

3.3.2.2 DPPH percent scavenging activity of Chaerophyllum macropodum *Boiss.*

DPPH percent scavenging activity of *Chaerophyllum macropodum* Boiss. crude extract was measured in different concentrations ranging between 0.01 and 1.4 mg/mL and results are given in **Figure 3.16.**

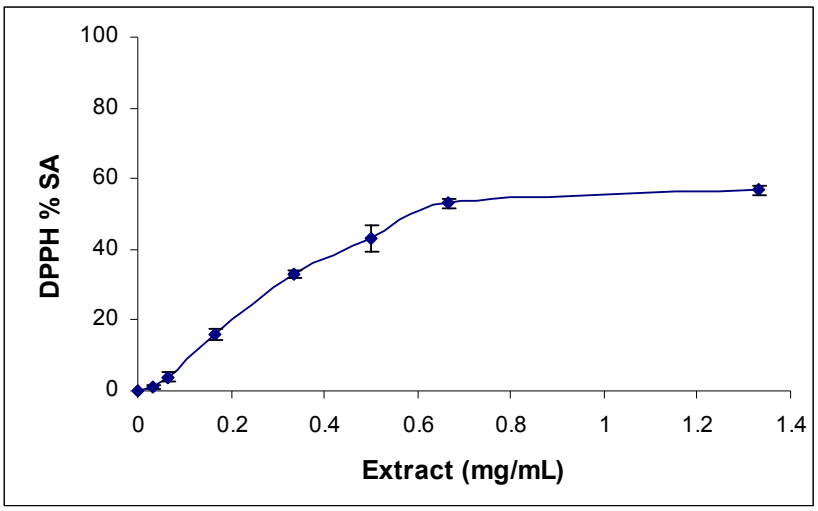


Figure 3.16. DPPH % scavenging activity of *Chaerophyllum macropodum* Boiss.

IC_{50} value of *Chaerophyllum macropodum* Boiss. DPPH percent scavenging activity was calculated as 0.623 mg/mL from the percent inhibition versus log extract concentration curve.

3.3.2.3. Lipid peroxidation inhibition capacity of Chaerophyllum macropodum Boiss.

Lipid peroxidation inhibition effect of *Chaerophyllum macropodum* Boiss. extract was tested using thiobarbituric acid test with 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations varying between 0.05 – 1mg/mL. Lipid peroxidation percent inhibition capacity of *Gundelia tournefortii* L. extract is shown in **Figure 3.17.**

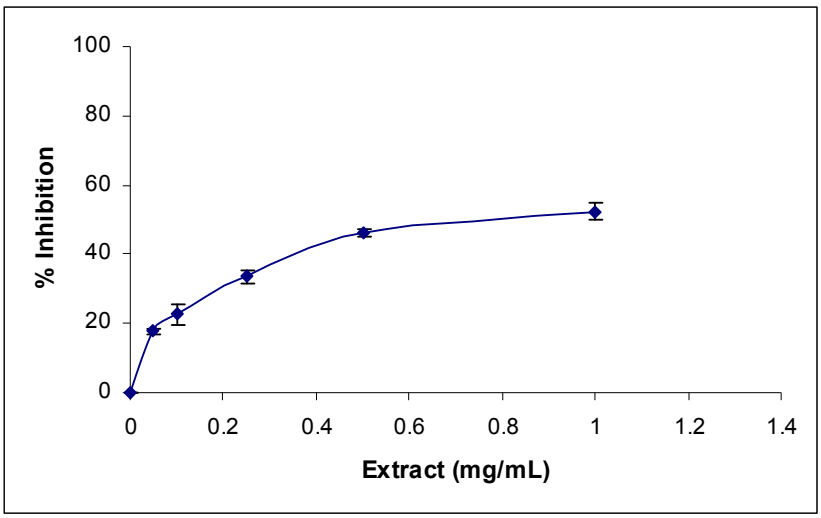


Figure 3.17. Microsomal lipid peroxidation percent inhibition capacity of *Chaerophyllum macropodum* Boiss.

The concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, IC_{50}, was calculated as 0.852mg/mL for *Chaerophyllum macropodum* Boiss extract.

3.3.2.4. Total phenolics content in Chaerophyllum macropodum Boiss.

Total phenolics contents in *Chaerophyllum macropodum* Boiss. crude extract was found to be 0.0340 ± 0.0070 mg in 1 mg of methanolic extract.

Chaerophyllum macropodum Boiss extract IC_{50} values 0.623 mg/mL for DPPH and 0.852 mg/mL for lipid peroxidation inhibition which are significantly higher than the IC_{50} values of standards, quercetin and α-tocopherol as 0.085mg/mL and 0.23 mg/mL, respectively . Hence, crude extract from the *Chaerophyllum macropodum* Boiss. could be considered as one of the ineffective plants as an antioxidant. Total phenol content of *Chaerophyllum macropodum* Boiss was also found to be very low, with 0.034 mg of GAE in mg of extract, which is in good correlation with low antioxidant activity.

In the literature, essential oils of *Chaerophyllum macropodum Boiss*. was reported by Nematollahi et.al, to be rich in monoterpene hydrocarbons (Nematollahi et.al., 2005).

3.3.3. Antioxidant effects of *Heracleum persicum* Desf. (*Apiaceae*)

Heracleum persicum Desf. is native to Asian temperate regions especially, to Iran and Turkey. In Iran, stem and seeds of this plant have been used in traditional medicine, for treatment of epilepsy. Sayyah et. al. have recently reported that the acetone extract of the seeds were efective in anticonvulsant activity (Sayyah et. al., 2005).

3.3.3.1 Extraction yield: 10 g air dried aerial parts of *Heracleum persicum* Desf. were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 599.00 ± 45.3 mg, and 5.99 % by weight.

3.3.3.2 DPPH percent scavenging activity of Heracleum persicum Desf.

DPPH percent scavenging activity of *Heracleum persicum* Desf. crude extract was measured in concentrations ranging between 0.01 and 0.7 mg/mL. The results are given in graph in **Figure 3.18.**

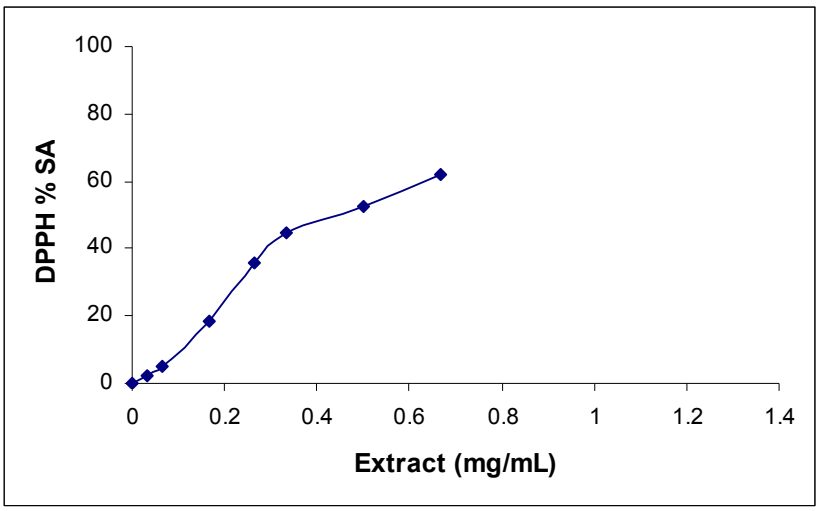


Figure 3.18. DPPH % scavenging activity of *Heracleum persicum* Desf.

IC_{50} for DPPH percent scavenging activity of *Heracleum persicum* Desf. Extract was found to be 0.438 mg/mL, as calculated from percent inhibition versus log extract concentration curve.

3.3.3.3 Lipid peroxidation inhibition capacity of Heracleum persicum Desf

Lipid peroxidation inhibition effect of *Heracleum persicum* Desf. extract was measured with thiobarbituric acid test using 1 mg/mL sheep liver microsomes with iron (II) induction. Extracts were applied between 0.01 – 1 mg/mL concentrations and the results are shown in **Figure 3.19.**

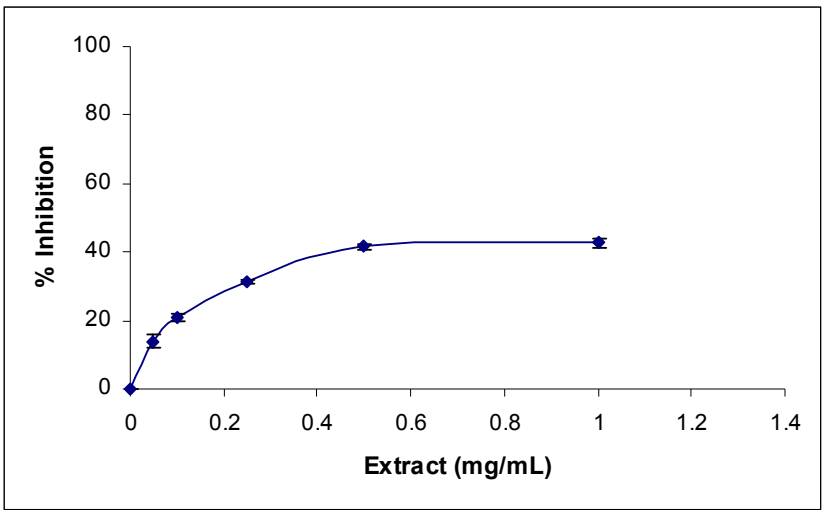


Figure 3.19. Microsomal lipid peroxidation percent inhibition capacity of *Heracleum persicum* Desf.

The concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, which is IC_{50}, was calculated as 1.586 mg/mL for *Heracleum persicum* Desf. crude extract.

3.3.3.4. Total phenolics content in Heracleum persicum Desf.

Amount of total phenolics content in *Heracleum persicum* Desf. extract was measured as 0.0596 ± 0.0028 mg in 1 mg of methanolic extract from the gallic acid standard curve.

Heracleum persicum Desf. extract IC_{50} value for DPPH scavenging activity was found to be 0.438 mg/mL, which is much higher than the IC_{50} value of standard quercetin, 0.085mg/mL. Furthermore, lipid peroxidation inhibition efect of the extract was also usatisfactory, with 1.586 mg/mL, in comparison to 0.23 mg/mL of α-tocopherol.

In the literature, preliminary phytochemical analysis showed the presence of alkaloids, terpenoids, triterpenes and steroids in the leaves extract of *Heracleum persicum* **(**Mojab and Nickavar, 2003)

Souri and colleques, described the bioactive component furanocoumarins found in fruits of *Heracleum persicum* Desf., with antioxidant activity (Souri et.al, 2004). Furanocoumarins are alos effective dermal photosensitizing agents and are widely used in thr treatment of leukoderma and in variouns sunten lotions (Aynehchi et al, 1978).

3.3.4. Antioxidant effects of *Prangos ferulacea* (*Apiaceae*)

Prangos ferulacea L. is native to Asian temperate regions. The genus *Prango*s, which belongs to the Umbelliferae family, consists of about 30 species. In Iran 15 species are present, among which five are endemic. Some *Prangos* species have been used in the folk medicine as emulient, carminative, tonic, antiflatulent, anthelmintic, antifungal and antibacterial agents (Ceylan, 1987).

3.3.4.1. Extraction yield: 10 g air dried seed samples from *Prangos ferulacea L.* were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 418.50 ± 85.6 mg and 4.18% of the total dry weight.

3.3.4.2. DPPH percent scavenging activity of Prangos ferulacea L.

DPPH percent scavenging activity of *Prangos ferulacea L.* extract was measured in 0.01 - 0.7 mg/mL concentrations. The results are shown below in **Figure 3.20.**

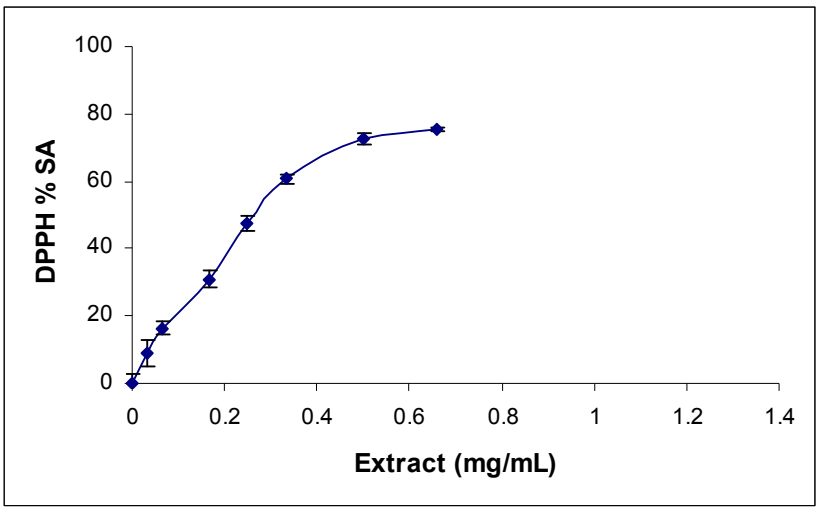


Figure 3.20. DPPH % scavenging activity of *Prangos ferulacea* L.

IC_{50} for DPPH percent scavenging activity of *Prangos ferulacea* L. extract was calculated from the percent inhibition versus log extract concentration curve as 0.242 mg/mL.

3.3.4.3. Lipid peroxidation inhibition capacity of Prangos ferulacea L.

Lipid peroxidation inhibition capacity of *Prangos ferulacea* L. extract was measured with thiobarbituric acid test by using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II) solution. Extracts were tested in concentrations between 0.05 – 1 mg/mL and the results are shown in **Figure 3.21.**

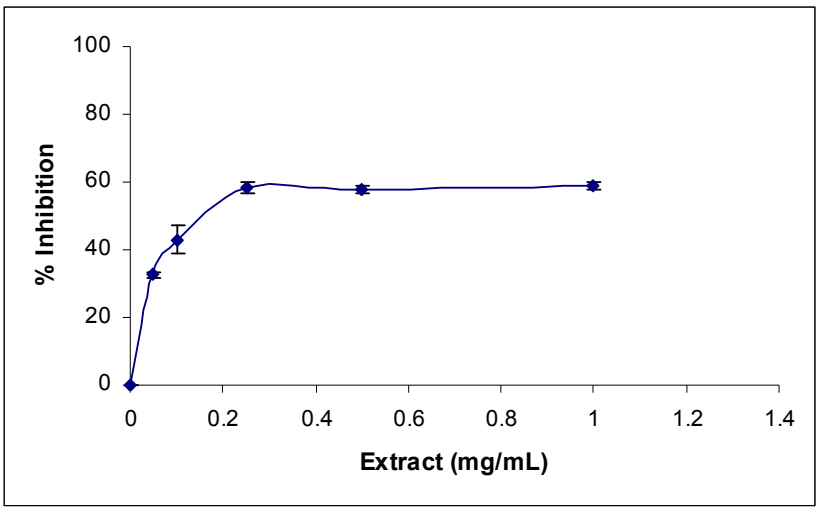


Figure 3.21. Microsomal lipid peroxidation percent inhibition capacity of *Prangos ferulacea* L

IC_{50} values of lipid peroxidation inhibition for *Prangos ferulacea* L. crude extract was calculated as 0.152 mg/mL.

3.3.4.4. Total phenolics content of Prangos ferulacea L.

Total phenolics content of *Prangos ferulacea* L. extracts were found to be 0.0651 ± 0.0064 mg in 1 mg of methanolic crude extract.

In this study, IC_{50} value of *Prangos ferulacea* L seed extract for lipid peroxidation inhibition was found to be 0.152 mg/mL, which was very active in comparison to the IC_{50} value 0.23 mg/mL of α-tocopherol a well-known standard. On the other hand, the IC_{50} value of the seed extract for DPPH scavenging was 0.242 mg/mL, which is not as quite effective as standard quercetin, with 0.085mg/mL value of IC_{50}.

In the literature, Mavi et.al., has reported the antioxidant Properties of Methanolic Extract of *Prangos ferulaceae* stems as IC_{50}RS= 201 mg/L for DPPH radical scavenging and IC_{50}=228 mg/L for peroxidation inhibition.

The amount of total phenolic compounds of methanolic extracts of *Prangos ferulacea* have also been reported as 0.024 mg per mg of extract (Mavi *et. al.,* 2004).

Chemical investigations on the components of the genus *Prangos* have resulted in the isolation of various coumarins, alkaloids, flavonoids and terpenoids (Sajjadi and Mehregan, 2003).

α-Pinene has been reported as the main constituent of oils of fruits of *P. ferulacea* (16.7%) (Baser *et. al.,* 1996)

Chaerophyllum macropodum Boiss, Heracleum persicum Desf. and *Prangos ferulacea L* species all belong to the same family of Apiaceae. When their antioxidant activities are compared, *Prangos ferulacea L* is found to be far better antioxidant than the others. *Prangos ferulacea L* is also a very effective GST inhibitor with an IC_{50} value of 79.25 µg/mL. *Chaerophyllum macropodum Boiss, Heracleum persicum* Desf. are the least effective antioxidants among all the plants studied, however, they are still considered as active antioxidants according to the literature.

3.3.5. Antioxidant effects of *Papaver bracteatum* Lindl.

Papaver bracteatum Lindl. is native to Asian-temperate regions. There are approximately 110 species in the genus *Papaver*, which belongs to the family Papaveraceae. *Papaver bracteatum Lindl.* is an economically important plant since it is used as both ornament environmentally (Goldblatt, 1974) and as a folkloric medicine (Duke et. al., 2002). Since ancient times, it was recognized with its important pharmacological actions, especially with its anesthetic activities. *P. bracteatum* Lindle, produces alkaloids with narcotic and psychotropic effects therefore its cultivation have been strictly controlled by the Narcotics and Psychotropics Control Law since 1990 in Japan (Hosokawa, 2004).

3.3.5.1 Extraction yield: 10 g dried samples from aerial parts of *Papaver bracteatum* Lindl were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50 ^{0}C. The amount of resulting crude extracts were 651.00 ± 62.2 mg which is 6.51% by weight**.**

3.3.5.2. DPPH percent scavenging activity of Papaver bracteatum Lindl

Antioxidant activity of *Papaver bracteatum* Lindl. was evaluated by measuring DPPH percent scavenging activity of the extract. 0.1 mL of *Papaver bracteatum* Lindl. crude extract was used in 0.1 – 1 mg/mL concentrations. The results are given in **Figure 3.22.**

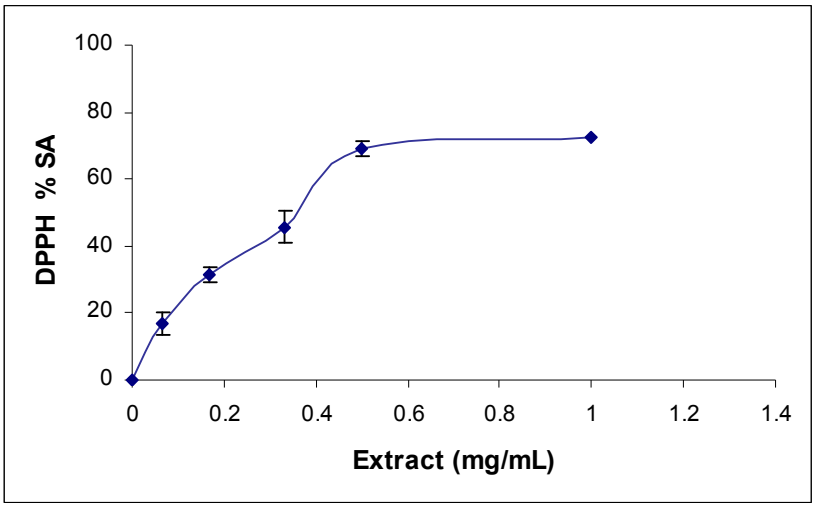


Figure 3.22. DPPH % scavenging activity of *Papaver bracteatum* Lindl

IC_{50} value for DPPH percent scavenging activity were calculated after constructing the percent inhibition versus log extract concentration curve and IC_{50} for DPPH percent scavenging activity of *Papaver bracteatum* Lindl extract was found to be 0.442 (mg/mL).

Antioxidant activity of the aerial parts for *Papaver bracteatum* Lindl. which was collected from Iran, have been studied by measuring the inhibition potential of

80% methanol extracts on linoleic acid peroxidation and the IC_{50} was calculated to be 3.51±0.10 µg while IC_{50} for α-tocopherol was 0.60 µg (Souri *et. al.*, 2004).

3.3.5.3. Lipid peroxidation inhibition capacity of Papaver bracteatum Lindl

Lipid peroxidation inhibition capacity of *Papaver bracteatum* Lindl. extract was evaluated with thiobarbituric acid test. 1 mg/mL sheep liver microsomes were used as the lipid source and lipid peroxidation was induced by incubation with iron (II). *Papaver bracteatum* Lindl extracts were tested in 0.1–1 mg/mL concentrations. Lipid peroxidation percent inhibition capacity of *Papaver bracteatum* Lindl. extract is given in **Figure 3.23.**

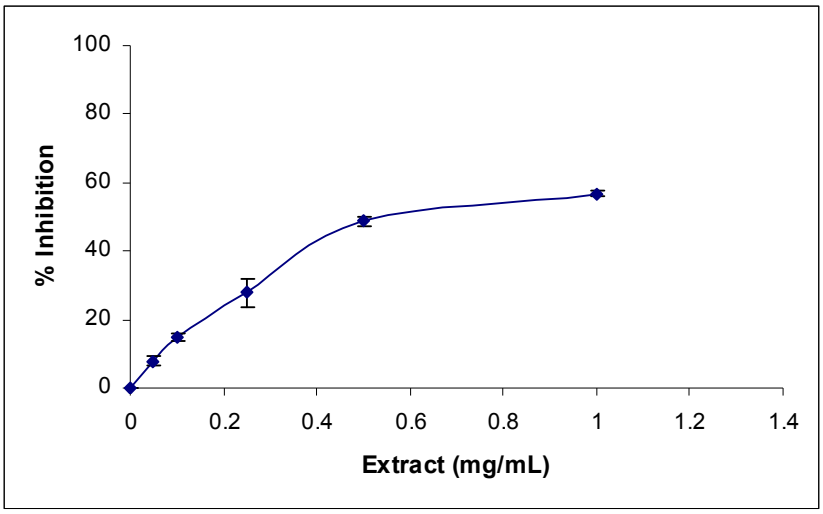


Figure 3.23. Microsomal lipid peroxidation percent inhibition capacity of *Papaver bracteatum* Lindl. 1 mg/mL sheep liver microsomes were used as lipid source.

IC_{50}, which is defined as the concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent was calculated as 0.678 mg/mL for *Papaver bracteatum* Lindl. extract.

3.3.5.4. Total phenolics content of Papaver bracteatum Lindl.

Amount of total phenolic compounds found in the *Papaver bracteatum* Lindl. were measured after constructing a calibration curve with gallic acid as the standard phenolic compound in 0.05 – 0.3 mg/mL concentrations. 0.1 mL of *Papaver bracteatum* Lindl. extract in 0.05, 0.1, 0.2, and 0.3 mg/mL concentrations were used for measuring total phenolics content and the total phenolics was found to be 0.2117 ± 0.0627 mg in 1 mg of crude methanolic extract of *Papaver bracteatum* Lindl.

When *Papaver bracteatum* Lindl. extract antioxidant activities are compared, it is not very active as a free radical scavenger with an IC_{50} of 0.444 mg/mL for DPPH versus quercetin which has IC_{50} value of 0.085 mg/mL. The same situation has been observed for lipid peroxidation inhibition capacity (IC_{50}=0.678 mg/mL), when compared with α-tocopherol (IC_{50} =0.23mg/mL). In fact, the total phenolics content of *Papaver bracteatum* Lindl. extract was quite high with the amount of 0.212 mg of GAE in mg of extract, even though the antioxidant activity of the extract was quite low.

The plant contains very important chemicals, some of which are bractamine and their derivatives, codeine, morphine, papaverine and thebaine (Duke, 1992).

Thebaine is the main alkaloid found in *Papaver bracteatum* Lindl. Many minor alkaloids are present of which 31 have been identified to date. The biological activity of the following alkaloids has been reported in the literature: codeine, thebaine, isothebaine, nuciferine, protopine, tetrahydropalmatine, and coptisine. Codeine is an established drug. Thebaine and isothebaine are both narcotics and analgesics. However, their therapeutic utility is limited because of their high toxicity and severe side-effects. Nuciferine is a CNS depressant. Protopine

has a sedative effect in test animals. It has bactericidal and cytotoxic properties, but is inactive against carcinomas *in vivo*. (Bosch *et. al*., 1981). Besides narcotic and psychotropic effects of thebaine and pain killer action, it is also used for whooping cough, pertussis (Vincent *et. al.*, 1979). Thus, most of the compounds found in the *Papaver bracteatum* Lindl. extract have narcotic and psychotropic effects rather than antioxidant actions. This can explain its high phenolics content but low antioxidant activity.

3.3.6. Antioxidant effects of *Urtica urens* L. (*Urticaceae*) Küçük Isırgan

Urtica urens L. has a has folkloric use as medicine and it is used commonly as food source like spinach in the East Anatolian Region.

3.3.6.1. Extraction yield: 10 g dried samples from aerial parts of *Urtica urens L.* were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 624.50 ± 51.6mg which is 6.4% by weight.

3.3.6.2. DPPH percent scavenging activity of Urtica urens L.

DPPH percent scavenging activity of *Urtica urens L.* crude extract was evaluated by using 0.1 mL extract in 0.1 – 1 mg/mL concentrations. The results are given in **Figure 3.24.**

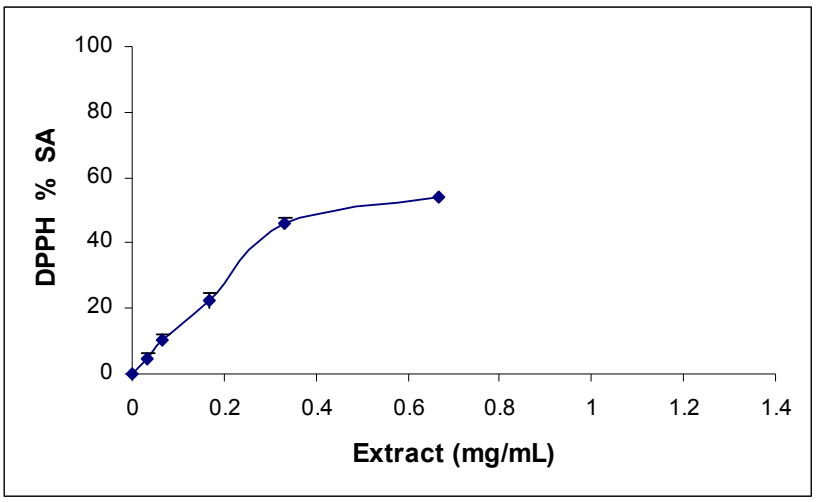


Figure 3.24. DPPH % scavenging activity of *Urtica urens L.*

IC_{50} value for DPPH percent scavenging activity were calculated after constructing the percent inhibition versus log extract concentration curve and IC_{50} for DPPH percent scavenging activity of *Urtica urens L.* extract was calculated as 0.201 mg/mL.

3.3.6.3. Lipid peroxidation inhibition capacity of Urtica urens L.

Thiobarbituric acid test was carried out for the evaluation of microsomal lipid peroxidation inhibition capacity of *Urtica urens L.* extract. 1 mg/mL sheep liver microsomes were used as the lipid source and iron (II) solution was used for induction of lipid peroxidation. The extracts were tested in 0.1 – 1 mg/mL concentrations. Effects of lipid peroxidation percent inhibition for *Urtica urens L* extract is shown in **Figure 3.25.**

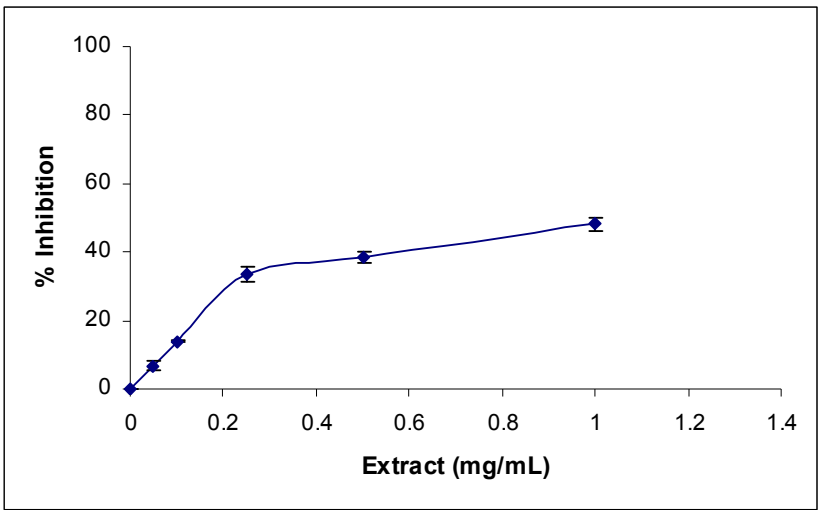


Figure 3.25. Microsomal lipid peroxidation percent inhibition capacity of *Urtica urens L.*

The extract concentration required to inhibit microsomal lipid peroxidation by 50 percent (IC_{50}) was calculated as 1.06 mg/mL for *Urtica urens L.*. extract.

3.3.6.4. Total phenolics content of Urtica urens L.

Total phenolics content in *Urtica urens L.* as measured after constructing a calibration curve with gallic acid were found to be 0.0542 ± 0.0027 mg in 1 mg of methanolic crude extract.

IC_{50} value of the *Urtica urens* L extract for DPPH scavenging is 0.201 mg/mL, while for the standard quercetin, 0.085 mg/mL, which is not a very effective free radical scavenger. IC_{50} value for lipid peroxidation inhibition is, 1.06mg/mL, in comparison to α-tocopherol, 0.23 mg/mL. Total phenolics content of *Urtica urens* L is lower than expected which was 0.054mg GAE.

The chemical composition of the aerial part of stinging nettle (*Urtica urens* L.) collected in Adigen region during flowering was investigated and caffeinic and chlorogenic acids and as 6-methoxy-7-hydroxycoumarin (scopoletin) were isolated (Kavtaradze, 2003)

3.3.7 Antioxidant effects of *Gundelia tournefortii* L. (*Asteraceae*)

Gundelia tournefortii L is a plant with thick flowering stem native to Asian-temperate zones of Western Asia such as Cyprus, Egypt, Iran; Iraq; Israel; Jordan Turkey, Azerbaijan and Turkmenistan. It is recorded that *Gundelia tournefortii* L. is used as gum and its f*lowers, leaves, root, seed and stem* are used as food sources (Ertuğ, 2000). In the Middle East, the young and still undeveloped flower buds is sold in the local markets. It is a sought after vegetable (Hedrick, 1972). Immature flowering heads were used like globe artichokes (Kunkel, 1984)

3.3.7.1 Extraction yield: 10 g dry samples from aerial parts of *Gundelia tournefortii* L. were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 630.00 ± 79.2 mg, that is 6.3% of its dry weight.

3.3.7.2. DPPH percent scavenging activity of Gundelia tournefortii L

DPPH percent scavenging activity of *Gundelia tournefortii* L. crude extract was measured in different concentrations ranging between 0.1 and 0.7 mg/mL. The results are given in graph in **Figure 3.26.**

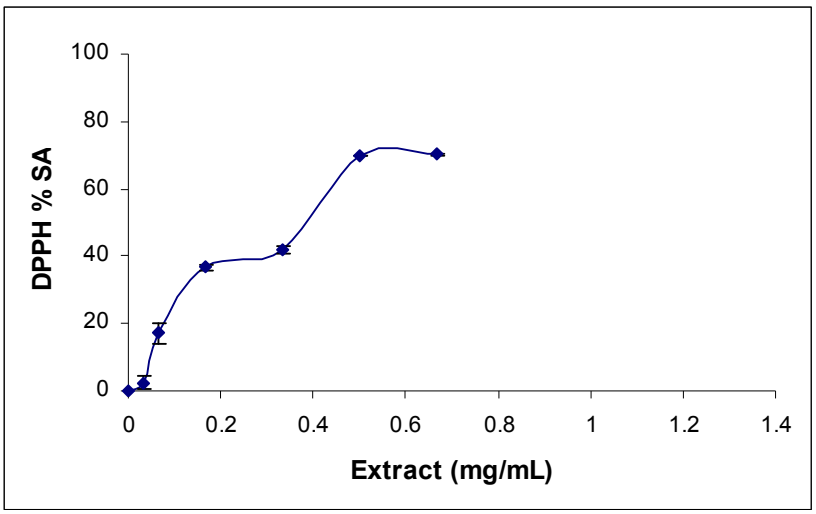


Figure 3.26. DPPH % scavenging activity of *Gundelia tournefortii* L.

IC_{50} value for DPPH percent scavenging activity were calculated from the percent inhibition versus log extract concentration curve and IC_{50} for DPPH percent scavenging activity of *Gundelia tournefortii* L. extract was found to be 0.293 mg/mL.

3.3.7.3. Lipid peroxidation inhibition capacity of Gundelia tournefortii L.

Lipid peroxidation inhibition capacity of *Gundelia tournefortii* L. extract was evaluated with thiobarbituric acid test using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations varying between 0.05 – 0.5 mg/mL. Lipid peroxidation percent inhibition capacity of *Gundelia tournefortii* L. extract is shown in **Figure 3.27.**

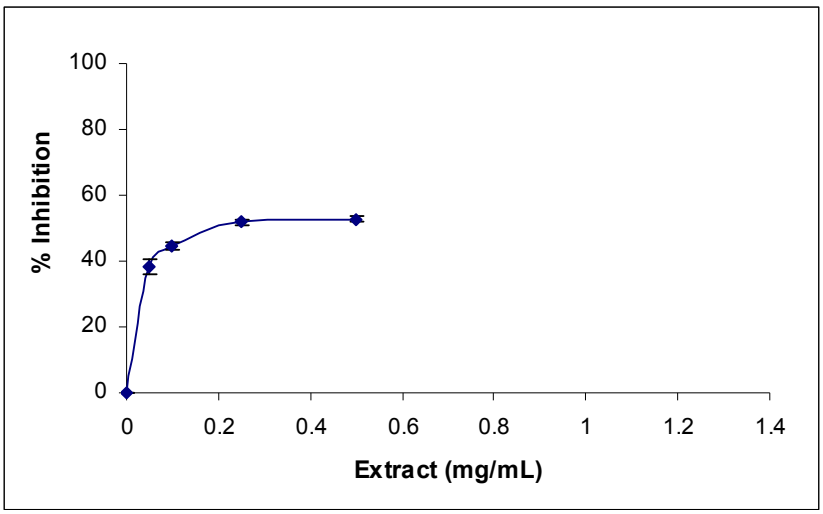


Figure 3.27. Microsomal lipid peroxidation percent inhibition capacity of *Gundelia tournefortii* L

The concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, IC_{50}, was calculated as 0.255 mg/mL for *Gundelia tournefortii* L. extract.

3.3.7.4. Total phenolics content of Gundelia tournefortii L.

The amount of total phenolic compounds found in *Gundelia tournefortii* L.crude extract was measured as 0.0644 ± 0.0048 mg in 1 mg of methanolic crude extract.

IC_{50} value for DPPH scavenging activity of *Gundelia tournefortii* L extracts was found to be 0.293mg/mL, which is 0.085 mg/mL for quercetin, explaining a lower activity in comparison to the standard. IC_{50} for lipid peroxidation inhibition activity was 0.255 mg/mL which is approximately similar to the value of the standard α-tocopherol, 0.23 mg/mL. When to total pheolics was considered with 6.44 % , it is reasonable in comparison to its antioxidant activity.

3.3.8. Antioxidant effects of *Aconitum cochleare* Woroschin (*Ranunculaceae*)

Aconitum is a genus of flowering plants belonging to the buttercup family Ranunculaceae. There are about 100 species, which are known as aconite, monkshood, or wolfsbane. *Aconitum cochleare* Woroschin has been recorded to be under a threat of extinction (Öztürk et.al, 2004)

3.3.8.1. Extraction yield: 10 g dried samples from aerial parts of *Aconitum cochleare* Woroschin were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 930.00 ± 76.4 mg and 9.3 % by dry weight which is significantly high compared to the extracts prepared from the shoot parts of the plants.

3.3.8.2. DPPH percent scavenging activity for Aconitum cochleare Woroschin

DPPH percent scavenging activity for *Aconitum cochleare* Woroschin crude extract was measured in different concentrations ranging between 0.001 and 0.5 mg/mL and the results are shown in the graph in **Figure 3.28.**

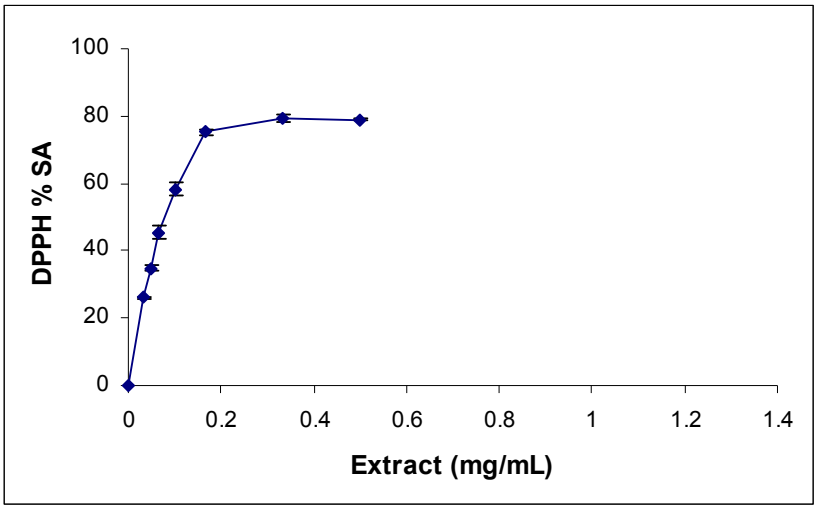


Figure 3.28. DPPH % scavenging activity of *Aconitum cochleare* Woroschin

IC_{50} value for DPPH percent scavenging activity *Aconitum cochleare* Woroschin crude extract were calculated as 0.076 mg/mL from the percent inhibition versus log extract concentration curve.

3.3.8.3. Lipid peroxidation inhibition capacity of Aconitum cochleare Woroschin

Lipid peroxidation inhibition capacity of *Aconitum cochleare* Woroschin extract was tetsed with thiobarbituric acid method using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations varying between 0.05 – 1 mg/mL and the results ara shown in **Figure 3.29.**

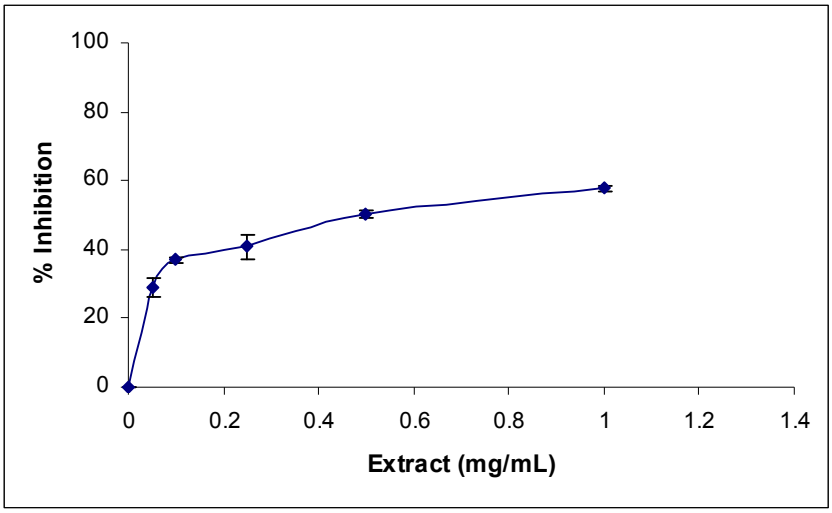


Figure 3.29. Microsomal lipid peroxidation percent inhibition capacity of *Aconitum cochleare* Woroschin

IC_{50} value for the *Aconitum cochleare* Woroschin methanol extract was calculated as 0.556 mg/mL.

3.3.8.4. T otal phenolics content of Aconitum cochleare Woroschin

The amount of total phenolic compounds in *Aconitum cochleare* Woroschin extract was found to be 0.0865 ± 0.0153 mg in 1 mg of methanolic crude extract.

IC_{50} value of the *Aconitum cochleare* Woroschin extract for DPPH scavenging is 0.076 mg/mL, while for the standard quercetin, 0.085 mg/mL, which can be regarded as a very effective antioxidant as a free radical scavenger.

IC_{50} value for lipid peroxidation inhibition is, 0.556 mg/mL, in comparison to α-tocopherol, 0.23 mg/mL, indicating relatively lower efficiency, but it has a considerable efficiency. Total phenolics content of *Urtica urens* L, 0.0865 mg GAE, is in accordance with its relatively moderate antioxidant activity.

3.3.9. Antioxidant effects of *Ferula rigidula* DC. (*Apiaceae*)

Ferula rigidula as one of the aromatic and flavouring plants, are added to make a special type of cheese in the Van region (Baser, 1997). The plant is also used as an aphrodisiac after its roots are thawed and mixed with honey in that region. Young stem is collected and used both for making cheese and added in many kind of meal. It was reported by the native people that the roots of the plant is used as a cure for infertility. It was also observed that the goats which eat the leaves of the plant are usually having twins. The plant is consumed after it is boiled and thus the bitter taste of the plant is ceased. Especially it is cooked with egg or prepared as pickle**.** Plants from the *Ferula* species have a long history of medicinal use and their hormonal effects are well documented in both the human and the veterinary practice (Singh, 1988).

3.3.9.1. Extraction yield: 10 g dried roots of *Ferula rigidula* were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 294.00 ± 32.5 mg, 29.4 % by dry weight.

3.3.9.2. DPPH percent scavenging activity of Ferula rigidula DC

DPPH percent scavenging activity of *Ferula rigidula* DC. crude extract was measured in concentrations ranging between 0.01 and 0.5 mg/mL. The results are given in graph in **Figure 3.30.**

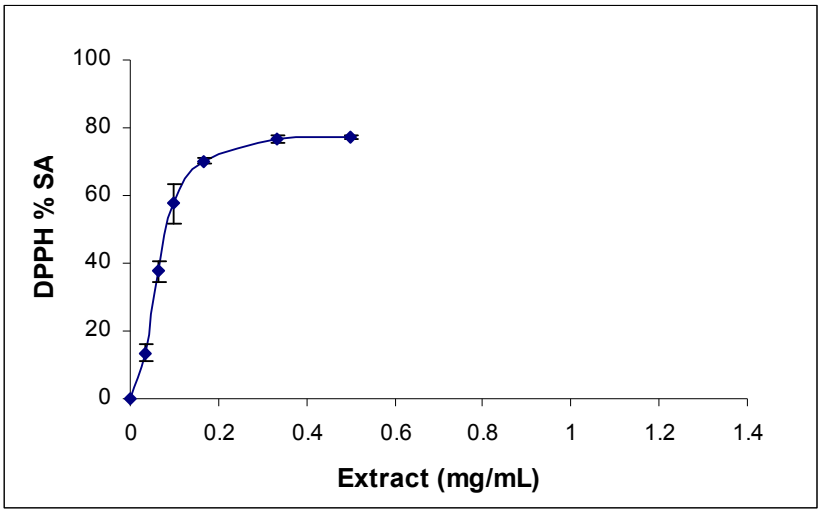


Figure 3.30. DPPH % scavenging activity of *Ferula rigidula* DC

IC_{50} values were calculated from the percent inhibition versus log extract concentration curve and IC_{50} for DPPH percent scavenging activity of *Ferula rigidula* DC extract was calculated as 0.0897 mg/mL.

3.3.9.3. Lipid peroxidation inhibition capacity of Ferula rigidula DC

Lipid peroxidation inhibition capacity of *Ferula rigidula* DC. extract was evaluated with thiobarbituric acid test using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations varying between 0.1 –1 mg/mL. Lipid peroxidation percent inhibition capacity of *Ferula rigidula* DC extract is shown in **Figure 3.31.**

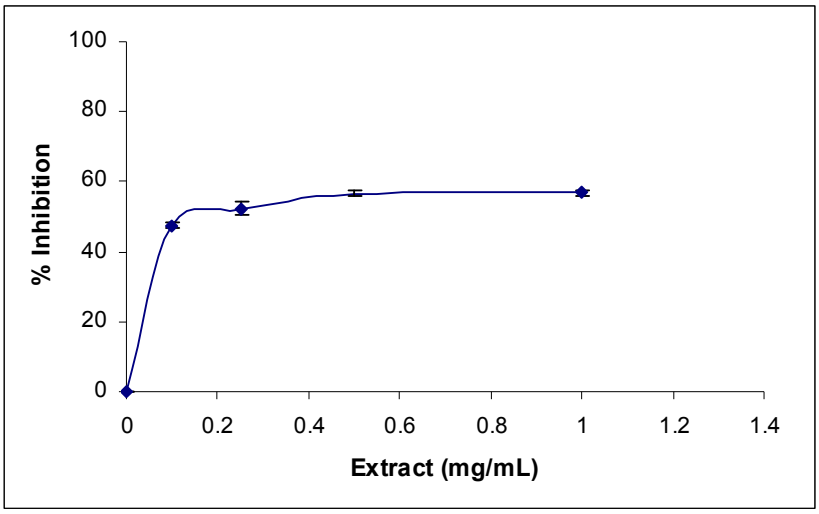


Figure 3.31. Microsomal lipid peroxidation percent inhibition capacity of *Ferula rigidula* DC

IC_{50} value, that is the concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, was calculated as 0.157 mg/mL for *Ferula rigidula* DC. extract.

3.3.9.4. Total phenolics content of Ferula rigidula DC.

Total phenolic comopunds content in *Ferula rigidula* DC were measured as 0.4170 ± 0.0762 mg in 1 mg of methanolic extract, by using gallic acid as standard for constructing a calibration curve.

IC_{50} value of the *Ferula rigidula* DC extract for DPPH scavenging is 0.0897 mg/mL, while for the standard quercetin, 0.085 mg/mL, which is as effective as the standard antioxidant in free radical scavenging. IC_{50} value for lipid peroxidation inhibition is, 0.157 mg/mL, in comparison to α-tocopherol, 0.23 mg/mL, indicating relatively higher efficiency. Total phenolics content of *Ferula rigidula* DC, 0.4170 mg GAE, is significantly high in spite of its moderate antioxidant activity.

Various biological activity in the ferula species is mainly attributable to ferutinin, an aromatic ester of a daucane alcohol with estrogenic activity and ferulic acid (Arnoldi, *et. al.,* 2004). One of the phenolic compounds ferulic acid, possess a variety of biological functions including antioxidative activities.In this study, high antioxidant activity and total phanolics content of *Ferula rigidula* could be attributed to its ferulic acid content.

3.3.10. Antioxidant effects of *Gundelia tournefortii* L. seeds

Dry seeds of *Gundelia tournefortii* L. are consumed as a type of coffee, which is known to be effective for the treatment of vitiligo disease, in Eastern Anatolia folk medicine. Fresh seeds are used in pickles and in fresh use, they are known to have diuretic effects.

3.3.10.1 Extract yield: 10 g of dry *Gundelia tournefortii* L. seeds were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 218.50 ± 61.5 mg and 2.18% by weight.

3.3.10.2. DPPH percent scavenging activity of Gundelia tournefortii L seeds

DPPH percent scavenging activity of *Gundelia tournefortii* L. seeds extract was measured in concentrations between 0.01 and 0.5 mg/mL. The results are shown in graph in **Figure 3.32.**

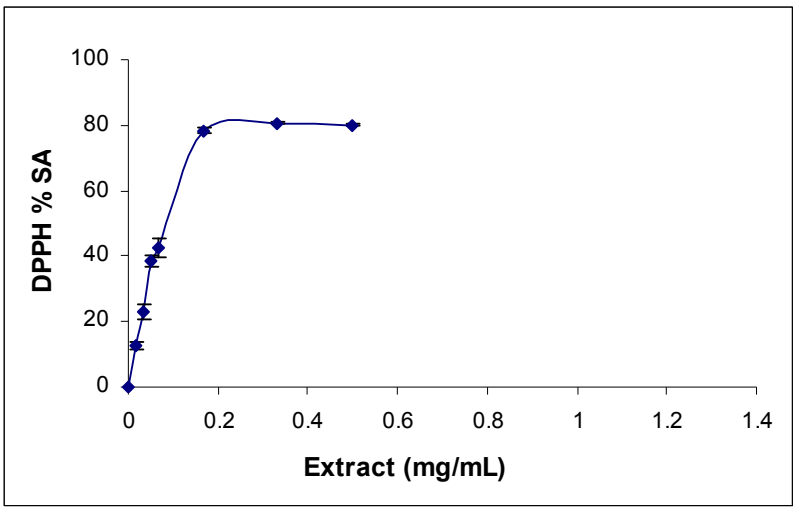


Figure 3.32. DPPH % scavenging activity of *Gundelia tournefortii* L.seeds

IC_{50} value for DPPH percent scavenging activity of *Gundelia tournefortii* L. seeds were calculated from the percent inhibition versus log extract concentration curve as 0.073 mg/mL.

3.3.10.3. Lipid peroxidation inhibition capacity of Gundelia tournefortii L. seeds

Microsomal lipid peroxidation inhibition capacity of *Gundelia tournefortii* L. seeds extract was measured with thiobarbituric acid test using 1 mg/mL sheep liver microsomes as lipid source. Extracts were tested in concentrations varying between 0.05 – 0.5 mg/mL. Effect of *Gundelia tournefortii* L. seeds extract on microsomal lipid peroxidation is shown in **Figure 3.33.**

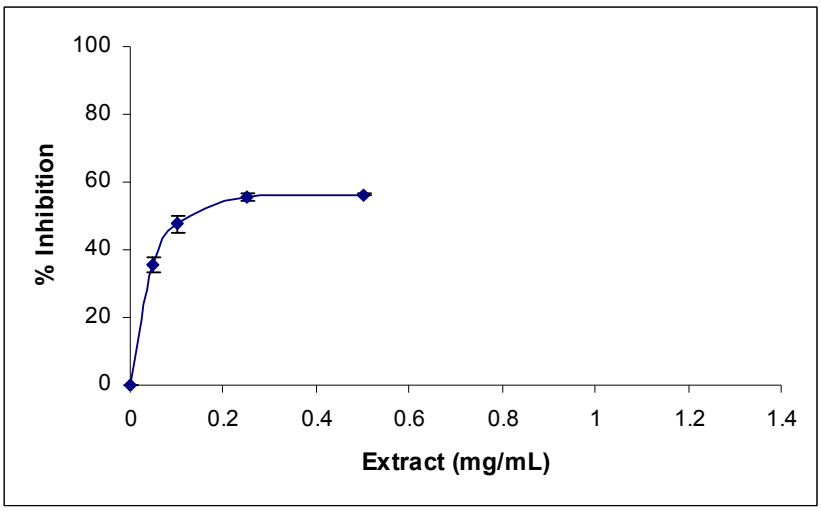


Figure 3.33. Microsomal lipid peroxidation percent inhibition capacity of *Gundelia tournefortii* L seeds

The concentration of *Gundelia tournefortii* L. seeds extract required to inhibit iron induced microsomal lipid peroxidation by 50 percent, was calculated as $IC_{50} =$ 0.146 mg/mL.

3.3.10.4. Total phenolics content of Gundelia tournefortii L. seeds

Total phenolics content found in *Gundelia tournefortii* L. seeds were calculated as 0.1051 ± 0.0087 mg in 1 mg of methanolic crude extract by using standard calibration curve constructed with gallic acid.

Gundelia tournefortii L. is found to be a very effective antioxidant in DPPH radical scavenging with the IC_{50} of 0.073 mg/mL in comparison to the standard quercetin, 0.085 mg/mL. Lipid peroxidation inhibition capacity is also very effective aith IC_{50} of 0.146, compared to that of α-tocopherol, 0.23 mg/mL. Its total phenolics content, with 0.1051 mg GAE can be considered as also quite high, being consistent with its antioxidant activity.

3.3.11 Antioxidant effects of *Rheum ribes* L. (*Polygonaceae*)

Rheum ribes L has been recorded to be under a threat of extinction (Öztürk et.al, 2004). It is native to Asian-temperate regions. It is reported to reduce blood glucose levels (Özbek *et. al.,* 2002). The roots consumed after boiling and mixing with honey. Having 21 different kind of species, the roots and the seedlings of *Rheum ribes* are consumed as a vegetable. The same parts of the plants are also used as a digestive and appetizer. The subterranean parts of the plants are used for the treatment of hemorrhoids and diabetes. It even has a role in treating jaundice as well as stomach and liver deseases (Özbek et.al. , 2004.)

3.3.11.1 Extraction yield: 10 g of *Rheum ribes* L. dried root samples were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 1346.45 ± 95.7 mg, 13.46 % by weight.

34.3.11.2. DPPH percent scavenging activity of Rheum ribes L.

DPPH percent scavenging activity of *Rheum ribes* L. extract was measured in different concentrations between 0.001 and 0.2 mg/mL and the results are shown in graph in **Figure 3.34.**

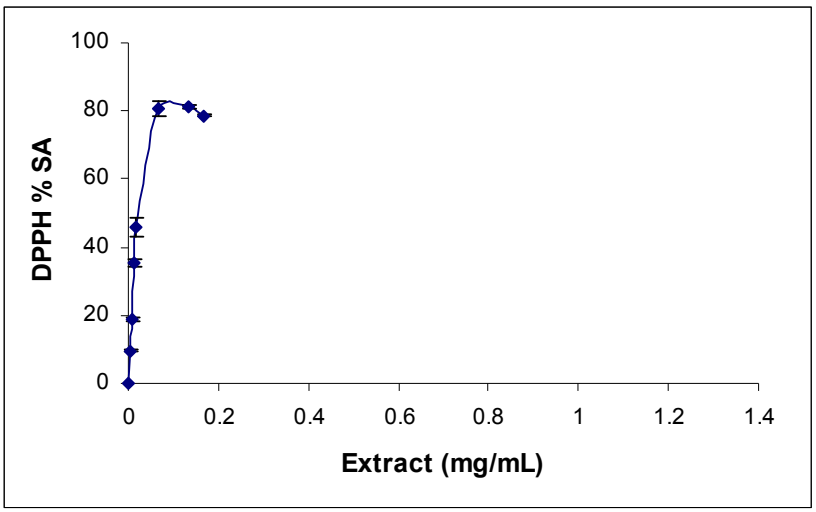


Figure 3.34. DPPH % scavenging activity of *Rheum ribes* L.

Percent inhibition versus log extract concentration curve was used for IC_{50} value for DPPH percent scavenging activity IC_{50} for *Rheum ribes* L. extract was found to 0.0205 mg/mL.

3.3.11.3. Lipid peroxidation inhibition capacity of Rheum ribes L.

Lipid peroxidation inhibition capacity of *Rheum ribes* L. extract was evaluated with thiobarbituric acid test using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations varying between 0.1 – 1 mg/mL and the results are shown below in **Figure 3.35.**

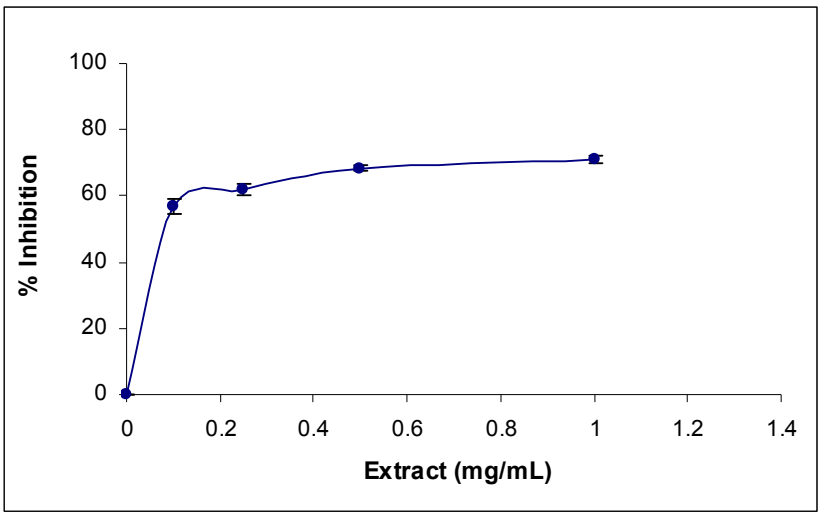


Figure 3.35. Microsomal lipid peroxidation percent inhibition capacity of *Rheum ribes* L.

The concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, IC_{50}, was calculated as 0.034 mg/mL for *Rheum ribes* L. curde methanol extract.

3.3.11.4. Total phenolics content of Rheum ribes L.

Total phenolics content in *Rheum ribes* L. crude extract was measures as 0.8026 ± 0.0725 mg in 1 mg of methanolic extract.

Rheum ribes L. is found to be a very effective antioxidant in DPPH radical scavenging with the IC_{50} of 0.02 mg/mL in comparison to the standard quercetin, 0.085 mg/mL. Lipid peroxidation inhibition capacity is also very effective aith IC_{50} of 0.034, compared to that of α-tocopherol, 0.23 mg/mL. Its total phenolics content, with 0.8 mg of phenolics per mg of extract, which is extremely high, being consistent with its very high antioxidant activity.

The roots of *Rheum ribe*s from Bingöl, reported to contain tannins (8%) and anthracene derivatives (0.025%). According to the results of the chemical study on material collected from Erzincan, chrysophanol, physcion, anthraquinone glycoside rhein, aloe-emodin, physcion-8-O-gluco-side, aloe-emodin-8-O-glucoside, sennoside A and rhaponticin were found in the subterranean parts of the plants (Baytop, 1999).

The roots of *Rheum ribes* is known as a very rich source for Vitamin C, which has an important contribution to the high antioxidant activity of *Rheum ribes extract* (Özbek et.al. , 2004.). It has shown that rhein dose-dependently inhibits superoxide anion production, chemotaxis and phagocytic activity of neutrophils, and macrophage migration and phagocytosis (Guo et al. 2002).

Rheum ribes was reported to show the most potent anti-herpes simplex virus activities and also active against Sindbis virus (Hudson et.al., 2000).

3.3.12 Antioxidant effects of *Rosa heckeliana* Tratt. (*Rosaceae*)

It is used for strengthening immune system, for flu, high fever, and infections. It is also reported to be efficient in many cases such as constipation, gall bladder, urinary bladder and kidney diseases, to remove weakness and tiredness, diabetes. To get rid of kidney stones, flu, burns, fever, gastric cramps, wound healing, rheumatism, haemmorhoids, cellulitis, cancer. The roots of *Rosa heckeliana* Tratt has nutritive and strengthening effects.

3.3.12.1 Extraction yield: 10 g of dried root samples from *Rosa heckeliana* Tratt. were extracted with 100 mL of methanol using a sample to solvent ratio of 1:10 for overnight at 50^0C. The amount of resulting crude extracts was 4934.50 ± 361.3 mg, 49.34% by dry weight, which is the highest percent among all the sample plants studied.

3.3.12.2. DPPH percent scavenging activity of Rosa heckeliana

DPPH percent scavenging activity of *Rosa heckeliana* crude methanol extract was measured in different concentrations ranging between 0.001 and 0.2 mg/mL. The results are given in graph in **Figure 3.36.**

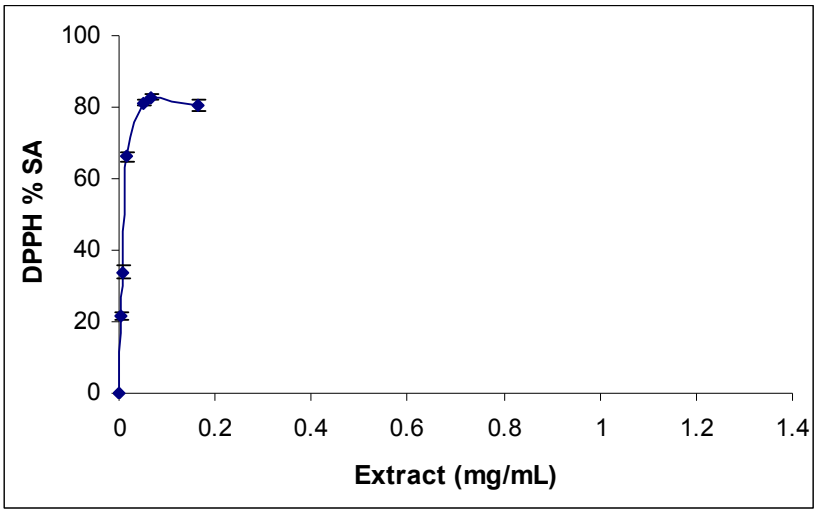


Figure 3.36. DPPH % scavenging activity of *Rosa heckeliana* Tratt.

IC_{50} was calculated from the percent inhibition versus log extract concentration curve for DPPH percent scavenging activity of *Rosa heckeliana* Tratt. extract as 0.0112 mg/mL.

3.3.12.3. Lipid peroxidation inhibition capacity of Rosa heckeliana Tratt

Lipid peroxidation inhibition capacity of *Rosa heckeliana* Tratt. extract was evaluated with thiobarbituric acid test, again using 1 mg/mL sheep liver microsomes as the lipid source and peroxidation was induced with iron (II). Extracts were tested in concentrations between 0.1 – 1 mg/mL and the results are given in **Figure 3.37.**

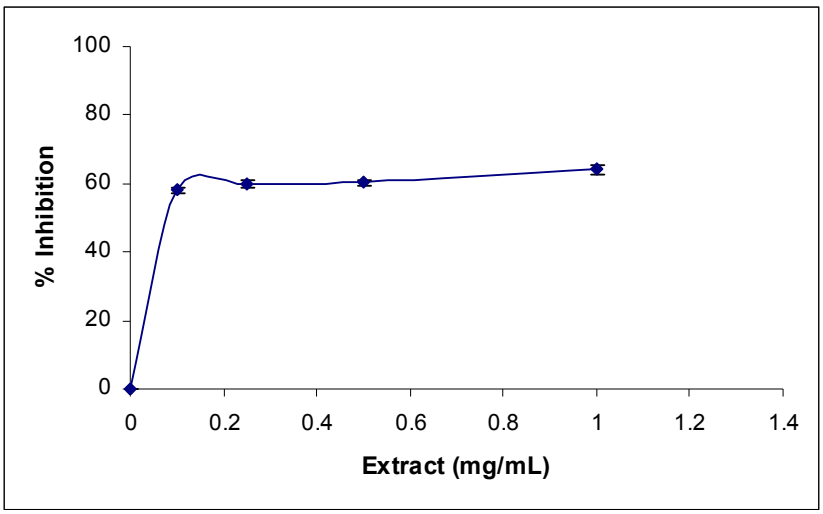


Figure 3.37. Microsomal lipid peroxidation percent inhibition capacity of *Rosa heckeliana* Tratt.

IC_{50}, that is the concentration of extract required to inhibit microsomal lipid peroxidation by 50 percent, was calculated as 0.0051mg/mL for *Rosa heckeliana* Tratt. extract.

3.3.12.4. Total phenolics content of Rosa heckeliana Tratt.

The amount of total phenolic compounds found in *Rosa heckeliana* Tratt.crude extract was measured as 0.7012 ± 0.0930 mg in 1 mg of methanolic crude extract. *Rosa heckeliana* Tratt. is found to be a very effective antioxidant in DPPH radical scavenging with the IC_{50} of 0.11 mg/mL in comparison to the standard quercetin, 0.085 mg/mL. Lipid peroxidation inhibition capacity is also very effective aith IC_{50} of 0.005 mg/mL compared to that of α-tocopherol, 0.23 mg/mL. Its total phenolics content, with 0.7 mg of phenolics per mg of extract, which is extremely high, being consistent with its very high antioxidant activity.

It highly contains Vitamin C, and other vitamins, A, B1, K, P, minerals, fruit acids, flavons, tannins and sugars .

When all the plant species are compared according to their IC_{50} values, as seen in **Table 3.4,** it is clear that all of the plant extracts have quite significant antioxidant activities.

Table 3.4. Comparison of antioxidant effects of studied plant extracts.

Plants	DPPH %SA IC_{50} (mg /mL)	LP % inhibition IC_{50} (mg/mL)	TP GAE (mg / mg of extract)
Allium vineale	0.79	1.097	0.0649 ± 0.0047
Chaerophyllum macropodum	0.623	0.852	0.0340 ± 0.0070
Heracleum persicum	0.438	1.586	0.0596 ± 0.0028
Prangos ferulacea	0.242	0.152	0.0651 ± 0.0064
Papaver bracteatum	0.442	0.678	0.2117 ± 0.0627
Urtica urens	0.201	1.06	0.0542 ± 0.0027
Gundelia tournefortii	0.293	0.255	0.0644 ± 0.0048
Aconitum cochleare	0.076	0.556	0.0865 ± 0.0153
Ferula rigidula	0.0897	0.157	0.4170 ± 0.0762
Gundelia tournefortii **seeds**	0.0727	0.146	0.1051 ± 0.0087
Rheum ribes	0.0205	0.034	0.8026 ± 0.0725
Rosa heckeliana	0.0112	0.0051	0.7012 ± 0.0930
Aesculus hippocastanum			
Bark	0.014	0.025	0.0607 ± 0.0131
Seeds	0.082	0.51	0.0207 ± 0.0042
Leaves	0.17	0.19	0.0747 ± 0.0167
Flowers	0.60	1.58	0.045 ± 0.008
Quercetin	0.085	ND	NA
Alpha-tocopherol	ND	0.23	NA

IC_{50} concentrations of extracts for DPPH % SA: DPPH Radical per cent scavenging activity; LP % Inhibition: Lipid peroxidation per cent inhibition activity and TP: Total phenolics content expressed as mg of Gallic Acid Equivalents; ND: Not determined; NA: Not applicable

Especially the extracts prepared from the root parts of the plants such as *Rheum ribes* L., *Ferula rigidula* DC., *Rosa heckeliana* Tratt. have very high activities compared to the rest of the studied samples which were from shoot parts of the plants. All three plants extracts have shown very high antioxidant activity both in inhibition of lipid peroxidation and radical scavenging assays. All three plant extracts are better antioxidants than the standard α-tocopherol as observed in lipid peroxidation inhibition activity.

Rosa heckeliana Tratt. has shown the highest lipid peroxidation inhibition activity among all the studied plant extracts. When IC_{50} values are compared, *Rosa heckeliana* Tratt. extracts are approximately 45 times more efficient than the standard α-tocopherol and 200 times more efficient than most of the plant extracts studied. Therefore, *Rosa heckeliana* Tratt extracts are considered as the most potential antioxidant extract by means of lipid peroxidation inhibition and DPPH radical scavenging activities.

Rheum ribes L. and *Rosa heckeliana* Tratt. extracts are even better antioxidants than the standard quercetin as radical scavengers while *Ferula rigidula* DC extracts seems to have quite similar efficiency as the standard.

When the total phenolics content of the root extracts are considered, all three of them have very high phenolics content, among all the plants studied. This might explain why *Rheum ribes* L., *Rosa heckeliana* Tratt and *Ferula rigidula* DC extracts have the highest antioxidant activity.

Gundelia tournefortii L. seeds and *Aconitum cochleare* Woroschin extracts were also as good as the standard quercetin for their radical scavenging capacities. The rest of the plant extracts still have considerable antioxidant activity by means of radical scavenging activity when compared to the standards.

When total phenolics contents of the extracts from aerial parts of the plants are considered, *Papaver bracteatum* Lindl. extracts are found to be rich in total phenolics content in spite of its low antioxidant activity.

Bark extract of *Aesculus hippocastanum L*. is still very efficient when compared among all the plant extracts studied. However, *Rosa heckeliana* is more active as a free radical scavenger (IC_{50}= 0.0112 mg/mL) than the bark extract of *Aesculus hippocastanum L*. with IC_{50}= 0.014 mg/mL.

Rosa heckeliana extract is the most potential antioxidant as lipid peroxidation inhibitor (IC_{50}= 0.0051 mg/mL), although bark extract of *Aesculus hippocastanum L*. is also close in efficiency (IC_{50}= 0.025 mg/mL) and followed by *Rheum ribes* extract (IC_{50}= 0.034mg/mL).

In comparison to the plant extracts from East Anatolian Region, bark extract of *Aesculus hippocastanum L*. can be considered as very efficient antioxidant since the total phenolics content of the bark extract is much lower than *Rosa heckeliana* extract and *Rheum ribes* extract.

3.4 Sheep Liver Cytosolic GST activity toward CDNB

Sheep liver cytosolic fractions prepared as given in "Materials and Methods" section, were used as the enzyme source. 0.2 M potassium phosphate buffer and final 1 mM GSH was used throughout the experiments. Enzyme activities were carried out at 25^{o}C. 21mg/mL sheep liver microsomes were used after 1/100 dilution with 10 mM potassium phosphate, buffer, pH 6.0. Concentrations ranging between 0.2 – 2 mM CDNB was used as substrate

The reactions were started by the addition of the enzyme. Incubation mixtures without the enzyme source were used as blanks (non-enzymatic reactions). Enzyme activities with 10 µl of DMSO were regarded as control. The enzyme activities were expressed in terms of percent specific activity. One unit of GSTs

activity is defined as the amount of enzyme producing µmole thioether formed per minute. The kinetic constants Km and Vmax were determined for GST activity after Lineweaver-Burk Plots were constructed. The enzyme activities were expressed in terms of percent specific activity. The extinction coefficient for CDNB conjugate at 340 nm is 0.0096 $\mu M^{-1}cm^{-1}$ (Habig *et. al,* 1974)

3.4.1 Effects of sheep liver cytosolic protein concentration on GST activities towards CDNB

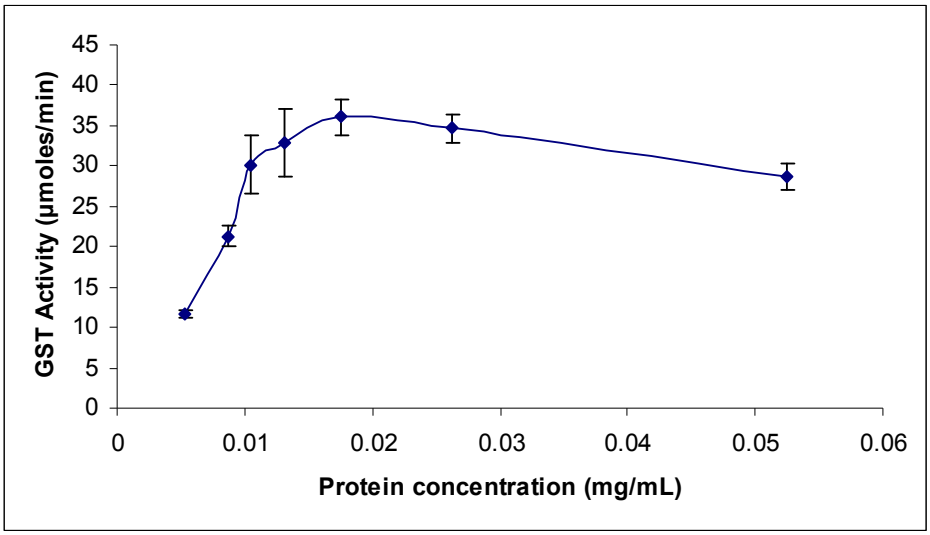


Figure 3.38. Effects of sheep liver cytosolic protein concentration on GST activity toward CDNB.

Effect of protein concentration on GST enzyme activity was evaluated by measuring the activity of the enzyme at 1/30, 1/50, 1/100, 1/150, 1/200, 1/300 dilutions from 21 mg/mL sheep liver cytosol. 50µL of diluted cytosolic suspension 1/100, final 0.0105 mg/mL protein concentration was used to carry out the experiments (**Figure 3.38**).

3.4.2 Effect of pH on GST Activity

Effect of pH on GST enzyme activity was evaluated by measuring the activity of the enzyme at varying pH values between pH 6 and pH 8.5 regarding to the literature about optimum GST activity (Farzad, 1994), using 0.2 M potassium phosphate buffer. During the reaction, 1 mM CDNB was used as the substrate and 1 mM GSH was used as co-substrate. Optimum pH for cytosolic GST activity is around 7.5 as shown in **Figure 3.39** and this pH is used for the rest of the GST activity studies.

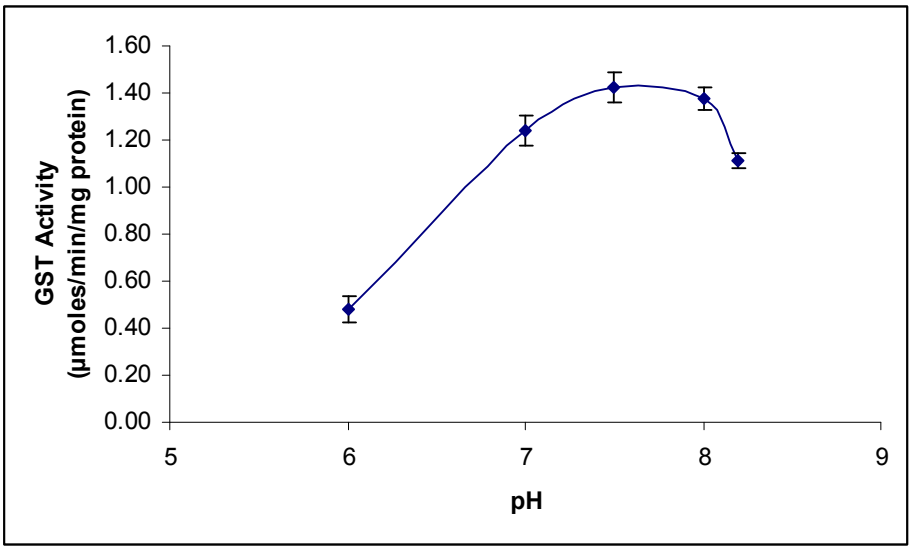


Figure 3.39. Effects of pH on sheep liver GSTs activity toward CDNB.

3.4.3 Effect of DMSO as solvent for the plant extracts on GST Activity

DMSO due to its water-miscible nature, and its efficiency to solubilize dense plant extracts, was used as a solvent for dissolving the dried plant extracts during the GST activity studies. Therefore, the effect of different concentrations of DMSO on GST activity was evaluated, as shown in **Figure 3.40**.

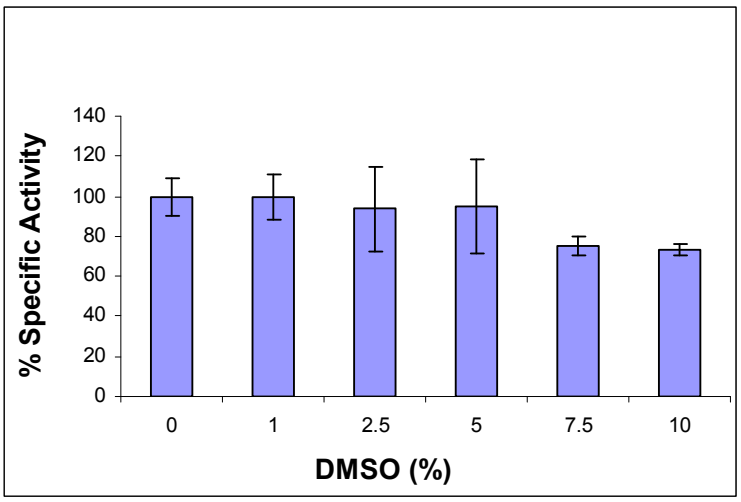


Figure 3.40. Effects of DMSO on sheep liver GSTs activity toward CDNB.

DMSO has shown an inhibitory effect on GST activity when it is used higher than 5% in the reaction solution. Therefore, 1% DMSO was used for the rest of the experiments.

3.4.4 Inhibitory Effects of Plant Extracts on GST Activity

Beyond dispute, cancer is the most studied and still investigated disease of the century, without a question many phytochemicals, antioxidants have been investigated for their potential usefulness as cancer chemopreventive agents by researchers. Unfortunately, development of drug resistance is a serious impediment to many chemotherapeutic treatments which requires more attantion. The role of GST enzymes in multidrug resistance is known therefore this stimulates the search for GST inhibitors (Armstrong, 1997; Zanden *et. al,* 2004).

Regarding to the importance of the role of GST enzymes in multidrug resistance, selected plant extracts were screened for their effects on GST activity besides their antioxidant efficiencies. % Inhibition of GST enzyme has been evaluated in the presence of extracts concentration in the range of 20 – 150 µg/mL.

GST enzyme inhibition studies have been carried out in 0.2 M potassium phosphate buffer and final concentration of 1 mM GSH, at 25°C. Combination buffer and the substrate solutions were prepared fresh (**Appendix B)**. Enzyme activities were carried out with 0.0105 mg/mL sheep liver microsomes. Concentrations ranging between 0.2 – 2 mM CDNB was used as substrate The reactions were started by the addition of the enzyme. Incubation mixtures without the enzyme source were used as blanks (non-enzymatic reactions). Enzyme activities with 10 µL of DMSO were regarded as control. The enzyme activities were expressed in terms of percent specific activity. One unit of GSTs activity is defined as the amount of enzyme producing µmole thioether formed per minute.

GST % inhibition grahics for Eastern Anatolian plant extracts have been displayed in groups of four, by considering their proximity in % inhibition values. First group of extracts are *Rosa heckeliana, Papaver bracteatum, Prangos ferulaceae* and *Rheum ribes* and their % inhibition graphs are shown in **Figure 3.41.**

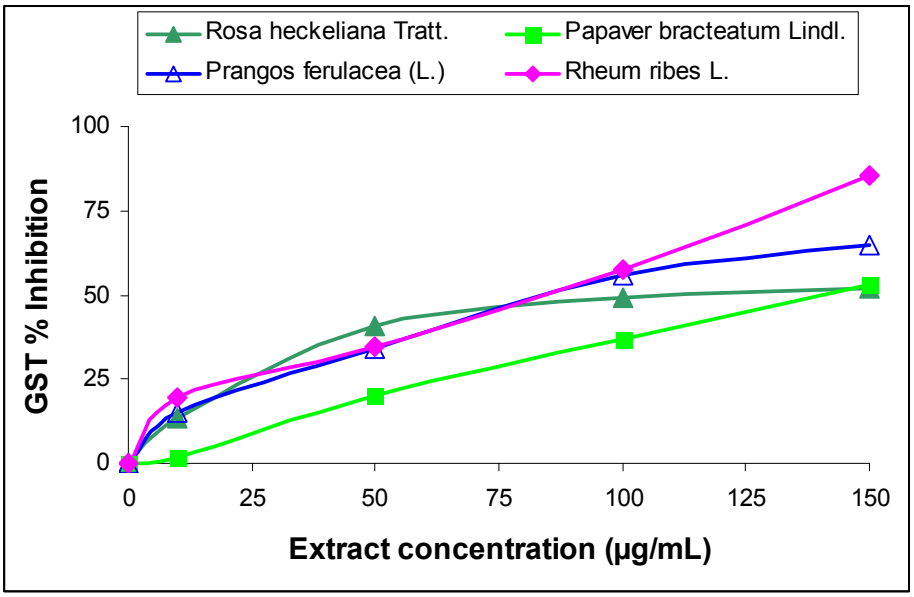


Figure 3.41. GST % inhibition plot for *Rosa heckeliana, Papaver bracteatum, Prangos ferulaceae* and *Rheum ribes.*

As shown in **Figure 3.41,** *Rosa heckeliana* extract have exerted a better inhibitory effect in the presence of 50 µg/mL extract.

Second group of extracts were chosen as *Ferula rigidula*, *Gundelia tournefortii, Urtica urens* and *Heracleum persicum* for GST % inhibition plotted as shown in **Figure 3.42.**

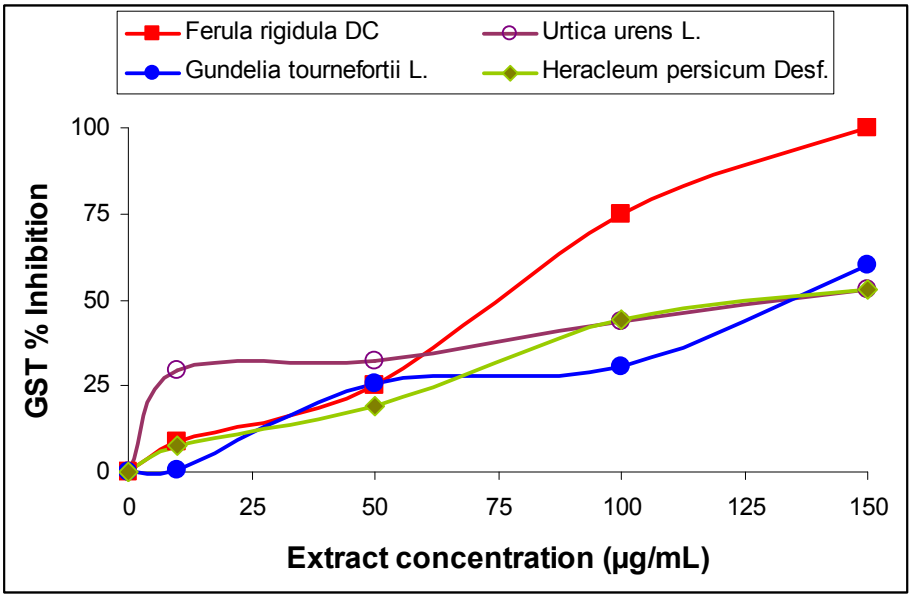


Figure 3.42. GST % inhibition plot for *Ferula rigidula*, *Gundelia tournefortii, Urtica urens* and *Heracleum persicum*

In the group of plants given in **Figure 3.42,** *Ferula rigidula* has exhibited the highest percent inhibition on GST activity.

Last group of plants, namely *Gundelia tournefortii* seeds, *Allium vineale*, *Chaerophyllum macropodum* and *Aconitum cochleare* were collected to display the percent inhibiton effects on GST activity as given in **Figure 3.43.**

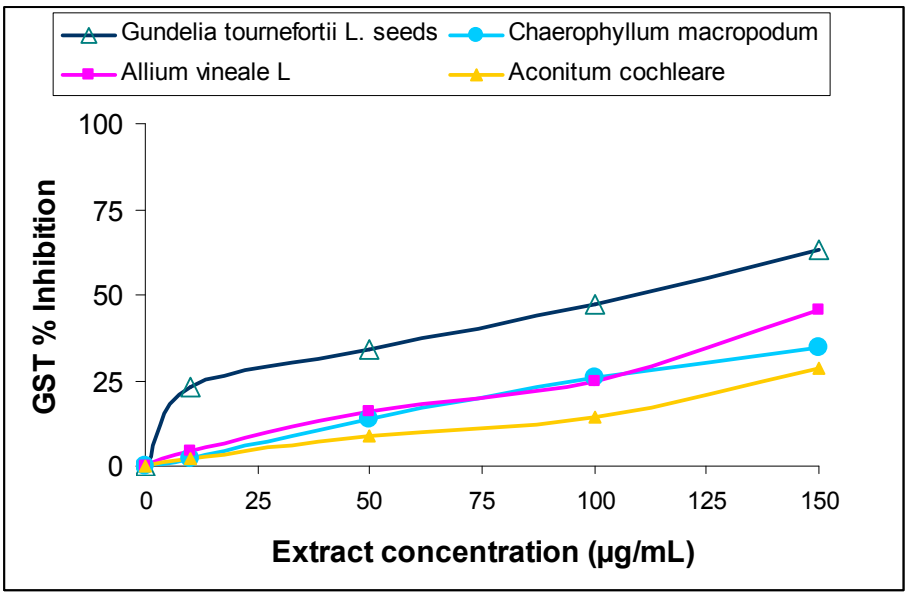


Figure 3.43 GST % inhibition plot for *Gundelia tournefortii* seeds, *Allium vineale*, *Chaerophyllum macropodum* and *Aconitum cochleare*

Among the third group of plants *Gundelia tournefortii* seeds have exhibited the highest inhibiton on GST activity.

Specific activitiy percent versus log [extract] plots were constructed to determine the concentration of extract necessary to inhibit GST activity by 50 % (IC_{50}). The lower the IC_{50} value, the more efficient the extract should be as an inhibitor. All the extracts were applied within 0.01-0.15 mg/mL final concentrations. At least 4 different concentrations of extracts were used and each data were obtained in triplicates with standard deviations less than 10%. IC_{50} values of extracts for GST activity are given in **Table 3.5.**

Table 3.5. Effects of plant extracts from East Anatolian Region on cytosolic total GST acitivity expressed as IC_{50} values.

Plants	IC_{50} (µg/mL)
Aconitum cochleare	4198.69
Allium vineale	2464.38
Chaerophyllum macropodum	350.70
Papaver bracteatum	179.95
Urtica urens	169.71
Gundelia tournefortii	155.21
Heracleum persicum	130.28
Rosa heckeliana	111.81
Gundelia tournefortii seeds	97.51
Rheum ribes	85.30
Prangos ferulacea	79.25
Ferula rigidula	48.95

When all the plant species are compared according to their IC_{50} values, as given in **Table 3.5**, *Ferula rigidula* DC. is the most effective inhibitor of cytosolic GST activity, with an IC_{50} value of 48.95 µg/mL. *Prangos ferulacea* (L.) Lindl., *Rheum ribes* L. and *Gundelia tournefortii* L. are also relatively effective inhibitors for GST activity. *Allium vineale* L. and *Aconitum cochleare* Woroschin are the least effective inhibitors with their high IC_{50} values of 2464.38 µg/mL and 4198.69 µg/mL respectively. All the other plant extracts can be considered as moderately good inhibitors of cytosolic GST activity toward CDNB.

The inhibitory effects of naturally occurring plant polyphenols such as tannic acid, ellagic acid, ferrulic acid, caffeic acid, silybin, quercetin, curcumin and chlorogenic acid against GST have been reported by researchers (Gyamfi e*t. al.,* 2004). For instance, thonningianin A, as a novel antioxidant isolated from the medicinal herb, *Thonningia sanguinea* on uncharacterized rat liver GST and human GST P1-Th was reported as a potent inhibitor of the conjugation of CDNB by crude cytosolic GSTs with IC_{50} of 1.1 µM (Gyamfi e*t. al.,* 2004).

3.4.5. GST Enzyme Kinetics

Kinetic properties of cytosolic GSTs were determined through measuring the initial rates of the enzyme at different CDNB substrate concentrations. Maximum rate of the enzyme (Vmax) and the Michaelis constant (Km) were determined from the Lineweaver-Burk plots, which gives more accurate results since the reciprocals of each values are used while plotting the graph.

The reactions were started by the addition of the enzyme. Incubation mixtures without the enzyme source were used as blanks (non-enzymatic reactions). Enzyme activities with 10 µl of DMSO were regarded as control. The enzyme activities were expressed in terms of percent specific activity. One unit of GSTs activity is defined as the amount of enzyme producing µmole thioether formed per minute. The extinction coefficient for CDNB conjugate at 340 nm is 0.0096 $\mu M^{-1}cm^{-1}$ (Habig *et. al,* 1974)

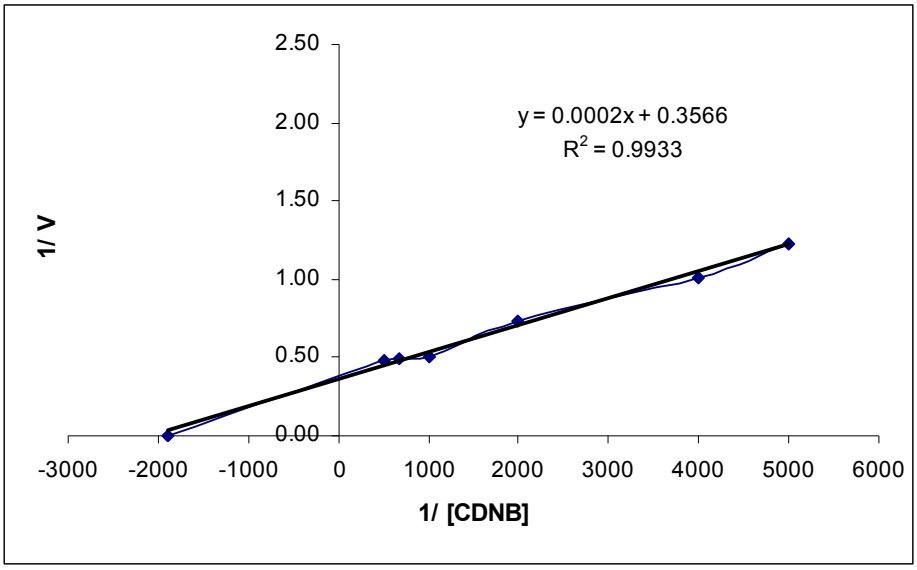


Figure 3.44. Lineweaver-Burk Plot of GST Activity toward CDNB. The enzyme activities are expressed as µmole/min/mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

Lineweaver-Burk Plot yields a straight line with an intercept of 1/Vmax on the y-axis, (1/V) and -1/Km can be derived from the x-axis (1/[CDNB]). The slope of the line gives Km/Vmax value. Vmax and Km values are determined as **2.8 µmoles/min/mg protein** and **$5.6x10^{-4}$** M, respectively (**Figure 3.44**).

In the literature, Vmax and Km values for sheep liver cytosolic GST activity towards CDNB were found to be 4.3 U/mg and $10.6x10^{-4}$ M, respectively (Alpturk, 2000).

3.4.6. Effect of Plant Extracts on GST Enzyme Kinetics

Selected extract samples were further studied for their effects on GST enzyme kinetics. Variations in the kinetic behaviour of an enzyme in the presence of an inhibitor, implies the type of inhibitor binding mode. Therefore the most efficient inhibitors and the least effective ones were considered for their effects on kinetic parameters, Vmax and Km for GST Activity.

3.3.6.1. Effect of Prangos ferulacea L. on GST Activity

In many studies in the literature, various biological activities in the ferula species were mainly found attributable to ferutinin, an aromatic ester of a daucane alcohol and estrogenic activity to ferulic acid (Arnoldi, *et. al.,* 2004) . Also ferulic acid among other phytochemicals, were reported to have varied effects on the enzyme activities such as cytochrome P450 (Kawabata, et. al, 2000).

IC_{50} for GST Inhibition of *Prangos ferulacea L* extract was found to be 79.25 µg/mL.

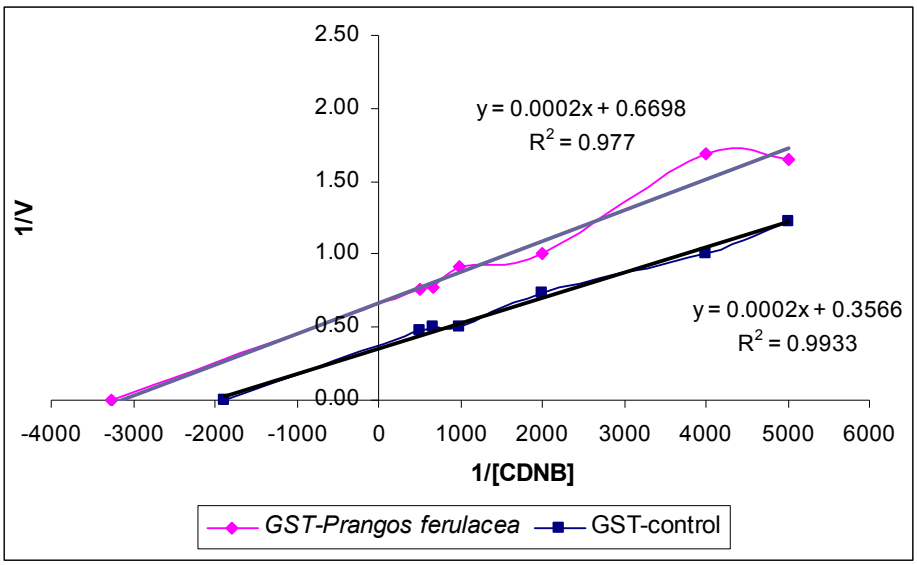


Figure 3.45. Lineweaver-Burk Plot of GST Activity toward CDNB in the presence of 50 µg/mL *Prangos ferulacea* extract. The enzyme activities are expressed as µmole/min/mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

Effcet of *Prangos ferulacea* seed extract on cytosolic GST activity was determined by the kinetic study carried out in the absence and presence of the extract. As given in **Figure 3.45,** Vmax for GST activity was measured as 1.49 µm/min/mg protein and Km as 2.98×10^{-4} in the presence of 50 µg/mL *Prangos ferulacea seed* extract.

In the **Figure 3.45,** double reciprocal plot. Vmax and Km both decrease, the ratio of Vmax /Km remains almost unchainged indicating an uncompetitive inhibition. This type of inhibiton occurs only with multisubstrate reactions as it is in GST enzyme. Uncompetitive inhibitors bind only to enzyme-substrate complex, not to free enzyme. In uncompetitive inhibition, Vmax is decreased and it is not reversed by the addition of more substrate.

A single concentration of 50 µg/mL *Prangos ferulacea seed* extract was used for all the kinetic studies and at this case, the concentration was very close to the

value of 50% inhibitory concentration (IC_{50}) for *Prangos ferulacea* extract, which was 79.25 µg/mL.

The inhibitory effects of naturally occurring plant polyphenols such as tannic acid, ellagic acid, ferrulic acid, caffeic acid, silybin, quercetin, curcumin and chlorogenic acid against GST have long been reported by many researchers (Gyamfi e*t. al.,* 2004).

3.3.6.2. Effect of Papaver bracteatum Lindl. on GST Activity

GST Inhibition IC_{50} = 179.95 µg/mL

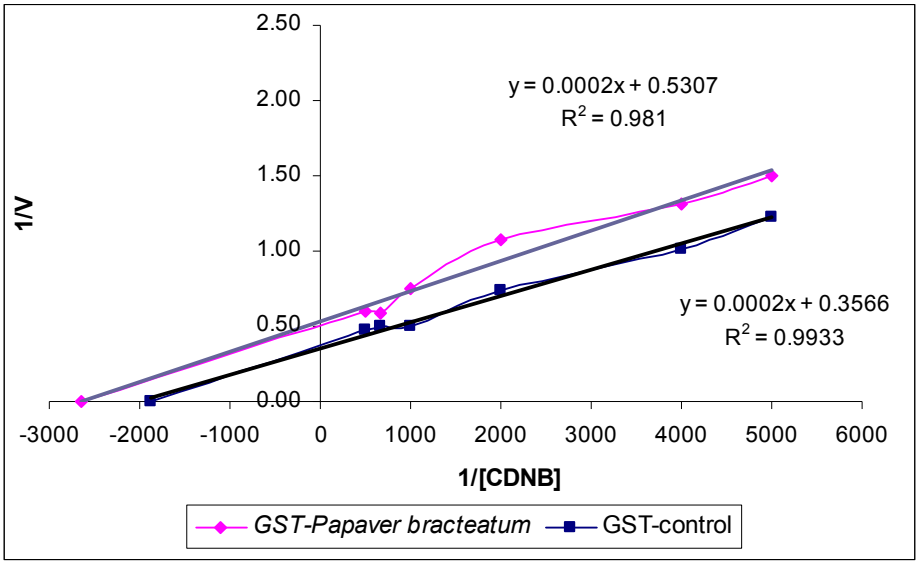


Figure 3.46. Lineweaver-Burk Plot of GST Activity toward CDNB in the presence of 50 µg/mL *Papaver bracteatum* extract. The enzyme activities are expressed as µmole/min/mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

Effcet of *Papaver bracteatum* extract on cytosolic GST activity was determined by the kinetic study carried out in the absence and presence of 50 µg/mL extract. As given in **Figure 3.46,** in the control graph, Vmax is calculated as

1.88 µmoles/min/mg protein and Km as $3.8x10^{-4}$ in the presence of 50 µg/mL *Papaver bracteatum* extract.

Single concentration of 50 µg/mL *Papaver bracteatum* extract was used for the kinetic studies, even though it is lower than 50% GST inhibitory concentration (IC_{50}) value of *Papaver bracteatum* extract, (179.95µg/mL), a proportional decrease in the Vmax and Km are still observable. However, the slope seems to be unchanged. This behaviour is describing an uncompetitive inhibition, which occurs only with multisubstrate reactions.

3.3.6.3. Effect of Aconitum cochleare Woroschin. on GST Activity

GST Inhibition IC_{50} = 4198.69 µg/mL

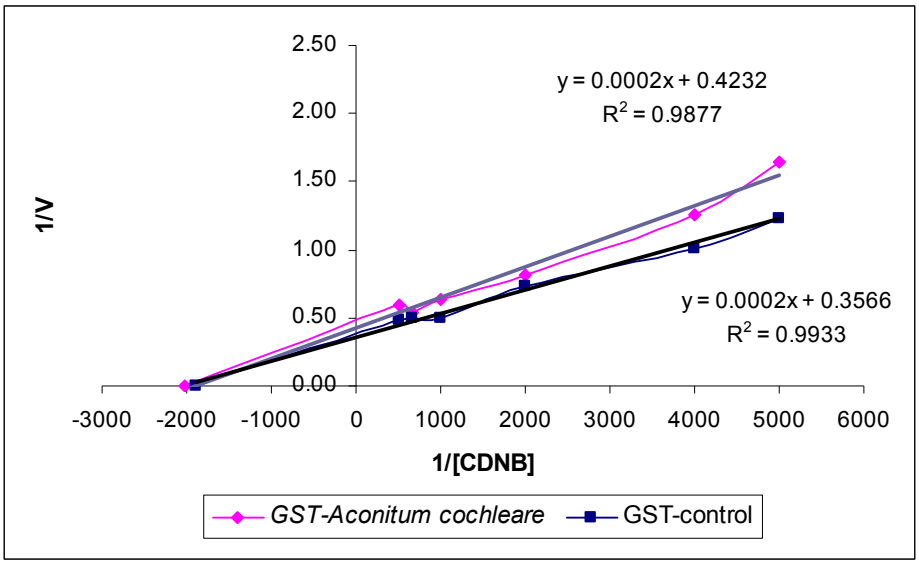


Figure 3.47. Lineweaver-Burk Plot of GST Activity toward CDNB in the presence of 50 µg/mL *Aconitum cochleare* extract. The enzyme activities are expressed as µmole/min/mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

When the activity of GST enzyme is measured in the presence of *Aconitum cochleare* extract, it is observed that even at low concentrations of inhibitor, compared to the IC_{50}= 4198.69 µg/mL, there is a decrease in the Vmax, since Vmax=2.36 µmoles/min/mg protein and the Km=4.72 $x10^{-4}$ seems to be constant.

The kinetic sheme in the **Figure 3.47** illustrates a non-competitive inhibition. When these inhibitors which do not structurally resemble to substrates bind to E and Es with equal affinity, the result is termed pure non-competitive inhibiton. This inhibition is characterized by a decrease in Vmax with no change in Km. On adouble reciprocal plot, the lines for pure non-competitive inhibition intersect at a point on the 1/S axis Pure non competitive inhibition is rare, but examples are known among alloseric enzymes. In these cases, the non-competitive inhibitor probabily alters the conformation of the enzyme to a shape that can still bind S, but can not catalyze any reaction.

3.3.6.4. *Effect of Ferula rigidula* DC *on GST Activity*

GST Inhibition IC_{50} = 48.95 µg/mL

One of the mechanisms of such chemopreventive action of ferulic acid on colon carcinogenesis is the suppression of metabolic activation and enhancement of detoxification.The phytochemicals, including ferulic acid, are reported to have varied effects on the enzyme activity of isoforms of cytochrome P450. Exposure of ferulic acid significantly increases the activities of detoxification enzymes GST. (Kawabata, 2000)

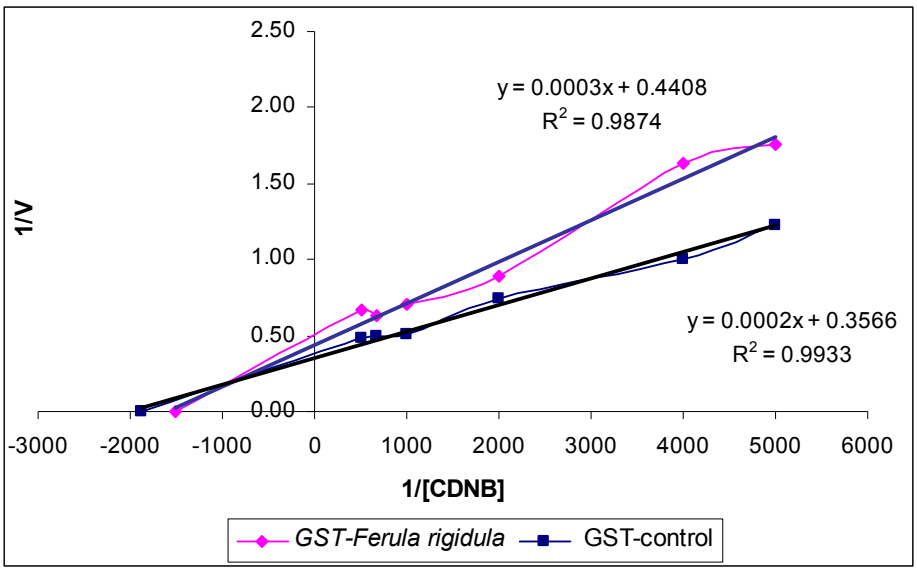


Figure 3.48. Lineweaver-Burk Plot of GST Activity toward CDNB in the presence of 50 µg/mL *Ferula rigidula* extract. Enzyme activities are expressed as µmole/min /mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

In the presence of 50 µg/mL *Ferula rigidula* extract, the kinetic parameters have changed with a decrease in Vmax to 2.27 micromoles/min/mg protein and increase in Km to 6.8×10^{-4} M for GST activity. As indicated before, IC_{50} value for *Ferula rigidula* extract was found to be 48.95 µg/mL for GST inhibition. Therefore, we can conclude that the concentration of *Ferula rigidula* extract used for the inhibiton kinetics experiments was sufficient.

The double reciprocal plot in **Figure 3.48** shows a in decrease Vmax and the lines intersect to the left of the y-axis, indicating an increase in Km which is observed when inhibitor has greater affinity for E than ES complex , Km increases. The result can be interpreted as mixed non-competitive inhibiton in the presence of *Ferula rigidula* extract

3.3.6.5. *Effect of Rheum ribes L. on GST Activity*

GST Inhibition IC_{50} = 85.30 µg/mL

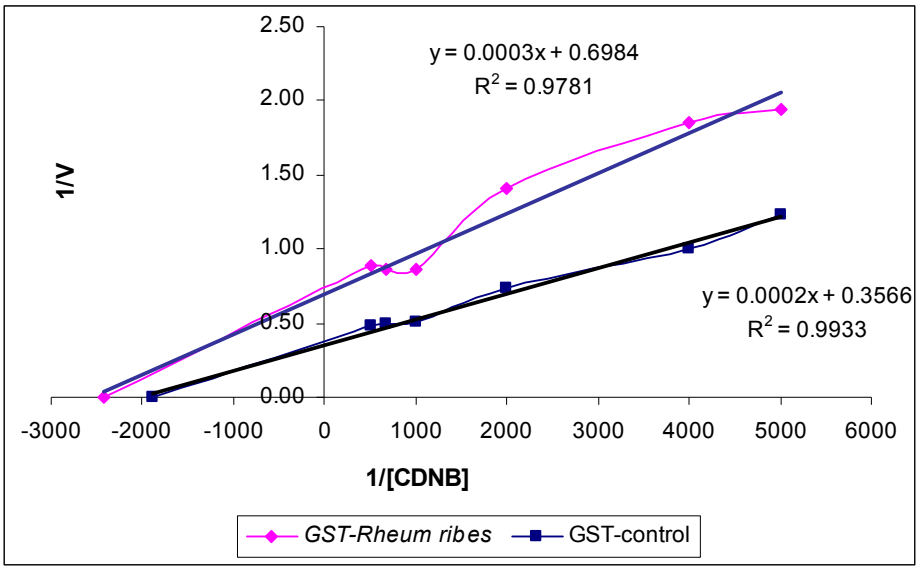


Figure 3.49. Lineweaver-Burk Plot of GST Activity toward CDNB in the presence of 50 µg/mL *Rheum ribes* L. extract. The enzyme activities are expressed as µmole/min/mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

50 µg/mL *Rheum ribes* L. extract effects the kinetic values for GST activity so that Vmax becomes 1.43 µmol/min/mg protein and Km= $4.3x10^{-4}$ M. From the double reciprocal plot, there is a decrease in Vax and decrease in Km. When inhibitor has greater affinity for the ES complex than E, Km decreases. Since IC_{50}= 85.30 µg/mL, concentration used for the inhibiton studies is sufficient to conclude that *Rheum ribes* L. extract shows a mixed type non-competitive inhibiton on GST activity. In this case, the result is termed mixed non-competitive inhibiton (**Figure 3.49**).

Compounds found in the *Rheum ribes* L. extract may binding at separate sites, but may bind to either enzyme to or the enzyme –substrate complex.

3.3.6.6. *Effect of Rosa heckeliana Tratt. on GST Activity*

GST Inhibition IC_{50} = 111.81 µg/mL

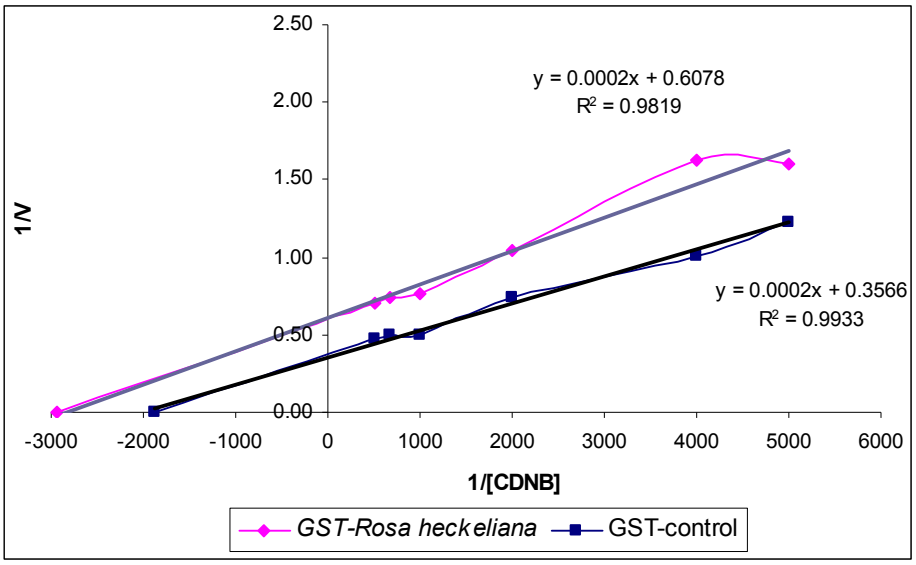


Figure 3.50 Lineweaver-Burk Plot of GST Activity toward CDNB in the presence of 50 µg/mL *Rosa heckeliana* Tratt. extract. The enzyme activities are expressed as µmole/min/mg of protein and CDNB concentration is expressed as M. 1 mM constant concentration of GSH is used as cosubstrate during the experiment.

When the GST activity is measured in the presence of 50 µg/mL *Rosa heckeliana* Tratt. extract, Vmax becomes 1.64 µmoles/min/mg protein and Km=3.3x10^{-4} (**Figure 3.50**). The concentration of *Rosa heckeliana* Tratt extract used for the inhibiton of GST enzyme is close to the IC_{50} value of extract, which is 111.81 µg/mL. Double recipocal plot indicates a decrease both in Vmax and Km values. In this case, it is difficult to differentiate between an uncompetitive (with unchanged slope) and mixed non-competitive (slope changes, intersection of lines below x-axis).

Among the studied plants, *Ferula rigidula* with a very low IC_{50} (48.95 µg/mL) has exhibited a non-competitive type of inhibiton by slightly decreasing Vmax of the enzyme from 2.8 µmoles/min/mg protein to 2.27 µmoles/min/mg protein however, it is increasing Km from 56mM to 68mM, therefore decreasing the affinity of the enzyme for CDNB.

Table 3.6. Effects of plant extracts on Vmax and Km values of GST actvity towards CDNB

Plants	Vmax (µmoles/min/mg protein)	Km (M)
GST Activity without any extract	2.8	$5.60x10^{-4}$
Rheum ribes	1.43	$4.30x10^{-4}$
Ferula rigidula	2.27	$6.80x10^{-4}$
Rosa heckeliana	1.64	$3.30x10^{-4}$
Papaver bracteatum	1.88	$3.80x10^{-4}$
Prangos ferulacea	1.49	$2.98x10^{-4}$
Aconitum cochleare	2.36	$4.72x10^{-4}$

Rosa heckeliana, Papaver bracteatum and Prangos ferulacea have all decrased Vmax of the enzyme from 2.8 µmoles/min/mg protein to 1.64µmoles/min/mg protein, 1.88µmoles/min/mg protein and 1.49µmoles/min/mg protein, respectively. They have all exerted a mixed non-competitive type of inhibition, although in their Lineweaver-Burk reciprocal plots, they all can be persieved as uncompetitive inhibitors, they are actually mixed non-competitive inhibitors, as well. All three plant extracts have decreased the Km value of the enzyme significantly, indicating mixed non-competitive type of inhibition of the enzyme.

Aconitum cochleare and *Rheum ribes* extracts both yields decrease in the Vmax of the enzyme however, the amount of decrease in the Vmax is differing because of the concentration of the extract, which is 50µg/mL, was sufficient for a reliable inhibition in case of *rheum ribes*, although it may not be sufficient for *Aconitum cochleare* due to their different IC_{50} values, 85.30 µg/mL and 4198.69 µg/mL, respectively. On the other hand, both of the Km values were lowered in the presence of extracts, indicating mixed type non-competitive inhibition.

CHAPTER 4

CONCLUSION

All of the plants selected for the screening of antioxidant capacity have displayed a varying range of considerable activity. *Aesculus hippocastanum* L.*, Rosa heckeliana* Tratt.*, Rheum ribes* L.*, Gundelia tournefortii* L. and *Ferula rigidula* DC. exhibited very high antioxidant efficiency, with a considerable correlation in radical scavenging capacity and lipid peroxidation inhibition capacities also with high total phenolics content. On the other hand, *Allium vineale* L., *Chaerophyllum macropodum* Boiss. and *Heracleum persicum* Desf extracts demonstrated comperatively low antioxidant capacities, correlating with all the applied methods for the analysis of antioxidant efficiency.

Regarding to the importance of GST enzymes in cellular defence mechanisms and in multidrug resistance, selected plant extracts were screened for their effects on GST activity along with their antioxidant efficiencies. Among the plant extracts studied for GST inhibition activity, *Prangos ferulacea* L. and *Ferula rigidula* DC. were the most efficient inhibitors with IC_{50} values 79.25µg/mL and 48.95 µg/mL for GST, respectively. Following the ferula species mentioned, *Rheum ribes* (IC_{50}=85.30 µg/mL)*, Rosa heckeliana* Tratt. (IC_{50}=111.81 µg/mL)*,* and *Heracleum persicum* Desf. (IC_{50}=130.28 µg/mL) were the next best GST inhibitors among the rest of the selected plants. *Gundelia tournefortii L. seeds* (IC_{50}=97.51µg/mL) has exhibited better inhibiton on GST activity than the aerial parts of *Gundelia tournefortii* L. (IC_{50}=155.21 µg/mL).

Outstandingly, *Ferula rigidula* extract exerted a very high inhibition with IC_{50} value of 48.95 µg/mL. In the presence of 50 µg/mL extract, Vmax of the enzyme slightly decreased from 2.8 µmoles/min/mg protein to 2.27 µmoles/min/mg protein, however, in the presence of extract, increased Km from 56mM to 68mM, therefore it was decreasing the affinity of the enzyme for CDNB, which was

completely different behaviour than the effect of all the other extracts. The most important effect of *Ferula rigidula* extract was its binding to free enzyme with more affinity than the substrate-bound form of the enzyme.

Kinetic behaviour of the enzyme was elucidated in the presence of 6 plant extracts used as inhibitors. *Rosa heckeliana, Papaver bracteatum and Prangos ferulacea, Aconitum cochleare* and *Rheum ribes* all acted as mixed type non-competitive inhibitors, which is observed generally in the case of multi-substrate binding enzymes. All the extracts have decreased Vmax and Km for the substrate CDNB. All these extracts were binding to substrate-bound form of the enzyme with more affinity than the free enzyme.

Turkey has a very rich and diverse flora being located in between two continents. Out of 9.000 different kind of species found in Turkey, approximately 3000 are endemic species. In this study, plants from the East Anatolian Region were explored having very little information if not in the literature. Several of these plants have revealed very high radical scavenging and lipid peroxidation inhibition capacities as well as inhibition on GST enzyme activity. Most of these plant extracts have exerted much higher antioxidant capacity than well-known antioxidant reference compounds such as α-tocopherol and quercetin.

Bioactivity in the plant extracts are predominantly attributed to their phenolics content, unfortunately that are found in low concentrations and needs to be effectively multiplied in the tissue-culture laboratories by the help of genetic engineering methods. Since the endemic species are endangered by direct consumption of the plants, consequently, they should be cultivated in large scales.

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APPENDICES

Appendix A: Preparation of the test tubes for the Lowry assay

Reagents:

Reagent I: 2% $CuSO_4.5H_2O$

Reagent II: 2% Na-K Tartrate

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Alkaline Copper Reagent: Mix reagents I, II and A in ratio 1: 1: 100 respectively.

Folin-Phenol Reagent: Dilute 2N Folin-Phenol reagent with distilled water at 1:1 ratio.

Tube Number	**Standards and Unknowns**	**dH_2O**	**ACR**	**Folin-Phenol Reagent**
0 – 0′ Blank	-	0.5 ml	2.5 ml	0.25 ml
1-1′	0.5 ml of 0.05 mg BSA/ml	-	2.5 ml	0.25 ml
2-2′	0.5 ml of 0.1 mg BSA/ml	-	2.5 ml	0.25 ml
3-3′	0.5 ml of 0.15 mg BSA/ml	-	2.5 ml	0.25 ml
4-4′	0.5 ml of 0.2 mg BSA/ml	-	2.5 ml	0.25 ml
5-5′	0.5 ml of 1:100 diluted microsomal suspension	-	2.5 ml	0.25 ml
6-6′	0.25 ml of 1:100 diluted microsomal suspension	0.25 ml	2.5 ml	0.25 ml
7-7′	0.1 ml of 1:100 diluted microsomal suspension	0.4 ml	2.5 ml	0.25 ml

Appendix B: The constituents of the reaction mixture for GSTs Assay

Constituents of the Reaction Mixture	**Volume Added (µl)**	**Final Concentration**
Combination Buffer - 20 mL of 0.2 M potassium phosphate buffer, pH 7.5 - 0.8 mL of 50 mM GSH - 13.2 mL H_2O	890	 0.1 M 1 mM
Substrate Solution - 20 mM CDNB	50	1 mM
Enzyme Source - Sheep Liver Cytosol (21mg/ml) 1/100 diluted with 10mM potassium phosphate, buffer, pH 6.0	50	
H_2O, DMSO and Plant extracts	10	

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Research Interests

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- BAP-07-02-DPT-2003-K120920-16, Biyoaktif I: Türkiye bitki örtüsünün Yüksek Verimli İlaç Tarama Tekniği uygulanarak, antioksidan ve biyoaktif maddeler açısından taranması
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National Conference Proceedings

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