

**THE EFFECTS OF PHENOLIC COMPOUND TANNIC ACID ON  
PHASE II AND CYTOCHROME P450 DEPENDENT ENZYMES IN  
RABBIT LIVER AND KIDNEY**

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PHASE II AND CYTOCHROME P450 DEPENDENT  
ENZYMES IN RABBIT LIVER AND KIDNEY**

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## ABSTRACT

### THE EFFECTS OF PHENOLIC COMPOUND TANNIC ACID ON PHASE II AND CYTOCHROME P450 DEPENDENT ENZYMES IN RABBIT LIVER AND KIDNEY

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Cancer is the second leading cause of death after cardiovascular diseases in the world. Many of the chemical carcinogens need metabolic activation that catalyzed by cytochrome P450 and Phase II enzymes in order to exert their genotoxic and carcinogenic effects. Hence one possible mechanism is that phenolic compounds may alter anticarcinogenic effects is through an interaction with these enzymes either by the inhibition or activation of certain forms, leading to a reduced production of the ultimate carcinogen. Therefore anti-carcinogen activity of tannic acid, a hydrolyzable plant polyphenol, has a crucial importance to prevent conversion of pro-carcinogens to their carcinogenic form. Tannic acid is produced from secondary metabolism of plants and is found in edible vegetables, fruits and nuts, especially tea, cocoa, coffee and wine.

In the present work, modulation of rabbit liver and kidney microsomal P450 dependent aniline 4-hydroxylase, N-nitrosodimethylamine N-demethylase and *p*-nitrophenol hydroxylase activities and cytosolic phase II enzymes; glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase:1 (NQO1) were studied in the presence of tannic acid at concentrations ranging from 0.5  $\mu$ M to 150  $\mu$ M in the reaction medium.

The results obtained in this study were shown that tannic acid significantly inhibited the activities of *p*-nitrophenol hydroxylase, aniline 4-hydroxylase, NDMA N-demethylase, glutathione S-transferase, NAD(P)H:quinine oxidoreductase 1. Tannic acid was found to be the most potent inhibitor of cytosolic glutathione S-transferase with IC<sub>50</sub> of 0.33 μM and the least potent inhibitor of microsomal aniline 4-hydroxylase. with IC<sub>50</sub> of 60.26 μM.

Effect of tannic acid on enzyme activities was further studied for both mode and type of inhibition. For this purpose various concentrations of the substrate were examined at various tannic acid concentrations. Lineweaver-Burk and Dixon plots were then generated from the resulting data sets. The K<sub>m</sub> value and inhibition constants (K<sub>I</sub>) were determined from double reciprocal and Dixon plot of the enzyme activity versus substrate and inhibitor concentration, respectively. Tannic acid was shown to be a noncompetitive inhibitor for liver cytosolic GST, NQO1 and microsomal aniline 4- hydroxylase enzymes with K<sub>I</sub> of 0.3 μM, 41 μM and 54.7 μM, respectively. On the other hand, in kidney tissues, tannic acid was an uncompetitive inhibitor of cytosolic GST, while it was noncompetitive inhibitor for cytosolic NQO1 with a K<sub>I</sub> of 12.6 μM.

These results indicate that tannic acid may modulate cytochrome P450 dependent and Phase II enzymes and influence the metabolic activation of xenobiotics mediated by these enzymes.

**Keywords:** Tannic acid, Cytochrome P450, Aniline 4-hydroxylase, Phase II ; GST, NQO1, Rabbit, Liver, Kidney.

## ÖZ

### FENOLİK BİLEŞİK TANNİK ASİTİN TAVŞAN KARACİĞER VE BÖBREĞİNDE FAZ II VE SİTOKROM P450 BAĞIMLI ENZİMLER ÜZERİNDEKİ ETKİLERİ

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Kanser, kardiyovasküler hastalıklardan sonra ölüme sebebiyet veren en önemli nedendir. Birçok kimyasal kanserojenin genotoksik ve kanserojenik etkilerini gösterebilmesi için sitokrom P450 ve faz II enzimleri tarafından aktive edilmesi gerekmektedir. Dolayısıyla anti kanserojenik etkilerinin değiştirilebileceği muhtemel bir mekanizma fenolik bileşiklerin bu enzimlerle etkileşime girerek gerek inhibe ederek gerekse de aktive ederek temel kanserojenin oluşumunun azalmasına yol açmasıdır. Bundan dolayı hidroliz edilebilir bir bitkisel polifenol olan tannik asidin pro-kanserojenlerin aktif kanserojen formlarına dönüşmelerini engelleyen anti-kanserojen aktivitesi büyük bir öneme sahiptir. Tannik asit bitkilerin ikincil metabolizmaları sonucu üretilir ve sebze, meyve ceviz ve fındık gibi kabuklularda ve özelliklede çay, kakao, kahve ve şarapta bulunmaktadır.

Bu çalışmada 0.5  $\mu$ M ile 150  $\mu$ M arasında değişen tannik asit konsantrasyonlarında tavşan karaciğer ve böbreğinde P450 bağımlı anilin 4- hidroksilaz, N-nitrosodimethylamine N-demetilaz, ve *p*-nitrofenol hidroksilaz aktiviteleri ile faz 2 enzimlerden glutation S-transferaz (GST), ve NAD(P)H:kinon oksidoreduktaz:1 (NQO1) enzimlerinin değişimi incelenmiştir.

Deneylemler sonucunda elde edililen sonular tannik asitin, *p*-nitrofenol hidroksilaz, aniline 4-hidroksilaz, NDMA N-demetilaz, glutation S-transferaz ve NAD(P)H:kinon oksidoreduktaz:1 aktivitesini nemli lde inhibe ettiėi gstermiřtir. Tannik asidin glutation S-transferaz aktivitesinde en kuvvetli inhibitr ( $IC_{50} = 0.33 \mu M$ ) olduėu ve mikrozomal anilin 4- hidroksilaz ( $IC_{50}=60.26 \mu M$ ) aktivitesinde ise en az etkiye sahip olduėu bulunmuřtur.

Tannik asidin enzim aktivitesine olan etkisi daha sonra inhibisyon tipi ve řekli aısından incelenmiřtir. Bu amala farklı substrat konsantrasyonları farklı tannik asit konsantrasyonlarında alıřılmıřtır. Lineweaver-Burk ve Dixon grafikleri elde edilen sonular doėrultusunda oluřturulmuřtur. Konsantrasyon sabiti ( $K_m$ ) ve inhibisyon sabiti ( $K_I$ ) enzim aktivitesine karřı sırasıyla substrat ve inhibitr konsantrasyonlarına karřın izilmesiyle bulunmuřtur. Tannik asitin karaciėer enzimlerinden sitozolik GST ve NQO1 ile mikrozomal anilin 4-hidroksilaz enzim aktivitesine karřı sırasıyla  $0.3 \mu M$ ,  $41 \mu M$  ve  $54.7 \mu M$   $K_I$  deėeriyle “noncompetitive“ inhibitr olduėu gsterilmiřtir. Diėer yandan bbrek dokularında tannik asitin  $12.6 \mu M$   $K_I$  deėeri ile sitozolik NQO1 iin “noncompetitive“ inhibitr iken sitozolik GST iin “uncompetitive“ inhibitr olduėu gsterilmiřtir.

Bu sonular gstermektedir ki tannik asit sitokrom P450E1 ve faz II enzimlerini deėiřtirebilmekte ve bu enzimler tarafından deėiřtirilen kimyasal maddelerin metabolik aktivasyonlarını etkilemektedir.

**Anahtar kelimeler:** Tannik asit, Sitokrom P450, Anilin 4-Hidroksilaz, Faz II, GST, NQO1, Tavřan, Karaciėer ve Bbrek

*Dedicated to my wife*



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## LIST OF SYMBOLS AND ABBREVIATIONS

<b>B[a]P</b>	Benzo[a]pyren
<b>BSA</b>	Bovine serum albumine
<b>CDNB</b>	1-chloro-2,4-dinitrobenzene
<b>CYP</b>	Cytochrome P450
<b><math>\epsilon</math>-ACA</b>	$\epsilon$ -Amino caproic acid
<b>EDTA</b>	Ethylenediamine tetra acetic acid
<b>Da</b>	Dalton
<b>DCPIP</b>	2,6 dichlorophenol-indophenol
<b>DNB-SG</b>	1-(S-glutathionyl)-2,4-dinitrobenzene
<b>FAD</b>	Flavine adenine dinucleotide
<b>FMO</b>	Flavin-containing monooxygenases
<b>GSH</b>	Glutathione reduced form
<b>GST</b>	Glutathione S-transferases
<b>HEPES</b>	N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid
<b>IC<sub>50</sub></b>	the concentration giving 50 % inhibition
<b>K<sub>I</sub></b>	Inhibition constant
<b>K<sub>m</sub></b>	Michaelis-Menten constant
<b>MFO</b>	Mixed function oxidases
<b>M<sub>w</sub></b>	Molecular weight
<b>NADH</b>	Nicotinamideadenine dinucleotide, reduced form
<b>NADP<sup>+</sup></b>	Nicotinamideadenine dinucleotide phosphate
<b>NADPH</b>	Nicotinamideadenine dinucleotide phosphate, reduced form
<b>NDMA</b>	N-nitrosodimethylamine
<b>NQO1</b>	NAD(P)H:Quinone Oxidoreductase 1
<b>PAH</b>	Poly aromatic hydrocarbons
<b>pAP</b>	<i>p</i> -aminophenol
<b>PMSF</b>	Phenylmethylsulfonyl fluoride
<b>PNPH</b>	<i>p</i> -nitrophenol hydroxylase
<b>TA</b>	Tannic Acid



<b>TCA</b>	Trichloro acetic acid
<b>TRIS</b>	Tris (hydroxymethyl) aminomethane
<b>V<sub>max</sub></b>	Maximum velocity
<b>ZTB</b>	Zero time blank

## **CHAPTER 1**

### **INTRODUCTION**

Diets rich in fruit and vegetables decrease the risk of mortality from major clinical conditions, including cancer and cardiovascular diseases. However it is not yet clear which components or combination of components in fruits and vegetables are protective and what is their mechanism of action. Plants produce thousands of phenolic and polyphenolic compounds and they have important place in human nutrition. However as yet there is little clear evidence that polyphenols are putative dietary factors with important health benefit. Tannic acid is one of the polyphenolic molecules of diverse tannins.

#### **1.1. Tannins**

The name 'tannin' is derived from 'tanin' in French and is used for a range of natural polyphenols (Khanbabaee and van Ree, 2001). Although making a precise definition for tannins is difficult since they are chemically diverse, the word tannin can be defined as any phenolic compound of sufficiently high molecular weight (500-3000 Da) containing hydroxyl and other suitable groups, such as carboxyl, to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions. It was shown that the low and high-molecular-weight phenolic compounds, molecular weight lower than 500 Da and molecular weight higher than 3000 Da, respectively, are ineffective tanning agents (Bate-Smith, 1962; Horvath, 1981).

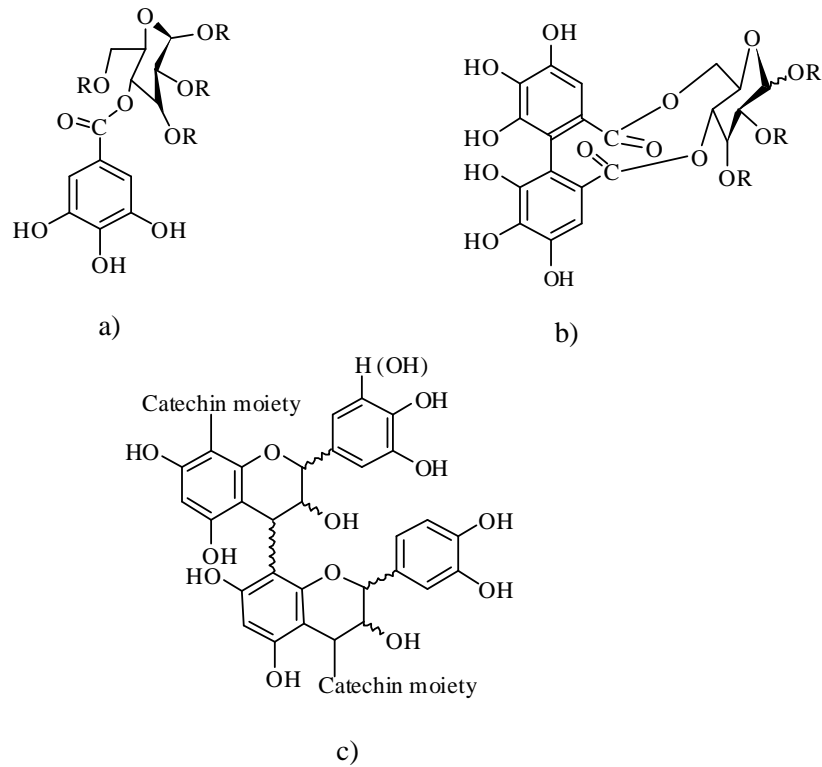
Variety of plants utilized as food and feed contain tannins. These include food grains such as sorghum, millets, barley, dry beans, faba beans, peas, carobs, pigeon peas, winged beans, and other legumes. Fruits such as apples, bananas, blackberries, cranberries, dates, grapes, hawthorns, peaches, pears, persimmons, plums, raspberries, and strawberries also contain an appreciable quantity of tannins. In addition to grapes and fruits phenolic compounds are also present in wine and tea. Forages such as crown vetch, lespedeza, lotus, sainfoin, and trefoil are also reported to contain tannins (Goldstein; 1963; Hoff, 1975; Sanderson *et al.*, 1975; Chavan *et al.*, 1977; Salunkhe *et al.*, 1982).

Plant polyphenols are secondary metabolites synthesized by several of the higher plant kingdom. They are distinguished by the following general features; a) Water solubility, b) Molecular weight, c) Structure and polyphenolic character, d) Intermolecular complexation and e) Structural characteristics. Polyphenols exert certain of their roles by virtue of three distinctive general characteristics which they all possess to a greater or lesser degree. These are their complexation with metal ions (Iron, copper, aluminum, calcium etc.), their antioxidant and radical scavenging activities and their ability to make complex with other molecules including macromolecules such as proteins and polysaccharides (Haslam, 1996).

Due to complex chemistry and non-uniform structure, systematic classification of tannins depends on their chemical properties and specific structural characteristics. Depending on their chemical and structural properties, tannins can be divided into two general groups; hydrolysable and non-hydrolysable (condensed tannins) (Haslam, 1981). Hydrolyzable tannins are present in plants as gallotannins or ellagitannins (Figure 1.1). Hydrolysable tannins can be fractionated hydrolytically into their components when they are treated with tannases or hot water. Tannins also contain a central core of polyhydric alcohol such as glucose, and hydroxyl groups. They are esterified either partially or wholly by gallic acid (gallotannins) in which galloyl units are bound to diverse polyol-, catechin- or triterpenoid units. Hexahydroxydiphenic acids (ellagitannins) are also composed of at least two galloyl units that are C–C

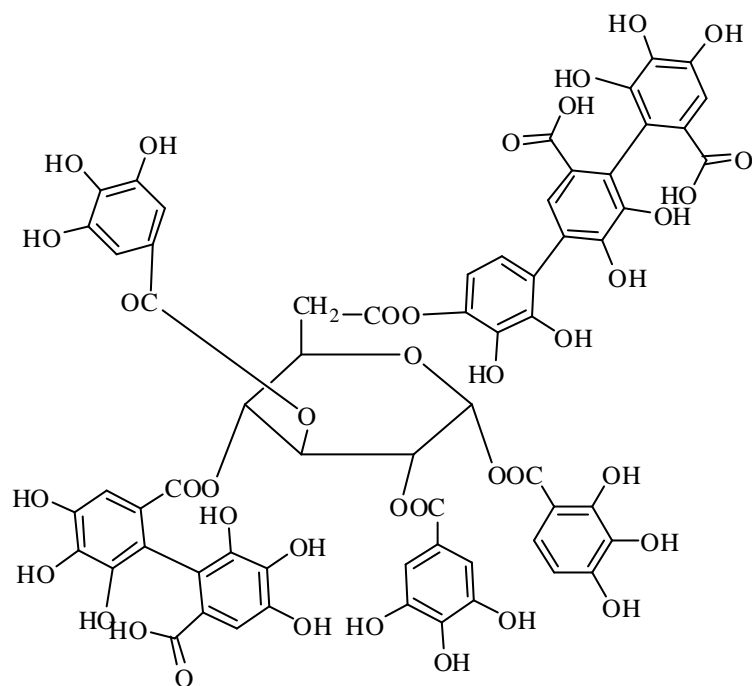
coupled to each other, and do not contain a glycosidically linked catechin unit (Khanbabaee and Ree, 2001). Hydrolysable tannins are present in plants as gallotannins or ellagitannins. Chinese tannin (tannic acid), Turkish tannin, Tara tannin, Acer tannin, and Hamamelis tannin are important examples of gallotannins while corilagin, chebulinic acid and chebulagic acid are the important ellagitannins.

After hydrolysis by acids, bases, or certain enzymes, gallotannins yield glucose and gallic acids. However condensed tannins are non-hydrolysable oligomeric and polymeric proanthocyanidins. Structure of condensed tannins as shown in Figure 1.1 is much more complex than hydrolysable tannins. Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of C-4 of one catechin with C-8 or C-6 of the next monomeric catechin (Khanbabaee and van Ree, 2001).



**Figure 1.1** Structures of hydrolysable tannins (a) Gallotannic , (b) Ellagitannin and non-hydrolysable tannins (condensed tannins) (c) catechin-(4 $\alpha$ →8)-epicatechin (procyanidin B4).

Tannic acid shown in Figure 1.2 is a hydrolysable polyphenol produced from the secondary metabolism of plants. Tannic acid enters to human body via consumption of polyphenol rich barks, fruits, vegetables and beverages. It is estimated that an average of 1–2 g/day of these components may be consumed in a human diet (Markham, 1989). Tea, cocoa, beans, grapes, strawberries and especially the bark of the oak species, sumac and myrobalan are tannic acid rich plants (Merck *et al.* 1976; Nepka *et al.*, 1999).



**Figure 1.2** Structure of tannic acid.

### 1.1.1 Therapeutic Effects of Tannic Acid

There is an inverse correlation between individuals who consume a diet rich in fruits and vegetables and their risk in developing cancers (Stich, 1984; Stoner, 1989). It was shown that stomach cancer incidence was significantly decreased with tea consumption (Stocks, 1970; Oguni *et al.*, 1988). Moreover oral consumption of green tea decrease tumor size in papilloma-bearing mice and also inhibits N-nitrosodimethylamine (NDMA)-induced tumorigenesis (Conney *et al.*, 1992; Wang *et al.*, 1993).

In 1989, Athar *et al.* showed that incidence of fore stomach and pulmonary tumors was decreased in the mice fed with tannic acid compared with the mice fed with B[a]P, and also they showed that glutathione S - transferase and NAD(P)H: quinone reductase activities were increased in these mice.

The inhibitory effect of tannins has been shown to be due to the reduction of enzyme activity, dysfunctioning of cell membrane and deprivation of substrate metal ions and minerals (Goel *et al.*, 2005).

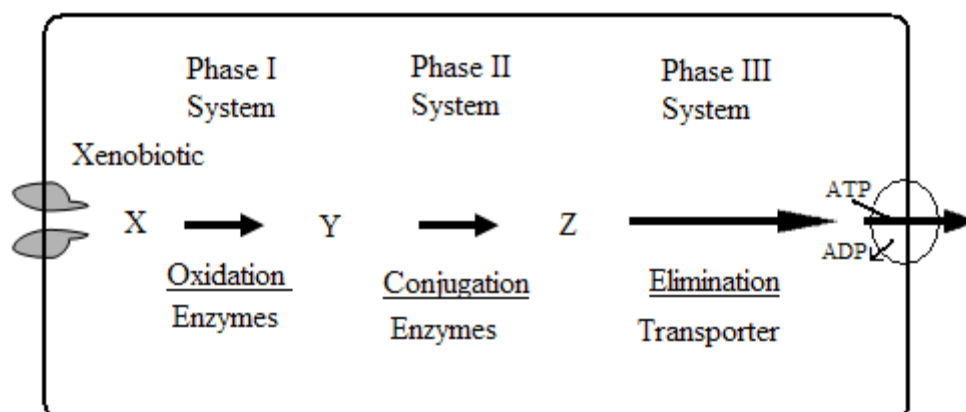
Like many polyphenols, tannic acid possesses antioxidant, antimutagenic and anticarcinogenic properties (Lopes *et al.*, 1999). In addition to its anticarcinogenic and antimutagenic activity, it was shown that tannic acid effectively neutralizes the activity of *Naja kaouthica* venom (Pithayanukul, 2007). Besides, tannic acid also has a toxic effect on some bacteria such as *Streptococcus pyogenes* which cause mild superficial skin infections to life-threatening systemic diseases and *Yersinia enterocolitica* which causes severe diarrhea in humans, along with Peyer's patch necrosis, chronic lymphadenopathy, and hepatic or splenic abscesses (Chung, 1993). Furthermore, it was shown that tannic acid inhibits growth of *Influenza virus* factor of flu and pneumonia (Green, 1948).

## 1.2 Detoxification Enzyme Systems

During lifetime, human beings may take several types of xenobiotics whether from pharmaceutical compounds or food additives. These chemicals can cause mutation, cancer and birth defects. In order to react with cellular macromolecules, chemical carcinogens require metabolic activation. Steps required for the carcinogenesis process include: *a*) metabolic activation of a carcinogen by cellular xenobiotic-metabolizing enzymes, *b*) binding of the active metabolite to DNA to produce a DNA adduct, *c*) faulty repair of the adduct to produce a gene mutation, *d*) cell replication to fix the mutation to the genome, and *e*) progression to a full neoplasm of the replicating cell containing the mutated genes (Sugimura, 1992).

Our body is capable of managing these environmental compounds by detoxifying them as illustrated in Figure 1.3. For this task, our body evolves a system. Metabolism of xenobiotics consists of two steps; first one is functionalization step in which oxygen is used to form reactive site and it is

known as Phase I metabolism, and the other step is conjugation step that results with addition of a water soluble group to the reactive site and it is known as Phase II metabolism (Liska, 1998).



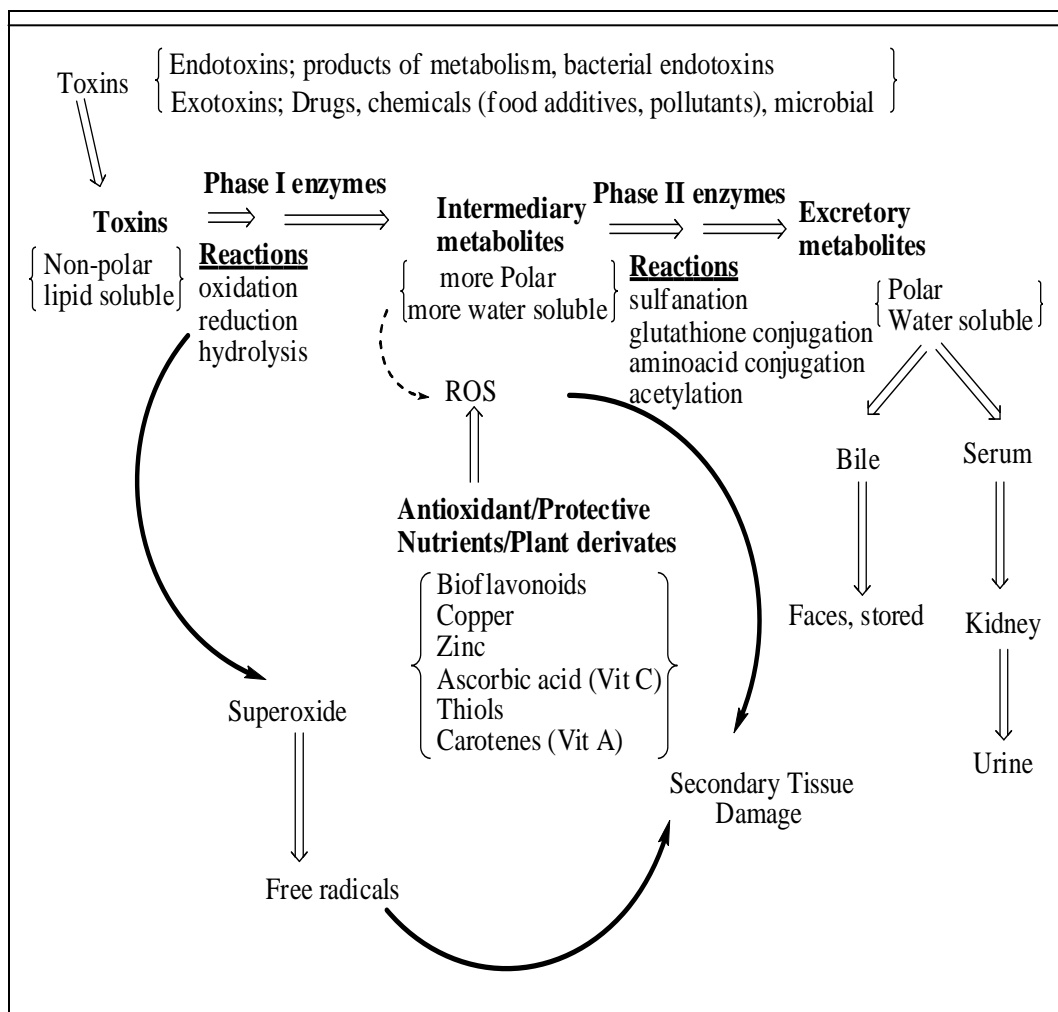
**Figure 1.3** Illustration of Phase I, Phase II and Phase III detoxification system (adapted from Nakata *et al.*, 2006).

Detoxification system may be manipulated via induction or inhibition depending on the presence of xenobiotic and dietary compounds, genetic, age, sex and lifestyle habit such as drinking alcohol (Guengerich, 1995; Park, 1996).

When the body exposes to a high dose of xenobiotic, Phase I and/or Phase II enzymes can be induced in order to detoxify these compounds. Inducers can be mono-functional or multi-functional (Park, 1996). Mono-functional inducers just affect one enzyme or one phase of detoxification system. Such as polycyclic hydrocarbons from cigarette smoke significantly induce CYP1A1 enzymes in Phase I activity but has little or no induction for Phase II enzymes (Kall, 1995). Multi-functional inducers affect multiple enzymes and activities. Tannins found in edible plants affect not only Phase I enzymes but also Phase II enzymes. Such as ellagic acid induces several Phase II enzymes while it was found to be potent inhibitor for certain Phase I enzymes (Barch, 1994).



Liver is the main organ for xenobiotic metabolizing process (Lue and West, 1980; Nebert and Gonzales, 1987). Cytochrome P450s are generally expressed in lower amounts in extrahepatic tissues such as gut, kidney, and lung (Arinç and Philpot, 1976; Arinç, 1993). As it is shown in Figure 1.4 lipophilic compounds are converted to a water-soluble compound via biotransformation and excreted in urine or feces as a result of Phase I and Phase II detoxification system.

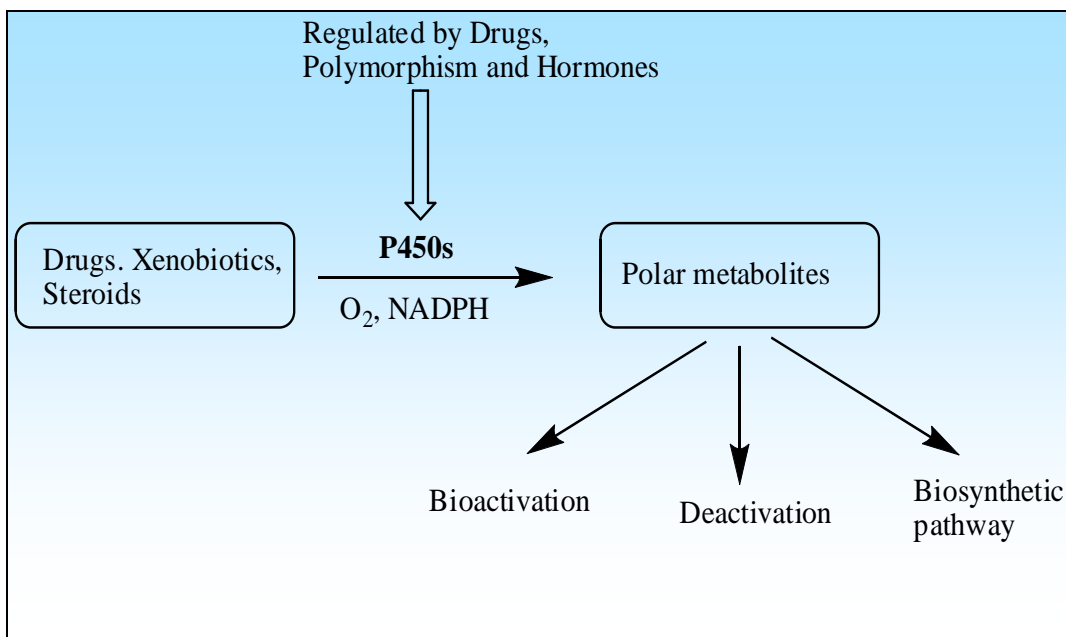


**Figure 1.4** Liver detoxification pathways and supportive nutrients (taken from DeAnn, 1998)

### 1.2.1 Phase I Detoxification / Activation System

Although Phase I detoxification system includes P450s, flavin-containing monooxygenases (FMO) and epoxide hydrolases, the main component of Phase I detoxification system is Cytochrome P450 system which contains very large groups of enzymes encoded by the P450 supergene family. The term P450 refers to a pigment that absorbs light at 450 nm when reduced form exposed to carbon monoxide (Omura and Sato, 1964)

P450s (CYPs) may be found in every living organism from bacteria to human (Nelson, 1996). CYPs are membrane bound proteins with an approximate molecular weight of 50 kDa and contain a heme moiety. Like other mixed function oxygenases CYPs are mainly found in the endoplasmic reticulum of the liver. The total CYP450 content of the livers ranged from 0.06 to 0.46 nmol/mg microsomal protein (Iyer and Sinz, 1999). By using NADPH and O<sub>2</sub>, P450 enzymes modify or insert new functional groups on a molecule by oxidation, reduction, and hydrolysis reactions. While some types of P450s are involved in the biosynthetic pathways of steroids, vitamin D and bile acid production, most of them metabolize foreign compounds or xenobiotics; including drugs, toxicants and chemical carcinogens as the first defense mechanism which is illustrated in Figure 1.5. On the other hand, certain chemicals are metabolically activated by P450s to reactive derivatives which cause cell toxicity and cancer. The P450s which activate procarcinogens to carcinogenic form are CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1 and CYP3A. These compounds which are more toxic than parent molecules have very short lifetime and may cause protein, DNA or RNA damages unless they are not conjugated by Phase II enzymes. Addition to this feature, they can add functional groups in order to moderately increase polarity and water solubility (Vermeulen, 1996; Iyer and Sinz, 1999; Guengerich, 2003).



**Figure 1.5** Cytochrome P450 enzymes and their regulation.

Depending on their sequence similarities P450 proteins can be categorized into families and subfamilies. At the amino acid level, sequences which are greater than 40 % identical belong to the same family. If this similarity ratio rises up to 55 % they are categorized in the same subfamily. More than 7700 cytochrome P450 sequence has been reported as of September 2007 (<http://drnelson.utmem.edu>). On the other hand all P450s have some similar properties; the ability to activate the O-O bond, the thiolate bond to the heme iron and similar overall structure and shape (Anzenbacher and Anzenbacherová, 2001). The standard naming system uses the abbreviation for Cytochrome P450 [**CYP**] followed by a number denoting the gene family [**2**], a letter indicating the subfamily [**E**], and another enzyme referring to the enzyme [**1**]; [such as **CYP2E1**]. Rabbit and mouse cytochrome P450 isozymes with certain properties are given in Table 1.1.

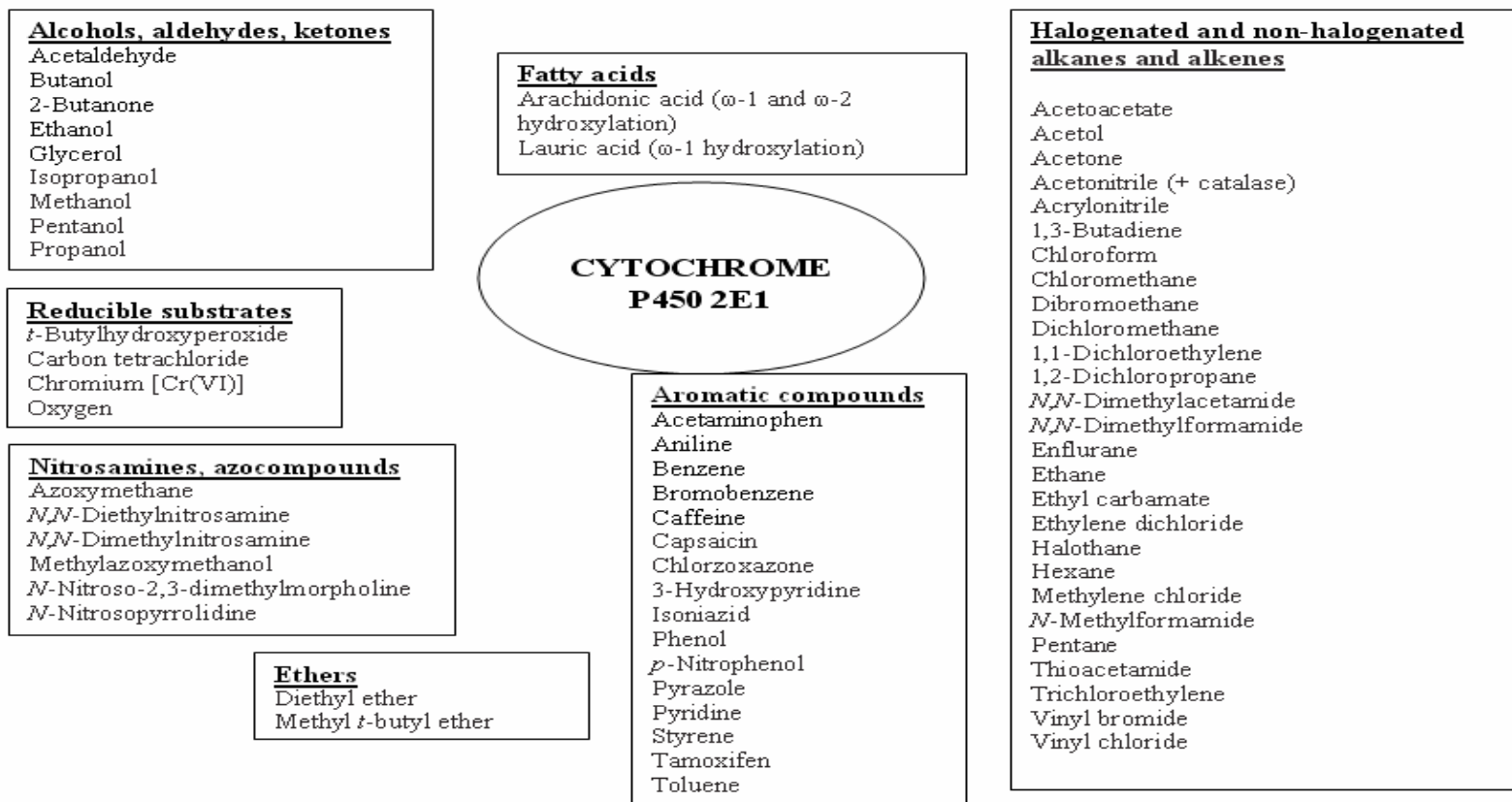
**Table 1.1** Rabbit and Mouse Cytochrome P450 (adapted from Norman *et al.*, 1978; Koop and Coon, 1984).

<b>Rabbit and Mouse Cytochrome P450</b>				
Gene category	Cytochrome P450	A <sub>max</sub> (nm) CO <sub>-reduced</sub>	Molecular Weight (Da)	Efficient Catalytic Reaction
<b>RABBIT P450</b>				
1A1	Form 6	448	57 500	Benzo( <i>a</i> )pyrene hydroxylation
1A2	Form 4	448	54 000	2-Acetylaminoflouren N-hydroxylation
2B4	Form 2	451	49 500	Benzphatamine N-demetylation
2C3	Form 3b	450	52 000	Progesterone 6β- hydroxylation
2E1	Form 3a	452	51 000	Ethanol oxidation
3A6	Form 3c	449	53 000	Steroid 6β- hydroxylation
4B1	Form 5	449	57 000	2-Aminoanthracene metabolism
-	Form 7	451	59 000- 60 000	Prostaglandin ω- hydroxylation
<b>MOUSE P450</b>				
1A1	P <sub>1</sub> 450	449	55 000	Arylhydrocarbon hydroxylation
1A2	P <sub>3</sub> 450	448	55 000	Acetanilide 4-hydroxylation
2A3	P450 15α	451	48 000	Testosterone 15α- hydroxylation
2D9	P450 16α	449	49 500	Testosterone 16α- hydroxylation

It is well established that cytochrome P450 monooxygenase system functions as a multi-component electron transport system. Lu and Coon (1968), for the first time, demonstrated that liver microsomal cytochrome P450 dependent monooxygenase system has three components; cytochrome P450, FAD and FMN containing NADPH dependent cytochrome P450 reductase and lipid. All three components of cytochrome P450 dependent monooxygenase system (NADPH, cytochrome P450 reductase, cytochrome P450 and lipid) are required to reconstitute the full hydroxylation activity (Lu and Coon, 1968; Arınc and Philpot, 1976; Arınc and Adalı, 1990; Arınc, 1993, 1995).

#### **1.2.1.1 CYP2E1**

CYP2E1 is one of the best conserved of the xenobiotic-metabolizing P450s involved in chemical activation of many carcinogens, procarcinogens, and toxicants. CYP2E1 first identified in rabbits is ethanol inducible form of P450. Beside to liver, CYP2E1 is found in extrahepatic tissues such as lung, kidney heart and bone marrow. Substrates of CYP2E1 are given in Figure 1.6. Especially CYP2E1 metabolize a large number of low-molecular weight compounds lower than 100 kDa such as chemical additives or industrial solvents like carbon tetrachloride, chloroform (Raucy, 1993). The highest level of CYP2E1 is found in the centrilobular zone of liver acinus (Ingelman-Sundberg *et al.*, 1988). Therefore toxins such as ethanol, acetaminophen, nitrosamines, and carbon tetrachloride, which are substrates of CYP2E1, preferentially destroy the centrilobular liver region. In liver, CYP2E1 is expressed mainly in the hepatocytes, but significant amounts are also found in the Kupffer cells; both hepatocyte and Kupffer cell CYP2E1s are inducible, e.g., by ethanol (Jimenez-Lopez and Cederbaum, 2005).



**Figure 1.6** Substrates Metabolized/Activated by CYP2E1 (Modified from Ronis *et al.*, 1996 and Rendic and Di Carlo, 1997)

Organic solvents such as benzene were demonstrated to increase CYP2E1 dependent activities (Arinç *et al.*, 1991). Moreover, it was established that pyridine treatment enhanced NDMA N-demethylase activity 6.9, 5.2 and 3.4 fold increased in rabbit liver, lung and kidney microsomes, respectively (Arinç *et al.*, 2000a, 2000b). Several halogenated anaesthetics (e.g. halothane, sevoflurane and isoflurane) and drugs such as acetaminophen, chlorzoxazone, trimetmethadione and acetylsalicylic acid (aspirin) are also mainly catalyzed by CYP2E1 (Wu and Cederbaum, 2001; Tanaka *et al.*, 2003; Gonzales, 2005; Khemawoot *et al.*, 2007). Increased activity of CYP2E1 has been associated with increased risk of cancer through an increased production of ROS and enhanced activation of a variety of procarcinogens. CYP2E1 level are influenced by a variety of hormones, e.g., hypophysectomy and T3 increase CYP2E1 protein and mRNA levels, whereas insulin lowers these levels (Peng and Coon, 1998; Hong *et al.*, 1990). Main CYP2E1 inhibitors and inducers were given in Table 1.2.

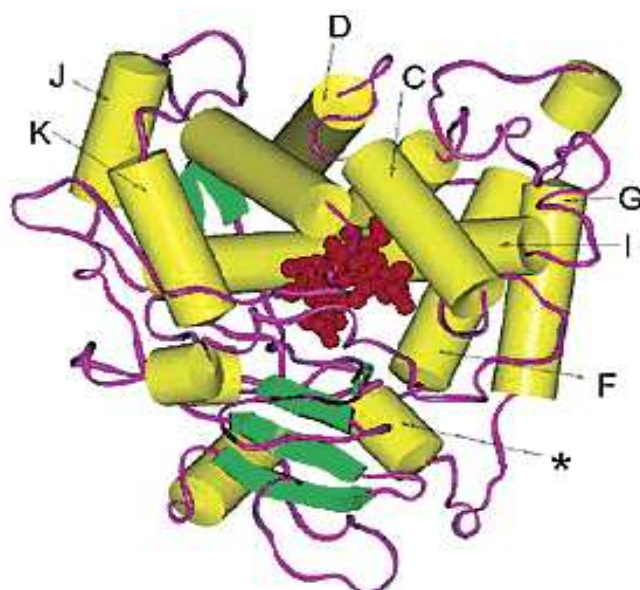
**Table 1.2** Cytochrome P450 CYP2E1 Inhibitor and Inducers (modified from Tanaka *et al.*, 2000).

<u>Inducer</u>	<u>Inhibitor</u>
❖ Ethanol	❖ Disulfiram,
❖ Isoniazid	❖ Cimetidine,
❖ Ritonavir	❖ Watercress,
❖ Acetone	❖ Diallylsulfone,
❖ Benzene	❖ Chlormethiazole,
❖ Isopropanol	❖ Diallylsulfide,
❖ Pyrazole	❖ 4-methyl-pyrazole,
❖ Pyridine	❖ Isothiocyanates
❖ Pathological states (such as diabetes or starvation)	❖ Insulin
❖ Hypophysectomy	

Since its substrates are moderately small, a neutral and relatively hydrophilic molecule, CYP2E1's active site shown in Figure 1.7 is not expected to be spacious, and capable of forming one or two hydrogen bonds with substrates close to the site of metabolism (Smith, 1997; Lewis, 2000). Since CYP2E1 is involved in the metabolism of several xenobiotics, including chemical carcinogens, its modulation can dramatically affect the compound's toxicity and carcinogenesis. It was shown that tannic acid is potent inhibitors of CYP2E1 activity in mouse liver microsomes (Mikstacka, 2002). Furthermore, Mikstacka showed that tannic acid was a mixed type ( $K_i = 1 \mu\text{M}$ ) *p*-nitrophenol hydroxylase inhibitor.

CYP2E1 can also be induced under a variety of metabolic or nutritional conditions, such as in obese rats and in rats fed a high-fat diet (Raucy *et al.*, 1991). On the other hand, levels of CYP2E1 in rats were also increased by fasting and by prolonged starvation (Hong *et al.*, 1987). Levels of acetone were conspicuously elevated under fasting conditions in CYP2E1 null mice, suggesting the critical role of CYP2E1 in acetone metabolism (Bondoc *et al.*, 1999). Diabetes have differential effects on CYP2E1 expression and associated drug metabolizing enzyme activities in rabbit liver, lung and kidney (Arınç *et al.*, 2005, 2007). CYP2E1 induction in diabetes may be related with the elevated production of ketone bodies (Bellward *et al.*, 1988). In rat hepatocyte cultures, addition of pyrazole or 4-MP slowed the decline in CYP2E1 protein and activity, without any effect on CYP2E1 mRNA levels (Wu *et al.*, 1990).





**Figure 1.7** Modelling of Active sites of CYP2E1. Helices are represented as yellow cylinders,  $\beta$ -sheets as green ribbons, and coils as narrow magenta ribbons. The heme is shown in as red CPK representations (Park and Harris, 2003).

### 1.2.2 Phase II Detoxification System

Activated hydrophobic compound by Phase I enzymes must be converted to hydrophilic forms. In order to excrete xenobiotics they must be converted to more water soluble compounds. This is done by Phase II enzyme systems via conjugation reactions with glutathione, sulfate or glucuronide (Table 1.3). Therefore it is possible to accept Phase II mechanism as a detoxification process of xenobiotics. Nevertheless the Phase II system is a critical step in the formation of genotoxic electrophiles. Furthermore accumulation of the resulting metabolites in cells can lead to a decrease in detoxification activity of the Phase II system. Phase II metabolites are eliminated by efflux pumps called as Phase III detoxification system (Ishikawa, 1992).

**Table 1.3** Major Phase II Detoxification Activities in Humans (taken from Vermeulen, 1996).

Reaction	Enzyme	Localization	Substrates
Glutathione	Glutathione transferases	Microsomes Cytosol	Electrophiles
Glucuronic acid (UDPGA) <sup>b</sup>	Glucuronyl transferases	Microsomes	Phenols, thiols, amines, Carboxylic acids
Sulfuric acid (PAPS) <sup>b</sup>	Sulfotransferase	Cytosol	Phenols, thiols, amines
Methyl Group (SAM) <sup>b</sup>	N- and O- methyl transferases	Cytosol Microsomes	Phenols, amines
Acetic acid (Acetyl-CoA) <sup>b</sup>	N-acetyl transferases	Cytosol	Amines
Amino acids (Acetyl-CoA, taurine, glycine)	Amino acid transferases	Microsomes	Carboxylic acids
<p>❖ Microsome refers to membrane-associated activities but these activities may be localized to the cellular membrane or to internal membranes; cytosol refers to soluble activities present in the cytosolic portion of the cell</p> <p>❖ <sup>b</sup> Abbreviations in brackets are co-substrates: UDPGA = uridine - 3', 5' - diphosphoglucuronic acid; PAPS = 3' -phosphoadenosine 5' - phosphosulfate; SAM = S - adenosylmethionine; CoA = coenzyme A.</p>			

### 1.2.2.1 Glutathione S-Transferase

Glutathione S-transferases (GSTs, EC 2.5.1.18) are soluble proteins with typical molecular masses of around 50 kDa. GSTs have two general functions; to remove toxic metabolites from the cell and to maintain cellular sulfhydryl groups in their reduced form (Bendich *et al.*, 1988). Besides, the resistance of cells and organisms to pesticides, herbicides and antibiotics was implicated in GST activities (Arca *et al.*, 1988; Fournier *et al.*, 1992; Wilce and Parker, 1994).

GSTs are multifunctional dimeric enzymes and play a key role in biotransformation and detoxification of potential alkylating agents and xenobiotics during phase II reactions. It was also demonstrated that bioactivation of toxic metabolites by GST may cause organ damages (Bladeren van, 1988; Sherratt *et al.*, 1997; Anders and Dekant, 1998).

Bioactivation role of GST on dichloromethane, haloalkenes, vicinal dihaloalkenes and haloacids which are toxic metabolites was demonstrated (Bladeren van, 1988; Sherratt *et al.*, 1997; Anders and Dekant, 1998). GSTs have been shown to be over-expressed in tumor cells hence it increases the resistance for chemotherapeutic drugs. Cancer chemotherapeutic agents such as adriamycin, 1,3-bis (2-chloroethyl)-1 nitrosourea, busulfan cyclophosphamid are detoxified by GST (Hayes and Pulford, 1995; Hamilton *et al.*, 2003). Therefore, enhanced GST-mediated conjugations of cytostatics due to increased GST expression used in cancer treatment have been suggested among the mechanisms of drug resistance. In this context, inhibition of GSTs may sensitize drug-resistant cells (Yang *et al.*, 1992; Horton *et al.*, 1999).

Depending on isoelectric point, inhibitor properties, antibody recognition, and N-terminal amino acid sequence, subtypes of glutathione S-transferases have been grouped into classes consisting of homo- and heterodimers grouped into species-independent families: alpha ( $\alpha$ ), pi ( $\pi$ ), mu ( $\mu$ ) and theta ( $\theta$ ) as well as a microsomal trimeric enzyme (Mannervik *et al.*, 1992; Beckett, 1993). The first three (alpha, pi and mu) are present only in animals and yeasts but absent in bacteria and plants while theta is present in yeasts, plants, bacteria, rats, humans, chickens, salmon, and non vertebrates (Pemble and Taylor, 1992; Taylor *et al.*, 1993).

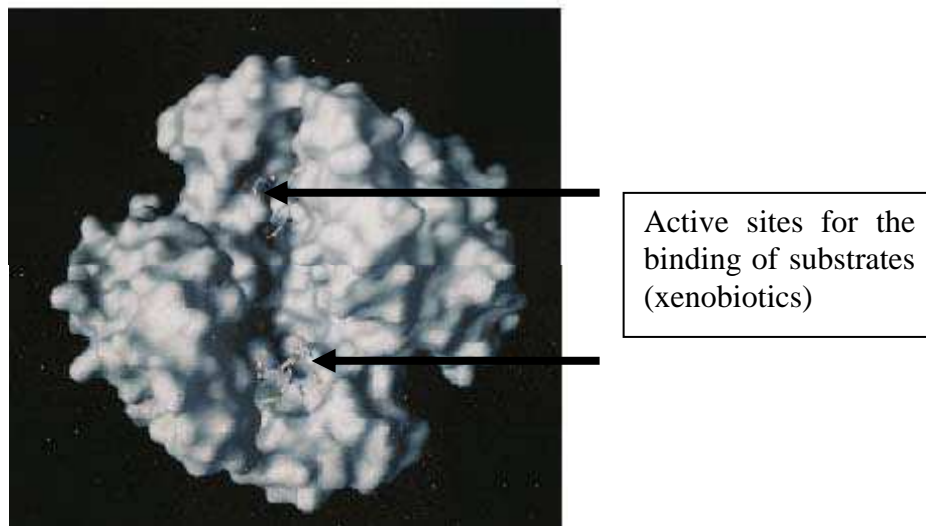
Mammalian cytosolic GSTs are all dimeric with subunits of 199–244 amino acids in length. Depending on amino acid sequence similarities, while seven classes of cytosolic GST are determined in mammalian species, designated Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta (Hayes and Pulford, 1995; Armstrong, 1997; Sheehan *et al.*, 2001), the other cytosolic GST subunits, namely Beta, Delta, Epsilon, Lambda, Phi, Tau, and the “U” class, have been identified in nonmammalian species (Sheehan *et al.*, 2001; Ding *et al.*, 2003; Edwards and Dixon, 2004).

In addition to catalyzes conjugation, reduction, and isomerization reactions, cytosolic GSTs bind, covalently and noncovalently, hydrophobic nonsubstrate ligands (Hayes and Pulford, 1995). This type of activity contributes to intracellular transport, sequestration, and disposition of xenobiotics and hormones. Such compounds include azo-dyes, bilirubin, heme, PAHs, steroids, and thyroid hormones (Hayes and Pulford, 1995).

The mammalian mitochondrial class Kappa GST isoenzymes are dimeric and consist of 226 amino acids. Distinct from cytosolic GSTs, mouse, rat, and human possess only a single Kappa GST (Ladner *et al.*, 2004; Robinson *et al.*, 2004; Jowsey *et al.*, 2003; Morel *et al.*, 2004). Although the majority of cytosolic GST isoenzymes are found in the cytoplasm of the cell, mouse and human Alpha-class GSTA4-4 can associate with mitochondria and membranes (Gardner and Gallagher, 2001; Raza *et al.*, 2002; Robin *et al.*, 2003). It has also been shown to

be located in peroxisomes (Morel *et al.*, 2004). The presence of GSTK1-1 in both organelles suggests it may be specifically involved in  $\beta$ -oxidation of fatty acids, either through its catalytic activity, some transport function, or interaction with a membrane pore (Hayes *et al.*, 2005)

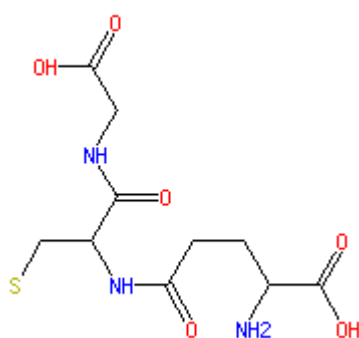
As shown Figure 1.8, GST subunit consists of two domains, each containing two binding sites, the G site and the H site. The highly conserved G site binds the tripeptide glutathione (GSH) and is largely composed of amino acid residues found in the N-terminal domain. The H-site or electrophilic substrate binding site is more variable in structure and is largely formed from residues at the C-terminus.



**Figure 1. 8** Structure of glutathione S-transferase. The dimeric molecule is globular with a prominent large cavity formed between the two subunits. The active site is located in a cleft situated between domains I and II and each subunit can binds two molecules (taken from Reinemer *et al.*, 1996; Schröder, 2001).

Glutathione S-transferase has a physiological role in initiating the detoxification of potential alkylating agents, including pharmacologically active compounds. These enzyme catalyze the reaction of such compounds with -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble (Habig *et. al.*, 1974).

Glutathione (GSH; C<sub>10</sub>H<sub>17</sub>N<sub>3</sub>O<sub>6</sub>S), shown in Figure 1.9 is a tripeptide (L-glutamyl-L-cysteinyl-glycine) and first reported in 1988 as a phytothion, distributed in the intracellular space of plants, animals, and microorganisms has two general functions: to remove toxic metabolites from the cell and to maintain cellular sulfhydryl groups in their reduced form (Bendich, 1988). The antioxidant function of GSH is mediated by the SH group of cysteine which upon oxidation, forms a disulfide bond with a second molecule of GSH to form oxidized glutathione. GSH can also form mixed disulfides with proteins or other thiols, such as coenzyme A. The negative redox potential (Eo' = -0.34 V) of GSH allows for efficient reduction of dehydroascorbate or disulfides bond in proteins (Alscher *et. al.*, 1993).



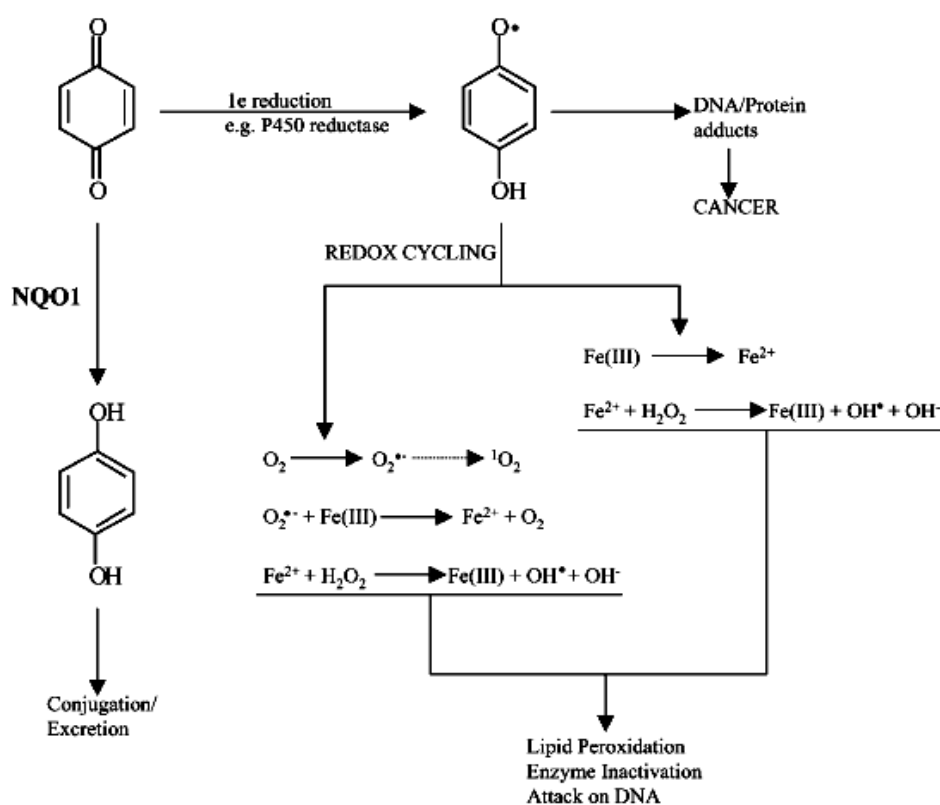
**Figure 1.9** Chemical structure of Glutathione. -SH is the most biochemically active thiol group that can react with nucleophiles.

GST serves as a mobile pool for long distance transport of reduced sulfur between different organs and a reservoir of reduced sulfur that can be remobilized under increased demand (Rennenberg, 2001). It was reported that in order to detoxify not only the xenobiotics but also endogenous compounds, glutathione S-transferase binds non-covalently with many organic anions as well as bilirubin, hormones, dyes and drugs and covalently binds with carcinogens and/or their metabolites (Litwack, 1971; Habig *et al.*, 1974; Ketley *et al.*, 1975).

#### **1.2.2.2 NAD(P)H:Quinone Oxidoreductase 1 (NQO1)**

NAD(P)H:quinone oxidoreductase 1 (NQO1; also termed as nicotinamide quinone oxidoreductase 1, DT-diaphorase, quinone reductase type 1, or menadione reductase; EC 1.6.99.2) is an obligate two-electron reductase that catalyzes reduction of a broad range of substrates including quinones, quinone-imines, and nitro-compounds (Ross *et al.*, 2000). NQO1 is a FAD-containing protein that exists as a homodimer and is biochemically characterized by its unique ability to use either NADH or NADPH as reducing cofactors and by its inhibition by the anticoagulant dicumarol (Ernster, 1967; Edwards *et al.*, 1980). The enzyme is generally considered as a detoxification enzyme because of its ability to reduce reactive quinones and quinone-imines to less reactive and less toxic hydroquinones. It was discovered by Ernster and his colleagues in Stockholm in the late fifties (Ernster *et al.*, 1958; Ernster and Navazio, 1958). Apart from other quinone reductases such as cytochrome P450 reductase or cytochrome b5 reductases which catalyze reduction via one electron mechanisms leading to the generation of semiquinone radicals and, in the presence of oxygen, NQO1 obligates two electron reductase which reduces two electrons of quinones and generate hydroquinones (Ernster, 1967; Ross, 1997).

The mechanism of NQO1 is illustrated in Figure 1.10. Actually quinones are not the only substrate of NQO1 but also quinone-imines, nitro and azo compounds can be mentioned in NQO1 substrates (Ernster, 1967; Lind *et al.*, 1990). Although NQO1 was described in numerous species, it was found at very low level in normal human livers (Aleksunes, 2006).



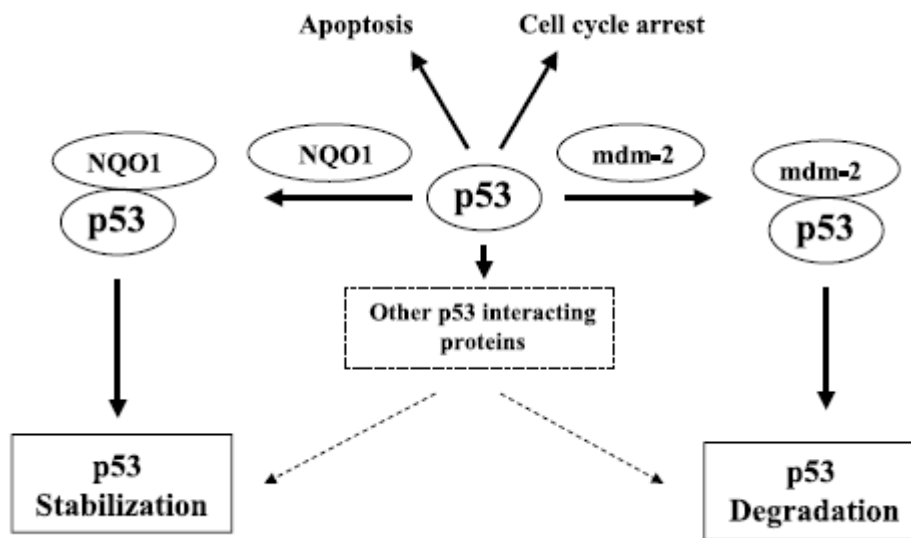
**Figure 1. 10** Proposed role of NQO1 in both chemoprotection/detoxification and bioactivation (taken from Nioi, 2004).

A hypothetical scheme for the metabolism of a simple quinone (1,4-benzoquinone) is shown in Figure 1.10. Single-electron reduction (by P450 reductase, for example) produces highly reactive semiquinone species in which an oxygen atom contains an unpaired electron. Due to this electrophilicity, semiquinones are capable of direct reaction with cellular macromolecules including protein and DNA, and this may ultimately lead to neoplasia. Redox-



cycling may also occur; the unpaired electron from the semiquinone can be used to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , which in turn drives the Fenton reaction leading to hydroxyl radical production. Alternatively, a semiquinone may reduce molecular oxygen, which can lead to generation of singlet oxygen and superoxide-driven Fenton reactions. ROS can cause lipid peroxidation, enzyme inactivation and they can attack DNA directly. The exact consequences of semiquinone production will depend upon the chemical in question. NQO1 catalyses the obligatory two-electron reduction of quinones, thus bypassing the semiquinone intermediate. Fully reduced hydroquinones are generally less toxic to the cell and are more readily conjugated and excreted.

Increased expression of NQO1 enhances three defensive strategies; a) direct catalytic action; single step detoxification of quinones, b) indirect effect; maintenance of endogenous antioxidants in their reduced and active forms, c) regulation of the stability of the p53 tumor suppressors protein (Figure 1.11) (Dinkova-Kostova, 2000; Ross, 2000; Talalay, 2004).



**Figure 1. 11** A proposed mechanism of p53 stabilization by NQO1 involving a protein–protein interaction (taken from Ross, 2004).

### 1.2.3 Effect of Tannic Acid on P450 Dependent Phase I and Phase II Enzymes

Tannic acid plant phenolic compound was reported to inhibit the mutagenicity of the direct acting mutagen benzo[a]pyrene-7,8-diol- 9,10-epoxide in *Salmonella typhimurium* and in Chinese hamster V-79 cells (Huang *et al.*, 1983). It is also an inhibitor of epidermal monooxygenase and inhibits the binding of benzo[a]pyrene and benzo[a]pyrene 7,8-dihydrodiol to DNA (Das *et al.*, 1987). Teel and Huynh (1998) showed that tannic acid was a potent inhibitor of not only BROD, MROD and EROD activities of hamster liver microsomes but also CYP 2C11, 1A and 2C6 enzyme activities. In their in vivo study, Krajka-Kuźniak *et al.* (2004) showed that tannic acid had no significant effect on Phase II enzymes; NQO1 in liver and also in kidney of 3-MC-induced rat, however the activity of NQO1 was reduced (about 43%) to control level by tannic acid pretreatment. Moreover, they expressed that tannic acid inhibited mouse hepatic cytochromes

P450 1A1, 1A2 and 2B mediated enzyme activities in vitro even to a greater extent ( $IC_{50}$  2.6 –7  $\mu$ M) than CYP2E1 mediated enzyme activity in the current study (Das *et al.*, 1987; Baer-Dubowska *et al.*, 1998).

Mikstacka *et al.* (2002) had investigated the effect of tannic acid on hepatic PNPB in acetone induced mouse microsomes and claimed that tannic acid was a mixed type inhibitor ( $K_I = 1 \mu$ M).

## Scope of This Study

Incidence rates of cancer continue to rise with a significant ratio in the last century. Even more, cancer is the second leading cause of death after cardiovascular diseases in the world. There is a link between certain cancers and human exposure to toxic chemicals in the environment. In order to generate mutagenic and carcinogenic metabolites, the majority of these chemicals require metabolic activation by the enzymes. Therefore anti-carcinogen activity of tannic acid, a hydrolysable plant polyphenol, may have a crucial importance to prevent conversion of pro-carcinogens to their carcinogenic form. There are several investigations mainly focus on laboratory animals such as rats and mice. However no data is available regarding the effects of tannic acid on rabbit Phase I and Phase II enzymes. It is necessary to carry out comparative studies using different laboratory animals in order to learn more about the species differences in the xenobiotic metabolism and effect of those molecules including plant polyphenolics. This study was undertaken to elucidate the possible *in vitro* effects of plant phenolic compound tannic acid for its ability to modulate cytochrome P4502E1 mediated enzyme activities of N-nitrosodimethylamine N-demethylase (NDMA N-demethylase), *p*-nitrophenol hydroxylase (PNPH) and aniline 4-hydroxylase involved in the metabolic activation and detoxification of known environmental agents including mutagens and carcinogens. In addition, *in vitro* effects of tannic acid on cytosolic phase II enzymes; Glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase:1 (NQO1) were also investigated.

In order to achieve these goals, first microsomal and cytosolic fractions from rabbit liver and kidney were prepared by differential centrifugation. Those fractions were preincubated in the presence of tannic acid (0.1 - 150  $\mu$ M) for 10 minutes at room temperature and Phase I and Phase II enzyme activities given above were determined by spectrophotometry. The induction/inhibition kinetics of Phase I and Phase II enzymes by tannic acid may provide insight into the prescription of anticarcinogenic drug development. To our knowledge, this study is the first concerning the effects of tannic acid on Phase I and Phase II enzymes of rabbit liver and kidney.

## CHAPTER 2

### MATERIAL AND METHODS

#### 2.1 Chemicals

Tannic acid (T0200), 2,6 dichlorophenol-indophenol (DCPIP; D1878), 1-chloro-2,4-dinitrobenzene (CDNB; C6396), ammonium acetate (A7672),  $\epsilon$ -amino caproic acid ( $\epsilon$ -ACA; A2504), bovine serum albumin (BSA; A7511), D-glucose-6-phosphate monosodium salt (G7879), D-glucose-6-phosphate dehydrogenase type IX (G8878), glutathione reduced form (GSH; G4251), N-2-hydroxyethylpiperazine-N-2,ethane sulfonic acid (HEPES; H3375), N-nitrosodimethylamine (NDMA; N3632), phenylmethane sulfonyl fluoride (PMSF; P7626), sodium potassium tartarate (Rochell salt; S2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T1378), were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Ethylene diamine tetra acetic acid disodium salt (EDTA; 08421), glacial acetic acid (0056), glycerol (04093), magnesium chloride ( $MgCl_2$ ; 05833), methanol (02500), *p*-nitrophenol (106798), potassium chloride (KCl 104935), potassium dihydrogen phosphate ( $KH_2PO_4$ ; 04871), di-potassium hydrogen phosphate ( $K_2HPO_4$ ; 05101), sodium carbonate (06398), sodium hydroxide (06462), trichloroacetic acid (TCA; 00256) were the products of E. Merck, Darmstadt, Germany.

Aniline (A0759) was obtained from Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, USA. Formaldehyde was obtained from Fluka A.G., Switzerland. Absolute ethanol (32221) and acetyl acetone (33005) were taken from Riedel, Germany.

$\beta$ -nicotinamide adenine dinucleotide phosphate reduced form (NADPH; A1395),  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>; N0505), were taken from Applichem, Darmstadt, Germany.

All the other chemicals used in this study were of analytical grade and were obtained from commercial sources at the highest grade of purity.

## **2.2 Method**

### **2.2.1 Preparation of Rabbit Liver and Kidney Cytosols and Microsomes**

2.5 months old adult male New Zealand white rabbits, initially weighing 1.5-2.2 kg. were purchased from Lameli Husbandary, Ankara. The rabbits were caged separately and maintained at 23-25 °C on a 12 h light / 12 h dark cycle with free access to water and commercial chow for 7 days before the beginning of the treatments. The procedures involving animals and their care carried out in accordance with the Declaration of Helsinki.

Rabbit liver and kidney microsomes were prepared as described by the method of Arınç and Adalı (1990). The livers and kidneys were removed immediately after killing the animals by decapitation. Gall bladders were removed from the livers to prevent release of its contents that are known to be inhibitory to monooxygenase activity. All subsequent steps were carried out at 0-4 °C. After removal of the connective and fatty tissues, livers and kidneys were washed with cold distilled water and then with 1.15% KCl solution several times to remove the excess blood. After blotting the tissues by the help of a filter paper, tissues were weighed and chopped with scissors. The resulting minced tissues were homogenized in 1.15 % KCl solution containing 2 mM EDTA pH 7.2, 0.25 mM  $\epsilon$ -ACA and 0.1 mM PMSF (described in Table 2.1) at a volume of equal to 3 times of weight of liver and 2.2 times weight of kidney by using Potter-Elvehjem glass homogenizer coupled with a motor (Black and Decker, V850, multispeed drill)-driven teflon pestle at 2 400 rpm. Fifteen passes were made for the homogenization of both liver and kidney tissues.

The resulting liver and kidney homogenate was centrifuged at 9 500 rpm (10 800 x g) (Sigma 3K30 Centrifuge, Osterode am Harz, Germany) with 12156 type rotor for 25 minutes to remove cell debris, nuclei and mitochondria.

The supernatant fraction containing endoplasmic reticulum and soluble fraction of the cell was filtered through double layers of cheese cloth in a Buchner funnel. The microsomes were sedimented from the supernatant by centrifugation at 45 000 rpm (145 215 x g) for 50 minutes using Sorval-Combi Ultracentrifuge (Dupond Company, Newton, Connecticut, USA) with T270 type rotor.

The supernatant fraction (cytosol) was removed and stored at -80 °C until use in determination of activity of cytosolic enzymes. The firmly packed microsomal pellet was suspended in 1.15% KCl solution containing 2 mM EDTA (described in Table 2.1) and resedimented by ultracentrifugation at 45 000 rpm (145 215 x g) for 50 minutes by using Sorval-Combi Ultracentrifuge (Dupond Company, Newton, Connecticut, USA) with T270 type rotor.

Then the washed microsomal pellet was resuspended in 25 % glycerol containing 1 mM EDTA at a volume of 0.5 ml for each gram of liver tissue and 0.3 ml for each gram of kidney tissue. In order to obtain a homogenous microsomal suspension, resuspended microsomes were homogenized manually using the glass-teflon homogenizer.

The microsomal suspensions containing approximately 25-40 mg of microsomal protein per milliliter were then gassed with nitrogen in Nalgene tubes and stored at -80 °C for enzymatic assays.

**Table 2. 1** The Constituents of Homogenization Mixture for Rabbit Liver and Kidney Tissues and Suspension of Liver and Kidney Microsomes.

Preparation of Stock KCl-EDTA Solution			
Constituents	Stock Concentration	Volume/Gram to be added	Final Concentration
EDTA pH 7.2	100 mM	20 ml	2 mM
KCl	-	11.5 g	1.15 % (w/v)
Final Volume is completed to 1 L with distilled water			

Tissue Homogenization Solution			
Constituents	Stock Concentration	Volume to be added	Final Concentration
PMSF	100 mM	0.1 ml	0.1 mM
$\epsilon$ -ACA	100 mM	0.25 ml	0.25 mM
Final volume is completed to 100 ml with 1.15 % (w/v) KCl-EDTA Solution			

Microsome Suspension Solution			
Constituents	Stock Concentration	Volume to be added	Final Concentration
EDTA pH 7.2	100 mM	0.5 ml	2.5 mM
Glycerol	-	5 ml	25 % (v/v)
Final volume is completed to 20 ml with distilled water			



### 2.2.2 Determination of Protein Concentration

Protein concentrations of microsomal and cytosolic fractions were determined according to the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

The principle of the Lowry method depends on the reactivity of the peptide nitrogen with the copper ions under alkaline conditions and the subsequent reduction of Folin reagent to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids (Lowry *et al.*, 1951).

#### **Reagents:**

Reagent I: 2% (w/v)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

Reagent II: 2% (w/v) Na-K tartarate ( $\text{NaKC}_4\text{H}_4\text{O}_6$ )

Reagent A: 2% (w/v)  $\text{Na}_2\text{CO}_3$  in 0.1 N NaOH

Lowry Alkaline Copper Reagent: Reagent I, Reagent II and Reagent A are mixed in the same order with the ratio of 1:1:100.

Folin-phenol reagent: 2 N Stock Folin reagent is diluted to 1 N with distilled water (store in dark bottle).

Before the preparation of the reaction tubes, initial dilution was done for microsomes and cytosols. 200 times dilution was done for both liver and kidney microsomes and also cytosols. In addition to initial dilutions, dilution within tube was carried out by taking 0.1 to 0.5 ml of initially diluted samples into reaction tubes and completed it to final volume of 0.5 ml with distilled water. After that 2.5 ml of Lowry Alkaline Copper Reagent was added to each tube. All tubes were mixed and let stand for 10 minutes at room temperature for copper reaction in alkaline medium. Then 0.25 ml of diluted Folin reagent (1 N) was added to the each tube and tubes mixed within 8 seconds by vortex. The tubes were incubated for 30 minutes at room temperature. The intensity of resulting color was measured at 660 nm. Standard tubes with five different protein concentrations (20, 50, 100, 150, 200  $\mu\text{g}$  BSA in 0.5 ml final volume) were prepared from crystalline bovine

serum albumin and same steps used for samples were carried out for standard tubes. A standard curve was drawn by using intensity readings of standards and slope of this curve was used for the calculation of protein amounts in the sample.

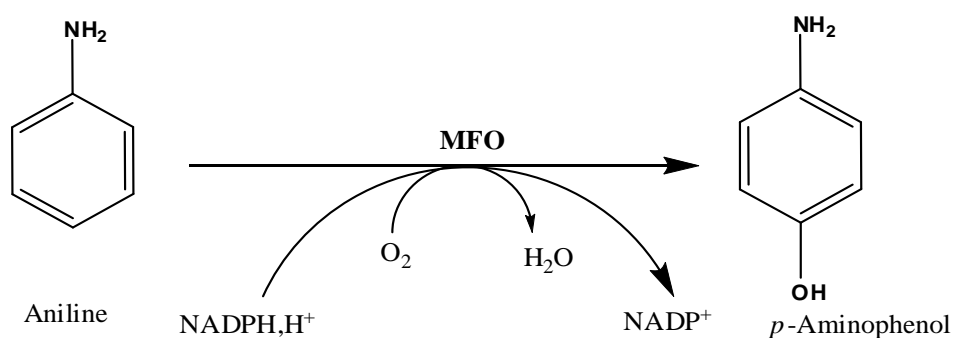
Protein concentration was calculated by the following formula: (2.1)

$$\text{Protein Concentration (mg/ml)} = \frac{\text{OD}_{660 \text{ nm}}}{\text{Slope of Standards}} \times \text{Dilution}_{\text{in tubes}} \times \text{Dilution}_{\text{initial}}$$

## 2.2.3 Determination of Mixed Function Oxidase (MFO) Enzyme Activities

### 2.2.3.1 Determination of Aniline 4-Hydroxylase Activity

Aniline 4-hydroxylase activity of rabbit liver and kidney microsomes was determined by measuring the quantity of *p*-aminophenol (pAP) formed according to the method of Imai *et al.* (1966). Aniline 4-hydroxylation reaction is given in Figure 2.1.



**Figure 2. 1** Aniline 4-hydroxylation reaction.

#### **Reagents:**

1. Hepes Buffer: 400 mM, pH 7.6
2. Aniline: 100 mM (Light sensitive)
3. Trichloro Acetic Acid (TCA): 20% (w/v) (Light sensitive)
4. Sodium carbonate: 20% (w/v)
5. 4 % Phenol in 0.4 N NaOH (Light sensitive)

A typical reaction mixture given in Table 2.2 contained 100 mM HEPES buffer pH 7.6, 10 mM aniline, 1.5 mg microsomal protein for liver, 4 mg microsomal protein for kidney and 0.5 mM NADPH generating system in a final volume of 1.0 ml.

NADPH generating system was prepared by adding 0.5 units of glucose 6-phosphate dehydrogenase into a test tube containing 2.5 mM glucose 6-phosphate, 2.5 mM MgCl<sub>2</sub>, 14.6 mM HEPES buffer pH 7.8 and 0.5 mM NADP<sup>+</sup>. Then the test tube containing generating system was incubated 37 °C for 5 minutes and finally kept in crushed ice. 1 unit of glucose 6-phosphate dehydrogenase is equal to the amount of enzyme reducing 1 µmole of NADP<sup>+</sup> in one minute at 25 °C.

The reaction was initiated by the addition of 0.15 ml of NADPH generating system to the microsomal incubation mixture and to zero time blank (ZTB) to which 0.5 ml of 20 % TCA was added before the addition of cofactor. Zero time blanks were prepared to measure non-enzymatic product formation. Reaction was carried out at 37 °C for 25 minutes aerobically with moderate shaking in a water bath (Nuve Instruments Ltd., 06640 Ankara-Turkey). At the end of the incubation time, the reaction was stopped by addition of 0.5 ml 20 % TCA. Then incubation mixture was transferred to ependorf tubes and denaturated proteins were removed by centrifugation at 13 100 rpm (16 000 x g) for 25 minutes by using Sigma 1-15 microcentrifuge. Finally, 1 ml of supernatant containing product, *p*-aminophenol, was removed and mixed with 0.5 ml of 20% Na<sub>2</sub>CO<sub>3</sub> and with 0.5 ml of 0.4 N NaOH solution containing 4 % phenol. The resulting mixture was incubated at 37 °C for 30 minutes. The intensity of blue color developed was measured at 630 nm using Shimadzu UV-1201 spectrophotometer (Schimadzu Corporation, Analytical Instruments Division, Kyoto- Japan).

*p*-Aminophenol solution was used as a standard. Since it is light sensitive, 0.5 mM *p*-aminophenol solution was prepared freshly and kept in the dark. Standards at four different *p*-aminophenol concentration (2.5, 5, 12.5, 25 nmoles) containing aniline and other incubation constituents were run under the same conditions as for reaction mixture. A standard curve of *p*-aminophenol was constructed and used for the calculation of enzyme activity.

The effect of tannic acid on aniline 4-hydroxylase activity was studied by adding various concentrations of tannic acid; 5 μM, 10 μM, 25 μM, 35μM, 50 μM, 75 μM, 100 μM, and 150 μM into the reaction mixture. Liver and kidney microsomes were preincubated in the presence of tannic acid for 10 minutes at room temperature and then the enzyme reaction was started by adding NADPH generating system into the reaction mixture. Tannic acid containing reaction medium was also run under the same conditions as given before.

Aniline 4-hydroxylase activity was determined according to the following formula: (2.2)

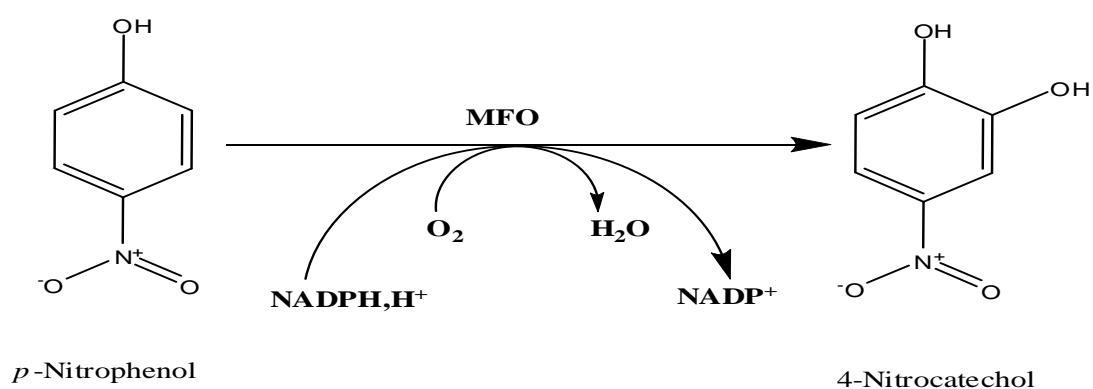
$$\text{Specific Enzyme Activity (nmol/min/mg)} = \frac{\text{OD}_{\text{Reaction}} - \text{OD}_{\text{ZTB}}}{6 \times 10^{-3} \mu\text{M}^{-1} \times \text{cm}^{-1}} \times \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{25 \text{ min}}$$

**Table 2.2** The Constituents of the Incubation Mixture for Determination of Aniline 4-Hydroxylase Activity in Rabbit Liver and Kidney Microsomes.

<b>Constituents</b>	<b>Stock Concentration</b>	<b>Volume to be added</b>	<b>Final Concentration</b>
Hepes buffer pH 7.6	400 mM	250 $\mu$ l	100 mM
Aniline	100 mM	100 $\mu$ l	10 mM
Microsomal fraction	-	-	1.5 mg for liver 4 mg for kidney
<b>NADPH generating system</b>			0.5 mM
Glucose 6-phosphate	100 mM	25 $\mu$ l	2.5 mM
MgCl <sub>2</sub>	100 mM	25 $\mu$ l	2.5 mM
Hepes Buffer pH 7.8	200 mM	73 $\mu$ l	14.6 mM
NADP <sup>+</sup>	20 mM	25 $\mu$ l	0.5 mM
Glucose 6-phosphate dehydrogenase	500 U	1 $\mu$ l	0.5 U
Final volume is completed to 1 ml with distilled water			

### 2.2.3.2 Determination of *p*-Nitrophenol Hydroxylase Activity

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol (1,2-dihydroxy-4-nitrobenzene) was determined using the method of Reinke and Moyer (1985). The assay conditions that were optimized for lung microsomal *p*-nitrophenol hydroxylase by Arınç and Aydoğmuş (1990) were also used for the determination of rabbit liver and kidney microsomal *p*-nitrophenol hydroxylase activity. The reaction of *p*-nitrophenol hydroxylation is given in Figure 2.2.



**Figure 2.2** *p*-Nitrophenol hydroxylation reaction.

#### **Reagents:**

1. Tris Buffer: 400 mM, pH 6.8
2. *p*-nitrophenol: 2.5 mM (Light sensitive)
3. Perchloric Acid: 0.75 N (Light sensitive)
4. NADPH Generating System: 0.5 mM (Prepared fresh)
5. NaOH: 10 N

The reaction medium given in Table 2.3 contained 100 mM Tris-HCl buffer pH 6.8, 0.25 mM *p*-nitrophenol, 1.5 mg and 4 mg of microsomal protein for liver and kidney, respectively and 0.5 mM NADPH generating system in a final volume of 1.0 ml.

*p*-Nitrophenol hydroxylation reaction was started by the addition of 0.15 ml NADPH generating system and carried out for 10 minutes at 37 °C with moderate shaking in a water bath. 0.5 ml of 0.75 N perchloric acid was added to zero time blank tubes just before addition of NADPH generating system. The enzyme reaction was terminated by the addition of 0.5 ml 0.75 N perchloric acid. The contents of mixture transferred to ependorf tubes and were centrifuged at 13 100 rpm (16 000 x g) for 25 minutes for removal of denatured proteins.

After centrifugation, 1.0 ml aliquot of the supernatant was taken and mixed with 0.1 ml of 10 N NaOH for complete ionization of 4-nitrocatechol. Then the mixture was centrifuged at 13 100 rpm (16 000 x g) for 5 minutes in order to remove NaOH precipitates. 4-nitrocatechol formed as a result of *p*-nitrophenol hydroxylase activity of microsomal enzymes was determined spectrally at 546 nm. The enzyme activity was determined by using extinction coefficient of 9.53 mM<sup>-1</sup> x cm<sup>-1</sup> (Koop *et al.*, 1986).

The effect of tannic acid on *p*-nitrophenol hydroxylase activity was studied by adding various concentrations of tannic acid in a final concentration of 1 μM, 5 μM, 10 μM, 25μM, 50 μM, and 100 μM, into the reaction mixture. Liver and kidney microsomes were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding NADPH generating system into the reaction mixture. Tannic acid containing reaction medium was also run under the same conditions as given before.

*p*-Nitrophenol hydroxylase activity was determined according to the following formula: (2.3)

$$\text{Specific Enzyme Activity } (\mu\text{mol/min/mg}) = \frac{\text{OD}_{\text{reaction}} - \text{OD}_{\text{ZTB}}}{9.53 \text{ mM}^{-1} \times \text{cm}^{-1}} \times \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{10 \text{ min.}}$$

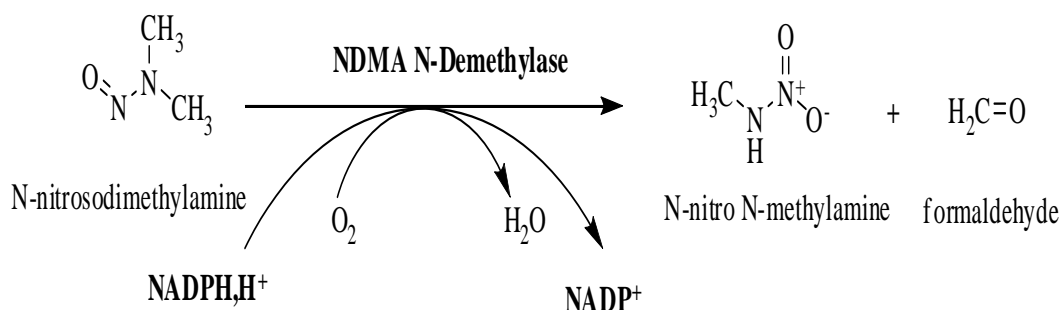


**Table 2.3** The Constituents of the Incubation Mixture for Determination of *p*-Nitrophenol Hydroxylase Activity in Rabbit Liver and Kidney Microsomes.

<b>Constituents</b>	<b>Stock Concentration</b>	<b>Volume to be added</b>	<b>Final Concentration</b>
Tris buffer, pH 6.8	400 mM	125 $\mu$ l	50 mM
<i>p</i> -nitrophenol	2.5 mM	100 $\mu$ l	0.25 mM
Microsomal fraction	-	-	1.5 mg for liver 4 mg for kidney
<b>NADPH generating system</b>			0.5 mM
Glucose 6-phosphate	100 mM	25 $\mu$ l	2.5 mM
MgCl <sub>2</sub>	100 mM	25 $\mu$ l	2.5 mM
Hepes Buffer, pH 7.8	200 mM	73 $\mu$ l	14.6 mM
NADP <sup>+</sup>	20 mM	25 $\mu$ l	0.5 mM
Glucose 6-phosphate dehydrogenase	500 U	1 $\mu$ l	0.5 U
Final volume is completed to 1 ml with distilled water			

### 2.2.3.3 Determination of N-Nitrosodimethylamine (NDMA) N-Demethylase Activity

NDMA N-demethylase activity of rabbit liver and kidney microsomes was determined according to the method of Gorsky and Hollenberg (1989) and formaldehyde formed was measured by the method Nash (1953) as modified Cochin and Axelrod (1959). NDMA N-demethylase reaction is shown in Figure 2.3.



**Figure 2.3** N-Nitrosodimethylamine (NDMA) N-demethylation reaction.

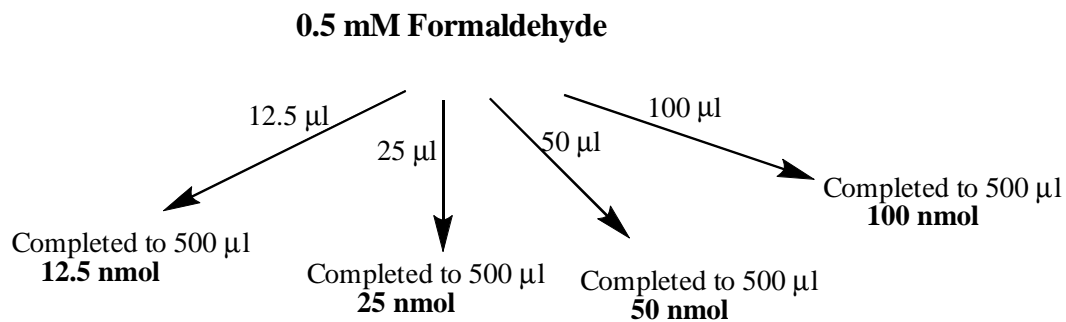
#### **Reagents:**

1. Hepes Buffer: 400 mM, pH 7.7
2. N-Nitrosodimethylamine (NDMA): 25 mM
3. Perchloric Acid: 0.75 N
4. NADPH Generating System : 0.5 mM
5. Nash Reagent: Prepared by the addition of 0.4 ml acetylacetone to the 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid.
6. Formaldehyde: 0.5 mM (Light sensitive)

A 0.5 ml of incubation mixture given in Table 2.4 contained 100 mM HEPES buffer pH 7.7, 2.5 mM NDMA, 0.75 mg microsomal protein for liver and 2 mg microsomal protein for kidney and 0.5 mM NADPH generating system in final concentration. The enzyme reaction was initiated by the addition of 0.075 ml NADPH generating system to incubation mixture and to zero time blank to which 0.5 ml of 0.75 N perchloric acid was added just before the cofactor. The reaction was carried out at 37 °C for 20 minutes under the air with constant moderate shaking in a water bath. The enzyme reaction was stopped by the addition of 0.5 ml 0.75 N perchloric acid at the end of 20 minutes. Then the contents of the tubes were transferred to the ependorf centrifuge tubes and denatured proteins were removed by the centrifugation at 13 100 rpm (16 000 x g) for 25 minutes. Finally, 0.5 ml aliquots of supernatant were taken and were mixed with freshly prepared 0.375 ml of Nash reagent. The mixture was incubated for 10 minutes at 50 °C in a water bath and the intensity of yellow color developed was measured at 412 nm using Shimadzu UV-1201 spectrophotometer.

The effect of tannic acid on NDMA N-demethylase activity was studied by adding various concentrations of tannic acid; 0.5 µM, 1 µM, 5 µM, 10 µM, 50 µM, and 100 µM, into the reaction mixture. Liver and kidney microsomes were preincubated in the presence of tannic acid for 10 minutes at room temperature and then the reaction was started by adding NADPH generating system into the reaction mixture. Tannic acid containing reaction medium was also run under the same conditions as given before.

A 0.5 mM freshly prepared formaldehyde solution was used as a standard as described in Figure 2.4. The tubes containing standards at four different concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity.



**Figure 2. 4** Preparation of formaldehyde standards.

N-Nitrosodimethylamine (NDMA) N-demethylase activity was calculated according to the following formula: (2.4)

$$\text{Specific Enzyme Activity (nmol/min/mg)} = \frac{\text{OD}_{\text{Reaction}} - \text{OD}_{\text{ZTB}}}{2.4 \times 10^{-3} \mu\text{M}^{-1} \times \text{cm}^{-1}} \times \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{20 \text{ min.}}$$

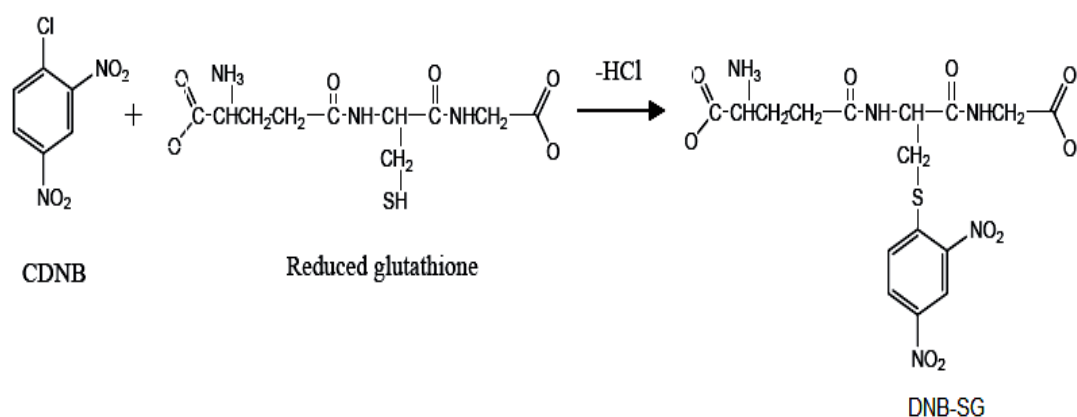
**Table 2.4** The Constituents of the Incubation Mixture for Determination of N-Nitrosodimethylamine (NDMA) N-Demethylase Activity in Rabbit Liver and Kidney Microsomes.

<b>Constituent</b>	<b>Stock Concentration</b>	<b>Volume to be added</b>	<b>Final Concentration</b>
Hepes buffer, pH 7.7	400 mM	125 $\mu$ l	100 mM
NDMA	25 mM	50 $\mu$ l	2.5 mM
Microsomal fraction	-	-	1.5 mg for liver 4 mg for kidney
<b>NADPH generating system</b>			0.5 mM
Glucose 6-phosphate	100 mM	12.5 $\mu$ l	2.5 mM
MgCl <sub>2</sub>	100 mM	12.5 $\mu$ l	2.5 mM
Hepes Buffer, pH 7.8	200 mM	36.5 $\mu$ l	14.6 mM
NADP <sup>+</sup>	20 mM	12.5 $\mu$ l	0.5 mM
Glucose 6-phosphate dehydrogenase	500 U	1 $\mu$ l	0.5 U
Final volume is completed to 0.5 ml with distilled water			

## 2.2.4 Determination of Phase II Enzyme Activities

### 2.2.4.1 Determination of Total Glutathione S -Transferase (GST) Activities

Glutathione S-transferase (GST) activity of rabbit liver and kidney cytosolic fractions was determined according to the method of Habig *et al.* (1974) with slight modifications. 1-Chloro-2,4-dinitrobenzene (CDNB) was used as a substrate for determination of total glutathione S-transferase activity. The rate of glutathione conjugate (1-(S-glutathionyl)-2,4-dinitrobenzene (DNB-SG)) formation was monitored by following the rate of increase in the absorbance at 340 nm. The reaction catalyzed by glutathione S-transferase is given in Figure 2.5.



**Figure 2.5** Reaction catalyzed by GST.

#### **Reagents:**

Potassium Phosphate Buffer: 50 mM, pH 7.0

\* GSH: 20 mM

\* CDNB: 20 mM (CDNB was first dissolved in absolute ethanol then completed to final volume with distilled water. Ethanol should not exceed to 3 % of assay mixture. Store in dark bottle)

\* It should be prepared fresh.

A typical assay mixture given in Table 2.5 contained 2500  $\mu\text{l}$  of 50 mM potassium phosphate buffer pH 7.0, 200  $\mu\text{l}$  of 20 mM GSH, 150  $\mu\text{l}$  of cytosolic fractions and 150  $\mu\text{l}$  of 20 mM 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in a final volume of 3 ml. Each time a blank cuvette (reaction with no enzyme) was also used. The reaction was started by addition of 150  $\mu\text{l}$  of 20 mM 1-chloro-2, 4-dinitrobenzene (CDNB). Then thioether formation was followed continuously at 340 nm for 2 minutes using Shimadzu UV-160A UV-visible spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). The absorbance obtained from blank cuvette was subtracted from absorbance of enzymatic reaction to eliminate non-enzymatic product formation.

The enzyme activity was calculated by using  $0.0096 \mu\text{M}^{-1} \times \text{cm}^{-1}$  as an extinction coefficient of thioether formed by GST.

The effect of tannic acid on GST activity was studied by adding various concentrations of tannic acid; 0.05  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , 0.2  $\mu\text{M}$ , 0.25  $\mu\text{M}$ , 0.3  $\mu\text{M}$ , 0.35  $\mu\text{M}$ , 0.4  $\mu\text{M}$ , 0.5  $\mu\text{M}$  and 1  $\mu\text{M}$  into the reaction mixture. Liver and kidney cytosols were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding CDNB into the reaction mixture. Tannic acid containing reaction medium was also run under the same conditions as given before.

GST activity was determined according to the following formula: (2.5)

$$\text{Enzyme Activity (nmol/min/ml)} = \frac{\Delta\text{OD}_{340 \text{ nm}}}{0.0096 \mu\text{M}^{-1} \times \text{cm}^{-1}} \times \frac{\text{Total Volume}}{\text{Volume of Sample}} \times \text{Dilution Factor}$$

**Table 2.5** The Constituents of the Reaction Mixture for Determination of GST Activity of Rabbit Liver and Kidney Cytosol.

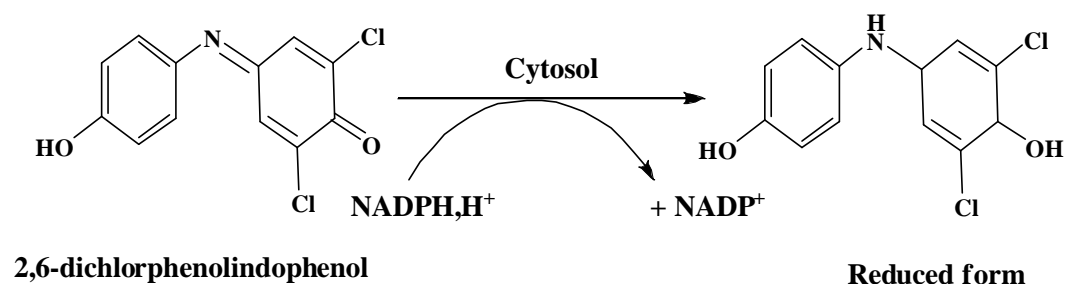
<b>Constituents</b>	<b>Stock Concentration (mM)</b>	<b>Volume to be added (ml)</b>	<b>Final Concentration (mM)</b>
Potassium Phosphate Buffer, pH 7.0	50	2.5	41.6
1-Chloro-2,4-dinitrobenzene (CDNB)	20	0.150	1
Glutathione reduced form (GSH)	20	0.200	1.3
Cytosol	-	0.150	-



#### 2.2.4.2 Determination of NAD(P)H Dehydrogenase, Quinone 1 (NQO1)

##### Activity

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) activity of rabbit liver and kidney cytosols was determined according to the method of Ernster (1960). 2,6-Dichlorophenolindophenol (DCPIP) was used as a substrate for NQO1 and DCPIP reduction was monitored continuously by following the decrease in the absorbance at 600 nm. The reaction catalyzed by NQO1 is given in Figure 2.6.



**Figure 2. 6** The reaction catalyzed by NQO1.

##### Reagents:

1. Potassium Phosphate Buffer: 0.1 M, pH7.4
2. NADPH: 10 mM (Prepared fresh)
3. 2,6-Dichlorophenolindophenol (DCPIP): 0.5 mM (Light sensitive, prepared fresh )
4. Dicoumarol: 0.5 mM (Prepared fresh)
5. BSA: 28 mg/ml

A typical assay mixture described in Table 2.6 contained, 250  $\mu\text{l}$  of 0.1 M potassium phosphate buffer pH 7.8, 25  $\mu\text{l}$  of 28 mg/ml BSA, appropriate amount of cytosolic fraction and 80  $\mu\text{l}$  of 0.5 mM 2,6-dichlorophenolindophenol (DCPIP) in a final volume of 1 ml. Reaction was started by adding 20  $\mu\text{l}$  of 10 mM NADPH to the reaction mixture and to zero time blank (ZTB) to which 40  $\mu\text{l}$  of 0.5 mM dicoumarol was added before the addition of cofactor. Zero time blank was prepared to measure the non-enzymatic product formation. DCPIP reduction reaction was followed continuously at 600 nm for 2 minutes against the blank containing only buffer by using Shimadzu UV-160A UV visible spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). The enzyme activity was calculated using an extinction coefficient of  $0.021 \mu\text{M}^{-1} \times \text{cm}^{-1}$  when DCPIP used as a substrate.

The effect of tannic acid on NQO1 activity was studied by adding various concentrations of tannic acid; 1  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 5  $\mu\text{M}$ , 7.5  $\mu\text{M}$ , 10  $\mu\text{M}$ , 25  $\mu\text{M}$ , 35  $\mu\text{M}$ , and 50  $\mu\text{M}$  into the reaction mixture. Hepatic and renal cytosols were preincubated in the presence of tannic acid for 10 minutes at room temperature and then the reaction was started by adding NADPH into the reaction mixture. Tannic acid containing reaction medium was also run under the same conditions as given before.

NQO1 activity was determined according to the following formula: (2.7)

$$\text{Enzyme Activity (nmol/min/ml)} = \frac{\Delta\text{OD}_{\text{Sample}} - \Delta\text{OD}_{\text{ZTB}}}{0.021 \mu\text{M}^{-1} \times \text{cm}^{-1}} \times \frac{\text{Total Volume}}{\text{Volume of Sample}} \times \text{Dilution Factor}$$

**Table 2.6** The Constituents of the Reaction Mixture for Determination of NQO1 Activity of Rabbit Liver and Kidney Cytosol.

<b>Constituents</b>	<b>Stock Concentration</b>	<b>Volume to be added</b>	<b>Final Concentration</b>
Potassium Phosphate Buffer, pH 7.8	100 mM	250 $\mu$ l	25 mM
Bovine Serum Albumin (BSA)	28 mg/ml	25 $\mu$ l	0.7 mg/ml
Cytosol	-	-	2 - 4 mg/ml
NADPH	10 mM	20 $\mu$ l	0.2 mM
2,6 Dichlorophenol-indophenol (DCPIP)	0.5 mM.	80 $\mu$ l	0.04 mM
Complete to final volume to 1 ml with distilled water			

## **CHAPTER 3**

### **RESULTS**

#### **3.1. Preparation of Rabbit Liver and Kidney Microsomes and Cytosols**

Liver and kidney tissues of rabbit were homogenized and then microsomal and cytosolic fractions were prepared by differential centrifugation. Average protein concentration of three rabbit liver microsomal and cytosolic preparations were found  $36.23 \pm 3.02$  mg/ml and  $35.033 \pm 0.448$  mg/ml, respectively. Moreover, average protein concentrations of three rabbit kidney microsomal and cytosolic preparations were found  $41.77 \pm 1.02$  mg/ml and  $29.28 \pm 1.45$  mg/ml, respectively.

#### **3.2. Effects of Tannic acid on Rabbit Liver and Kidney Mixed Function Oxidase Enzyme Activities**

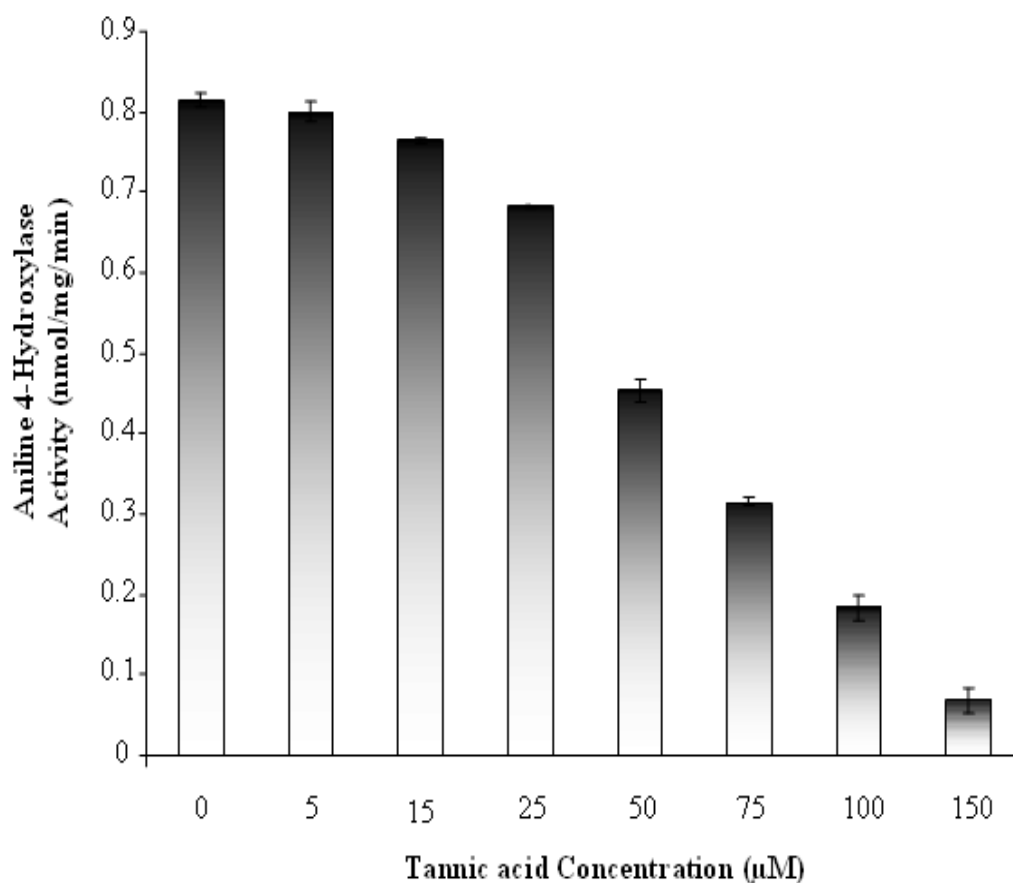
Modulation of rabbit liver and kidney microsomal P450 dependent aniline 4-hydroxylase, NDMA N-demethylase and p-nitrophenol hydroxylase activities were studied in the presence of tannic acid at concentrations ranging from  $0.5 \mu\text{M}$  to  $150 \mu\text{M}$  in the reaction medium.

### **3.2.1 Effect of Tannic Acid on Rabbit Liver and Kidney Microsomal Aniline 4-Hydroxylase Activity**

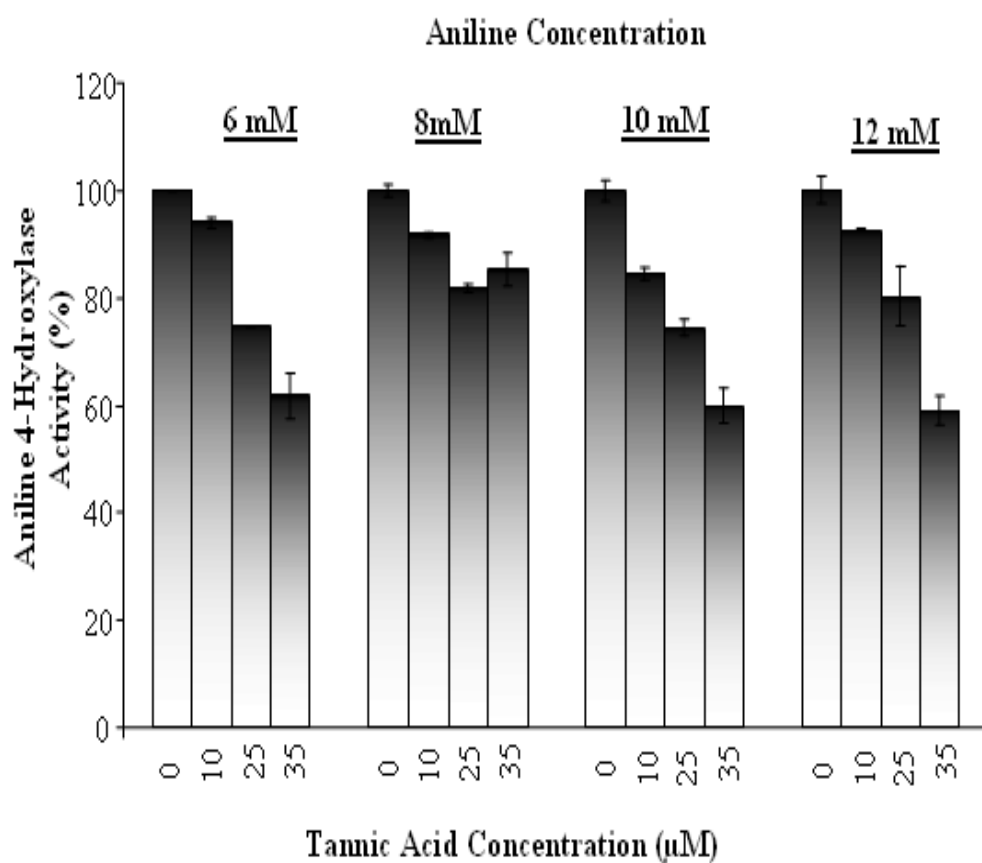
Rabbit liver and kidney microsomal aniline 4-hydroxylase activity in the presence of different concentrations of tannic acid was determined using the method of Imai *et al.* (1966). A range of tannic acid concentration changing from 5  $\mu\text{M}$  to 150  $\mu\text{M}$  was used against various concentrations of substrate aniline from 6 mM to 12 mM to investigate the effect of tannic acid on rabbit microsomal aniline 4-hydroxylase activity.

Figure 3.1 shows that rabbit liver aniline 4-hydroxylase activity was inhibited by increasing the concentration of tannic acid. Although the enzyme reaction was inhibited at all concentrations of tannic acid, inhibition was not significant up to 25  $\mu\text{M}$  tannic acid present in the reaction medium. While half of the liver aniline 4-hydroxylase activity was inhibited at 60  $\mu\text{M}$  tannic acid, only less than 8 % of initial activity remained when liver microsomes preincubated with 150  $\mu\text{M}$  tannic acid.

Modulation kinetic of rabbit liver microsomal aniline 4-hydroxylase activity by tannic acid was studied by increasing the tannic acid concentration from 10  $\mu\text{M}$  to 35  $\mu\text{M}$  in the reaction medium containing four different fixed concentrations of aniline (6 mM, 8 mM, 10 mM and 12 mM). As shown in Figure 3.2, aniline 4-hydroxylase activity decreased with an increased concentration of tannic acid. 40 % of the initial activity was inhibited when 35  $\mu\text{M}$  of tannic acid used at all concentrations of aniline.



**Figure 3.1** Effect of tannic acid on rabbit liver microsomal aniline 4- hydroxylase activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given in “Method”. Liver microsomes were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 0.5 mM NADPH generating system into the reaction mixture.  $\text{IC}_{50}$  was calculated to be 60  $\mu\text{M}$ .



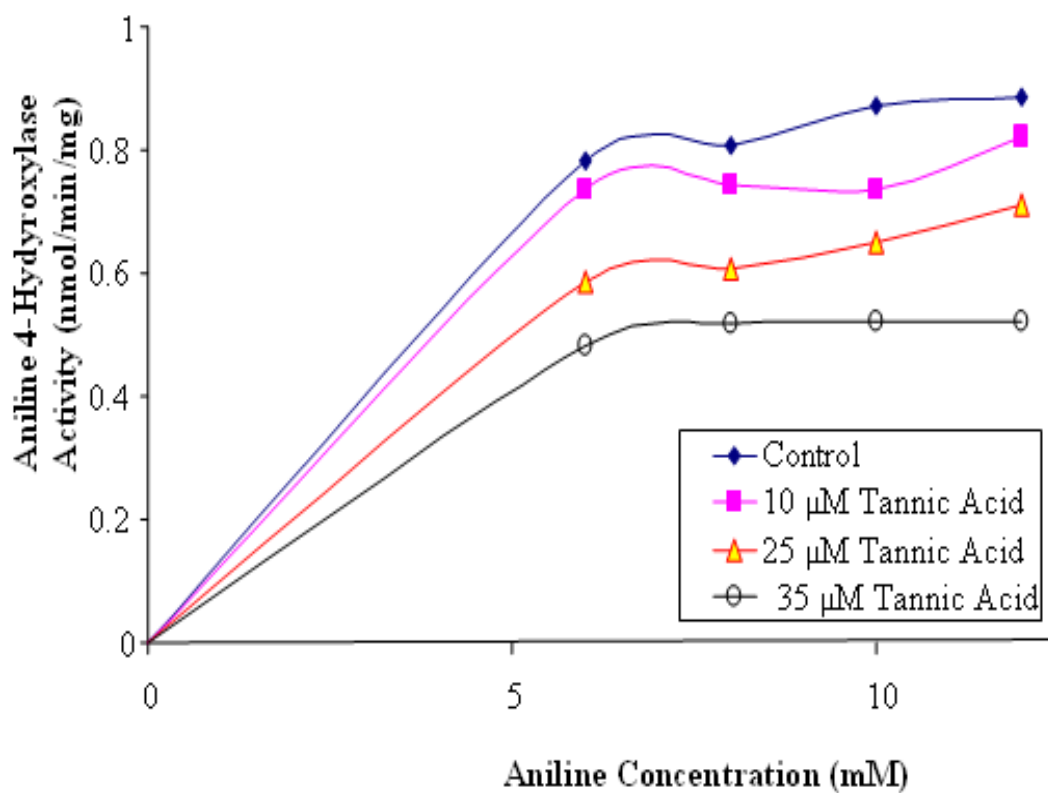
**Figure 3.2** Modulation of rabbit liver microsomal aniline 4-hydroxylase activity by tannic acid. Reaction medium contained indicated amount of tannic acid and aniline.

Michaelis – Menten,  $V$  versus  $[S]$ , and Lineweaver - Burk,  $1/V$  versus  $1/[S]$ , plots in the presence of three different concentrations of tannic acid were shown in Figure 3.3 and Figure 3.4, respectively. The Lineweaver – Burk plot indicated that the Michaelis – Menten constant ( $K_m$ ) remained unchanged by the presence of different concentrations of tannic acid, while  $V_{max}$  (maximum velocity) decreased with increasing tannic acid concentration. The apparent  $K_m$  value was found to be 1.72 mM for aniline in all concentrations of tannic acid present in the reaction medium. However,  $V_{max}$  values of the enzyme reaction were decreased from 1.005 nmol/min/mg (no tannic acid) to 0,615 nmol/min/mg (35  $\mu$ M tannic acid) with increasing tannic acid concentration.

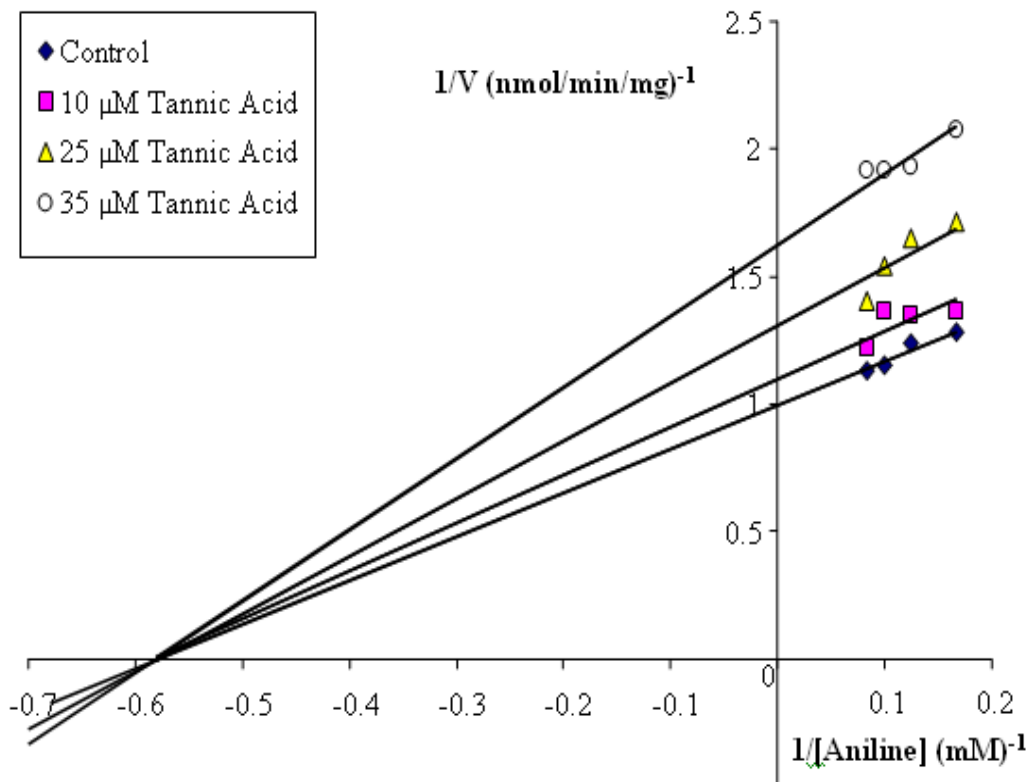
Figure 3.5 shows Dixon plot,  $1/V$  versus  $1/[tannic\ acid]$ , in the presence of different fix concentrations of aniline (from 6 mM to 12 mM). The apparent  $K_I$  value for tannic acid was calculated to be 54.7  $\mu$ M. This figure informed the type of inhibition of P450 dependent aniline 4-hydroxylation by tannic acid.  $K_I$  value remained same while  $V_{max}$  value differed for each concentration of aniline. Therefore, the inhibition of rabbit liver microsomal aniline 4-hydroxylase activity by tannic acid was found to be noncompetitive type of inhibition.

Since aniline 4-hydroxylase activity of kidney microsomes was very low, modulatory mechanism of tannic acid on enzyme activity could not be studied using different concentrations of substrate, aniline. Figure 3.6 shows the effect of tannic acid, in the range of 0-150  $\mu$ M, on kidney microsomal aniline 4-hydroxylase activity when only 10 mM aniline used in the reaction medium. Although the kidney aniline 4-hydroxylase was inhibited at all concentrations of tannic acid, inhibition was not significant up to 100  $\mu$ M tannic acid present in the reaction medium. While half of the renal aniline 4-hydroxylase activity was inhibited at 100  $\mu$ M tannic acid, only less than 20 % of initial activity remained when renal microsomes preincubated with 150  $\mu$ M tannic acid.

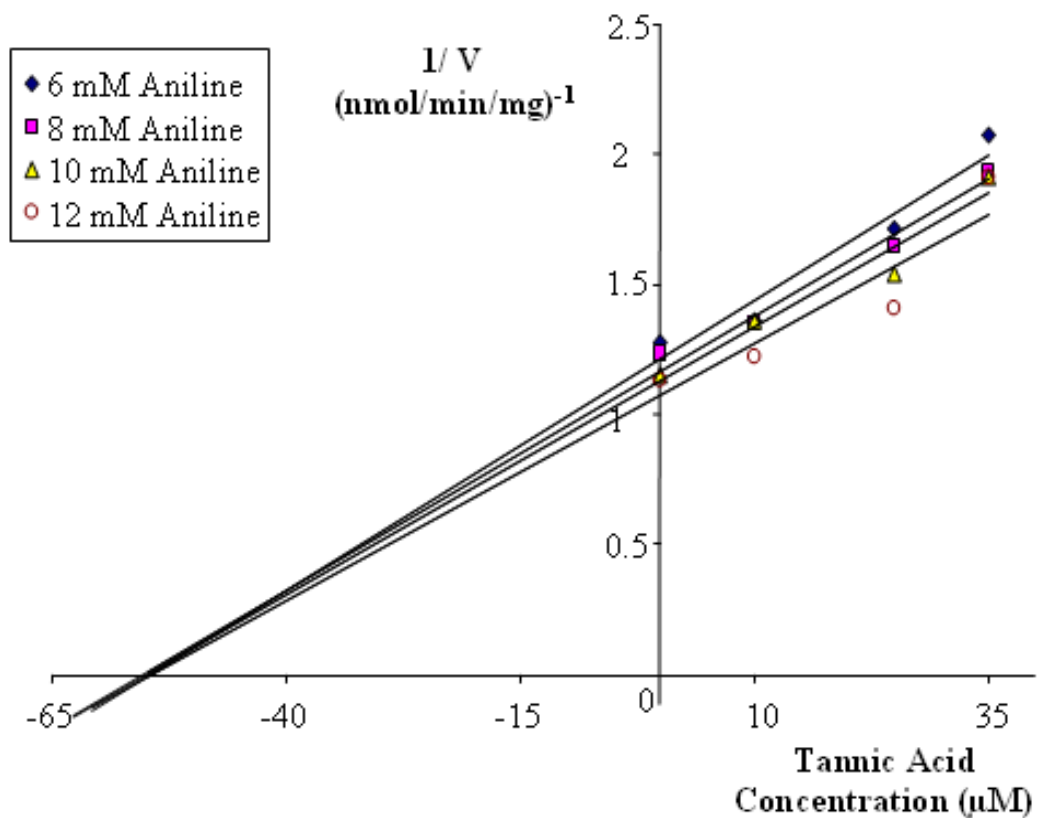




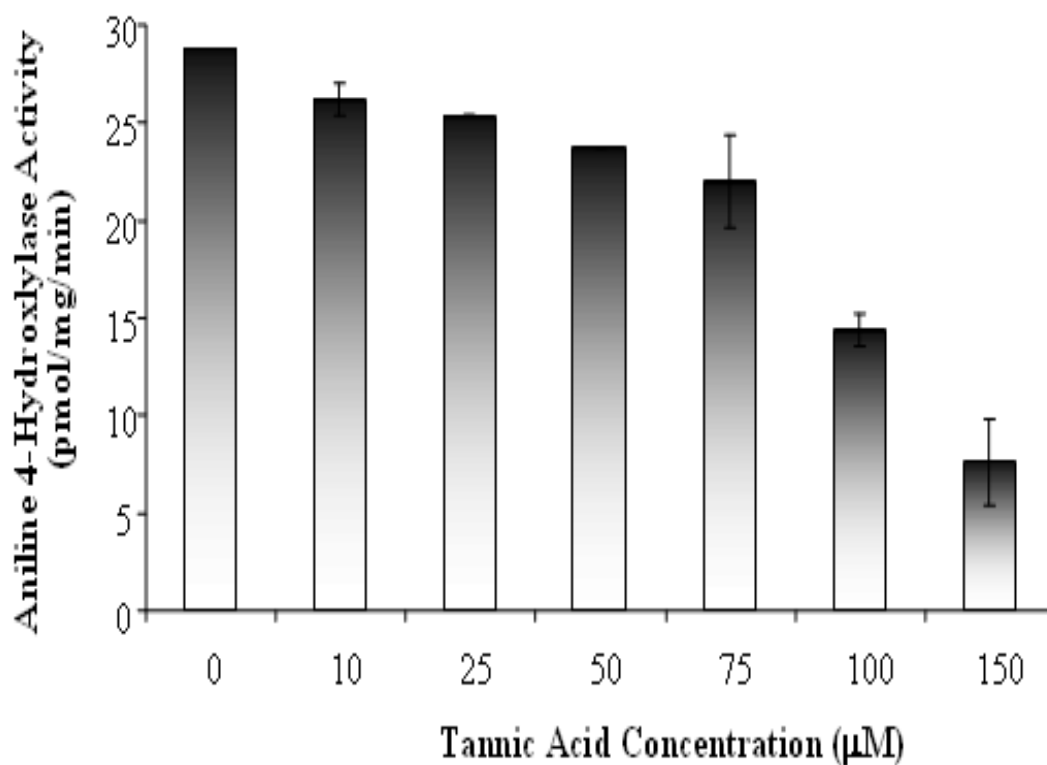
**Figure 3.3** Michaelis-Menten plot for rabbit liver microsomal aniline 4-hydroxylase activity. Effect of tannic acid concentration on rabbit liver microsomal aniline 4-hydroxylase activity was measured using different concentrations of aniline (6 mM-12 mM).



**Figure 3. 4** Lineweaver-Burk plot for rabbit liver microsomal aniline 4-hydroxylase activity in the presence of different concentrations of tannic acid. The apparent  $K_m$  was 1.72 mM and  $V_{max}$  values of the enzyme reaction were decreased from 1.005 nmol/min/mg to 0.615 nmol/min/mg increasing tannic acid concentration.



**Figure 3.5** Dixon plot for rabbit liver microsomal aniline 4-hydroxylase activity,  $1/V$  versus [tannic acid], in the presence of different fixed concentrations of substrate aniline (6 mM - 12 mM). The apparent  $K_I$  value for tannic acid was calculated to be 54.7  $\mu\text{M}$ .



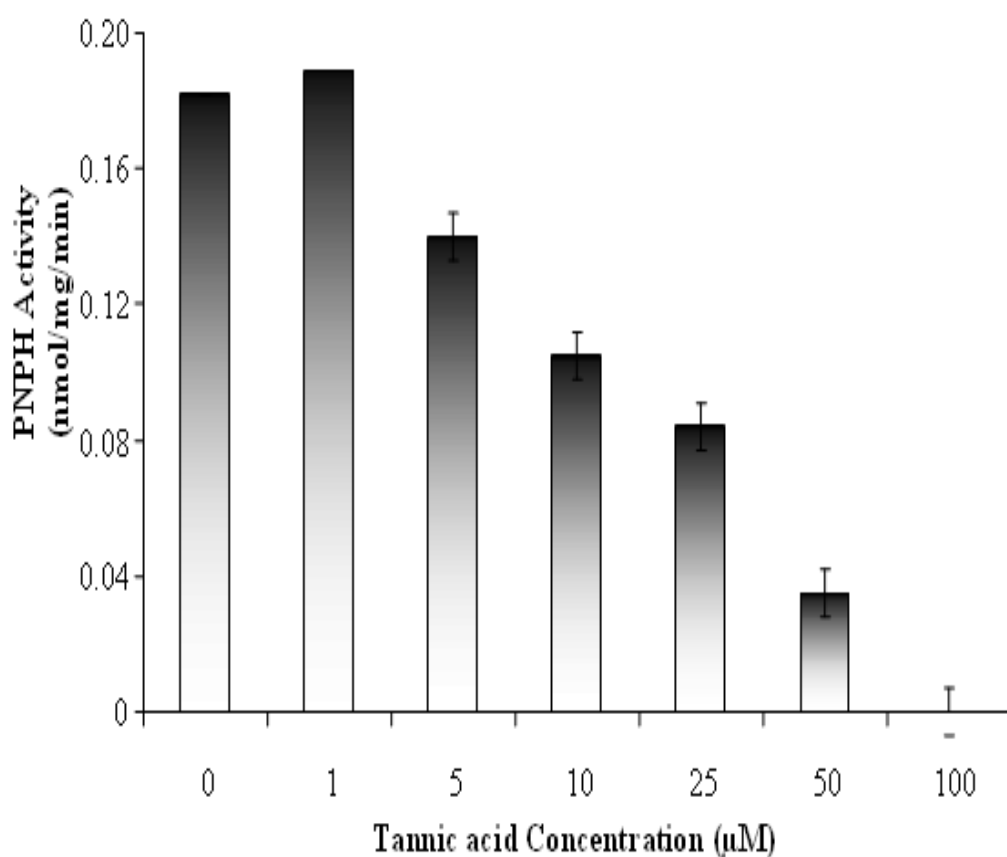
**Figure 3. 6** Effect of tannic acid on rabbit kidney microsomal aniline 4-hydroxylase activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given in “Method”. Renal microsomes were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 0.5 mM NADPH generating system into the reaction mixture.  $\text{IC}_{50}$  was calculated to be 100  $\mu\text{M}$ .

### **3.2.2 Effect of Tannic Acid on Rabbit Liver Microsomal *p*-Nitrophenol Hydroxylase Activity**

Rabbit liver microsomal *p*-nitrophenol hydroxylase activity was determined by the method of Reinke and Moyer (1985) which was later modified by Arınç and Aydoğmuş (1990).

The effect of tannic acid on rabbit liver microsomal *p*-nitrophenol hydroxylase was studied by adding various concentrations of tannic acid into the assay system containing fixed concentration of substrate *p*-nitrophenol (0.25 mM).

Since *p*-nitrophenol hydroxylase activity of rabbit liver microsomes was very low, modulatory mechanism of tannic acid on enzyme activity could not be studied using different concentrations of substrate, *p*-nitrophenol. Figure 3.7 shows the effect of tannic acid, in the range of 0-100  $\mu$ M, on rabbit liver microsomal *p*-nitrophenol hydroxylase activity when only 0.25 mM *p*-nitrophenol used in the reaction medium. The enzyme reaction was inhibited at all, except 1  $\mu$ M concentrations of tannic acid. The  $IC_{50}$  value (the concentration giving 50 % inhibition) of tannic acid was estimated to be 26  $\mu$ M. Rabbit liver microsomal *p*-nitrophenol hydroxylase activity was completely inhibited at 100  $\mu$ M tannic acid concentration.



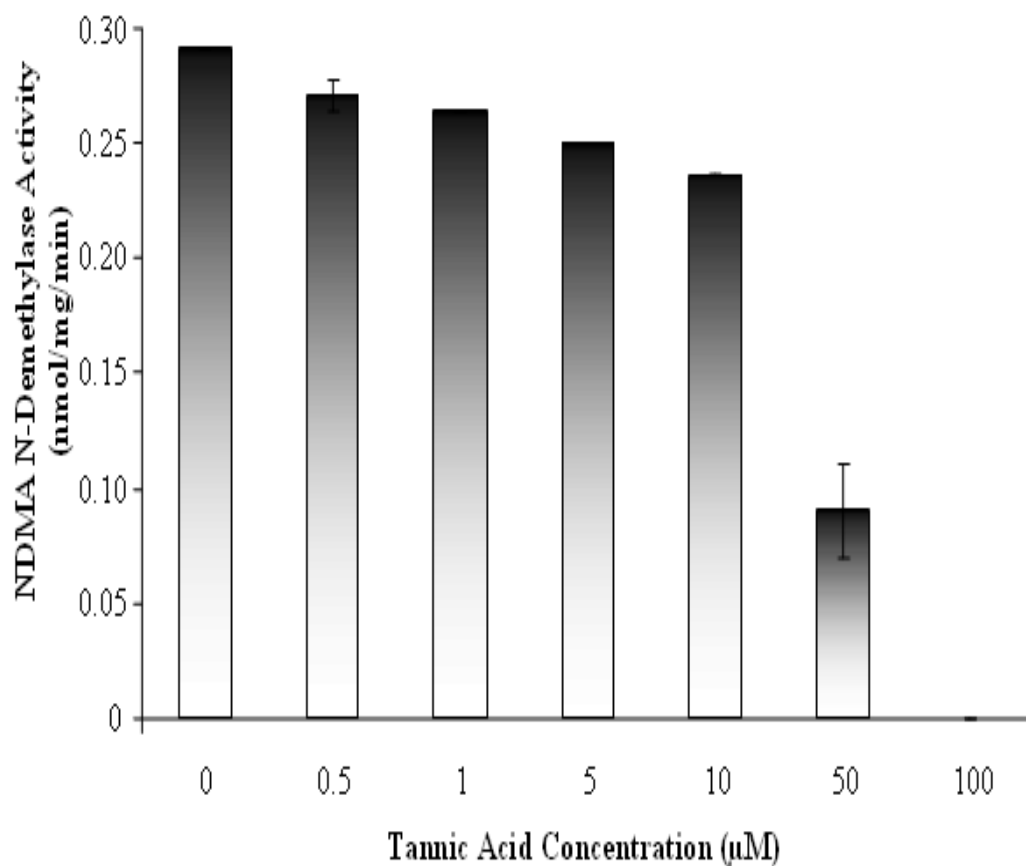
**Figure 3.7** Effect of tannic acid on liver PNP activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given “Method”. Liver microsomes were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 0.5 mM NADPH generating system into the reaction mixture.  $\text{IC}_{50}$  was calculated to be 26  $\mu\text{M}$ .

### 3.2.3 Effect of Tannic Acid on Rabbit Liver NDMA N-Demethylase Activity

Rabbit liver microsomal NDMA N-demethylase activity was determined as described by Gorsky and Hollenberg (1989).

The effect of tannic acid on rabbit liver microsomal NDMA N-demethylase was studied by adding various concentrations of tannic acid into the assay system containing fixed concentration of substrate NDMA (2.5 mM).

Figure 3.8 shows the effect of tannic acid, in the range of 0.5-100  $\mu$ M, on rabbit liver microsomal NDMA N-demethylase activity when only 2.5 mM N-nitrosodimethylamine used in the reaction medium. Although the enzyme reaction was inhibited at all concentrations of tannic acid, inhibition was not significant up to 50  $\mu$ M tannic acid present in the reaction medium. While half of the rabbit liver NDMA N-demethylase activity was inhibited at 37  $\mu$ M tannic acid, enzyme activity was completely inhibited at 100  $\mu$ M tannic acid concentration. Since NDMA N-demethylase activity of rabbit liver microsomes was very low, modulatory mechanism of tannic acid on enzyme activity could not be studied using different concentrations of substrate, N-nitrosodimethylamine.



**Figure 3.8** Effect of tannic acid on NDMA N-demethylase activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given “Method”. Liver microsomes were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 0.5 mM NADPH generating system into the reaction mixture.  $\text{IC}_{50}$  was calculated to be 37  $\mu\text{M}$ .



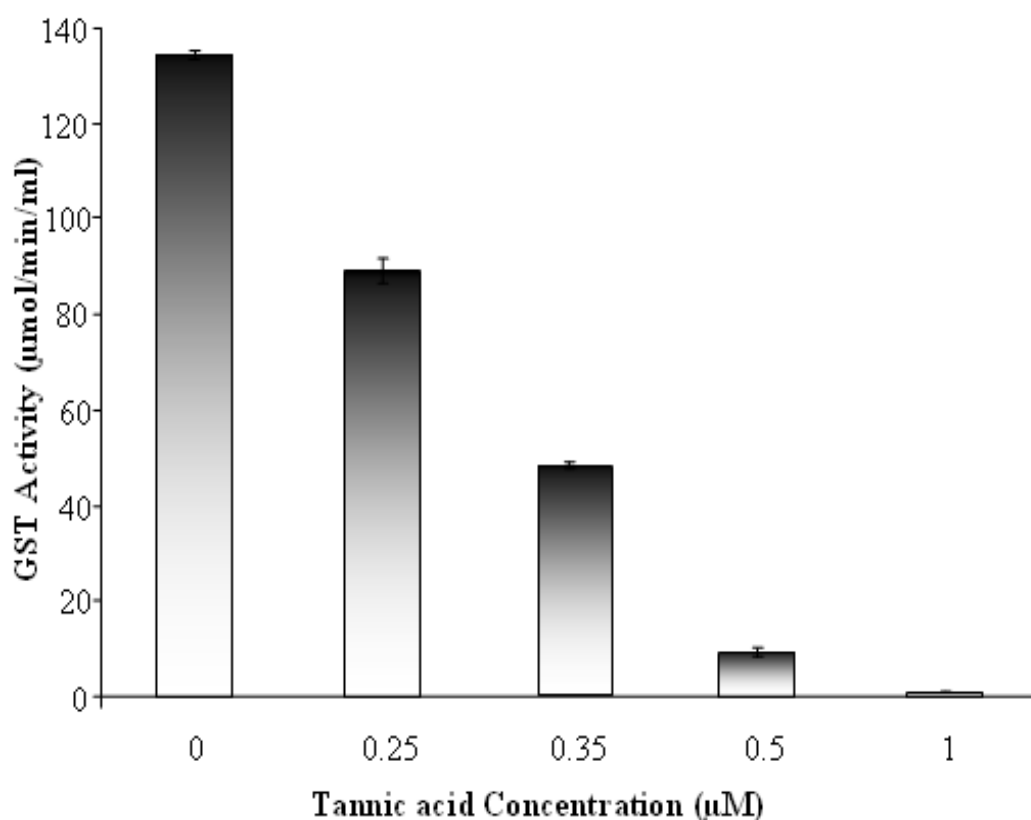
### **3.3 Effects of Tannic acid on Phase II Enzyme Activities**

Effect of tannic acid on rabbit liver and kidney Phase II enzymes; Glutathione S-Transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) were studied in the presence of tannic acid at concentration ranging from 0.25  $\mu\text{M}$  to 50  $\mu\text{M}$  in the reaction medium.

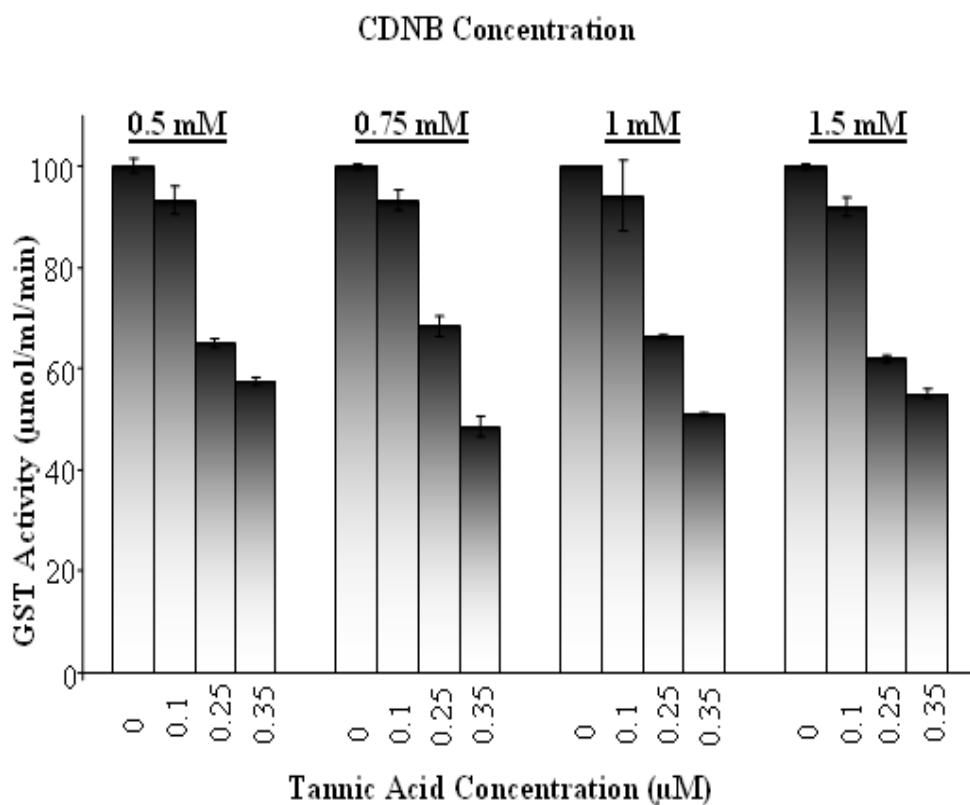
#### **3.3.1 Effect of Tannic Acid on Rabbit Liver and Kidney Cytosolic Glutathione S -Transferase (GST) Activity**

Rabbit liver and kidney cytosolic glutathione S-transferase activity in the presence of different concentration of tannic acid was determined by the method of Habig *et al.* (1974). A range of tannic acid concentration changing from 0.05  $\mu\text{M}$  to 1  $\mu\text{M}$  was used against various concentrations of substrate CDNB from 0.2 mM to 1.5 mM to investigate the effect of tannic acid on rabbit cytosolic GST activity.

Figure 3.9 and Figure 3.10 showed that rabbit liver cytosolic glutathione S-transferase activity was inhibited by increasing the concentration of tannic acid. The enzyme reaction was inhibited at all concentrations of tannic acid used. The  $\text{IC}_{50}$  value of tannic acid was estimated to be 0.33  $\mu\text{M}$  when the assay was carried out using 1 mM CDNB. Rabbit liver cytosolic glutathione S-transferase activity was completely inhibited at 1  $\mu\text{M}$  tannic acid concentration.



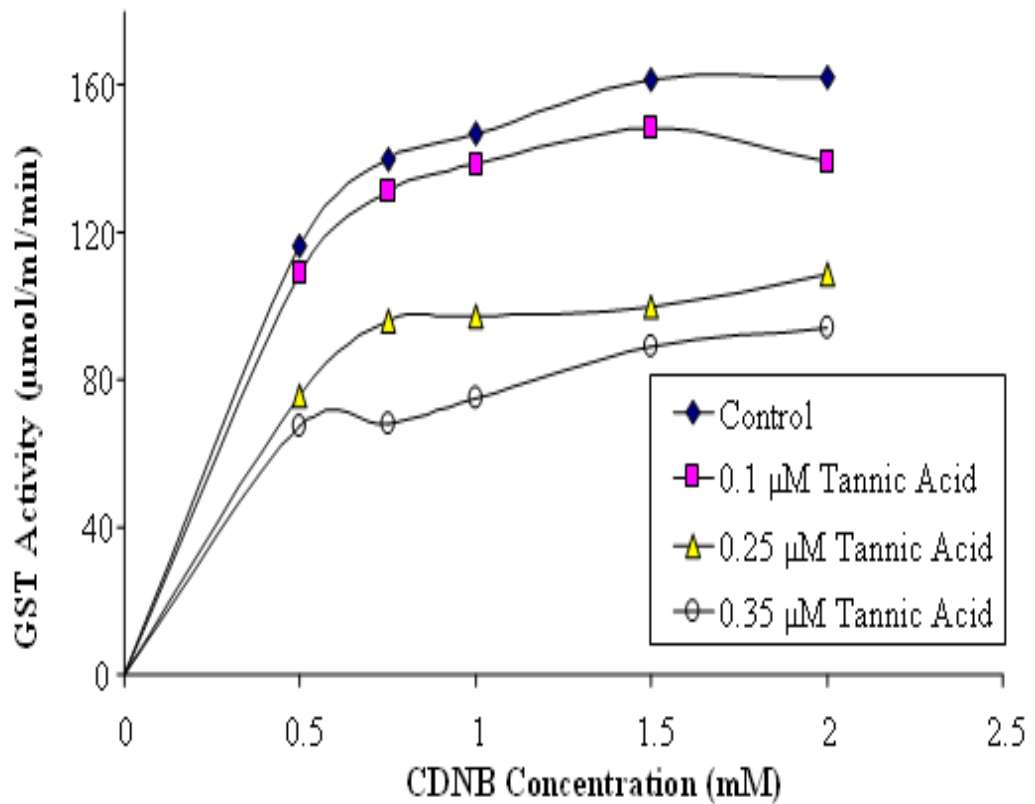
**Figure 3.9** Effect of tannic acid on rabbit liver cytosolic GST activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given under ‘Methods’. Liver cytosols were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 1 mM CDNB into the reaction mixture in a final volume of 3.0 ml.  $IC_{50}$  was calculated to be 0.33  $\mu$ M.



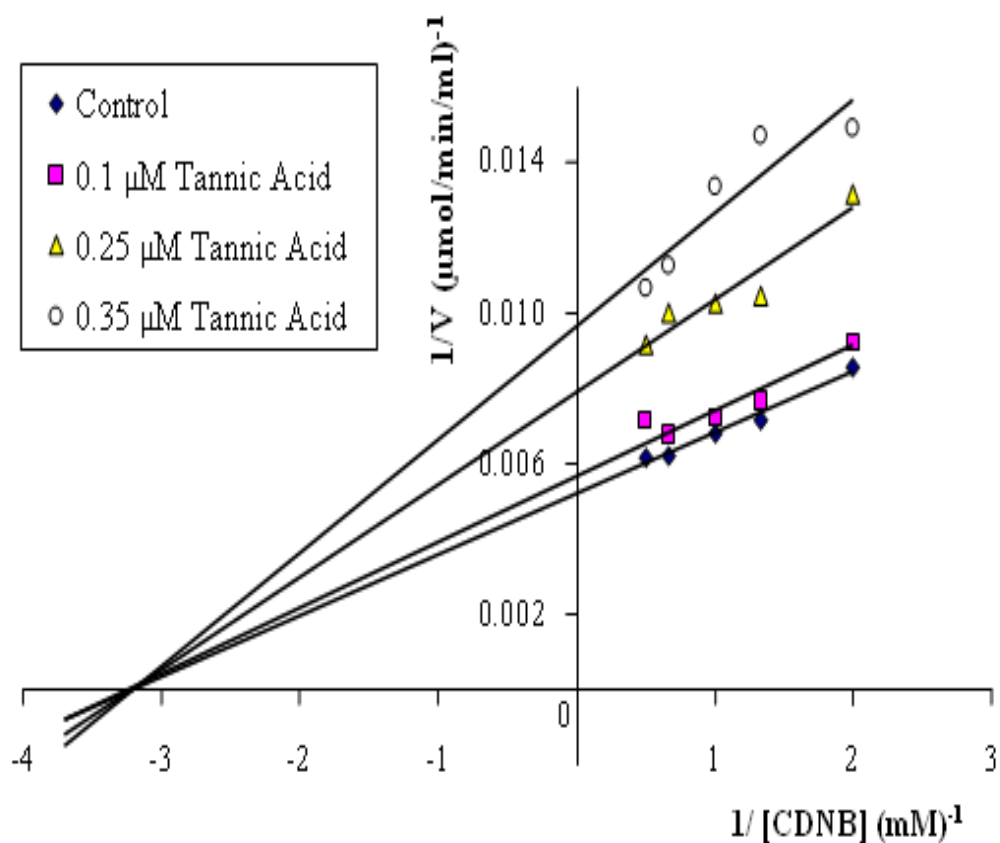
**Figure 3. 10** Modulation of rabbit liver cytosolic glutathione S-transferase activity by tannic acid. Reaction medium contained indicated amount of tannic acid and CDNB.

Michaelis – Menten,  $V$  versus  $[S]$ , and Lineweaver - Burk,  $1/V$  versus  $1/[S]$ , plots in the presence of three different concentrations of tannic acid were shown in Figure 3.11 and Figure 3.12, respectively. The Lineweaver – Burk plot indicated that the Michaelis – Menten constant ( $K_m$ ) remained unchanged by the presence of different concentrations of tannic acid, while  $V_{max}$  (maximum velocity) decreased with increasing tannic acid concentration. The apparent  $K_m$  value was found to be 0.3 mM for CDNB in all concentrations of tannic acid present in the reaction medium. However,  $V_{max}$  values of the enzyme reaction were decreased from 192.3  $\mu\text{mol/ml/min}$  (no tannic acid) to 104.2  $\mu\text{mol/ml/min}$  (0.35  $\mu\text{M}$  tannic acid) with increasing tannic acid concentration.

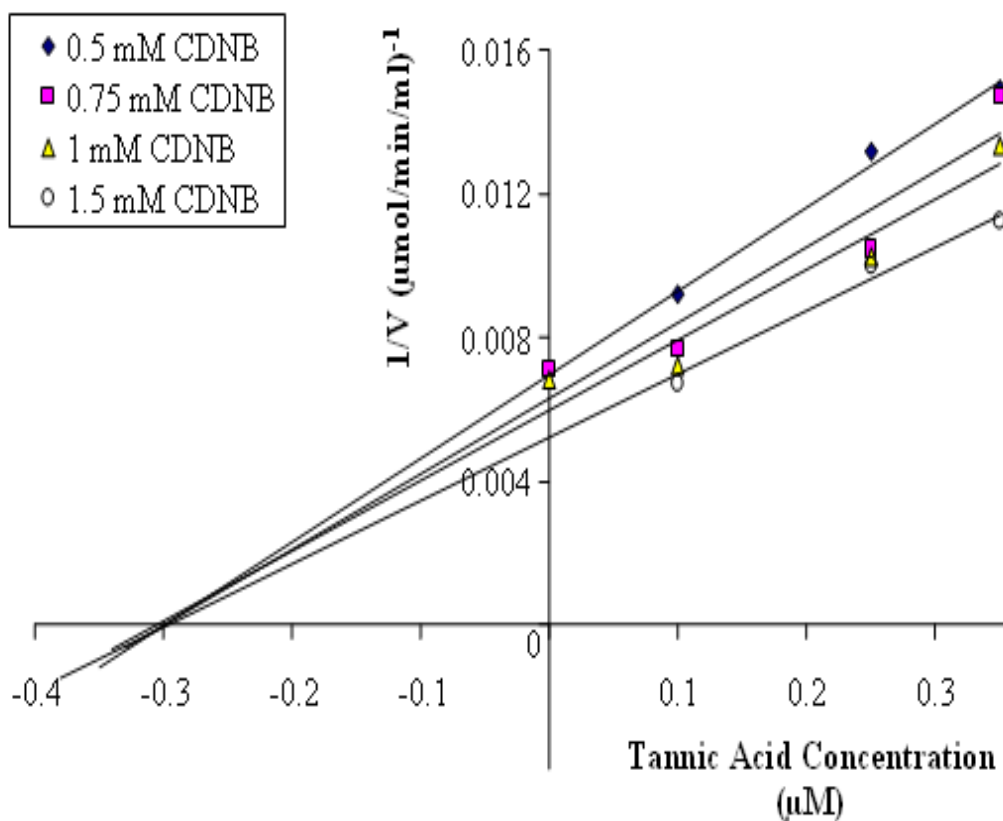
Figure 3.13 shows Dixon plot,  $1/V$  versus  $1/[\text{tannic acid}]$ , in the presence of different fix concentrations of CDNB (from 0.5 mM to 1.5 mM). From the intersecting point of the four lines, the  $K_I$  value was determined to be 0.3  $\mu\text{M}$  for rabbit liver cytosolic GST.  $K_I$  value remained same while  $V_{max}$  value differed for each concentration of CDNB. Therefore the plot was suggesting the inhibitor manner to be apparently noncompetitive.



**Figure 3. 11** Michaelis - Menten plot for rabbit liver cytosolic GST activity. Effect of tannic acid concentration on rabbit liver cytosolic GST activity was measured using different concentrations of CDNB (0.5 mM-2 mM).



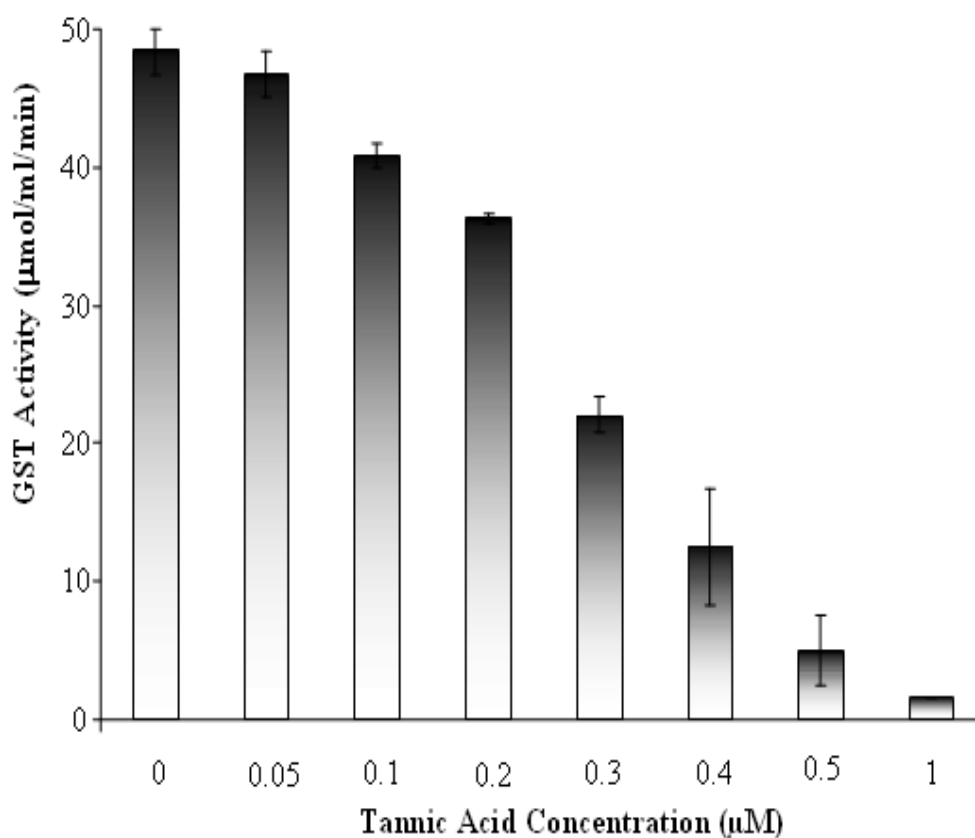
**Figure 3. 12** Lineweaver-Burk plot for GST activity of rabbit liver cytosol preincubated with different concentrations of tannic acid. The apparent  $K_m$  was 0.3 mM and  $V_{max}$  values of the reaction were decreased from 19.2  $\mu\text{mol/ml/min}$  to 10.3  $\mu\text{mol/ml/min}$  increasing tannic acid concentration.



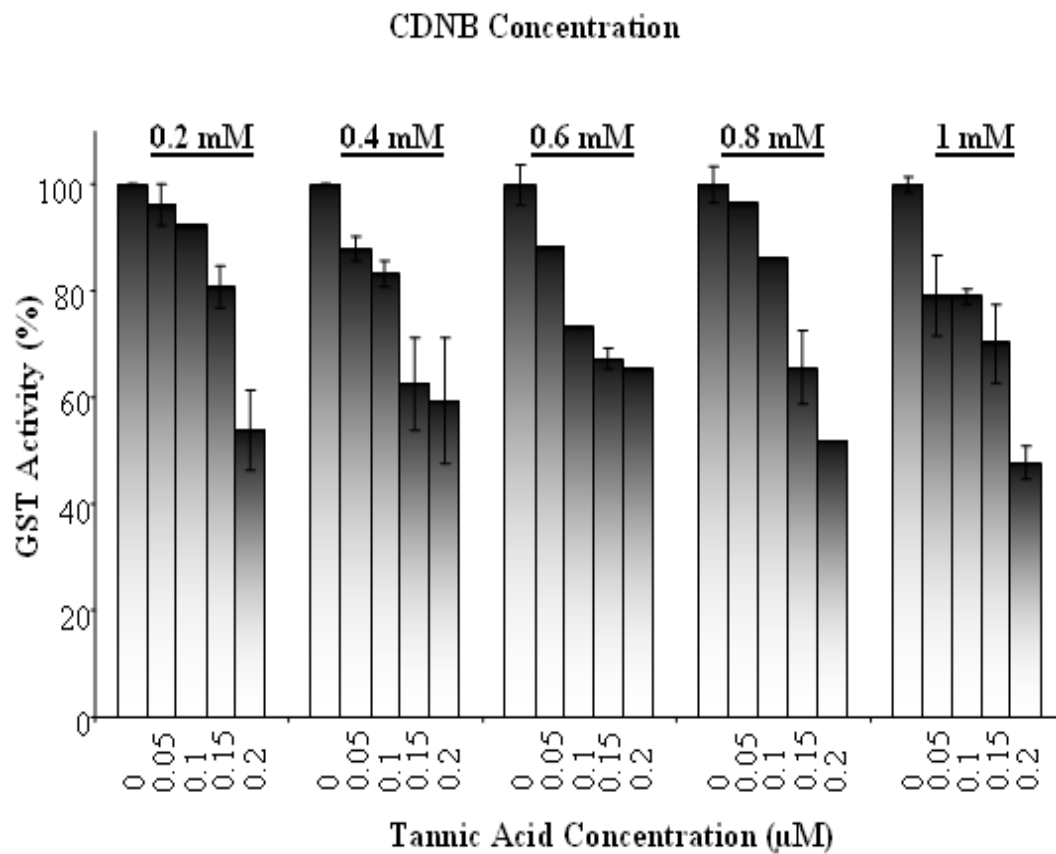
**Figure 3. 13** Dixon plot for rabbit liver cytosolic GST activity,  $1/V$  versus [tannic acid], in the presence of different fixed concentrations of substrate CDNB. The apparent  $K_I$  value for tannic acid was calculated to be 0.3  $\mu\text{M}$ .

Figure 3.14 shows the effect of tannic acid, in the range of 0.05 - 1  $\mu\text{M}$ , on rabbit kidney glutathione S-transferase activity when 1 mM CDNB used as a substrate in the reaction medium. Tannic acid has no significant inhibitory effect on renal GST activity up to 0.1  $\mu\text{M}$ . The half of the initial renal GST activity was inhibited with 0.24  $\mu\text{M}$  tannic acid while the total activity was inhibited at 1  $\mu\text{M}$  tannic acid concentration. Modulation kinetic of rabbit kidney cytosolic GST activity by tannic acid was studied by increasing the tannic acid concentration from 0.05  $\mu\text{M}$  to 0.2  $\mu\text{M}$  in the reaction medium containing five different fixed concentrations of CDNB (0.2 mM, 0.4 mM, 0.6 mM, 0.8 and 1 mM) (Figure 3.15).



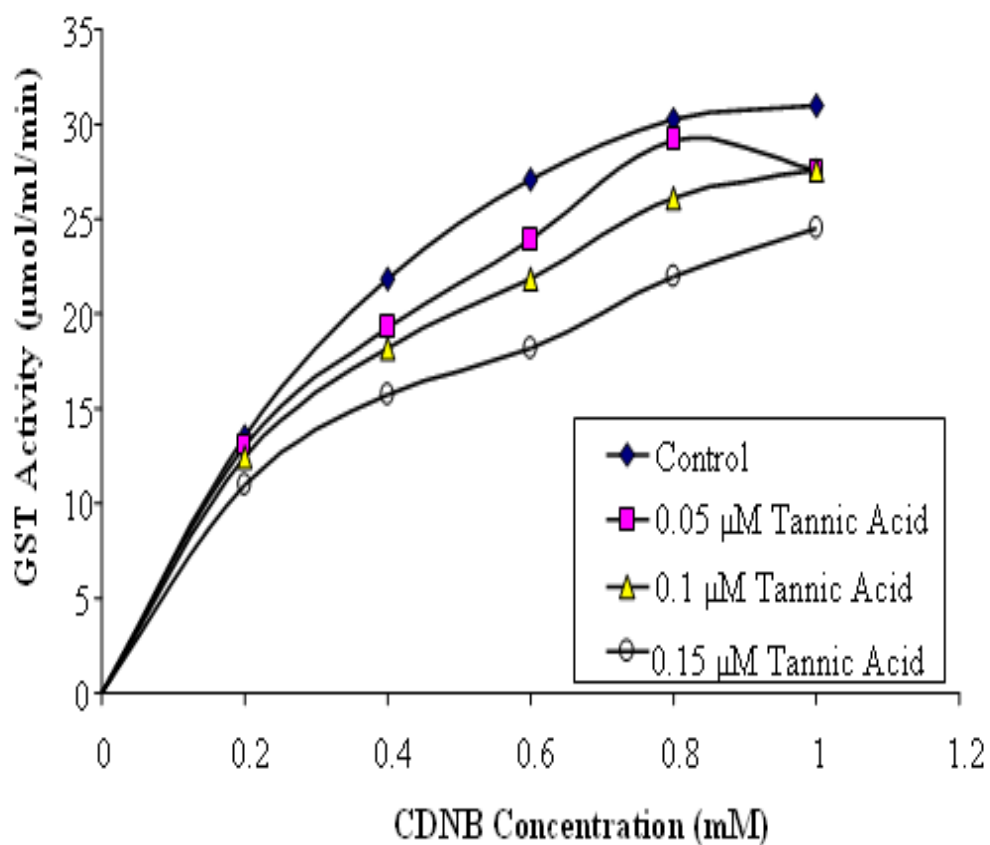


**Figure 3. 14** Effect of tannic acid on rabbit kidney cytosolic GST activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given under ‘Methods’. Kidney cytosols were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 1 mM CDNB into the reaction mixture in a final volume of 3.0 ml. IC<sub>50</sub> was calculated to be 0.24 µM.

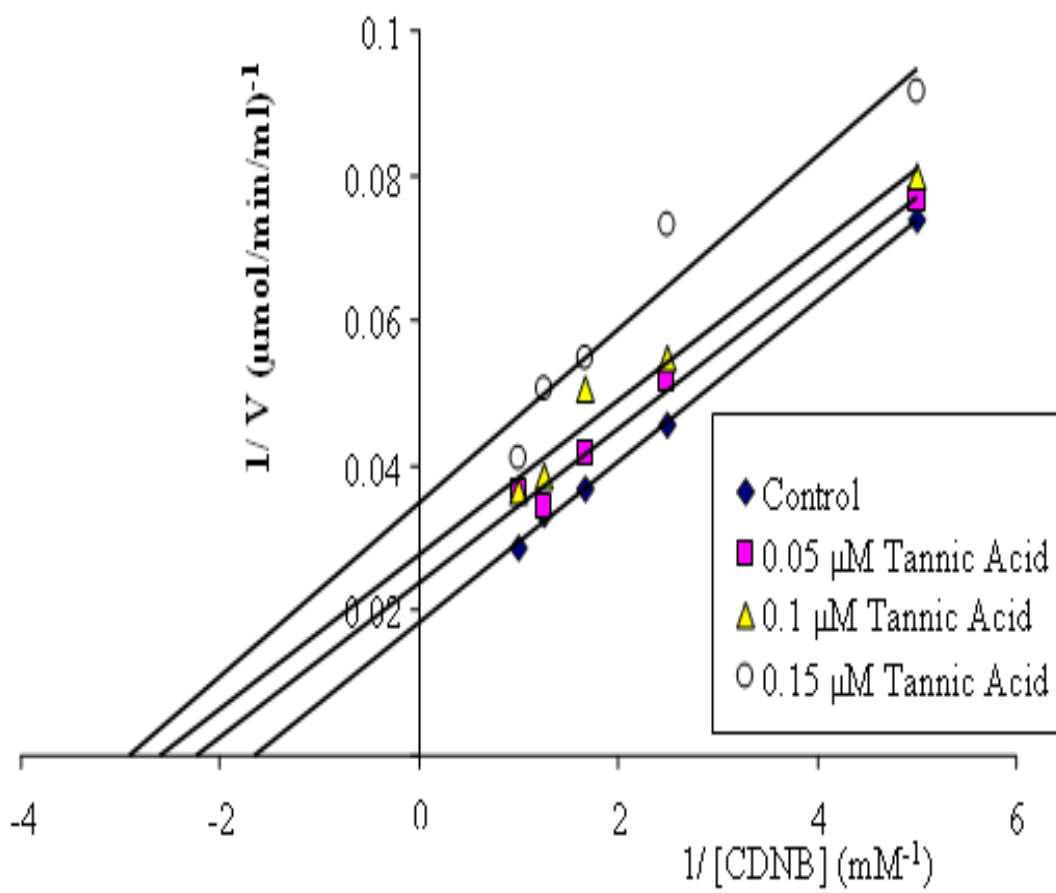


**Figure 3.15** Modulation of rabbit kidney cytosolic glutathione S-transferase activity by tannic acid. Reaction medium contained indicated amount of tannic acid and CDNB.

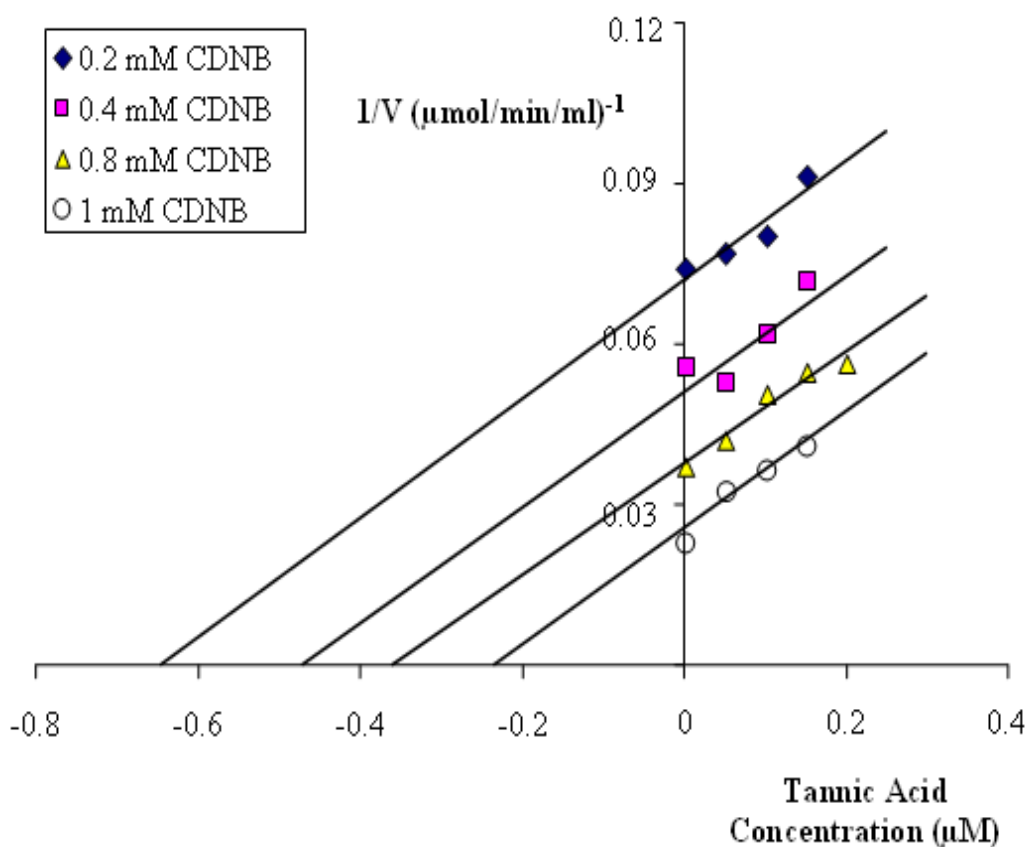
In the determination of the inhibitory mechanism of tannic acid on rabbit kidney cytosolic glutathione S-transferase, as given in Figure 3.16, the concentration of tannic acid was held three different values (0.05  $\mu\text{M}$ , 0.1  $\mu\text{M}$  and 0.15  $\mu\text{M}$ ) in the assay mixture, while the substrate (CDNB) concentration were varied (0.2 mM, 0.4 mM, 0.8 mM and 1 mM). When Lineweaver – Burk plot ( $1/V$  versus  $1/[S]$ ) was plotted at four different substrate concentration, the four lines were not crossed and become parallel to each other. The Lineweaver – Burk plot indicated that both Michaelis constant ( $K_m$ ) and  $V_{\text{max}}$  decreased with increasing tannic acid concentration, from 0.6 mM to 0.34 mM and 54.64  $\mu\text{mol/ml/min}$  to 28.65  $\mu\text{mol/ml/min}$ , respectively (Fig 3.17). Dixon plot was also plotted since it is used to identify the type of inhibition and to determine the  $K_I$  value. As it was shown in Figure 3.18, tannic acid was uncompetitive inhibitor for rabbit kidney cytosolic glutathione S-transferase with decreased  $K_I$  (from 0.615 to 0.252).



**Figure 3. 16** Michaelis - Menten plot for rabbit kidney cytosolic GST activity. Effect of tannic acid concentration on rabbit liver cytosolic GST activity was measured using different concentrations of CDNB (0.2 to 1 mM).



**Figure 3. 17** Lineweaver-Burk plot for GST activity of rabbit kidney cytosol preincubated with different concentrations of tannic acid. The apparent  $K_m$  decreases from 0.6 mM to 0.34 mM and  $V_{\max}$  of the reaction were decreased from 54.64  $\mu\text{mol}/\text{ml}/\text{min}$  to 28.65  $\mu\text{mol}/\text{ml}/\text{min}$ .



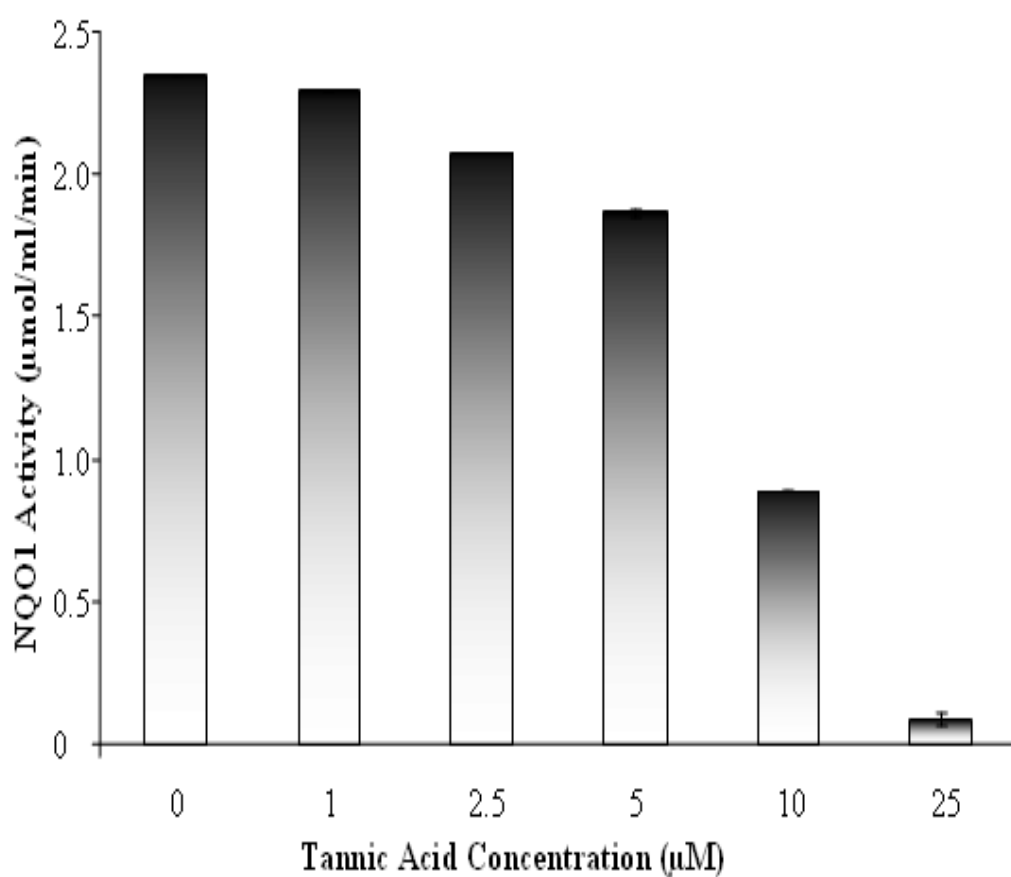
**Figure 3. 18** Dixon plot for rabbit kidney cytosolic GST activity,  $1/V$  versus [tannic acid], in the presence of different fixed concentrations of substrate CDNB (0.2 mM - 1 mM). The apparent  $K_I$  values for increased tannic acid concentrations were decreased from 0.615  $\mu\text{M}$  to 0.252  $\mu\text{M}$ .

### **3.3.2 Effects of Tannic acid on NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) Activity**

Rabbit liver and kidney cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) activity in the presence of different concentration of tannic acid was determined by the method of Ernster (1960).

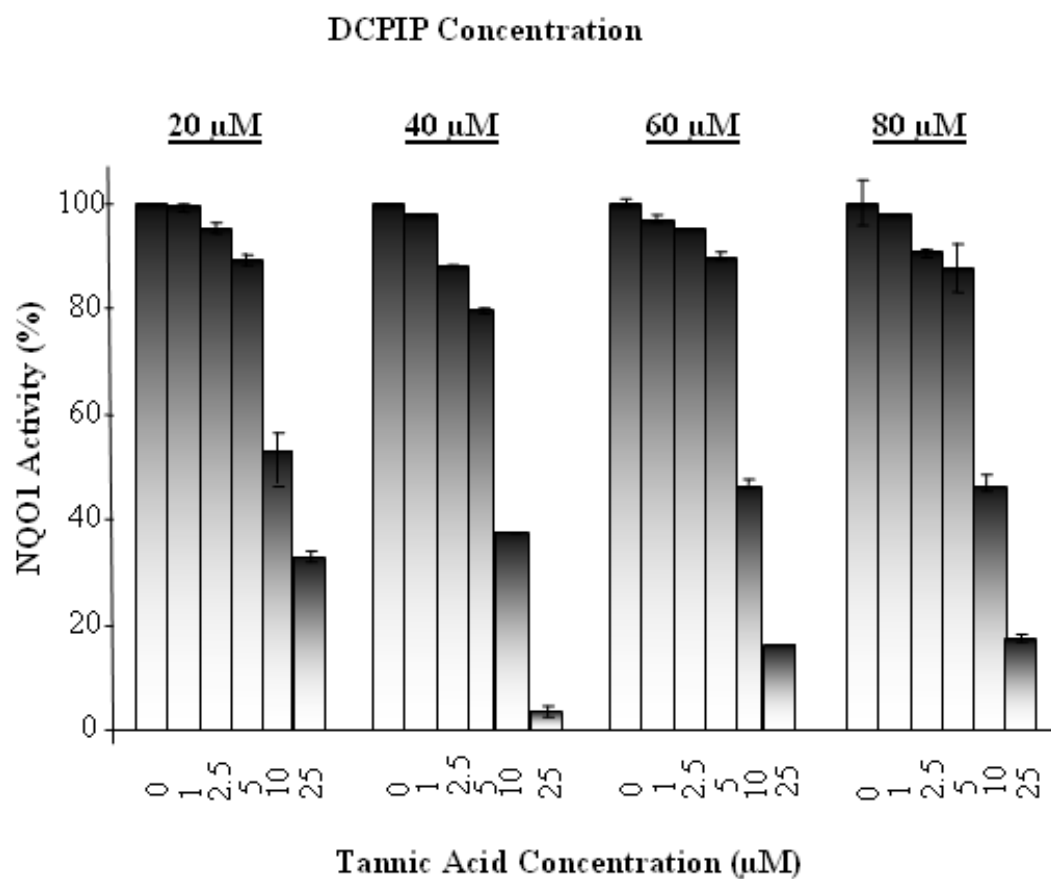
The effect of tannic acid on rabbit liver cytosolic NQO1 was studied by adding various concentrations of tannic acid (1  $\mu\text{M}$  - 50  $\mu\text{M}$ ) into the assay system containing fixed concentration of substrate DCPIP (40  $\mu\text{M}$ ).

As it was shown in Figure 3.19, tannic acid showed an inhibitory effect on rabbit liver cytosolic NQO1 activity. Although the enzyme reaction was inhibited at all concentrations of tannic acid, inhibition was not significant up to 10  $\mu\text{M}$  tannic acid present in the reaction medium. The  $\text{IC}_{50}$  value of tannic acid was estimated to be 7.25  $\mu\text{M}$ . At 25  $\mu\text{M}$  tannic acid concentration, hepatic cytosolic NQO1 activity was almost completely (97 %) inhibited. Modulation mechanism of rabbit liver cytosolic NQO1 activity by tannic acid was studied by increasing tannic acid concentration from 1  $\mu\text{M}$  to 25  $\mu\text{M}$  in the reaction medium in which four different concentrations of DCPIP (20  $\mu\text{M}$ , 40  $\mu\text{M}$ , 60  $\mu\text{M}$ , and 80  $\mu\text{M}$ ) were used (Figure 3.20).



**Figure 3. 19** Effect of tannic acid on rabbit liver cytosolic NQO1 activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given under ‘Methods’. Liver cytosols were preincubated with tannic acid for 10 minutes at room temperature.  $IC_{50}$  was calculated to be 7.25  $\mu$ M.

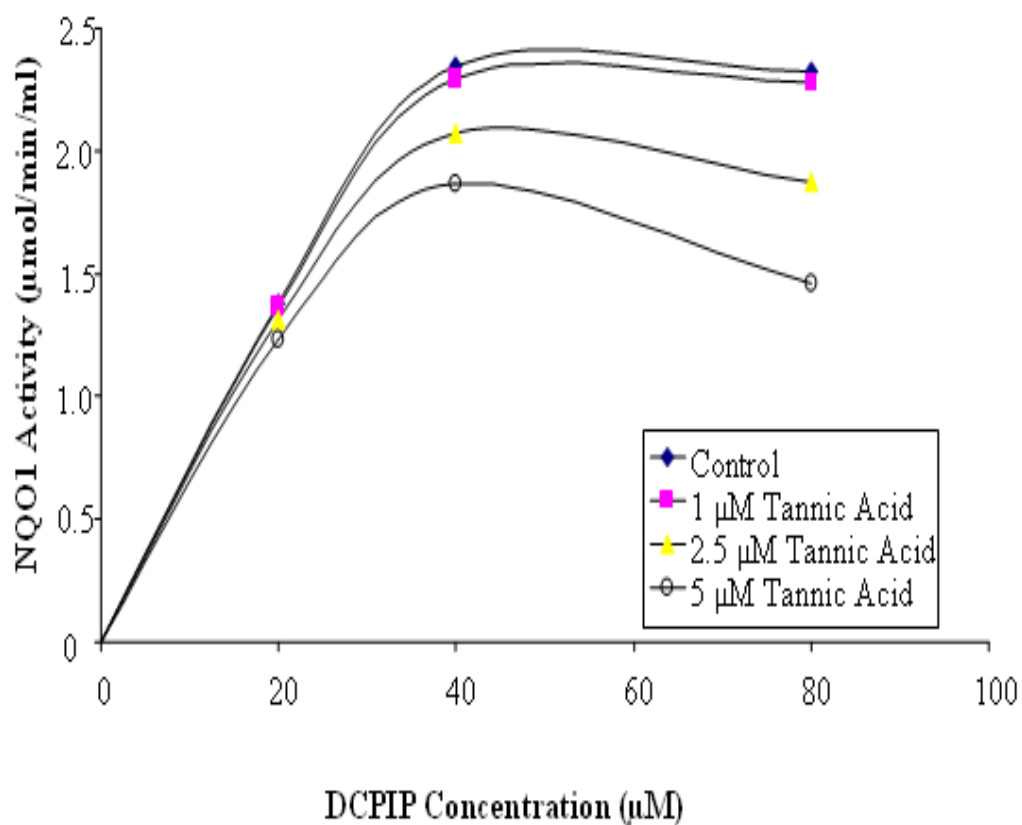




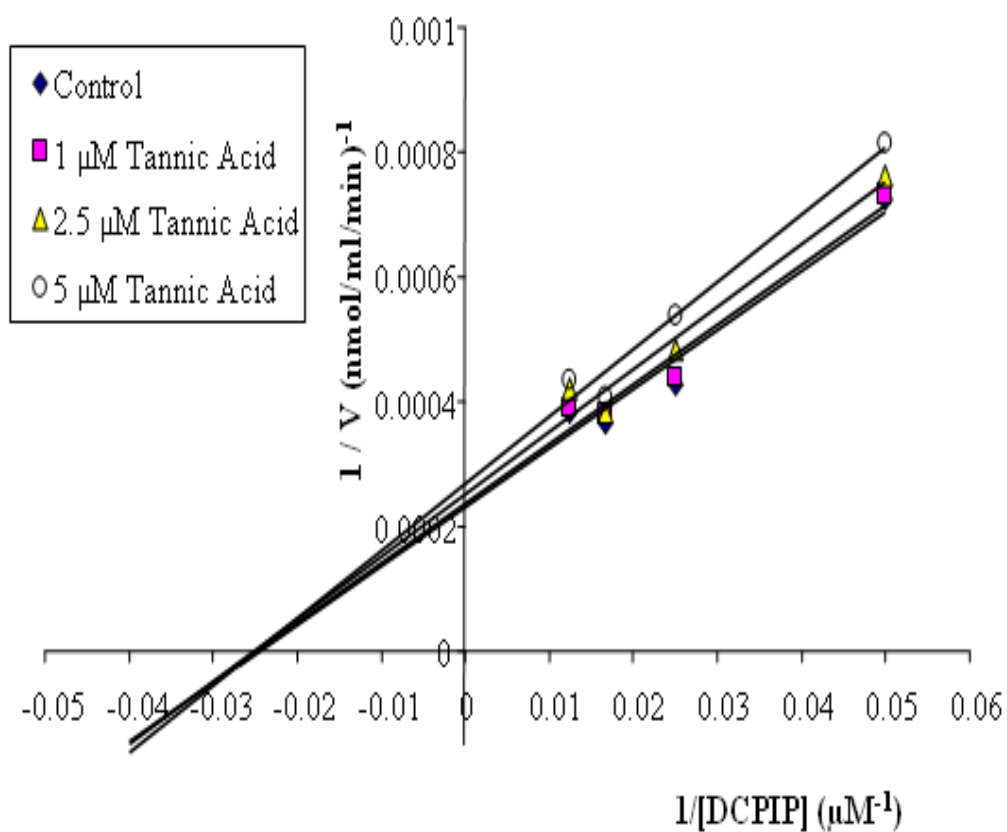
**Figure 3. 20** Modulation of rabbit liver cytosolic NQO1 activity by tannic acid. Reaction medium contained indicated amount of tannic acid and DCPIP.

Michaelis – Menten,  $V$  versus  $[S]$ , and Lineweaver - Burk,  $1/V$  versus  $1/[S]$ , plots in the presence of three different concentrations of tannic acid were shown in Figure 3.21 and Figure 3.22, respectively. The Lineweaver – Burk plot indicated that the Michaelis – Menten constant ( $K_m$ ) remained unchanged by the presence of different concentrations of tannic acid, while  $V_{max}$  (maximum velocity) decreased with increasing tannic acid concentration. The apparent  $K_m$  value was found to be  $40 \mu\text{M}$  for DCPIP in all concentrations of tannic acid present in the reaction medium. However,  $V_{max}$  values of the enzyme reaction were decreased from  $4.2 \mu\text{mol/ml/min}$  (no tannic acid) to  $3.7 \mu\text{mol/ml/min}$  ( $5 \mu\text{M}$  tannic acid) with increasing tannic acid concentration.

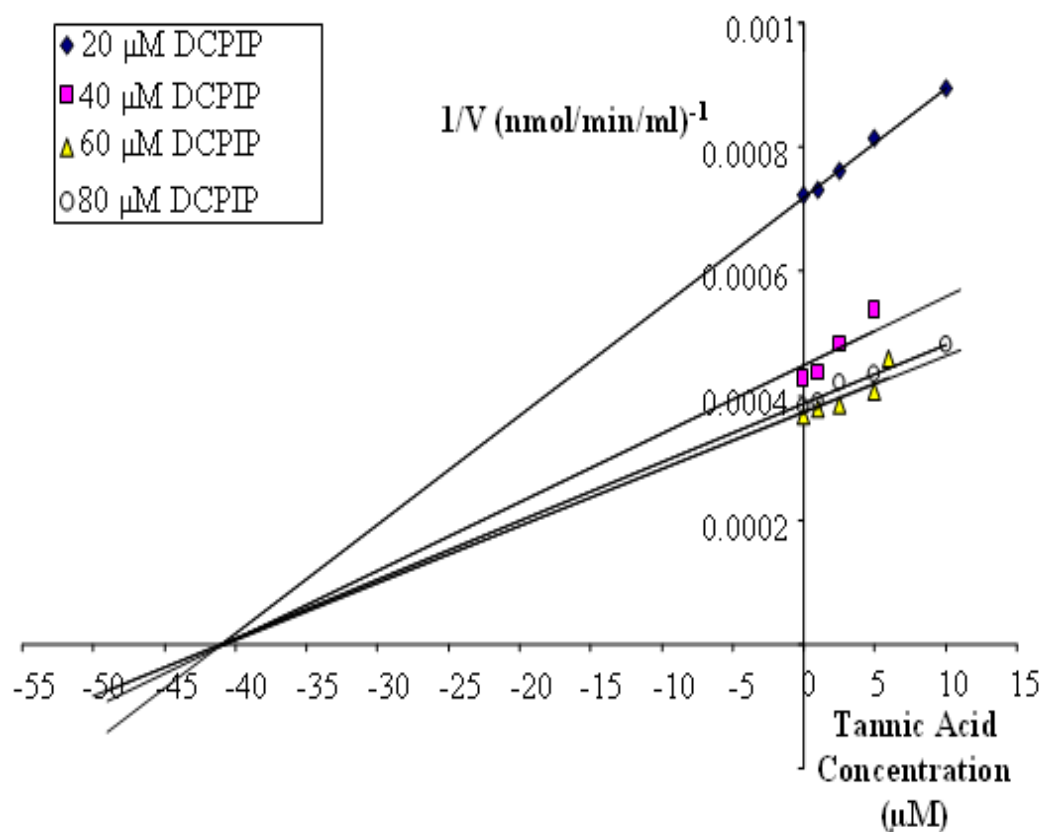
Figure 3.23 shows Dixon plot,  $1/V$  versus  $1/[\text{tannic acid}]$ , in the presence of different fix concentrations of DCPIP (from  $20 \mu\text{M}$  to  $80 \mu\text{M}$ ). From the intersecting point of the four lines, the  $K_I$  value was determined to be  $41 \mu\text{M}$  for rabbit liver cytosolic NQO1.  $K_I$  value remained same while  $V_{max}$  value differed for each concentration of CDNB. Therefore the plot was suggesting the inhibitor manner to be apparently noncompetitive.



**Figure 3. 21** Michaelis - Menten plot for rabbit liver cytosolic NQO1 activity. Effect of tannic acid concentration on rabbit liver cytosolic NQO1 activity was measured using different concentrations of DCPIP (20 µM -80 µM).



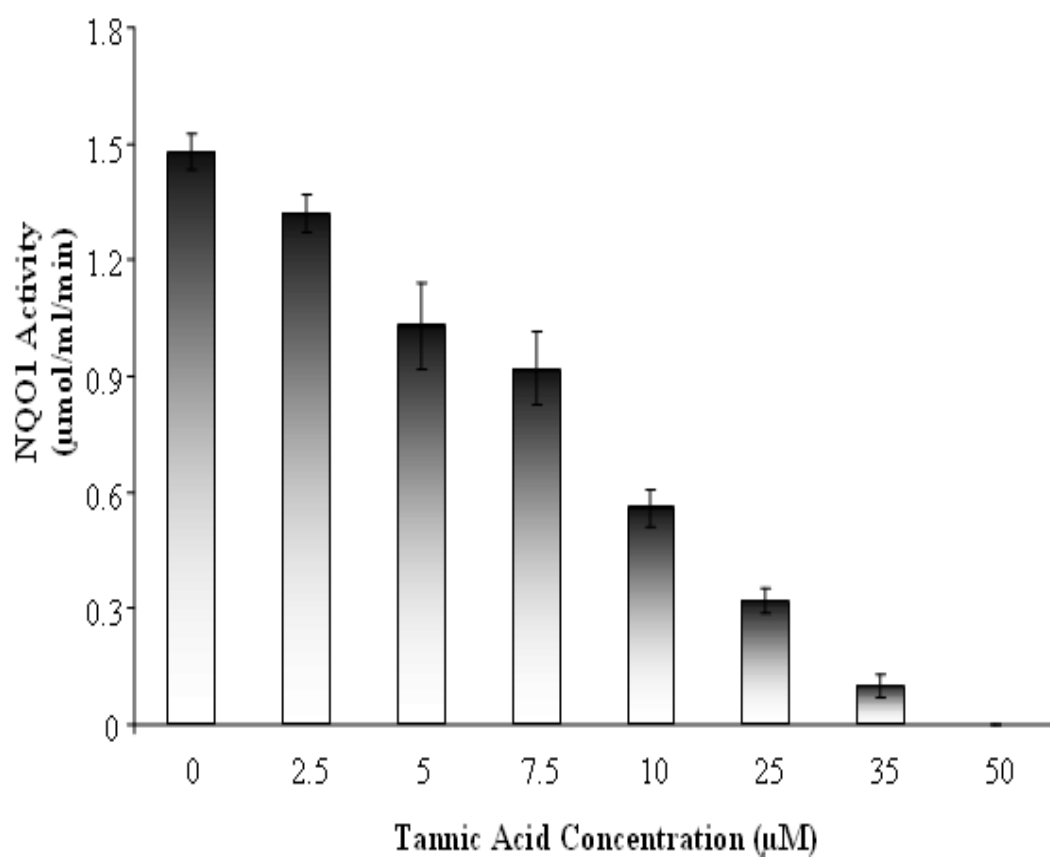
**Figure 3. 22** Lineweaver-Burk plot for NQO1 activity of rabbit liver cytosol preincubated with different concentrations of tannic acid. The apparent  $K_m$  was 40 μM and  $V_{max}$  values of the reaction were decreased from 4.2 μmol/ml/min to 3.7 μmol/ml/min increasing tannic acid concentrations.



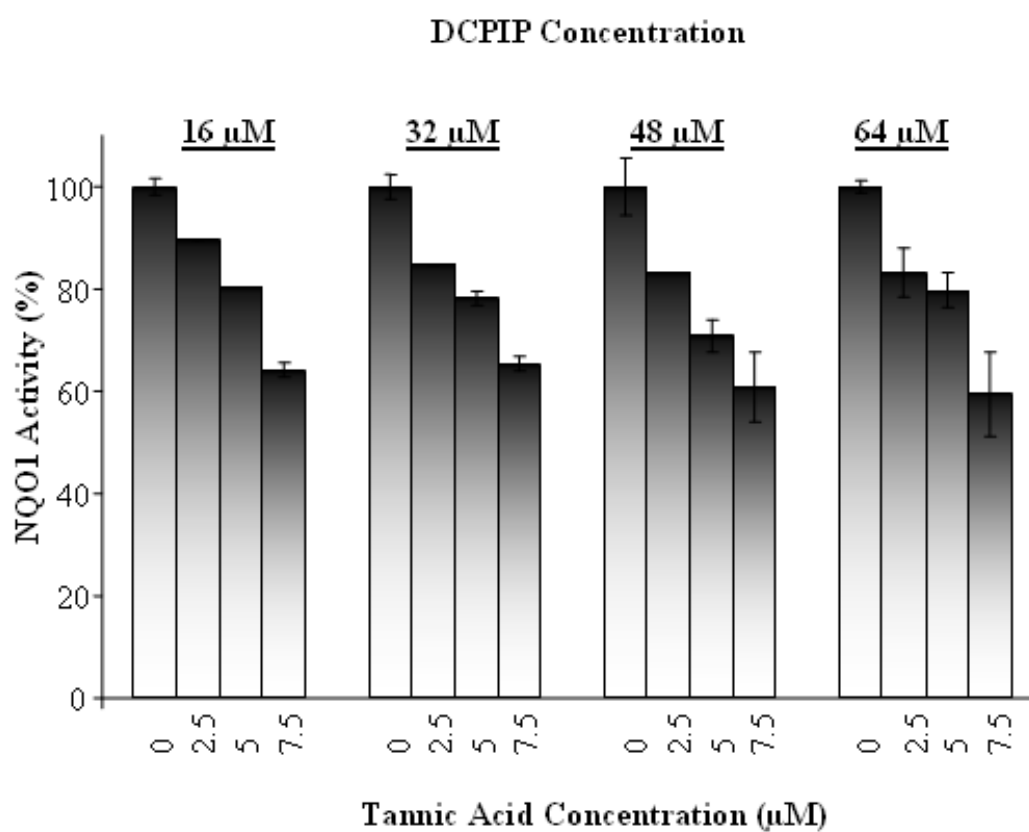
**Figure 3.23** Dixon plot for rabbit liver cytosolic NQO1 activity,  $1/V$  versus [tannic acid], in the presence of different fixed concentrations of substrate DCPIP. The apparent  $K_I$  value for tannic acid was calculated to be  $41 \mu\text{M}$ .

Figure 3.24 shows the effect of tannic acid, in the range of 2.5 - 50  $\mu\text{M}$ , on rabbit kidney NQO1 activity when 40  $\mu\text{M}$  DCPIP used as a substrate in the reaction medium. Tannic acid has a concentration dependent inhibitory effect on rabbit kidney NQO1 activity. The half of the initial renal NQO1 activity was inhibited at 8.6  $\mu\text{M}$  tannic acid concentration while the total activity was inhibited at 50  $\mu\text{M}$  tannic acid.

Modulation kinetic of rabbit kidney cytosolic NQO1 activity by tannic acid was studied by increasing the tannic acid concentration from 2.5  $\mu\text{M}$  to 7.5  $\mu\text{M}$  in the reaction medium containing four different fixed concentrations of DCPIP (16  $\mu\text{M}$ , 32  $\mu\text{M}$ , 48  $\mu\text{M}$  and 64  $\mu\text{M}$ ) (Figure 3.25).



**Figure 3. 24** Effect of tannic acid on rabbit kidney cytosolic NQO1 activity. The reaction medium contained indicated amount of tannic acid in addition to standard assay mixture constituents given under ‘Methods’. Kidney cytosols were preincubated with tannic acid for 10 minutes at room temperature and then the reaction was started by adding 40 μM 2,6-dichlorophenolindophenol (DCPIP) into the reaction mixture in a final volume of 1.0 ml. IC<sub>50</sub> was calculated as 8.6 μM.

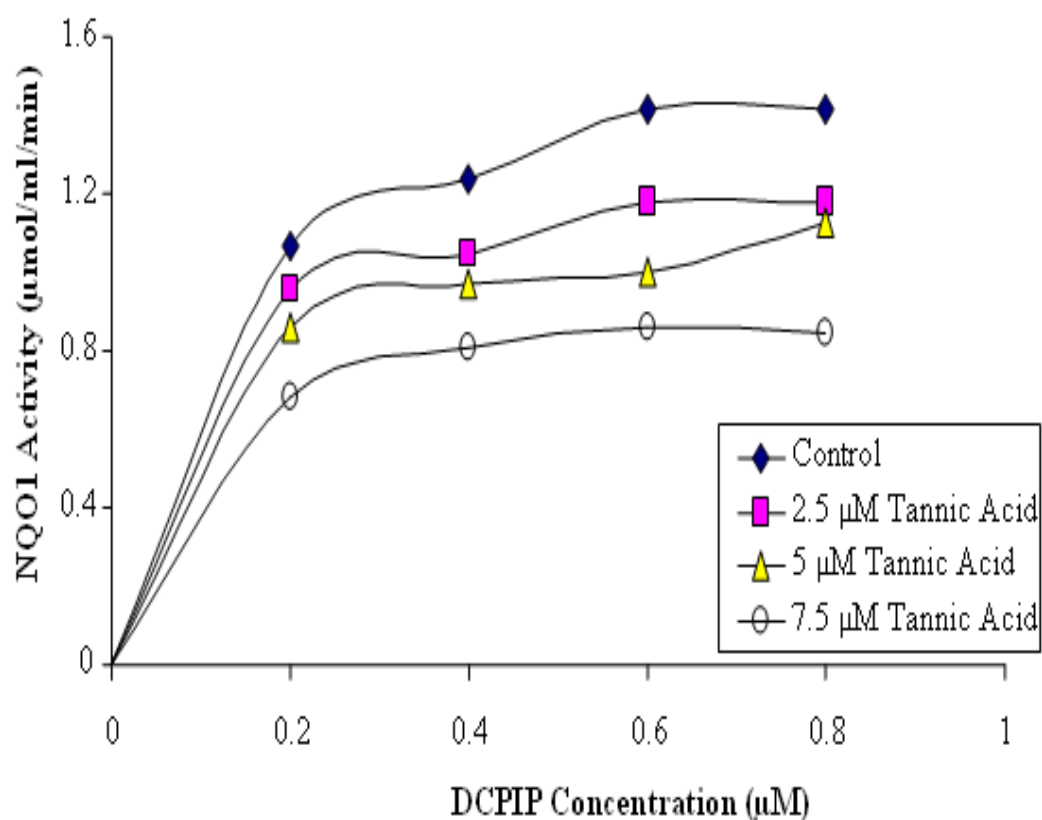


**Figure 3. 25** Modulation of rabbit kidney cytosolic NQO1 activity by tannic acid. Reaction medium contained indicated amount of tannic acid and DCPIP.

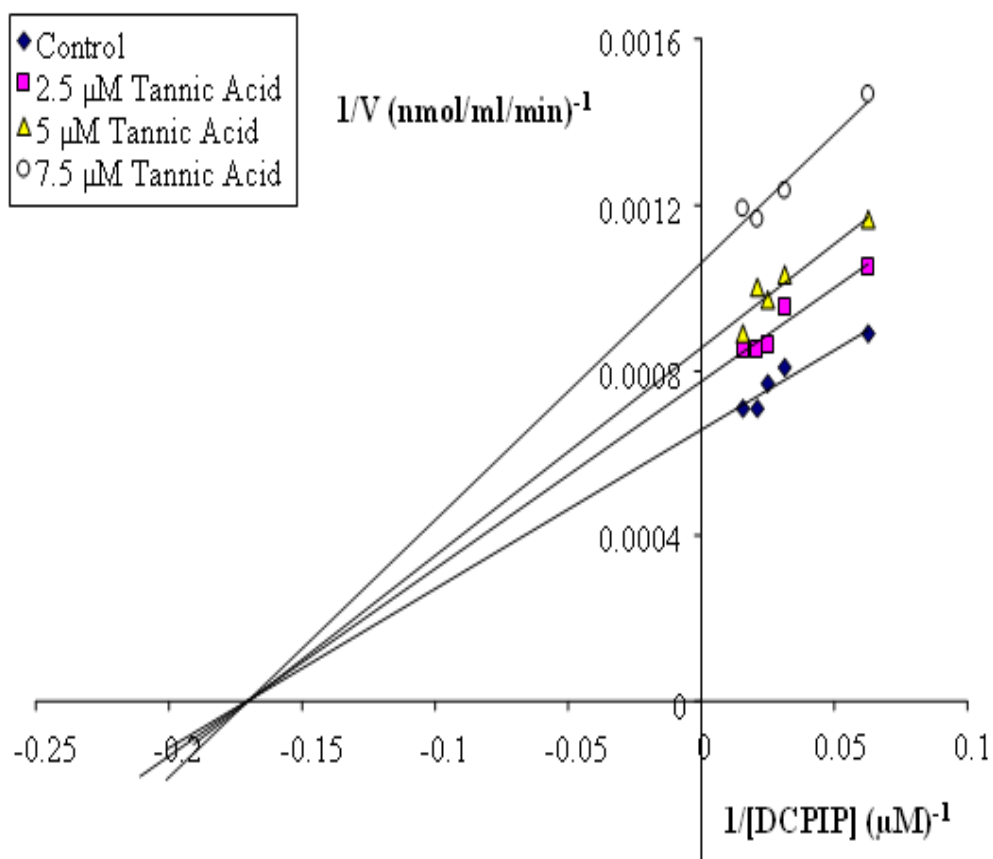


The relationship between the rate of substrate conversion by enzyme and substrate concentration (Michaelis-Menten kinetic,  $V$  versus  $[S]$ ) was described in Figure 3.26. When  $1/V$  was plotted against  $1/[S]$  (Lineweaver-Burk plot) at five different substrate concentrations, the four lines crossed apparently at a point on the X-axis which indicated that Michaelis-Menten constant ( $K_m$ ) remained unchanged ( $5.9 \mu\text{M}$ ), while  $V_{\text{max}}$  (maximum velocity) decreased from  $1.4 \mu\text{mol/ml/min}$  to  $0.9 \mu\text{mol/ml/min}$  for rabbit kidney cytosolic NQO1 with increasing tannic acid concentration (Figure 3.27).

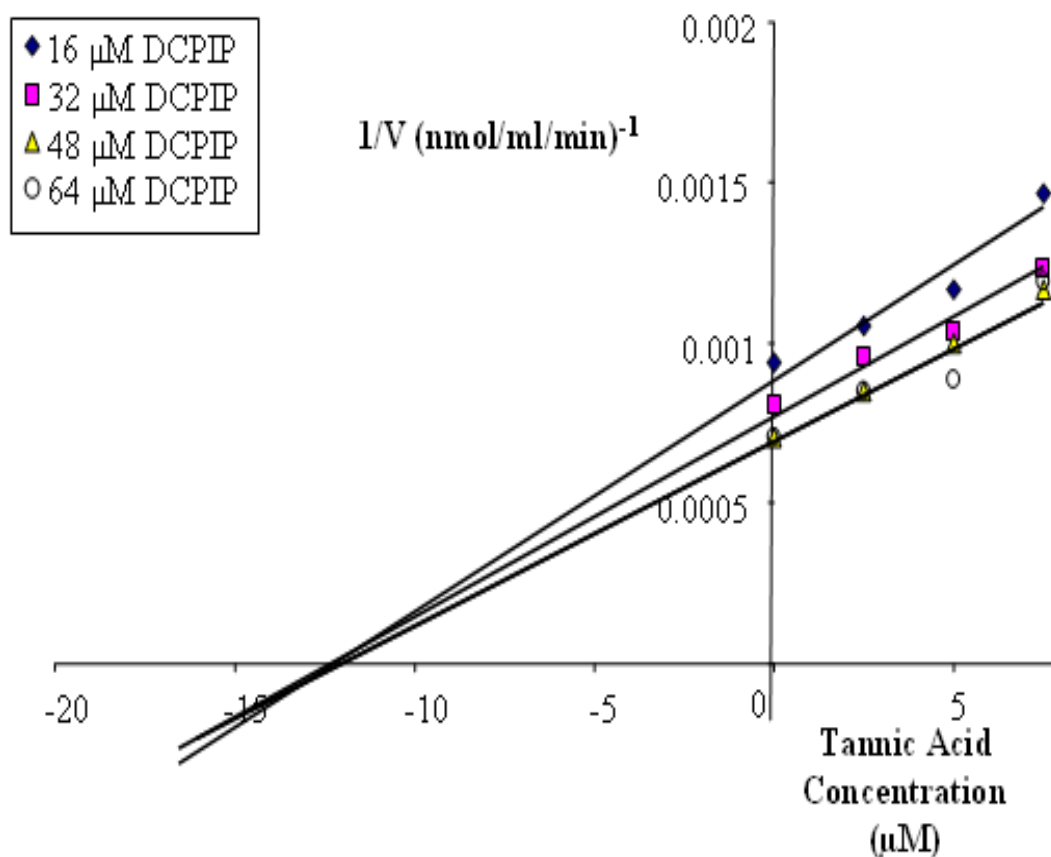
$1/V$  was also plotted against tannic acid concentration (Dixon plot) since Dixon plot is used to identify the type of inhibition and to determine the  $K_I$  value. As shown in Figure 3.28, the plot suggests the inhibition manner to be apparently noncompetitive. From the intersecting point of four lines, the  $K_I$  value was determined to be  $12.6 \mu\text{M}$ .



**Figure 3. 26** Michaelis - Menten plot for rabbit kidney cytosolic NQO1 activity. Effect of tannic acid concentration on rabbit kidney cytosolic NQO1 activity was measured using different concentrations of DCPIP (20 µM – 80 µM).



**Figure 3. 27** Lineweaver-Burk plot for NQO1 activity of rabbit kidney cytosol preincubated with different concentrations of tannic acid. The apparent  $K_m$  was 5.9  $\mu\text{M}$  and  $V_{\text{max}}$  values of the reaction were decreased from 1.4  $\mu\text{mol/ml/min}$  to 0.9  $\mu\text{mol/ml/min}$ .



**Figure 3.28** Dixon plot for rabbit kidney cytosolic NQO1 activity,  $1/V$  versus [tannic acid], in the presence of different fixed concentrations of substrate DCPIP. The apparent  $K_I$  value for tannic acid was calculated to be 12.6  $\mu\text{M}$ .

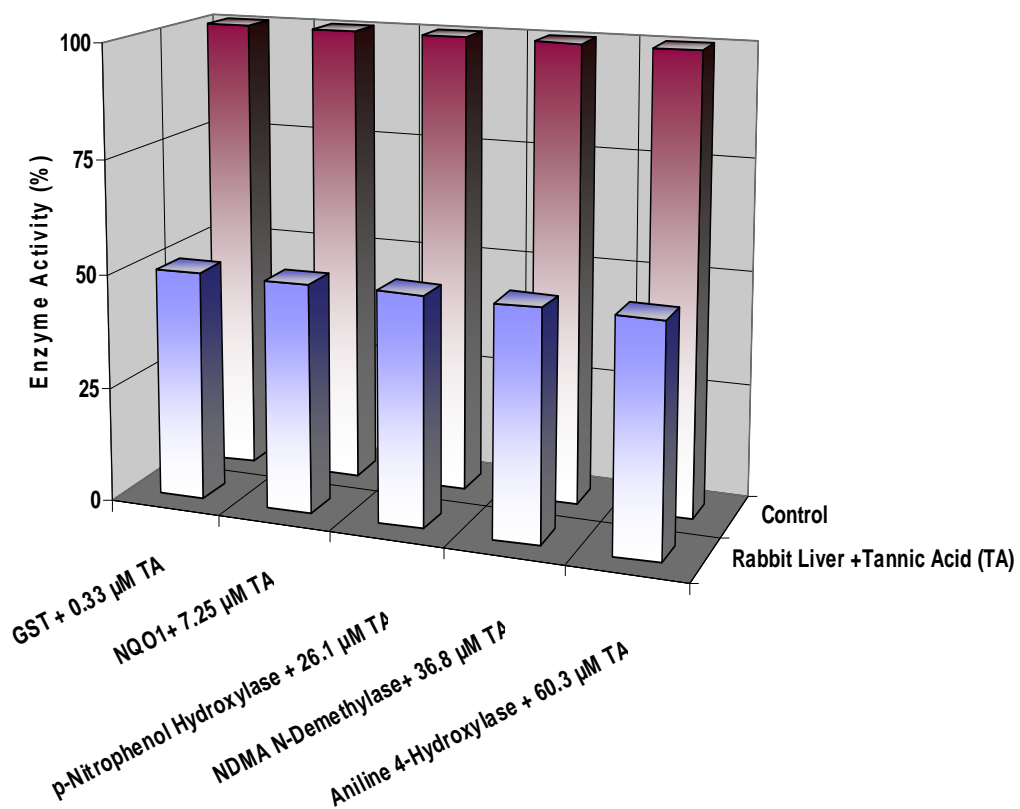
## CHAPTER 4

### DISCUSSION

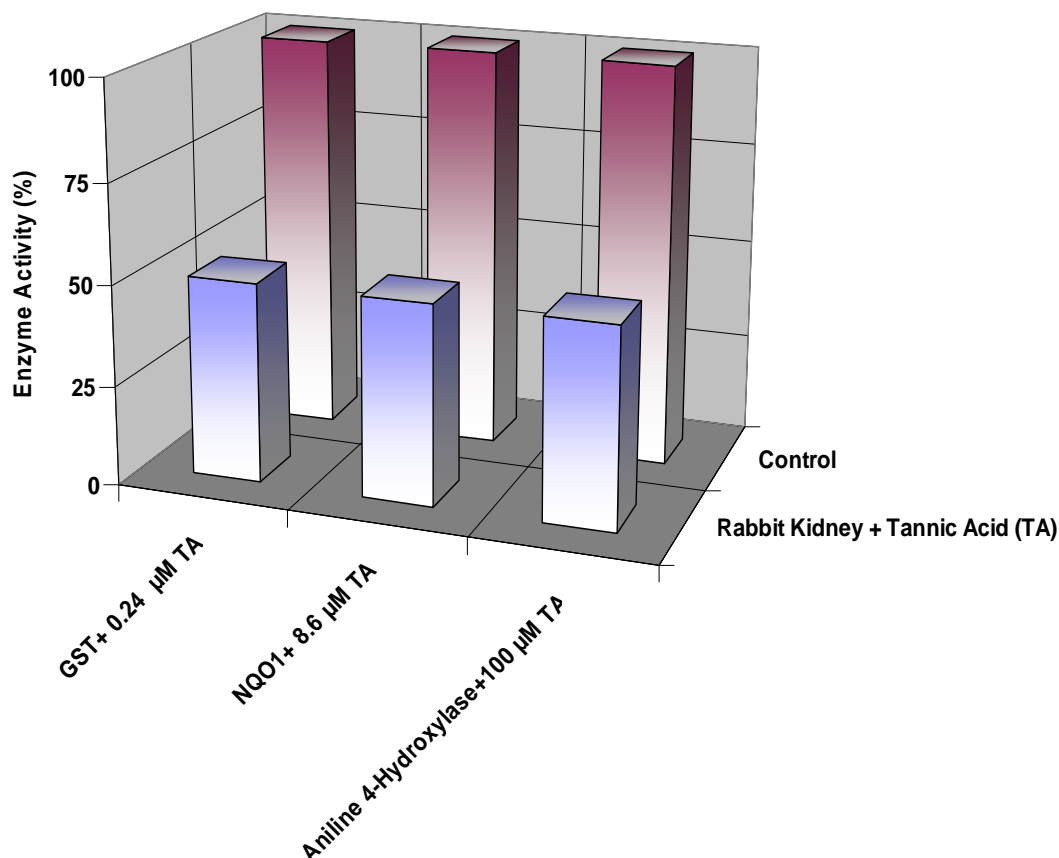
One of the major causes of death across the world has been determined as cancer over the past decades. The best way to protect from cancer is to prevent it before it starts, which can be possible by modulation of dietary factors. Phenolic compounds, abundant in vegetables and fruits ubiquitous in diet, were described to play an important role as chemopreventive agents. It is known that approximately 90 % of human cancer is due to environmental chemicals. Fortunately our bodies are capable of managing environmental exposure by detoxifying them via detoxification enzymes. These enzyme systems generally function adequately to minimize the potential of damage from xenobiotics. The detoxification systems are highly complex. Such as conjugation reaction of Phase II enzymes does not have only protective activity but also activating activity on chemical carcinogens. Although GST has an important role in detoxification of several chemicals, it activates some other chemicals such as 1,2-dihaloethanes, dichloromethane, haloalkenes, vicinal dihaloalkenes and haloacids (Rannug, *et al.*, 1978; Bladeren van, 1988; Sherratt *et al.*, 1997; Anders and Dekant, 1998). Therefore modulation of this enzyme system has a crucial importance for chemoprotection.

In this concept, tannic acid, hydrolysable polyphenol produced from the secondary metabolism of plants, passes one step further due to its ability to modulate Phase I and Phase II enzymes. Tannic acid was suggested to be anticarcinogenic (Chunk *et al.*, 1998). Variety of edible vegetables and fruits possess tannic acid such as sorghum, grapes, strawberries lotus, nut wines and tea (Goldstein; 1963; Hoff, 1975; Sanderson *et al.*, 1975; Chavan *et al.*, 1977; Salunkhe *et al.*, 1982).

Up to now, several compounds have been reported as selective inhibitors of cytochrome(s) P450, including both reversible and mechanism-based inactivators. Such as coumarins was demonstrated to have a structure-activity relationship for P450 CYP1A1 selectivity and the type or mode of inhibition (Cai *et al.*, 1993). Diallyl sulfide and phenethyl isothiocyanate from *Allium sp.* and *Brassicaceae* vegetables, respectively, have selective inhibition on CYP2E1 (Guyonnet *et al.*, 2000). Most studies with plant polyphenols mainly focused on small laboratory animals such as rats and mice. However no data is available concerning effects of tannic acid on rabbit Phase I and Phase II enzymes. In this in vitro study we have demonstrated that tannic acid has an inhibitory effect on rabbit liver and kidney cytochrome P450 dependent Phase I enzyme activities (aniline 4-hydroxylase, p-nitrophenol hydroxylase and NDMA N-demethylase) and also Phase II enzyme activities (GST and NQO1) as shown in Figure 4.1 and Figure 4.2, respectively.



**Figure 4.1** The effects of tannic acid on rabbit liver Phase I and Phase II enzyme activities. Control values are given as 100 %. The names of the enzyme and the amount of tannic acid for inhibition of 50 % of initial activity were given.



**Figure 4. 2** The effects of tannic acid on rabbit kidney Phase I and Phase II enzyme activities. Control values are given as 100 %. The names of the enzymes are given with required amount of tannic acid for inhibition of 50 % of initial activity.

P4502E1, a member of cytochrome P450 superfamily, is involved in the metabolism of both exogenous and endogenous compounds. Exogenous substrates and also inducers of CYP2E1 are industrial chemicals, drugs and procarcinogens/carcinogens. Among them are nitrosamines including NDMA (Gorro *et al.*, 1981; Arınç *et al.*, 2007), heterocyclic compounds like pyridine (Arınç *et al.*, 2000a, b), small molecular weight hydrocarbons such as benzene and styrene (Guengerich, 1995). Activation of these chemicals into more toxic or carcinogenic form is catalyzed by CYP2E1. Moreover CYP2E1 reduces molecular oxygen, resulting in the formation of  $H_2O_2$  and  $O_2^{\cdot-}$  radicals (Gorsky *et al.*, 1984; Elkstran and Ingelman-Sundberg, 1989; Persson *et al.*, 1990). Therefore



Cytochrome P4502E1 has received a great deal of attention in recent years because of its vital role in the activation of many toxic chemicals. Its possible role in the activation of xenobiotics to electrophilic, potentially mutagenic metabolites and in tumor development has been demonstrated in studies with benzene (Mehlman, 1991) and nitrosamines (Yoo *et al.*, 1988; Yang *et al.*, 1990).

The inhibition of CYP2E1 dependent enzyme activities observed in our study might be important for anticarcinogenic activity of tannic acid. The inhibition of CYP2E1 is expected to prevent both the toxicity and the carcinogenicity of compounds given above. In order to study type and mode of inhibition of CYP2E1 enzymes in detail, preinduction of these enzymes with chemical carcinogens such as benzene is required. However we have used untreated rabbits to study the effects of tannic acid on CYP2E1 dependent aniline 4-hydroxylase, *p*-nitrophenol hydroxylase and NDMA N-demethylase enzymes.

Phase II enzymes are generally assumed to possess an important protective properties due to their detoxification action. However it is demonstrated that these enzymes such as GST and NQO1 also activates some carcinogenic compounds (Ernster, 1967; Lind *et al.*, 1990; Anders and Dekant, 1998; Xu and Thornalley, 2001). Therefore, inhibition of these enzymes on one hand contributes to suicide of the cell but on the other hand, prevention of carcinogenic and mutagenic compounds. In this respect, the present study is the first concerning the effects of tannic acid on Phase I and Phase II enzymes of rabbit liver and kidney. The results obtained in this study have shown that tannic acid significantly inhibited the activities of rabbit microsomal *p*-nitrophenol hydroxylase, aniline 4-hydroxylase, N-nitrosodimethylamine N-demethylase and cytosolic glutathione S-transferase and NAD(P)H: quinone oxidoreductase 1 enzymes.

Tannic acid was found to be the most potent inhibitor with IC<sub>50</sub> of 0.33 µM on the activity of rabbit liver cytosolic glutathione S-transferase and the least potent inhibitor with IC<sub>50</sub> of 60.26 µM on the activity of rabbit liver microsomal aniline 4-hydroxylase. Tannic acid has distinct inhibitory mechanism on cytosolic GST activities of rabbit liver and kidney. Moreover, tannic acid is found as an

uncompetitive inhibitor of rabbit kidney GST activity while it is a noncompetitive inhibitor for hepatic GST activity. These results obtained from rabbit were different from in vivo mouse and rat studies of Krajka-Kuźniak *et al.* (2003) and Nijhoff *et al.* (1993), respectively. Krajka-Kuźniak *et al.* (2003) showed that tannic acid acted as an activator in mouse kidney while Nijhoff *et al.* (1993) did not find any change in the overall GST activity in rat liver as a result of the tannic acid dietary treatment. Therefore, the above studies together with ours indicate that the experimental model (in vitro, in vivo), the method of animal treatment (injection, dietary) and the species' and tissue's specific response affect the enzyme activity by this polyphenol, tannic acid. Inhibition of GST activity has a crucial importance since it may increase efficacy of chemotherapeutic drug. In order to prevent its adverse effects on normal tissues it may be used with tumor specific monoclonal antibodies as combinational therapy.

As it can be seen in Table 4.1, inhibitory effect of tannic acid on rabbit liver Phase I and Phase II enzyme activities can be ordered from more to less as GST > NQO1 > PNPB > NDMA N-demethylase > aniline 4-hydroxylase. Due to very low enzyme activity of PNPB and NDMA N-demethylase measured in rabbit kidney microsomes, it can not be possible to study these enzymes in detail. But, a similar inhibitory effect of tannic acid in kidney enzymes (GST > NQO1 > aniline 4-hydroxylase) was observed.

**Table 4. 1** Effects of tannic acid on Phase I and Phase II enzymes of rabbit liver and kidney. IC<sub>50</sub> is the amount of tannic acid for inhibition of 50 % of total enzyme activity.

Name of Enzyme	IC <sub>50</sub>	
	Liver	Kidney
GST	0.33 μM	0.24 μM
NQO1	7.25 μM	8.6 μM
PNPB	26.1 μM	ND
NDMA N-Demethylase	36.8 μM	ND
Aniline 4-Hydroxylase	60.3 μM	100 μM

ND; not determined

Inhibition kinetics and mechanisms were studied in the presence of different concentrations of tannic acid and substrates using Michaelis – Menten, V versus [S], Lineweaver - Burk, 1/V versus 1/[S], and Dixon, 1/V versus 1/ [tannic acid] plots It was shown that tannic acid has distinct inhibitory mechanism on enzyme activities depending on the type of enzyme and the type of tissue as shown in Table 4.2.

**Table 4. 2**  $K_I$  values and type of inhibition of Phase I and Phase II enzymes by tannic acid.

Name of Enzyme	Liver		Kidney	
	$K_I$	Type	$K_I$	Type
GST	0.3 $\mu$ M	Noncompetitive	0.615 $\mu$ M to 0.252 $\mu$ M	Uncompetitive
NQO1	41 $\mu$ M.	Noncompetitive	12.6 $\mu$ M.	Noncompetitive
PNPH	ND	ND	ND	ND
NDMA N-Demethylase	ND	ND	ND	ND
Aniline 4-Hydroxylase	54.7 $\mu$ M	Noncompetitive	ND	ND

ND; not determined

Aniline ( $C_6H_5NH_2$ ) and its derivatives are widely used in chemical, pharmaceutical, and agricultural industries (Belitz *et al.*, 2004). Moreover these compounds have been introduced into the environment directly through industrial discharges and may cause environmental pollution (Fishbein, 1984). Experimental studies with laboratory animals have shown that aniline and its metabolites have a very acute and chronic toxicity. Such as aniline metabolite, acetaminophen causes both liver necrosis while it is shown to be an acute nephrotoxin of the pars recta of the proximal tubule (Gartland *et al.*, 1989, 1990; Thomas, 1993; Fu *et al.*, 2004).

Aniline 4-hydroxylase activity is known to be mainly catalyzed by enzymes belonging to the P450 2E1 gene subfamily (Chen *et al.*, 1996). As given before, CYP2E1 is involved in the metabolism of several xenobiotics, including chemical carcinogens. Hence, its modulation can dramatically affect the compound's toxicity and carcinogenesis. It was shown that aniline 4-hydroxylase, a member of mixed-function oxidase, has catalyzed transformation of aniline to nephrotoxic *p*-aminophenol (Calder *et al* 1979, Crove *et al*, 1979). Therefore inhibition of aniline 4-hydroxylase activity has a crucial importance to prevent toxic effects of the metabolites of aniline. The incidence of kidney toxicity due to aniline metabolism by this enzyme may be prevented through inhibition by tannic acid. In order to prevent substrate inhibition, aniline concentrations used in this study were chosen under 15 mM (Arinc and Iscan, 1983). Gaillard (1974) showed that tannic acid given to rats in a dose of 25 mg/kg/day for 4 days caused a significant inhibition of rat aniline 4-hydroxylation. Kinetic studies on kidney enzymes were not carry out due to low expression of P450s including P4502E1 in this tissues. In the present work, tannic acid was shown to be a noncompetitive inhibitor of rabbit liver microsomal aniline 4-hydroxylase with  $K_I$  of 54.7  $\mu$ M and it binds to the enzyme at a site other than the active site. Presence of the tannic acid may cause a change in the structure and shape of the enzyme. This change in shape reduces the concentration of active enzyme resulting in a decrease in the  $V_{max}$  (1.005 nmol/min/mg to 0.615 nmol/min/mg) without affecting the binding affinity,  $K_m$  (1.72 mM) of the substrate. There is no competition between the tannic acid and aniline, so increasing the concentration of the substrate still does not allow the maximum enzyme activity rate to be achieved.

PNPH and NDMA N-demethylase are other enzymes to evaluate tannic acid for its ability to modulate cytochrome P450 2E1 mediated metabolism. In their study, Mikstacka *et al.* (2002) showed that tannic acid is potent inhibitor of CYP2E1 dependent mouse liver microsomal PNPH activity with  $IC_{50} = 29.6 \mu$ M. The results obtained in this study ( $IC_{50}$  is 26.1  $\mu$ M) were found similar to those. Moreover they studied for both the mode and the type of inhibition and they found that tannic acid is a mixed ( $K_I = 1 \mu$ M) inhibitor for acetone induced mice liver PNPH. Since, in this study we have obtained a very low PNPH enzyme activity

(0.18 nmol/mg liver/min) in rabbit microsomes, it is not possible to study inhibition kinetic of PNPH both in liver and kidney. Therefore induction of PNPH is required in order to study inhibition kinetic. Krajka-Kuźniak *et al.* (2003) also showed in their in vivo study that the activity of both mouse hepatic and renal PNPH was reduced by ~ 50% as result of treatment of 80 mg/kg tannic acid.

*N*-nitrosodimethylamine (NDMA) is the simplest dialkyl nitrosamine (ATSDR, 1989). The main NDMA sources are the manufacture of pesticides, rubber tires, alkylamines, and dyes. Moreover it can be formed under natural conditions in air, water, and soil as a result of chemical, photochemical, and biological processes and has been detected in drinking-water and in automobile exhaust (Ayanaba & Alexander, 1974). The presence of NDMA has been demonstrated in some foods, most frequently in beer, cured meat, fish products, and some cheeses (OME, 1998; Goff *et al.*, 1980). There is great evidence that NDMA is mutagenic and clastogenic (reviewed in IARC, 1978; ATSDR, 1989). Increased tumor incidences were reported at concentrations of NDMA of about 5 mg/L in drinking-water and 10 mg/kg in the diet. Increased incidences of nasal, hepatic, pulmonary, and renal tumors were observed in rats exposed to NDMA via inhalation (Moiseev & Benemanskii, 1975; Klein *et al.*, 1991). In order to show mutagenic, carcinogenic and clastogenic effects, NDMA required metabolic activation since it is a procarcinogen. CYP2E1 dependent monooxygenases, specifically known as NDMA N-demethylase is responsible for NDMA metabolism. As a result of the metabolism of unstable hydroxylated metabolites methyl (hydroxymethyl) nitrosamine is formed. Then it is converted to the methylated methyldiazonium ion which alkylates biological macromolecules such as DNA, RNA, and proteins (Souliotis *et al.*, 1995; Yang and Hong, 1995; Liteplo and Meek, 2001). Due to an adverse effect of NDMA on human health, modulation of NDMA N-demethylase has a crucial importance. In this study it was demonstrated that tannic acid has an inhibitory effect on rabbit liver NDMA N-demethylase activity with IC<sub>50</sub> of 36.84 μM. Moreover it was detected that hepatic NDMA N-demethylase activity is higher than renal. This is due to metabolism of xenobiotics accomplished mainly in liver. Since, in this study we have measured a very low NDMA N-demethylase enzyme activity in rabbit

microsomes, it is not possible to study inhibition kinetic of NDMA N-demethylase both in liver and kidney. Therefore induction of this enzyme is necessary in order to study inhibition kinetic.

Glutathione S-transferases (GSTs, EC 2.5.1.18) are multifunctional dimeric enzymes which have a crucial role in the biotransformation and detoxification of potential alkylating agents and xenobiotics during phase II reactions. It was demonstrated that bioactivation of toxic metabolites by GST may cause organ damages. Furthermore the resistance of cells and organisms to pesticides, herbicides and antibiotics was implicated in GST activities. GSTs have been shown to be overexpressed in tumor cells hence it increases the resistance for chemotherapeutic drugs. In this study in vitro effect of tannic acid, hydrolysable polyphenol, produced from the secondary metabolism of plants, for its ability to modulate rabbit liver and kidney cytosolic glutathione S-transferase activity was determined. It was shown that tannic acid is the potent inhibitor of the rabbit cytosolic glutathione S-transferase activity in organs, liver and kidney with  $IC_{50} = 0.33 \mu\text{M}$  and  $IC_{50} = 0.24 \mu\text{M}$ , respectively. Rabbit cytosolic GSTs are the most effected enzymes in the presence of tannic acid compared to other Phase I and Phase II enzymes studied in this work. Tannic acid inhibits hepatic and renal GST activities with different mechanisms. In liver, tannic acid was shown to be a noncompetitive inhibitor of GST with  $K_I$  of  $0.3 \mu\text{M}$  ( $K_m$  remained unchanged while  $V_{max}$  decreased). In hepatic tissue, tannic acid binds other site of GST apart from catalytic site and binding causes a conformational change in GST resulting decrease of  $V_{max}$ . However, tannic acid was found to be uncompetitive inhibitor of renal GST (both  $K_m$  and  $V_{max}$  were changed). In kidney, tannic acid can bind only to the complex formed between GST and CDNB (the ES complex). On the other hand, Krajka-Kuźniak *et al.* (2003) showed in their in vivo study with mouse that tannic acid increases (24 %) the GST activity in kidney. Moreover, Nijhoff *et al.* (1993) did not find any change in the overall GST activity in rat liver as a result of the tannic acid dietary treatment.

Inhibition of GST may have a crucial importance for treatment of tumor cells in which chemotherapeutic drug resistance are increased due to high expression of GST. On the other hand, tannic acid's inhibitory effect on GST activity of normal cells may cause problems for detoxification system of those cells. Therefore it was suggested that tannic acid may be introduced to the tumor cells inside liposomes bound to specific monoclonal antibodies for tumor cells. It may be used in combinational therapy with other chemotherapeutic drugs in order to increase their efficacy.

Another detoxification enzyme studied in this work is NAD(P)H:quinone oxidoreductase 1 (NQO1; EC 1.6.99.2). It is a homodimeric, cytosolic flavoprotein. It is able to perform two-electron reduction of quinones and nitro aromatics (Ernster, 1958; Ross, 2004). NQO1 is accepted to having important protective properties, not only by detoxifying some carcinogenic compounds but also by preventing the generation of oxygen radicals via catalyzation of two-electron transfer from both reduced pyridine nucleotides to some redox azo dyes and quinones (Smitskamp-Wilms *et al.*, 1996; Begleiter *et al.*, 1997). On the other hand, this enzyme may provide not only a cellular detoxifying system, but also, with some substrates, an activating mechanism. These comprise significant mutagens and carcinogens, such as heterocyclic amines (De Flora *et al.*, 1988; Kitamura *et al.*, 1999). NQO1 catalyzes the conversion of 4-nitroquinoline N-oxide to 4-hydroxyamino-quinoline N-oxide, an obligatory proximate metabolite (Nagao and Sugimura, 1976). Therefore on one hand the inhibition of NQO1 contribute to the anticarcinogenic activity of tannic acid towards amine derivatives, but on the other hand, it may accelerate the formation of the reactive oxygen species through the semiquinones pathway. The major NQO1 inhibitor is dicoumarol which competes with the binding of NAD(P)H (Ernster, 1960). Dicoumarol has distinct effect on NQO1 activity in in-vivo and in vitro studies. It was shown that 1.6  $\mu$ M dicoumarol inhibits 50 % of activity for in vivo studies while it decreases to 0.5 nM for in vitro studies (Lee *et al.*; 2005). In this study we have demonstrated that tannic acid dramatically reduced the activity of rabbit cytosolic NQO1. Krajka-Kuźniak *et al.* (2003) showed in their in vivo study that 90 % decrease of hepatic enzyme activity was observed in mice after treatment

with tannic acid at the doses of 60 and 80 mg/kg. This is contradictory to the reported beneficial effects of these phytochemical. Like dicoumarol, in vivo inhibition of NQO1 by flavonoids does not occur at the same concentration causing inhibition in in vitro studies. In their study Lee *et al.* (2005) showed that several flavonoids that inhibit NQO1 activity in in-vitro studies did not inhibit in living cells. This is due to high level of NAD(P)H concentration in living cells. We have found that degree of inhibition of rabbit cytosolic NQO1 activity with dicoumarol was increased with increasing tannic acid concentration. It was previously also demonstrated that dicoumarol competes with the binding of NAD(P)H (Ernster, 1960). We demonstrated that tannic acid was a noncompetitive inhibitor for hepatic and renal NQO1s with  $K_I$  of 41  $\mu$ M and 12  $\mu$ M, respectively. Therefore it is suggested that tannic acid may cause conformational changes in NQO1's active site where dicoumarol binds, which decrease the binding possibilities of dicoumarol and therefore NAD(P)H has chance to bind there. Binding of dicoumarol to the NQO1 inhibits the electron transfer from NAD(P)H to FAD (Huang *et al.*, 1987). Any conformational changes due to tannic acid prevent this inhibition effect of dicoumarol and increase the enzyme activity. Moreover it may be possible to increase the activity by increasing NAD(P)H concentration.

The results of the present study indicate that this phenolic compound, tannic acid can modulate carcinogens activating and detoxifying pathways, particularly those in which CYP2E1, GST and NQO1 are involved. This effect depends on the species and tissues used and type of enzyme determined. The results of the studies in literature together with ours indicate that the experimental model (in vitro, in vivo), the method of animal treatment (injection, dietary) and the species' and tissue's specific response affect the enzyme activity differently by this polyphenol, tannic acid. Since humans are exposed to tannic acid in their diet, it is important to understand its pharmacological and toxicological functions and also to understand how it might interact and modulate carcinogen-metabolizing enzymes in different laboratory animals.



## CHAPTER 5

### CONCLUSION

Chemical carcinogens need metabolic activation that catalyzed by cytochrome P450 and Phase II enzymes in order to exert their genotoxic and carcinogenic effects. Hence one possible mechanism is that phenolic compounds may alter anticarcinogenic effects is through an interaction with these enzymes either by the inhibition or activation of certain forms, leading to a reduced production of the ultimate carcinogen. Therefore anti-carcinogen activity of tannic acid, a hydrolyzable plant polyphenol, has a crucial importance to prevent conversion of pro-carcinogens to their carcinogenic form. Tannic acid is produced from secondary metabolism of plants and is found in edible vegetables, fruits and nuts, especially tea, cocoa, coffee and wine.

In the present work, modulation of rabbit liver and kidney microsomal cytochrome P450 (CYP2E1) dependent aniline 4-hydroxylase, NDMA N-demethylase and *p*-nitrophenol hydroxylase activities and cytosolic phase II enzymes; glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase:1 (NQO1) were studied in the presence of tannic acid at concentrations ranging from 0.5  $\mu$ M to 150  $\mu$ M in the reaction medium.

The results obtained in this study have shown that tannic acid significantly inhibited the activities of *p*-nitrophenol hydroxylase, aniline 4-hydroxylase, NDMA N-demethylase, glutathione S-transferase, NAD(P)H:quinone oxidoreductase 1. Tannic acid was found to be the most potent inhibitor with IC<sub>50</sub> of 0.33  $\mu$ M on the activity of cytosolic glutathione S-transferase and the least potent inhibitor with IC<sub>50</sub> of 60.26  $\mu$ M on the activity of microsomal aniline 4-hydroxylase.

Effect of tannic acid on enzyme activities was further studied for both the mode and the type of inhibition. For this purpose various concentrations of the substrate were examined at various tannic acid concentrations. Lineweaver-Burk and Dixon plots were then generated from the resulting data sets. The inhibition constants ( $K_I$ ) were determined from double reciprocal and Dixon plots of the enzyme activity versus substrate and inhibitor concentration, respectively. Tannic acid was shown to be a noncompetitive inhibitor for liver cytosolic GST, NQO1 and microsomal aniline 4- hydroxylase enzymes with  $K_I$  of 0.3  $\mu$ M, 41  $\mu$ M and 54.7  $\mu$ M, respectively. On the other hand, in renal tissues, tannic acid was an uncompetitive inhibitor of cytosolic GST, while it was noncompetitive inhibitor for cytosolic NQO1 with a  $K_I$  of 12.6  $\mu$ M.

The results of the present study indicate that this phenolic compound, tannic acid can modulate carcinogens activating and detoxifying pathways, particularly those in which CYP2E1, GST and NQO1 are involved. This effect depends on the tissue used and type enzyme determined. The results of the studies in literature together with ours indicate that the experimental model (in vitro, in vivo), the method of animal treatment (injection, dietary) and the species' and tissue's specific response affect the enzyme activity differently by this polyphenol, tannic acid. Since humans are exposed to tannic acid in their diet, it is important to understand its pharmacological and toxicological functions and also to understand how it might interact and modulate carcinogen-metabolizing enzymes in laboratory animals. More detailed studies, such as in vivo and cell culture, are required to further clarify the effect of tannic acid on CYP2E1 and Phase II enzymes.

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