# MECHANISM OF INHIBITION OF CYTOCHROME P4501A1 ASSOCIATED 7-ETHOXYRESORUFIN O-DEETHYLASE (EROD) ACTIVITY AND GLUTATHIONE S-TRANSFERASE (GST) ACTIVITIES IN FISH LIVER BY PHENOLIC COMPOUNDS/FLAVONOIDS

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# ABSTRACT

MECHANISM OF INHIBITION OF CYTOCHROME P4501A1 ASSOCIATED 7-ETHOXYRESORUFIN O-DEETHYLASE (EROD) ACTIVITY AND GLUTATHIONE S-TRANSFERASE (GST) ACTIVITIES IN FISH LIVER BY PHENOLIC COMPOUNDS/FLAVONOIDS

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Flavonoids, present in fruits, vegetables and beverages derived from plants, have been described as health-promoting, disease-preventing dietary supplements, and have activity as cancer preventive agents. The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including modulating enzyme activities resulting in the decreased carcinogenicity of xenobiotics. Cytochrome P4501A1 (CYP1A1) is a Phase I enzyme which is known to be involved in the activation of procarcinogens and Glutathione S-Transferase (GST) is a Phase II enzyme which is largely responsible for the detoxification of carcinogens. In this study, it was aimed to investigate the mechanisms of inhibition of CYP1A1 and GST activities of fish by phenolic compounds/flavonoids. Leaping mullet (*Liza saliens*), captured from highly polluted sites of İzmir Bay, expressing high levels of CYP1A, were used in order to investigate these effects. It was demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on both CYP1A1 associated 7-Ethoxyresorufin-O-deethylase (EROD) activity and GST activities of fish, although the degree of inhibition was varied with the flavonoid used. Of the flavonoids tested, the most potent inhibitor of CYP1A1 associated EROD activity was found to be quercetin. The potency of the phenolic compounds/flavonoids to inhibit CYP1A1 associated EROD activity follow the sequence of quercetin > resveratrol > naringenin > hesperidin > rutin

with IC50 values of 1.32 μM, 3.59 μM, 9.78 μM, 98.5 μM and 0.64 mM respectively. Quercetin, resveratrol, hesperidin and rutin were found to inhibit EROD activity in a competitive manner, on the other hand, naringenin was found to inhibit EROD activity in a non-competitive manner. Inhibition constant (Ki) values of quercetin, resveratrol, naringenin, hesperidin and rutin were calculated from Dixon plots as 0.12 μM, 0.67 μM, 2.63 μM, 18 μM and 0.1 mM, respectively.

In the case of GST enzyme, it was demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on both total GST and GST-Mu activities of fish. Of the flavonoids tested, the most effective inhibitor of total GST activity was found to be resveratrol. The potency of the phenolic compounds/flavonoids to inhibit total GST activity follow the sequence of resveratrol > quercetin > rutin > naringenin > hesperidin with IC50 values of 7.1 μM, 24.5 μM, 89 μM, 116 μM and 118 μM respectively. Resveratrol, quercetin and hesperidin were found to inhibit total GST activity in a competitive manner, on the other hand, rutin and naringenin were found to inhibit GST activity in a mixed type manner. Ki values of resveratrol, quercetin, hesperidin, naringenin and rutin were calculated from Dixon plots as 3.2 μM, 12.5 μM, 45 μM, 128 μM and 150 μM respectively. In the case of GST-Mu activity, the most potent inhibitor was found to be rutin. The potency of the phenolic compounds/flavonoids to inhibit GST-Mu activity follow the sequence of rutin > resveratrol > quercetin > naringenin > hesperidin with IC50 values of 66.5 μM, 72.3 μM, 113.5 μM, 135.5 μM and 196 μM, respectively.

In conclusion, this study indicated that flavonoids were the strong inhibitors of CYP1A1 associated EROD activity and GST activities of mullet liver. The modulation of drug-metabolizing enzymes by flavonoids is important in terms of human health, since these enzymes can activate or inactivate carcinogens. The potential role of xenobiotic metabolizers CYP1 family in the activation of carcinogens and inactivation of chemotherapeutics suggests a potential therapeutic benefits in inhibiting these enzymes. The results of the present study support the hypothesis that flavonoids may be involved in the prevention of malignant transformation, by reducing the formation of carcinogens through inhibition of enzymes such as CYP1A1 which is known to be involved in carcinogen activation.

Keywords: Flavonoids, CYP1A1, EROD, GST, carcinogenicity, chemoprevention

# ÖZ

FENOLİK BİLEŞİKLERİN/FLAVONOİDLERİN BALIK KARACİĞERİNDEKİ SİTOKROM P4501A1'E BAĞLI 7-ETOKSİRESORUFİN O-DEETİLAZ (EROD) VE GLUTATYON S-TRANSFERAZ (GST) AKTİVİTELERİ ÜZERİNDEKİ İNHİBİSYON MEKANİZMASI

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Flavonoidler meyvelerde, sebzelerde ve bitkilerden elde edilen içeceklerde mevcut olup sağlık teşvik edici, hastalık önleyici diyet takviyeleri olarak tanımlanmıştır ve kanser önleyici aktiviteleri bulunmaktadır. Flavonoidlerin kanser koruyucu etkileri, ksenobiyotik metabolize eden enzimlerin aktivitelerini modüle ederek ksenobiyotiklerin karsinojenitesini azaltmaya dayalı mekanizmayı da içeren birçok çeşitli mekanizmaya atfedilmiştir. Sitokrom P4501A1 (CYP1A1) pro-kanserojenlerin aktivasyonundan sorumlu olduğu bilinen bir faz I enzimidir. Glutatyon S-Transferaz (GST) büyük ölçüde kanserojen detoksifikasyonundan sorumlu olan bir faz II enzimidir. Bu çalışmada, balık CYP1A1 ve GST aktivitelerinin fenolik bileşikler/flavonoidler tarafından inhibisyon mekanizmasının araştırılması amaçlanmıştır. Bu etkileri incelemek üzere, İzmir Körfezinin oldukça kirli noktalarından toplanan ve yüksek CYP1A1 düzeyi gösteren kefal balığı (*Liza saliens*) kullanılmıştır. Kullanılan flavonoidle birlikte inhibisyon derecesindeki farklılıklara rağmen, kullanılan tüm fenolik bileşiklerin/flavonoidlerin hem CYP1A1 bağımlı 7-etoksiresorufin O-deetilaz (EROD) aktivitesi hem de GST enzim aktiviteleri üzerindeki rolü inhibe edici olarak bulunmuştur. Test edilen flavonoidlerden, CYP1A1 bağımlı EROD aktivitesinin en etkili inhibitörü kuersetin olarak bulunmuştur. Fenolik bileşiklerin/flavonoidlerin CYP1A1 bağımlı EROD aktivitesini inhibe etmekteki etkinlik sırası quercetin > resveratrol > narinjenin > hesperidin > rutin şeklindedir. IC50 değerleri sırasıyla 1.32 μM, 3.59 μM, 9.78 μM, 98.5 μM and 0.64 mM olarak

bulunmuştur. Kuersetin, resveratrol, hesperidin ve rutinin EROD aktivitesini yarışmacı şekilde inhibe ettiği bulunmuştur, öte yandan narinjeninin EROD aktivitesini yarışmacı olmayan biçimde inhibe ettiği bulunmuştur. Kuersetin, resveratrol, narinjenin, hesperidin ve rutin için inhibisyon sabiti (Ki) değerleri Dixon grafiğinden sırasıyla 0.12 μM, 0.67 μM, 2.63 μM, 18 μM and 0.1 mM olarak hesaplanmıştır.

GST enzim aktivitesi çalışmalarında, kullanılan tüm fenolik bileşiklerin/flavonoidlerin hem total GST hem de GST-Mu aktiviteleri üzerindeki etkileri inhibe edici olarak bulunmuştur. Test edilen flavonoidlerden, total GST aktivitesinin en etkili inhibitörü resveratrol olarak bulunmuştur. Fenolik bileşiklerin/flavonoidlerin total GST aktivitesini inhibe etmekteki etkinlik sırası resveratrol > quercetin > rutin > narinjenin > hesperidin şeklindedir. IC50 değerleri sırasıyla 7.1 μM, 24.5 μM, 89 μM, 116 μM and 118 μM olarak bulunmuştur. Resveratrol, kuersetin ve hesperidinin total GST aktivitesini yarışmacı şekilde inhibe ettiği bulunmuştur, öte yandan rutin ve narinjenin total GST aktivitesini karışık biçimde inhibe ettiği bulunmuştur. Resveratrol, quercetin, hesperidin, narinjenin ve rutin için Ki değerleri Dixon grafiğinden sırasıyla 3.2 μM, 12.5 μM, 45 μM, 128 μM ve 150 μM olarak hesaplanmıştır. GST-Mu aktivitesi durumunda, en etkili inhibitor rutin olarak bulunmuştur. Fenolik bileşiklerin/flavonoidlerin GST-Mu aktivitesini inhibe etmekteki etkinlik sırası rutin > resveratrol > quercetin > narinjenin > hesperidin şeklindedir. IC50 değerleri sırasıyla 66.5 μM, 72.3 μM, 113.5 μM, 135.5 μM ve 196 μM olarak bulunmuştur.

Sonuç olarak bu çalışma, flavonoidlerin kefal CYP1A1 bağımlı EROD aktivitesinin ve GST aktivitelerinin etkin inhibitörleri olduğunu göstermektedir. İlaç metabolize eden enzimler kanserojenleri etkin veya etkisiz hale getirdiğinden, bu enzimlerin flavonoidler tarafından modülasyonu sağlık açısından önemlidir. Ksenobiyotik metabolize eden CYP1 ailesinin kanserojenlerin aktivasyonundaki ve kemoteröpatiklerin etkisizleştirilmesindeki potansiyel rolü bu enzimlerin inhibe edilmesinin potansiyel teröpatik fayda sağlayacağını ileri sürmektedir. Bu çalışmanın sonuçları, flavonoidlerin kanserojen aktivasyonuna katıldığı bilinen CYP1A1 gibi enzimleri inhibe ederek kanserojen oluşumunu azaltıp malignant transformasyonu önlemeye katılabileceği hipotezini desteklemektedir.

Anahtar Kelimeler: Flavonoidler, CYP1A1, EROD, GST, karsinojenisite, kemoprevansiyon

**To My Mother**

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# TABLE OF CONTENTS

# LIST OF TABLES

TABLES

# LIST OF FIGURES

FIGURES

# LIST OF SYMBOLS

| | |
|---|---|
| ε-ACA | ε-Amino caproic acid |
| 3-MC | 3-Methylcholanthrene |
| AHR | Aryl hydrocarbon receptor |
| ALP | Alkaline phosphatase |
| APS | Ammonium per sulphate |
| ARNT | Ah receptor nuclear translocator |
| B(a)P | Benzo(a)pyrene |
| BCIP | Bromochloroindoylphosphate |
| BSA | Bovine serum albumin |
| BIS | N, N'-Methylene bisacrylamide |
| BNF | β-Naphthoflavone |
| BTE | Basal transcriptional elements |
| CDNB | 1-chloro-dinitrobenzene |
| CYP | Cytochrome P450 |
| DCNB | 1,2-dichloro-4-nitrobenzene |
| DMSO | Dimethylsulfoxide |
| DNB-SG | 1-(S-glutationyl)-2,4-dinitrobenzene |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra acetic acid |
| EROD | 7-Ethoxyresorufin O-deethylase |
| FAD | Flavin adenine dinucleotide |
| FMO | Flavin-containing monooxygenases |
| GSH | Glutathione reduced form |
| GST | Glutathione S-transferase |
| HEPES | N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid |
| IC50 | The concentration giving 50% inhibition |
| KCl | Potassium chloride |
| Ki | Inhibition constant |
| Km | Michaelis-Menten constant |

| | |
|---|---|
| KPi | Potassium phosphate |
| MFO | Mixed function oxidases |
| NADH | Nicotinamideadenine dinucleotide, reduced form |
| $NADP^+$ | Nicotinamideadenine dinucleotide phosphate |
| NADPH | Nicotinamideadenine dinucleotide phosphate, reduced form |
| NBT | Nitrotetrazolium blue |
| NQO1 | NADPH-quinone oxidoreductase |
| Pab | Polyclonal antibody |
| PAGE | Polyacrylamide gel electrophoresis |
| PAH | Polycyclic aromatic hydrocarbons |
| PCB | Polychlorinated biphenyl |
| PMSF | Phenylmethylsulfonyl fluoride |
| SDS | Sodium dodecyl sulfate |
| TCDD | 2,3,7,8-tetrachlorodibenzo-p-dioxin |
| TEMED | N-N-N'-N'-tetramethylenediamine |
| TRIS | Tris(hydroxymethyl) aminomethane |
| Vmax | Maximum velocity |
| XRE | Xenobiotic response element |
| ZTB | Zero time blank |

# CHAPTER 1

# INTRODUCTION

With the high technological and industrial development, exposure to chemical substances is inevitable in modern life. The body deals with these compounds through a system of xenobiotic metabolizing enzymes, called Phase 1 and Phase 2 enzymes. These enzymes work together to metabolize any foreign substance that enter or make contact with the body. The metabolism of the foreign substances can result in toxification or detoxification -the activation or deactivation of the potential carcinogens. If body activates the potential carcinogens, cancer may occur as a result. Cancer is the second leading cause of death worldwide, accounting for 23.1% of all deaths in US (American Cancer Society, US Mortality Data 2006, National Center for Health Statistics, Centers for Disease Control and Prevention, 2009.). World Health Organization reported that deaths from cancer worldwide are projected to continue rising with an estimated 12 million deaths in 2030. It is well known that diet has a very important role in reducing the risk for cancer. Several epidemiological studies provide support for a protective effect of the consumption of fresh fruits and vegetables against cancer (Block *et al.,* 1992; Ingram *et al.,* 1997; Birt *et al.,* 2001; Galati and O'Brien, 2004, Kale *et al.,* 2008; Taylor *et al*., 2009; Bobe *et al.,* 2009). Fruits and vegetables do play a preventive role due to a variety of constituents, including vitamins, minerals, fiber, and numerous phenolic compounds, including flavonoids. Because of the putative beneficial health effects of fruits and vegetables, together with the finding that fruits, vegetables and various beverages contain considerable amounts of phenolic compounds and flavonoids (Kuhnau, 1976; Hertog *et al.,* 1992; King and Young, 1999; Cheynier, 2005; Crozier *et al*., 2009; Meulenberg, 2009), several researchers have focused their attention on these compounds.

## 1.1 Phenolic Compounds

Phenolic compounds comprise one of the largest and most ubiquitous group of plant metabolites. All plant phenolic compounds arise from the common intermediate, phenylalanine, or its close precursor, shikimic acid. They are formed to protect the plant from photosynthetic stress, reactive oxygen species, wounds, and herbivores. Phenolic compounds are an important part of the human diet. The most commonly occurring ones in foods are flavonoids.

### 1.1.1 Flavonoids

Flavonoids are a subclass of naturally occurring polyphenolic compounds and represent one of the most prevalent classes in vegetables, nuts, fruits and beverages such as coffee, tea, and red wine (Hollman and Katan, 1997). It has been estimated that humans consume approximately 1 g of mixed flavonoid per day (Middleton, 1984). Flavonoids are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge. This bridge consists of three carbons that combine with an oxygen and two carbons of one of the aromatic rings (A ring) to form a third 6-member ring (C ring). Figure 1.1 represents general structure and numbering pattern for common food flavonoids.

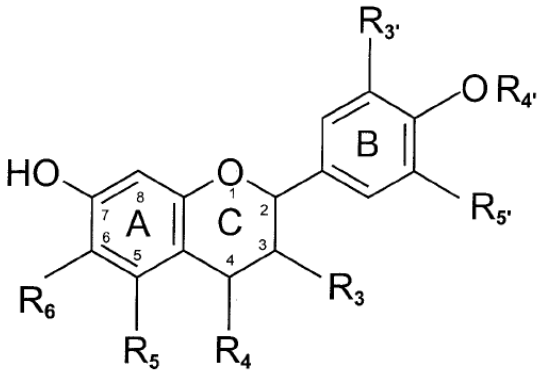



**Figure 1.1** General structure and numbering pattern for common food flavonoids (Beecher, 2003)

The flavonoids are further divided into subclasses based on the connection of the B ring to the C ring, as well as the oxidation state and functional groups of the C ring. Table 1.1 shows the chemical structures and major food sources of the 6 major flavonoid subclasses.

**Table 1.1** The chemical structures and major food sources of the 6 flavonoid subclasses (Moon *et al*., 2006)

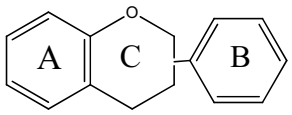
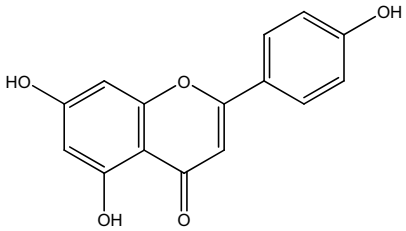
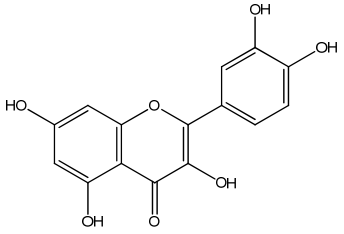
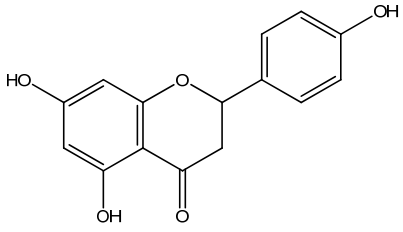
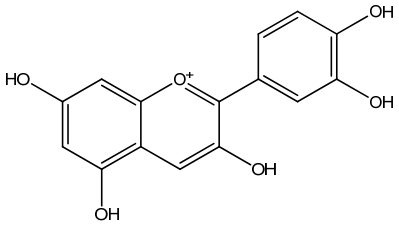
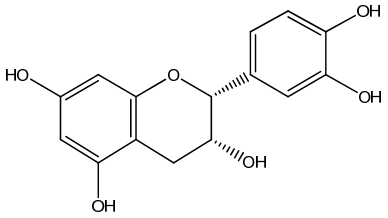
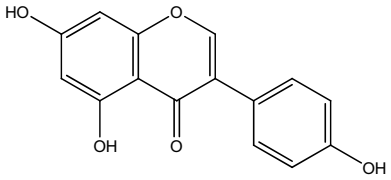
| | Structure | Example | Major food source |
|---|---|---|---|
| Basic structure of flavonoid |   | | |
| Flavone |   Apigenin | Acacetin<br>Apigenin<br>Baicalein<br>Chrysin<br>Diosmetin<br>Luteolin<br>Tangeretin | Parsley, thyme, celery, sweet red peppers, honey, propolis |
| Flavonol |   Quercetin | Galangin<br>Kaempferol<br>Morin<br>Myricetin<br>Quercetin | Onions, kale, broccoli, apples, cherries, berries, tea, red wine |
| Flavanone |   Naringenin | Eriodictyol<br>Hesperetin<br>Naringenin | Citrus |

**Table 1.1** The chemical structures and major food sources of the 6 flavonoid subclasses (continued)

| | Structure | Example | Major food source |
|---|---|---|---|
| Anthocyanin |  Cyanidin | Cyanidins Pigmented Compounds | Cherries, grapes, berries, red cabbage |
| Flavanol |  Epicatechin | Catechin Epicatechin | Cocoa, chocolate, green tea, red wine, hawthorn, bilberry, motherwort and other herbs |
| Isoflavone |  Genistein | Biochanin A Genistein Diadzein Formononetin Equol | Red flower, alfalfa, peas, soy and other legumes |

More than 5000 subclasses of flavonoids were identified by 1990 (Harbourne, 1993). This research will concern 5 of the most common phenolic compounds/ flavonoids namely; quercetin, resveratrol, rutin, naringenin and hesperidin. These phenolic compounds/flavonoids will be discussed in details in section 1.3.

#### 1.1.1.1 Therapeutic Effects of Flavonoids

Many epidemiological studies have indicated that consumption of some plant-derived foodstuffs with high phenolic content is associated with the prevention of some diseases and that these compounds may have similar properties to antioxidants,

antimutagenic agents, antithrombotic agents, anti-inflammatory agents, anti-HIV-1, and anticancer agents (Artico *et al.,* 1998; Son and Lewis, 2002; Montpied *et al.,* 2003).

The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify carcinogens, and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters (Canivenc-Lavier *et al*., 1996; Shih *et al*., 2000).

Flavonoids also have inhibitory effect on the activities of many enzymes, including β-glucuronidase (Kim *et al*., 1994), lipoxygenase (Laughton *et al*., 1991; Schewe *et al*., 2002), cyclooxygenase (Laughton *et al*., 1991), inducible nitric oxide synthase (Raso *et al*., 2001), cytochrome P4501A, 3A3 and 3A4 dependent monooxygenases (Siess *et al*., 1995), thyroid peroxidase (Doerge and Chang, 2002), xanthine oxidase (Sheu *et al*., 1998), mitochondrial succinoxidase and NADH oxidase (Hodnick *et al*., 1994), phosphodiesterase (Picq *et al*., 1989), phospholipase $A_2$ (Gil *et al*., 1994), and protein kinase (Cushman *et al*., 1991).

Figure 1.2 illustrates the effects of flavonoids in different stages of carcinogenesis. Flavonoids can interfere with different steps of multistage process of carcinogenesis. Flavonoids can inhibit the metabolic activation of procarcinogens to their ultimate electrophlic species by phase I enzymes (predominantly cytochrome P450s), or their subsequent interaction with DNA. Therefore these agents block tumor initiation (Blocking agents). Flavonoids can also inhibit the promotion and progression steps in carcinogenesis by affecting cell cycle, angiogenesis, invasion and apoptosis, preventing the malignant tumor formation (Suppressing agents). Alternatively, flavonoids can stimulate the detoxification of carcinogens by inducing phase II enzymes, leading to their elimination from the body.

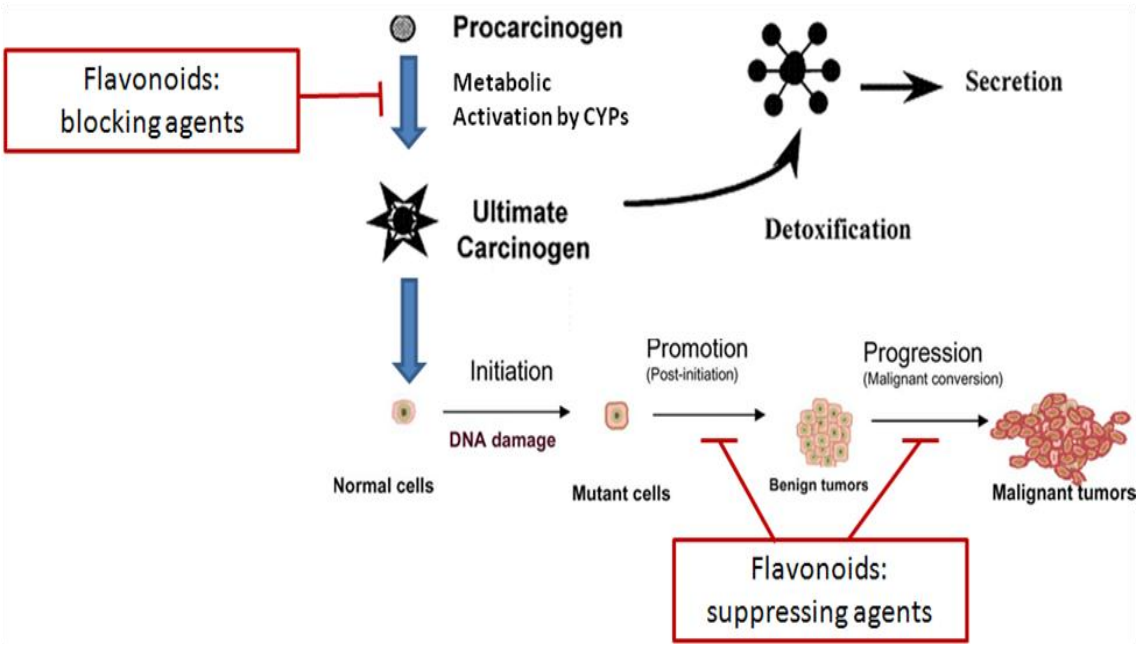



**Figure 1.2** The effects of flavonoids in different stages of carcinogenesis (Adapted from Moon *et al*., 2006)

The focus of this research is examining the effects of phenolic compounds/flavonoids and on a cytochrome P450 (CYP) and a phase II enzyme, CYP1A1 and GST respectively, which are key enzymes involved in the metabolism of xenobiotics.

### 1.2 Phase I and Phase II Xenobiotic Metabolizing Enzymes

Phase I and Phase II xenobiotic metabolizing enzymes are the major enzymes responsible for the biochemical metabolic alterations of xenobiotics and drugs. These enzymes work together to convert lipophilic compounds to more water soluble compounds via biotransformation to be excreted. The reactions mainly take place in the liver; however these reactions also occur in lung, kidney, skin and gastrointestinal tracts, adrenal testis, ovary, placenta and brain of mammalian tissues in different amounts and functions.

### 1.2.1 Phase I Xenobiotic Metabolizing Enzymes

Phase I reactions mainly convert xenobiotics or drugs to a more polar metabolite by introducing or revealing a functional group such as -OH, -$NH_2$, -SH groups. These phase I reactions involve oxidation, reduction or hydrolysis reactions. Phase I reactions generally convert lipophilic xenobiotics to hydrophilic compounds. This system usually serves as a route of detoxification as well as as a route of metabolic activation to yield metabolites which initiates toxic and carcinogenic events (Nebert and Gonzalez, 1987; Schenkman, 1999).

Phase I enzymes include hydrolytic enzymes such as amidases, esterases and epoxide hydrolases; reductive enzymes such as azoreductases, disulfide reductases, NADPH-quinone oxidoreductase (NQO1), aldo-keto reductases, nitroreductases; oxidative enzymes such as alcohol and aldehyde dehydrogenases, amine oxidases, flavin-containing monooxygenases (FMOs) and Cytochrome P450 monooxygenases (Schenkman, 1999). Among all of them, Cytochrome P450 monooxygenases are the major phase I xenobiotic metabolizing enzymes.

#### 1.2.1.1 Cytochrome P450s

Cytochrome P450 monooxygenases are a superfamily of heme proteins that are the terminal oxidases of the mixed function oxidase system. These enzymes are localized predominantly in the smooth endoplasmic reticulum of the liver and other tissues. They play critical roles in the metabolism of exogenous substrates including drugs, dietary and environmentally derived toxicants and carcinogens and also in the metabolism of several endogenous compounds, such as fatty acids and steroids (Lu and Levin, 1974; Arınç and Philpot, 1976; Arınç and Adalı, 1983; Lieber *et al.,* 1997; Gonzalez and Kimura, 2003). The name cytochrome P450 (CYP) derives from the spectroscopic observation that when a drug is bound to the reduced heme enzyme ($Fe^{+2}$), carbon monoxide can bind to the complex and absorb light at a characteristic and distinctive 450 nm (Omura and Sato, 1964).

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 two components; cytochrome P450 and FAD and FMN containing NADPH dependent cytochrome P450 reductase. Later, it was shown that three components,

cytochrome P450, FAD and FMN containing NADPH dependent cytochrome P450 reductase and lipid, phosphatidylcholine dilauryl are required to reconstitute the full hydroxylation activity (Lu and Coon, 1968; Lu and Levin, 1974; Arınç and Philpot, 1976; Black and Coon, 1986; Adalı and Arınç, 1990; Arınç 1993). In the microsomal systems, FAD and FMN containing flavoprotein, NADPH dependent cytochrome P450 reductase catalyzes electron transfer from NADPH to cytochrome P450. Lipid is involved in the transfer of electrons from NADPH-cytochrome P450 reductase to cytochrome P450 in the monooxygenase system. The mechanism postulated by cytochrome P450s is illustrated in Figure 1.3. The cycle is initiated by substrate binding to oxidized form of cytochrome P450 which shifts the equilibrium between the low and high spin configuration of cytochrome P450 towards the high spin state. The cytochrome P450-substrate complex is, then, reduced to ferrous form ($Fe^{+2}$) with the electron donated by the NADPH via the flavoprotein ($F_{PT}$) cytochrome P450 reductase. Later, the complex is oxygenated and the oxyferrous cytochrome P450 is formed. This is followed by the second electron flow from NADPH which converts the bound oxygen into superoxide anion ($O^{-}_{2}$) form. After an internal oxidoreduction reactions, hydroxylated substrate is released and cytochrome P450 is regenerated in its oxidized ($Fe^{+3}$) form.

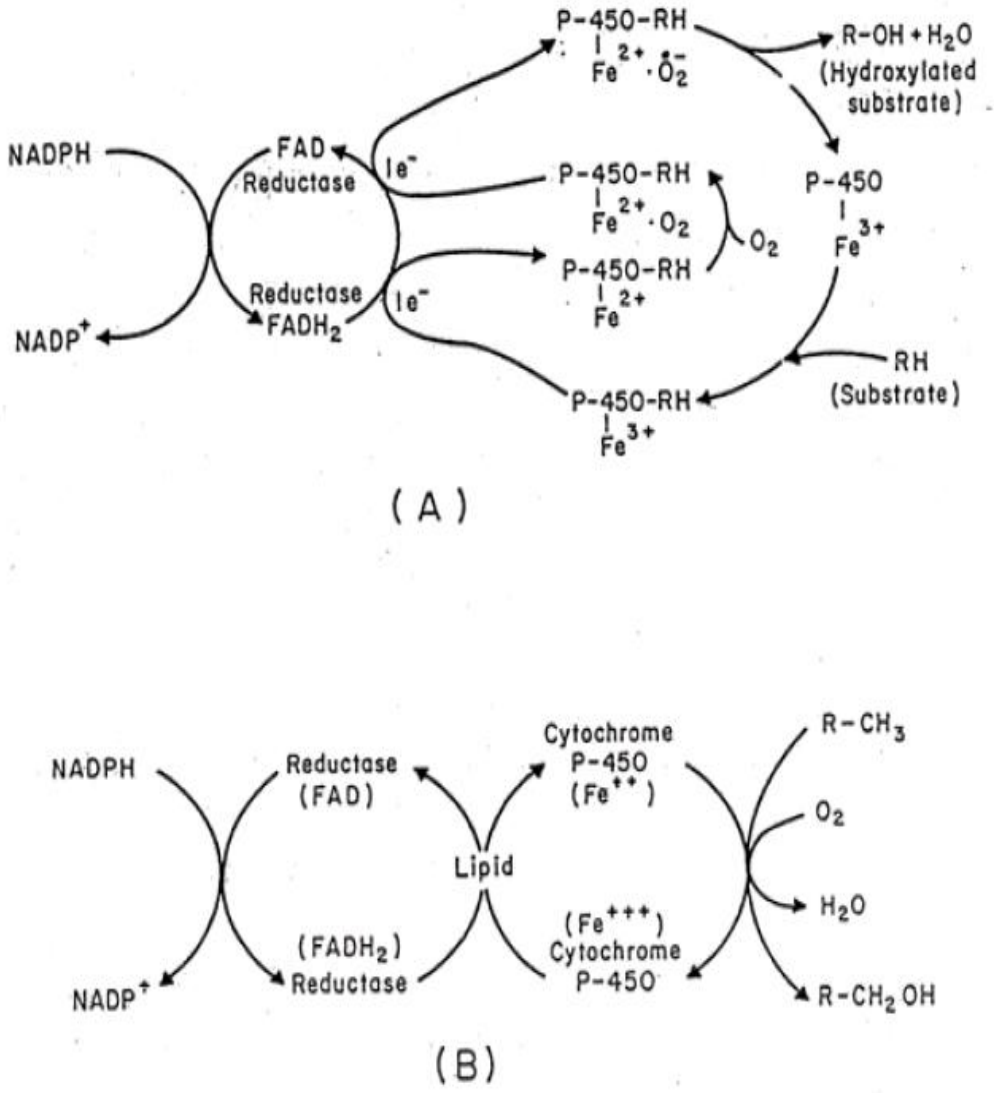



**Figure 1.3** (A) Mechanism postulated for the hydroxylation of organic substrates by the liver microsomal cytochrome P450 system (taken from Lehninger, 1975)

(B) The role of lipid in mechanism

The reaction catalyzed by P450s involves the oxidation of a substrate (R) where there is a heterocyclic ring, an aromatic ring, an alkane, or an alkene substituent, in the presence of atmospheric oxygen ($O_2$) and reduced cofactor (NADPH, $H^+$). The general reaction mechanism catalyzed by P450s is as follows.

$$\text{NADPH, } H^+ + \mathbf{O_2} + \text{RH} \longrightarrow \text{NADP}^+ + \mathbf{H_2O} + \text{R-}\mathbf{O}\text{H}$$

Only one atom of oxygen is incorporated into the substrate in this reaction, with the other atom being reduced to water, thus the cytochrome P450 dependent enzymes are generally classified as monooxygenases. Depending on the particular reaction and the nature of various unstable intermediates, different reactions can occur. These include oxidative and reductive dehalogenation; N-hydroxylation and N-oxidation; oxidative deamination; S-, N-, and O-dealkylation; and aliphatic and aromatic hydroxylation (Schenkman, 1991; Guengerich, 1993).

Substrates of cytochrome P450s include the majority of drugs and other xenobiotics, together with several types of endogenous compounds (Gonzalez, 1989; Anzenbacher and Anzenbacherova, 2001; Danielson, 2002; Hsu *et al*., 2007). The endogenous substrates of P450s include saturated and unsaturated fatty acids, eicosanoids, sterols and steroids, bile acids, vitamin $D_3$ derivatives, retinoids, and uroporphyrinogens. Also, many cytochrome P450 enzymes can metabolize various xenobiotics including drugs, plant- or fungal-derived secondary metabolites consumed with food, and thousands of environmental pollutants like halogenated hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), arylamines, ingredients of combustion, industrial complex mixtures, herbicides, and pesticides, resulting in their detoxification (Gonzalez, 1989). Besides, the action of P450 enzymes can also generate toxic metabolites that contribute to increased risks of cancer, birth defects, and other toxic effects (Nebert and Russell, 2002). Such P450 substrates include PAHs (e.g. benzopyrene), nitrosamines, hydrazines, and arylamines.

It is well known that certain chemicals can “induce” (increase the level) some of cytochrome P450s in the tissues of organisms when exposed to those chemicals called inducers. Because of their role in metabolism and activation of aromatic hydrocarbon carcinogens and other toxic chemicals, the members of the CYP1A subfamily and their

response to various exogenous inducers such as β-Naphthoflavone (BNF), PAHs, 2,3,7,8-Tetrachloridibenzo-p-dioxin (TCDD) are among the best characterized and well studied in whole cytochrome P450 superfamily (Nebert and Gonzalez, 1987; Nebert *et al*., 1991, 2004).

P450s have been characterized in many species of organisms, including bacteria, fungi, plants, fish, birds, reptiles, insects and mammalian systems (Lu and Levin, 1974; Philpot *et al*., 1975; Arınç *et al*., 1976; Arınç and Philpot, 1976; Coon *et al*., 1978; Nebert and McKinnon, 1994; Şen and Arınç, 1997, 1998a,b). As of August 20, 2009; a total of 11270-cytochrome P450 sequences were identified from various organisms, so that 3282 animals, 4266 plants, 2570 fungi, 905 bacteria, and 247 other eukaryotic CYPs were counted (http://drnelson.utmem.edu/CytochromeP450.html).

Presence of so many P450s in various organisms created a need for systemic nomenclature for cytochrome P450s, and it has been devised in 1987 by Nebert and coworkers and it is now being used worldwide (Nebert *et al*., 1987). This systemic nomenclature is based on amino acid sequence similarities between P450 proteins. According to this system, the cytochrome P450 superfamily is categorized into respective families and subfamilies. Enzymes that share at least 40% sequence homology are assigned to a family designated by an Arabic numeral, whereas those sharing at least 55% homology makeup a particular subfamily designated by a letter (Nebert and McKinnon, 1994; Nelson *et al*., 1996). Single members of a subfamily represent a particular enzyme and are designated by the number following the subfamily description (e.g. CYP1A1). The example given below explains the system of nomenclature.

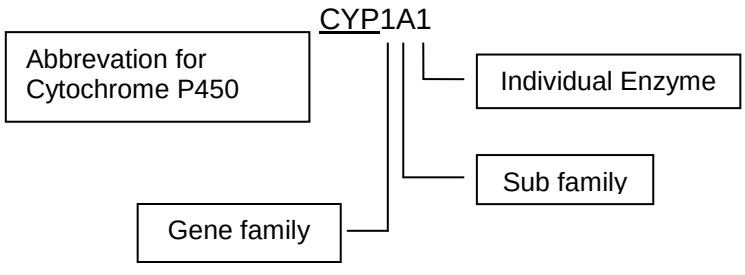



**Figure 1.4** Example of CYP nomenclature

As stated earlier, P450s have been characterized in many species of organisms, including fish. Cytochrome P450 dependent mixed function oxidase system in fish is explained in details below.

#### 1.2.1.1.1 Cytochrome P450 Dependent Mixed Function Oxidase System in Fish

Although, Brodie and Maickel in 1962 suggested that fish lacked the required enzymes for the metabolism of xenobiotics, it is now well established that a liver MFO system, with the capability of metabolizing a variety of chemicals exists in both freshwater and marine fish (Arınç *et al*., 1976; Arınç and Adalı, 1983; Arınç and Şen, 1993a; Stegeman 1993, 1995; Stegeman *et al*., 1997). Resolution of the fish liver microsomal MFO system was first described by Arınç *et al.* in 1976. Little skate liver microsomal MFO system was resolved into three components: cytochrome P450, NADPH cytochrome P450 reductase and lipid. Thus, MFO systems in fish and invertebrates appear to be multicomponent systems similar to the microsomal cytochrome P450 dependent electron transport systems in mammals. The major MFO system in fish, as in mammals, is associated with microsomes from hepatic tissues, although it has been observed in virtually all tissues examined. A number of reviews have appeared on these topics (Stegeman 1993; Andersson and Förlin, 1992; Bucheli and Fent, 1995; Buhler, 1995; Stegeman, 1995).

Defining the function of MFO system and linking a given characteristic of MFO system to the action of environmental chemicals require knowing the features associated with the varied biological or physiological conditions. In mammals, sex, diet and age are among the factors known to influence MFO system (Arınç and Şen, 1999a). Marked sex differences also occur in cytochrome P450 and/or MFO activity of fish. Moreover, in fish, there are changes in MFO activity associated with season and gonadal status (for reviews see Andersson and Förlin, 1992; Bucheli and Fent, 1995). The implication is that MFO of marine species might be regulated by hormonal factors, as well as that MFO system participates in hormone metabolism. As in the mammal system, multiple forms of cytochrome P450 belonging to the families of CYP1A, CYP2B, CYP2E, CYP2M, CYP2K, CYP3A, CYP11A, CYP17, and CYP19 are found in fish. There are about 51 cytochrome P450 forms reported to be purified, partially purified or cloned from aquatic species (Stegeman 1993; Stegeman *et al*., 1997; Andersson and Förlin, 1992; Bucheli and Fent, 1995; Buhler, 1995; Buhler, 1995; Stegeman, 1995; Şen and Arınç, 1997, 1998b; Bozcaarmutlu and Arınç, 2008).

Among the purified fish cytochrome P450 forms, P4501A1 homologues hold the priority due to its role in metabolism of carcinogens, mutagens and environmental pollutants. So the occurrence and functions of cytochrome P4501A forms in diverse organisms are being investigated vigorously (Arınç and Şen, 1999a).

#### 1.2.1.1.2 Cytochrome P4501A

CYP1A subfamily consists of two genes, CYP1A1 and CYP1A2, present in all mammalian species studied up to now. These forms are previously called as cytochromes P448. Both CYP1A1 and CYP1A2 are involved in oxidative metabolism and activation of exogenous chemicals such as aromatic hydrocarbons, aromatic amines and other toxic chemicals. A number of planar compounds including 3-methyl-cholanthrene (3-MC), benzo(a)pyrene (B(a)P), TCDD, dibenzofurans, polychlorinated biphenyls (PCBs) are known to be potent common inducers of these two forms of cytochrome P450.

CYP1A1 and CYP1A2 subfamilies share 75% identical amino acid sequences, a very similar gene organization and chemical properties (Sogawa *et al*., 1985; Gonzalez *et al*., 1990) but their catalytic properties for carcinogenic chemicals and mechanism of transcriptional regulation show clear differences. For example, in animals, such as rat, rabbit and mouse cytochrome P4501A1 had a markedly high specific B(a)P hydroxylase activity which resulted in carcinogenicity, while CYP1A2 had a limited activity (Yamazoe and Kato, 1992; Ioannides and Parke, 1993). Expression of CYP1A1 had been correlated with development of polycyclic aromatic hydrocarbon-associated cancers and other disorders in rodents (Nebert and Jones, 1989). Another difference between mammalian CYP1A1 and CYP1A2 forms is that, they show very different patterns of tissue expression. CYP1A2 is expressed primarily in the liver. On the other hand, CYP1A1 is expressed primarily in extrahepatic tissues such as the lungs, lymphocytes and placenta (Arınç, 1993; Ding and Kaminsky, 2003; Shimada *et al*., 2003; Ionnides and Lewis, 2004; Bièche *et al*., 2007; Özkarslı *et al*., 2008).

In animals, CYP1A1 gene is inducible expressed in liver and extrahepatic tissues such as kidney, lung and skin while inducible expression of cytochrome P4501A2 gene is exclusively limited to the liver. In humans, cytochrome P4501A1 gene is not appreciably expressed in liver, but inducible expressed in extrahepatic tissues and in placenta. Many cell lines derived from hepatocytes and other tissues such as Hepa-1 and HeLa cells exhibit inducible expression of CYP1A1 in response to the

inducer, similar to those in livers of experimental animals, while cell lines with the ability of inducible expression of CYP1A2 have not been reported. Primary cultures of hepatocytes also rapidly lose the inducibility of CYP1A2, whereas that of CYP1A1 is retained long time.

Cytochrome P4501A studies were extensively carried out in aquatic organisms due to its central role in assessing the toxic effects of chemicals both from the point of view of organism and from that of chemical. Furthermore, inducibility of the enzyme system by only certain class of chemicals in fish could provide a valuable indication of the presence of such compounds in the field and an assessment of early toxic effects on living organisms.

#### 1.2.1.1.2.1 Cytochrome P4501A in Fish

In all fish species studied so far a single gene or purified protein with the properties related to the CYP1A subfamily in the mammals have been found. (Williams *et al*, 1982; Klotz *et al*., 1983; Williams *et al*.,1984; Goksoyr *et al*., 1985; Heilman *et al*,1988; Zhang *et al*., 1991; Morrison *et al.*, 1995; Şen and Arınç, 1998b). Heilmann *et al*. (1988) cloned cytochrome P4501A cDNA from trout liver and found that trout P4501A had 57-59% of the amino acid residues identical to various mammalian P4501A1 forms, and 51-53% identical to mammalian P4501A2 forms. Based on these results, they have decided that the trout cytochrome P4501A is a P4501A1 (Heilmann *et al*., 1988).

Cytochrome CYP1A1 has been extensively studied in fish due to its role in the biotransformation of environmentally persistent aromatic hydrocarbons (e.g. TCDD, PAHs) and their relationship with disease process resulting from exposure to compounds of this nature (Hahn and Stegeman, 1994). It was purified from several teleosts species, from freshwater species rainbow trout (Williams and Buhler 1984, Miranda *et al*., 1989; 1990; Andersson, 1992), and perch (Zhang *et al*., 1991) and from marine species scup (Klotz *et al*., 1983; 1986 Stegeman *et al*., 1990), and cod (Goksoyr, 1985; Goksoyr *et al*., 1986). Şen and Arınç (1998a) have reported the purification of cytochrome P4501A in a homogenous form from the leaping mullet (*Liza saliens*) caught from the most polluted part of İzmir Bay. Its catalytic, spectral, and electrophoretic and immunological properties have shown the characteristics of P4501A1. It gave a single band on SDS-PAGE, having a monomer of *M*r 58000 ± 500. 7-ethoxyresorufin O-deethylation (EROD) activity was reconstituted in the presence of

purified mullet P4501A, purified mullet NADPH cytochrome P450 reductase, and lipid (Şen and Arınç, 1998a,b). The CO-difference spectrum of dithionite-reduced cytochrome P4501A showed a peak at 448 nm and its absolute absorption spectrum had a major peak at 417.5 nm. Purified *L. saliens* liver microsomal cytochrome P4501A1 showed strong cross-reactivity with the antibodies directed against the cytochrome P4501A1 homologues purified from other teleost species such as rainbow trout and scup. Spectral, electrophoretic, immunochemical and biocatalytic properties of the purified cytochrome P4501A1 strongly suggested that it is the CYP1A1 in the *L. saliens* liver (Şen and Arınç, 1998a,b).

In a study, Şen *et al*. (2001) performed sequence analysis of leaping mullet CYP1A1 cDNA. In this study, alignment of 13 fish species demonstrated that there were totally 248 aminoacid identical with the fish species examined. Among the overall conserved 248 aminoacids in the 13 fish species, 184 of them (74.2%) match with the corresponding aminoacids in human CYP1A1 (Şen *et al*., 2001).

It is well known that, the level of CYP1A1 in unexposed and/or untreated fish is very low, even is barely detectable. However CYP1A1 are highly inducible by various PAH, PCB, TCDD and other halogenated compounds. Table 1.2 shows the xenobiotics that induce fish cytochrome P4501A1.

Liver CYP1A1 induction in fish by certain classes of chemicals described above has been applied extensively as a biomarker in the field studies (Arınç *et al*., 2000). Examples of recent field studies employing CYP1A and/or associated enzyme activities in fish liver as a biomarker are given in Table 1 of Arınç *et al*., 2000. Most studies compare CYP1A concentrations and enzyme activities in fish from suspected sites with those in fish from reference sites. Most of the earlier field studies employed the induction of liver BPH activity in biomonitoring. The use of this assay has been declining because of the carcinogenic property of the substrate, benzo(a)pyrene as well as the possibility of substrate cross reactions with other CYP isozymes. CYP1A associated enzyme activity has been determined by using 7-ethoxyresorufin as a substrate. The measurement of 7-ethoxyresorufin O-deethylase (EROD) activity appears to be the most sensitive and the most widely used catalytic probe for determining induction response of CYP1A in fish. The advantages of using EROD activity as a biomarker are the specificity for CYP1A in fish, high sensitivity, feasibility and simplicity of its measurement (Bucheli and Kent, 1995; Arınç and Şen, 1994; Arınç and Şen 1999b; Bozcaarmutlu *et al*., 2009).

**Table 1.2** Examples of xenobiotics that induce CYP1A1 in fish

| |
|---|
| Benzo(a)pyrene |
| 2,3,7,8-Tetrachloridibenzo-p-dioxin (TCDD) |
| 3-Methylcholanthrene |
| Polychlorinated biphenyls |
| Ethoxyquin |
| β-Naphthoflavone |
| Hexabromobenzene |
| Endosulfan |
| Butylatedhydroxytoluene |
| Pyrene, Chrysene |
| Butylated hydroxyanisole |
| 7,12-dimethylbenz(a)-anthracene |
| Tert-Butylhydroxyquinone |

When CYP1A concentrations and enzyme activities in fish from suspected sites were compared with those in fish from reference sites, it was found that leaping mullet from the highly urbanized and industrial section of İzmir Bay, Pasaport, showed highly elevated enzyme activities which were about 56 to 62 times higher than the value at the reference site (Arınç and Şen, 1999a; Arınç and Şen 1999b; Arınç *et al.,* 2000).

The induction of cytochrome P4501A1 by xenobiotics is an interesting example of an adaptive cellular response to an altered chemical environment. As described in more detail below, induction of cytochrome P4501A1 is an interesting model response for analyzing the mechanism by which a lipophilic chemical signal activates transcription of a specific gene. Such analyses provide insights into the regulation of mammalian transcription in general.

### 1.2.1.1.2.2 Regulation of Cytochrome P4501A1 Induction

Regulation of induction of CYP1 family is the best studied and well characterized among the cytochrome P450 families because of its association with the etiology of several cancers that are thought to arise through CYP1-mediated bioactivation of procarcinogens.

There are at least two types of regulatory DNA sequences found in the cell. The first one is designated as xenobiotic responsible elements (XRE) that works as an inducible enhancer in response to inducers. This cis-acting regulatory DNA element is important in terms of inductive response (Fujisava-Sehara *et al*., 1987; Whithlock *et al*.,

1996). The other regulatory element is named as basal transcription element (BTE) that is involved in the constitutive expression of the gene (Yanagida *et al*., 1990; Fuji-Kuriyama *et al*., 1992). It has been shown that these regulatory elements exist in human CYP1A1 gene.

XRE works via a trans-acting regulatory factor which is found in the cytosol. It is identified and named as XRE-binding factor or aromatic hydrocarbon receptor (AhR) (Denison *et al*., 1986; Fujisawa-Sehera *et al*., 1987; Jonson and McKnight, 1989; Elferink *et al*., 1990). The AhR is a ligand-activated transcription factor that controls several dozen genes, including up-regulation of all CYP1 genes (Nebert *et al*., 2004). Ligands for the AhR include dioxin, PAHs, polyhalogenated aromatic hydrocarbons, indoles and tryptophan-derived endogenous ligands, and benzoflavones found especially in cruciferous plants (Denison and Nagy, 2003). The AhR gene exists in all vertebrates and even in *Caenorhabditis elegans* (Hahn, 2002). The AhR participates in cell cycle control and apoptosis that is cell type- or tissue-specific (Nebert *et al*., 2000). To date, at least nine mutations in and near the human AhR gene (Daly *et al*., 1998; Smart and Daly, 2000; Wong *et al*., 2002) and a staggering 2,213 mutations in and near the mouse Ahr gene spanning ~16 kb from 13 inbred strains (Thomas *et al*., 2002) have been reported. The Ahr(-/-) knockout mouse exhibits lowered viability and fertility and defects in liver development (Fernandez-Salguero *et al*., 1995; Lahvis *et al*., 2000; Shimizu *et al*., 2000). The Ahr(-/-) mouse lacks constitutive and inducible CYP1 expression and is resistant to TCDD-induced toxicity (Fernandez-Salguero *et al.,* 1996), topical BaP-induced skin tumors (Shimizu *et al*., 2000), and benzene-induced hemotoxicity (Yoon *et al*., 2002). The Ahr(-/-) mouse generated in Japan (Shimada *et al*., 2002) appears to have high constitutive CYP1A2 levels in liver but not in lung.

It was found that AhR exists as cryptic form in the cytoplasm of the untreated cells and associates with the inducers when it is available (Denison *et al*., 1986; Cuthill *et al*., 1987; Hapgood *et al*., 1989). In the presence of an inducer, the inducer or the lipophilic ligand crosses the plasma membrane and binds to the AhR. The AhR has been characterized as a ligand-activated transcription factor and member of the bHLH:PAS (basic Helix-Loop-Helix:Per-Arnt-Sim) family. AhR is normally present in the cytosol as a heterotetrameric 9S complex with two heat shock Hsp90 protein molecules and other not well identified protein(s) (P: immunophilin related and:or pp60Src). Hsp90 acts as a chaperone protein system that prevents transcriptional activation of the AhR but keeps the receptor in a conformation that facilitates ligand binding. After ligand

binding, Hsp90 are released, AhR is translocated to the nucleus by an unknown process and binds to its partner, designated as the Ah receptor nuclear translocator (Arnt) (Hoffman *et al*., 1991). This heterodimer interacts with 5'-GCGTG-3' DNA sequence, the core binding motif of the Xenobiotic Responsible Element (XRE). Binding of AhR:Arnt leads to changes in the chromatin structure allowing transactivation (TA) of AhR-controlled genes (Ah-gene-battery). An initiation complex forms at the promoter and the transcription of CYP1A1 mRNA initiates. Consequently, messenger RNA synthesis is increased, resulting in elevated protein level. The model mechanism for the induction of CYP1A1 is given in Figure 1.4.

Studies with teleost species (Kloepper-Sams and Stegeman, 1987; Haasch *et al*., 1989; Lorenzo and Okey, 1990) showed that regulation of CYP1A1 is a receptor mediated system as seen in mammals. An Ah receptor similar to the mammalian Ah receptor has been found in cytosol of several fish (Heilmann *et al*., 1988; Lorenzo and Okey, 1990). Moreover, XRE occurs in inducible gene CYP1A1 gene in fish but it is nature is unknown (Devaux and Pesonen, 1992). On the other hand, while the general mechanism seems to be similar, the process of the CYP1A1 induction in fish has several distinctions when compared to mammals such as greater lag time between mRNA increase and translation, posttranslational suppression by estradiol (Stegeman, 1995).

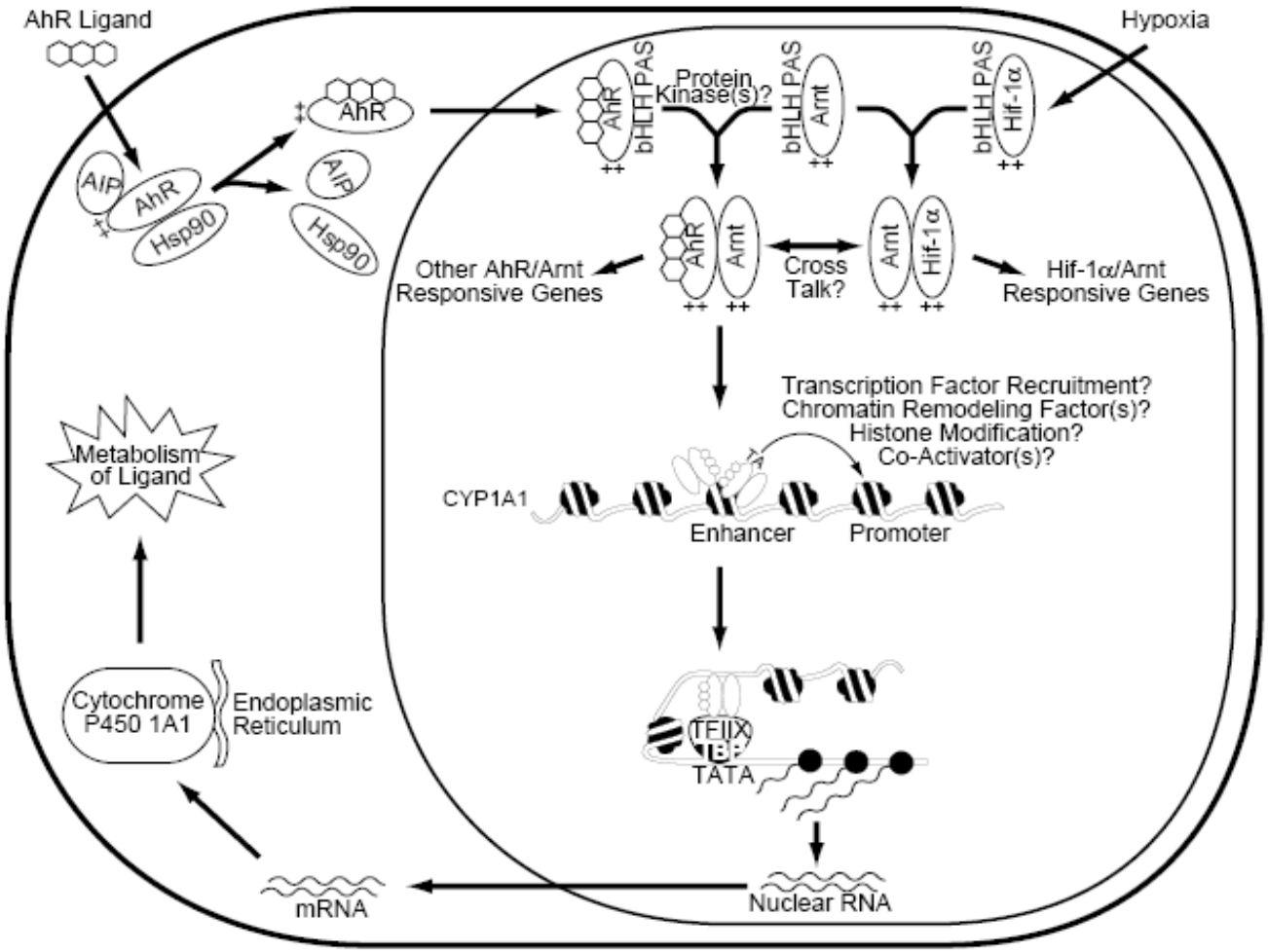



**Figure 1.5** Working model for the induction of cytochrome P4501A1 (taken from Whitlock *et al*., 1996)

### 1.2.2 Phase II Xenobiotic Metabolizing Enzymes

The Phase II xenobiotic metabolizing enzymes mainly participate in the conjugation (addition) reactions. In most conjugation reactions, a hydrogen atom present in a –OH, $-NH_2$ or –SH group is replaced by the addition of conjugating subgroups. This functional group such as –OH, $-NH_2$ or –SH groups may either be present on the parent xenobiotic or drug, or they may have resulted from a Phase I reaction of oxidation, reduction or hydrolysis. The conjugating subgroups include glucuronate, acetate, glutathione, glycine, sulfate and methyl groups. The conjugation reactions mainly involve glucuronidation, acetylation, glutathione conjugation, glycine conjugation, sulfate conjugation, methylation. Thus, in these conjugation reactions which are mediated by Phase II xenobiotic metabolizing enzymes; the xenobiotic or drug becomes linked to an endogenous moiety through one or more functional groups (Schenkman, 1999).

Most of the Phase II enzymes include specific transfer enzymes such as UDP-glucuronyl acid transferase, N-Acetyl transferase, ACYL-Co-A glycine transferase, and sulfotransferases. In addition, transmethylases are other Phase II xenobiotic metabolizing enzymes. With the exception of acetylation and methylation, Phase II xenobiotic metabolizing enzymes result in a high increase in xenobiotic/drug hydrophillicity and promote the excretion of foreign chemicals.

#### 1.2.2.1 Glutathione S-Transferases (GSTs)

The glutathione S-transferase (GST) enzymes are supergene family of dimeric enzymes that catalyze the conjugation of a variety of compounds with the endogenous tripeptide, glutathione (GSH), on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom (Strange *et al*., 2000; Hayes *et al*., 2005). They are involved in the cellular detoxification and excretion of many physiological and xenobiotic substances. GST enzymes are expressed in probably all life forms. Three major families of proteins, cytosolic, mitochondrial and microsomal GSTs, are widely distributed in nature and exhibit glutathione transferase activity. The cytosolic and mitochondrial GSTs comprise soluble enzymes that are only distantly related. The three families share the feature that they catalyze conjugation of GSH with 1,2-dichloro-4-

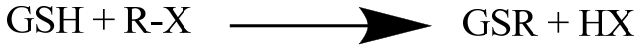
nitrobenzene (CDNB) (Jakobbson *et al*., 1999; Ladner *et al*., 2004; Robinsson *et al*., 2004).

The majority of GST substrates are either xenobiotics or products of oxidative stress. Besides detoxifying electrophilic xenobiotics, such as chemical carcinogens, environmental pollutants, and antitumor agents, glutathione S-transferases inactivate endogenous α,β-unsaturated aldehydes, quinones, epoxides, and hydroperoxides formed as secondary metabolites during oxidative stress. GST-catalyzed endogenous and exogenous compounds are given in Table 1.3.

**Table 1.3** Exogenous and endogenous substrates of GSTs (Adapted from Hayes *et al*., 2005; Coles and Kaldbular, 2003)

| Exogenous Substrates | | Endogenous Substrates |
|---|---|---|
| Chemotherapeutic agents | Adriamycin<br>Busulfan<br>Carmustine<br>Chlorambucil<br>Cis-platin<br>Cyclophosphamide<br>Ethacrynic acid<br>Mitozantrone<br>Thiotepa | α,β-unsaturated aldehydes<br>quinones<br>epoxides<br>hydroperoxides |
| Environmental and Chemical Carcinogens | Acrolein<br>Atrazine<br>DDT<br>Inorganic arsenic<br>Lindane<br>Malathion<br>Methyl parathion<br>Muconaldehyde<br>Tridiphane | |

The generalized detoxification reaction catalyzed by GST is shown below.




Mercapturic acids (N-acetylcysteine conjugates) are the ultimate excreted metabolites formed by further metabolism of glutathione conjugates of organic molecules.

In addition to their role in detoxification, GSTs also carry out a range of other functions. They have peroxidase and isomerase activities, they can inhibit the Jun N-terminal kinase (thus protecting cells against $H_2O_2$-induced cell death), and they are able to bind non-catalytically a wide range of endogenous and exogenous ligands. These enzymes are also intimately involved in the biosynthesis of leukotrienes, prostaglandins, testosterone, and progesterone, as well as the degradation of tyrosine.

The GSTs comprise a complex and widespread enzyme superfamily that has been subdivided further into an ever-increasing number of classes based on a variety of criteria, including amino acid/nucleotide sequence, and immunological, kinetic and tertiary/quaternary structural properties. Mammalian cytosolic GSTs are all dimeric with subunits of 199–244 amino acids in length. Cytosolic GSTs of mammals have been particularly well characterized, and were originally classified into seven classes based on their amino acid sequence similarities. These classes are called Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta. Alpha class GST have been shown to be the predominant isoenzymes in human hepatocytes with the microsomal and mu class GST present in small quantities. Mu class GST is subject to polymorphism and is present in the livers of only about half of the Caucasian adults (Hussey *et al.*, 1987). Pi class GST in the adult human liver is only expressed in the biliary epithelium. In many animal models of hepatocellular carcinoma there is overexpression of pi class GST. The explanation for the heterogeneity of distribution of the various GST isoenzymes within and between tissues is unclear, but one possible reason is that they subserve different functions in different tissues, sometimes acting as carrier proteins rather than being metabolically active. The overexpression of certain isoenzymes, principally pi class

GST in intestinal and pancreatic tumours and alpha class GST in hepatocellular carcinoma, may simply represent clonal expansion of those cells expressing these isoenzymes.

Non-mammalian GSTs have been much less well characterized. In non-mammalian species other classes are designated as Beta, Delta, Epsilon, Lambda, Tau and 'U' class. In human and rodents, cytosolic GST isoenzymes within a class typically share >40% identity, and those between classes share <25% amino acid sequence identity (Sheehan *et al*., 2001; Edwards and Dixon, 2004).

Phase II enzymes are also inducible by exposure to certain foreign compounds (Hammock and Ota, 1983; Anderson *et al*., 1985; George 1994). Altered GST activity indicates defensive response of the organism to enhanced pollution stress (Roy and Hänninen 1993). Benzo[a]pyrene as well as other PAHs and PCBs have been shown to cause significant alterations in the activities of antioxidant enzymes in laboratory experiments with mollusks and fish (Otto and Moon, 1995; Van der Oost *et al*., 2003). Changes in the levels of phase II enzymes, glutathione S-transferase and antioxidant enzymes have also been proposed as biomarkers of contaminants in a variety of marine organisms, including invertebrates, mussels, and fish (Otto and Moon, 1995; Livingstone, 2001; Orbea *et al*., 2002; Sen and Kirikbakan, 2004; Ferreira *et al*., 2004; Martínez-Gómez *et al*., 2006).

However as compared with cytochrome P450 MFOs, the induction response of phase II enzymes are generally less pronounced (Anderson *et al*., 1985; George, 1994) so that they may be masked by natural variability factors such as sex, maturity, nutrition, season, temperature, etc. For these reasons they have more restricted use as biomarker.

### 1.3 The Effects of Phenolic Compounds/Flavonoids on Phase I and Phase II Xenobiotic Metabolizing Enzymes

As stated earlier, flavonoids are a subclass of phenolic compounds present in foods of plant origin. Due to antioxidant properties *in vitro* and their inhibitory role in various stages of tumor development in animal studies, flavonoids may contribute to the protective effects of vegetables and fruits. Below, the chemical and biological properties

of 5 of the most common phenolic compounds/flavonoids and their effects on phase I and phase II xenobiotic metabolizing enzymes will be discussed in details.

### 1.3.1 Quercetin

Quercetin is the main flavonol in the human diet, found abundantly in apples, onions, black tea, and red wine (Hollman *et al*., 1997). The estimated average daily dietary intake of quercetin by an individual in the United States is 25 mg (National Toxicology Program Technical Report, 1991). Figure 1.5 represents the chemical structure of quercetin.

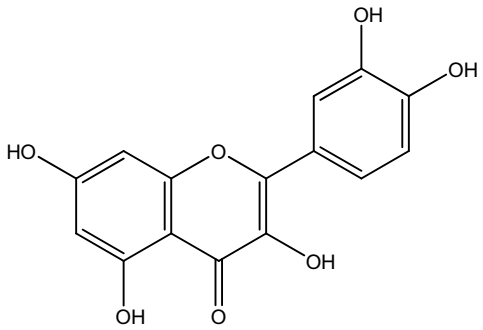



**Figure 1.6** Structure of quercetin

Because of its interesting chemical and biological properties, quercetin has been one of the most studied flavonoids. In the last decades, many claims on the beneficial health effects of quercetin have been stated, including protection against various forms of cancer, cardiovascular diseases and neurodegenerative diseases (Formica and Regelson, 1995; Middleton *et al*., 2000). Furthermore, anti-inflammatory, antibacterial and muscle-relaxating activities have been ascribed to quercetin (Galvez *et al*., 1993; Mata *et al*., 1997; Shoskes, 1998; Harborne and Williams, 2000).

Among all of the beneficial health effects of quercetin, protection against various forms of cancer is of great importance. In both *in vitro* and *in vivo* studies, quercetin has demonstrated a protective role in breast (Choi *et al*., 2001), lung (Khanduja *et al*.,

1999; Marchand *et al*., 2000), liver (Denda *et al*., 1998), ovarian (Scambia, 1994), and colon cancers (Huber *et al*., 1997). The protective effect of quercetin was the strongest against squamous cell carcinoma (a cell type specifically associated with subjects carrying the CYP1A1*2 allele) and was dependent on the CYP1A1 genotype, suggesting that CYP1A1 may play a role in this association. It was suggested that foods rich in certain flavonoids may protect against certain forms of lung cancer and that decreased activation of carcinogens by inhibition of CYP1A1 should be the underlying mechanism (Marchand *et al*., 2000).

Much research exists on the role of quercetin in regulating Phase 1 and Phase 2 enzymes, although the results of these studies are not always consistent (Table 1.4). The effectiveness of quercetin as a modulator of these carcinogen-metabolizing enzymes varies, depending on several factors. In order to elucidate the mechanisms of action by which quercetin may decrease the risk for specific cancers, many studies using various models and tissues, several doses and forms of quercetin, at different time points must be conducted and the results analyzed, in order to form a strong conclusion.

**Table 1.4** Previous human, animal, and cell culture studies on the effect of the flavonoid quercetin on Phase 1 (Cytochrome P4501A1) and Phase 2 (Glutathione S-transferase) enzyme activities

| Author | Model used | Quercetin Dose | GST | CYP1A1 |
|---|---|---|---|---|
| Sousa *et al.* 1985 | Rat liver microsomes | 10-250 nM | N.D | Dose dependent decrease in EROD activity |
| Siess *et al*. 1990 | Human liver and rat liver microsomes | 0.006-300 μM | N.D | Dose dependent decrease in EROD activity |
| Kang *et al.* 1999 | HepG2 human liver carcinoma cell line | 0.1-10 μM | No effect | Dose dependent decrease in EROD activity |
| Ciolino *et al*. 1999 | MCF-7 cells | 0.5-10 μM | N.D | Concentration and time dependent increase in EROD activity |
| Chaudhary *et al*. 2006 | 22Rv1 prostate cell | 0.5-5 μM | N.D | Dose dependent decrease in EROD activity |
| Apáti *et al*. 2000 | HepG2 cell line | 250 mM | 30% GST inhibited | N.D |
| Fiander *et al*. 2000 | Human neuronal cell line IMR-32 | 3 μM | 3% increase in GST activity | N.D |

N.D [Not Determined]

### 1.3.2 Resveratrol

The great interest in resveratrol has been spurred by the dietary anomaly referred to as the 'French paradox' (Dolnick, 1990; Safer, 1991). This paradox refers to the correlation of a high-fat diet with a lower incidence of coronary heart disease (CHD) found in Mediterranean cultures contrasted with a higher incidence of CHD among most Western cultures. A significant dietary difference was the moderate consumption of red wine by the Mediterranean population (St. Leger *et al*., 1979; Renaud and de Lorgeril, 1992). Of all the many components of wine, resveratrol, which is a natural component specifically present in wine, has been identified as being mainly responsible for the health-promoting properties.

Resveratrol, 3,5,4′-trihydroxy-trans-stilbene, is a phytoalexin found in grapes, peanuts, mulberries and red wine (Soleas *et al*., 1997; Sanders *et al*., 2000; Piver *et al*., 2003). Resveratrol exists in cis- and trans- isomeric forms but the cis-isomer has never been identified in grape extract. The structure of trans-resveratrol is given in Figure 1.6.

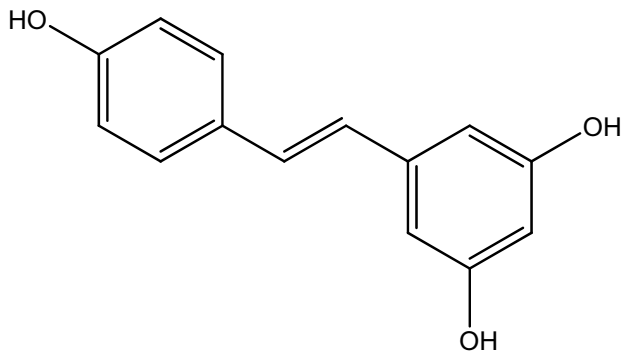



**Figure 1.7** Structure of trans-resveratrol

There are several studies indicating the beneficial effects of resveratrol. It has been showned that resveratrol has protective effect against coronary heart diseases (Goldberg, 1996; Soleas *et al*., 1997; Constant, 1997; Szmitko and Verma, 2005; Penumathsa *et al*., 2006). The protective effect of resveratrol toward coronary heart disease has been attributed to the antioxidant activity (Fauconneau *et al.,* 1997),

modulation of the synthesis of hepatic apolipoprotein and lipids (Frankel *et al.,* 1993), inhibition of platelet aggregation (Pace-Asciak *et al.,* 1995; Pace-Asciak *et al.,* 1995), and eiconanoid productions in human platelets and neutrophils (Kimura *et al.,* 1985; Pace-Asciak *et al.,* 1995). The beneficial effects of resveratrol are not limited to cardiovascular disease prevention and its anti-coagulant property. Resveratrol is also an anti-cancer agent.

In various *in vitro* and *in vivo* models, resveratrol has proved to be capable of retarding or preventing various steps of carcinogenesis. Resveratrol induced quinone reductase in cultured mouse hepatoma cells, inhibited cyclooxygenase and hydroperoxidase, and induced cell differentiation of HL-60 human promyelocytic leukemia cells. Moreover, it has shown to have an anti- tumorigenic effect in a two-stage mouse skin cancer model (Jang *et al.,* 1997).

The effects of resveratrol on cytochrome P4501A1 expression were previously reported, but the results were controversial (Ciolino *et al.,* 1998; Frötschl *et al.,* 1998). Resveratrol inhibited the increase in P4501A1 mRNA transcription caused by TCDD in cultured human HepG2 cells (Ciolino *et al.,* 1998) but induced P4501A1 mRNA in human HeLa cell cultures (Frötschl *et al.,* 1998).

In a study conducted by Mollerup *et al.*, human bronchial epithelial cells were treated either with benzo[a]pyrene or 2,3,7,8-tetrachlorodibenzo-p-dioxin in the presence or absence of resveratrol. It was found that, resveratrol inhibited both the constitutive and the induced expression of CYP1A1 and CYP1B1 in a dose-dependent manner (Mollerup *et al.,* 2001). In contrast, the expression of the microsomal epoxide hydrolase (mEH) gene was increased in response to resveratrol and no change in the expression of GSTP1 was found. The altered gene expression in response to resveratrol was reflected in a reduced overall level of benzo[a]pyrene metabolism (Mollerup *et al.,* 2001). These data indicated that resveratrol may exert lung cancer chemopreventive activity through altering the expression of genes involved in the metabolism of polycyclic aromatic hydrocarbons, resulting in altered formation of carcinogenic benzo[a]pyrene metabolites in human bronchial epithelial cells.

### 1.3.3 Rutin

The flavonoid rutin (quercetin-3-O-rutinoside) is a flavonol glycoside comprised of the flavonol quercetin and the disaccharide rutinose. Rutin is found in buckwheat, many vegetables, fruits, and plant-derived beverages such as tea and wine (Manach *et al.,* 1997). Other rich dietary sources of rutin include apple peels and a number of commercial citrus species. The structure of rutin is given in Figure 1.7.

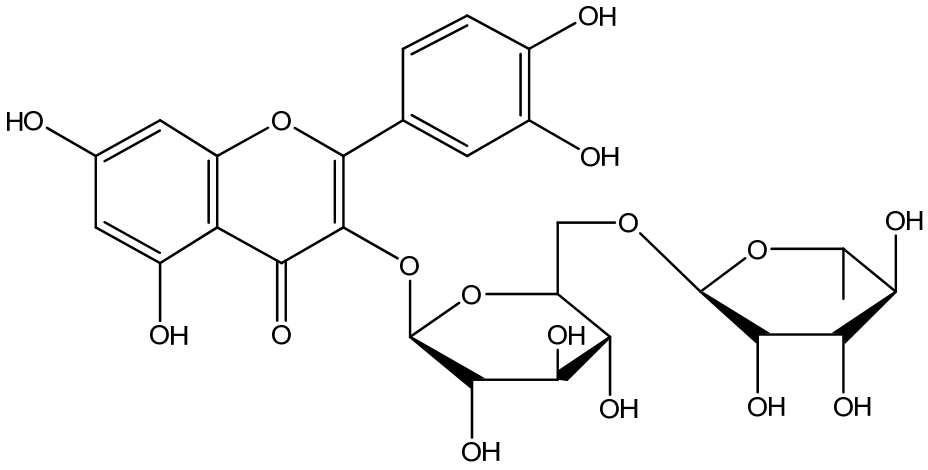



**Figure 1.8** Structure of Rutin

Rutin has antiplatelet, antiviral, and antihypertensive properties, as well as strengthing the capillaries, which is the result of its high radical scavenging activity and antioxidant capacity (Guo *et al.,* 2007). In addition, hypolipidemic, cytoprotective antispasmotic and anticarcinogenic activities have also been reported.

Rutin is hydrolyzed by intestinal bacteria to quercetin in humans. However in a number of studies, rutin is showned to be less effective compared to quercetin in terms of inhibiting CYP1A1. In a recent study, Chaudhary *et al*. (Chaudhary and Willett, 2006) investigated some flavonoids for their inhibition of recombinant human CYP1A1 and showed that rutin did not inhibit CYP1A1 at concentrations up to 10 μM. They also showed that compared to other flavonoids tested (myricetin, apigenin, kaempferol,

quercetin, amentoflavone, and quercitrin) rutin was the least effective inhibitor of CYP1A1 activity.

Besides its inhibitory effect on CYP1A1 activity, the effect of rutin on GST activity is reported to be mainly activatory. In a study, rutin gave dose-dependent increases in GST activity in HepG2 cells, with a 50% increase at 250 mM (Apáti *et al.,* 2000).

### 1.3.4 Naringenin

Naringin is the predominant flavonone in grapefruit (*Citrus paradisi*) (up to 10% of the dry weight) and has a multitude of pharmacological effects including, antithrombotic (Corvazier and Maclouf, 1985), anti-inflammatory (Middleton and Kandaswami, 1992), anti-estrogenic (Kao *et al.,* 1998), as well as chemopreventive (Guthrie and Carrol, 1998) actions.

Naringenin is the aglycon form of naringin. Orally administered naringin is hydrolyzed by enterobacteria to naringenin before being absorbed. The structure of naringenin is given in Figure 1.8.

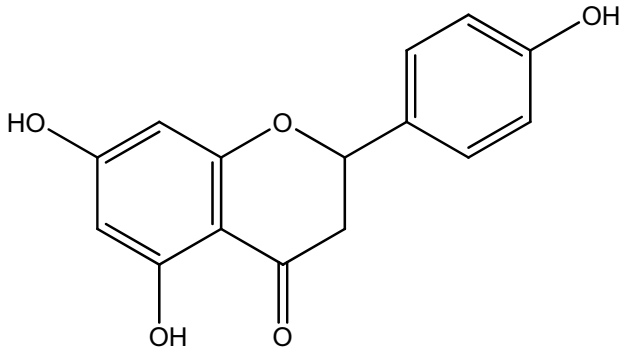



**Figure 1.9** Structure of Naringenin

Naringenin has been shown to possess antioxidant (Rice-Evans *et al.,* 1996), anti-inflammatory (Franke *et al.,* 2005), blood lipid and cholesterol lowering activities (Lee *et al.,* 1999; Santos *et al.,* 1999) as well as anticarcinogenic activities (Kanno *et*

*al.,* 2005). However the mechanism of the inhibitory effect on tumor growth by naringenin has not been elucidated yet.

It was suggested naringenin may modulate cytochrome P450-dependent monooxygenase, the primary enzyme involved in the metabolism of many xenobiotics (Ueng *et al.*,1999). In a recent study, Kim *et al.* investigated the effect of naringenin on TCDD-inducible CYP1A1 gene expression in mouse hepatoma Hepa-1c1c7 cells and showed that naringenin alone did not affect CYP1A1-specific EROD activity. In contrast, the TCDD-inducible EROD activities were markedly reduced upon concomitant treatment with TCDD and naringenin in a dose dependent manner (Kim *et al.*, 2004).

### 1.3.5 Hesperidin

Hesperidin is a flavanone glycoside consisting of the flavone hesperitin bound to the disaccharide rutinose. It is found mainly in citrus fruits. The structure of hesperidin is given in Figure 1.9.

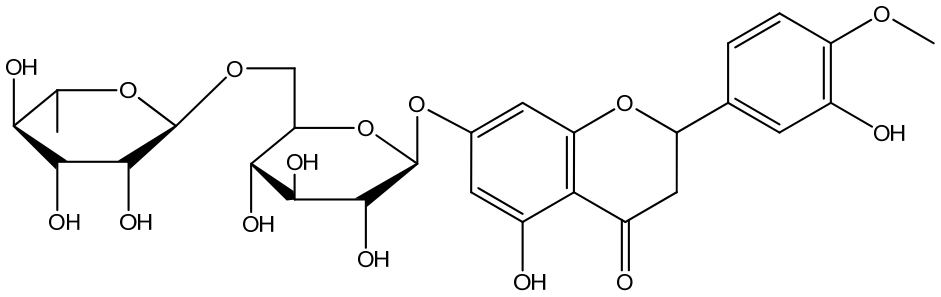



**Figure 1.10** Structure of Hesperidin

Hesperidin has several biological functions such as antioxidant, anti-inflammatory, prostaglandin-synthesis inhibition, anti-mutagenic and anti-carcinogenic activity (Huang *et al.*, 1983; Fujiki *et al.*, 1986; Ratty and Das, 1988; Galati *et al.*, 1994). Anticarcinogenic activity of hesperidin has been demonstrated in the inhibition of skin tumorigenesis (Berkarda *et al.*, 1998) and inhibition of carcinogenesis in the bladder (Yang *et al.*, 1997). Additionally, hesperidin suppresses cell proliferation in azoxymethane-induced rat colon carcinogenesis (Tanaka, 1997). Modulation of drug-

metabolizing enzymes may be one of the mechanisms which provides anticarcinogenic activity of hesperidin. In a recent study, interaction of hesperetin with human CYP1 enzymes were studied (Doostdar, 2000). The data indicated that hesperetin is a selective substrate of human CYP1A1 and CYP1B1 in the lymphoblastoid cell line AHH-1, and it is a competitive inhibitor of CYP1B1 (Doostdar *et al.*, 2000).

### 1.4 Aim of the Study

In daily life, people are continuously exposed exogenously to varying amounts of chemicals that have been shown to have carcinogenic or mutagenic properties in experimental systems. Approximately 70% of human cancers are caused by these chemicals. Fortunately; defense mechanisms have evolved to protect against toxic insults. The cytochrome P450 family of enzymes plays critical roles in the biotransformation of drugs, carcinogens, steroid hormones, and environmental toxicants. However the metabolism of chemicals by cytochrome P450 enzymes does not always result in detoxification of these chemicals. Sometimes as a result of metabolic bioactivation, these chemicals may turn into even more toxic substances. Especially CYP1A1 and 1A2 play critical roles in the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines/amides (HAAs) to electrophilic reactive intermediates, leading to toxicity and cancer. Therefore; an obvious strategy for chemoprevention would be the inhibition of CYP1As. Flavonoids, a family of naturally occurring polyphenolic compounds, have thought to show promise in this respect.

In this study we used leaping mullet caught from highly polluted sites of İzmir Bay, therefore express high levels of CYP1A and associated enzyme activities; in order to investigate the possible *in vitro* effects of phenolic compounds/flavonoids on EROD activity. In addition, *in vitro* effects of flavonoids on total GST and GST-Mu activities of mullet liver were also examined. To study the mode of inhibition exerted by phenolic compounds/flavonoids on the mullet liver EROD and GST activities was another purpose of this study.

Another aim of this study was to compare flavonoids in terms of their potencies and selectivities for inhibition of CYP1A1 and GST. This may have important

implications for cancer prevention, as well as other pharmacological and toxicological effects of these compounds.

This study could provide an insight into flavonoids inhibiting CYP1A1 and GST with potential to serve as a model for the development of therapeutic drugs in combating cancer.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals

Resveratrol was kindly provided by Mikrogen Pharma, İstanbul, Turkey. Resorufin (23015-4) was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA. Quercetin (Q0125), rutin (R5143), naringenin (N5893), hesperidin (H5254), bovine serum albumin (BSA; A7511), ethoxyresorufin (E3763), 1-chloro-2,4 dinitrobenzene (CDNB; C6396), glutathione reduced form (G4251), sodium potassium tartrate (S2377), phenyl methane sulfonyl fluoride (PMSF), ε-amino caproic acid (ε-ACA; A2504), acrylamide (A8887), 5-bromo-4-chloro-3-indolyl phosphate (BCIP; B8503), N,N-dimethylformamide (D8654), β-mercaptoethanol (M6250), N,N'-methylene bisacrylamide (BIS; M7256), nitroblue tetrazolium (NBT; N6876), polyxyethylene sorbitan monolaurate (Tween 20; P1376), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris, T1378), anti-rabbit IgG-ALP conjugate (A3687), phenazine methosulfate (PMS; P9625), glycine (G7126), were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Ammonium persulfate (161-0700), N, N, N', N' tetrametylethylene diamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, California, USA.

1,2-dichloro-4-nitrobenzene (DCNB; D85662), copper (II) sulfate-pentahydrate ($Cu_2SO_4$; 02790), ethylene diamine tetra acetic acid disodium salt (EDTA; 08421), potassium chloride (KCl; 04935), glycerol (04093), potassium dihydrogen phosphate ($KH_2PO_4$; 05101), dipotassium hydrogen phosphate ($K_2HPO_4$; 04871), sodium chloride (NaCl; 13423), sodium hydroxide (NaOH; 06462), sodium carbonate ($Na_2CO_3$; 06398), dimethylsulfoxide (DMSO; 02951), magnesium chloride ($MgCl_2$; 05833), methanol

(02500), sodium dodecyl sulfate (SDS; 822050), zinc chloride ($ZnCl_2$; 08515) were purchased from E. Merck, Darmstadt, Germany.

β-nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH; A1395) was purchased from Applichem Biochemica, Chemica Synthesis Services, Darmstadt, Germany. Ethanol (32221) was obtained from Riedel, Germany.

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

### 2.1.2 Sample Collection

The fish species used in this study was leaping mullet (*Liza saliens*). The fish used in this study were collected in our laboratory within the project of TUBİTAK (Project No-102Y044) in November 2002 and October 2003 from the different sites of İzmir Bay, on the Aegean Coast of Turkey which is one of the most polluted areas of Turkey. Domestic and industrial wastes, urban and agricultural run off, discharges from ships, sediments and contaminated waters of rivers have cumulatively had significant adverse effects on the water quality of the Bay. Fish caught in these sites exhibited elevated levels of CYP1A and associated enzyme activities (Arınç *et al*., 2000).

In this study microsomes of leaping mullets having elevated EROD activities were selected to prepare microsomal pools. These selected microsomes were belonged to leaping mullet (*Liza saliens*) caught by fish net from Pasaport, Üçkuyular port site, and Hekim Island site of İzmir Bay.

## 2.2 Methods

### 2.2.1 Preparation of Fish Liver Microsomes and Cytosol

The fish liver microsomes used in this study were prepared by Tuğba Boyuneğmez Tümer in November 2002 and October 2003 during her master's degree study (Boyuneğmez T, 2004). The fish liver cytosols used in this study were kindly provided by Azra Bozcaarmutlu. In the following, preparation of microsomes and cytosol will be described.

The method described by Arınç and Şen (1993a) was used for the preparation of liver microsomes. Fish were killed by decapitation and the livers, weighing

approximately 2-5 grams were removed immediately. The gall bladder was removed carefully with scissors in order to avoid the spillage of its contents that are known to be inhibitory to monooxygenase activities. The livers were first wrapped by freezing bags and covered by aluminum foil and frozen by putting into liquid nitrogen. Freshly frozen fish livers were transported in liquid nitrogen from İzmir to university laboratory in Ankara.

Each microsome was prepared from one fish liver. In the laboratory, the livers were taken from liquid nitrogen and thawed on ice. All subsequent steps were carried out in 0-4°C ice bath. Livers were washed first with cold distilled water, and then with cold 1.15% KCl solution to remove as much blood as possible. After draining on a paper towel, tissues were weighed and cut into small pieces with scissors. The resulting tissue mince was homogenized in 1.15% KCl solution containing 10 mM EDTA pH 7.7, 0.25 mM ε-ACA, 0.1 mM PMSF at a volume equal to 2.5 times the weight of liver tissue using Potter-Elvehjem glass homogenizer packed in crushed ice, coupled motor (Black & Decker, V850 multispeed drill)-driven Teflon pestle at 2400 rpm, twelve passes were made for homogenization.

The homogenate was centrifuged at 13300xg (Sigma 3K30 Refrigerated Centrifuge, Saint Louis, Missouri, USA) by using 12156 rotor for 40 minutes to remove cell debris, nuclei and mitochondria. The supernatant fraction containing endoplasmic reticulum and other fractions of the cell was filtered through two layers of cheesecloth. The microsomes were sedimented from the supernatant solution by centrifugation at 45000 rpm (70000xg) for 60 minutes using Sorvall-Kombi ultracentrifuge, Ivan Sorvall Inc., Newton, Connecticut, 06740 USA with T-880 rotor. The supernatant fraction (cytosol) was taken and shocked with liquid nitrogen and then stored at -80°C to use later. The packed microsomal pellet was suspended in 1.15% KCl solution containing 10 mM EDTA and resedimented by ultracentrifugation at 45000 rpm (70000xg) for 50 minutes. The supernatant fraction was discarded. Then the washed microsomal pellet was resuspended in 10% glycerol containing 10 mM EDTA pH 7.7 at a volume of 0.5 ml for each gram of liver tissue for leaping mullet. Resuspended microsomes were homogenized manually using the Teflon-glass homogenizer in order to obtain homogenous suspension.

Microsomes were separated into small aliquots and put into eppendorf tubes, stored in liquid nitrogen after gassing with nitrogen and kept within liquid nitrogen since 2002 till now.

#### 2.2.1.1 Preparation of Fish Liver Microsome Pool for This Study

Two mixed pools of fish liver microsomes were prepared to study the *in vitro* effects of phenolic compounds/flavonoids on CYP1A1 activity of fish liver.

Pool 1 was prepared from 8 different microsomal preparations obtained from livers of *Liza saliens* caught from Pasaport, Üçkuyular port site, and Hekim Island site. Pool 2 was prepared from 9 different microsomal preparations obtained from livers of *Liza saliens* caught from Pasaport and Hekim Island site. Pools were prepared by pouring the microsomes into the glass homogenizing vessel packed in crushed ice and homogenizing manually using the Teflon pestle in order to obtain a totally homogenous suspension. Twenty passes were made for each homogenization in order to get a totally homogenized microsomal pool. After homogenization, the suspensions were separated into small aliquots and put into eppendorf tubes. During each aliquot separation, the suspensions were mixed by vortex in order to prevent the microsomes from settling down to the bottom of the vessel. The eppendorf tubes were then stored in liquid nitrogen till use.

### 2.2.2 Protein Determinations

The protein concentrations of both microsome pools and cytosol were determined by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a standard. The microsome pool and cytosol were initially diluted 200 times. In addition to initial dilutions, dilutions within test tubes were performed by taking aliquots of 0.1 ml, 0.25 ml, and 0.5 ml from the initially diluted samples into the test tubes. Their volumes were then completed to a final volume of 0.5 ml with distilled water. Then, they were mixed with 2.5 ml of alkaline copper reagent (prepared by mixing 2% copper sulfate, 2% sodium potassium tartrate and 0.1 N NaOH containing 20% sodium carbonate in a ratio of 1:1:100 in their written order). All the tubes were mixed by vortex and let stand for 10 minutes at room temperature for the copper reaction in alkaline medium.

Then, 2 N Folin Phenol Reagent was diluted 1:1 ratio with distilled water and 0.25 ml of this reagent was added to each tube and mixed by vortex within 8 seconds. The tubes were then incubated for 30 minutes at room temperature. The intensity of color developed was measured at 660 nm. Standard tubes at five different concentrations (20, 50, 100, 150, 200 μg of BSA/ml) were prepared from crystalline bovine serum albumin and the same steps were performed for the standard tubes. A standard curve was constructed from the intensity readings of standards and this standard curve was used in the calculation of protein amount in the samples. Protein concentration was calculated by the following formula.

$$\text{Protein Concentration (mg/ml)} = \frac{OD_{660\ nm}}{\text{Slope of Standard Curve}} \times \text{Tube Dilution Factor} \times \text{Initial Dilution Factor}$$

Average protein concentration was found to be 24 mg/ml for pool 1 and 36.54 mg/ml for pool 2. Average protein concentration was found to be 24.7 mg/ml for cytosol.

### 2.2.3 Western Blot Analysis-Protein Blotting

In previous studies carried out in our laboratory, it was demonstrated that leaping mullet from the highly urbanized and industrial section of İzmir Bay, Pasaport, showed highly elevated enzyme activities which were about 62 times higher than the value at the reference site, Outer Bay (Arınç *et al.*, 2000). Cytochrome P4501A protein level was determined using the polyclonal antibodies produced in our laboratory against purified leaping mullet liver cytochrome P4501A (Arınç and Şen, 1999). In this study, antibodies raised against purified leaping mullet CYP1A were used to detect liver CYP1A protein level in microsomal pool 2 prepared from the leaping mullet caught from the polluted sites, Pasaport and Hekim Island site of İzmir Bay. Liver cytochrome P4501A protein levels were determined by Towbin *et al.* (1979) with some modifications.

First microsomal proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS, in a discontinuous buffer system as described by Laemmli

(1970) by using 4% stacking gel and 8.5% separating gel. The separating and stacking gel solutions were prepared just before use as given in Table 2.1 in the given order.

**Table 2.1** Components of separating and stacking gel solutions

| **Constituents** | **Separating Gel (8.5%) (0.375 M Tris, pH 8.8)** | **Stacking Gel (4%) (0.125 M Tris, pH 6.8)** |
|---|---|---|
| Gel solution (ml) | 8.5 | 1.3 |
| Distilled water (ml) | 13.55 | 6.1 |
| Separating gel buffer (ml) | 7.5 | - |
| Stacking Gel buffer (ml) | - | 2.5 |
| 10% SDS (ml) | 0.3 | 0.1 |
| Ammonium persulfate (ml) | 0.15 | 0.05 |
| Temed (ml) | 0.015 | 0.01 |
| Total volume | 30 | 10 |

Vertical slab gel electrophoresis was carried out using Scie-plasV10-CDC vertical electrophoresis unit (Southam, England). Polyacrylamide slab gels were prepared using the gel sandwich. The gel sandwich was prepared between two glass plates leaving 1 mm space between plates and central gel running module was assembled. The module was placed in melted agar and both sides were sealed with agar to prevent a leakage of separating gel and stacking gel solution. First separating gel solution was transferred to the center of gel sandwich until the desired height of the solution in the sandwich was obtained. The top of the gel polymerizing solution was covered with a layer of isobutanol to ensure the formation of smooth gel surface. After polymerization of separating gel, the layer of alcohol was poured off completely. The stacking gel polymerization solution was prepared and poured into the center of gel sandwich over the separating gel until the sandwich was filled completely. After adding stacking gel, a 1.0 mm Teflon comb with 12 wells was inserted into stacking gel

solution. After polymerization, Teflon comb was carefully removed without tearing the wells.

Wells were filled with electrode running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) using syringe with a fine needle to remove any air bubbles and unpolymerized chain particles. Then gel running module was filled with a necessary volume of electrode running buffer.

After preparing gel setup, liver microsomes were diluted 1:3 (3 part sample and 1 part buffer) with 4x sample dilution buffer containing 0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.01% bromophenol blue and were boiled in a boiling water bath for 1 minutes and 10 seconds. The sample was applied in different amounts to different wells by Hamilton syringe.

After application of the samples, gel set up was placed in the main buffer tank filled with an appreciate amount of electrode running buffer. The electrophoresis unit was connected to the power supply Bio-Rad model 2 (Bio-Rad Laboratories, Richmond, California, USA) and electrophoresis was run at 8 mA and 100 V in stacking gel and at 20 mA and 200 V in separating gel. When electrophoresis was completed, gel was removed from the cell for western blot analyses which was carried out as described by Towbin *et al.* (1979) with some modifications. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 15 minutes with constant shaking in order to adjust the final size of gel and remove the buffer salts and SDS which were used in the SDS-Polyacrylamide gel electrophoresis. Nitrocellulose membrane was cut 1 cm larger than the dimension of the gel and two pieces of filter paper (Whatman #1) were cut to a dimension a little bit larger than the membrane. Nitrocellulose membrane, two filter paper and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Western blot sandwich was prepared as seen in Figure 2.1. A test tube was rolled gently over the sandwich in order to remove air bubbles between the layers. This step is very critical because any air bubbles between gel and membrane will block the transfer of proteins present at this point. Later, the sandwich was put into the Bio-Rad Trans-Blot Cell and the cell was filled with cold transfer buffer. Voltage and current were set to 90 V and 400 mA, respectively. Transfer process was carried out at cold room (4°C) for 90 minutes. At the end of this period, the membrane having the transferred protein on it, “blot”, was obtained and taken from the cell and placed into a plastic dish in such a way that protein side facing up and washed with

TBST (Tris Buffered Saline plus Tween 20: 20mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 0.05% Tween 20) for 10 minutes in order to remove the salts and buffers of transfer medium.

Then the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBST) for 60 minutes in order to fill the empty spaces between bound proteins by this way prevent the non-specific binding of antibodies on the membrane.

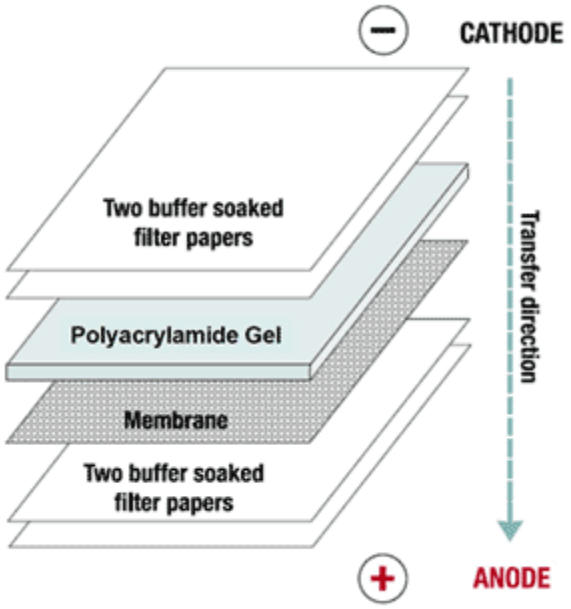



**Figure 2.1** Cross-section of the Western blot sandwich

The blot was incubated with primary antibody for 2 hours. As primary antibody, polyclonal antibodies produced against purified leaping mullet liver cytochrome P4501A in rabbits were used and dissolved in blocking solution 1:5000 ratio. Then the blot was washed 3 times with 100 ml TBST for 5 minutes each. The washing steps are necessary to remove excess antibody from the membrane. The blot was then incubated with secondary antibody conjugated to marker enzyme-alkaline phosphatase (anti-rabbit IgG-ALP conjugate, 1:10.000 ratio) for 1 hour. The blot was washed three times with TBST for 5 minutes each to remove excess antibody. Since the excess antibody

will give reaction with substrate solution nonspecifically the complete removal of the excess antibody between each washing steps are extremely important. Finally, blot was incubated with substrate solution given in Table 2.2 as described by Ey and Ashman (1986) to visualize the specifically bound antibodies. The final image was photographed by using computer based gel imaging instrument (Infinity 3000-CN-3000 darkroom) (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) by using Infinity-Capt Version 12.9 software. Then the final image was dried under air, covered by filter paper and stored at dark.

**Table 2.2** Preparation of substrate solution for immunodetection

| |
|---|
| **Solution A:**<br>2.67 ml of 1.5 M Tris-HCl, pH 8.8<br>4.0 ml of 1M NaCl<br>0.82 ml of 100 mM $MgCl_2$<br>0.04 ml of 100 mM $ZnCl_2$<br>0.096 ml of DEA<br>12.2 mg NBT<br>Distilled water to 40 ml<br>(pH of the solution was adjusted to 9.55 with saturated Tris before completing to final volume) |
| **Solution B:** 2 mg/ml phenazine methosulfate in distilled water |
| **Solution C:** 5.44 mg/0.136 ml N, N-dimethyl formamide |
| Finally NBT/BCIP substrate solution was prepared by mixing solution A with solution C and 0.268 ml of solution B. |

### 2.2.4 Determination of 7-Ethoxyresorufin-O-deethylase (EROD) Activity in Leaping Mullet (*Liza saliens*) Liver Microsomes

7-Ethoxyresorufin-O-deethylase (EROD) activity is most commonly associated with cytochrome P4501A (CYP1A) isoenzymes which are inducible by polyaromatic

hydrocarbons. An increase of the level of EROD was the most sensitive biochemical marker of environmental pollution and exposure to polycyclic aromatic hydrocarbons (Arınç and Şen, 1999a,b; Arınç *et al*., 2000). Figure 2.2 shows the ethoxyresorufin O-deethylase reaction catalyzed by monooxygenases in the presence of molecular oxygen and NADPH.

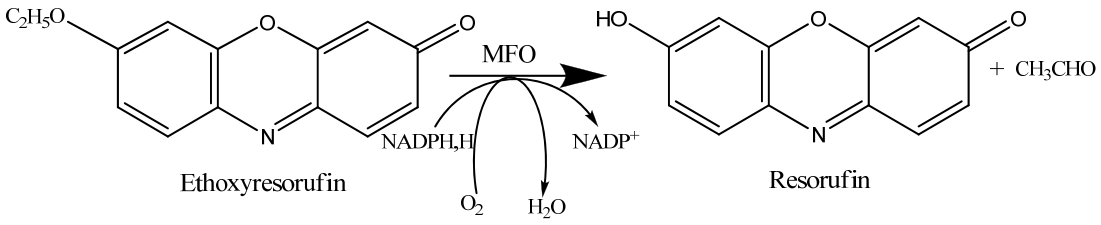



**Figure 2.2** Ethoxyresorufin O-deethylase reaction

EROD activity of the leaping mullet (*Liza saliens*) liver microsomes was determined by the method of Burke and Mayer (1974) with some modifications. Assay conditions optimized for gilthead seabream liver microsomes by Arınç and Şen (1994) was also used for fish species in this study. The principle of the assay depends on measuring the formation of resorufin from 7-ethoxyresorufin in microsomal preparations. This dealkylation reaction was measured by spectrofluorometric method.

The constituents of the reaction mixture for the determination of EROD activity in fish liver microsomes is given in Table 2.3. As it is shown in Figure 2.3, a typical reaction mixture contained 0.1 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl, 1.2 mg BSA, 1.5 µM 7-Ethoxyresorufin, 100 µg microsomal protein, 0.5 mM NADPH in a final volume of 2 ml in a fluorometer cuvette. The assay was performed at room temperature (25ºC). The reaction was initiated by the addition of NADPH and followed for 2 minutes in spectrofluorometer (Hitachi F-2000, Hitachi ltd., Tokyo, Japan) at 535 nm (excitation) and 585 nm (emission). Finally a known amount of resorufin was added as a standard to the reaction mixture and the increase in fluorescence was recorded. Enzyme activity was calculated using fluorescence increase caused by the addition of resorufin.

**Table 2.3** The constituents of the reaction mixture for the determination of EROD activity in fish liver microsomes

| **Constituents** | **Stock Solutions** | **Volume to be taken (ml)** | **Final Concentration in 2 ml reaction mixture** |
|---|---|---|---|
| Potassium Phosphate Buffer pH 7.8 containing 0.2 M NaCl | 0.2 M | 1 | 0.1 M |
| BSA | 12 mg/ml | 0.2 | 1.2 mg/ml |
| Ethoxyresorufin | 10 μM | 0.3 | 1.5 μM |
| NADPH | 10 mM | 0.1 | 0.5 mM |
| Fish liver microsomes | ---- | Depending on the protein concentration of microsomes | 100 μg of microsomal protein |
| Distilled water | | to 2 ml | |

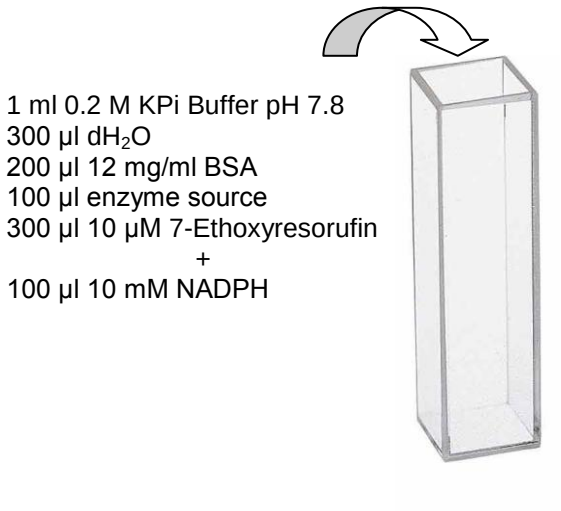



**Figure 2.3** Reaction constituents for the measurement of Ethoxyresorufin O-deethylase (EROD) activity in fish liver microsome

### 2.2.4.1 Determination of the Effect of Phenolic Compounds/Flavonoids on CYP1A1 Associated EROD Activity

In this study, the effects of phenolic compounds/flavonoids on CYP1A dependent EROD activity of fish liver microsome were investigated. All the phenolic compounds/flavonoids used (quercetin, resveratrol, rutin, naringenin, hesperidin) in this study were dissolved in 50% (v/v) DMSO. The final concentration of DMSO in the assay mixture was 0.5% and it had a 5-10% inhibitory effect on fish liver EROD activity. Since the stock solutions of flavonoids were prepared in DMSO, inhibition by flavonoid was compared with DMSO-Control measurements.

The effect of the flavonoid of interest was studied by adding various concentrations of flavonoid to the reaction mixture shown in Table 2.3. After the addition of the flavonoid, the reaction mixture was mixed and incubated for 5 minutes at room temperature (25ºC). Finally reaction is initiated with the addition of NADPH and followed for 2 minutes at room temperature in spectrofluorometer (Hitachi F-2000, Hitachi ltd., Tokyo, Japan) at 535 nm (excitation) and 585 nm (emission). IC50 values (inhibitor concentration which produces 50% inhibition) were determined by linear regression (% of control) against logarithmic inhibitor (flavonoid) concentrations. After the IC50 values were calculated, three different flavonoid concentrations were chosen to study the inhibition mechanism. All these parts will be covered in “Results” in details.

## 2.2.5 Determination of Glutathione S-Transferase (GST) Activity in Leaping Mullet Liver Cytosols

### 2.2.5.1 Determination of Total GST Activity

Total GST Activity of fish liver cytosol was measured according to the method of Habig *et al*. (1974) with some modifications. 1-chloro-2,4-dinitrobenzene (CDNB) was used as a substrate for determination of total GST Activity. The reaction is monitored by following the increase in absorbance at 340 nm due to the formation of glutathione conjugate 1-glutathione 2,4-dinitrobenzene (DNB-SG). The reaction catalyzed by GST is given in Figure 2.4.

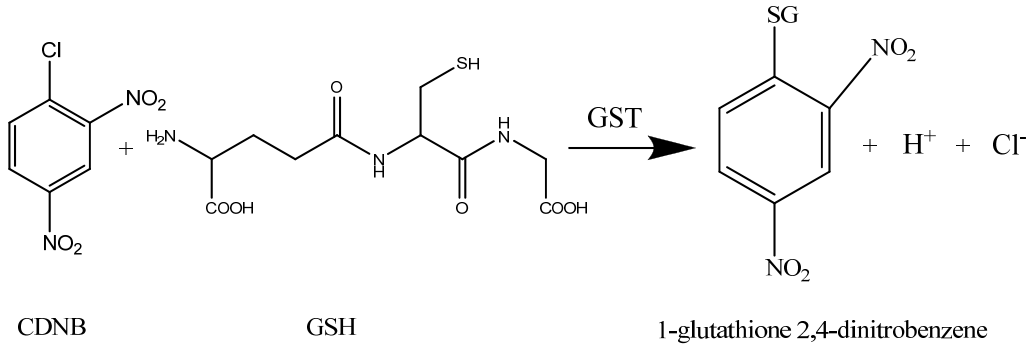



**Figure 2.4** Glutathione S-Transferase reaction

The typical assay mixture used for the determination of total GST activity is given in Table 2.4. Before GST assay, cytosol sample was centrifuged one more time at 13100 rpm (16000 xg) (Sigma 3K30 Refrigerated Centrifuge, Saint Louis, Missouri, USA) by using 12156 rotor. The assay was performed in a 3 ml quartz cuvette at room temperature (25ºC). Into 3 ml quartz cuvette, 2.5 ml of 50 mM potassium phosphate buffer pH 7.0 containing 5% (v/v) glycerol, 200 μl of 20 mM GSH, 150 μl of 20 mM CDNB were added and the reaction was started by the addition of 150 μl of 1:250 diluted fish liver cytosols. Then, thioether formation was followed for 3 minutes at 340 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, there were blank readings (reaction with no enzyme) in order to subtract non-enzymatic product formation from the GST assay.

Then, the enzymatic activity was calculated as the amount of thioether (μmol) formed in one minute by using 0.0096 $\mu M^{-1}.cm^{-1}$ as an extinction coefficient of thioether formed by GST. The following formula was used for the calculation of GST activity.

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

**Table 2.4** The constituents of the reaction mixture for determination of total GST activity of fish liver cytosol

| **Constituents** | **Stock Concentrations (mM)** | **Volume to be taken (ml)** | **Final Concentration in 3 ml quartz cuvette (mM)** |
|---|---|---|---|
| Potassium Phosphate Buffer pH 7.0 containing 5% (v/v) glycerol | 50 | 2.5 | 41.6 |
| Glutathione reduced form (GSH) | 20 | 0.2 | 1.3 |
| 1-Chloro-2,4-dinitrobenzene (CDNB)* | 20 | 0.15 | 1 |
| Cytosol | --- | 0.15 | - |

* CDNB should be freshly prepared and after weighing the proper amount of CDNB it should be dissolved first in ethanol then the proper amount of distilled water should be added (ethanol/d$H_2O$:3/2). It should be stored in dark

### 2.2.5.1.1 Determination of the Effect of Phenolic Compounds/Flavonoids on Total GST Activity

In this study the effects of phenolic compounds/flavonoids on GST activity of fish liver cytosol was investigated. All the phenolic compounds/flavonoids used (quercetin, resveratrol, rutin, naringenin, hesperidin) in this study were dissolved in 50% (v/v) DMSO. The final concentration of DMSO in the assay mixture was 1.67% and it had no effect on fish liver GST activity. Since the stock solutions of flavonoids were prepared in DMSO, inhibition by flavonoid was compared with DMSO-Control measurements.

The effect of the flavonoid of interest was studied by adding various concentrations of flavonoid to the reaction mixture shown in Table 2.4. The reaction is initiated by the addition of 150 μl of 1:250 diluted fish liver cytosols. Then, thioether formation was followed for 3 minutes at 300 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, there were blank readings (reaction with no enzyme) in order to subtract non-enzymatic product formation from the GST assay. IC50 values (inhibitor concentration which produces 50% inhibition) were determined by linear regression (% of control) against logarithmic

inhibitor (flavonoid) concentrations. After the IC50 values were calculated, three different flavonoid concentrations were chosen to study the inhibition mechanism. All these parts will be covered in “Results” in details.

### 2.2.5.2 Determination of Mu Isozymes of GST Activity

Class mu glutathione S-transferases are important in the detoxification of epoxides generated by oxidative metabolism. However the mu-class GSTs are present in the livers of only about half of the Caucasian adults where the major mu-class subunit M1 is deleted (Hussey *et al.*, 1987). The GSTM1 deficiency may increase an individual susceptibility to certain environmentally derived compounds (Board *et al.*, 1990) and previous data suggest a link between the GSTM1 null allele (lack of GSTM1 activity) and the potential risk to lung cancer (Hirvonen *et al.*, 1993).

GST-Mu activity was measured according to the method of Habig *et. al*., (1974) with some modifications.1,2-dichloro-4-nitrobenzene (DCNB) is a substrate of mu isozymes of GST.

DCNB was used as a substrate for determination of GST-Mu activity in the presence of the cofactor reduced glutathione (GSH) by monitoring the thioether formation at 345 nm.

The typical assay mixture used for the determination of GST-Mu activity is given in Table 2.5. The assay was performed in a 1 ml quartz cuvette at room temperature (25ºC). Into 1 ml quartz cuvette, 800 μl of 120 mM potassium phosphate buffer pH 6.25, 100 μl of 20 mM GSH, 50 μl of 20 mM DCNB were added and the reaction was started by the addition of 50 μl of fish liver cytosol sample (no dilutions were performed). Then the activity increase was followed for 3 minutes at 345 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, there were blank readings (reaction with no enzyme) in order to subtract non-enzymatic product formation from the GST assay. Then, the enzymatic activity was calculated as the amount of thioether (μmol) formed in one minute by using 0.0085 $\mu M^{-1}.cm^{-1}$ as an extinction coefficient of thioether formed by GST. The following formula was used for the calculation of GST-Mu activity.

$$\text{Enzyme Activity (µmol/min/ml)} = \frac{\Delta OD_{345\ nm}}{0.0085\ \mu M^{-1} \times cm^{-1}} \times \frac{\text{Total Volume}}{\text{Volume of Sample}}$$

**Table 2.5** The constituents of the reaction mixture for determination of Mu isozymes of GST activity of fish liver cytosol

| **Constituents** | **Stock Concentrations (mM)** | **Volume to be taken (µl)** | **Final Concentration in 1 ml quartz cuvette (mM)** |
|---|---|---|---|
| Potassium Phosphate Buffer pH 6.25 | 120 | 800 | 96 |
| Glutathione reduced form (GSH) | 20 | 100 | 2 |
| 1,2-dichloro-4-nitrobenzene (DCNB)* | 20 | 50 | 1 |
| Cytosol | --- | 50 | - |

* DCNB should be freshly prepared and after weighing the proper amount of DCNB it should be dissolved first in ethanol then the proper amount of distilled water should be added (ethanol/d$H_2O$:3/2). It should be stored in dark.

### 2.2.5.2.1 Determination of the Effect of Phenolic compounds/Flavonoids on GST-Mu Activity

In this study the effects of phenolic compounds/flavonoids on GST-Mu activity of fish liver cytosol was investigated. All the phenolic compounds/flavonoids used (quercetin, resveratrol, rutin, naringenin, hesperidin) in this study were dissolved in 50% (v/v) DMSO. The final concentration of DMSO in the assay mixture was 1.67% and it had no effect on fish liver GST-Mu activity. Since the stock solutions of flavonoids were prepared in DMSO, inhibition by flavonoid was compared with DMSO-Control measurements.

The effect of the flavonoid of interest was studied by adding various concentrations of flavonoid to the reaction mixture shown in Table 2.5. The reaction was started by the addition of 50 µl of fish liver cytosol sample (no dilutions were

performed). Then the activity increase was followed for 3 minutes at 345 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, there were blank readings (reaction with no enzyme) in order to subtract non-enzymatic product formation from the GST assay. Then, the enzymatic activity was calculated as the amount of thioether (μmol) formed in one minute by using 0.0085 $\mu M^{-1}.cm^{-1}$ as an extinction coefficient of thioether formed by GST.

IC50 values (inhibitor concentration which produces 50% inhibition) were determined by linear regression (% of control) against logarithmic inhibitor (flavonoid) concentrations. These parts will be covered in “Results” in details.

# CHAPTER 3

# RESULTS

In daily life, humans are constantly exposed to potential carcinogens. Many of these xenobiotics are metabolized by the cytochrome P450 enzymes to either biologically inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA producing carcinogenicity (Conney, 2003). It is well recognized that, among the cytochromes P450, cytochrome P4501A plays an important role in the bioactivation, and thus increased toxicity of chemicals. In this regard, inhibiting CYP1A activity has a crucial importance to prevent conversion of pro-carcinogens to their carcinogenic forms. Phenolic compounds/flavonoids are considered to be promising chemoprevention agents in this respect. In this study, *in vitro* effects of phenolic compounds/flavonoids quercetin, resveratrol, rutin, naringenin and hesperedin were investigated on fish liver microsomal CYP1A1 associated EROD and cytosolic GST activities. The fish species used throughout this study, leaping mullet (*Liza saliens*), were caught in November 2002 and October 2003, from the different sites of İzmir Bay, on the Aegean Coast of Turkey which is one of the most polluted areas of Turkey. Therefore, mullet used in this study exhibited elevated levels of CYP1A and associated enzyme activities. The effects of flavonoids on micosomal CYP1A1 associated EROD activity and cytosolic GST activities of fish liver were found to be inhibitory type and the mode of inhibition was also investigated.

## 3.1 CYP1A1 Associated EROD Activity and CYP1A1 Protein Content of Fish Liver Microsomes Used in This Study

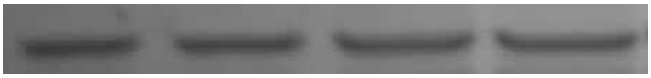
### 3.1.1 CYP1A1 Associated EROD Activity of Leaping Mullet

As previously described in “Materials and Methods”, 8 different microsomal preparations obtained from livers of *Liza saliens* caught from Pasaport, Üçkuyular port site, and Hekim Island site having high CYP1A1 associated EROD activity were used to

construct a microsomal pool to be used in this study. This pool was named as Pool 1 and average EROD activity of Pool 1 was determined to be 1826 pmol/min/mg protein. Before investigating the effects of flavonoids on EROD activity, many preliminary studies were performed to find the appropriate microsome concentration to be used in the kinetic study and to examine the stability of the diluted microsome. These preliminary studies built up the basis of the inhibition kinetic experiments. In stability experiments, CYP1A1 associated EROD activity of diluted Pool 1 microsomal pool was determined at certain time intervals and it was found that there was no significant activity loss within 90 minutes. It was very important not to lose any significant activity within this period, because the inhibition kinetic experiments took as much time as 90 minutes. In order to find the appropriate microsome concentration to be used in the kinetic experiments, two different types of dilutions were performed in the microsomal pool so that the tested final microsome concentration in the cuvette was 50 µg and 100 µg. Different 7-Ethoxyresorufin concentrations were tested in these diluted microsomes. It was found that final 100 µg of protein concentration in the cuvette was more suitable for the kinetic studies. At the end of each experiment, control activity was measured again and compared with the control activity which was measured at the beginning of the experiment. By this way, stability of the diluted microsome was continuously re-checked. At the end of these preliminary studies, Pool 1 was used up, so another pool, Pool 2, was constructed from 9 different microsomal preparations obtained from livers of *Liza saliens* caught from Pasaport and Hekim Island site. The average EROD activity of Pool 2 was determined to be 1223 pmol/min/mg protein. Both Pool 1 and Pool 2 were used to investigate the effect of flavonoids on CYP1A1 associated EROD activity of fish liver.

### 3.1.2 CYP1A1 Protein Content of Leaping Mullet (Western-Blot Analysis)

CYP1A1 protein levels in hepatic microsomes of leaping mullet (Pool 2) caught from highly polluted sites of İzmir Bay were determined by western blotting coupled with immunodetection. Polyclonal antibodies produced against purified leaping mullet liver cytochrome P4501A in rabbits was used as primary antibody and anti-rabbit IgG-ALP conjugate was used as secondary antibody. Figure 3.1 demonstrates the western blot analysis of Pool 2.



10 µg 10 µg 25 µg 25 µg

**Figure 3.1** Immunochemical detection of liver microsomal cytochrome P4501A of leaping mullet caught from polluted sites of İzmir Bay.

Polyclonal anti-mullet P4501A1 showed strong cross-reactivity with liver microsomal pool (Pool 2) of the leaping mullet caught from highly polluted sites of İzmir Bay. By this way, high EROD activity of Pool 2 was also shown with western blot analysis.

### 3.2 Determination of the Effect of Phenolic Compounds/Flavonoids on CYP1A1 Associated EROD Activity

In this study, the effects of quercetin, resveratrol, rutin, naringenin and hesperidin on CYP1A1 associated EROD activity of fish liver microsomes were investigated.

#### 3.2.1 Effect of Resveratrol on Fish Liver CYP1A1 Associated EROD Activity

Fish liver EROD activity in the presence of different concentrations of resveratrol was determined as described in “Methods”. Substrate 7-Ethoxyresorufin concentration was held at 1.5 μM while resveratrol concentration was changed from 1 to100 μM.

Figure 3.2 and Figure 3.3 show the effect of resveratrol on EROD activity of fish liver microsomes. Resveratrol inhibited EROD activity in a dose dependent manner. 100 μM of resveratrol concentration caused 99% inhibition of EROD activity.

IC50 value was determined by plotting EROD activity percent against logarithmic resveratrol concentration as shown in Figure 3.4. From the equation of the plot, IC50 value of resveratol is calculated to be 3.59 μM.

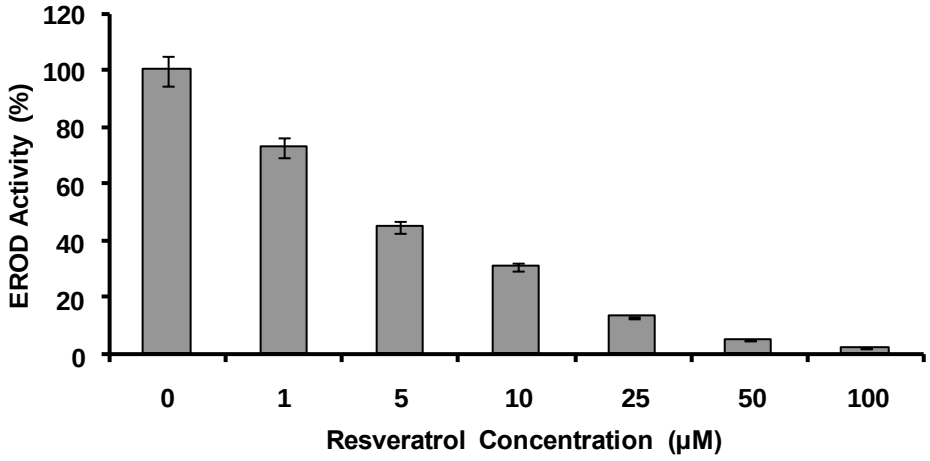



**Figure 3.2** Effect of resveratrol on fish liver CYP1A1 mediated EROD activity.

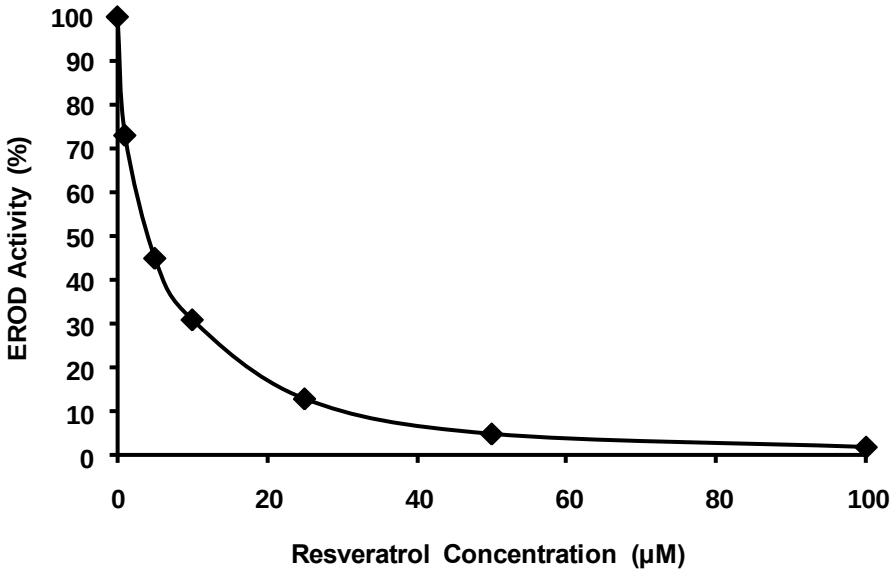



**Figure 3.3** Effect of resveratrol on fish liver CYP1A1 mediated EROD activity.

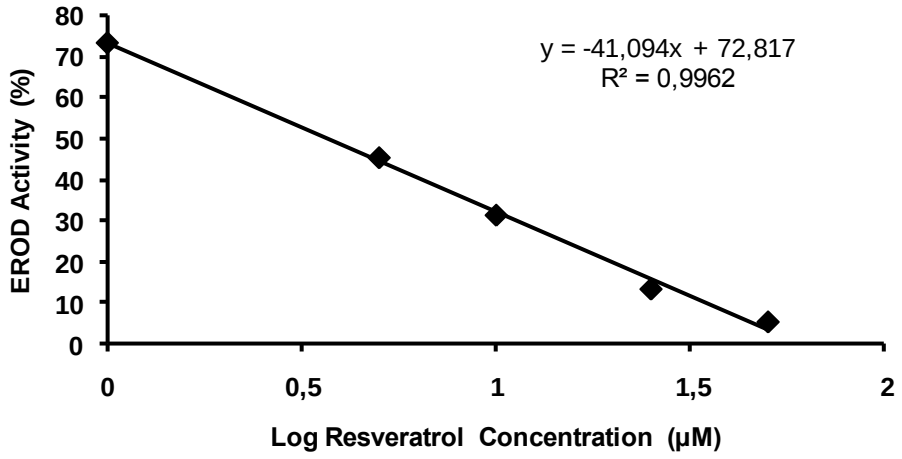



**Figure 3.4** Determination of IC50 value for resveratrol. Inhibition experiments of EROD activity to determine IC50 values were performed at a substrate concentration of 1.5 μM. Resveratrol caused a dose dependent inhibition of EROD activity with an IC50 of 3.59 μM.

In the determination of inhibition mechanism of resveratrol on fish liver EROD activity, the concentration of resveratrol was held at three different concentrations (0.5 µM, 1 µM, 1.5 µM) in activity assay mixture, while the substrate 7-Ethoxyresorufin concentration was varied (0.2 μM, 0.4 μM and 0.8 μM).

Michaelis-Menten, [v] versus [S], and Linewear-Burk, 1/[v] versus 1/[S], plots in the presence of three different fixed concentrations of resveratrol were shown in Figure 3.5 and Figure 3.6 respectively. The Linewear-Burk plot indicated that the Vmax remained unchanged by the presence of different concentrations of resveratrol, while apparent Km increased with increasing resveratrol concentration. The highest concentration of resveratrol (1.5 μM) significantly increased *K*m from 0.33 μM (DMSO-Control) to 1.17 μM without significantly changing *V*max. This suggests the inhibition manner to be apparently competitive.

Figure 3.7 shows Dixon plot, 1/v versus resveratrol concentration, in the presence of three different fixed concentrations of 7-Ethoxyresorufin. From the intersecting point of three lines in Dixon plot, Ki value was determined to be 0.67 µM. Similarly, when calculated from the Linewear-Burk plot, Ki value was found as 0.87 µM.

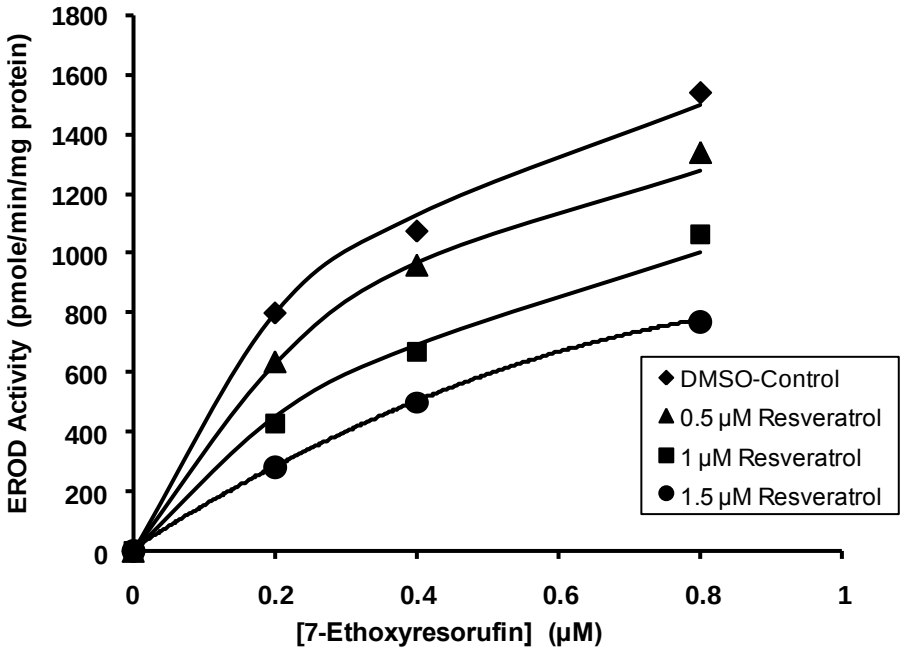



**Figure 3.5** Michaelis-Menten plot for fish liver CYP1A1 mediated EROD activity, v versus [7-Ethoxyresorufin] plot in the presence and in the absence of different fixed concentrations of resveratrol.

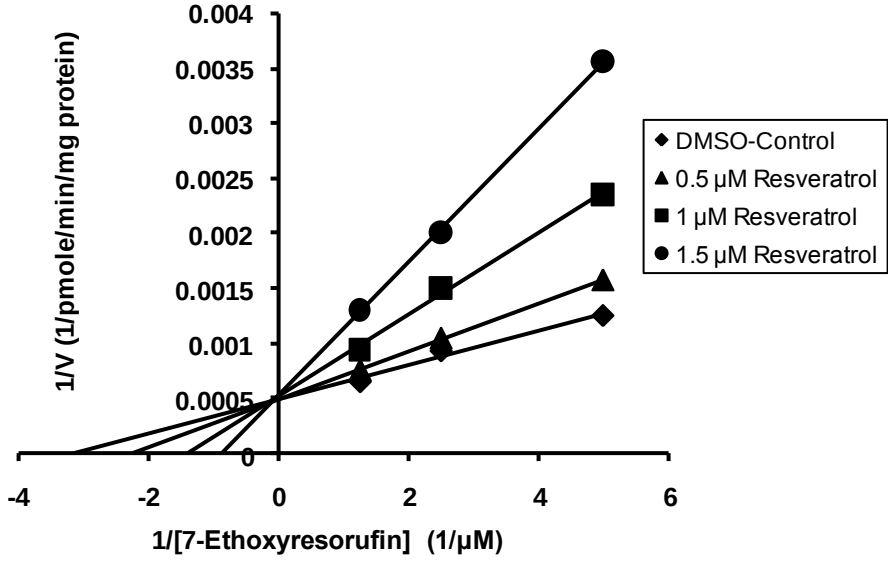



**Figure 3.6** Lineweaver-Burk plot for fish liver CYP1A1 mediated EROD activity, 1/v versus 1/[7-Ethoxyresorufin] plot in the presence of different fixed concentrations of resveratrol.

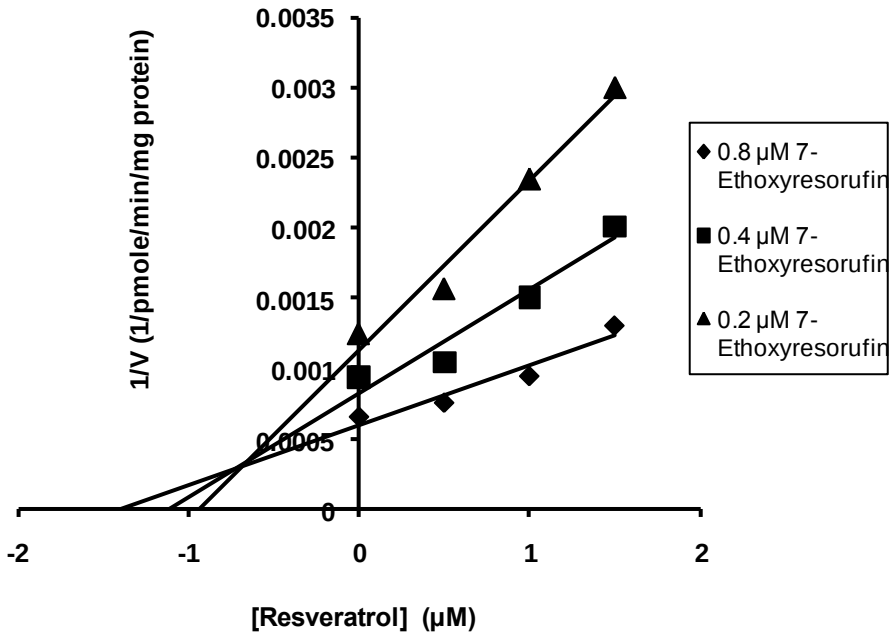



**Figure 3.7** Dixon plot for fish liver CYP1A1 mediated EROD activity, 1/v versus [Resveratol] in the presence of different fixed concentrations of substrate 7-Ethoxyresorufin. Ki value was calculated to be 0.67 µM.

### 3.2.2 Effect of Quercetin on Fish Liver CYP1A1 Associated EROD Activity

Fish liver EROD activity in the presence of different concentrations of quercetin was determined as described in "Methods". Substrate 7-Ethoxyresorufin concentration was held at 1.5 µM while quercetin concentration was changed from 0.075 to 10 µM.

Figure 3.8 shows EROD Activity (%) in the presence of different concentrations of quercetin. It is indicated that fish liver EROD activity was inhibited by increasing concentrations of quercetin in a dose dependent manner. IC50 value of quercetin was calculated as described in resveratrol and found to be 1.32 µM. When liver microsomes were preincubated with 10 µM quercetin, only 8% of initial activity remained.

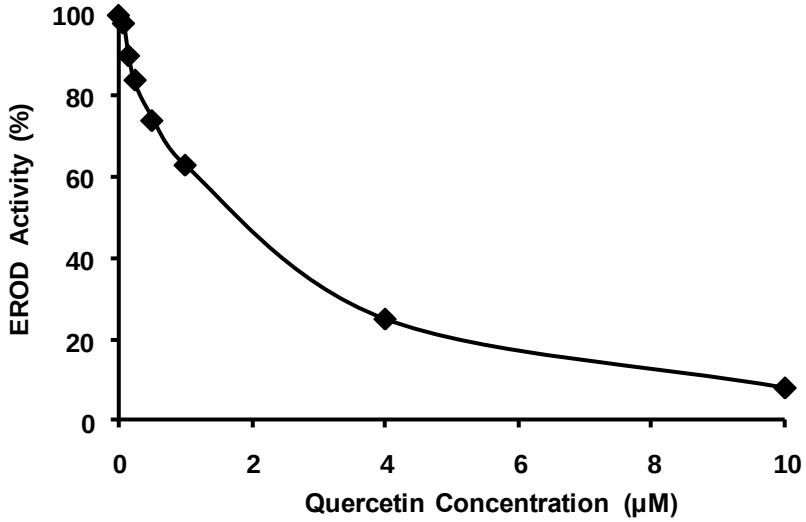



**Figure 3.8** Effect of quercetin on fish liver CYP1A1 mediated EROD Activity. Inhibition experiments of EROD activity to determine IC50 values were performed at a substrate concentration of 1.5 μM. Quercetin caused a dose dependent inhibition of EROD activity with an IC50 of 1.32 μM.

In the determination of the inhibition mechanism of quercetin on fish liver EROD activity, the concentration of the quercetin was held at three different values (0.075, 0.15, and 0.25 μM) in activity assay mixture, while the substrate concentration was varied (0.2 μM, 0.4 μM and 0.8 μM).

Michaelis-Menten, [v] versus [S], and Linewear-Burk, 1/[v] versus 1/[S], plots in the presence of three different fixed concentrations of resveratrol were shown in Figure 3.9 and Figure 3.10 respectively. Lineweaver-Burk plot indicated that the maximum velocity (Vmax) remained unchanged by the presence of different concentrations of quercetin while the Michaelis-Menten constant (Km) increased with increasing quercetin concentration. The highest concentration of quercetin (0.25 μM) significantly increased Km from 0.48 μM (DMSO-Control) to 1.83 μM without significantly changing Vmax. Therefore; the inhibition of fish liver EROD activity by quercetin was estimated to be competitive type of inhibition.

Figure 3.11 shows Dixon plot in the presence of different fixed concentrations of substrate 7-Ethoxyresorufin. The inhibitor constant, Ki, was calculated to be 0.12 μM from the intersection point of three lines in Dixon plot. Similarly, Ki value was calculated from Lineweaver-Burk plot as 0.10 μM.

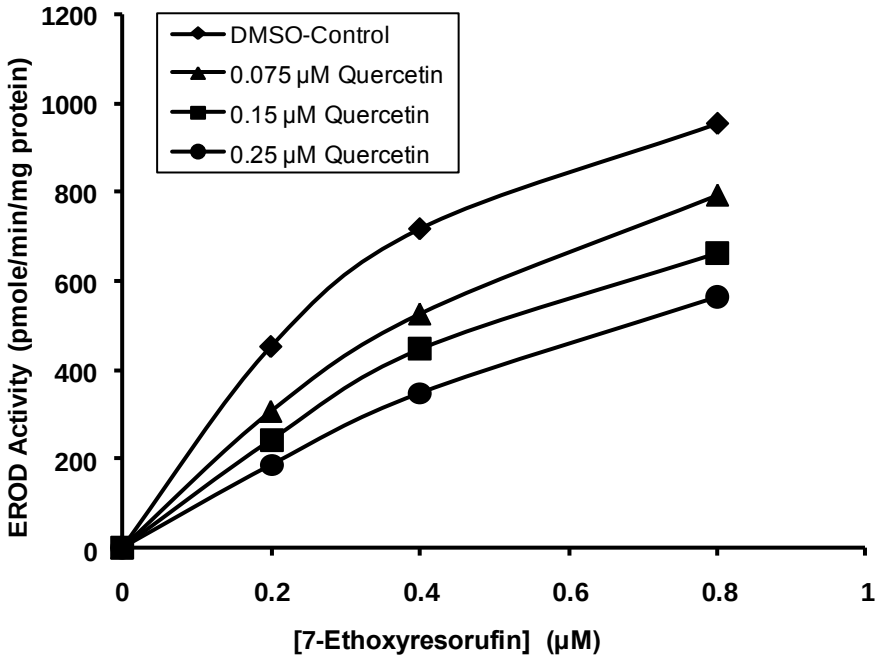



**Figure 3.9** Michaelis-Menten plot for fish liver CYP1A1 mediated EROD activity, v versus [7-Ethoxyresorufin] plot, in the presence and in the absence of different fixed concentrations of quercetin.

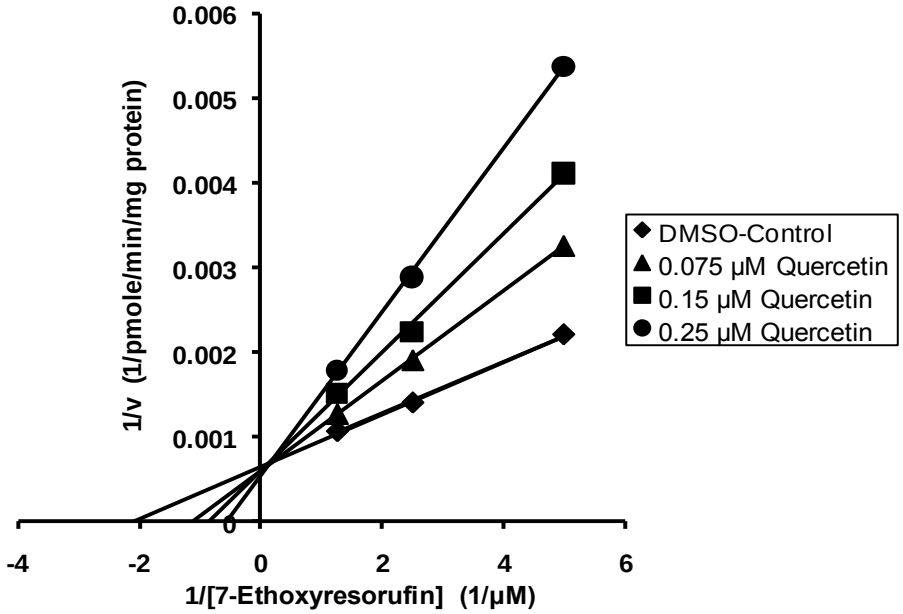



**Figure 3.10** Lineweaver-Burk plot for fish liver CYP1A1 mediated EROD activity, 1/v versus 1/[7-Ethoxyresorufin] plot, in the presence of different fixed concentrations of quercetin.

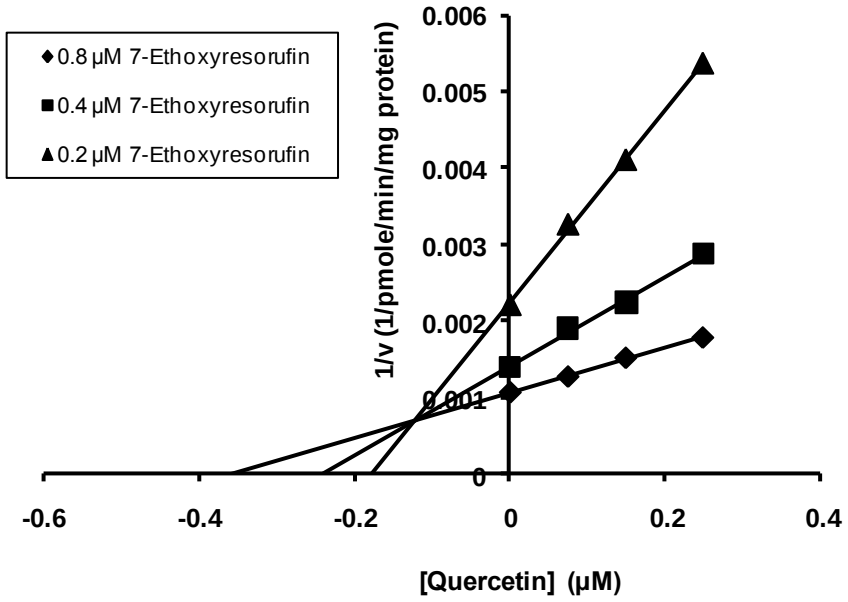



**Figure 3.11** Dixon plot for fish liver CYP1A1 mediated EROD activity, 1/v versus [Quercetin], in the presence of different fixed concentrations of substrate 7-Ethoxyresorufin. Ki value was calculated to be 0.12 μM.

### 3.2.3 Effect of Rutin on Fish Liver CYP1A1 Associated EROD Activity

Fish liver EROD activity in the presence of different concentrations of rutin was determined as described in “Methods”. Substrate 7-Ethoxyresorufin concentration was held at 1.5 μM while rutin concentration was changed from 0.2 to 1 mM.

The effect of increasing concentrations of rutin on fish liver EROD activity is given in Figure 3.12. It is indicated that fish liver EROD activity was inhibited by increasing concentrations of rutin. IC50 value of rutin was calculated as described in resveratrol and found to be 0.64 mM.

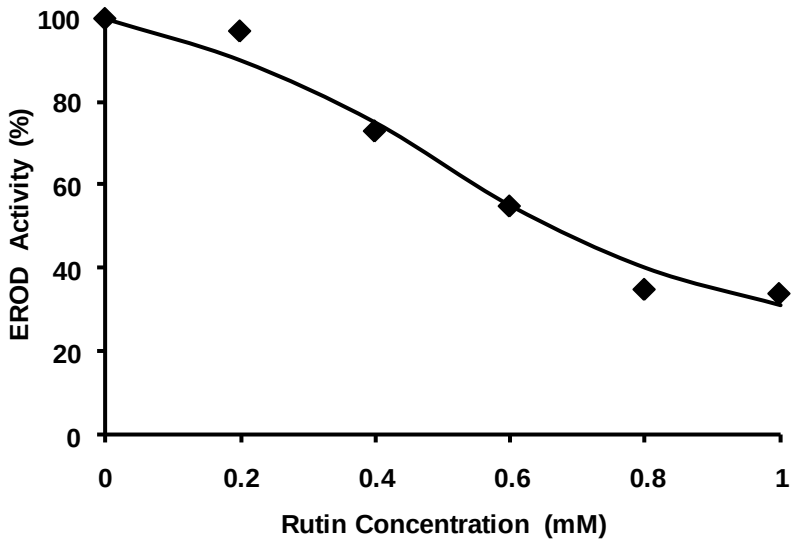



**Figure 3.12** Effect of rutin on fish liver CYP1A1 mediated EROD activity. Inhibition experiments of EROD activity to determine IC50 values were performed at a substrate concentration of 1.5 μM. Rutin did inhibited EROD activity in a dose dependent manner with an IC50 of 0.64 mM.

Considering the IC50 value, three different concentrations of rutin (0.4 mM, 0.6 mM and 0.8 mM) were selected to study the inhibition kinetics at three different 7-Ethoxyresorufin concentrations (0.2 μM, 0.4 μM and 0.8 μM). Figure 3.13 and Figure 3.14 represents the Michaelis-Menten and Lineweaver-Burk plots respectively, in the presence and absence of three different fixed concentrations of rutin. Lineweaver-Burk

plot indicated that, as rutin concentration increases, the intercept on the 1/[S] axis moves closer to the origin, that is, apparent Km continually increases without changing Vmax. The highest concentration of rutin (0.8 mM) significantly increased Km from 0.4 μM (DMSO-Control) to 4.25 μM without significantly changing Vmax. This type of inhibition is depicted as competitive type of inhibition. Ki value was calculated to be 0.1 mM from the Dixon plot (Figure 3.15). Similarly, Ki value was found as 0.12 mM from Lineweaver-Burk plot.

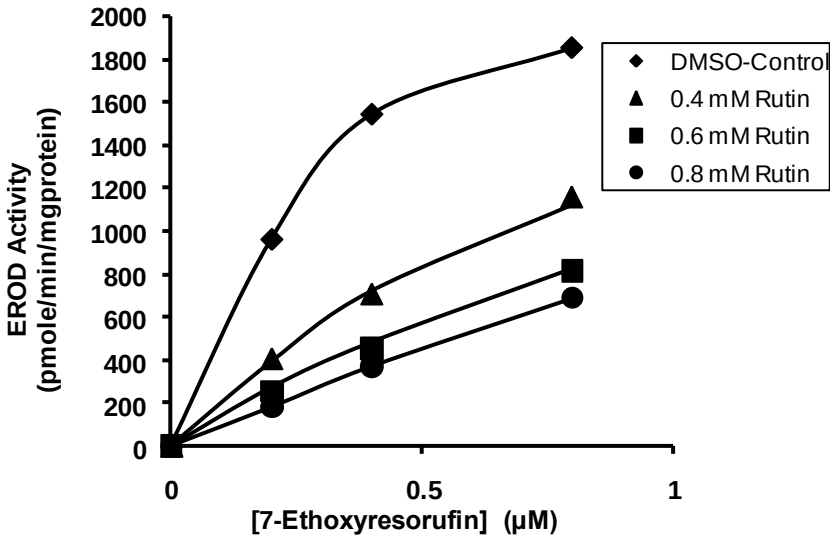



**Figure 3.13** Michaelis-Menten plot for fish liver CYP1A1 mediated EROD activity, v versus [7-Ethoxyresorufin] plot in the presence and in the absence of different fixed concentrations of rutin.

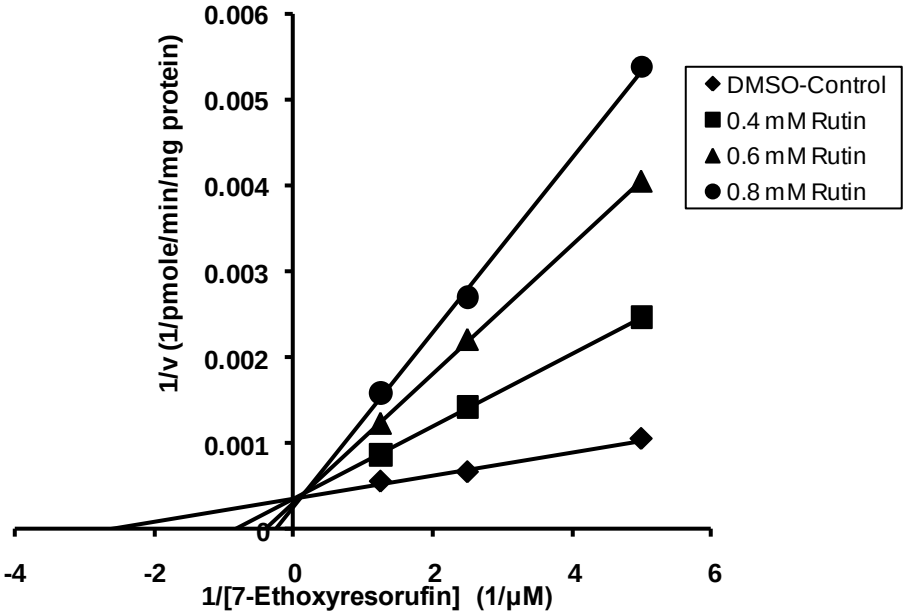



**Figure 3.14** Lineweaver-Burk plot for fish liver CYP1A1 mediated EROD activity, 1/v versus 1/[7-Ethoxyresorufin] plot in the presence of different fixed concentrations of rutin.

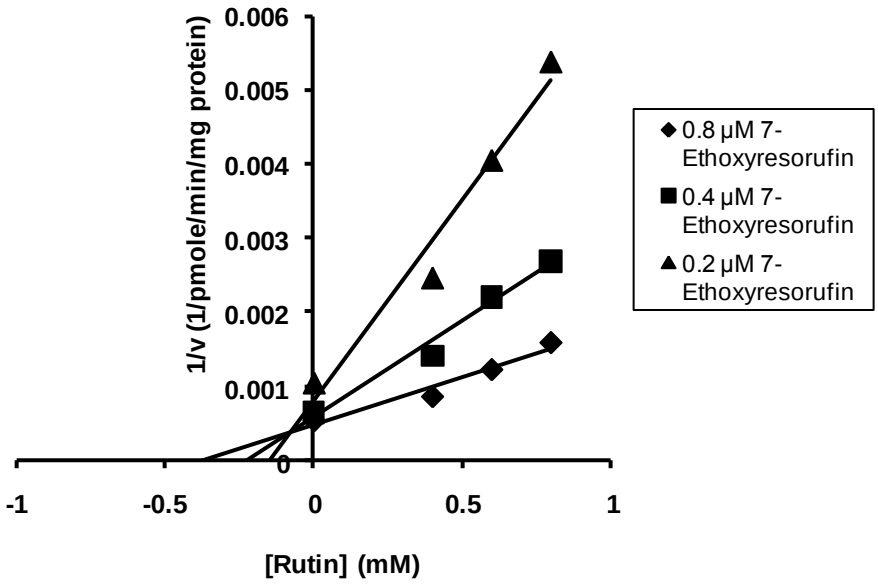



**Figure 3.15** Dixon plot for fish liver CYP1A1 mediated EROD activity, 1/v versus [Rutin] in the presence of different fixed concentrations of substrate 7-Ethoxyresorufin. Ki value was calculated to be 0.1 mM.

### 3.2.4 Effect of Naringenin on Fish Liver CYP1A1 Associated EROD Activity

Fish liver EROD activity in the presence of different concentrations of naringenin was determined as described in “Methods”. Substrate 7-Ethoxyresorufin concentration was held at 1.5 μM while naringenin concentration was changed from 0.25 to 5 μM.

Figure 3.16 shows the effect of increasing concentration of naringenin on fish liver EROD activity. It is indicated that fish liver EROD activity was inhibited by increasing concentrations of naringenin. IC50 value of naringenin was calculated as decribed in resveratrol and found to be 9.78 μM.

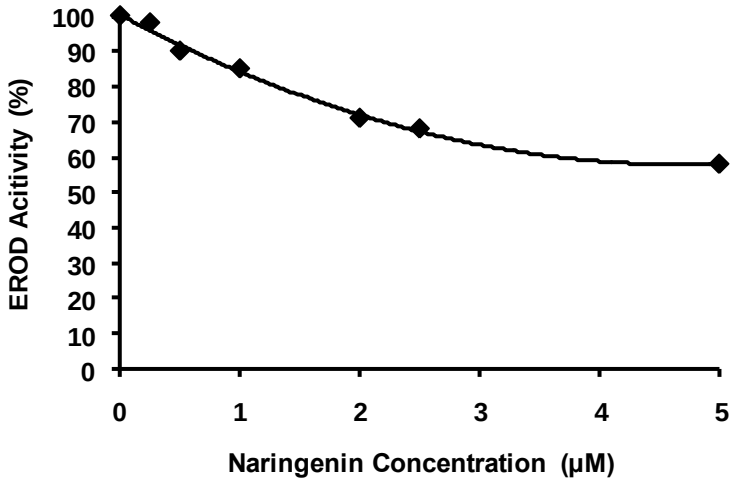



**Figure 3.16** Effect of naringenin on fish liver CYP1A1 mediated EROD activity. Inhibition experiments of EROD activity to determine IC50 values were performed at a substrate concentration of 1.5 μM. IC50 value is calculated to be 9.78 μM.

In the determination of inhibition mechanism of naringenin on fish liver EROD activity, the concentration of naringenin was held at three different concentration (0.5 μM, 1 μM, 2 μM) in the reaction medium, while the substrate 7-Ethoxyresorufin concentration was varied (0.2 μM, 0.4 μM and 0.8 μM).

Michaelis-Menten and Lineweaver-Burk plots are given in figures 3.17 and 3.18 respectively. The Linewear – Burk plot indicated that the Michaelis-Menten constant

(Km) remained unchanged by the presence of different concentrations of naringenin, while Vmax (maximum velocity) decreased with increasing naringenin concentration. The apparent Km value was found to be 0.64 µM for 7-Ethoxyresorufin in all concentrations of naringenin present in the reaction medium. However, Vmax values of the enzyme reaction were decreased from 4000 pmol/min/mg (DMSO-Control) to 2381 pmol/min/mg (2 µM naringenin) with increasing naringenin concentration. This suggests that naringenin caused a non-competitive type of inhibition in fish liver EROD activity.

Figure 3.19 represents Dixon plot in the presence of different fixed concentrations of 7-Ethoxyresorufin . Dixon plot indicated that Ki value remained the same (2.63 µM) while Vmax value differed for each concentration of 7-Ethoxyresorufin. So Dixon plot also confirmed that the inhibition of fish liver EROD activity by naringenin is non-competitive type. Ki value was calculated from Lineweaver-Burk plot as 2.85 µM, similar to 2.63 µM which was calculated from Dixon plot.

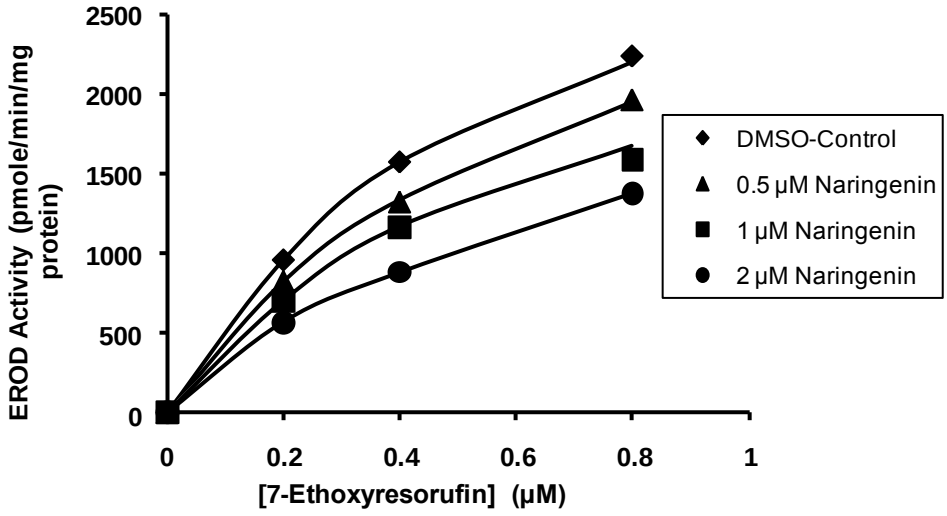



**Figure 3.17** Michaelis-Menten plot for fish liver CYP1A1 mediated EROD activity, v versus [7-Ethoxyresorufin] plot in the presence and in the absence of different fixed concentrations of naringenin.

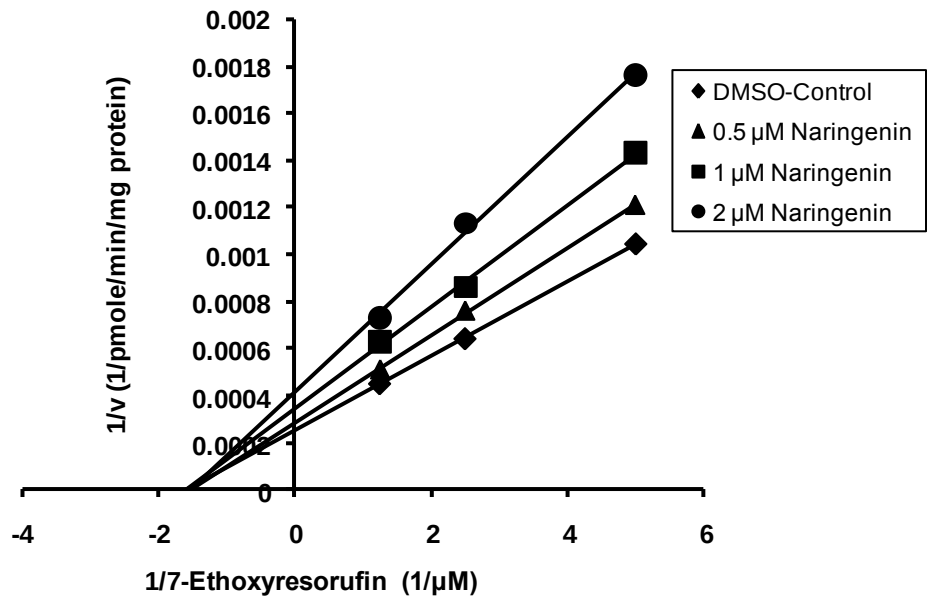



**Figure 3.18** Lineweaver-Burk plot for fish liver CYP1A1 mediated EROD activity, 1/v versus 1/[7-Ethoxyresorufin] plot in the presence of different fixed concentrations of naringenin.

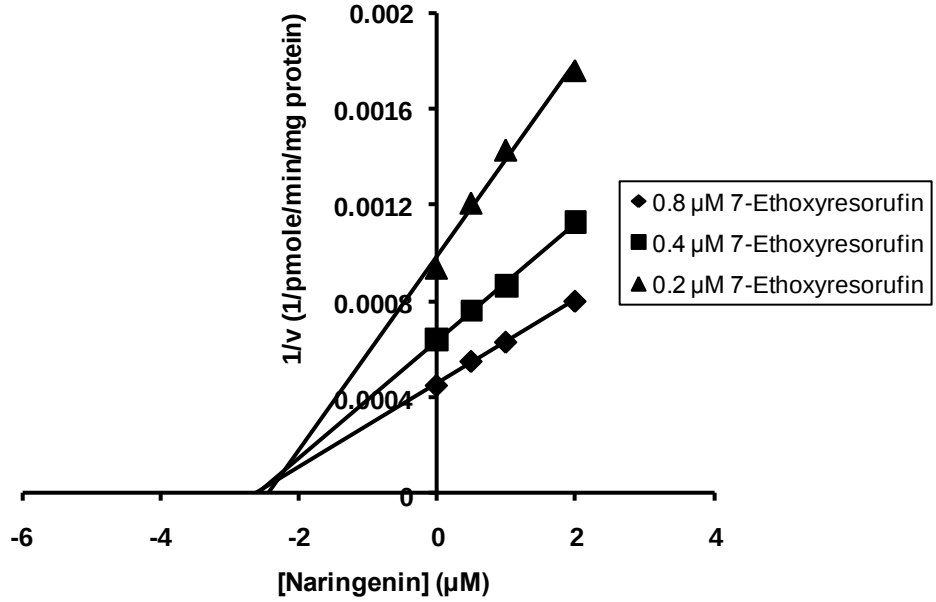



**Figure 3.19** Dixon plot for fish liver CYP1A1 mediated EROD activity, 1/v versus [Naringenin] in the presence of different fixed concentrations of substrate 7-Ethoxyresorufin. Ki value was calculated to be 2.63 μM.

### 3.2.5 Effect of Hesperidin on Fish Liver CYP1A1 Associated EROD Activity

Fish liver EROD activity in the presence of different concentrations of hesperidin was determined as described in “Methods”. Substrate 7-Ethoxyresorufin concentration was held at 1.5 μM while hesperidin concentration was changed from 1 to 200 μM.

The effect of increasing concentration of hesperidin on fish liver EROD activity is given in Figure 3.20. It is indicated that hesperidin inhibited EROD activity in a dose dependent manner. IC50 value of hersperidin was calculated as described in resveratrol and found to be 98.5 μM.

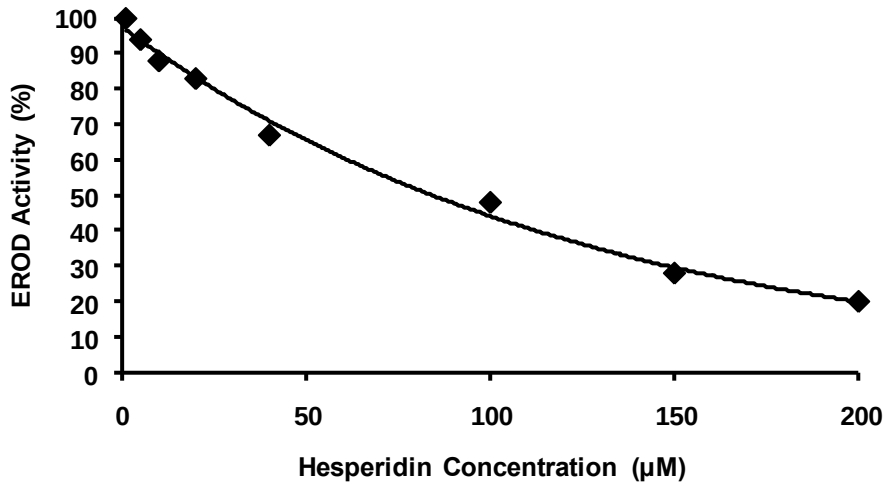



**Figure 3.20** Effect of hesperidin on fish liver CYP1A1 mediated EROD activity. Inhibition experiments of EROD activity to determine IC50 values were performed at a substrate concentration of 1.5 μM. Hesperidin caused a dose dependent inhibition of EROD activity with an IC50 of 98.5 μM.

In order to determine the inhibition mechanism of hesperidin on fish liver EROD activity, the concentration of hesperidin was held at four different concentrations (5 μM, 10 μM, 25 μM, 30 μM) in activity assay mixture, while the substrate 7-Ethoxyresorufin concentration was varied (0.2 μM, 0.4 μM, 0.8 μM).

Figures 3.21 and 3.22 represent Michaelis-Menten and Lineweaver-Burk plots respectively for fish liver EROD activity in the presence and absence of different fixed concentrations of hesperidin. Lineweaver-Burk plot indicated that the maximum velocity (Vmax) remained unchanged by the presence of different concentrations of hesperidin while the Michaelis-Menten constant (Km) increased with increasing hesperidin concentration. The highest concentration of hesperidin (30 μM) significantly increased *K*m from 0.5 μM (DMSO-Control) to 1.62 μM without significantly changing *V*max. This suggests that hesperidin caused a competitive type of inhibition in fish liver EROD activity.

Figure 3.23 shows Dixon plot, 1/v versus hesperidin concentration, in the presence of three different fixed concentrations of 7-Ethoxyresorufin. Ki value was calculated to be 18 μM from the intersection point in Dixon plot. Similarly, Ki value was calculated as 14.4 μM from Lineweaver-Burk plot.

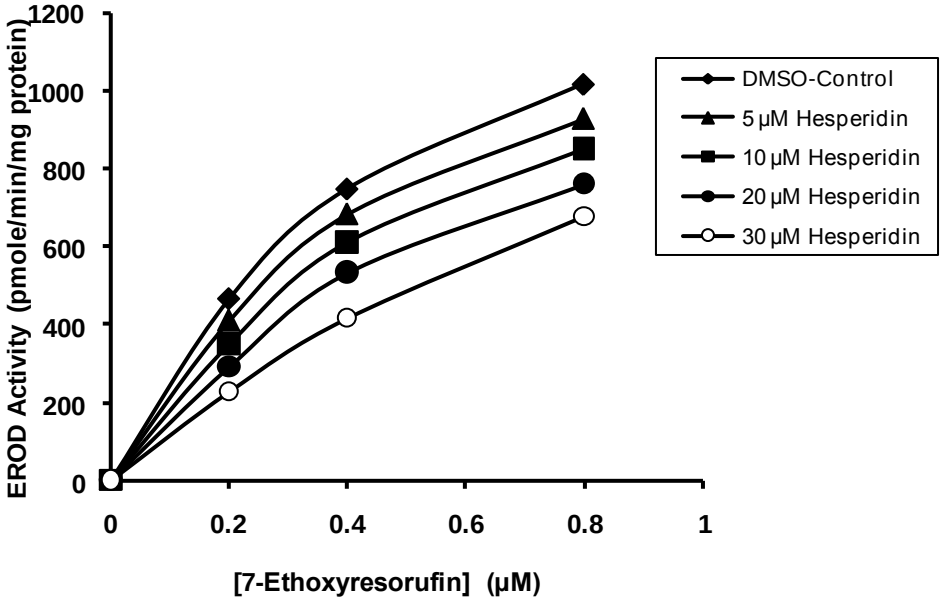



**Figure 3.21** Michaelis-Menten plot for fish liver CYP1A1 mediated EROD activity, v versus [7-Ethoxyresorufin] plot in the presence and in the absence of different fixed concentrations of hesperidin.

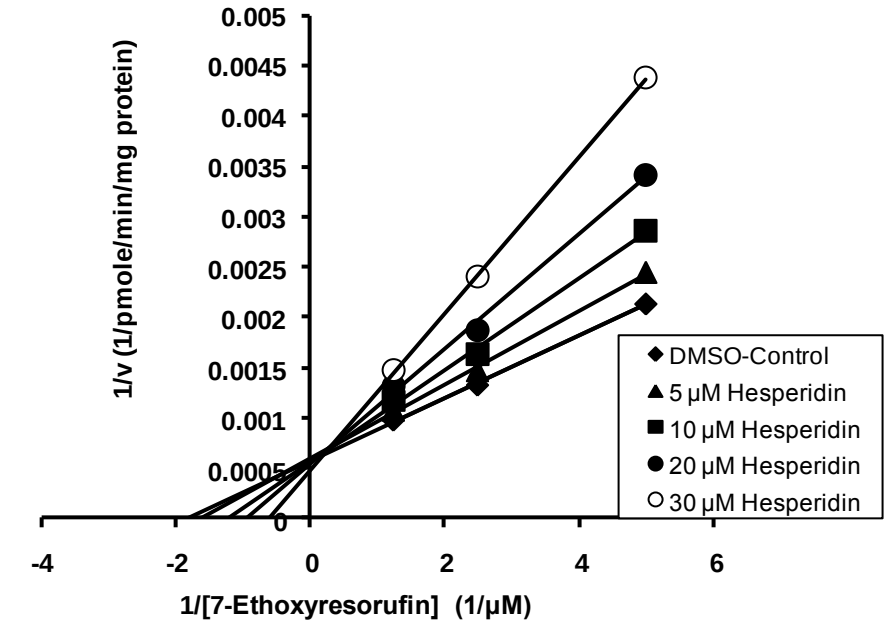



**Figure 3.22** Lineweaver-Burk plot for fish liver CYP1A1 mediated EROD activity, 1/v versus 1/[7-Ethoxyresorufin] plot in the presence of different fixed concentrations of hesperidin.

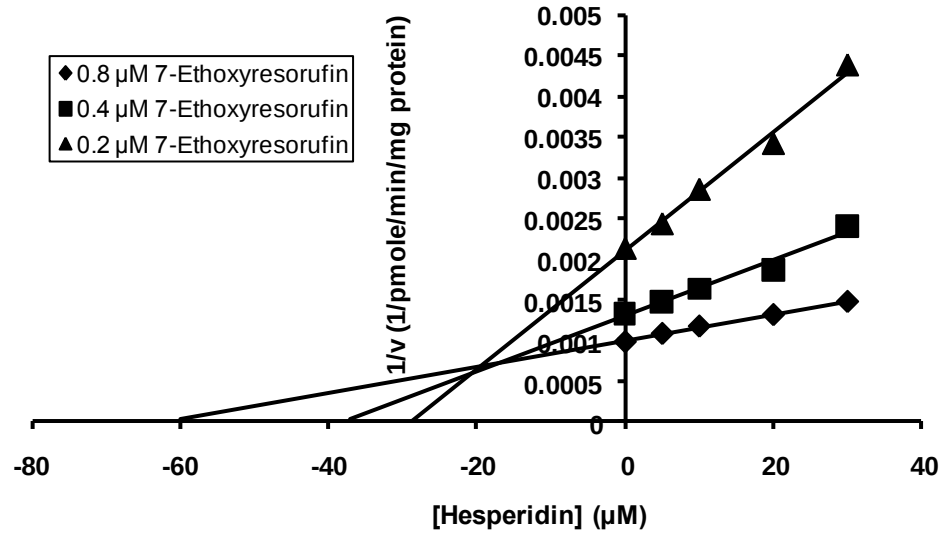



**Figure 3.23** Dixon plot for fish liver CYP1A1 mediated EROD activity, 1/v versus [Hesperidin] in the presence of different fixed concentrations of substrate 7-Ethoxyresorufin. Ki value was calculated to be 18 μM.

Table 3.1 represents all the phenolic compounds/flavonoids studied and their inhibition types on CYP1A1 associated EROD activity of fish liver.

**Table 3.1** The inhibition types of phenolic compounds/ flavonoids on CYP1A1 associated EROD activity of fish liver.

| Phenolic Compound/Flavonoid | Inhibition Type |
|---|---|
| Resveratrol | Competitive |
| Quercetin | Competitive |
| Rutin | Competitive |
| Naringenin | Non-competitive |
| Hesperidin | Competitive |

## 3.3 Determination of the Effect of Phenolic Compounds/Flavonoids on Cytosolic Total GST Activity

In this study, the effects of quercetin, resveratrol, rutin, naringenin and hesperidin on cytosolic total GST activity of fish liver were investigated.

### 3.3.1 Effect of Quercetin on Fish Liver Total GST Activity

Fish liver cytosolic total GST activity in the presence of different concentrations of quercetin was determined as described in “Methods”. Substrate CDNB concentration was held at 1 mM and GSH concentration was held at 1.33 mM while quercetin concentration was changed from 5 μM to 33 μM to investigate the effect of quercetin on fish liver total GST activity.

Figure 3.24 represents the effect of increasing concentrations of quercetin on fish liver total GST activity. It is shown that quercetin inhibited fish liver GST activity in a dose dependent manner.

IC50 value of quercetin was determined by plotting total GST activity percent against logarithmic quercetin concentration as shown in Figure 3.25. From the equation of the plot, IC50 value for quercetin was calculated to be 24.5 μM.

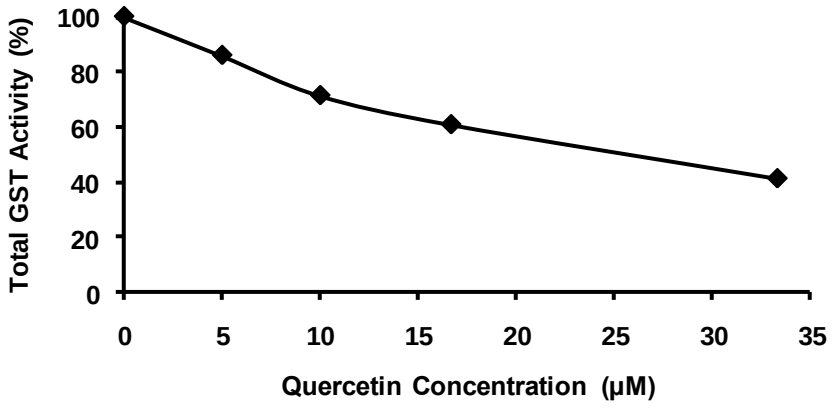



**Figure 3.24** Effect of quercetin on fish liver total GST Activity. Inhibition experiments of GST activity to determine IC50 values were performed at a substrate CDNB concentration of 1 mM and GSH concentration of 1.33 mM. Quercetin inhibited fish liver total GST activity in a dose dependent manner with an IC50 value of 24.5 µM.

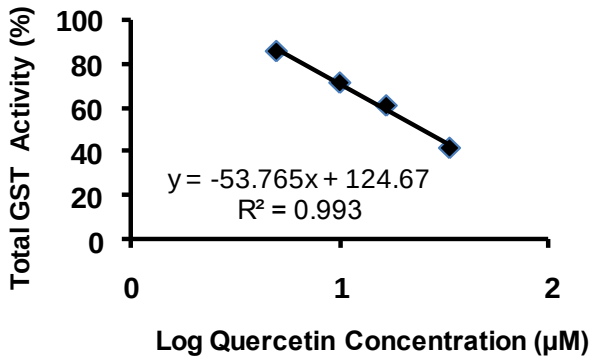



**Figure 3.25** Determination of IC50 value for quercetin.

In order to determine the mode of inhibition of quercetin on GST, the concentration of quercetin was held at three different concentrations (5 µM, 10 µM, 25 µM) in activity assay mixture, while the substrate CDNB concentration was varied (0.25 mM, 0.50 mM, 0.75 mM).

Figures 3.26 and 3.27 represent Michaelis-Menten and Lineweaver-Burk plots respectively for fish liver GST activity in the presence and absence of different fixed

concentrations of quercetin. Lineweaver-Burk plot indicated a competitive type of inhibition with increasing apparent Km without significantly affecting Vmax.

Figure 3.28 shows Dixon plot, 1/v versus quercetin concentration in the presence of three different fixed concentrations of CDNB. Ki value was calculated to be 12.5 μM from Dixon plot and 10.1 μM from Linewever-Burk plot.

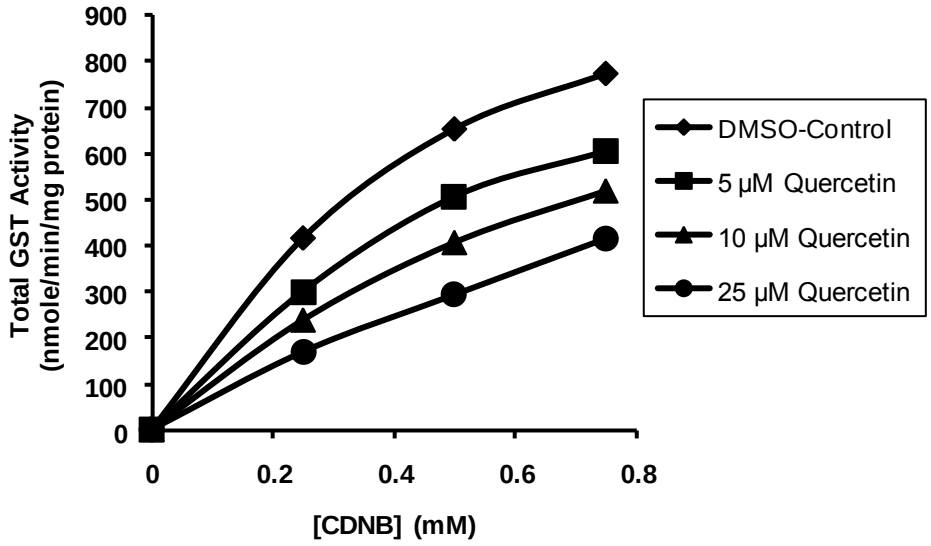



**Figure 3.26** Michaelis-Menten plot for fish liver total GST activity, v versus [CDNB] plot in the presence and in the absence of different fixed concentrations of quercetin.

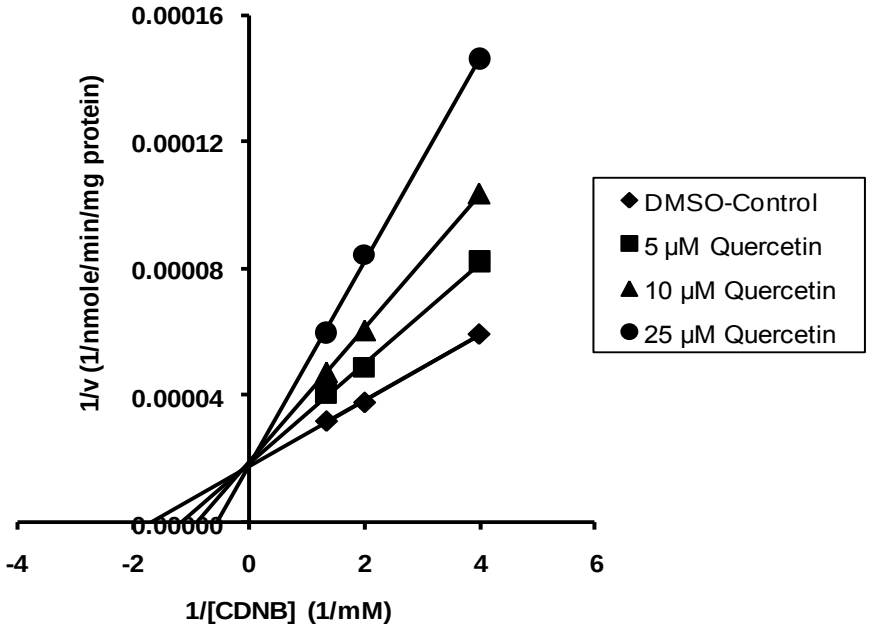



**Figure 3.27** Lineweaver-Burk plot for fish liver total GST activity, 1/v versus 1/[CDNB] plot in the presence of different fixed concentrations of quercetin.

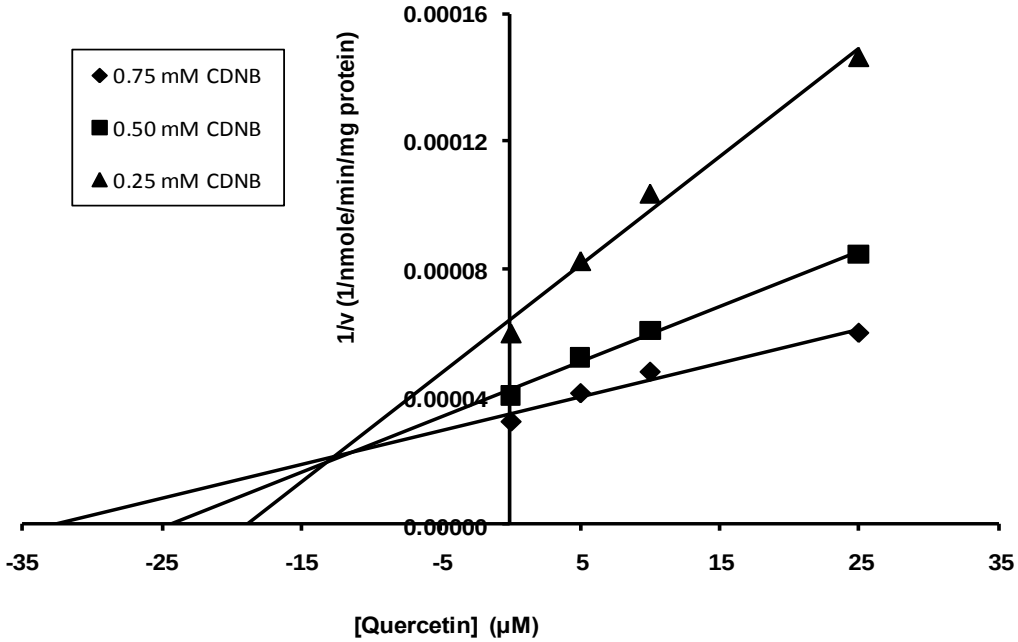



**Figure 3.28** Dixon plot for fish liver total GST activity, 1/v versus [Quercetin] in the presence of different fixed concentrations of substrate CDNB. Ki value was calculated to be 12.5 µM.

### 3.3.2 Effect of Resveratrol on Fish Liver Total GST Activity

Fish liver cytosolic total GST activity in the presence of different concentrations of resveratrol was determined as described in “Methods”. Substrate CDNB concentration was held at 1 mM and GSH concentration was held at 1.33 mM while resveratrol concentration was changed from 1 µM to 10 µM to investigate the effect of resveratrol on fish liver total GST activity.

The effect of increasing concentrations of resveratrol on fish liver total GST activity is given in Figure 3.29. It is shown that resveratol inhibited fish liver total GST activity in a dose dependent manner. IC50 value of resveratrol was calculated as described in quercetin and found to be 7.1 µM.

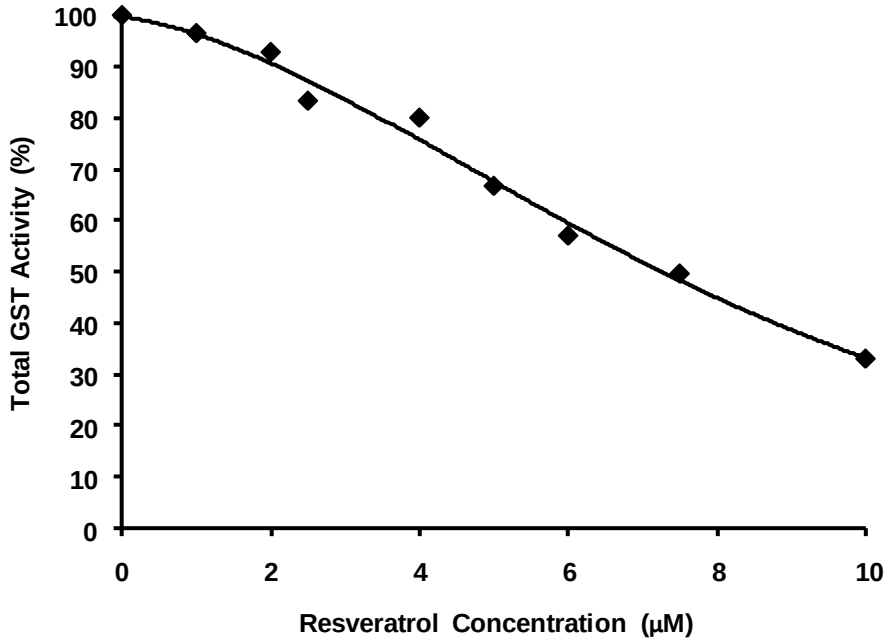



**Figure 3.29** Effect of resveratrol on fish liver total GST activity. Inhibition experiments of GST activity to determine IC50 values were performed at a substrate CDNB concentration of 1 mM and GSH concentration of 1.33 mM. Resveratol inhibited fish liver GST activity in a dose dependent manner with an IC50 value of 7.1 µM.

The inhibition mechanism of resveratrol on fish liver total GST activity was studied by holding the concentration of resveratrol at three different concentrations (2 μM, 4 μM, 6 μM) in activity assay mixture, while the substrate CDNB concentration was varied (0.25 mM, 0.50 mM, 0.75 mM).

Michaelis-Menten, v versus [S], and Linewear-Burk, 1/[v] versus 1/[S], plots in the presence of three different fixed concentrations of resveratrol were shown in Figure 3.30 and Figure 3.31 respectively. The Linewear-Burk plot indicated that the Vmax remained unchanged by the presence of different concentrations of resveratrol, while apparent Km increased with increasing resveratrol concentration. The highest concentration of resveratrol (6 μM) significantly increased Km from 0.4 mM (DMSO-Control) to 1 mM without significantly changing Vmax. This suggests that resveratrol was a competitive inhibitor of GST.

Figure 3.32 shows Dixon plot, 1/v versus resveratrol concentration, in the presence of three different fixed concentrations of CDNB. Ki value was calculated to be 3.2 μM from Dixon plot and 4.86 μM from Lineweaver-Burk plot.

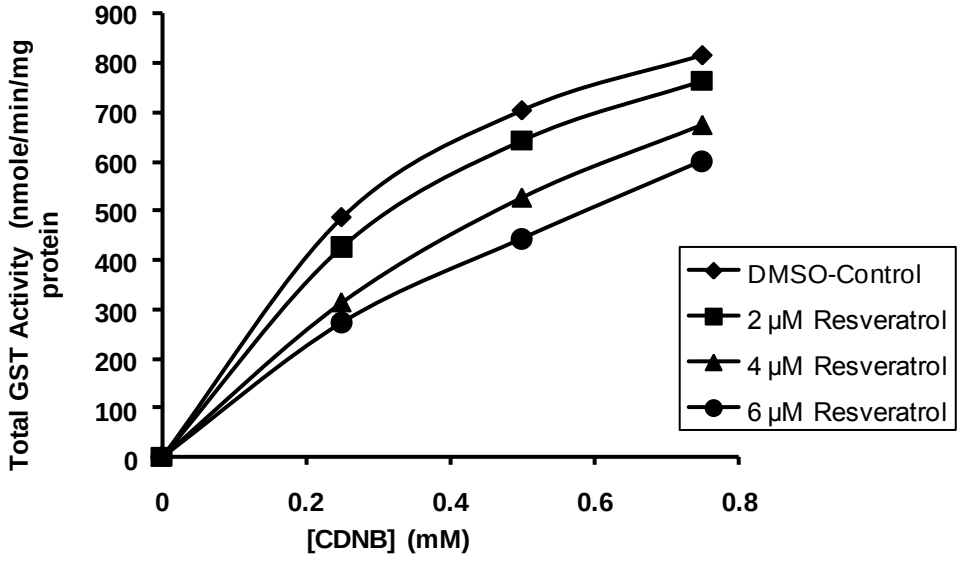



**Figure 3.30** Michaelis-Menten plot for fish liver total GST activity, v versus [CDNB] plot in the presence and in the absence of different fixed concentrations of resveratrol.

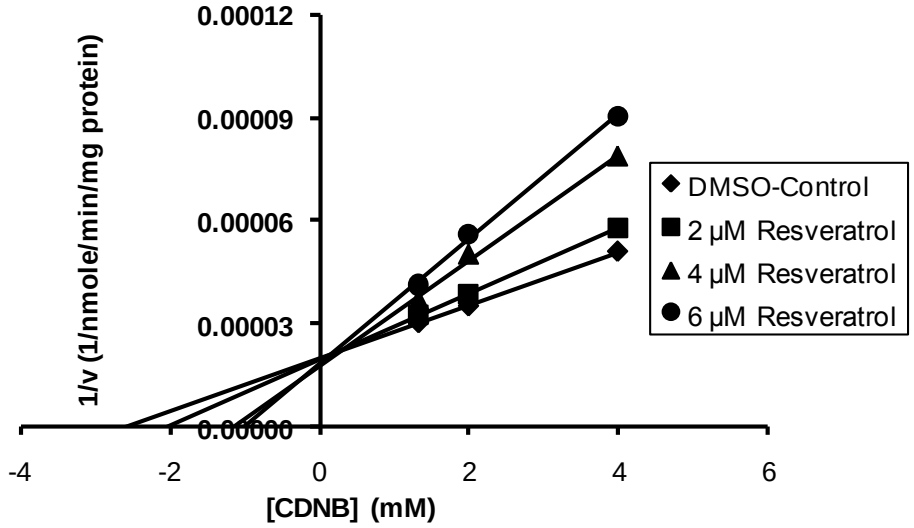



**Figure 3.31** Lineweaver-Burk plot for fish liver total GST activity, 1/v versus 1/[CDNB] plot in the presence of different fixed concentrations of resveratrol.

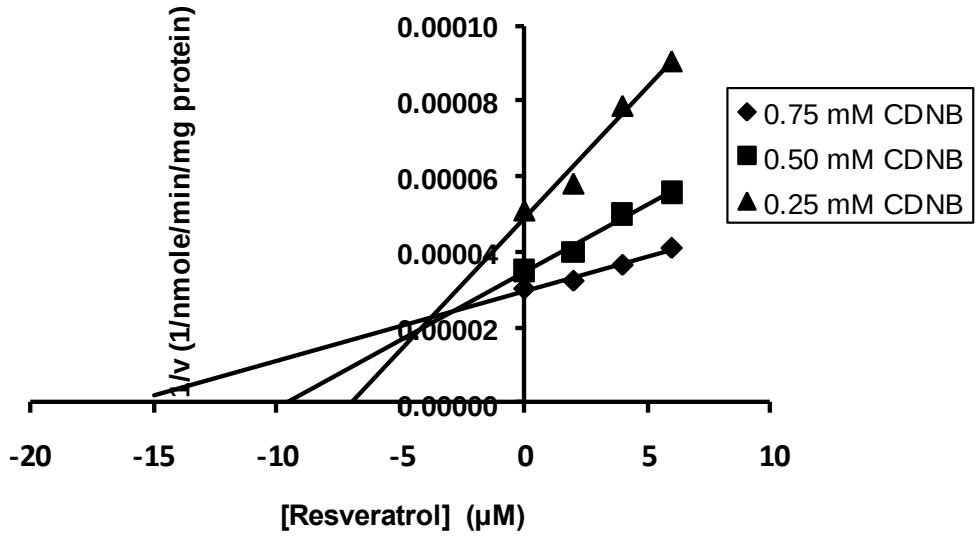



**Figure 3.32** Dixon plot for fish liver total GST activity, 1/v versus [Resveratrol] in the presence of different fixed concentrations of substrate CDNB. Ki value was calculated to be 3.2 µM.

### 3.3.3 Effect of Rutin on Fish Liver Total GST Activity

Fish liver cytosolic total GST activity in the presence of different concentrations of rutin was determined as described in “Methods”. Substrate CDNB concentration was held at 1 mM and GSH concentration was held at 1.33 mM while rutin concentration was changed from 25 µM to 200 µM to investigate the effect of rutin on fish liver total GST activity.

Figure 3.33 represents the effect of rutin on fish liver total GST activity. IC50 value of rutin was calculated as described in quercetin and found to be 89 µM. GST enzyme activity was completely inhibited at 200 µM rutin concentration.

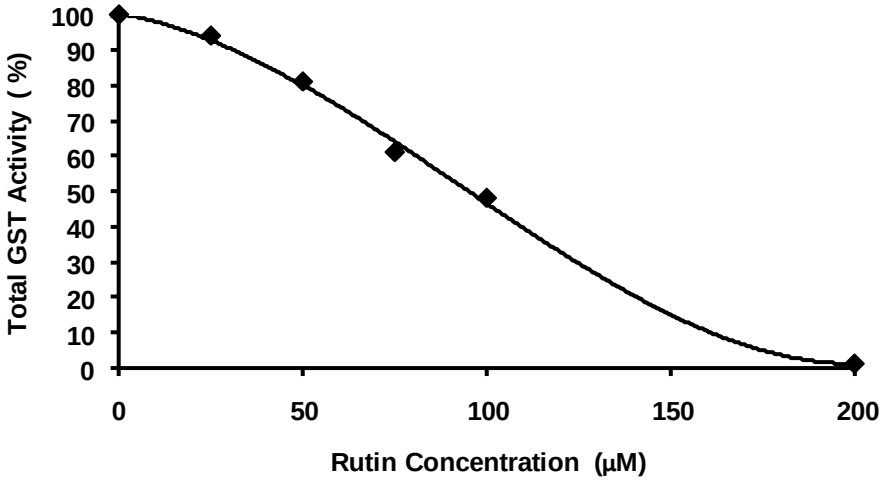



**Figure 3.33** Effect of rutin on fish liver total GST activity. Inhibition experiments of GST activity to determine IC50 values were performed at a substrate CDNB concentration of 1 mM and GSH concentration of 1.33 mM. Rutin inhibited fish liver total GST activity in a dose dependent manner with an IC50 value of 89 µM.

In order to determine the inhibition mechanism of rutin on fish liver total GST activity, the concentration of rutin was held at three different concentrations (30 µM, 50 µM, 75 µM) in activity assay mixture, while the substrate CDNB concentration was varied (0.25 mM, 0.50 mM, 0.75 mM).

Figure 3.34 and 3.35 represent Michaelis-Menten and Lineweaver-Burk plots respectively for fish liver total GST activity in the presence and absence of different fixed concentrations of rutin. Lineweaver-Burk plot indicated straight lines meeting at a point between the horizontal and vertical axis. This type of inhibition has been interpreted to be of mixed type, where both competitive and noncompetitive interactions are responsible for the inhibition of the enzyme. This inhibition was characterized by a concentration-dependent increase in Km values as well as a decrease in Vmax value.

Figure 3.36 shows Dixon plot for fish liver total GST activity, 1/v versus rutin concentration in the presence of different fixed concentrations of substrate CDNB. Ki value was calculated to be 150 µM.

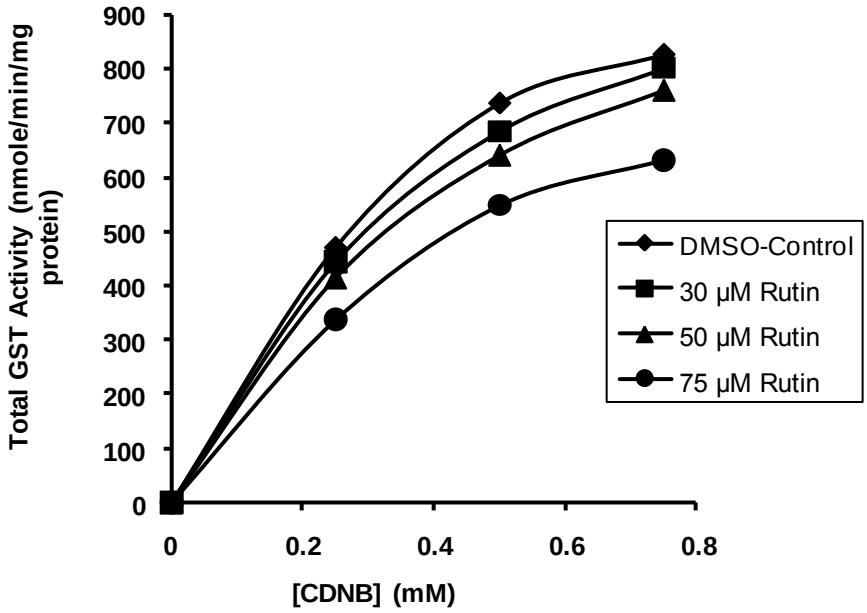



**Figure 3.34** Michaelis-Menten plot for fish liver total GST activity, v versus [CDNB] plot in the presence and in the absence of different fixed concentrations of rutin.

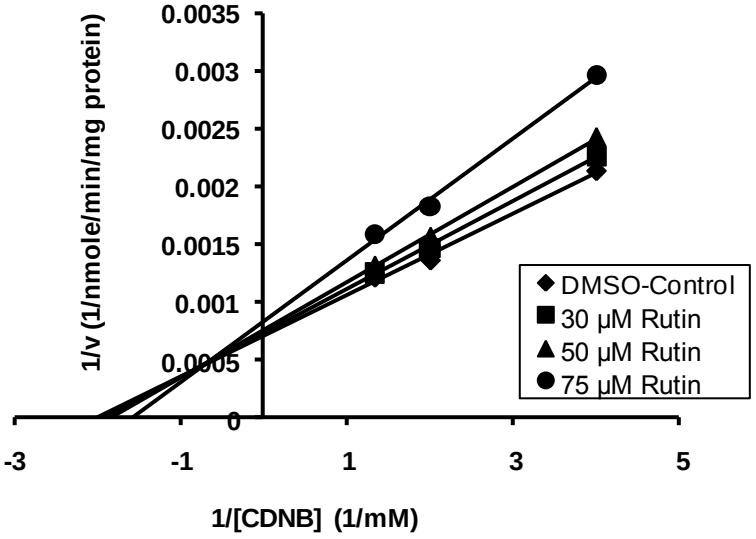



**Figure 3.35** Lineweaver-Burk plot for fish liver total GST activity, 1/v versus 1/[CDNB] plot in the presence of different fixed concentrations of rutin.

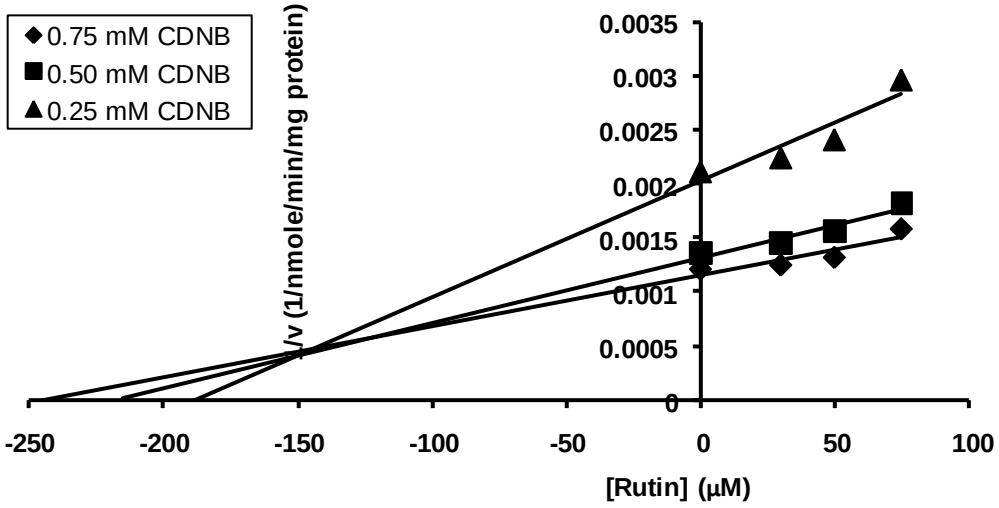



**Figure 3.36** Dixon plot for fish liver total GST activity, 1/v versus [Rutin] in the presence of different fixed concentrations of substrate CDNB. Ki value was calculated to be 150 µM.

### 3.3.4 Effect of Naringenin on Fish Liver Total GST Activity

Fish liver cytosolic total GST activity in the presence of different concentrations of naringenin was determined as described in “Methods”. Substrate CDNB concentration was held at 1 mM and GSH concentration was held at 1.33 mM while naringenin concentration was changed from 5 µM to 150 µM to investigate the effect of naringenin on fish liver total GST activity.

Figure 3.37 exhibits the effect of varying concentrations of naringenin on fish liver total GST activity. It is shown that naringenin inhibited fish liver total GST activity in a dose dependent manner. IC50 value of naringenin was calculated as described in quercetin and found to be 116 µM.

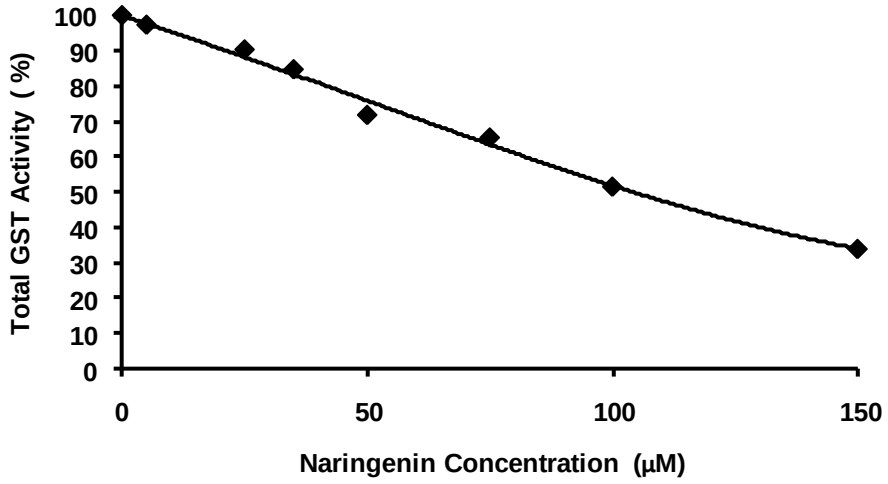



**Figure 3.37** Effect of naringenin on fish liver total GST activity. Inhibition experiments of GST activity to determine IC50 values were performed at a substrate CDNB concentration of 1 mM and GSH concentration of 1.33 mM. Naringenin inhibited fish liver total GST activity in a dose dependent manner with an IC50 value of 116 µM.

In order to determine the mechanism of inhibition of naringenin on fish liver total GST activity, naringenin concentration was held at three different concentrations (30 μM, 60 μM, 80 μM) in activity assay mixture while substrate CDNB concentration was varied (0.25 mM, 0.50 mM, 0.75 mM).

Figure 3.38 and Figure 3.39 show Michaelis-Menten and Lineweaver-Burk plots respectively in the presence and in the absence of different fixed concentrations of naringenin. Lineweaver-Burk revealed that naringenin inhibited fish liver total GST activity in a mixed type manner.

Figure 3.40 illustrates the Dixon plot 1/v versus Naringenin concentration in the presence of different fixed concentrations of substrate CDNB. From the intersecting point of three lines in the Dixon plot, the Ki value of naringenin was determined to be 128 μM. Similarly, Ki value was calculated as 135 μM from Lineweaver-Burk plot.

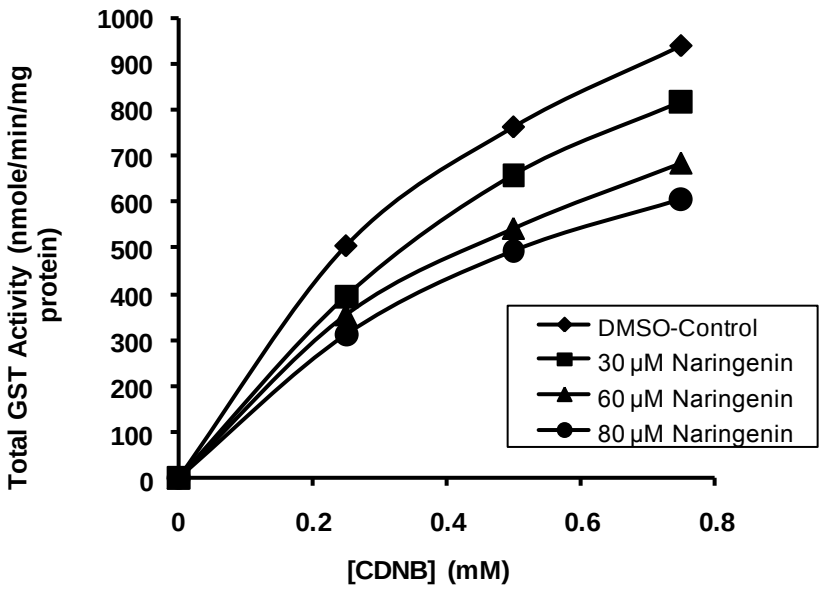



**Figure 3.38** Michaelis-Menten plot for fish liver total GST activity, v versus [CDNB] plot in the presence and in the absence of different fixed concentrations of naringenin.

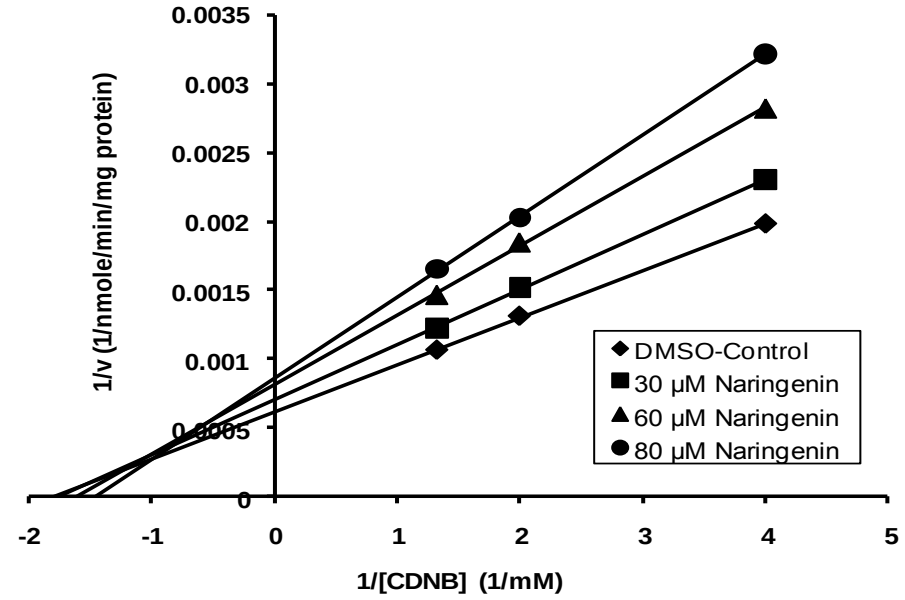



**Figure 3.39** Lineweaver-Burk plot for fish liver total GST activity, 1/v versus 1/[CDNB] plot in the presence of different fixed concentrations of naringenin.

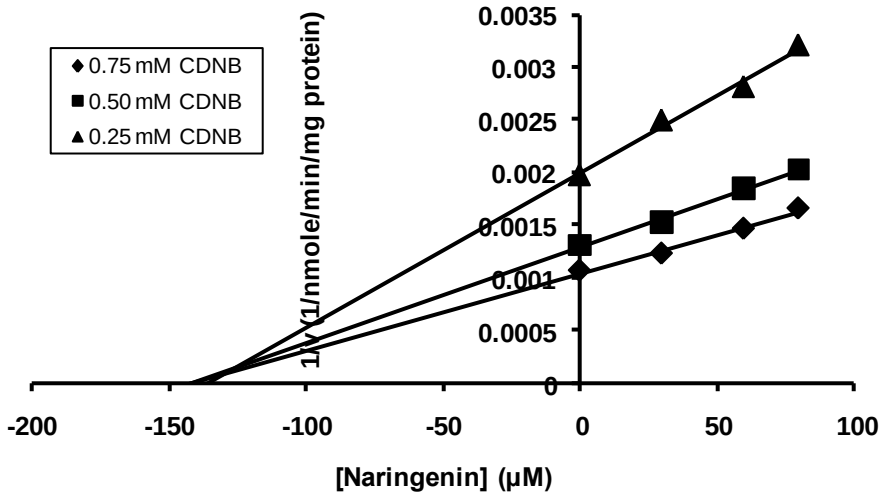



**Figure 3.40** Dixon plot for fish liver total GST activity, 1/v versus [Naringenin] in the presence of different fixed concentrations of substrate CDNB. Ki value was calculated to be 128 µM.

### 3.3.5 Effect of Hesperidin on Fish Liver Total GST Activity

Fish liver cytosolic total GST activity in the presence of different concentrations of hesperidin was determined as described in “Methods”. Substrate CDNB concentration was held at 1 mM and GSH concentration was held at 1.33 mM while hesperidin concentration was changed from 5 μM to 200 μM to investigate the effect of hesperidin on fish liver total GST activity.

As shown in Figure 3.41, hesperidin showed an inhibitory effect on fish liver total GST activity in a concentration dependent manner. IC50 value of hesperidin was calculated as described in quercetin and found to be 118 μM.

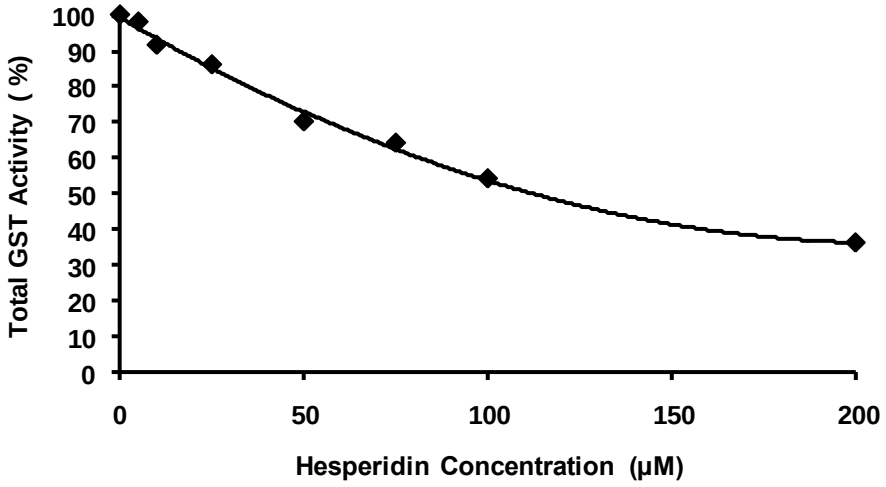



**Figure 3.41** Effect of hesperidin on fish liver total GST activity. Inhibition experiments of GST activity to determine IC50 values were performed at a substrate CDNB concentration of 1 mM and GSH concentration of 1.33 mM. Hesperidin inhibited fish liver total GST activity in a dose dependent manner with an IC50 value of 118 μM.

In the determination of inhibition mechanism of hersperidin on fish liver total GST activity, the concentration of hesperidin was held at three different concentrations (25 μM, 50 μM, 75 μM) in activity assay mixture, while the substrate CDNB concentration was varied (0.25 mM, 0.50 mM, 0.75 mM).

Figure 3.42 and 3.43 illustrate Michaelis-Menten and Lineweaver-Burk plots respectively in the presence and in the absence of different fixed concentrations of hesperidin. When 1/[v] (1/µmol/min/ml) was plotted against 1/[S] (1/[CDNB]) (Lineweaver–Burk plot) at three different substrate concentrations, the three lines crossed apparently at a point on the *Y*-axis. Lineweaver–Burk plot indicated that *V*max (maximum velocity) remained unchanged by the presence of different concentrations of hesperidin, while Michaelis constant (*K*m) increased with increasing hesperidin concentrations. As shown in Figure 3.44, 1/v was also plotted against inhibitor concentration (Dixon plot) . Both Lineweaver-Burk and Dixon plots suggest inhibition manner to be apparently competitive. From the intersecting point of the three lines in dixon plot, the *K*i value was determined to be 45 µM in Dixon plot. Similarly, Ki value was found as 38.6 µM from Lineweaver-Burk plot.

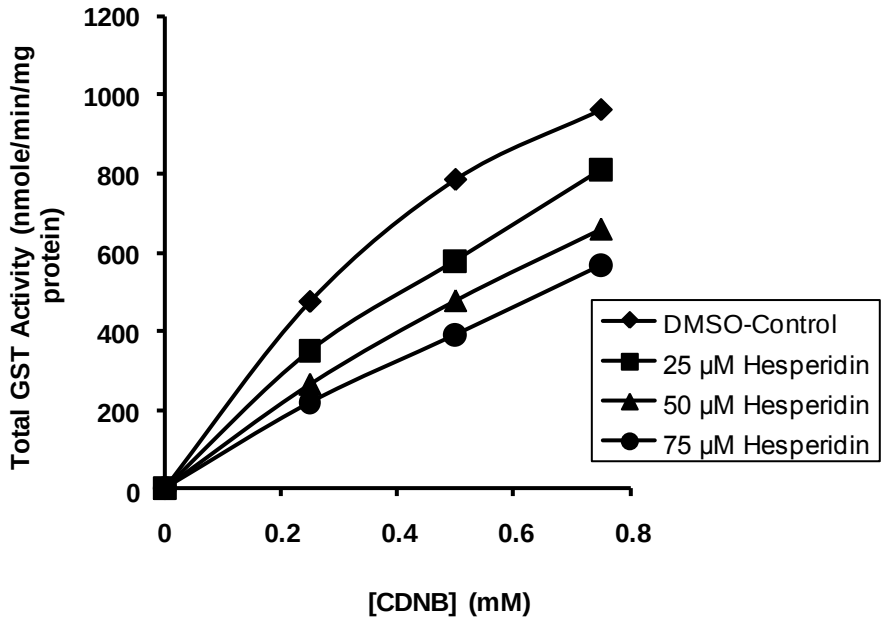



**Figure 3.42** Michaelis-Menten plot for fish liver total GST activity, v versus [CDNB] plot in the presence and in the absence of different fixed concentrations of hesperidin.

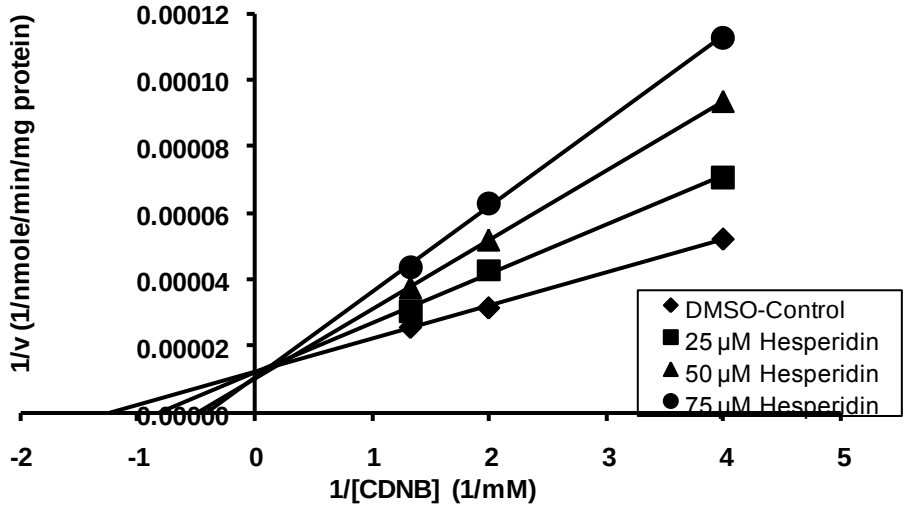



**Figure 3.43** Lineweaver-Burk plot for fish liver total GST activity, 1/v versus 1/[CDNB] plot in the presence of different fixed concentrations of hesperidin.

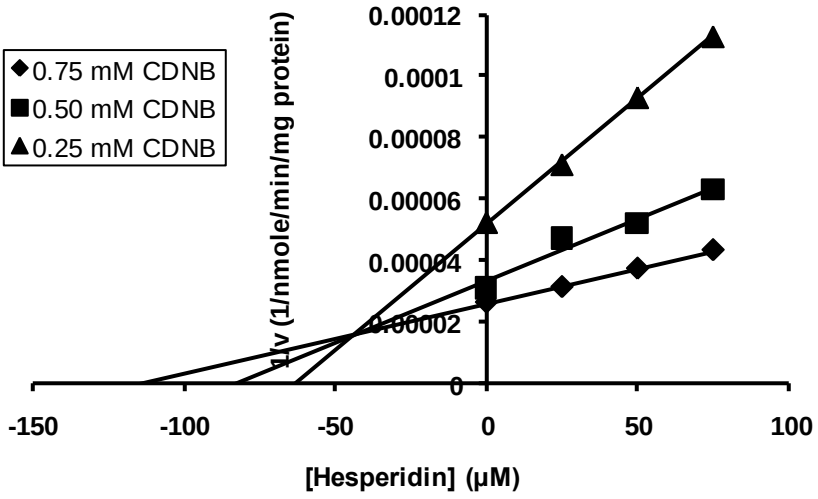



**Figure 3.44** Dixon plot for fish liver total GST activity, 1/v versus [Hesperidin] in the presence of different fixed concentrations of substrate CDNB. Ki value was calculated to be 45 µM.

Table 3.2 represents all the phenolic compounds/flavonoids studied and their inhibition types on total GST activity of fish liver.

**Table 3.2** The inhibition types of phenolic compounds/flavonoids on total GST activity of fish liver.

| Phenolic Compound/Flavonoid | Inhibition Type |
|---|---|
| Quercetin | Competitive |
| Resveratrol | Competitive |
| Rutin | Mixed-type |
| Naringenin | Mixed-type |
| Hesperidin | Competitive |

## 3.4 Determination of the Effect of Phenolic Compounds/Flavonoids on GST-Mu Activity

In this study, the effects of quercetin, resveratrol, rutin, naringenin and hesperidin on cytosolic GST-Mu activity of fish liver were investigated. GST-Mu activity was determined by using DCNB as a substrate. DCNB concentration was held at 1 mM and GSH concentration was held at 2 mM. Since GST-Mu activity of fish liver cytosols was very low, modulatory mechanism of phenolic compounds/flavonoids on enzyme activity could not be studied using different concentrations of substrate, DCNB.

### 3.4.1 Effect of Quercetin on Fish Liver GST-Mu Activity

Quercetin concentration was changed from 50 μM to 300 μM to investigate the effect of quercetin on fish liver GST activity.

Figure 3.45 represents the effect of increasing concentrations of quercetin on fish liver GST-Mu activity. It is shown that quercetin inhibited fish liver GST-Mu activity in a dose dependent manner.

IC50 value of quercetin was determined by plotting GST-Mu activity percent against logarithmic quercetin concentration as shown in Figure 3.46. From the equation of the plot, IC50 value of quercetin is calculated to be 113.5 µM.

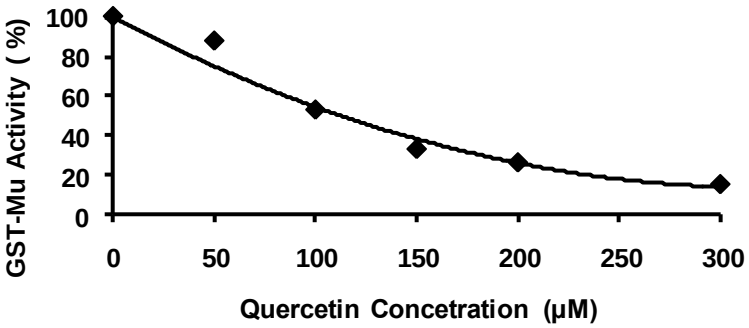



**Figure 3.45** Effect of quercetin on fish liver GST-Mu activity. Inhibition experiments of GST-Mu activity to determine IC50 values were performed at a substrate DCNB concentration of 1 mM and GSH concentration of 2 mM. Quercetin inhibited fish liver GST-Mu activity in a dose dependent manner with an IC50 value of 113.5 µM.

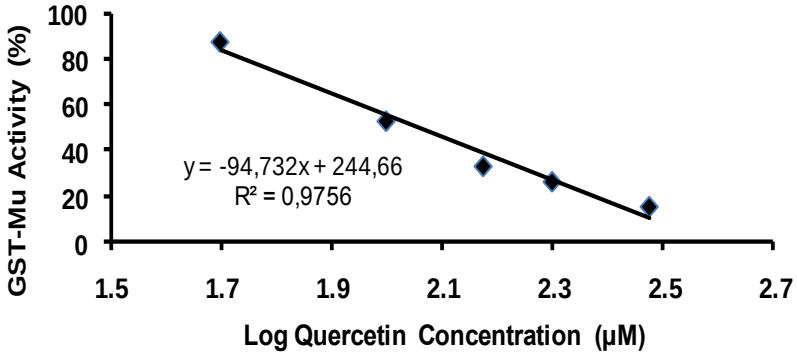



**Figure 3.46** Determination of IC50 value for quercetin.

### 3.4.2 Effect of Resveratrol on Fish Liver GST-Mu Activity

Resveratrol concentration was changed from 7 μM to 160 μM to investigate the effect of resveratrol on fish liver GST activity.

The effect of varying concentrations of resveratrol on fish liver GST-Mu activity is given in Figure 3.47. IC50 value of resveratrol was calculated as described in quercetin and it is shown that, resveratrol caused 50% inhibition at a concentration of 72.3 μM.

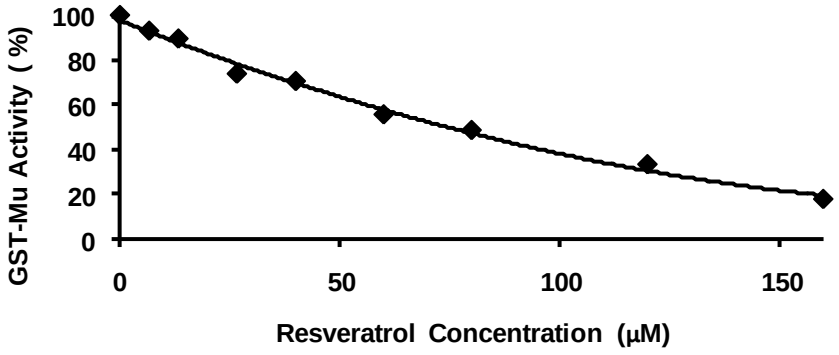



**Figure 3.47** Effect of resveratrol on fish liver GST-Mu activity. Inhibition experiments of GST-Mu activity to determine IC50 values were performed at a substrate DCNB concentration of 1 mM and GSH concentration of 2 mM. Resveratrol inhibited fish liver GST-Mu activity in a dose dependent manner with an IC50 value of 72.3 μM.

### 3.4.3 Effect of Rutin on Fish Liver GST-Mu Activity

Rutin concentration was changed from 30 μM to 120 μM to investigate the effect of rutin on fish liver GST activity.

Figure 3.48 shows the effect of rutin on fish liver GST-Mu activity. It is illustrated that rutin inhibited fish liver GST-Mu activity in a dose dependent manner. IC50 value of rutin was calculated as described in quercetin and found to be 66.5 μM.

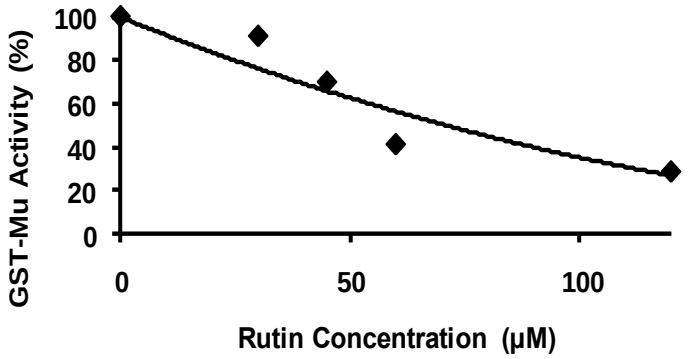



**Figure 3.48** Effect of rutin on fish liver GST-Mu activity. Inhibition experiments of GST-Mu activity to determine IC50 values were performed at a substrate DCNB concentration of 1 mM and GSH concentration of 2 mM. Rutin inhibited fish liver GST-Mu activity in a dose dependent manner with an IC50 value of 66.5 µM.

### 3.4.4 Effect of Naringenin on Fish Liver GST-Mu Activity

Naringenin concentration was changed from 30 µM to 240 µM to investigate the effect of naringenin on fish liver GST activity.

Figure 3.49 shows the effect of naringenin on fish liver GST-Mu activity. Naringenin inhibited fish liver GST-Mu activity in a concentration dependent manner. IC50 value of naringenin was calculated as described in quercetin and estimated to be 135.5 µM.

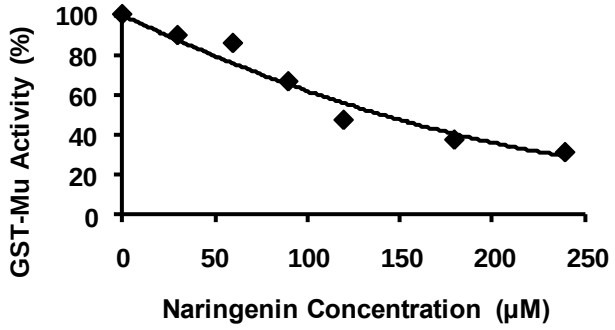



**Figure 3.49** Effect of naringenin on fish liver GST-Mu activity. Naringenin inhibited fish liver GST-Mu activity in a dose dependent manner with an IC50 value of 135.5 µM.

### 3.4.5 Effect of Hesperidin on Fish Liver GST-Mu Activity

Hesperidin concentration was changed from 100 µM to 400 µM to investigate the effect of hesperidin on fish liver GST activity.

Figure 3.50 represents the effect of hesperidin on fish liver GST-Mu activity. IC50 value of hesperidin was calculated as described in quercetin and found to be 196 µM. Less than 15% of GST-Mu activity remained when 400 µM of hesperidin was used.

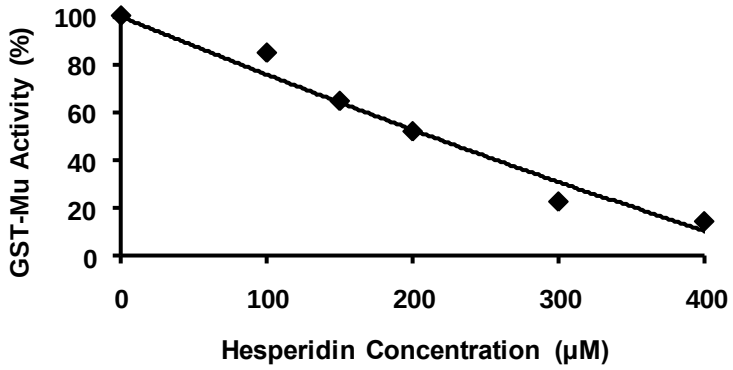



**Figure 3.50** Effect of hesperidin on fish liver GST-Mu activity. Inhibition experiments of GST-Mu activity to determine IC50 values were performed at a substrate DCNB concentration of 1 mM and GSH concentration of 2 mM. Hesperidin inhibited fish liver GST-Mu activity in a dose dependent manner with an IC50 value of 196 µM.

# CHAPTER 4

# DISCUSSION

Cytochrome P450s are a large family of heme-containing enzymes, catalyzing a variety of reactions including hydroxylations, epoxidations, N-dealkylations, O-dealkylations and S-oxidation. They play critical roles in the metabolism of exogenous substrates including drugs, dietary and environmentally derived toxicants and carcinogens and also in the metabolism of several endogenous compounds, such as fatty acids and steroids. Usually the outcome of P450 metabolism is inactivation of the substrate; paradoxically, however, in some cases the consequence of P450 metabolism is activation to a reactive intermediate which can lead to cancer. Because of the important role of the CYPs in the bioactivation and inactivation of both carcinogens and anticancer drugs, they play an important role in the etiology of cancer diseases (Oyama *et al.*, 2004; Rooseboom *et al.*, 2004). Targeting of these enzymes with natural or synthetic small molecules offers potential benefits in cancer prevention and therapy.

So far, the most successful example of targeting CYP enzymes is presumably the introduction of aromatase (CYP19) inhibitors for the treatment of hormone dependent breast cancer. It is well known that estrogen plays an important role in breast cancer development. Upon binding to estrogen, an estrogen receptor activates transcription of its target genes, which are responsible for cancer cell proliferation in estrogen-dependent breast tumors. The ovaries are the principal source of circulating estrogens in premenopausal women. However, the majority of patients with breast cancer are postmenopausal women and 75% of these patients have estrogen-dependent tumors defined by estrogen receptor positivity (McGuire, 1980). After menopause the ovaries stop to produce estrogens, but these steroids continue to be synthesized in nonovarian tissue (Grodin *et al*., 1973) including the breast tissue (O'Neill *et al*., 1988) through aromatization of circulating androgens (namely, androstenedione and testosterone) to estrogens (estrone and estradiol). This reaction is catalyzed by the enzyme aromatase, which mediates the rate limiting step in estrogen

biosynthesis. Peripherally synthesized estrogen acts locally in an intracrine or paracrine manner, without being released into the circulation. In such cases, the concentrations of estrogens in the breast are found to be four to six times higher than in the serum and equivalent to breast tissue levels in premenopausal women (van Landeghem *et al.,* 1985). Furthermore, two-thirds of breast carcinomas contain aromatase activity (Lipton *et al*., 1987) and estrogen levels in malignant tissue are found to be higher than those in normal breast tissue (Blankenstein *et al*., 1998). Therefore; the development of aromatase inhibitors represents a new strategy in the treatment of breast cancer. The first successful aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA, Formestane), was demonstrated by Harry and Angela Brodie and colleagues in 1977 and early pre-clinical studies with this compound showed that it inhibits estrogen synthesis in peripheral tissues (Brodie *et al*., 1977). Subsequently, clinical trials demonstrated significant responses to formestane in postmenopausal advanced breast cancer patients (Coombes *et al*., 1984). However, despite these encouraging results, further analysis revealed that formestane was not able to fully block aromatase activity (up to 90% inhibition at best (MacNeill *et al*., 1995)) thus leading to the development of more potent and selective inhibitors. Since then, several steroidal and non-steroidal selective inhibitors of aromatase have been developed. The current third generation aromatase inhibitors were developed in the early 1990s. A great deal of work is still being done in this field to optimize the efficacy of aromatase inhibitors both alone and in combination with other treatments. Despite the obstacles that lay ahead, it seems clear that aromatase inhibitors represent the first successful class of cancer therapeutics specifically designed to target a CYP enzyme.

As stated earlier, among the cytochrome P450s, CYP1 family are widely known with their role in metabolism and activation of aromatic hydrocarbon carcinogens and other toxic chemicals. The human CYP1 family contains three members, CYP1A1, CYP1A2, and CYP1B1. These enzymes have been studied extensively because of their roles in chemical carcinogenesis. These CYP enzymes catalyze the activation of procarcinogens. CYP1A1 is well known as an aryl hydrocarbon hydroxylase, and is capable of catalyzing a number of oxidations of polycyclic aromatic hydrocarbons. CYP1A1 is expressed, only in trace amounts, in the human liver, and mainly in extrahepatic tissues. Among the different reactions catalyzed by CYP1A1, hydroxylation at a vacant position of an aromatic ring is considered to be the hallmark for the initiation

of carcinogenesis, through the formation of highly reactive conversion products that can cause oncogenic mutations in experimental animals and humans (Wei *et al*., 1996; Buterin *et al*., 2000). The transcriptional activation of the CYP1A1 gene is mediated by the binding of environmental pollutants and inhalation chemicals, notably substrates of the CYP1A1 enzyme, to the cytosolic receptor AhR and is also mediated by its translocation to the nucleus and subsequent formation of a dimer, which interacts with the corresponding xenobiotic response elements to activate transcription (Hankinson, 1995). Human CYP1A2 is known to be capable of activating arylamines and heterocyclic amines, such as those found in broiled meats (Shimada and Fujii-Kuriyama, 2004; Tang M-shong *et al.*, 2000). Human CYP1A2 is constitutively expressed in human liver, but not in extrahepatic tissues. CYP1A2 gene expression is also regulated by the Ah receptor-mediated signal pathway. Human CYP1B1 is characterized by its ability to metabolically activate PAHs, such as benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (DMBA). CYP1B1 can also be induced by TCDD, similarly to the other members of CYP1 family (Shimada *et al*., 2001; 2002). CYP1A1 and 1A2 share 80% amino acid sequence homology and are 40% identical to the CYP1B1 found in humans (Shimada *et al*., 1996; Shimizu *et al*., 2000). Generally the substrate specificities of the CYP1 family members towards various pro-carcinogens and pro-mutagens are found to be very similar. Therefore; targeting CYP1 enzymes is of great importance for cancer prevention and therapy. Many types of compounds have been tested to find the isoform-specific inhibitors of the CYP1 subfamily. Flavonoids, a large class of phenolic compounds present in plants, were considered to be promising agents in this respect.

Phenolic compounds comprise one of the largest and most ubiquitous group of plant metabolites. They are formed to protect the plant from photosynthetic stress, reactive oxygen species, wounds, and herbivores. Phenolic compounds are an important part of the human diet. The most commonly occurring ones in foods are flavonoids. Flavonoids are the largest class of phenolic compounds; over 5000 compounds have been described. They are mainly classified into flavones, flavanols (catechins), isoflavones, flavonols, flavanones, and anthocyanins. All are structurally related to the parent compound, flavone (2-phenylbenzopyrone). Flavonoids have been described as health-promoting, disease-preventing dietary supplements, and have activity as cancer preventive agents. Additionally, they are extremely safe and

associated with low toxicity, making them excellent candidates for chemopreventive agents. The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including the modulation of enzymes (such as CYP1A1) which metabolize the procarcinogens or carcinogens to more carcinogenic compounds or induction of detoxification enzymes such as GSTs.

In this study we used leaping mullet caught from highly polluted sites of İzmir Bay, therefore express high levels of CYP1A and associated enzyme activities; in order to investigate the possible *in vitro* effects of phenolic compounds/flavonoids on CYP1A1 associated EROD activity of fish liver. In general, studies investigating the possible inhibitors of CYP1A1 involve the induction of cell line or animal with a carcinogenic compound at first. By this way, high CYP1A1 levels and elevated CYP1A associated EROD activities can be obtained to study the mode of inhibition. However in this study we used fish caught from highly polluted areas, therefore exposed to carcinogenic compounds naturally, thus; having high CYP1A1 levels. High CYP1A1 levels were also demonstrated by western blot analysis (Figure 3.1). Quercetin, rutin, naringenin, hesperidin and resveratrol, being the most common phenolic compounds/flavonoids in human diet, were selected to study this effect. We demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on CYP1A1 associated EROD activity of fish.

The affinities of all the flavonoids to CYP1A1 were not at the same degree. Of the flavonoids tested, quercetin exhibited much higher inhibitory effect at low concentrations; so it was evidently a more potent inhibitor than others. The potency of the phenolic compounds/flavonoids to inhibit CYP1A1 enzyme follow the sequence of quercetin > resveratrol > naringenin > hesperidin > rutin. Table 4.1 summarizes the inhibitory effect of phenolic compounds/flavonoids of CYP1A1 associated EROD activity and their IC50 values

**Table 4.1** Inhibitory effects of phenolic compounds/flavonoids on CYP1A1 associated EROD activity

| Phenolic compound/ Flavonoid | % inhibition at 50 µM flavonoid concentration | IC50 (µM) |
|---|---|---|
| Quercetin | 100 | 1.32 |
| Resveratrol | 97 | 3.59 |
| Naringenin | 72 | 9.78 |
| Hesperidin | 38 | 98.5 |
| Rutin | No inhibition | 640 |

Both Lineweaver–Burk plot and Dixon plots were used to determine the mode of inhibition exerted by the phenolic compounds/flavonoids. It was shown that; all phenolic compounds/flavonoids except for naringenin inhibited CYP1A1 associated EROD activity in a competitive manner. In competitive type of inhibition, the inhibitor and the substrate competes for the same site for binding to the enzyme. Thus, a competitive inhibitor acts only to increase the apparent Km for the substrate, it does not affect Vmax. Naringenin, on the other hand, showed a non-competitive type of inhibition. In non-competitive type of inhibition, substrate and inhibitor bind reversibly, randomly and independently at different sites. A non-competitive inhibitor lowers Vmax but does not affect Km. Ki values of quercetin, resveratrol, naringenin, hesperidin and rutin were calculated from Dixon plots as 0.12 µM, 0.67 µM, 2.63 µM, 18 µM and 0.1 mM, respectively. The calculated Ki values more clearly stated that the most effective inhibitor was quercetin. Table 4.2 represents the mechanism of inhibition of CYP1A1 associated EROD activity by phenolic compounds/flavonoids and their apparent Ki values.

**Table 4.2** Mechanism of inhibition of CYP1A1 associated EROD activity by phenolic compounds/ flavonoids and their apparent Ki values

| Phenolic compound/ Flavonoid | Ki (μM) | Type of inhibition |
|---|---|---|
| Quercetin | 0.12 | Competitive |
| Resveratrol | 0.67 | Competitive |
| Naringenin | 2.63 | Non-competitive |
| Hesperidin | 18 | Competitive |
| Rutin | 100 | Competitive |

Quercetin is one of the most abundant flavonoids in fruits, vegetables, tea and wine, with principal sources being onions, apples, tea, and therefore readily available in the daily diet. In a recent case–control study quercetin consumption has been associated with reduced lung cancer risk in Hawaii (Marchand *et al.*, 2000). In the present study, among the phenolic compounds/flavonoids tested, quercetin was found to be the most effective inhibitor of CYP1A1 associated EROD activity with an IC50 value of 1.32 μM (Figure 3.8). In the present study, Lineweaver Burk and Dixon plots demonstrated that quercetin showed a competitive type of inhibition. Ki value of quercetin was calculated as 0.10 μM from Lineweaver Burk plot (Figure 3.10) and 0.12 μM from Dixon plot (Figure 3.11). In previous studies, quercetin was shown to be an agonist of AhR (Ciolino *et al.*, 1999), thus; it may compete for the binding of AhR and reduce the expressions of CYP1A1. Quercetin appears to be a potent dietary phytochemical in the protection against PAH toxicity. Several studies showed that quercetin could be readily absorbed in human subjects and can reach micromolar concentrations in the plasma and urine (Hollman *et al.*, 1997; Conquer *et al.*, 1998; Meng *et al.*, 2004). Moreover, quercetin was shown to be eliminated slowly from the blood (elimination half-life of approximately 24 h), suggesting that repeated intake would lead to a build-up of quercetin to even higher levels (Hollman and Katan, 1998). Thus, these plasma concentrations are roughly in the range of the IC50 values of

quercetin for inhibition by CYP1A1 reported herein. Taken together, oral dietary quercetin can be absorbed and reach tissues and plasma where inhibiting concentrations could be reached; supporting our hypothesis that quercetin may be involved in the prevention cancer, by reducing the formation of carcinogens through inhibition of enzymes, such as CYP1A1. However, additional studies will be needed to determine whether quercetin and its metabolites can influence carcinogen-activating CYP1A1 activity in vivo.

Resveratrol is a phytoalexin compound found in juice and wine produced from dark-skinned grape cultivars and reported to have anti-inflammatory and anticarcinogenic activities. In various *in vitro* and *in vivo* models, resveratrol has proved to be capable of retarding or preventing various steps of carcinogenesis. In the present study, resveratrol was found to inhibit fish CYP1A1 associated EROD activity with an IC50 value of 3.59 μM (Figure 3.4) and the inhibition type was found to be competitive from Lineweaver Burk and Dixon plots. This suggests that resveratrol competes for substrate binding site with 7-Ethoxyresorufin. Ki value of resveratrol was found to be 0.87 μM from Lineweaver Burk plot (Figure 3.6) and 0.67 μM from Dixon plot (Figure 3.7). In a previous study, it has been showned that resveratrol exhibited potent inhibition of human P4501A1 in a dose-dependent manner with IC50 of 23 μM for EROD and IC50 of 11 μM for methoxyresorufin O-demethylation (MROD), and the inhibition manner is found to be mixed-type (competitive-noncompetitive) (Chun *et al.*, 1999). Based on these results Chun *et al.*, concluded that resveratrol is a selective human P4501A1 inhibitor, and may be considered for use as a strong cancer chemopreventive agent in humans. This conclusion also supports the data presented in here. The difference in IC50 values and the mode of inhibition may be due to species difference.

Naringenin and hesperidin were the citrus flavonoids that have been shown to exert inhibitory effect on CYP1A1 associated EROD activity of fish liver. As shown in Figure 3.16, naringenin was shown to inhibit CYP1A1 associated EROD activity with an IC50 value of 9.78 μM. The mode of inhibition of naringenin was found to be non-competitive type from Lineweaver Burk and Dixon plots. The noncompetitive mode of inhibition of CYP1A1 associated EROD activity suggests that the affinity of CYP1A1 to its substrate, 7-ethoxyresorufin, does not change in the presence of naringenin. Ki value of naringenin was calculated as 2.85 μM from Lineweaver Burk plot (Figure 3.18)

and 2.63 μM (Figure 3.19) from Dixon plot. In this study, it was demonstrated that hesperidin inhibited CYP1A1 associated EROD activity in a competitive manner with an IC50 value of 98.5 μM (Figure 3.20). Ki value for hesperidin was found to be 14.4 μM from Lineweaver Burk plot (Figure 3.22) and 18 μM from Dixon plot (Figure 3.23).

The flavonoid rutin (quercetin-3-O-rutinoside) is a flavonol glycoside comprised of the flavonol quercetin and the disaccharide rutinose Compared to the other flavonoids tested, rutin was the least effective inhibitor of CYP1A1 activity with an IC50 value of 0.64 mM (Figure 3.12). Rutin was shown to inhibit CYP1A1 associated EROD activity in a competitive manner. Ki value of rutin was found to be 120 μM from Lineweaver Burk plot (Figure 3.14) and 100 μM from Dixon plot (Figure 3.15). Similar to our work, in a recent study, Chaudhary *et al.* investigated some flavonoids for their inhibition of recombinant human CYP1A1 and showed that compared to other flavonoids tested (myricetin, apigenin, kaempferol, quercetin, amentoflavone, and quercitrin) rutin was the least effective inhibitor of CYP1A1 activity (Chaudhary and Willett, 2006). Similarly, it did not inhibit CYP1-mediated 7,8-diol-BaP epoxidation (Schwarz and Roots, 2003) or protect from the mutagenicity of aromatic amines (Lee *et al.*, 1994). Rutin has a disaccharide substituent and the bulk or polarity of this moiety may prohibit interaction with P450s.

In this study, *in vitro* effects of phenolic compounds/flavonoids were also studied on GSTs. As stated earlier GSTs are a group of phase II enzymes which have a crucial role in the cellular detoxification and excretion of many physiological and xenobiotic substances. Glutathione transferases are of interest to pharmacologists and toxicologists because they provide targets for antiasthmatic and antitumor drug therapies (Evans *et al.*, 1991; Matsushita *et al.*, 1998; Jakobsson *et al.*, 1999; Ruscoe *et al.*, 2001), and they metabolize cancer chemotherapeutic agents, insecticides, herbicides, carcinogens, and by-products of oxidative stress. Overexpression of GST in mammalian tumor cells has been implicated with resistance to various anticancer agents and chemical carcinogens (Hayes and Pulford, 1995).

In this study, the effects of phenolic compounds/flavonoids were investigated also on the GST activities of fish liver. We demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on GST activities of fish although the degree of inhibition varied with the flavonoid used.

In the case of GST activities, the most potent inhibitor is found to be resveratrol with an IC50 value of 7.1 μM (Figure 3.29). The potency of the phenolic compounds/flavonoids to inhibit GST enzyme follow the sequence of resveratrol > quercetin > rutin > naringenin > hesperidin. Table 4.3 summarizes the inhibitory effect of phenolic compounds/flavonoids of GST activity and their IC50 values.

**Table 4.3** Inhibitory effects of phenolic compounds/ flavonoids on total GST activity

| **Phenolic compound/ Flavonoid/** | **% inhibition at 50 μM flavonoid concentration** | **IC50 (μM)** |
|---|---|---|
| Resveratrol | 100 | 7.1 |
| Quercetin | 67 | 24.5 |
| Rutin | 16.55 | 89 |
| Naringenin | 27 | 116 |
| Hesperidin | 29 | 118 |

The inhibition manners of the phenolic compounds/flavonoids were decided by plotting Lineweaver-Burk and Dixon plots. Resveratrol, quercetin and hesperidin were found to inhibit GST activity in a competitive manner with Ki values of 3.2 μM, 12.5 μM and 45 μM respectively (Figure 3.32, Figure 3.28 and Figure 3.44 respectively). On the other hand, rutin and naringenin were found to inhibit GST activity in a mixed type manner with Ki values of 150 μM and 128 μM respectively (Figure 3.36 and Figure 3.39 respectively). The calculated Ki values more clearly stated that the most effective inhibitor was resveratrol. Table 4.4 represents the mechanism of inhibition of GST activity by phenolic compounds/flavonoids and their apparent Ki values.

**Table 4.4** Mechanism of inhibition of total GST activity by phenolic compounds/ flavonoids and their apparent Ki values

| Phenolic compound/ Flavonoid | Ki (μM) | Type of inhibition |
|---|---|---|
| Resveratrol | 3.2 | Competitive |
| Quercetin | 12.5 | Competitive |
| Hesperidin | 45 | Competitive |
| Naringenin | 128 | Mixed-type |
| Rutin | 150 | Mixed type |

Unlike the consistency of literature data on the effects of phenolic compounds/flavonoids on CYP1A1 associated EROD activity, the data regarding the effects of phenolic compounds/flavonoids on GST activity is limited and controversial in literature.

Similar to our work, in a previous study Zhang and Das examined the effects of plant polyphenols on rat liver glutathione S-transferases and found that quercetin inhibited GST with an IC50 of 18.732 μM (Zhang and Das, 1993). However in another study, it has also been reported that flavanones and flavones increase the activity of GSTs (Canivenc-Lavier *et al*., 1996). In a recent study, Apati *et al*., showed that the glycoside rutin gave dose-dependent increase in GST activity of HepG2 cells, with a 50% increase at 250 mM, while the aglycone quercetin inhibited the enzyme by 30% at 250 mM (Apati *et al.*, 2000). In the case of resveratrol, an increase in GST activity was observed in mammalian intestinal cells when stimulated with carcinogenic agents (Sengottuvelan *et al*., 2006). The increased GST activity observed in ciliates incubated with resveratrol is also consistent with the observed increase in activity of this enzyme in human lymphocytes and cultured aortic smooth muscle cells incubated with resveratrol (Yen et al., 2003; Li et al., 2006). However in this study, resveratrol, like all of the flavonoids used, was shown to inhibit GST activity of fish liver. The inconsistency

of data in literature on the effects of flavonoids on GST activities may be due to several reasons. It is because the effect of flavonoids as a modulator of carcinogen-metabolizing enzymes varies, depending on several factors: 1) the model used (*in vitro* vs. *in vivo* studies), 2) the tissue involved (such as lung, liver, kidney, colon, or breast), 3) the doses of flavonoid used for treatment, 4) the form of flavonoid given in vivo (glycone or aglycone), and 5) the time period in which the flavonoid was administered (before, after, or in the absence of a carcinogen). In order to elucidate the mechanisms of action by which flavonoids may decrease the risk for specific cancers, many studies using various models and tissues, several doses and forms of flavonoids, at different time points must be conducted and the results analyzed, in order to form a strong conclusion.

As stated earlier, cytosolic GSTs of mammals were originally classified into seven classes based on their amino acid sequence similarities. These classes were called Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta. Proteins related to mammalian mu, alpha, pi, and theta GSTs have also been described in various fish species including *Liza saliens* (leaping mullet) (George, 1994). GST isoenzymes appear to be expressed to varying extent in tissues from different species and even in tissues within the same species. Although these enzymes have been mainly found in liver, GST activities have been demonstrated in gills, kidney, and intestinal ceca of several fish species. The differential expression of GST isoenzymes in different organs is of particular interest as it might be a factor in the differential susceptibility of tissues to the toxic effects of xenobiotics. The GST expression levels in many species can be significantly increased by exposure to foreign compounds, suggesting that they form part of an adaptive response to chemical stress, which is used as effective biomarkers of aquatic contamination (Hayes and Pulford, 1995). In this study, we also aimed to investigate the effects of phenolic compounds/flavonoids on GST mu, alpha and pi subclasses. However, only GST-Mu activities could be measured. Even though the effect of phenolic compounds/flavonoids on GST-Mu activity could be studied and IC50 values could be calculated, GST-Mu activity of fish liver was very low in order to study the modulatory mechanism of flavonoids on enzyme activity. It was demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on GST-Mu activities of fish. The most potent inhibitor of GST-Mu was found to be rutin. Table 4.5

summarizes the IC50 values of phenolic compounds/flavonoids on GST-Mu activity of fish liver.

**Table 4.5** Inhibitory effects of phenolic compounds/flavonoids on GST-Mu activity

| Phenolic compound/ Flavonoid | % inhibition at 50 µM flavonoid concentration | IC50 (µM) |
|---|---|---|
| Rutin | 37 | 66.5 |
| Resveratrol | 37 | 72.3 |
| Quercetin | 16 | 113.5 |
| Naringenin | 10.5 | 135.5 |
| Hesperidin | No inhibition | 196 |

The results of the present study demonstrate that phenolic compounds/flavonoids can modulate carcinogen activating and detoxifying pathways particularly those in which CYP1A1 and GST are involved. The results of the studies in literature together with ours indicate that phenolic compounds/flavonoids inhibit CYP1A1 associated EROD activity and GST activities in different species, therefore may be considered as potential chemopreventive agents. So far, inhibitory effects of quercetin, rutin, naringenin, hesperidin and resveratrol on CYP1A associated enzyme activity (EROD) and phase II enzyme glutathione S-transferase (GST) have been reported in different studies (Doostdar *et al.,* 2000; Birt et al., 2001; Kuo *et al.,* 2004; Moon *et al.,* 2006). However, there is no reported study about the effects of these five flavonoids in the same species and in any fish species. Recently, fish has been used as model organism in many different toxicology and pharmacology studies. As in the case of small laboratory animals, fish do not expose to flavonoids. However, information gained from fish studies would be useful for comparative xenobiotic metabolism. The results of this study indicated that quercetin, rutin, naringenin, hesperidin and

resveratrol inhibit the naturally induced EROD and GST activities of fish. In addition, the effects of five flavonoids were compared in the same species in this study. We gained information about the potencies of these flavonoids. The results of this study supported also chemopreventive role of these flavonoids. In this study, the mechanism of inhibition was also determined for the first time in fish.

# CHAPTER 5

# CONCLUSION

It is well known that approximately 70% of human cancers are caused by chemicals that in daily life people are continuously exposed to. These chemicals are metabolized by xenobiotic metabolizing enzymes called Phase I and Phase II enzymes. Sometimes as a result of metabolic bioactivation, these chemicals may turn into even more toxic substances. CYP1 family, especially CYP1A1, is known to play critical roles in the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons (PAHs) and other pro-carcinogens to electrophilic reactive intermediates, leading to toxicity and cancer. Therefore; targeting CYP1 enzymes is of great importance for cancer prevention and therapy. Flavonoids are especially promising candidates for cancer prevention. They have shown many biological properties that may account for cancer chemoprevention.

In this study we used leaping mullet caught from highly polluted sites of İzmir Bay, therefore express high levels of CYP1A and associated enzyme activities; in order to investigate the possible *in vitro* effects of phenolic compounds/flavonoids on CYP1A1 associated EROD activity of fish liver. It was demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on CYP1A1 associated EROD activity of fish. Of the flavonoids tested, the most potent inhibitor of CYP1A1 associated EROD activity was found to be quercetin with an IC50 value of 1.32 μM. Ki value of quercetin is calculated from Dixon plot as 0.12 μM. Quercetin was shown to inhibit EROD activity in a competitive manner. The least effective inhibitor of EROD activity was found to be rutin with an IC50 value of 0.64 mM and a Ki value of 0.1 mM. Like quercetin, rutin was also shown to inhibit EROD activity in a competitive manner. Of the flavonoids tested, only naringenin was shown to inhibit EROD activity in a non-competitive manner. The presence of naringenin causes reduction in the maximum activity of CYP1A1, which indicates an alteration in the structure of enzyme.

In this study, *in vitro* effects of phenolic compounds/flavonoids on total GST and GST-Mu activities of fish liver were also investigated. It was demonstrated that all of the phenolic compounds/flavonoids used, exert an inhibitory effect on both total GST and GST-Mu activities of fish. Of the flavonoids tested, the most effective inhibitor of total GST activity was found to be resveratrol with an IC50 value of 7.1 μM and a Ki value of 3.2 μM. Resveratrol was found to inhibit total GST activity in a competitive manner. Of the flavonoids tested, the most effective inhibitor of GST-Mu activity was found to be rutin with an IC50 value of 66.5 μM. The mode of inhibition of flavonoids on GST-Mu activity could not be studied, since GST-Mu activities were very low.

In conclusion, this study indicated that flavonoids were the strong inhibitors of CYP1A1 associated EROD activity and GST activities of mullet liver. The modulation of drug-metabolizing enzymes by flavonoids is important in terms of human health, since these enzymes can activate or inactivate carcinogens. The potential role of xenobiotic metabolizers CYP1 family in the activation of carcinogens and inactivation of chemotherapeutics suggests a potential therapeutic benefits in inhibiting these enzymes. Additionally, flavonoids may also interact with chemotherapeutic drugs used in cancer treatment through the induction or inhibition of their metabolism. An extensive study on structure–function relationships of flavonoid activities is required in terms of providing a valuable inspiration for a rational drug and/or chemopreventive agent design of future pharmaceuticals in the prevention and/or treatment of cancer. It is evident that further research regarding whether this *in vitro* study can be extrapolated to human situations because of the dose and bioavailability issue is required. For more precise information on the role of dietary polyphenols in cancer prevention in humans, reliable biomarkers for the consumption of specific polyphenols are needed, in addition to the use of dietary questionnaires. The association between the consumption of a specific type of polyphenol (or food item) and lowered cancer risk needs to be observed consistently in different studies, if these plant derived products are to be used as therapy. Nonetheless, our results support the hypothesis that flavonoids may be involved in the prevention of malignant transformation, by reducing the formation of carcinogens through inhibition of enzymes such as CYP1A1 which is known to be involved in carcinogen activation.

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