INVESTIGATION OF THE EFFECTS OF FOLK MEDICINAL PLANT

_EPILOBIUM HIRSUTUM_ L. ON CYTOCHROME P450 DEPENDENT AND
ANTIOXIDANT ENZYMES: A MOLECULAR APPROACH

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF FOLK MEDICINAL PLANT EPILOBIUM HIRSUTUM L. ON CYTOCHROME P450 DEPENDENT AND ANTIOXIDANT ENZYMES: A MOLECULAR APPROACH

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The genus *Epilobium* sp. (Onagraceae) is widely distributed all over the world and consists of nearly 200 species. *Epilobium hirsutum* L. (*EH*) one of the members of this genus is known as its analgesic, anti-microbial and anti-proliferative activity and it is used in our country as an alternative medicine. The pharmacological effect of *EH* could be explained by the presence of polyphenolics including steroids, tannins and flavonoids in the aerial parts. Cytochrome P450s (CYPs) known as phase I enzymes have important function on the metabolism of xenobiotics including drugs and other chemicals. Several procarcinogens require metabolic activation by CYP enzymes in order to show their toxic and carcinogenic effects. This toxic effect can be eliminated by phase II enzymes known as conjugation or antioxidant enzymes. In the first part of the thesis study, *EH* was collected, by using aerial parts of the plant extract was prepared and lyophilised then active substances were identified and quantitated by LC-MS MS. In the second part of the study, the water extract of *EH* was dissolved in ddH₂O and 37 mg extract/kg body weight/day was intraperitoneally (i.p.) injected to 4 months old male Wistar Albino rats for a period of nine days to investigate in vivo therapeutic/toxic effects of *EH* on rat liver enzymes. To our knowledge, this is the first study which investigates the effects of *EH* on liver phase I, phase II and antioxidant enzymes activities, as well as mRNA and protein levels of those in rats. Spectrophotometric analysis of lactate dehydrogenase activity was shown that *EH* does not cause any cytotoxicity on rat liver (1.01-fold). *EH* has an inhibitory effect on rat liver cytochrome P450 dependent enzyme activities; CYP1A1 catalyzed ethoxyresorufin-o-demethylase (1.46-fold) p<0.0003, CYP2B1 dependent benzphetamine N-demethylase (2.12-fold) p<0.0001 and ethylmorphine N-demethylase (8.68-fold) p<0.0001 and CYP2E1 dependent aniline 4-hydroxylase (4.93-fold) p<0.0001 and NDMA N-demethylase (1.2-fold) p<0.05. Similar to cytochrome P450s, total GST and its isoforms GST mu and GST theta were also inhibited significantly (p<0.0001)
when rats were injected with *EH*. On the other hand, phase II enzyme NQO1 and antioxidant enzymes SOD and GPx activities significantly increased (p<0.0001) upon treatment of animals with *EH*. In order to investigate the causes of the alteration of enzyme activities, protein and mRNA expressions of those enzymes were determined by western blot and qRT-PCR, respectively. Western blotting analysis have shown that CYP1A1, CYP2B1, CYP2E1 and CYP3A1 and GST-mu protein expressions significantly decreased (p<0.0001) in *EH* treated rats as compared to control animals. In contrast to these findings, treatment of rats with *EH* caused induction of the protein expressions of NQO1 and GPx as 2.97-fold and 1.93-fold, respectively. Analysis of mRNA expressions by qRT-PCR demonstrated that mRNA expressions of CYP1A1, CYP2B1, CYP2E1, CYP3A1 GST-mu and GSTT1 were significantly decreased (p<0.0001) In contrast to these, *EH* treatment increased the GPx and NQO1 mRNA expressions as 4.97-fold and 3.5-fold (p<0.0001) compared to control, respectively. Cross analyses of enzyme activity versus protein and mRNA expressions showed that there is a correlation between mRNA and protein expression levels and corresponding enzyme activities in *EH* treated samples. Inhibition of CYP enzymes may affect the metabolism of many chemicals and drug molecules. Since procarcinogens require metabolic activation by CYP enzymes in order to show their toxic and carcinogenic effects, inhibition of the enzyme system prevents the formation of hazardous metabolites. Prodrugs or active drugs’ metabolism may be modulated by *EH* which resulted in toxicity or enhanced response. The presented evidences point out that EH alters the activity and expression of enzymes involved in xenobiotics activation/detoxification pathways. Therefore, modulatory effect of *EH* on these enzymes suggests an inherent chemopreventive action against a group of chemicals including carcinogens and drugs. However, necessary precautions such as medical advice should be taken regarding the usage of this plant in replacement treatments since it reveals possible interactions with drugs and dietary foods.

**Keywords:** *Epilobium hirsutum* L., Medicinal Plant, Cytochrome P450s (CYPs), NQO1, GST, Antioxidant enzymes, Xenobiotic Metabolism, Western Blot, qRT-PCR, rat liver
ÖZ

**TIBBİ BİTKİ **EPILOBİUM HIRSUTUM’UN SİTOKROM P450 BAĞIMLI VE ANTIOKSIDANT ENZİMLER ÜZERİNE ETKİLERİİNİN ARAŞTIRILMASI: MOLEYÜLER YAKLAŞIM

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*Epilobium* sp. cinsi üyeleri dünya üzerinde geniş bir şekilde yapılmış olup 200 kadar tür sahiptir. Bu cinsin bir üyesi olan *Epilobium hirsutum* L. analjezik, antimikrobiyal ve tümör hücrelerinin büyümesini önleyici özelliği ile tanınmaktadır ve ülkenizde alternatif ilaç olarak kullanılmaktadır. *Epilobium hirsutum* L.’ nin farmakolojik etkisi onun sahip olduğu steroidler, tanenler ve flavonoidlere dayanmaktadır. Sitokrom P450 enzimleri birçok ilaç ve kimyasalın metabolizmasından sorumludurlar. Birçok önçül karsinojen madde toksik ya da karsinojenik etki gösterebilirmeleri için sitokrom P450 enzimleri tarafından metabolize edilmeleri gerekmektedir. Bu toksik etki faz II enzimleri tarafından katalizlenen konjugasyon reaksiyonları ya da antioksidan enzimler tarafından önlenebilir. Tez çalışmanın ilk kısmında *EH* örnekleri toplandı bitkinin taop rak üstü kısımları ile hazırlanan sulu ekstre liyofilize edildi ve aktif bileşikleri ve bunların miktari LC-MS/MS tekniği yardımıyla saptandı. İkinci kısımda ise *EH*’un suyu ektresi suda çözüldük 37,5 mg ekstre/kg vücut ağırlığı/gün olarak şekilde intraperitoneal yolla 3 aylık erkek Wistar Albino sıçanlara 9 gün boyunca enjekte edilerek *EH* su sıkan karaciğer faz I, faz II ve antioksidan enzimler üzerinde teröpatik ve toksik etkileri araştırıldı. Bu çalışma *EH* su sıkan karaciğer faz I, faz II ve antioksidan enzim aktiviteleri, mRNA ve protein ekspresyonlarının incelendiği ilk çalışma olmaktadır. Spektrofotometrik analizler *EH* su sıkan karaciğer dokuları üzerine her hangi bir sitotoksik etkisi olmadığını göstermiştir (1.01-kat). *EH* enjeksiyonu sonucunda sıkan karaciğer faz I, faz II ve antioksidan enzim aktivitelerinde meydana gelen değişimler incelenmiş ve *EH* su sitokrom P450 enzim aktiviteleri üzerinde önemli ölçüde inhibe edici etkisi olduğu bulunmuştur; CYP1A1 bağımlı EROD (1.46-kat) p<0.0003, CYP2B1 bağımlı benzefatin N-demetilaz (2.12-kat) p<0.0001, CYP2E1 bağımlı anilin 4-hidroksilaz (4.93-kat) p<0.0001 ve NDMA N-demetilaz (1.2-kat) p<0.05
ve CYP3A4 bağımlı etilmorfın N-demetilaz (8.68-kat) p<0.0001. Sitokrom P450 enzim aktivitesine benzer şekilde, toplam GST ve onun izoformları olan GST-mu ve GST-theta enzim aktivitelerinde de önemli ölçüde bir inhibisyon gözlemmiştir (p<0.0001). Diğer bir yandan faz II enzimlerinden NQO1 ve antioksidan enzimlerden SOD ve GPx enzim aktivitelerinde kontrol gruplarına göre kıyaslandığında önemli bir artış saptanmıştır (p<0.0001). Bu değişimlerin kaynağıneh bulmak için protein ve mRNA ekspresyonları.

Western blot tekniği ve qRT-PCR teknikleri yardımıyla incelenmiştir. Western blot çalışmaları sonucunda CYP1A1, CYP2B1, CYP2E1, CYP3A1 ve GST-mu protein ekspresyonlarında ciddi bir azalma (p<0.0001) gözlemlenirken NQO1 ve GPx protein ekspresyonlarında sırasıyla 2.97-kat ve 1.93-kat artma saptanmıştır. qRT-PCR çalışmaları sonucunda EH'un karsinojen maddelerin aktif hale gelmeleri için sitokrom P450 enzimleri tarafından metabolize edilmeleri gerektiginden, bu enzim sisteminde meydana gelen inhibisyon zararlı metabolitlerin oluşumunu da engellemektedir. Ayrıca sitokrom P450 enzimleri tarafından metabolize edilen öncül veya aktif toksisite ya da arttırmış etkinlikleri de bu enzim sisteminde meydana gelen inhibisyon sonucunda değişime uğramaktadır. Dolaysıyla özellikle CYP2B6 ve CYP3A4 enzimleri tarafından metabolize edilen ilaçların kullanımında EH alınımına dikkat etmek gereklidir. Ayrıca sitokrom P450 enzim sistemleri tarafından katalizlenen reaksiyonlar sonucunda ROS bu sistemdeki inhibisyon dolaylı yolla da olsa ROS miktarında azalmaya neden olacaktır. Ayrıca oluşan ROS'ların tüketime sağlanan NQO1, SOD ve GPx enzim aktivitelerindeki artış da ROS'ların insan vücuduna vereceği zararı azaltmaktadır. Aynı zamanda kanser tedavisi sırasında kullanılan bazı kemoteröpatik ilaçlar GST enzimi tarafından metabolize edilerek hücreden dışarı atılmakta ve etkinliklerini gösterememektedirler. EH'un GST enzim aktivitesi üzerindeki inhibe edici özelliği kemoteröpatik ilaçların etkinliklerini arttırabileceği ve kanser tedavisiinde destek madde olarak kullanılabilir. Bu çalışma EH'ın ksenobiyoitik maddelerin aktivasyonu ve detoksifikasyonu yolunda rol alan enzimlerin ekspresyonlarına ve aktivitelerine değişirdirdiğini göstermektedir. Bundan dolayı EH'un düzenleyici bu etkisi onu birçok karsinojen kimyasal maddelerle karşı ya da kullanılan ilaçların metabolizmasını etkilediklerinden koruyucu özellikleri olduğuunu göstermektedir. Ancak bu bitki tarafından ekspresyonları değişirilen enzimler tarafından metabolize edilen ilaçları kullanı plaklama ve ilaç-gida etkileşimlerinden dolayı kullanılmalıdır ve dikkat edilmesi gerekmektedir.

**Anahtar kelimeler:** *Epilobium hirsutum L.*, Tibbi Bitki, Sitokrom P450 (CYPs), NQO1, GST, Antioksidan Enzimler, Ksenobiyoitik metabolizmasi, Western Blot, RT-PCR.

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To my beloved wife and sons
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LIST OF SYMBOLS AND ABBREVIATIONS

B[a]P Benzo[a]pyren
BCA Bicinchoninic Acid
BCIP 5-Bromo-4-chloro-3-indolyl phosphate
BSA Bovine serum albumin
CDNB 1-chloro-2,4-dinitrobenzene
CYP Cytochrome P450
Da Dalton
DCPIP 2,6 dichlorophenol-indophenol
DEA Diethanolamine
DEPC diethylpyrocarbonate
ε-ACA ε-Amino caproic acid
EDTA Ethylenediamine tetra acetic acid
EHT Epilobium hirsutum Treated
GSH Glutathione reduced form
HEPES N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid
MFO Mixed function oxidases
Mw Molecular weight
NADH Nicotinamide adenine dinucleotide, reduced form
NADP+ Nicotinamide adenine dinucleotide phosphate
NADPH Nicotinamide adenine dinucleotide phosphate, reduced form
NBT nitro blue tetrazolium chloride
NDMA N-nitrosodimethylamine
NQO1 NAD(P)H:Quinone Oxidoreductase 1
OD Optical Density
PAH Poly aromatic hydrocarbons
pAP p-aminophenol
PCR Polymerase Chain Reaction
PMSF Phenylmethylsulfonyl fluoride
ROS Reactive oxygen Species
SDS sodium dodecyl sulfate
SEM Standard Error of Means
TBE Tris borate EDTA
TBS Tris-buffered saline
TBST Tris-buffered saline Tween 20
TCA Trichloro acetic acid
TE Tris EDTA
TRIS Tris (hydroxymethyl) aminomethane
ZTB Zero time blank
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CHAPTER 1

INTRODUCTION

1.1. Taxonomy of *Epilobium hirsutum* L.

Division: Magnoliophyta Cronquist,
Class: Magnoliopsida Brongn.
Subclass: Rosidae Takht.
Order: Myrtales Lindl.
Family: Onagraceae Juss.
Genus: *Epilobium* L.
Species: *Epilobium hirsutum* L.

**Common Name(s):** Hairy willow-herb, hairy willow-weed, willow herb (English, United States), tüylü yakı otu (Turkey)

*Epilobium hirsutum* L. (Figure 1.1) is a member of Division Magnoliophyta. It is found in moist waste ground of the Mediterranean region, Europe, Asia, and Africa. It has a wide distribution in Turkey. *Epilobium hirsutum (EH)* is a perennial, and it spreads by seeds and by rhizomes. Flower buds develop after 10 to 12 weeks of growth. Side shoots also produce flowering stems, and the whole plant is flowering by mid-summer (July–August) (Stuckey 1970). Self-pollination is possible, but seed production is reduced by self-pollination. Seeds are ripe and begin to disperse 4 to 6 weeks after flowering. Each seed is oblong and flattened, with a tuft of long white hairs. The plant has a thick rhizome and intensive hairy lanceolate leaf. EH has pink colored flowers and 4-10 cm length capsules in the flowers. Length of stem can vary between 30 and 210 cm. EH spreads mainly waterfront. In Turkey, the genus *Epilobium* is called →yakı otu” and the species *Epilobium hirsutum* L. is called →tıylü yakı otu” (Baytop 1994; Baytop 1999).
1.2. Biochemical and Medical Studies of *Epilobium hirsutum* L.

Naturally produced plant origin chemicals have gained crucial importance over the last decades because of their multiple biological effects in cancer, aging, cardiovascular diseases and their antioxidant activity. Herbs can be used in many domains, including flavoring, nutrition, beverages, dyeing, cosmetics and medicine. It is an indisputable fact that plants have been used as medicine for human health from the time immemorial and also today they are used as alternative medicine commonly. *Epilobium*, the largest genus of Onagraceae family, is widely distributed all over the world and consists of approximately 200 species. The species, *Epilobium hirsutum* L. (*EH*) possess polyphenolics including steroids, tannins and flavonoids (Barakat *et al.* 1997; Ivancheva *et al.* 1992). Total phenolic content of the *EH* was found as 4.03 ± 0.12 mg of GAE/100 g of dry weight (Wojdylo *et al.* 2007). *EH* extract was exhibited to have highest antioxidant activity (69.6 μM trolox/100 g dw) and highest antioxidant concentration (2020.51 μM TEAC/100 g dw) in 32 plant extracts (Wojdylo *et al.* 2007). The body of the plant has been reported as anti-inflammatory and oedema preventer (Hiermann *et al.* 1986). In addition to this, cytotoxicity studies of *EH* extract on human skin fibroblast cells showed no cytotoxic effect, which is important because of the frequent external use of those extract (Kiss *et al.* 2011). *EH* was used to prevent rectal bleeding by Native Americans and for treatment of menstrual disorders by Chinese (Gruenwald 1998). In Anatolia, the leaves and roots of *EH* have been used as antifebrile drug and for treatment of constipation and prostate (Everest and Ozturk 2005; Tita *et al.* 2001). Furthermore, *EH* has been demonstrated to regulate cancer development. This is
because of inhibitory effect of EH on activity of lipoygenase, important for growth-related signal transduction (Kiss et al. 2011). EH is also used as wound healing factor because of its inhibitory effect on hyaluronidase activity (Pogrel et al. 1993) Hyaluronic acid has a function of viscoelastic behavior of synovial fluid and its subsequent degradation by the hyaluronidase is the triggering event for the subsequent stages of wound healing that lead to complete repair (Pogrel et al. 1993). An anti-inflammatory effect of EH was also demonstrated (Almagboul et al. 1988). That might because of inhibitory effect of EH on myeloperoxidase activity. Myeloperoxidase produces HOC1, powerful oxidant generated by neutrophils, from H_{2}O_{2} and chloride. It may play an important role as an inflammatory mediator in bactericidal and fungicidal activities (Kiss et al. 2011). Moreover, EH has also been found to prevent benign prostate tumors (Vitalone et al. 2001; Vitalone et al. 2003). Aqueous extract of the plant has been shown as an effective oedema and tumor preventive on rat and reported as its antimicrobial effect especially on *Pseudomonas pyocyanea*, *Candida albicans*, *Staphylococcus aureus* (Battinelli et al. 2001).

1.3. Phase I and Phase II Xenobiotic Metabolizing Enzymes

1.3.1. Phase I Xenobiotic Metabolizing Enzymes

Detoxification and activation of chemical compounds including drugs mainly performed by phase I enzymes which include cytochrome P450s, flavin-containing monoxygenases (FMO) and epoxide hydrolases. These enzymes catalyze the reactions of exogenous compounds and convert them more polar forms, thus facilitating direct excretion by oxidation, reduction and hydrolysis. Cytochrome P450s also catalyze the chemicals to more active forms which bind to macromolecules such as DNA and proteins resulting in mutagenesis and carcinogenesis.

1.3.1.1. Cytochrome P450 Enzymes

Cytochromes P450 (CYPs) constitute a superfamily of heme enzymes found from bacteria to humans involved in a variety of metabolic and biosynthetic processes. The term P450 refers to a pigment that absorbs light at 450 nm when reduced form exposed to carbon monoxide (Omura and Sato 1964). CYPs are membrane bound proteins with an approximate molecular weight of 50 kDa and contain a heme moiety. Like other mixed function oxygenases CYPs are mainly found in the endoplasmic reticulum of the liver. The total CYP450 content of the livers ranged from 0.06 to 0.46 nmol/mg microsomal protein ([Iyer and Sinz 1999). The outside of the enzyme there is an expanse of protein framework of many hundreds of amino acid residues. On the surface of the enzyme, there are some channels from where substrate can pass through in order to bind to active side. Although not all P450 substrates are entirely hydrophobic, inside the enzyme, there is a hydrophobic pocket that consists of many amino acids that can bind a molecule by a number of weak interactions. Such as Van der Walls' forces, hydrogen bonding or pi-pi bond stacking, these provide a grip on the substrate in a number of places in the molecule, which prevents excessive movement. Below the hydrophobic pocket, a haem structure is also called ferriprotoporphyrin 9 (F-9) is present (Figure 1.2). F-9 supports a CYP iron molecule, which is the core of the enzyme. The iron molecule is normally found in the Fe^{2+}, ferrous, form. This part of the CYP is the same for all CYP enzymes.
Figure 0.2 The structural features of ferriprotoporphyrin 9 (F-9), showing the iron anchored in five positions.

Cytochromes P450 has an important role in the metabolism of xenobiotics such as drugs, dietary and environmentally derived toxicants and carcinogens, and endogenous compounds such as steroids, vitamin D, fatty acids and bile acid. By using NADPH and O₂, P450 enzymes modify or insert new functional groups on a molecule by oxidation, reduction, and hydrolysis reactions (Figure 1.3).

Figure 0.3 Oxidation of xenobiotics by CYPs.
The main function of cytochrome P450 enzymes is to insert an oxygen molecule into a stable and hydrophobic molecule. There are five main features of the process, which illustrated in Figure 1.4 whereby the following equation is carried out:

\[
\text{Hydrocarbon (-RH) + O}_2 + 2 \text{e}^- + 2 \text{H}^+ \rightarrow \text{Alcohol (-ROH) + H}_2\text{O}
\]

1. Substrate binding
2. Oxygen binding
3. Oxygen splitting
4. Insertion of oxygen into substrate
5. Release of product

![Diagram of the P450 Catalytic Cycle (Fang et al., 1997).](image)

In the first step, substrate (-RH) binds to P450, which causes a lowering of the redox potential, which makes the transfer of an electron favorable from NADH or NADPH. This is accompanied by a change in the spin state of the haem iron at the active site, (Fe$^{+3}$-RH). Once the substrate has been bound, the next stage is to receive the first of two electrons from CYP reductase, so reducing the iron (Fe$^{+2}$-RH). Second stage is the binding of the molecular oxygen to iron/substrate complex (O$_2$- Fe$^{+2}$-RH). In the third stage, oxygen molecule splits into two atoms, which cause rearrangement of the Fe$^{+2}$O$_2$ complex to form O$_2$Fe$^{+3}$RH. Next, a second electron from NADPH via the CYP reductase feeds into the complex and forms O$_2$Fe$^{+2}$-RH. This step is the rate-limiting step of the cycle, whether the substrate will be oxidized or not depends on this step. In the fourth stage, one oxygen atom of the complex is transferred to 2 H$^+$ atom, which produce 1 mole of water and RH (Fe-O)$^{+3}$ complex. In order to bind oxygen to substrate, CYP removes hydrogen from part of the substrate molecule. The abstracted hydrogen is closest to the carbon to be oxidized. R$^+$(Fe-OH)$^{+3}$ complex is produced and active carbon atom...
of the substrate reacts with the hydroxyl group, yielding the alcohol (R-OH). In the fifth and last step, due to structural and physicochemical differences, the product is released from active site of the CYP.

The microsomal cytochrome P450 monooxygenase system has a central role in toxicity and carcinogenesis by bio-activating a variety of xenobiotics such as drugs, food additives, industrial solvents, and pollutants to highly reactive and mutagenic metabolites. Although the main function of these enzymes is the detoxification of those compounds, they may catalyze the metabolic activation of pro-carcinogens to their ultimate carcinogenic forms (Guengerich and Shimada 1991). Metabolism of substrate by cytochrome P450 enzymes sometimes resulted with the formation of superoxide anion, H$_2$O$_2$, and hydroxyl radical.

The different forms of P450 found in a given organism were referred as P450 isoforms. P450 proteins can be categorized into families and subfamilies depending on their sequence similarities. At the amino acid level, sequences that are greater than 40 % identical belong to the same family. If this similarity ratio rises up to 55 %, they are categorized in the same subfamily. Up to now, more than 7700 P450 sequences in 866 families have been identified, of which 2740 sequences are found in animals and 2675 sequences in plants (Hamdane et al. 2008). On the other hand, all P450s have some similar properties; the ability to activate the O-O bond, the thiolate bond to the heme iron and similar overall structure and shape (Anzenbacher and Anzenbacherová 2001). The standard naming system uses the abbreviation for Cytochrome P450 [CYP] followed by a number denoting the gene family [1], a letter indicating the subfamily [A], and another enzyme referring to the enzyme [1]; [such as CYP1A1].

There are three main families of CYPs (CYP 1, 2 and 3) and 23 individual CYP enzymes that are expressed in a human liver (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A7,CYP2A13,CYP2B6,CYP2C8,CYP2C9,CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP2W1, CYP3A4, CYP3A7, CYP3A43.) It is known that 9 of the 10 drugs in use today are metabolized by five of these isoforms; CYPs 1A2, 2C9, 2C19, 2D6, and 3A4/5.
1.3.1.1.1. CYP1A1

Cytochrome P450 1A1 (CYP1A1) is one of the three members of the CYP1 family and localized to the endoplasmic reticulum. It is found mainly in extrahepatic tissues and located on chromosome 15. CYP1A1 has a wide distribution in animal kingdom. The overall sequence similarity of human CYP1A1 to rat was found as 78% in amino acids and 80% in coding nucleotide sequence (Adali et al. 1996; Kawajiri and Fujii-Kuriyama 1991). CYP1A1 participates the metabolism of huge number of xenobiotics especially; it converts polycyclic aromatic hydrocarbons to their mutagenic metabolites via hydroxylation at a vacant position of an aromatic ring, which is important for the initiation of carcinogenesis via production of highly reactive products which may cause oncogenic mutation. Polycyclic aromatic hydrocarbons (PAHs) such as Benzo[a]pyrene (BaP) which are the major substrate of CYP1A1 enzymes play a major role in lung cancer development (Arinc et al. 1991; Hecht 1999a). Although BaP is not directly mutagenic, its metabolism via CYP1A1 enzymes resulted with the formation of a highly reactive BaP-7,8-diol-9,10-epoxide that forms covalent DNA adducts, primarily with deoxyguanosine (Figure 1.5) (Androutsopoulos et al. 2009).

![Diagram of metabolic activation of B[a]P by CYP1A1](image)

**Figure 0.5** Metabolic activation of B[a]P to the carcinogenic metabolite by CYP1A1.
Modulation of CYP1A1 gene on the transcriptional level might be due to aryl hydrocarbon receptor (AHR), a ligand activated transcription factor. As illustrated in Figure 1.6, under normal circumstances, AHR is located in the cytoplasm with molecular chaperones; heat-shock protein, ARA9 and p53. Binding of environmental pollutants such as, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and inhalation chemicals, particularly substrates of the CYP1A1 enzyme, leads AHR to translocate into the nucleus and form a dimer with AHR nuclear translocator (ARNT). AHR/ARNT heterodimer binds to the xenobiotic response elements (XREs), which increases CYP1A1 transcription (Hayes et al. 2009).

**Figure 0.6** Transcriptional regulation of CYP1A1.
1.3.1.1.2. CYP2B1

CYP2B1 is the one of the eight enzymes of CYP2B family and mainly involved in metabolism of xenobiotics especially drugs such as benzodiazepines, tamoxifen, cyclophosphamide and ifosfamid. The isoforms CYP2B1 and CYP2B2 have 97% amino acid sequence homology, and CYP2B1 has nearly 75% identity with the human CYP2B6 (Haduch et al. 2008). CYP2B1 does not exist only in liver but also found in adrenal gland, kidney, lung brain endometrium, bronchoalveolar macrophages, and peripheral blood lymphocytes (Ding and Kaminsky 2003; Gervot et al. 1999). In the liver, CYP2B1 is expressed across zone 1 and zone 3 in the parenchymal cells throughout the hepatic lobules (Murray et al. 1990). Beside the metabolism of drugs, CYP2B1 has an important function on the catalysis of natural and synthetic procarcinogens such as aflatoxinB1, 6-aminochrysene, benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, and dibenz[a,h]anthracene (Figure 1.7) (Massey et al. 1995; Mugford and Kedderis 1998).

![Chemical structures](image)

**Figure 0.7** Metabolism of endogen and xenobiotic compounds by CYP2B1.
Constitutive expression and the induction of CYP2B1 on the transcriptional level is mainly regulated by androstane receptor (CAR) and pregnane X receptor (PXR) under the control of thyroid hormones and growth hormone (Figure 1.8). CYP2B1 is highly inducible by phenobarbital, benzodiazepines, dexamethasone and rifampicin. CYP2B1 was thought to be relevant with Parkinson’s disease because of its induction by nicotine in the brain not in the liver (Hidestrand et al. 2001). Predominantly, CAR is found in the cytoplasm as a phosphorylated form in a complex with Heat Shock Protein 90 (Hsp90) and cytoplasmic CAR retention protein (CCRP) in the absence of inducers and binding of the inducer such as phenobarbital, removes phosphate group and let the CAR to translocate to the nucleus (Kawamoto et al. 1999). Therefore, one of the mechanisms to prevent activation of CYP2B genes is hold the CAR in the cytoplasm. In the nucleus, phenobarbital removes from CAR and CaM kinase (CK) add a new phosphate group to the CAR. Phosphorylated CAR binds to retinoid X receptor (RXR). That complex then binds to phenobarbital-responsive enhancer module (PBREM) and initiates the transcription of target genes such as CYP2B and CYP3A. CYP2B expression was found to be elevated in the alcoholic and smokers compared with the non-alcoholic and non-smoking. Furthermore, high level of CYP2B expression was increase the coincidence of neural damage in brain (Miksys et al. 2003).

![Diagram](image.png)

**Figure 0.8** Transcriptional regulation of CYP2B genes.
1.3.1.1.3. CYP2E1

CYP2E1, the ethanol-inducible form, is constitutively expressed in the endoplasmic reticulum of hepatocytes of the liver but significant amounts are also found in most organs, including the brain, lung, kidney, bone narrow (Hansson et al. 1990). The human CYP2E1 gene is located on chromosome 10 and encodes an mRNA that is 1.6 kb in length. The sequence analysis of cDNA showed that human CYP2E1 cDNA shares 81% similarity over the coding region with rat CYP2E1. CYP2E1 metabolizes many low–molecular weight toxicants and carcinogens, such as acetaminophen, nitrosamines, phenol, benzene, 4-nitrophenol, carbon tetrachloride, chloroform, pyrazole and vinyl chloride (Gonzalez 2005).

CYP2E1 has an important function on the metabolism of drugs, but also it activates many pre-carcinogens and pre-toxins including ethanol, carbon tetrachloride, acetaminophen, and N-nitrosodimethylamine. For instance, metabolism of N-nitrosamines which is found in tobacco smoke and foodstuffs and several industrial and endogenous carcinogens by CYP2E1 resulted with formation of methyl nitrosamine which causes DNA methylation and carcinogenesis (Arinc et al. 2000a; Arinc et al. 2000b).

The CYP2E1 gene is under transcriptional control during development. The transcription of CYP2E1 in mammalian cells starts a few hours after birth and reach to its maximum in one day (Umeno et al. 1988). In rats, immediately after birth, CYP2E1 expression is activated, and maximal transcription occurs within the first week (Kessova and Cederbaum 2003). Constitutive CYP2E1 transcription is regulated by the transcription factor hepatocyte nuclear factor-1 (HNF-1) in the liver (Ueno and Gonzalez 1990). It was shown that transcription of CYP2E1 significantly decreased in HNF-1 knockout mice (Matsunaga et al. 2008). On the other hand, major CYP2E1 regulation is thought to be occurred at the level of protein where substrates bind to the heme group of the enzyme and prevent its degradation (Abdulla et al. 2006).

Expression of CYP2E1 can be influenced by many factors including drugs; acetaminophen, isoniazid, chemicals; benzene, pyridine, age and foods; high-fat diet or fasting for as short a period as 8 h can cause induction of CYP2E1 (Arinc et al. 1991; Arinc et al. 2000b). Hormones including T3 increase CYP2E1 protein and mRNA levels, while insulin lowers these. Pathological conditions such as diabetes and obesity increase the expression of CYP2E1 (Peng and Coon 1998; Yang et al. 1990). During diabetes or fasting, concentration of ketone bodies, hydroxybutyrate and acetoacetate increases which leads to stabilization of CYP2E1 protein and increase the concentration of CYP2E1 (Woodcroft et al. 2002).

CYP2E1 has a wide range of substrates, over than 70, including industrial chemicals such as alkanes, alkenes, other solvents, and aromatic and halogenated hydrocarbons and drugs such as disulfiram, chlorzoxazone and acetaminophen (Bolt et al. 2003; Carriere et al. 1993; Peter et al. 1990). The substrates, inducers and inhibitors of CYP2E1 were given in Figure 1.9 (Rendic and Di Carlo 1997; Ronis et al. 1998).
Figure 0.9  The substrates, inducers and inhibitors of CYP2E1.
1.3.1.1.4. CYP3A4

CYP3 family contains only one subfamily; CYP3A (Nelson et al. 1996). CYP3A is the most predominant subfamily of CYP enzymes in the keys of drug metabolism since 50% of the currently used drugs including anticancer, antiepileptic, anticoagulant, metabolized by the members of this family. CYP3A family has 4 different isoform in human being; CYP3A4, CYP3A5, CYP3A7, and CYP3A43. The most abundantly expressed CYP3A gene in humans is CYP3A4, approximetly 30-40% of the total hepatic CYP content consists of CYP3A4 (Imaoka et al. 1996). Beside this, it is also most abundantly expressed in the gut, where it can metabolize a large number of orally administered drugs (Kolars et al. 1994). The substrates for CYP3A4 include drugs, such as quinidine, nifedipine, diltiazem, lidocaine, lovastatin, erythromycin, cyclosporin, triazolam, and midazolam, and endogenous substances, including testosterone, progesterone, and androstenedione (Guengerich 1999). In addition, CYP3A4 has function on the activation of procarcinogens, including aflatoxin B1, polycyclic aromatic hydrocarbons (PAHs), 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone, and 6-aminochrysene (Aoyama et al. 1990; Hecht 1999b; Yamazaki et al. 1995).

Transcriptional regulation of CYP3A genes is mainly controlled by pregnane X receptor (PXR) (Fig. 1.10). PXR is a 50-kDa protein with approximately 434 amino acids and primarily expressed in the liver and intestine (Lehmann et al. 1998). PXR has a wide variety of ligand including low-affinity exogenous and endogenous compounds such as progesterone, estrogen, corticosterone, androstanol, or dietary compounds such as coumestrol and carotenoids (Blumberg et al. 1998; Moore et al. 2000). Binding of the ligand stimulates PXR translocate to the nucleus and lets it to form a heterodimer with the retinoid X receptor. Then, PXR/RXR complex binds to 6-nucleotide space (ER6), which increases transcription of CYP3A4 gene (Waxman 1999).
Figure 0.10  Transcriptional regulation of CYP3A genes.
1.3.2. Phase II Enzymes

Detoxification of therapeutic drugs and various carcinogens by conjugation reactions are catalyzed by the members of the phase II enzymes; Glutathione S-transferases, NAD(P)H:Quinone Oxidoreductase I. Conjugation with phase II enzymes increases hydrophilicity of compounds, and facilitate excretion in the bile and/or the urine. On the other hand, conjugation sometimes could result in activated metabolites and increase the toxicity.

1.3.2.1. Glutathione S-Transferase

The Glutathione S-transferases (GSTs) (EC.2.5.1.18) are enzymes that participate in cellular detoxification of endogenous as well as foreign electrophilic compounds.

Glutathione-S-Transferase has a function on the conjugation of xenobiotics with glutathione (GSH, γ-glu-cys-gly) (Figure 1.11). In 1961, two independent research groups reported that GST found in cytosolic fraction of rat liver catalyzed GSH conjugation of xenobiotics; bromosulfophthalein (Combes and Stakelum 1961) and halobenzenes (Al-Kassab et al. 1963). The cofactor for reactions catalyzed by these enzymes is the tripeptide glutathione (GSH), which is composed of glycine, glutamic acid, and cysteine. The enzymes catalyze the reaction of the nucleophilic sulfhydryl of glutathione with compounds containing electrophilic carbon atoms. The reaction of the glutathione thiolate anion (GS−) results in formation of a thioether bond between the carbon atom and the sulfhydryl group of glutathione. Compounds that are substrates for the glutathione S-transferases share the following three basic features:

1. They must be hydrophobic to some degree.
2. They must contain an electrophilic carbon atom.
3. They must react nonenzymatically with glutathione at some measurable rate.

These enzymes are ubiquitous; the greatest activity occurs in the testis, liver, intestine, kidney, and adrenal gland. Electrophiles, produced by the action of phase I enzymes, may possess cytotoxic, mutagenic and carcinogenic effects and their conjugation to GSH is the cell’s major defense against them. GSTs serve as transporters of potentially harmful substances out of the cell with different electrophilic species (Jemth and Mannervik 1999). Following conjugation, these generally harmless GSH adducts, or their mercapturic metabolites, are secreted into the bile or urine.
Figure 0.11  Structure of GSH.

All GST isozymes are known to use reduced GSH as an acceptor species, but they differ in the specificity with which different substrates are transferred to the thiol group of cysteine of GSH. The GSTs are found in all eukaryotes and prokaryotic systems, in the cytoplasm, in the microsomes, and in the mitochondria. The cytoplasmic activities are generally 5 to 40 times greater than the microsomal activity. Soluble GSTs are dimers of approximately 25 kDa subunits. Isolated monomers would presumably be catalytically inactive. An individual GST subunit is built of two domains (Figure 1. 12). The N-terminal domain comprises the first 80 residues and is essential an α/β structure. This domain harbors the GSH-binding site and the binging pocket for GSH has been called the —Gite”. In many GST classes, a tyrosine residue located within its hydroxyl group within hydrogen bonding distance from the GSH sulfur atom is important for catalysis while it is serine for other GST classes; Theta, zeta, Phi and Tau. G site of GSTs has been conserved during evolution. The second domain is an α-structure, composed of 5-6 α-helices. This domain contributes most of the interactions responsible for binding of the electrophilic substrate, and this hydrophobic binding site is called as —Hite”. In contrast to the G-site, H-site varies significantly from enzyme to enzyme. Moreover, the peptide segments forming the H-site are the primary determinants of substrate specificity, exchange of H-site amino acids by site directed mutagenesis enhance GST A1-1 affinity to other substrate which are the substrate of GST A4-4 (Nilsson et al. 2000). The glutathione conjugates are subsequently cleaved to cysteine derivatives, primarily by enzymes located in the kidney. These derivatives are then acetylated to give the N-acetylcysteine (mercapturic acid) conjugates that are readily excreted in urine.
Figure 0.12  Structure of GST Enzyme, Grey, monomer 1; yellow, monomer 2; green, G-site; red, H-site (Bhat et al. 2010).

Depending on the amino acid sequence similarities, physical structure of the genes, and immunological cross-reactivity seven classes of cytosolic GSTs were created named as alpha (α), mu (μ), kappa (κ), pi (π), theta (θ), omega (Ω), and zeta (δ) (Hayes et al. 2005).
1.3.2.2. NAD(P)H:Quinone Oxidoreductase I

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) is a FAD-containing, homodimer protein, each monomer consist of 273 amino acids, has been described in numerous mouse, rat, human and zebra fish (Ross et al. 2000). It is mainly located in cytosol (>90%) but also present in microsomes and mitochondria. Expression of NQO1 is mainly occurred in epithelium, endothelium, and adipocytes. NQO1 is characterized by its capacity for utilizing either NADH or NADPH as reducing cofactors. (Ross et al. 2000). One homodimer of NQO1 present with one molecule of FAD (Figure 1.13). The mechanism of catalysis has been proposed to involve a hydride transfer between the NADH and FAD cofactors and from FADH to the quinone substrate. Not only ortho quinones but also para quinones are substrates for NQO1 (Segura-Aguilar and Lind 1989).

Figure 0.13 Polypeptide structure of NQO1 (Colucci et al. 2008).

NQO1 is able to perform two-electron reduction of which catalyzes reduction of a wide range of substrates including quinones, quinone-imines, glutathionyl-substituted napthoquinones, dichlorophenolindolphenol, methylene blue, azo and nitro compounds and reduce them to less reactive and less toxic hydroquinones (Fig .1.14). Two electron reduction of NQO1 prevent formation of semiquinone, which prevents the generation of reactive oxygen species such as O$_2^-$ or hydrogen peroxide (Lind et al. 1990; Siegel et al. 1997). Beside this, two electron reduction of NQO1 leads to formation of antioxidant compounds such as vitamin E quinone, coenzyme Q10 and their complement hydroquinones (Beyer et al. 1996; Siegel et al. 1997). Moreover, NQO1 has been shown to stabilize p53, tumor suppressor protein (Anwar et al. 2003). Therefore, NQO1 can be categorized as an antioxidant and cancer preventive enzyme.
Figure 0.14 Two electron reduction of 1,4-benzoquinone to hydroquinone by NQO1.

In human, NQO1 is over-expressed in a variety of solid tumors comprising those of the adrenal gland, bladder, breast, colon, liver, lung, ovary and thyroid (Schlager and Powis 1990). Moreover NQO1 has a function on bioactivation of heterocyclic amines which are mutagenic and carcinogenic (Kitamura et al. 1999).

Investigation of cDNA of human, rat and mouse for NQO1 showed that NQO1 is a single copy gene and is located on human chromosome 16q22.1 (Ross et al. 2000). NQO1 gene has six exons and five introns for an approximate length of 20 kb (Robertson et al. 1986). There is extensive homology (85%) between the rat and human NQO1 coding regions.

NQO1 activity is induced by a wide range of chemicals including polycyclic aromatic hydrocarbons, azo dyes and phenolic antioxidants. Regulation of the transcription of NQO1 is done by nuclear transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2) (Dhakshinamoorthy and Jaiswal 2000). In the case of oxidative stress, NRF2 binds to antioxidant response element (ARE) in the promoter region and increased NQO1 transcription (Nioi et al. 2003). ARE and XRE have been shown to mediate NQO1 induction as well as repression, in many cellular systems. Not only the oxidative stress causes NQO1 transcription but presence of quinone also stimulates NQO1 transcription. In Parkinson’s and Alzheimer’s diseases, NQO1 expression has been reported to be elevated as an adaptive response against dopamine quinone metabolites’ potential accumulation (Raina et al. 1999; van Muiswinkel et al. 2004)
1.3.3. Antioxidant Enzymes

Transfer of electrons from one atom to another, which called oxidation, is the essential part of aerobic life and normal metabolism because oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP. On the other hand, during these processes free radicals, atoms, molecules, or ions with unpaired electrons on an open shell configuration, which cause them to be highly chemically reactive, are produced. In biological systems, the oxygen-centered free radicals are known as reactive oxygen species, ROS such as superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (·OH) (Fig 1.15-A). Oxidative stress occurs when there is an imbalance between production of ROS and cellular antioxidant capacity or when there is a decrease in this capacity. The imbalance between consumption and production of ROS may be very damaging, since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, to induce oxidations, which cause membrane damage, protein modification including enzymes and DNA damage. Oxidative stress has been found to be related with many diseases from cancer, diabetes, cardiovascular and chronic kidney diseases to neurodegenerative diseases. A number of major cellular antioxidant defense mechanisms exist to neutralize the damaging effects of free radicals. Living organisms have evolved with antioxidant systems to protect against free radicals (Fig 1.15-B). These systems include some antioxidants produced in the body, namely endogenous, and others supplied from the diet, namely exogenous. In particular the antioxidants such as reduced glutathione (GSH) and ascorbic acid (AA) and the antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR) are implicated in antioxidant cellular response.

Figure 0.15  Defense mechanism against ROS. (A) Schematic and simplified presentation of the processes leading to ROS and the defense against them. (B) Antioxidant enzymes involved in the defense against ROS (Johnson and Giulivi 2005).
1.3.3.1. Superoxide Dismutase

Superoxide dismutases (SODs) are metalloenzymes found widely distributed in prokaryotic and eukaryotic cells and first reported in 1969 by McCord and Fridovich. They constitute an enzyme family that catalyzes the conversion of superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$). As shown in Figure 1.16, the two-step chemical reaction of superoxide anion with the prosthetic group of SOD begins with the oxidized form of the enzyme ($Fe^{3+}$, $Cu^{2+}$ and $Mn^{3+}$ respectively) binding superoxide anion, acquiring a proton and releasing molecular oxygen. The reduced form of the enzyme ($Fe^{2+}$, $Cu^{+}$ and $Mn^{2+}$) then binds a second superoxide anion and proton, to liberate $H_2O_2$ and return to its oxidized state. Despite the metal ion centre becoming more negatively charged, the binding of the second superoxide anion is possible in an environment of neutral pH, by the total charge of the active site remaining the same.

![Figure 0.16](image)

**Figure 0.16** Mechanism of Superoxide dismutases (Culotta et al. 2006).

Depending on species, there may be up to three different metal-containing SOD enzymes present which, taken together, make up the major superoxide scavenging system in the mitochondrion, nucleus, cytoplasm and extracellular spaces. These SODs are the products of different genes and are historically designated, in higher eukaryotes, by their primary location as follows: SOD1 (cytoplasmic), SOD2 (mitochondrial) and SOD3 (extracellular). SOD1 contains copper (Cu) and zinc (Zn) within the active site (also known as CuZn SOD), and is mainly found in cell cytoplasm. SOD2 or manganese (Mn) SOD has an active site that contains manganese and is located in mitochondria. SOD2 is the only antioxidant enzyme known to be present within the mitochondria and this has important implications because this is a major site for the production of ROS during normal cellular metabolism. SOD3 or extracellular (EC) SOD also has Cu and Zn within the active site and is the least studied of the three SOD isoforms. The SOD3 may bind to cell surfaces by its interaction with polyanions, such as heparan sulphate. In humans, the SOD family members are either dimeric (SOD1; 32 kD, (McCord and Fridovich, 1969) or tetrameric (SOD2; 89 kD, (McCord, 1976), SOD3; 135 kD, (Marklund, 1982). They are also defined between kingdoms by virtue of their bound metal ion, namely the Cu, Zn-SOD and Mn-SOD class or the Fe-SOD class, the latter of which exists in prokaryotic cells and some green plants (Figure 1.17). In humans, 111 SNPs have been identified for SOD1, 190 for SOD2 and 100 for SOD3 (Crawford et al. 2012).
Figure 0.17  3D-structures of SOD with various metal ligands required for its activity and/or preservation of structure (Johnson and Giulivi 2005).

The SODs are one part of a suite of enzymes that catalyzes reactive oxygen species (ROS), produced as minor by-products of metabolism, to less reactive species (Figure 1.18). Oxidative stress arises because of an enhanced production of ROS or a deficient anti-oxidant system (enzymatic and non-enzymatic). This leads to the chemical modification of key cellular components. Some known and well-studied consequences of oxidative stress are alteration of membrane integrity, DNA instability and decline in enzymatic activities. In terms of the whole organism, increased oxidative stress has been associated with much acute and chronic pathology. An example of an acute pathology is ischemia-reperfusion injury (Crack and Taylor 2005) whereas for chronic pathologies, it may be vascular damage in patients with diabetes mellitus (Oberley 1988). Oxidative stress is also associated with the aging process.
Figure 0.18  Role of superoxide dismutase (SOD) in the reactive oxygen species scavenging pathway (Johnson and Giulivi 2005).

It was clear that SOD played a critical role considering (i) its fast reaction with superoxide anion, (ii) its presence in every organ and almost all intracellular compartments and, (iii) the high concentration of SOD compared to its substrate i.e. SOD concentrations are 106-times higher than that of superoxide anion (Johnson and Giulivi 2005).
1.3.3.2. Glutathione Peroxidase

The enzyme Glutathione peroxidase (GPx) (EC. 1.11.1.9), a selenocysteine-containing protein, was found both in cytosol and the mitochondria. GPx has a central role in controlling the level of reactive oxygen species (ROS) and therefore buffering oxidative stress. This antioxidant enzyme has a variety of function in the mammals as it reduces fatty acid hydroperoxides, \( \text{H}_2\text{O}_2 \), phospholipid hydroperoxides and cholesterol hydroperoxides (Imai et al. 1998). GPx knockout mice were found extremely susceptible to oxidative stressors like paraquat (de Haan et al. 1998). Furthermore its deficiency has been found to be associated with cardiovascular diseases and stroke (Freedman et al. 1996). There are 7 proteins exhibiting glutathione peroxidase activity GPx1-GPx7. Each GPx is encoded by a different gene and differs in its structure and localization. These enzymes catalyze the reduction of \( \text{H}_2\text{O}_2 \) and alkyl hydroperoxides by oxidizing glutathione (GSH), as well as reducing a variety of organic hydroperoxides. The classical GPxs are localized in the cytosol of a cell and in the mitochondrial matrix.

GPxs catalyze the following reactions:

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad \text{and} \quad 2\text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}.
\]

In these reactions, GSH reduced \( \text{H}_2\text{O}_2 \) and organic ROOH to water or hydroxy-derivatives and converted into the oxidized disulfide form, GSSG.

Enzyme exhibits board specificity towards oxidized substrates, which include: \( \text{H}_2\text{O}_2 \), almost all aliphatic and cyclic organic peroxides (ROOH) (e.g. tert-butyl and cumene hydroperoxides), polyunsaturated fatty acids, certain steroids, prostaglandin F2α and prostaglandin G2. Lipid peroxides and cholesterol 25-OOH represent the only exceptions.

All GPx reduce \( \text{H}_2\text{O}_2 \) (in contrast to GT) and organic ROOH to water and alcohols (ROH), respectively. The antioxidant reactions are realized in the four lines of defense as illustrated in Figure 1.19 (Imai et al. 1998).

1) Reduction of to \( \text{H}_2\text{O}_2 \) catalyzed by SOD
2) Reduction of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) by catalase and GPx (the role of GPx is more important than that of catalase)
3) Reduction of various ROOH catalyzed by GPx and GT
4) Detoxification of toxic aldehydes (especially 4-hydroxynonenal) by condensation with GSH catalyzed by GT or (more rarely) oxidation of aldehydes to acids.
Figure 0.19  Four lines of antioxidant defense. Designations: Black arrows indicate reaction of reactive oxygen species formation; empty arrows indicate reactions decreasing ROS reactivity; numbers in brackets indicate the number of defense. R(O)—4-hydroxy-2,3- nonenal and other toxic aldehydes; HR(O)SG—conjugates with GSH.

1.3.3.3.  Arylesterase (ARE)

Arylesterase (EC 3.1.1.2) catalyzes the hydrolysis of organophosphorus esters and mainly found in the blood and liver. That ability of arylolesterases has a crucial role in the detoxification of organophosphorous compounds in mammalian systems which are important factor to determine the resistance of organism against organophosphate toxicity (Mackness 1989). Beside this, human serum Arylesterase was reported to be associated with high density lipoproteins (HDL). There are two isozymes of arylolesterase present: isozymes A and B which define three phenotypes: A, AB and B (respective genotypes AA, AB and BB), the B isozyme being the most active one (Musayev et al. 1995).
1.4. **Scope of the Study**

Increasing popularity towards traditional medicine such as herbal remedies or dietary supplements leads investigators to examine the actions of phytochemicals on xenobiotic metabolism and their antioxidant, antitumor, anticarcinogenic and antimutagenic effects. *EH* has a worldwide distribution and is being used as a folk medicine in Asia, America and Europe. *EH* contains many biologically active compounds, including polyphenolic and flavonoid compounds, which are known their biological effects such as anti-oxidant activities of polyphenols, anti-noniceptive and anti-inflammatory activities of flavonoids. It has been reported that *EH* extract exhibited the highest antioxidant activity and highest antioxidant concentration among 32 plants extracts (Wojdyło et al. 2007). On the other hand, up now, there is no data available in the literature for the possible effects of *EH* on xenobiotic metabolizing enzymes such as drug and carcinogen metabolizing CYPs, phase II and antioxidant enzymes.

In this study, effects of *Epilobium hirsutum* L. on xenobiotic metabolizing enzymes and its drug-diet interaction potentials were investigated by biochemical and molecular approaches.

1) This study may come into prominence since it is the first study that investigates relationship between *EH* and the xenobiotic metabolizing enzymes. Although there are some studies related with *EH*, this is the first comprehensive study considering phase I, phase II and antioxidant enzymes.

2) This is the first study that investigates the effects of *EH* in broad perspective regarding enzyme activity, gene expression, and protein expression.

3) The result obtained from the study has universal value since *EH* spreads worldwide, not only in Turkey but also in European and USA.

4) The data obtained from this study may supply valuable information about usage of this plant in replacement treatment since it reveals possible interactions of *EH* with drugs and foods.

5) It may contribute to economy since the study has a potential to identifying possible curative, healing, toxic or side effects of the plant.

In order to achieve these purposes, the plants were collected and their water extracts were prepared. Polyphenolic content of *EH* was determined by using LC-MS/MS. The water extract of *EH* was dissolved in ddH₂O and 37.5 mg extract/kg body weight/day was intraperitoneally (i.p) injected to 4 months old male Wistar Albino rats (n=30) (*Rattus norvegicus*) for a period of nine consecutive days. After decapitation of the animals, blood samples were collected in order to determine cytotoxic effect of *EH* by measuring LDH enzyme activities. The livers were removed and S1.5, cytosolic and microsomal fractions were prepared by homogenization and differential centrifugation. Protein concentration was determined by using BCA method. Determination of enzyme activities was performed spectrophotometrically in a manner of continuous and discontinuous enzyme assays. Effects of *EH* on mRNA and protein expressions were determined by quantitative real-time PCR (qRT-PCR) using cDNA synthesized from total RNA of rat livers and western-blot using specific primary and secondary antibodies.
To our knowledge, this study is the first concerning the effects of medicinal plant *Epilobium hirsutum* L. on rat liver drug and carcinogen metabolizing CYPs, phase II and antioxidant enzyme activities as well as protein and mRNA expressions.
CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

2,6-dichlorophenol-indophenol (DCPIP; D1878), bicinchoninic acid (D8284), 1-chloro-2,4-dinitrobenzene (CDNB; C6396), ammonium acetate (A7672), \( \varepsilon \)-amino caproic acid (\( \varepsilon \)-ACA; A2504), bovine serum albumin (BSA; A7511), D-glucose–6-phosphate monosodium salt (G7879), D-glucose–6-phosphate dehydrogenase type IX (G8878), glutathione reduced form (GSH; G4251), N-2-hydroxyethylpiperazine-N-2,ethane sulfonic acid (HEPES; H3375), N-nitrosodimethylamine (NDMA; N3632), phenylmethane sulfonil fluoride (PMSF; P7626), sodium potassium tartrate (Rochell salt; S2377), 2-amino-2( hydroxymethyl)-1,3-propanediol (Tris; T1378), acrylamide (A-8887), ammonium per sulfate (APS; A-3678), bromophenol blue (B5525), diethanolamine (D-2286), diethylpyrocarbonate (DEPC; D5758), ficoll (F2637), glycogen (G5516), glycine (G-7126), \( \beta \)-mercaptoethanol (M6250), methanol (34885), N\(^{-}\)-N\(^{-}\)-bis-methylene-acrylamide (M7256), N,N-dimethylformamide (D-8654), phenazine methosulfate (P9625), secondary antibody AP mouse (A3562), secondary antibody AP rabbit (A3687), sodium dodecyl sulfate (SDS; L4390), sodium-potassium (Na-K) tartrate (S-2377), tween 20 (P1379), xylene cyanol (X4126) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Ethylene diamine tetra acetic acid disodium salt (EDTA; 08421), glacial acetic acid (0056), glycerol (04093), magnesium chloride (MgCl\(_2\); 05833), methanol (02500), \( \rho \)-nitrophenol (106798), potassium chloride (KCl 104935), potassium dihydrogen phosphate (KH\(_2\)PO\(_4\); 04871), di-potassium hydrogen phosphate (K\(_2\)HPO\(_4\); 05101), sodium carbonate (06398), sodium hydroxide (06462), trichloroacetic acid (TCA; 00256), boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate (CuSO\(_4\).5H\(_2\)O; A894987 605), folin-phenol reagent (1.09001.0500), magnesium chloride (MgCl\(_2\); Art.5833), potassium chloride (KCl; 4935), sodium carbonate (Na\(_2\)CO\(_3\); 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), zinc chloride (ZnCl\(_2\); 108815) were the products of E. Merck, Darmstadt, Germany.

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

\( \beta \)-nicotinamide adenine dinucleotide phosphate reduced form (NADPH; A1395), \( \beta \)-nicotinamide adenine dinucleotide phosphate (NAD\(^{+}\); N0505), were taken from Applichem, Darmstadt, Germany.
5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821), dithiothreitol (DDT; R0861), gene rulerTM 50 bp DNA ladder (SM0371), light cycler-fast start DNA Master<sup>®</sup> SYBR green I (K0252), Maloney murine leukemia virus reverse transcriptase (M-MuLu-RT; K1622), pre-stained protein ladder (SM0671) were purchased from MBI Fermentas, USA.

Non-fat dry milk (170-6404) and tetra methyl ethylene diamine (TEMED; 161-0801) were the products of Bio-Rad Laboratories, Richmond, California, USA.

Ethylene diamine tetra acetic acid (EDTA; A5097) and nitrotetrazolium blue chloride (NBT; A1243) were acquired from Applichem GmbH, Germany.

The CYP1A1 (sc-20772), CYP2B1/2 (sc-73546), CYP2E1 (sc-133491), GPx (sc-133160) antibodies were purchased from Santa Cruz (Santa Cruz, CA) and NQO1 (ab-34173) primary antibody was purchased from Abcam (Cambridge, UK). Primers were made by Bio Basic Inc (ON, Canada).

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

2.2. Collection of *Epilobium hirsutum* L.

The location of *Epilobium hirsutum* L. was detected depending on the reports of Davis (1978) and Davis (1984); and in the direction of blooming time, distribution, habitat and elevation of the plant, land search plans were done. Plant samples were collected from unpolluted field, whose location was described below in Table 2.1.

**Table 2.1 Plant Information**

<table>
<thead>
<tr>
<th>The date of the collection of plant material:</th>
<th>17 / 07 / 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>The address of the collection of plant material:</td>
<td>On the way of Saçmalıpınar village from Kisa Mahmut neighborhood, Gölyaka, Düzce</td>
</tr>
</tbody>
</table>
| The coordinates of the collection of plant material: | N 40° 44′ 19.9″
E 30° 56′ 28.2″ |
| The altitude of the collection of plant material: | 563 m |
| The herbarium (Ankara University Faculty of Pharmacy Herbarium, AEF) number of the collected plant materials: | AEF 25 812 |
After collecting plants, samples were pressed depending on the herbarium rules before losing the morphology. Plants were identified depending on the flora of Turkey and East Aegean Islands (Davis 1972; Davis 1982) and a voucher specimen (No: AEF 25812) deposited in Faculty of Pharmacy Herbarium Ankara University. Samples have been dried, pressed and stored at room temperature until use in the laboratory.

2.3. Preparation of *Epilobium hirsutum* L. Extracts

Air dried and powdered flowering aerial parts of plant material has been subjected to active maceration in ddH2O by using Heidolph mechanic shaker at 300 rpm/min at room temperature for 8 hours. The extract obtained was filtered from filter paper and dried in the freeze dryer (Christ Gamma 2-16 LSC) and weighed. The extract was gassed with nitrogen to prevent oxygen interaction, and stored at -20 °C for injection to rats.

2.3.1. Identification and quantification of Active Substances of *Epilobium hirsutum* L.

The total concentration of phenolic compounds in *EH* extracts was determined using a series of gallic acid standards as described by the method of Singleton and Rossi (1965) and the results were given as mg gallic acid equivalents (GAE) per 100 g of extract. Identification and quantitation of phenolic compounds in *EH* was performed by using Liquid Chromatography Mass Spectrometry (LC-MSMS). For reversed phase chromatographic separation, a Zorbax SB-C18 (2.1 x 50 mm x 1.8 μm) column (Agilent Technologies, Palo Alto, CA) was used. The mobile phase consisted of (A) 0.5% formic acid with 5 mM ammonium formate and (B) methanol. The spectra of eluted substances were recorded between 200 and 500 nm. Mass spectrometric analyses were performed in an Agilent 6460 triple quadrupole mass spectrometer (Agilent Corporation, US) equipped with an electrospray ionization (ESI) source plus Agilent Jet Stream that in the positive ionization (PI) mode. The capillary and nozzle voltages were 4000 and 500 V, respectively. The flow rate and temperature of the sheath gas were 10 ml/min and 350 °C, respectively. Sample acquisition was performed in the multiple reaction monitoring (MRM) mode.

2.3.2. Identification of active substance groups of *Epilobium hirsutum* L.

The reactions, whose details given below were accomplished in order to determine the group of compounds of *Epilobium hirsutum* L. extract qualitatively.

2.3.3. Identification of Alkaloids

After boiling of 0.5 g of crushed powder of *Epilobium hirsutum* L. in 10 ml of 70 % ethanol containing 6 % H2SO4 for 1 minute, the solution were cooled and let to precipitate. Little amount of supernatant were poured into 2 tubes. The formation of precipitation was checked by adding Mayer and Dragendorff reactant. The appropriate amount of 25 % Na2CO3 was added in this ethanol-extract mixture in order to make the solution alkaline. The solution was mixed with 15 ml chloroform that causes separation of layers from each other and after separation chloroform was depleted via 15 ml of 10 % acetic acid. The acetic acid layer was taken into 3 tubes; first tube was for control,
second and third tubes mixed with Mayer and Dragendorff reactant, respectively in order to check precipitation.

2.3.4. Identification of Cardioactive Heterosides

2 g of powder of *Epilobium hirsutum* L. was filtered after boiling in 10 ml of 70% of ethanol for 2 minutes in water bath. The filtrate was diluted 2 times volume of water and 1 ml of concentrated lead subacetate was added to solution and filtered. The filtrate was mixed with 10 ml of chloroform. The solution was taken into 3 capsules and following reactions were applied.

2.3.4.1. Keller - Kiliani Test:

The solution in the capsule was evaporated until dried phase obtained. 3 ml of FeCl₃ solution containing 3.5 % of glacial acetic acid was added into the capsule and incubated for 1 minute. Then obtained solution was poured carefully into a test tube containing 2 ml of H₂SO₄ in order to form a layer and obtained color was monitored.

2.3.4.2. Baljet Test:

The solution in the capsule was evaporated and the dried material was dissolved in 1 ml of ethanol. Baljet reactant was dropped on this solution and formation of color was monitored.

2.3.4.3. Liebermann - Burchard Test:

The solution in the capsule was evaporated and the dried material was dissolved in 1 ml of glacial acetic acid. The solution was poured into a test tube and 1-2 drop of concentrated H₂SO₄ was added and formation of color was monitored.

2.3.5. Identification of Saponosides

0.5 g of powdered material was put in a test tube with 10 ml of hot water and let to cool. Then the test tube was mixed harshly. In the presence of saponoside, foam which was minimum 10 minutes stable, 1-10 cm height and was not disappeared with 1-2 drop 2 N HCl was observed.

2.3.6. Identification of Flavonoids

2 % decoction of plant powder was prepared. This extract then separated into 3 parts and following reactions were applied:

- 1-2 drop of 10% ammonia was added and the color was observed.
- Lead subacetate solution was added and the color was observed.
- FeCl₃ solution was added drop by drop and the color was observed.
- Cyanide reaction: plant powder dissolved in 5 ml ethanol via heating and extract was obtained. After filtration, 0.5 ml of concentrated HCl and a spatula end amount of Mg powder was added on the filtrate. Hydrogen production and color formation in the foam were observed.

2.3.7. Identification of Anthocyanins

The plant powder was shaken in % 50 of ethanol and warmed then filtrated. The filtrate was separated into 5 parts and following reactions were applied:
- Color was observed after adding concentrated H₂SO₄.
- First NaOH was added and then HCl was added and color was observed.
- Precipitation was observed after adding 10% of lead subacetate solution.
- Amyl alcohol was added and mixed. Color was observed in the layers.
- Heated with diluted H₂SO₄ and put on to cool. Then mixed with amyl alcohol and color was observed in the amyl alcohol layers.

2.3.8. Identification of Cyanogenic Glycosides

1 g of plant powder was put in a 100 ml erlenmeyer flask and moisturized by water. A Na₂CO₃ wetted and picric acid impregnated filter paper was hanged down by the help of cork stopper. Then erlenmeyer flask was heated gently and formation of color in the filter paper was observed.

2.3.9. Identification of Tannins

5% of infusion was prepared from plant powder. Following reactions were applied on this infusion:

- The precipitation with heavy-metal salts was observed.
- The color with 5% FeCl₃ solution was observed.
- The precipitation with salty gelatin solution was observed.
- The precipitation with bromic water was observed.
- The precipitation with Stiasny reactant (formol + concentrated HCl) was observed.

2.3.10. Identification of Anthracene Derivatives

0.1 g of plant powder was boiled in 5 ml of H₂SO₄ for 2 minutes. The solution was filtrated while it was hot. The filtrate was cooled and mixed with a little amount of benzene. The benzene layer on the upper site was taken and mixed with 10% of ammoniac. The color of ammoniac found in bottom was observed.

2.3.11. Identification of Coumarins

1 g of plant powder mixed with 10 ml of 1 N H₂SO₄. The solution was boiled under cooling system and filtrated while it was hot. The filtrate was mixed with 15 ml of chloroform and shaked in separating funnel. Chloroform phase was taken and 5 ml of this phase mixed with 5 ml of 10% NH₃ and mixed. 5 minutes later, the florescence of the ammoniac phase at UV366 nm was observed.

2.3.12. Identification of Volatile Oil

The formation of odor was monitored after rubbing of plant materials between fingers. Same materials powder was also investigated with Sartur reactant. Mild heat was applied to the slide and diluted ethanol was poured corner of slide and disappearance of oil was monitored.
2.4. Animal Studies

Male Wistar Albino rats (12 weeks old) weighing 200–250 g were used. They were housed at the University Animal House in standard conditions and fed with a standard diet with water ad libitum. All experimental procedures in animals such as the administration of substances by intraperitoneally (i.p.), collection of blood and tissue, etc., were performed to the national standards under appropriate regimes with veterinary services and licensed projects. Rats were randomly assorted into the following two groups: Group I (control I, 10 rats) rats were treated with water i.p. daily for 9 days; Group II (EH, 30 rats) rats were treated with EH water extract 37.5 mg/kg, i.p. daily for nine consecutive days. At the end of the experimental period and following 16 h of fasting, the animals were sacrificed. Blood samples were taken from the aorta to determine the serum enzymes. The livers were isolated and rinsed with cold physiological saline and stored at -80 °C until analyzed. This part of the study was carried out with the collaboration of Pamukkale University, Biology Department.

2.5. Preparation of Rat Liver S1.5, Cytosolic and Microsomal Fractions

The livers were removed immediately after killing the animals by decapitation. All subsequent steps were carried out at 0-4 °C. After removal of the connective and fatty tissues, livers were washed with cold distilled water and then with 1.15% KCl solution several time to remove the excess blood. The blood obtained from rats was centrifuged at 3000 rpm for 5 minutes at 4 °C in order to obtain serum. Liver and serum samples were stored at -80 °C.

Rat liver S1.5, cytosolic and microsomal fractions were prepared by the method of Schenkmam and Cinti (1978) as optimized by Şen and Kırıkbakan (2004). After blotting the tissues by the help of a filter paper, tissues were weighed and chopped with scissors. The resulting minced tissues were homogenized in homogenization solution containing 3 mM EDTA pH 7.8, 50 mM Tris-HCl, pH7.5, 0.3 mM e-ACA,0.5 mM PMSF, 0.15 mM BTH, %10 glycerol and % 0.15 Triton X-100 at a volume of equal to 3 times of weight of liver by using Potter-Elvehjem glass homogenizer coupled with a motor-driven teflon pestle at 2600 rpm. Ten passes were made for the homogenization of liver tissue. The resulting liver homogenate was centrifuged at 1500 x g for 10 minutes to obtain S1.5 fraction. The pellet was resuspended with same volume of homogenization solution and recentrifuged at 12000 x g for 25 minutes and the supernatant fraction containing endoplasmic reticulum and soluble fraction of the cell was filtered through double layers of cheese cloth in a Buchner funnel. The supernatant was mixed 0.5 times cold 0.16 M CaCl₂ and centrifuged at 32000 x g for 25 minutes. The supernatant fraction (cytosol) was removed and stored at -80 °C until use in determination of activity of cytosolic enzymes.

The firmly packed microsomal pellet was suspended in homogenization solution and resedimented at 32000 x g for 20 minutes. Supernatant was discarded and pellet was resuspended in resuspension solution (10 % glycerol containing 2 mM EDTA at a volume
of 0.5 ml for each gram of liver tissue. The microsomal suspensions were stored at -80 °C for enzymatic assays.

2.6. Determination of Protein Concentration

Protein concentrations of rat liver S1.5, microsomal and cytosolic fractions were determined by the BCA (Bicinchonicin Acid) described by Smith et al., (1985) using crystalline bovine serum albumin as a standard, and the results were given in Table 3. BCA reaction was illustrated in Figure 2.2.

![Figure 2.1](image)

Figure 2.1 Mechanism of BCA reaction.

In BCA method, 100 µl of sample and BSA standards were added into the 96-well plate and 100 µl of BCA solution; BCA, alkaline CuSO4 and Na-K tartrate were added on it. After incubation at 60 °C for 15 minutes the absorbance of samples were read at 562 nm with ELISA-reader spectrophotometer. The intensity of color is proportional with protein concentration.
2.7. Determination of Enzyme Activities

Drug and carcinogen metabolizing cytochrome P450, phase II and antioxidant enzyme activities were spectrophotometrically determined. Enzyme activity determination was performed with continuous and discontinuous assays.

2.7.1. Determination of Cytochrome P450 Dependent Enzyme Activities

2.7.1.1. Determination of Ethoxyresorufin-O-Demethylase Activity

CYP1A1 dependent 7-Ethoxyresorufin-O-deethylase (EROD) activity was determined by the method of Burke and Mayer (1974) as modified according to the method of Arıç and Sen (1994). Formation of resorufin from 7-ethoxyresorufin was measured by spectrofluorometric method. Ethoxyresorufin reaction was given in Figure 2.3.

![Figure 2.2 Ethoxyresorufin-O-deethylase reaction.](image)

Typical reaction mixture for determination of ethoxyresorufin-O-demethylase activity was given in Table 2.2, contained 0.1 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl, 1.2 mg BSA, 1.5 mM 7-ethoxyresorufin, 100 μg microsomal protein, 0.5 mM NADPH in a final volume of 2 ml in a fluorometer cuvette. 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). Product formation was quantified by the addition of resorufin. The increase in the fluorescence was recorded to calculate enzyme activity.

Ethoxyresorufin-O-demethylase activity was calculated according to the following formula: (1)

\[
\text{Specific Enzyme Activity} \quad \frac{\text{pmol}}{\text{min}} \quad \text{mg}^{-1} = \frac{\text{OD}_{\text{Reaction}} - \text{OD}_{\text{ZTB}}}{2.4 \times 10^{-3} \mu \text{M}^{-1} \times \text{cm}^{-1}} \times \frac{1}{\text{Protein Amount} (\text{mg})} \times \frac{1}{10 \text{ min}}
\]
Table 2.2  The Constituents of the Incubation mixture for the determination of Ethoxyresorufin-O-demethylase activity of rat liver microsome.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Phosphate Buffer pH 7.8 containing 0.2 M NaCl</td>
<td>200 mM</td>
<td>1 ml</td>
<td>100 mM</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>12 mg/ml</td>
<td>200 µl</td>
<td>1.2 mg/ml</td>
</tr>
<tr>
<td>Ethoxyresorufin</td>
<td>10 µM</td>
<td>300 µl</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>NADPH</td>
<td>10 mM</td>
<td>100 µl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>-</td>
<td>-</td>
<td>100 µg for liver</td>
</tr>
</tbody>
</table>

Final volume was completed to 2 ml with distilled water

2.7.1.2. Determination of Benzphetamine N-Demethylase Activity

CYP2B1 dependent benzphetamine N-demethylase activity of rat liver microsomes was determined colorimetrically by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959) and Adalt and Arinç (1990).

The benzphetamine N-demethylation reaction was given in Figure 2.4.

![Benzphetamine N-demethylation reaction](image)

**Figure 2.3**  Benzphetamine N-demethylase reaction.
A typical assay mixture used for the determination of benzphetamine N-demethylase activity is given in Table 2.3, contained 100 mM HEPES buffer pH 7.7, 1.5 mM benzphetamine, 0.5 mg microsomal protein for rat liver, and finally 0.5 mM NADPH generating system in 0.5 ml final volume. Benzphetamine N-demethylation reaction was initiated by the addition of 0.075 ml of NADPH generating system to incubation mixture and to zero time blanks to which 0.5 ml of 0.75 N perchloric acid was added just before the addition of the cofactor. Then, the reaction was carried out at 37 °C for 10 minutes with constant moderate shaking in a shaking water bath. After 10 minutes, the enzyme reaction was finalized by the addition of 0.5 ml 0.75 N perchloric acid solution. The denatured proteins was transferred to the eppendorf centrifuge tubes, and centrifuged at 16000 x g at microcentrifuge for 20 minutes at 4 °C. After centrifugation, 0.7 ml aliquots was mixed with 0.525 ml Nash reagent (prepared by the addition of 0.4 ml acetyl acetone, just before use, to 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid) again in eppendorf tubes and the mixture was incubated for 10 minutes at 50 °C in a water bath. Then, the formaldehyde amount was determined by measuring the absorbance at 412 nm using spectrophotometer.

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

Benzphetamine N-demethylase activity was calculated according to the following formula: (2)

\[
\text{Specific Enzyme Activity}\quad \text{ (nmol/min)/mg)} = \frac{\text{OD}_{\text{Reaction}} - \text{OD}_{\text{ZTB}}}{2.4 \times 10^{-3} \text{M}^{-1} \times \text{cm}^{-1}} \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{10 \text{ min}}
\]
Table 2.3  The Constituents of the Incubation mixture for the determination of Benzphetamine N-demethylase activity of rat liver microsomes.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes buffer, pH 7.7</td>
<td>400 mM</td>
<td>125 μl</td>
<td>100 mM</td>
</tr>
<tr>
<td>Benzphetamine</td>
<td>7.5 mM</td>
<td>100 μl</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>-</td>
<td>-</td>
<td>0.5 mg for liver</td>
</tr>
<tr>
<td><strong>NADPH generating system</strong></td>
<td></td>
<td></td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>100 mM</td>
<td>12.5 μl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgCl2</td>
<td>100 mM</td>
<td>12.5 μl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Hepes Buffer, pH 7.8</td>
<td>200 mM</td>
<td>36.5 μl</td>
<td>14.6 mM</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>20 mM</td>
<td>12.5 μl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>500 U</td>
<td>1 μl</td>
<td>0.5 U</td>
</tr>
</tbody>
</table>

Final volume was completed to 0.5 ml with distilled water.
2.7.1.3. Determination of Aniline 4-Hydroxylase Activity

CYP2E1 dependent aniline 4-hydroxylase activity of rat liver microsomes was determined by measuring the quantity of p-aminophenol (pAP) formed according to the method of Imai et al. (1966). Aniline 4-hydroxylation reaction is given in Figure 2.5.

![Aniline 4-hydroxylation reaction](image)

**Figure 2.4** Aniline 4-hydroxylation reaction.

**Reagents:**

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

A typical reaction mixture given in Table 2.4 contained 100 mM HEPES buffer pH 7.6, 10 mM aniline, 1.5 mg microsomal protein for liver, and 0.5 mM NADPH generating system in a final volume of 1.0 ml.

NADPH generating system was prepared by adding 0.5 units of glucose 6-phosphate dehydrogenase into a test tube containing 2.5 mM glucose 6-phosphate, 2.5 mM MgCl₂, 14.6 mM HEPES buffer pH 7.8 and 0.5 mM NADP⁺. Then the test tube containing generating system was incubated 37 °C for 5 minutes and finally kept in crushed ice. 1 unit of glucose 6-phosphate dehydrogenase is equal to the amount of enzyme reducing 1 μmole of NADP⁺ in one minute at 25 °C.

The reaction was initiated by the addition of 0.15 ml of NADPH generating system to the microsomal incubation mixture and to zero time blank (ZTB) to which 0.5 ml of 20 % TCA was added before the addition of cofactor. Zero time blanks was prepared to measure non-enzymatic product formation. Reaction was carried out at 37 °C for 25 minutes aerobically with moderate shaking in a water bath. At the end of the incubation time, the reaction was stopped by addition of 0.5 ml 20 % TCA. Then incubation mixture was transferred to ependorf tubes and denaturated proteins were
removed by centrifugation at 13 100 rpm (16 000 x g) for 25 minutes by using Sigma 1-15 microcentrifuge. Finally, 1 ml of supernatant containing product, p-aminophenol, was removed and mixed with 0.5 ml of 20% Na₂CO₃ and with 0.5 ml of 0.4 N NaOH solution containing 4 % phenol. The resulting mixture was incubated at 37 °C for 30 minutes. The intensity of blue color developed was measured at 630 nm using Schimadzu UV-1201 spectrophotometer.

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

Aniline 4-hydroxylase activity was determined according to the following formula: (3)

$$\text{Specific Enzyme Activity} = \frac{\text{OD}_{\text{Reaction}} - \text{OD}_{\text{ZTB}}}{6 \times 10^{-3} \text{µM}^{-1} \times \text{cm}^{-1} \times \frac{1}{\text{Protein Amount}} \times \frac{1}{25 \text{ min}}}$$

Table 2.4 The Constituents of the Incubation Mixture for Determination of Aniline 4-Hydroxylase Activity in Rat Liver Microsome.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes buffer pH 7.6</td>
<td>400 mM</td>
<td>250 µl</td>
<td>100 mM</td>
</tr>
<tr>
<td>Aniline</td>
<td>100 mM</td>
<td>100 µl</td>
<td>10 mM</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>--------</td>
<td>--------</td>
<td>1.5 mg for liver</td>
</tr>
<tr>
<td><strong>NADPH generating system</strong></td>
<td></td>
<td></td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>100 mM</td>
<td>25 µl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>100 mM</td>
<td>25 µl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Hapes Buffer pH 7.8</td>
<td>200 mM</td>
<td>73 µl</td>
<td>14.6 mM</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>20 mM</td>
<td>25 µl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>500 U</td>
<td>1 µl</td>
<td>0.5 U</td>
</tr>
</tbody>
</table>

Final volume was completed to 1 ml with distilled water
2.7.1.4. Determination of N-Nitrosodimethylamine (NDMA) N-Demethylase Activity

CYP2E1 dependent NDMA N-demethylase activity of rat liver microsomes was determined according to the method of Gorsky and Hollenberg (1989) and formaldehyde formed was measured by the method Nash (1953) as modified Cochin and Axelrod (1959). NDMA N-demethylase reaction was shown in Figure 2.6.

![NDMA N-Demethylase Reaction](image)

**Figure 2.5** N-Nitrosodimethylamine (NDMA) N-demethylation reaction.

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

A 0.5 ml of incubation mixture given in Table 2.5 contained 100 mM HEPES buffer pH 7.7, 2.5 mM NDMA, 0.75 mg microsomal protein for liver and 0.5 mM NADPH generating system in final concentration. The enzyme reaction was initiated by the addition of 0.075 ml NADPH generating system to incubation mixture and to zero time blank to which 0.5 ml of 0.75 N perchloric acid was added just before the cofactor. The reaction was carried out at 37 °C for 20 minutes under the air with constant moderate shaking in a water bath. The enzyme reaction was stopped by the addition of 0.5 ml 0.75 N perchloric acid at the end of 20 minutes. Then the contents of the tubes were transferred to the ependorf centrifuge tubes and denatured proteins were removed by the centrifugation at 13 100 rpm (16 000 x g) for 25 minutes. Finally, 0.5 ml aliquots of supernatant were taken and mixed with freshly prepared 0.375 ml of Nash reagent. The mixture was incubated for 10 minutes at 50 °C in a water bath and the intensity of yellow color developed was measured at 412 nm using spectrophotometer.
A 0.5 mM freshly prepared formaldehyde solution was used as a standard as described in Figure 2.7. The tubes containing standards at four different concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity.

![0.5 mM Formaldehyde](image)

**Figure 2.6** Preparation of formaldehyde standards.

N-Nitrosodimethylamine (NDMA) N-demethylase activity was calculated according to the following formula: (4)

\[
\text{Specific Enzyme Activity} = \frac{OD_{\text{Reaction}} - OD_{\text{ZTB}}}{2.4 \times 10^{-3} \mu M^{-1} \times \text{cm}^{-1}} \times \frac{1}{\text{Protein Amount} (\text{mg})} \times \frac{1}{20 \text{ min}}
\]
Table 2.5 The Constituents of the Incubation Mixture for Determination of N-Nitrosodimethylamine (NDMA) N-Demethylase Activity in Rat Liver Microsome.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes buffer, pH 7.7</td>
<td>400 mM</td>
<td>125 µl</td>
<td>100 mM</td>
</tr>
<tr>
<td>NDMA</td>
<td>25 mM</td>
<td>50 µl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>--------</td>
<td>--------</td>
<td>0.75 mg for liver</td>
</tr>
<tr>
<td><strong>NADPH generating system</strong></td>
<td></td>
<td></td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>100 mM</td>
<td>12.5 µl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgCl2</td>
<td>100 mM</td>
<td>12.5 µl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Hepes Buffer, pH 7.8</td>
<td>200 mM</td>
<td>36.5 µl</td>
<td>14.6 mM</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>20 mM</td>
<td>12.5 µl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>500 U</td>
<td>1 µl</td>
<td>0.5 U</td>
</tr>
</tbody>
</table>

Final volume was completed to 0.5 ml with distilled water
2.7.1.5 Determination of Ethylmorphine N-Demethylase Activity

CYP3A4 dependent ethylmorphine N-demethylase activity of rat liver microsomes was determined colorimetrically according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). Figure 2.8 shows the N-demethylation reaction catalyzed by mixed function oxidase.

![Chemical structure of Ethylmorphine and Norethylmorphine]

**Figure 2.7** Ethylmorphine N-demethylation Reaction.

A 0.5 ml of incubation mixture given in Table 2.6 contained 100 mM HEPES buffer pH 8.0, 15 mM Ethylmorphine-HCl, 0.5 mg microsomal protein for liver and 0.5 mM NADPH generating system in final concentration. The enzyme reaction was initiated by the addition of 0.075 ml NADPH generating system to incubation mixture and to zero time blank to which 0.5 ml of 0.75 N perchloric acid was added just before the cofactor. The reaction was carried out at 37 °C for 15 minutes under the air with constant moderate shaking in a water bath. The enzyme reaction was stopped by the addition of 0.5 ml 0.75 N perchloric acid at the end of 15 minutes. Then the contents of the tubes were transferred to the ependorf centrifuge tubes and denatured proteins were removed by the centrifugation at 14 000 x g for 15 minutes. Finally, 0.5 ml aliquots of supernatant were taken and mixed with freshly prepared 0.375 ml of Nash reagent. The mixture was incubated for 10 minutes at 50 °C in a water bath and the intensity of yellow color developed was measured at 412 nm using spectrophotometer.

Ethylmorphine N-demethylase activity was calculated according to the following formula: (5)

\[
\text{Specific Enzyme Activity (nmol/min)/mg)} = \left( \frac{\text{OD}_{\text{Reaction}} - \text{OD}_{\text{ZTB}}}{2.4 \times 10^{-3} \mu M^{-1} \times \text{cm}^{-1}} \right) \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{15 \text{ min}}
\]
**Table 2.6** The Constituents of the Incubation mixture for the determination of Ethylmorphine N-demethylase activity of rat liver microsome.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes buffer, pH 8.0</td>
<td>400 mM</td>
<td>125 μl</td>
<td>100 mM</td>
</tr>
<tr>
<td>Ethylmorphine HCl</td>
<td>75 mM</td>
<td>100 μl</td>
<td>15 mM</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>—</td>
<td>—</td>
<td>0.5 mg for liver</td>
</tr>
<tr>
<td><strong>NADPH generating system</strong></td>
<td></td>
<td></td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>100 mM</td>
<td>12.5 μl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgCl2</td>
<td>100 mM</td>
<td>12.5 μl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Hepes Buffer, pH 7.8</td>
<td>200 mM</td>
<td>36.5 μl</td>
<td>14.6 mM</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>20 mM</td>
<td>12.5 μl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>500 U</td>
<td>1 μl</td>
<td>0.5 U</td>
</tr>
</tbody>
</table>

Final volume is completed to 0.5 ml with distilled water
2.7.2. Determination of Phase II Enzyme Activities

2.7.2.1. Determination of Total GST, GST-Mu and GST-Theta Activities

Glutathione S-transferase (GST) activity of rat liver cytosolic fractions was determined according to the method of Habig et al. (1974) with slight modifications. 1-Chloro-2,4 dinitrobenzene (CDNB) was used as a substrate for determination of total glutathione S-transferase activity. The rate of glutathione conjugate (1-(S-glutationyl)-2,4-dinitrobenzene (DNB-SG)) formation was monitored by following the rate of increase in the absorbance at 340 nm. The reaction catalyzed by glutathione S-transferase is given in Figure 2.9.

\[
\text{CDNB} + \text{Reduced glutathione} \rightleftharpoons \text{Reduced glutathione conjugate (DNB-SG)}
\]

**Figure 2.8** Reaction catalyzed by GST.

Reagents:

Potassium Phosphate Buffer: 50 mM, pH 7.0

GSH: 20 mM

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

A typical assay mixture given in Table 2.7 contained 2500 μl of 50 mM potassium phosphate buffer pH 7.0, 200 μl of 20 mM GSH, 150 μl of cytosolic fractions and 150 μl of 20 mM 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in a final volume of 3 ml. Each time a blank cuvette (reaction with no enzyme) was also used. The reaction was started by addition of 150 μl of 20 mM 1-chloro-2,4-dinitrobenzene (CDNB). Then thioether formation was followed continuously at 340 nm for 2 minutes using spectrophotometer. The absorbance obtained from blank cuvette was subtracted from absorbance of enzymatic reaction to eliminate non-enzymatic product formation. The enzyme activity was calculated by using 9.6 x 10^3 μM⁻¹ x cm⁻¹ as an extinction coefficient of thioether formed by GST.
Total GST activity was determined according to the following formula: (6)

$$\text{Specific Enzyme Activity} \ (\text{nmol/min)/mg}) = \frac{\Delta \text{OD}_{340}}{\text{Extinction coefficient}} \times \frac{\text{Total Volume}}{\text{Protein Amount} \ (\text{mg/ml})} \times \text{DF}$$

**Table 2.7** The Constituents of the Reaction Mixture for Determination of Total GST Activity of Rat Liver Cytosol.

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

Glutathione S-transferase-Mu activity of rat liver cytosolic fractions was determined according to the method of Habig et al. (1974) with slight modifications. 1,2-dichloro-4-nitrobenzene (DCNB) was used as a substrate for determination of glutathione S-transferase mu activity. Formation of thioether was monitored by following the rate of increase in the absorbance at 360 nm.

**Reagents:**

**KPi buffer:** 120 mM, pH 6.5

**GSH:** 20 mM

1,2-dichloro-4-nitrobenzene (DCNB) in ethanol/dH₂O (3/2 v/v); 20 mM (DCNB was first dissolved in absolute ethanol then completed to final volume with distilled water. Ethanol should not exceed to 3 % of assay mixture. Store in dark bottle) It should be prepared fresh.

A typical assay mixture given in Table 2.8 contained 800 µl of 120 mM potassium phosphate buffer pH 6.5, 100 µl of 20 mM GSH, 50 µl of cytosolic fractions and 50 µl of 20 mM 1,2-dichloro-4-nitrobenzene (DCNB) as a substrate in a final volume of 1 ml. Each time a blank cuvette (reaction with no enzyme) was also used. The reaction was started by addition of 50 µl of 20 mM DCNB. Then thioether formation was followed continuously at 360 nm for 2 minutes using spectrophotometer. The absorbance obtained...
from blank cuvette was subtracted from absorbance of enzymatic reaction to eliminate non-enzymatic product formation. The enzyme activity was calculated by using $8.5 \times 10^{-3}$ μM$^{-1}$ x cm$^{-1}$ as an extinction coefficient of thioether formed by GST.

GST-Mu activity was determined according to the following formula: (7)

$$\text{Specific Enzyme Activity} = \frac{\Delta \text{OD}_{360}}{0.0085} \cdot \frac{\text{Total Volume}}{\text{Protein Amount (mg/ml)}}$$

Table 2.8  The Constituents of the Reaction Mixture for Determination of GST-Mu Activity of Rat Liver Cytosol.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Stock Concentration (mM)</th>
<th>Volume to be added (μl)</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Phosphate Buffer, pH 6.5</td>
<td>120</td>
<td>800</td>
<td>96</td>
</tr>
<tr>
<td>1,2-dichloro-4-dinitrobenzene (DCNB)</td>
<td>20</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>Glutathione reduced form (GSH)</td>
<td>20</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Cytosol</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
</tbody>
</table>

Glutathione S-transferase-Theta activity of rat liver cytosolic fractions was determined according to the method of Habig et al. (1974) with slight modifications. 1, 2-epoxy-3 (paranitrophenoxy) propane (EPNP) was used as a substrate for determination of glutathione S-transferase theta activity. Formation of thioether was monitored by following the rate of increase in the absorbance at 360 nm.

**Reagents:**

**Acetate buffer:** 100 mM, pH 6.0

**GSH:** 20 mM

**1, 2-epoxy-3 (paranitrophenoxy) propane (EPNP):** 20 mM (EPNP was first dissolved in absolute ethanol then completed to final volume with distilled water. Ethanol should not exceed to 3 % of assay mixture. Store in dark bottle) It should be prepared fresh.
A typical assay mixture given in Table 2.9 contained 800 μl of 100 mM potassium phosphate buffer pH 6.0, 100 μl of 20 mM GSH, 50 μl of cytosolic fractions and 50 μl of 20 mM EPNP as a substrate in a final volume of 1 ml. Each time a blank cuvette (reaction with no enzyme) was also used. The reaction was started by addition of 50 μl of 20 mM EPNP. Then thioether formation was followed continuously at 360 nm for 2 minutes using spectrophotometer. The absorbance obtained from blank cuvette was subtracted from absorbance of enzymatic reaction to eliminate non-enzymatic product formation. The enzyme activity was calculated by using $0.5 \times 10^{-3} \, \mu M^{-1} \times cm^{-1}$ as an extinction coefficient of thioether formed by GST theta.

GST theta activity was determined according to the following formula: (8)

$$\text{Specific Enzyme Activity} = \frac{\Delta \text{OD}_{360}}{0.0005 \times \text{Protein Amount}}$$

**Table 2.9** The Constituents of the Reaction Mixture for Determination of GST theta Activity of Rat Liver Cytosol.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Stock Concentration (mM)</th>
<th>Volume to be added (μl)</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate Buffer, pH 6.0</td>
<td>100</td>
<td>800</td>
<td>80</td>
</tr>
<tr>
<td>1, 2-epoxy-3 (paranitrophenoxy) propane (EPNP)</td>
<td>20</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>Glutathione reduced form (GSH)</td>
<td>20</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Cytosol</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
</tbody>
</table>
2.7.2.2. Determination of NAD(P)H:Quinone Oxidoreductase 1 (NQO1) Activity

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) activity of rat liver cytosol was determined according to the method of Ernster (1967). 2,6-Dichlorophenolindophenol (DCPIP) was used as a substrate for NQO1 and DCPIP reduction was monitored continuously by following the decrease in the absorbance at 600 nm. The reaction catalyzed by NQO1 was given in Figure 2.10.

![Figure 2.9](image)

**Figure 2.9** The reaction catalyzed by NQO1.

**Reagents:**

1. **Potassium Phosphate Buffer:** 0.1 M, pH 7.4
2. **NADPH:** 10 mM (Prepared fresh)
3. **2,6-Dichlorophenolindophenol (DCPIP):** 0.5 mM (Light sensitive, prepared fresh)
4. **Dicoumarol:** 0.5 mM (Prepared fresh)
5. **BSA:** 28 mg/ml

A typical assay mixture described in Table 2.10 contained, 250 µl of 0.1 M potassium phosphate buffer pH 7.8, 25 µl of 28 mg/ml BSA, appropriate amount of cytosolic fraction and 80 µl of 0.5 mM 2,6-dichlorophenolindophenol (DCPIP) in a final volume of 1 ml. Reaction was started by adding 20 µl of 10 mM NADPH to the reaction mixture and to zero time blank (ZTB) to which 40 µl of 0.5 mM dicoumarol was added before the addition of cofactor. Zero time blank was prepared to measure the non-enzymatic product formation. DCPIP reduction reaction was followed continuously at 600 nm for 2 minutes against the blank containing only buffer by using spectrophotometer. The enzyme activity was calculated using an extinction coefficient of 0.021 µM⁻¹ x cm⁻¹ when DCPIP used as a substrate.

NQO1 activity was determined according to the following formula: (9)

\[
\text{Specific Enzyme Activity (nmol/min)/mg) = \frac{\Delta OD_{Sample} - \Delta OD_{ZTB}}{2.1 \times 10^{-2} \text{µM}^{-1} \times \text{cm}^{-1} \times \text{Total Volume (mg/ml)} \times \text{Protein Amount (mg/ml)} \times \text{DF}}
\]
<table>
<thead>
<tr>
<th>Constituents</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Phosphate Buffer, pH 7.8</td>
<td>100 mM</td>
<td>250 µl</td>
<td>25 mM</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>28 mg/ml</td>
<td>25 µl</td>
<td>0.7 mg/ml</td>
</tr>
<tr>
<td>Cytosol</td>
<td>--------</td>
<td>--------</td>
<td>2 - 4 mg/ml</td>
</tr>
<tr>
<td>NADPH</td>
<td>10 mM</td>
<td>20 µl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>2,6 Dichlorophenol-indophenol (DCPIP)</td>
<td>0.5 mM.</td>
<td>80 µl</td>
<td>0.04 mM</td>
</tr>
</tbody>
</table>

Complete to final volume to 1 ml with distilled water
2.7.3. Determination of Antioxidant Enzyme Activities

2.7.3.1. Determination of Total SOD Activity

Superoxide dismutase (SOD) activity was determined using the method described by Marklund and Marklund (1974), where the superoxide radical is generated by the autoxidation of pyrogallol under alkaline pH with the action of atmospheric oxygen. As superoxide builds in the solution, the formation of yellow chromophore of oxidized pyrogallol accelerates because superoxide reacts with pyrogallol (Fig. 2.11). Increased level of SOD activity decreases the level of oxidized pyrogallol and this change was followed at 440 nm for 3 min.

![Structure of Pyrogallol molecule.](image)

**Figure 2.10** Structure of Pyrogallol molecule.

**Reagents:**
1. Tris-EDTA, pH 8.2 buffer:
2. Pyrogallol: 10 mM (Prepared fresh)

Standard enzyme assay mixture was prepared by adding 2.8 ml Tris-EDTA, pH 8.2 buffer and varied volume (5, 10, 15, 20, 25, 30, 40, 50 µl) 1000 x g cytosolic fractions into the 3 ml cuvettes. Final volume was completed to 2.925 ml with TE buffer pH 8.2. The assay was initiated by adding 75 µl pyrogallol and the rate of autoxidation was followed at 440 nm for 3 minutes. Enzyme blank was used to determine uninhibited autoxidation.

Percent inhibition of pyrogallol autoxidation was calculated according to equation given below for each protein amount that was added to the reaction medium: (10)

\[
\text{% Inhibition} = 100 \times \frac{\Delta \text{OD}_{\text{Blank}} - \Delta \text{OD}_{\text{Sample}}}{\Delta \text{OD}_{\text{Blank}}}
\]

Percent inhibition versus mg protein graphs was constructed and converted into percent inhibition versus log protein graphs. Then, 1 unit total SOD activity was calculated as the amount of protein causing 50% inhibition of pyrogallol autoxidation.

In order to find out the amount of total SOD activity in 1 mg protein containing homogenate, the following equation was applied: (11)

\[
\text{Specific Enzyme Activity (Unit/mg)} = \frac{1}{\text{Protein Amount (50% Inhibition of Autoxidation of Pyrogallol)}}
\]
2.7.3.2. Determination of Glutathione Peroxidase Activity

Glutathione Peroxidase (GPx) activity was determined using the method described by Paglia and Valentine (1967), where the rate of NADPH oxidation was followed with the decrease in the absorbance at 340 nm. As shown in Figure 2.12, GPx produces oxidized glutathione and the degree of NADPH reduction is directly proportional to GPx activity.

![Figure 2.11: GPx reaction.](image)

Standart 3ml enzyme assay mixture was prepared as given in Table 2.11 and contained 85 mM Tris-HCl, pH 8.0, 2 mM GSH, 0.24 U glutathione reductase, 100 µl of cytosol, and 0.066 mM NADPH. The assay was initiated by adding 100 µl NADPH into the reaction medium and the reduction of absorbance was monitored at 340 nm for 3 min. A molar extinction coefficient of 6220 M⁻¹ x cm⁻¹ was used for calculation of enzyme activity. Glutathione peroxidase activity was described as the amount of NADPH consumed in 1 minute by 1 mg protein containing cytosolic fraction.

Glutathione Peroxidase (GPx) activity was determined according to the following formula: (12)

\[
\text{Specific Enzyme Activity} = \frac{\Delta OD}{6220 \text{ M}^{-1} \times \text{cm}^{-1}} \times \frac{\text{Total Volume}}{\text{Protein Amount}} \times \text{DF}
\]

\[
\text{(µmol/min)/mg)
\]

54
### Table 2.11

The Constituents of the Reaction Mixture for Determination of GPx Activity of Rat Liver Cytosol.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 8.0</td>
<td>0.1 M</td>
<td>2525 µl</td>
<td>85 mM</td>
</tr>
<tr>
<td>Reduced Glutathione</td>
<td>80 mM</td>
<td>75 µl</td>
<td>2 mM</td>
</tr>
<tr>
<td>Glutathione Reductase</td>
<td>--------</td>
<td>100 µl</td>
<td>0.24 U</td>
</tr>
<tr>
<td>Cytosol</td>
<td>--------</td>
<td>100 µl</td>
<td>40 fold diluted</td>
</tr>
<tr>
<td>NADPH</td>
<td>2 mM</td>
<td>100 µl</td>
<td>0.066 mM</td>
</tr>
</tbody>
</table>

Final volume was completed to 3 ml.

#### 2.7.3.3. Determination of Lactate Dehydrogenase (LDH) Activity

Lactate dehydrogenase (LDH) activity determination of blood serum was carried out using the spectrophotometric method of Wroblewski and La Due (1955). The activity of LDH was measured by monitoring the rate at which the substrate, pyruvate was reduced to lactate. The reduction was coupled with the oxidation of NADH, which was followed spectrophotometrically in terms of reduced absorbance at 340 nm. LDH reaction was shown in Figure 2.13

![Figure 2.12](image)  
**Figure 2.12**  
Lactate dehydrogenase reaction.

The reaction mixture was contained 2.85 ml of 0.1 M phosphate buffer, pH 7.5, 0.05 ml serum and 0.2 mg NADH. All the tubes were mixed and left in 25 °C for 20 minutes. Then 0.1 ml of 22.7 mM Na-pyruvate in 0.1 M phosphate buffer, pH 7.5 was added to the tubes. After mixing the tubes, the reaction was monitored at 340 nm for 3 minutes against water as reference.
The LDH activity was determined according to the following formula: (13)

\[
\text{Serum LDH Activity (Unit/ml)} = \frac{\Delta \text{OD}_{340}}{(0.001 \times \text{Volume of Serum})}
\]

Where: 0.001 = OD\text{\textsubscript{340}} equivalent to 1 unit of LDH activity in a 3 ml volume with 1 cm light path at 25 °C.

2.7.3.4. Determination of Arylesterase (ARE) Activity

Arylesterase activity (PON1) was determined using the method described by Rodrigo et al. (1997) where the rate of phenylacetate hydrolysis by arylesterase was followed by the formation of hydrolysis product phenol (Figure 2.14).

![Phenol (270 nm)](image)

**Figure 2.13** The reaction catalyzed by arylesterase.

Standart 3ml enzyme assay mixture was prepared as given in Table 2.12 and contained 9 mM Tris-HCl, pH 8.0, 0.9 mM CaCl\textsubscript{2}, 20 μl microsome, and 3.6 mM phenylacetate. The assay was initiated by adding 100 μl phenylacetate solution and the formation of phenol was monitored by its absorbance at 270 nm and at 37 °C with spectrophotometer. A molar extinction coefficient of 1310 M\textsuperscript{-1} x cm\textsuperscript{-1} was used for calculation of enzyme activity, which was expressed in unit per liter (U/ml). One unit of arylesterase activity is defined as 1μmol phenol formed per minute under the given conditions.

Arylesterase activity was determined according to the following formula: (14)

\[
\text{Specific Enzyme Activity (\mu mol/min)/mg) = } \frac{\Delta \text{OD}}{1.31 \text{ mM}^{-1} \times \text{cm}^{-1}} \times \frac{\text{Total Volume}}{\text{Protein Amount (mg/ml)} \times DF}
\]
<table>
<thead>
<tr>
<th>Constituents</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 8.0</td>
<td>10 mM</td>
<td>2700 µl</td>
<td>9 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>50 mM</td>
<td>54 µl</td>
<td>0.9 mM</td>
</tr>
<tr>
<td>Microsome</td>
<td>--------</td>
<td>20 µl</td>
<td>--------</td>
</tr>
<tr>
<td>dH₂O</td>
<td>--------</td>
<td>126 µl</td>
<td>--------</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>108 mM</td>
<td>100 µl</td>
<td>3.6 mM</td>
</tr>
</tbody>
</table>
2.8. Determination of Protein Expressions by Western Blotting Technique

Effects of *Epilobium hirsutum* L. on protein expression of rat liver proteins were analyzed by Western blot analysis, essentially as described by Towbin et al. (1979). Before western blotting, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by using 4% stacking gel and 8.5-12% separating gels (depending on the molecular weight of proteins) in a discontinuous buffer system as described by Laemmli (1970). Separating and stacking gel solutions was prepared just before use as given in Table 2.13 in a given order.

Table 2.13 Components of separating and stacking gel solutions.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Separating Gel 0.375 M Tris, pH 8.8</th>
<th>Stacking Gel 0.125 M Tris, pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer Conc.</td>
<td>8.5 %</td>
<td>12 %</td>
</tr>
<tr>
<td>Gel solution (ml)</td>
<td>8.5</td>
<td>12</td>
</tr>
<tr>
<td>dH₂O (ml)</td>
<td>13.55</td>
<td>10.035</td>
</tr>
<tr>
<td>Separating gel buffer (ml)</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Stacking gel buffer (ml)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>10% ammonium per sulfate (ml)</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>TEMED (ml)</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>Total volume (ml)</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

SDS–PAGE was performed on 8.5% (for CYP isoforms) and 12% (for GSTs, NQO1 and GPx) separating gels in a discontinuous buffer system. Vertical slab gel electrophoresis was carried out using Mini-PROTEAN tetra cell mini trans blot module (Bio-Rad, Richmond, CA). Sandwich unit of module was set up by using two glass plates with 1 cm space. Separating gel solution was prepared as given Table 2.13 and immediately transferred into the sandwich unit. The top of the separating gel solution was covered with isopropanol alcohol in order to obtain smooth gel surface as well as fast polymerization. After polymerization of separating gel solution, the alcohol was poured
off completely and then freshly prepared stacking gel solution was poured into the plates and 1 mm teflon comb with 15 wells was inserted into stacking gel solution. After polymerization, comb was removed and wells were filled with electrode running buffer (25 mM Tris, 192 mM glycine and 0.1 %) using syringe to remove air bubbles and unpolymerized gel particles. Then the running module was filled with electrode running buffer. Protein samples were diluted with 4X sample dilution buffer (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 90 seconds. Each sample was applied to different wells. After application, gel-running module was placed in the main buffer tank filled with electrode running buffer. The electrophoresis unit was connected to the power supply Bio-Rad power pac basic power supply and electrophoresis was run at 10 mA-100V in stacking gel and 20 mA-200V in separating gel. When electrophoresis was completed, gel was removed for western bolt.

Before western blot, gel was equilibrated in transfer buffer (25 mM Tris, 192 mM, glycine and 20 % methanol) for 15 minutes in order to adjust final size of gel and remove buffer salts and SDS which was used in SDS-PAGE. Then transfer sandwich was prepared as given in Figure 2.15.

![Figure 2.14](image)

**Figure 2.14** Preparation of western blot sandwich.
Proteins were transferred from the gel to a nitrocellulose membrane by using the trans-blot electrophoretic transfer cell (Bio-Rad, Richmond, CA) containing Tris-glycine buffer, pH 8.3 and methanol. The transfer was carried out at 0–4 °C for 90 min at 90 V (400 mA). After transferring the protein to membrane, membrane was equilibrated in TBS (20 mM Tris pH 7.4, 500 mM NaCl) for 10 minutes. Then nitrocellulose membrane was incubated in blocking solution (TBS with 5% non-fat dried milk). Immunochemical staining of the separated proteins on the nitrocellulose sheet was done by using the diluted anti-rat primary antibodies (1:1000) in TBS containing 5% non-fat dried milk and nitrocellulose sheet was incubated at room temperature for 2 h with shaking. After three washes with TBST (20 mM Tris pH 7.4, 500 mM NaCl, 0.05% Tween 20), the blot was further incubated with alkaline phosphatase or HRP conjugated secondary antibodies (1:5000) for 1 h and then washed three times with TBST. In order to detect antibody-bound immunoreactive proteins, nitrocellulose sheets were incubated with alkaline phosphatase substrate solution given in Table 2.14 as described by Ey and Ashman (1986) or Pierce ECL Western Blotting HRP Substrate solution which was prepared by mixing 1 volume of solution A and 1 volume of solution B. The gels were photographed using a computer-based gel imaging instrument and analyzed using the Image J software (Bethesda, MA).

Table 2.14 Preparation of alkaline phosphatase substrate solution for immunodetection.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stock Concentration</th>
<th>Volume to be added</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8,8</td>
<td>2,67 ml</td>
<td></td>
</tr>
<tr>
<td>1 M NaCl</td>
<td></td>
<td>4 ml</td>
</tr>
<tr>
<td>100 mM MgCl2</td>
<td></td>
<td>0,82 ml</td>
</tr>
<tr>
<td>100 mM ZnCl2</td>
<td></td>
<td>0,04 ml</td>
</tr>
<tr>
<td>DEA</td>
<td></td>
<td>0,096 ml</td>
</tr>
<tr>
<td>NBT</td>
<td></td>
<td>12,2 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>Complete to 40 ml</td>
</tr>
<tr>
<td><strong>Solution B</strong></td>
<td>phenazine methosulfate</td>
<td>2 mg/ml in distilled water</td>
</tr>
<tr>
<td><strong>Solution C</strong></td>
<td>N,N-dimethylformamide</td>
<td>5,44 mg/ 0,136 ml N,N-dimethyl formamide</td>
</tr>
</tbody>
</table>

Finally NBT/BCIP substrate solution is prepared by mixing **solution A** with **solution C** and 0,268 ml of **solution B**.
2.9. Determination of mRNA Expressions by Quantitative-Real Time PCR

2.9.1. Total RNA Isolation from Rat Liver

Total RNA was isolated from rat liver by the method of Chomczynski and Sacchi (2006) using TRizol with some modifications. All plastic and glass equipments for RNA isolation were treated with distilled water containing 0.1 % (v/v) diethylpyrocarbonate (DEPC) in order to inhibit RNAase activity. Excess DEPC was evaporated under hood for overnight and autoclaved before use. Distilled water for preparing solutions was also treated with 0.1 % (v/v) DEPC and then autoclaved.

70 mg of rat liver tissue was homogenized at room temperature (RT) with 0.7 ml TRizol in a glass-teflon homogenizer 5 seconds for 2-3 times. The homogenate was transferred into a 2 ml eppendorf tube and incubated for 5 min at room temperature. Then 0.1 ml cold chloroform was added and the tube was shaked vigorously by hand and incubated at room temperature for 2-3 min. Following phenol- chloroform extraction, the tube was centrifuged at 12000 x g for 15 min at +4 °C. After centrifugation, three layers were formed. The lower red part is the phenol-chloroform phase; the interphase; and the colorless upper phase. RNA was remained exclusively in the upper aqueous phase. The upper aqueous phase was transferred to a clean tube and same amount of cold isopropanol was added and mixed by hand. The mixture was incubated at RT for 10 min and centrifuged at 13 000 x g for 5 min at +4 °C. After centrifugation supernatant was removed and 1 ml cold 75 % ethanol was added and the pellet was resuspended by hand. The tube then centrifuged at 7500 x g for 5 min at +4°C. After centrifugation the supernatant was removed and the pellet was dried in the hood for 30 min. Then 75 μl of RNAse/DNAse free water was added into the tube and mixed by pipetting. Finally, the tube was incubated at 50 °C for 10 min and after incubation it was stored at -20 °C.

2.9.2. Determination of RNA Concentration

The isolated RNA was quantified by measuring the absorbance at 260 nm. Purity assessed by the 260 nm/280nm ratio. The ratio of OD<sub>260</sub> / OD<sub>280</sub> must be between 1.8 and 2.2. Below 1.8 refers the DNA contamination and above 2.2 refers the protein contamination. The optical density of 1.0 corresponded to the 40 μg/ml for RNA. The concentration and purity of the RNA were measured at Nano drop.

2.9.3. Qualification of RNA by Agarose Gel Electrophoresis

Presence and purity of RNA was checked on 1 % (w/v) agarose gel by using horizontal agarose gel electrophoresis unit. 1 % (w/v) agarose was prepared by mixing 0.5 g of agarose with 50 ml 0.5 X TBE (Tris-Borate-EDTA) buffer, pH 8.3. The grains of agarose were dissolved in microwave oven. The solution was cooled approximately 60 °C and 5 μl from 10 mg/ml ethidium bromide solution was added and the solution was mixed thoroughly. Agarose gel solution was poured into electrophoresis tray and comb was placed in order to well formation. After solidification, gel tank was filled with 0.5 X TBE buffer. The comb was removed. 5 μl of RNA solution was mixed with 1 μl of loading buffer (6X Loading dye) and the mixture was loaded into the wells.
Electrophoresis was performed at 90 mV for 1 hour. The gel was observed under UV light and photographed.

2.9.4. cDNA Synthesis

Reverse transcription of RNA to cDNA was carried out using Moloney-Murine Leukemia Virus Reverse Transcriptase and Oligo (dT) 18 Primer (Fermentas, Hanover, MD, USA). 20 μL of reverse transcription reaction contained 2 μg total RNA, 4 μL of buffer, 1μL of oligo dT (18), 2 μL of 10 mM dNTP, 1μl of ribolock. The mixture was incubated at 70 °C for 5 minutes, and then 1μL of Moloney-Murine Leukemia Virus Reverse Transcriptase was added. The resulting mixture was initially incubated at 42 °C for 60 minutes, and then heating the mixture to 70 °C for 10 minutes inactivated the reverse transcriptase. cDNA obtained was stored at -80 °C until further use.

2.9.5. Q-RT PCR

The effect of *Epilobium hirsutum* L. on gene expressions was analyzed by quantitative Real Time PCR (qRT-PCR) using Corbett Rotor Gene 6000 (Corbett Life Science, PO Box 435, Concorde, NSW 2137). The final reaction mixture (25μl) contained 500 ng cDNA, 0.5mM reverse and forward primers and 1X Maxima® SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD) and RNase free ddH2O. In order to detect DNA contamination NTC (no template control) was used during the reaction. As an internal standard, _housekeeping_ gene GAPDH was used. DNA was amplified in a reaction mixture containing the primers as given in Table 2.15. The qRT-PCR program consisted of the following temperature profile; initial denaturation at 95 °C for 10 min, 40 cycles of melting at 95 °C for 20 second, annealing temperature varied depending on the gene as shown in Table 2.15, for 30 sec, and extension at 72 °C for 30 second. The primers were designed using Primer 3 program and entered into the NCBI Blast to confirm the specificity for *Rattus norvegicus*. Each set of primer was carefully chosen to avoid primer dimerization and to ensure specificity of amplification. Specific annealing temperatures were verified by the use of a gradient thermal cycler.

Fold of inhibition/induction was calculated by the following formula: $2^{ΔΔCt}$,

Where $ΔΔCt$: $ΔCt$ (Treatment) - $Ct$(Control);

$ΔCt$ (Treatment): $ΔCt$ (Target Gene) - $ΔCt$(GAPDH);

$ΔCt$(control): $ΔCt$ (Target Gene) - $ΔCt$(GAPDH).
<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Reference Sequence</th>
<th>Sequence (5'→3')</th>
<th>Annealing Temp. (°C)</th>
<th>Product size (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>NM_012540.2</td>
<td>F→ GGAACTCCTTGATGGATCACCCR→ ATGCCAATGTCCAGTCTCT</td>
<td>58</td>
<td>393</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>NM_001134844.1</td>
<td>F→ CAGTGCTCCACAGGATTTCA R→ TGGACCTTCTCTGCAACGTG</td>
<td>51</td>
<td>201</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>NM_031543.1</td>
<td>F→ GTCTGAGGCTCATGATTTG R→ TCTGAAACTCATGGCTG</td>
<td>55</td>
<td>628</td>
</tr>
<tr>
<td>CYP3A1</td>
<td>NM_013105.2</td>
<td>F→ AATTCGATGTGGAGTGCCAT R→ CGGATAGGGCTGTATGAGATTC</td>
<td>56</td>
<td>696</td>
</tr>
<tr>
<td>GPx</td>
<td>NM_183403.2</td>
<td>F→ ACCGATCCAAGCTCATCA R→ CTCAAAGTTCAGGACACATCTG</td>
<td>54</td>
<td>64</td>
</tr>
<tr>
<td>GSTMu</td>
<td>NM_020540.1</td>
<td>F→ GAACGTTCGCGGACTTACTCA R→ ACGTATCTTTCTCTCCTCATAGTTTGAATCT</td>
<td>56</td>
<td>78</td>
</tr>
<tr>
<td>GSTT1</td>
<td>NM_053293.2</td>
<td>F→ ATGGTCTTGAAACTGTACR→ AGTACAGGTCAGCCAGG</td>
<td>58</td>
<td>510</td>
</tr>
<tr>
<td>NQO1</td>
<td>NM_017000.3</td>
<td>F→ TCCGCCCAACTTCTG R→ TCTGGTGCCGCAATCA</td>
<td>56</td>
<td>63</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008.3</td>
<td>F→ TGATGACATCAAGAAGGGTGAG R→ TCCTGGAGGCACATGTCG</td>
<td>60</td>
<td>240</td>
</tr>
</tbody>
</table>
2.10. **Statistical Analysis**

Statistical analyses were performed by student t-test using GraphPad Prism 5.0 statistical software package for Windows (La Jolla, CA). All results were expressed as means with their Standard Error of Means (SEM). P<0.05 was taken as the minimum level of significance.
CHAPTER 3

RESULTS

3.1. Yield of EH Plant Extract

*EH* extract prepared on October 2009: 5.5 g of lyophilized powder was obtained from 50 g plant.

Yield (%) = 5.5 x 100 / 50 = 11 %

*EH* extract prepared on November 2009: 7.5 g of lyophilized powder was obtained from 50 g plant.

Yield (%) = 7.5 x 100 / 50 = 15 %

Plant extract obtained in the form of powder after lyophilization was dissolved in dH₂O or related buffer solution.

3.1.1. Qualitative Determination of Active Substance Groups of *EH*

Active substance groups of *EH* (lyophilized powder of *EH*) were determined using a number of reactions explained in the Material and Method and the results were given in Table 3.1.

**Table 3.1** Active substance groups of Epilobium hirsutum L.

<table>
<thead>
<tr>
<th>Active Substance Groups</th>
<th>Reaction Type</th>
<th>Epilobium hirsutum (Herbal) AEF 25 812</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylic Heterosides</td>
<td>Keller - Kiliani Test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Baljet Test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Liebermann - Burchard Test</td>
<td>+</td>
</tr>
<tr>
<td>Saponozits</td>
<td>Stable foam</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>10 % of NH₃</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Alkaline Lead subacetate</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>FeCl₃ solution</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Cyanide reaction</td>
<td>+</td>
</tr>
<tr>
<td>Antosiyanozits</td>
<td>Diluted H₂SO₄</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NaOH-HCl</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lead subacetate</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Amyl alcohol</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Diluted H₂SO₄ - Amyl alcohol</td>
<td>+</td>
</tr>
<tr>
<td>Cyanogenetic Glycoside</td>
<td>Formation of brick-red color in Sodium picric paper</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>heavy-metal salts</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5 % FeCl₃ solution</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1 % salty gelatin solution</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>bromic water</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Stiasny reactant</td>
<td>-</td>
</tr>
<tr>
<td>Anthracene</td>
<td>Formation of yellow-red color in ammoniac phase</td>
<td>-</td>
</tr>
<tr>
<td>Coumarin</td>
<td>High florescence of ammoniac phase at UV₂₆₆ nm</td>
<td>+</td>
</tr>
<tr>
<td>Volatile Oil</td>
<td>Odor</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Reaction was observed; ++ Strong Reaction was observed; - No reaction
3.1.2. Total Phenolic Content and LC-MS/MS Analyses of Phenolic Compounds of *Epilobium hirsutum* L.

Total phenolic content of the *EH* was found as 4.03 ± 0.12 mg of GAE/100 g of dry weight. In the present study, gallic acid, vanilic acid, coumaric acid, p-coumarin, kaempferol, myricetin, quercetin and ellagic acid were used as standart compounds in order to identify and quantitate the polyphenolic substances of *EH*. *EH* and standart compounds were dissolved in dH₂O except ellagic acid which was dissolved in DMSO. LC-MS/MS analyses of *EH* showed that plant extract consists of 0.47 ± 0.0006 ppm p-coumarin, 36.61 ± 0.63 ppm gallic acid, 0.044 ± 0.0002 ppm kaempferol, 8.8 ± 0.11 ppm ellagic acid and also low concentration of vanilic acid, coumaric acid, myricetin and quercetin (≤ 0.01 ppm) (Figure 3.1 and Figure 3.2).

![Figure 3.1](image.png)

**Figure 3.1** LC-MS/MS profile of *EH* water extract. 10 ppm standards and 10 mg/ml *EH* were applied to column. Peak assignment: 1: Gallic acid, 2: Valinic acid, 3: Coumaric acid, 4: p-coumarin, 5: Kaempferol, 6: Myricetin, 7: Quercetin.
Figure 3.2  LC-MS/MS profile of EH water extract. 10 ppm standard and 10 mg/ml 
EH were applied. Peak assignment: 1: Ellagic acid.

3.2.  Protein Concentration of Rat Liver Fractions

Rat liver post mitochondrial (S1.5), cytosolic and microsomal protein
concentrations were determined by BCA method. BSA standards ranging from 0 to 200
µg were used in order to construct standard curve. Protein concentrations in rat liver post
mitochondrial (S1.5), cytosolic and microsomal fractions were given in Figure 3.3.

Figure 3.3  Protein concentrations in rat liver post mitochondrial (S1.5), cytosolic and
microsomal fractions.
3.3. Cytotoxic Effect of *Epilobium hirsutum* L. on Rat Liver

Cytotoxic effect of *Epilobium hirsutum* L. was determined by measuring blood serum lactate dehydrogenase activity (LDH) using the method of Wroblewski and La Due (1955). In this method, formation of reduced NADH was measured continuously at 340 nm for 3 minutes. Spectrophotometric analysis of LDH was shown that *EH* does not cause any cytotoxicity on rat liver (1.01 fold, p=0.387) as given in Figure 3.4.

![Graph showing lactate dehydrogenase activity](image)

**Figure 3.4** The cytotoxic effect of *EH* on rat liver. Cytotoxicity was determined by measuring blood serum lactate dehydrogenase activity. Values are mean ± SD for triplicate determinations.
3.4. Effects of *Epilobium hirsutum* L. on Cytochrome P450 Dependent Phase I, Phase II and Antioxidant Enzyme Activities

Effects of i.p. administered *EH* extract on rat liver Phase I enzymes; CYP1A1 dependent EROD, CYP2B6 dependent benzphetamine N-demethylase, CYP2E1 dependent aniline 4-hydroxylase and NDMA N-demethylase, CYP3A4 dependent ethylmorphine N-demethylase, Phase II enzymes; total, theta and Mu glutathione S-transferase (GST, GSTT and GSTMu), NADPH:Quinone oxidoreductase 1 (NQO1) and antioxidant enzymes; superoxide dismutase (SOD), glutathione peroxidase (GPx) and arylesterase (ARE) activities were determined by spectroscopic techniques using enzyme specific substrates.

3.4.1. Effects of *Epilobium hirsutum* L. on Cytochrome P450 Dependent Phase I Enzyme Activities

3.4.1.1. Effects of *Epilobium hirsutum* L. on Ethoxyresorufin-O-Deethylase (EROD) Activity

CYP1A1 catalyzed ethoxyresorufin-O-deethylase (EROD), an important CYP isoform for the formation of mutagenic metabolites, was measured by spectrofluorometry using 7-ethoxyresorufin as a substrate. Nine days of 37.5 mg/kg *EH* injection of male Wistar Albino rats significantly inhibited (1.46 fold) liver EROD activity from 6.54 ±1.21 pmol/min/mg to 4.48 ± 1.67 pmol/min/mg (p=0.0003). The effect of *EH* on rat liver microsomal EROD activity was shown in Figure 3.5.

**Figure 3.5** The effect of *EH* on rat liver CYP1A1 dependent EROD activity. Male Wistar rats were divided into two groups; Control rats (*n*=10) and *Epilobium hirsutum* treated rats (EHT) (*n*=30). Experiments were repeated at least 3 times. **Significantly different from respective control value (p=0.0003).
3.4.1.2. Effects of *Epilobium hirsutum* L. on Benzphetamine N-Demethylase Activity

CYP2B associated microsomal benzphetamine N-demethylase activity was determined colorimetrically by measuring the amount of formaldehyde formed using benzphetamine as a substrate according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). *EH* treatment of rats significantly inhibited (2.12 fold) liver microsomal benzphetamine N-demethylase activity from 1.42 ± 0.53 nmol/min/mg to 0.67 ± 0.34 nmol/min/mg (p<0.0001). The effect of *EH* on rat liver microsomal benzphetamine N-demethylase activity was given in Figure 3.6.

**Figure 3.6** The effect of *EH* on rat liver CYP2B1 dependent benzphetamine N-demethylase activity. Male Wistar rats were divided into two groups; Control rats (*n*=10) and *Epilobium hirsutum* treated rats (*EHT*) (*n*=30). Experiments were repeated at least 3 times. **Significantly different from respective control value (p<0.0001).**
3.4.1.3. Effects of *Epilobium hirsutum* L. on Aniline 4-Hydroxylase Activity

Rat liver CYP2E1 dependent microsomal aniline 4-hydroxylase activity was determined colorimetrically by measuring the quantity of *p*-aminophenol (pAP) formed using aniline as a substrate according to the method of Imai et al. (1966). Injection of *EH* extract to animals significantly inhibited (4.93 fold) rat liver microsomal aniline 4-hydroxylase activity from 0.537 ± 0.011 nmol/min/mg to 0.109±0.01 nmol/min/mg (p<0.0001). The effect of *EH* on rat liver microsomal aniline 4-hydroxylase activity was shown in Figure 3.7.

![Graph](image)

**Figure 3.7** The effect of *EH* on rat liver CYP2E1 dependent aniline 4-hydroxylase activity. Male Wistar rats were divided into two groups; Control rats (n=10) and *Epilobium hirsutum* treated rats (EHT) (n=30). Experiments were repeated at least 3 times. **Significantly different from respective control value (p<0.0001).**
3.4.1.4. Effects of *Epilobium hirsutum* L. on N-Nitrosodimethylamine (NDMA) N-Demethylase Activity

CYP2E1 dependent microsomal NDMA N-demethylase activity was determined colorimetrically by measuring the amount of formaldehyde formed using NDMA as a substrate according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). *EH* injection of rats slightly inhibited (1.2 fold) liver microsomal NDMA N-demethylase activity from 0.192 ± 0.011 nmol/min·mg to 0.159±0.012 nmol/min·mg (p<0.05). The effect of *EH* on rat liver microsomal NDMA N-demethylase activity was given in Figure 3.8.

![Graph showing the effect of EH on rat liver CYP2E1 N-demethylase activity](image)

**Figure 3.8** The effect of *EH* on rat liver CYP2E1 dependent NDMA N-demethylase activity. Male Wistar rats were divided into two groups; Control rats (n=10) and *Epilobium hirsutum* treated rats (EHT) (n=30). Experiments were repeated at least 3 times. *Significantly different from respective control value (p<0.05).
3.4.1.5. **Effects of *Epilobium hirsutum* L. on Ethylmorphine N-Demethylase Activity**

Microsomal ethylmorphine N-demethylase activity (CYP3A1 dependent) was determined colorimetrically by measuring the amount of formaldehyde formed using ethylmorphine-HCl as a substrate according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). *EH* injection of rats significantly inhibited (8.68 fold) liver microsomal ethylmorphine N-demethylase activity from $2.43 \pm 0.43 \text{nmol/min/mg}$ to $0.28\pm0.04 \text{nmol/min/mg}$ ($p<0.0001$). The effect of *EH* on rat liver microsomal ethylmorphine N-demethylase activity was shown in Figure 3.9.

![Bar chart](image.png)

**Figure 3.9** The effect of *EH* on rat liver CYP3A1 dependent ethylmorphine N-demethylase activity. Male Wistar rats were divided into two groups; Control rats ($n=10$) and *Epilobium hirsutum* treated rats (EHT) ($n=30$). Experiments were repeated at least 3 times. ***Significantly different from respective control value ($p<0.0001$).
3.4.2. Effects of *Epilobium hirsutum* L. on Rat Liver Phase II Enzyme Activities

3.4.2.1. Effects of *Epilobium hirsutum* L. on Total GST, GST-Mu and GST-Theta Activities

Rat liver total GST and its isoforms GST Mu and GST Theta enzyme activities were determined according to the method of Habig et al. (1974) by using 1- Chloro-2,4 dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and 1,2-epoxy-3 (paranitrophenoxy) propane (EPNP) as a substrate, respectively. Reactions were started by adding substrate into the reaction medium and glutathione conjugate formation was monitored by following the rate of increase in the absorbance at 340 nm for 3 minutes. 9 days of 37.5 mg/kg *EH* injection of 4 months old male Wistar Albino rats significantly inhibited liver cytosolic total GST (2.16 fold), GST Mu (1.73 fold) and GST Theta (1.65) enzyme activities from 756.2 ± 42.92 nmol/min/mg to 348.6 ± 18.16 nmol/min/mg (p<0.0001), from 14.20 ± 1.669 nmol/min/mg to 8.173 ± 0.6375 nmol/min/mg (p=0.0002) and from 28.37 ± 1.973 nmol/min/mg to 17.17 ± 0.9839 nmol/min/mg (p<0.0001), respectively. Effects of *EH* on rat liver cytosolic total GST, GST Mu and GST Theta enzyme activities were given in Figure 3.10 - 3.12.

![Graph showing the effect of *EH* on rat liver cytosolic total GST activity.](image)

**Figure 3.10** The effect of *EH* on rat liver cytosolic total GST activity. Male Wistar rats were divided into two groups; Control rats (n=10) and *Epilobium hirsutum* treated rats (EHT) (n=30). Experiments were repeated at least 3 times. ***Significantly different from respective control value (p<0.0001).
**Figure 3.11**  The effect of \( EH \) on rat liver cytosolic GST Mu activity. Male Wistar rats were divided into two groups; Control rats (\( n=10 \)) and *Epilobium hirsutum* treated rats (EHT) (\( n=30 \)). Experiments were repeated at least 3 times. ***Significantly different from respective control value (\( p=0.0002 \)).

**Figure 3.12**  The effect of \( EH \) on rat liver cytosolic GST theta activity. Male Wistar rats were divided into two groups; Control rats (\( n=10 \)) and *Epilobium hirsutum* treated rats (EHT) (\( n=30 \)). Experiments were repeated at least 3 times. ***Significantly different from respective control value (\( p<0.0001 \)).
3.4.2.2. Effects of *Epilobium hirsutum* L. on NAD(P)H:Quinone Oxidoreductase 1 (NQO1) Activity

Rat liver cytosolic NQO1 activity was determined according to the method of Ernster (1967) as modified by Karakurt and Adali (2011) and Karakurt et al. (2013) using 2,6-dichlorophenol-indophenol (DCPIP) as a substrate. Reaction was started by adding substrate into the reaction medium and DCPIP reduction was monitored continuously by following the decrease in the absorbance at 600 nm for 3 minutes. EH injection of rats significantly increased liver cytosolic NQO1 activity (2.68 fold) from 175.8 ± 23.52 nmol/min/mg to 472.5 ± 45.44 nmol/min/mg (p<0.0001). The effect of *EH* on rat liver cytosolic NQO1 enzyme activity was shown in Figure 3.13.

![Figure 3.13](image)

**Figure 3.13** The effect of *EH* on rat liver cytosolic NQO1 activity. Male Wistar rats were divided into two groups; Control rats (n=10) and *Epilobium hirsutum* treated rats (EHT) (n=30). Experiments were repeated at least 3 times. ***Significantly different from respective control value (p<0.0001).
3.4.3. Effects of *Epilobium hirsutum* L. on Rat Liver Antioxidant Enzyme Activities

3.4.3.1. Effects of *Epilobium hirsutum* L. on Total SOD Activity

Rat liver post mitochondrial (S1.5) superoxide dismutase (SOD) activity was determined according to the method of Marklund and Marklund (1974), using pyrogallol as a substrate. *EH* injection of animals significantly increased liver SOD activity (1.4 fold) from $2.12 \pm 0.16$ Unit/mg to $2.97 \pm 0.10$ Unit/mg ($p<0.0001$). The effect of *EH* on rat liver SOD enzyme activity was given in Figure 3.14.

![Graph showing SOD Activity](image)

**Figure 3.14** The effect of *EH* on rat liver SOD enzyme activity. Male Wistar rats were divided into two groups; Control rats ($n=10$) and *Epilobium hirsutum* treated rats (EHT) ($n=30$). Experiments were repeated at least 3 times. ***Significantly different from respective control value ($p<0.0001$).
3.4.3.2. Effects of *Epilobium hirsutum* L. on Glutathione Peroxidase Activity

Rat liver cytosolic glutathione peroxidase (GPx) activity was determined according to the method of Paglia and Valentine (1967) using NADPH and glutathione as substrates. Production of oxidized glutathione and reduction of NADPH was followed at 340 nm for 3 minutes. Injection of *EH* extract to rats significantly increased liver cytosolic GPx activity (1.55 fold) from 0.069 ± 0.006 nmol/min/mg to 0.107 ± 0.007 nmol/min/mg (p<0.0001). The effect of *EH* on rat liver cytosolic GPx enzyme activity was given in Figure 3.15.

![Graph showing GPx activity](image)

**Figure 3.15** The effect of *EH* on rat liver cytosolic GPx enzyme activity. Male Wistar rats were divided into two groups; Control rats (n=10) and *Epilobium hirsutum* treated rats (EHT) (n=30). Experiments were repeated at least 3 times. ***Significantly different from respective control value (p<0.0001).
3.4.3.3. Effects of *Epilobium hirsutum* L. on Rat Liver Arylesterase (ARE) Activity

Rat liver microsomal arylesterase (ARE) activity was determined according to the method of Rodrigo et al. (1997) as modified by Can Demirdöğen et al (2008) using phenylacetate as a substrate. *EH* injection of rats significantly decreased liver microsomal ARE activity (3.32 fold) from 7.03 ± 0.66 nmol/min/mg to 2.12 ± 0.11 nmol/min/mg (p<0.0001). The effect of *EH* on rat liver microsomal ARE enzyme activity was shown in Figure 3.16.

![Arylesterase Activity Graph](image)

**Figure 3.16** The effect of *EH* on rat liver microsomal ARE enzyme activity. Male Wistar rats were divided into two groups; Control rats (n=10) and *Epilobium hirsutum* treated rats (EHT) (n=30). Experiments were repeated at least 3 times. ***Significantly different from respective control value (p<0.0001).
3.5. Effects of *Epilobium hirsutum* L. on Protein Expressions

Effects of *EH* extract injection on rat liver Phase I CYP450s; CYP1A1, CYP2B6, CYP2E1 and CYP3A1, Phase II enzymes; glutathione S-transferase Mu isoform (GSTMu) and NADPH:Quinone oxidoreductase 1 (NQO1) and antioxidant enzyme; glutathione peroxidase (GPx) protein expressions were determined by Western blotting using protein specific antibodies. Beta actin (43 kDa) and GAPDH (37 kDa) were used as protein loading controls (internal standards).

3.5.1. Effects of *Epilobium hirsutum* L. on Rat Liver CYP1A1 Protein Expression

The effect of *EH* on rat liver microsomal CYP1A1 (56 kDa) protein expression was analyzed by Western blotting using 1/1000 diluted CYP1A1 mouse monoclonal IgG1 and then 1/5000 diluted rabbit anti-mouse IgG. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that CYP1A1 protein expression significantly decreased (3.4-fold) from 8519 ± 3466 R.P.A to 2528 ± 362 R.P.A (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver microsomal CYP1A1 protein expression (Mean±SDV) was given in Figure 3.17.

![Figure 3.17](image)

**Figure 3.17** Effect of *EH* treatment on CYP1A1 protein level of rat liver.  
(A) Representative immunoblot of liver microsomal CYP1A1 protein in experimental Control (Lane 1-2) and EHT (Lane 3-9) groups.  
(B) Comparison of CYP1A1 protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3). ***<Significantly different from respective control value (p<0.0001)
3.5.2. Effects of *Epilobium hirsutum* L. on Rat Liver CYP2B1 Protein Expression

The effect of *EH* on rat liver microsomal CYP2B1 (50 kDa) protein expression was analyzed by Western blotting using 1/500 diluted CYP2B1/2B2 mouse monoclonal IgG1 and then 1/2000 diluted goat anti-mouse IgG, antibodies. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that CYP2B1 protein expression significantly decreased (1.9-fold) from 4362 ± 426 R.P.A to 2247 ± 281 R.P.A (p<0.0001) in *EH* treated rats (n=30) as compared to control animals (n=10). The effect of *EH* on rat liver microsomal CYP2B1 protein expression (Mean±SDV) was shown in Figure 3.18.

![Western Blot](image1)

**Figure 3.18**  Effect of *EH* treatment on CYP2B1 protein level of rat liver.
(A) Representative immunoblot of liver microsomal CYP2B1 protein in experimental Control (Lane 1-6) and EHT (Lane 7-13) groups.
(B) Comparison of CYP2B1 protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).

***<Significantly different from respective control value (p<0.0001)
3.5.3. Effects of *Epilobium hirsutum* L. on Rat Liver CYP2E1 Protein Expression

The effect of *EH* on rat liver microsomal CYP2E1 (57 kDa) protein expression was analyzed by Western blotting using 1/1000 diluted CYP2E1 rabbit monoclonal IgG IgG and then 1/5000 diluted goat anti-rabbit IgG. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that CYP2E1 protein expression significantly decreased (2.6-fold) from 214 ± 27 R.P.A to 80 ± 12 R.P.A (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver microsomal CYP2B1 protein expression (Mean±SDV) was given in Figure 3.19.

![Western Blot](image)

**Figure 3.19** Effect of *EH* treatment on CYP2E1 protein level of rat liver.  
**(A)** Representative immunoblot of liver microsomal CYP2E1 protein in experimental Control (Lane 1-6) and EHT (Lane 7-13) groups.  
**(B)** Comparison of CYP2E1 protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).  
***<Significantly different from respective control value (p<0.0001)
3.5.4. Effects *Epilobium hirsutum* L. on Rat Liver CYP3A1 Protein Expression

The effect of *EH* on rat liver microsomal CYP3A1 (51 kDa) protein expression was analyzed by Western blotting using 1/1000 diluted CYP3A1 rabbit monoclonal IgG1 and then 1/5000 diluted goat anti-rabbit IgG antibodies. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that CYP3A1 protein expression significantly decreased (7.27-fold) from 17440 ± 10.47 R.P.A to 2399 ± 344 R.P.A (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver microsomal CYP3A1 protein expression (Mean±SDV) was shown in Figure 3.20.

![Western blot analysis](image)

**Figure 3.20** Effect of *EH* treatment on CYP3A1 protein level of rat liver.  
(A) Representative immunoblot of liver microsomal CYP3A1 protein in experimental Control (Lane 1-2) and EHT (Lane 3-9) groups.  
(B) Comparison of CYP3A1 protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).  
***<Significantly different from respective control value (p<0.0001)
3.5.5. Effects of *Epilobium hirsutum* L. on Rat Liver GST-Mu Protein Expression

The effect of *EH* on rat liver cytosolic GST-Mu (26 kDa) protein expression was analyzed by Western blotting using 1/1000 diluted GST-Mu rabbit monoclonal IgG1 and then 1/5000 diluted goat anti-rabbit IgG antibodies. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that GST-Mu protein expression significantly decreased (9.5-fold) from 8135 ± 1019 R.P.A. to 857.4 ± 138.6 R.P.A. (p<0.0001) in *EH* treated rats (n=30) as compared to control animals (n=10). The effect of *EH* on rat liver cytosolic GST-Mu protein expression (Mean±SDV) was given in Figure 3.21.

![Figure 3.21](image)

**Figure 3.21**  Effect of *EH* treatment on GST-Mu protein level of rat liver.
(A) Representative immunoblot of liver microsomal GST-Mu protein in experimental Control (Lane 1-6) and EHT (Lane 7-14) groups.
(B) Comparison of GST-Mu protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).

***Significantly different from respective control value (p<0.0001)
3.5.6. Effects of *Epilobium hirsutum* L. on Rat Liver NQO1 Protein Expression

The effect of *EH* on rat liver cytosolic NQO1 (31 kDa) protein expression was analyzed by Western blotting using 1/1000 diluted NQO1 rabbit monoclonal IgG1 and then 1/5000 diluted goat anti-rabbit IgG antibodies. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that NQO1 protein expression significantly increased (2.97-fold) from 3352 ± 569.7 R.P.A to 9955 ± 1111 R.P.A (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver cytosolic NQO1 protein expression (Mean±SDV) was shown in Figure 3.22.

![Western Blot](image)

**Figure 3.22** Effect of *EH* treatment on NQO1 protein level of rat liver.

(A) Representative immunoblot of liver microsomal NQO1 protein in experimental Control (Lane 1-4) and EHT (Lane 5-14) groups.

(B) Comparison of NQO1 protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).

***=Significantly different from respective control value (p<0.0001)
3.5.7. Effects of *Epilobium hirsutum* L. on Rat Liver GPx Protein Expression

The effect of *EH* on rat liver cytosolic GPx protein expression was analyzed by Western blotting using 1/1000 diluted GPx rabbit monoclonal IgG1 and then 1/5000 diluted goat anti-rabbit IgG antibodies. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that GPx protein expression significantly increased (1.93-fold) from $3222 \pm 63$ R.P.A to $6203 \pm 301$ R.P.A (p<0.0001) in *EH* treated rats (n=30) as compared to control animals (n=10). The effect of *EH* on rat liver cytosolic GPx protein expression (Mean±SDV) was given in Figure 3.23.

![Western blot analysis](image)

**Figure 3.23** Effect of *EH* treatment on GPx protein level of rat liver.
(A) Representative immunoblot of liver microsomal GPx protein in experimental Control (Lane 1-3) and EHT (Lane 4-10) groups.
(B) Comparison of GPx protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).

***<Significantly different from respective control value (p<0.0001)
3.5.8. Effects of *Epilobium hirsutum* L. on Rat Liver Internal Standard Proteins; Beta Actin and GAPDH Expressions

Effects of *EH* on rat liver cytosolic beta actin (43 kDa) and GAPDH (37 kDa) protein expressions were analyzed by Western blotting using 1/1000 diluted beta actin or GAPDH rabbit monoclonal IgG1 and then 1/5000 diluted goat anti-rabbit IgG antibodies. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A.) by Image J software program. Blot analysis revealed that beta actin and GAPDH protein expressions were not varied between *EH* treated rats (n=30) and control animals (n=10). The effects of *EH* on rat liver cytosolic beta actin and GAPDH protein expressions (Mean±SDV) were shown in Figure 3.24 and Figure 3.25, respectively.

![Western Blot Image](image)

**Figure 3.24** Effect of *EH* treatment on beta actin protein level of rat liver.
(A) Representative immunoblot of liver microsomal beta actin protein in experimental Control (Lane 1-6) and EHT (Lane 7-14) groups.
(B) Comparison of beta actin protein expression of the Control (n=10) and EHT treated (n=30) groups. Experiments were repeated at least 3 times (n ≥ 3).
M, Prestained Protein Ladder.
***p<Significantly different from respective control value (p<0.0001)
Figure 3.25  Effect of $EH$ treatment on GAPDH protein level of rat liver.

(A) Representative immunoblot of liver microsomal GAPDH protein in experimental Control (Lane 1-6) and EHT (Lane 7-14) groups.

(B) Comparison of GAPDH protein expression of the Control ($n=10$) and EHT treated ($n=30$) groups. Experiments were repeated at least 3 times ($n \geq 3$).

M, Prestained Protein Ladder.

***<Significantly different from respective control value ($p<0.0001$)
3.6. Effects of *Epilobium hirsutum* L. on mRNA Expressions

Effects of EH extract injection on rat liver Phase I CYP450s; CYP1A1, CYP2B1, CYP2E1 and CYP3A1, Phase II enzymes; glutathione S-transferase Mu isoform (GSTMu) and NADPH: Quinone oxidoreductase 1 (NQO1), antioxidant enzyme; glutathione peroxidase (GPx) and also internal standards; beta actin and GAPDH mRNA expressions were determined by quantitative Real Time PCR (qRT-PCR) using gene specific primers. The primers were designed using Primer 3 program and entered into the NCBI Blast to confirm the specificity for *Rattus norvegicus*. Each set of primer was carefully chosen to avoid primer dimerization and to ensure specificity of amplification. Specific annealing temperatures were verified by the use of a gradient thermal cycler.

In order to detect synthesis of each sample, first a melt curve was plotted as shown in Figure 3.26. Ct values of rat liver samples were calculated by using standart curve which was plotted by using several dilutions of control cDNA ranging from 10X to 5000X (Fig. 3.27-Fig. 3.28).

Fold of inhibition was calculated by the following formula: $2^{-\Delta \Delta Ct}$, Where $\Delta \Delta Ct$: $\Delta Ct$ (Treatment) - $\Delta Ct$(Control); $\Delta Ct$(Treatment): $\Delta Ct$ (Target Gene) - $\Delta Ct$(GAPDH); $\Delta Ct$(control): $\Delta Ct$ (Target Gene)- $\Delta Ct$(GAPDH).

**Figure 3.26** A representative Melt Curve obtained for GAPDH. No contaminating products are present which would show up as an additional peak separate from the desired amplicon peak.
Figure 3.27  Standard Curve was generated using serially diluted cDNA standards (from 10x to 5000x). The standard curve produces a linear relationship between Ct and initial amounts of cDNA, allowing the determination of the concentration of unknowns based on their Ct values.

Figure 3.28  The PCR amplification curve charts the accumulation of fluorescent emission at each reaction cycle. The curve can be broken into four different phases: the linear ground, early exponential, log-linear, and plateau phases. Data gathered from these phases are important for calculating background signal, cycle threshold (Ct), and amplification efficiency.
3.6.1. Effects of *Epilobium hirsutum* L. on Rat Liver CYP1A1 mRNA Expression

The effect of *EH* on rat liver CYP1A1 mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that CYP1A1 mRNA expression significantly decreased (6.7-fold) from 1.0 ± 0.01 to 0.15 ± 0.03 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver CYP1A1 mRNA expression (Mean±SDV) was given in Figure 3.29.

![Figure 3.29](image)

**Figure 3.29** The effect of *EH* treatment on rat liver CYP1A1 mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
3.6.2. Effects of *Epilobium hirsutum* L. on rat liver CYP2B1 mRNA Expression

The effect of *EH* on rat liver CYP2B1 mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that CYP2B1 mRNA expression significantly decreased (7.14-fold) from 1.00 ± 0.01 to 0.139 ± 0.012 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver CYP2B1 mRNA expression (Mean±SDV) was shown in Figure 3.30.

![Figure 3.30](image)

**Figure 3.30** The effect of *EH* treatment on rat liver CYP2B1 mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
3.6.3. Effects of *Epilobium hirsutum* L. on rat liver CYP2E1 mRNA Expression

The effect of *EH* on rat liver CYP2E1 mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that CYP2E1 mRNA expression significantly decreased (1.49 fold) from 1.00 ± 0.07 to 0.67 ± 0.03 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver CYP2E1 mRNA expression (Mean±SDV) was given in Figure 3.31.

![Figure 3.31](image)

**Figure 3.31** The effect of *EH* treatment on rat liver CYP2E1 mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
3.6.4. Effects of *Epilobium hirsutum* L. on CYP3A1 mRNA Expression

The effect of *EH* on rat liver CYP3A1 mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that CYP3A1 mRNA expression significantly decreased (12.5-fold) from 1.00 ± 0.02 to 0.08 ± 0.001 (*p*<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver CYP3A1 mRNA expression (Mean±SDV) was shown in Figure 3.32.

![Figure 3.32](image)

**Figure 3.32** The effect of *EH* treatment on rat liver CYP3A1 mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (*n* ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
3.6.5. Effects of *Epilobium hirsutum* L. on Rat Liver GST-Mu mRNA Expression

The effect of *EH* on rat liver GST-Mu mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that GST-Mu mRNA expression significantly decreased (11.1-fold) from 1.00 ± 0.015 to 0.096 ± 0.009 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver GST-Mu mRNA expression (Mean±SDV) was given in Figure 3.33.

![Figure 3.33](image)

The effect of *EH* treatment on rat liver GST-mu mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
3.6.6. Effects of *Epilobium hirsutum* L. on Rat Liver GST-Theta mRNA Expression

The effect of *EH* on rat liver GST-Theta mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that GST-Theta mRNA expression significantly decreased (3.57-fold) from 1.00 ± 0.01 to 0.280 ± 0.012 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver GST-Theta mRNA expression (Mean±SDV) was given in Figure 3.34.

![Figure 3.34](image)

**Figure 3.34**  The effect of *EH* treatment on rat liver GST-Theta mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.

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3.6.7. Effects of *Epilobium hirsutum* L. on NQO1 mRNA Expression

The effect of *EH* on rat liver NQO1 mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method”. qRT-PCR analysis revealed that NQO1 mRNA expression significantly increased (4.97-fold) from 1.00 ± 0.05 to 4.97 ± 0.06 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver NQO1 mRNA expression (Mean±SDV) was given in Figure 3.35.

![Figure 3.35](image)

Figure 3.35  
The effect of *EH* treatment on rat liver NQO1 mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
3.6.8. Effects of *Epilobium hirsutum* L. on GPx mRNA Expression

The effect of *EH* on rat liver GPx mRNA expression was analyzed by qRT-PCR. As an internal standard, “housekeeping” gene GAPDH was used. The qRT-PCR profile and reaction mixture were the same as mentioned in “Material and Method.” qRT-PCR analysis revealed that GPx mRNA expression significantly increased (3.5-fold) from 1.00 ± 0.02 to 3.50 ± 0.01 (p<0.0001) in *EH* treated rats (n=30) as compared with that in control animals (n=10). The effect of *EH* on rat liver GPx mRNA expression (Mean±SDV) was shown in Figure 3.36.

![Graph showing the effect of EH treatment on rat liver GPx mRNA expression.](image)

**Figure 3.36** The effect of *EH* treatment on rat liver GPx mRNA expression. Alteration in mRNA expression was analyzed by using qRT-PCR. Results are presented as the mean from three independent experiments (n ≥ 3) and expressed as relative mean ± SD. The effect of *EH* on mRNA level of the tested gene was normalized to housekeeping GAPDH mRNA.
CHAPTER 4

DISCUSSION

Herbal medicine represents traditional medicines such as a variety of efficacious plants or plant extracts, which have been known to enhance healing of various diseases for thousands of years. Increasing popularity towards traditional medicine; herbal remedies or dietary supplements leads the investigators to study the actions of phytochemicals on xenobiotic metabolism and their antioxidant, antitumour, anticarcinogenic and antimutagenic effects. Interviews with USA cancer patients (n: 31044) showed that 62 % of those were used at least one type of complementary and alternative medicine in a year (Barnes et al. 2004). Moreover, in the last 5 decades, besides chemotherapy, patients were used herbal drugs as a complementary therapy in order to alleviate clinical symptoms, prolong survival time, and minimize the adverse effects of chemotherapy in Asia (Cho 2010). The growing popularity of these remedies is fueled by increasing scientific interest in herbal medicines. On the other hand, due to lack of enough scientific research with those plants, efficacy and safety problems are appeared. Although those herbal medicines have been sold in the markets, less scientific research on their mechanism, safety and possible adverse effect make them dangerous for human health. It is an undeniable fact that plants have been used as treatment for human health from time immemorial.

One of the reputed family in medicinal plants is Onagraceae has numerous medicinal species such as Ludwigia octovalvis used for treatment of diarrhea and dysentery, Oenothera sp. for treatment of asthma, gastrointestinal disorders, neuralgia, skin diseases, and hepatic and kidney diseases, Epilobium angustifolium; has anti inflammatory activity (Kadum Yakob et al. 2012; Kiss et al. 2011; Singh et al. 2012). Epilobium sp., the largest genus of Onagraceae family, is widely distributed all over the world and consists of approximately 200 species. It is found in moist waste land of the Mediterranean region, Europe, Asia, and Africa. One of the members of this medicinal plant family is Epilobium hirsutum L (EH). The species EH possesses polyphenolics including steroids, tannins and flavonoids (Barakat et al. 1997; Ivancheva et al. 1992). The amount of total phenolic compounds found in the EH extract used in the present study was 13.96 mg of GAE/100 g of dry weight. It was reported that EH extract exhibited to have highest antioxidant activity (69.6 μM trolox/100 g dw) and highest antioxidant concentration (2020.51 μM TEAC/100 g dw) among 32 plants extracts (Wojdylo et al. 2007). HPLC analyses of EH revealed that plant extract contains gallic, protocatechuic, ellagic, valonieic dilactone, p-coumaric acids, methyl gallate, p-methoxy gallic acid methyl ester, kaempferol, quercetin and myricetin (Barakat et al. 1997). In the present study, the LC-MS/MS analysis of EH extract showed that plant extract consists of 0.47 ± 0.0006 ppm p-coumarin, 36.61 ± 0.63 ppm gallic acid, 0.044 ± 0.0002 ppm kaempferol, 8.8 ± 0.11 ppm ellagic acid and also low concentration of vanillic acid, coumaric acid, myricetin and quercetin (≤ 0.01 ppm). Cytotoxicity studies of EH
extract on human skin fibroblast cells showed no cytotoxic effect, which is important because of the frequent external use of those extract (Kiss et al. 2011). Cytotoxicity of EH was also studied in our study by measuring rat blood serum lactate dehydrogenase (LDH) activity. Spectrophotometric analysis of LDH showed that EH does not cause any cytotoxicity on rat liver (1.01-fold) as given in Table 4.1.

EH was also found important for its regulatory effect on cancer development. This might be due to its inhibitory effect on lipoygenase activity, important for growth-related signal transduction (Kiss et al. 2011). EH is also used as wound healing factor because of its inhibitory effect on hyaluronidase activity (Pogrel et al. 1993). Hyaluronic acid has a function of viscoelastic behavior of synovial fluid and its subsequent degradation by the hyaluronidase is the triggering event for the subsequent stages of wound healing that lead to complete repair (Pogrel et al. 1993). An anti-inflammatory effect of EH was also demonstrated (Almagboul et al. 1988). That might because of inhibitory effect of EH on myeloperoxidase activity. Myeloperoxidase produces HOC1, powerful oxidant generated by neutrophils, from H2O2 and chloride. It may play an important role as an inflammatory mediator in bactericidal and fungicidal activities (Kiss et al. 2011). Although EH was used as an alternative drug for the treatment of several diseases in ancient times, there is no report published in order to show molecular background of its action. Hence, we have undertaken this study to elucidate the effects of EH on several phase I, phase II and antioxidant enzyme systems.

Phase I reactions are catalyzed mainly by members of cytochrome P450 (CYP) superfamily. CYP-drug, CYP-food and CYP-chemical interactions lead to several results. A number of CYP catalyzed reactions result in detoxification (inactivation) in majority of cases. On the other hand, these enzymes can also bioactivate several prodrugs to their active form. In addition many environmental chemicals are activated to their mutagenic and carcinogenic forms by different CYP isoforms. CYP enzymes metabolise not only various xenobiotics but also many endogenous substrates including steroids (cholesterol, sex hormones), fatty acids, eicosanoids and vitamin D. Recently, 57 genes have been classified into 18 families and 44 subfamilies according to a degree of homology. CYPs are localized in the mitochondria and the endoplasmic reticulum. Mitochondrial CYPs are involved in the metabolism of endogenous substrates whereas microsomal CYPs metabolise both endogenous and exogenous substrates. The isoform composition of cytochrome P450 in human being may differ in animal tissues. Therefore, selection of CYP isoform has a crucial importance while working with other species to make right correlation to human being. In our study, we have chosen CYP1A1, CYP2B1, CYP2E1 and CYP3A1 isoforms in rat liver tissue which correspond to human drug metabolizing enzymes; CYP1A1, CYP2B6, CYP2E1, and CYP3A4 (Kobayashi et al. 2002).

In this study, effects of EH extract on rat liver Phase I CYP450s: CYP1A1 dependent EROD, CYP2B6 dependent benzyphenamine N-demethylase, CYP2E1 dependent aniline 4-hydroxylase and NDMA N-demethylase, CYP3A4 dependent ethylmorphine N-demethylase; Phase II enzymes: Total, theta and Mu glutathione S-transferases (GST, GSTT and GSTMu), NADPH:Quinone oxidoreductase 1 (NQO1) and antioxidant enzymes; superoxide dismutase (SOD), glutathione peroxidase (GPx) and
arylsterase (ARE) activities were investigated. In addition protein expressions as well as mRNA expressions of those were determined in control and EH treated rat liver.

In protein and mRNA expression studies, internal standards beta actin and GAPDH were used. Numerous studies have shown that rodent and human CYP1A1, CYP2E1, NQO1 and GPx enzymes catalyze similar reactions. Sequencing analysis of cDNA and corresponding protein showed that human CYP1A1, CYP2B1, CYP2E1, CYP3A1, NQO1 and GPx share over than 80% similarity with rat counterparts. Therefore, in the present study, the rat is used as a model organism for screening these enzymes.

The data presented in this study demonstrated that intraperitoneal administration of 37.5 mg/kg body weight water extract of EH to rats caused remarkable changes in xenobiotic metabolizing phase I, phase II and antioxidant enzymes in rat liver. In the first part of the study, we have shown that EH significantly decreased the CYP1A1 dependent EROD (1.46 fold, p=0.0003), CYP2B1 dependent benzphetamine N-demethylase (2.12 fold, p<0.0001), CYP2E1 dependent aniline 4-hydroxylase (4.93 fold, p<0.0001) and NDMA N-demethylase (1.2 fold, p<0.05), CYP3A1 dependent ethylmorphine N-demethylase (8.68 fold, p<0.0001), Total GST and its isoforms; GST Mu and GST Theta (2.16 fold, 1.73 fold and 1.65 fold, respectively p<0.0001) and arylesterase (3.32 fold, p<0.0001) activities. On the other hand, rat liver phase II and antioxidant enzyme activities were significantly increased with EH treatment. NQO1, SOD and GPx enzyme activities were increased 2.68 fold (p<0.0001), 1.4 fold (p<0.0001) and 1.55 fold (p<0.0001), respectively (Table 4.1).
Table 4.1  Effects of *Epilobium hirsutum* L. on Cytochrome P450 Dependent Phase I, Phase II and Antioxidant Enzyme Activities.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control</th>
<th>EHT</th>
<th>Fold Decrease / Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1 Dependent Ethoxyresorufin-o-demethylase (EROD) (nmol/min/mg)**</td>
<td>6.54 ±1.21</td>
<td>4.48 ± 1.67</td>
<td>1.46↓</td>
</tr>
<tr>
<td>CYP2B1 Dependent Benzphetamine N-demethylase (nmol/min/mg)***</td>
<td>1.42 ± 0.53</td>
<td>0.67 ± 0.34</td>
<td>2.12↓</td>
</tr>
<tr>
<td>CYP2E1 Dependent Aniline 4-hydroxylase (nmol/min/mg)***</td>
<td>0.537 ± 0.01</td>
<td>0.109 ± 0.01</td>
<td>4.93↓</td>
</tr>
<tr>
<td>CYP2E1 Dependent NDMA N-demethylase (nmol/min/mg)*</td>
<td>0.192 ± 0.011</td>
<td>0.159±0.012</td>
<td>1.2↓</td>
</tr>
<tr>
<td>CYP3A4 Dependent Ethylmorphine N-demethylase (nmol/min/mg)***</td>
<td>2.43 ± 0.43</td>
<td>0.28±0.04</td>
<td>8.68↓</td>
</tr>
<tr>
<td>Total GST (nmol/min/mg)***</td>
<td>756.2 ± 42.92</td>
<td>348.6 ± 18.16</td>
<td>2.16↓</td>
</tr>
<tr>
<td>GST Mu (nmol/min/mg)***</td>
<td>14.20 ± 1.669</td>
<td>8.173 ± 0.6375</td>
<td>1.73↓</td>
</tr>
<tr>
<td>GST Theta (nmol/min/mg)***</td>
<td>28.37 ± 1.973</td>
<td>17.17 ± 0.9839</td>
<td>1.65↓</td>
</tr>
<tr>
<td>NQO1 (nmol/min/mg)***</td>
<td>175.8 ± 23.52</td>
<td>472.5 ± 45.44</td>
<td>2.68↑</td>
</tr>
<tr>
<td>SOD (U/mg)***</td>
<td>2.12 ± 0.16</td>
<td>2.97 ± 0.10</td>
<td>1.4↑</td>
</tr>
<tr>
<td>GPx (nmol/min/mg)***</td>
<td>0.069 ± 0.006</td>
<td>0.107 ± 0.007</td>
<td>1.55↑</td>
</tr>
<tr>
<td>Arylesterase (nmol/min/mg)***</td>
<td>7.03 ± 0.66</td>
<td>2.12 ± 0.11</td>
<td>3.32↓</td>
</tr>
<tr>
<td>LDH (U/mg)</td>
<td>619.5 ± 46.30</td>
<td>687.6 ± 52.89</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Male wistar rats were divided into two groups. Control group (n=10) was not treated with any chemicals and EHT group (n=30) was intraperitoneally administered with *Epilobium hirsutum* L. extract (37.5 mg/kg).
Results are analyzed by GraphPad Prism 5.0 software and values are mean ± SD for triplicate determinations.
Significantly different from control by two-tailed Student’s t -test, *p<0.05, ** p=0.0003, *** p<0.0001.
It was well known that cytochrome P450 enzyme family (CYPs) are involved in the biotransformation of many endogenous and exogenous substances including drugs. CYP1A1 is the main member of cytochrome P4501A family, which could be induced by PHAs and involved in the metabolic activation of PAHs and heterocyclic amines (McManus et al. 1990). Metabolism of PAHs by CYP1A1 produce reactive products which are irreversibly bind to protein and DNA leading to toxic and carcinogenic events (Miller and Miller 1966). The risk assessment for many diseases especially respiratory and cancer, susceptible to the emission of PAH, might decrease with the inhibition of CYP1A1 playing role in the formation of such reactive products and those inhibitory mechanisms might be able to suppress the tumor formation induced by PAHs and other carcinogens. Hence inhibition of CYP1A1 dependent EROD activity due to the treatment of rats with EH may prevent formation of DNA adducts, which may protect the cells from environmental procarcinogens. CYP2E1 has a wide variety of substrates which are mainly exogenous such as industrial solvents, protoxins and procarcinogens (Gonzalez 2005). Hence, inhibition of CYP2E1 may prevent conversion of such pro-carcinogens to their carcinogenic forms such as nitrosamine, acrylamide, phenol and benzene. Benzene is one of the important environmental pollutants and its exposure causes a variety of haematotoxic effects including anemia and leucopenia (Hayes et al. 1997). Metabolism of benzene by CYP2E1 is resulted with the formation of benzoquinone which causes necrosis (Cheung et al. 2005). Furthermore, drugs used by human such as acetaminophen, chlorzoxazone and methoxyflurane have been also shown to be metabolized by CYP2E1. Metabolism of chlorzoxazone and acetaminophen by CYP2E1 is resulted with the formation of alkylating metabolites; N-acetyl-p-benzo-quinone imine and 6-hydroxy-chlorzoxazone, respectively (Gonzalez 2007). Moreover, as a result of CYP1A1 and CYP2E1 catalyzed processes, the oxygen-centered free radicals also known as reactive oxygen species, (ROS) such as superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (•OH) can be produced. In the case of any imbalance between production of ROS and cellular antioxidant capacity or when there is a decrease in this capacity, oxidative stress may occur. Oxidative stress may be very damaging, since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, to induce oxidations, which cause membrane damage, protein modification including enzymes and DNA damage. Therefore, inhibition of those enzymes might be one mechanism in order to prevent formation of ROS. Approximately 95 % of clinically used drugs encounter metabolism of CYP enzymes especially, CYP2 (45%) and CYP3 (50 %) families (Hidestrand et al. 2001; Rendic 2002). CYP2B subfamily members are mainly involved in the metabolism of xenobiotics including many therapeutic drugs such as cyclophosphamides, bupropion, ifosfamide, benzodiazepines and efavirenz (Ekins and Wrighton 1999; Ward et al. 2003). Indubitably, CYP3A is the most predominant family of CYP enzymes in the keys of drug metabolism since 50 % of the currently used drugs including anticancer, antiepileptic, anticoagulant, metabolized by hepatic CYP3A4. It was showen that CYP enzymes can be modulated by several kinds of plant extracts such as Hypericum Perforatum, Hydrastis canadensis and Thonningia sanguinea (Gurley et al. 2005; Gyamfi et al. 2000; Obach 2000; Zhou et al. 2003). Modulation of CYPs resulted with important clinical and toxicological consequences because of their action on the metabolism of drugs and toxicants. Beside of herb-drug interaction, CYPs are concerned
with a high ratio of drug-drug interactions (Lin and Lu 1998). For example, diazepam (muscle relaxant) is the substrate of CYP2C19 but on the other hand, it is the inhibitor of CYP2B6. When it is used with bupropion (antidepressant), metabolized to hydroxybupropion by the CYP2B6 enzyme, it prevents metabolism of bupropion and decrease hydroxybupropion in blood. Therefore modulation of drug metabolizing enzymes may affect the efficacy and toxicity of drugs because of its effect on drug metabolism and clearance. Increased level of parent drug or its metabolite may be harmful or quick metabolism/clearance of the drug may decrease its efficacy. One of the most important issues for drug metabolism is the drug-drug interactions which resulted with the alteration of metabolism of drugs. Pharmacological action required for transformation of pro-drugs to active metabolites can be eliminated with the inhibition of specific CYP enzyme. Paroxetine, SSRI antidepressant used during breast cancer to reduce hot flushes, is the inhibitor of CYP2D6. Co-used with tamoxifen, used in breast cancer treatment, reduce tamoxifen action (Teh and Bertilsson 2011). Like drug-drug interaction, drug-herb interaction is also important. Therefore *Epilobium hirsutum* L. should be used with caution in patients who use drugs metabolized by especially CYP2B6 and CYP3A4. Administration of water extract of *Epilobium hirsutum* L may modulate currently active used drug metabolism because of its potential inhibitory effect on cytochrome P450 dependent drug metabolizing enzymes.

Although the majority of glutathione S-transferase-linked reactions serve the function of detoxification via removing toxic metabolites from the cell and maintaining cellular sulfhydryl groups in their reduced form, in some cases toxicity is increased by formation of a GSH conjugate with dichloromethane, haloalkenes, vicinal dihaloalkenes and haloacids (Sherratt et al. 1997; van Bladeren and van Ommen 1991). Besides, the resistance of cells and organisms to pesticides, herbicides and antibiotics was implicated in GST activities (Arca et al. 1988; Fournier et al. 1992). Moreover, GSTs have been shown to be over-expressed in tumor cells hence it increases the resistance for chemotherapeutic drugs. Cancer chemotherapeutic agents such as adriamycin, 1,3-bis (2-chloroethyl)-1 nitrosourea, busulfan cyclophosphamide are detoxified by GST (Hayes and Pulford 1995). Therefore, enhanced GST-mediated conjugations of cytostatics due to increased GST expression used in cancer treatment have been suggested among the mechanisms of drug resistance. In this context, inhibition of GSTs may sensitize drug-resistant cells (Horton et al. 1999; Yang et al. 1992). Although there are many attempts have been made to develop GST inhibitors, many of those GST inhibitors such as synthetic analogues and glutathione conjugates are either too toxic to be used in vivo or are effective only in vitro. Furthermore, lacking of stability and selectivity, as well as their poor pharmacological profile pose major problems to their use (Burg and Mulder 2002; Lucente et al. 1998). Therefore inhibitory effect of EH on total GST activity and its isoforms; GST-Mu and GST-Theta seems to be a good strategy to prevent the formation of hazardous effects of their metabolites. One of the crucial enzyme in the defense system against ROS as well as neoplasia is NQO1 (Karakurt and Adali 2011). NQO1 has not only had a function on the detoxification of ubiquinone but also had a function on the antioxidant and cancer preventing processes (Landi et al. 1997). Increased level of NQO1 enzyme activities may be resulted with the inhibition of ROS and formation/stabilization
of tumor suppressor proteins such as p53 (Asher et al. 2001). Moreover, NQO1 induction might decrease the formation of the semiquinone radicals which are the product of quinones as if they are metabolized by cytochrome P450 enzymes not NQO1. Besides elevated NQO1 activity was shown to decrease oxidative DNA damage and inhibit the estrogen-induced breast cancer via decreasing the levels of 17β-estradiol (E2)-quinones capable of interacting with DNA by converting E2-quinones to E2-catechols (Singh et al. 2011).

In addition to phase I and phase II enzymes, in vivo effect of EH on antioxidant enzymes; SOD, GPx and ary lesterase were also investigated. Administration of EH extract to rats significantly increased SOD and GPx enzyme activities (p<0.0001). On the other hand ary lesterase enzyme activity was decreased 3.32-fold (p<0.0001). In order to prevent ROS-induced cell injury, our body generated an antioxidant system which includes antioxidants such as glutathione, alpha tocopherol, ascorbic acid and the main antioxidant enzymes such superoxide dismutase (SOD) and glutathione peroxidase (GPx). The first line of the defense against ROS is SOD. SODs catalyze dismutation of superoxide anion (O2−) to hydrogen peroxide (H2O2) and localize at distinct compartments; cytosol, mitochondria and extracellular matrix, they participate in compartmentalized redox signaling to regulate many vascular function. Moreover, decreased SOD activity was found to be associated with postmenopausal breast cancer (Kumar et al. 1991). Furthermore, increased level of SOD showed to have a protective role against atherosclerosis (Stocker and Keaney 2004). Since SODs are the master keys for controlling cellular ROS levels, SOD and SOD mimetics have been accepted have a potential uses as a therapeutic agents in oxidative stress-related diseases. Moreover, increased level of SOD expression was found to suppress the malignant phenotype of human melanoma cells (Shopova et al. 2009). GPx enzymes reduce H2O2 and organic ROOH to water and alcohols (ROH), respectively. Increased level of GPx activity may enhance the resistance against reactive oxygen species. GPx1-overexpressing [GPx1(+/+)] mice were found to be more resistant to paraquat-induced lethality than GPx1 knockout mice [GPx1(−/−)] (Cheng et al. 1998). Moreover, polymorphism of GPx1 gene at codon 198 was found to be associated with the increased risk for several types of cancers such as breast, colon, lung and prostate (Zhuo and Diamond 2009). Beside these, increased level of GPx is also protective against cardiovascular diseases since reactive oxygen species such as superoxide radicals or hydrogen peroxides causes any changes in the vascular tone and structure (Battin and Brumaghim 2009).

In addition to allosteric control mechanisms of enzymes, expression of cytochrome P450 enzymes as well as phase II and antioxidant enzymes were known to be controlled at the transcriptional and translational levels. Therefore, in this study, in addition to activity studies of those enzymes, in order to clarify the mode of control mechanism of those liver enzymes at the transcriptional and translational levels, protein and of mRNA expression analysis were accomplished using western blot and qRT-PCR techniques.

Immunoblot analysis, shown in Table 4.2, indicated that protein expressions have been altered via EH administration to rats. CYP1A1, CYP2B1, CYP2E1, CYP3A1, GSTMu protein expressions were significantly decreased (p<0.0001) while NQO1 and
GPx protein expressions significantly increased 2.97-fold and 1.93-fold, respectively compared to control group (p<0.0001).

Table 4.2  Effects of *Epilobium hirsutum* L. on Expression of Cytochrome P450 Dependent Phase I, Phase II and Antioxidant Proteins.

<table>
<thead>
<tr>
<th>Protein Expression</th>
<th>Control</th>
<th>EHT</th>
<th>Fold Decrease / Increase</th>
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<tr>
<td>CYP1A1***</td>
<td>8519 ± 3466</td>
<td>2528 ± 362</td>
<td>3.4↓</td>
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<tr>
<td>CYP2B1***</td>
<td>4362 ± 426</td>
<td>2247 ± 281</td>
<td>1.9↓</td>
</tr>
<tr>
<td>CYP2E1***</td>
<td>214 ± 27</td>
<td>80 ± 12</td>
<td>2.6↓</td>
</tr>
<tr>
<td>CYP3A1***</td>
<td>17440 ± 10.47</td>
<td>2399 ± 344</td>
<td>7.27↓</td>
</tr>
<tr>
<td>GSTMu***</td>
<td>8135 ± 1019</td>
<td>857.4 ± 138.6</td>
<td>9.5↓</td>
</tr>
<tr>
<td>NQO1***</td>
<td>3352 ± 569.7</td>
<td>9955 ± 1111</td>
<td>2.97↑</td>
</tr>
<tr>
<td>GPx***</td>
<td>3222 ± 63</td>
<td>6203 ± 301</td>
<td>1.93↑</td>
</tr>
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Densitometric analysis of protein bands were performed using Image J software. Results are analyzed by GraphPad Prism 5.0 software and presented as the mean from three independent experiments and expressed as relative mean ± STD, n:10 (control); n:30 (EHT). Significantly different from control by two-tailed Student’s t-test, p<0.0001.

Moreover, cross analyses of protein expression versus enzyme activity showed that there is a correlation between protein expression levels and corresponding enzyme activities in *EH* treated and control groups as shown in Figure 4.1–Figure 4.4.
**Figure 4.1** Correlation between liver microsomal EROD activity and relative CYP1A1 protein expression in rat. The correlation coefficient \( r = 0.88, p<0.0001 \) was calculated by the least squares linear regression method. The solid line represents the line of best fit.

**Figure 4.2** Correlation between liver microsomal aniline 4-hydroxylase and relative CYP2E1 protein expression in rat. The correlation coefficient \( r = 0.71, p<0.0001 \) was calculated by the least squares linear regression method. The solid line represents the line of best fit.
**Figure 4.3** Correlation between liver cytosolic glutathione peroxidase activity and relative GPx protein expression in rat. The correlation coefficient \( r = 0.75, \ p < 0.0001 \) was calculated by the least squares linear regression method. The solid line represents the line of best fit.

**Figure 4.4** Correlation between liver cytosolic NQO1 activity and relative NQO1 protein expression in rat. The correlation coefficient \( r = 0.88, \ p < 0.0001 \) was calculated by the least squares linear regression method. The solid line represents the line of best fit.
Alterations in protein expressions due to EH extract treatment of rats leads us to further analyze the source of this variation. Therefore, mRNA expression of phase I, phase II and antioxidant genes were analyzed by qRT-PCR. The results obtained from qRT-PCR showed that EH extract has remarkable effects on mRNA expressions of CYP1A1, CYP2B1, CYP2E1, CYP3A1, GSTMu, GSTT1, NQO1 and GPx genes (p<0.0001) (Table 4.3).

Table 4.3  Effects of Epilobium hirsutum L. on mRNA expressions of Rat Liver Phase I, Phase II and Antioxidant Genes.

<table>
<thead>
<tr>
<th>mRNA Expression</th>
<th>Control</th>
<th>EHT</th>
<th>Fold Decrease / Increase</th>
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<tr>
<td>CYP1A1***</td>
<td>1.00 ± 0.01</td>
<td>0.150 ± 0.03</td>
<td>6.7↓</td>
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<tr>
<td>CYP2B1***</td>
<td>1.00 ± 0.01</td>
<td>0.139 ± 0.01</td>
<td>7.14↓</td>
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<tr>
<td>CYP2E1***</td>
<td>1.00 ± 0.07</td>
<td>0.670 ± 0.03</td>
<td>1.49↓</td>
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<tr>
<td>CYP3A1***</td>
<td>1.00 ± 0.02</td>
<td>0.080 ± 0.01</td>
<td>12.5↓</td>
</tr>
<tr>
<td>GSTMu***</td>
<td>1.00 ± 0.02</td>
<td>0.096 ± 0.01</td>
<td>11.1↓</td>
</tr>
<tr>
<td>GSTT1***</td>
<td>1.00 ± 0.01</td>
<td>0.280 ± 0.01</td>
<td>3.57↓</td>
</tr>
<tr>
<td>NQO1***</td>
<td>1.00 ± 0.05</td>
<td>4.97 ± 0.06</td>
<td>4.97↑</td>
</tr>
<tr>
<td>GPx***</td>
<td>1.00 ± 0.02</td>
<td>3.50 ± 0.01</td>
<td>3.5↑</td>
</tr>
</tbody>
</table>

The effects of EH on the mRNA levels of the tested genes were normalized to housekeeping GAPDH mRNA, were analyzed by GraphPad Prism 5.0 software and were presented as the relative mRNA expression compared to control groups. n:10 (control); n:30 (EHT). Fold of inhibition was calculated by the following formula: $2^{-\Delta\Delta CT}$.

***Significantly different from control by two-tailed Student’s t -test, p<0.0001.

The results of the present study showed that CYP1A1 protein (3.4-fold) and mRNA (6.7-fold) expressions were decreased significantly (p<0.0001) when compared with control group. Inhibition of CYP1A1 on the transcriptional level might be due to aryl hydrocarbon receptor (AHR), a ligand activated transcription factor. Under normal circumstances, AHR is located in the cytoplasm with molecular chaperones; heat-shock protein, ARA9 and p53. In the case of any ligand binding, AHR is translocated into the nucleus and form a dimer with AHR nuclear translocator (ARNT). AHR/ARNT
heterodimer binds to the xenobiotic response elements (XREs), which increases CYP1A1 transcription (Hayes et al. 2009). In addition to receptor mediated control mRNA expression of CYP1A1 might be due to elevated level of microRNA (miRNA) and methylation. Interestingly, both hypermethylation and hypomethylation of CYP1A1 promotes carcinogenesis since hypermethylation causes less active CYP1A1, which altered carcinogen metabolism, and hypomethylation leads more active enzyme resulted with higher activation of procarcinogens (Tamási et al. 2011).

In this study, protein and mRNA expression of CYP2B1 was decreased 2.2-fold and 7.14-fold, respectively (p<0.0001) when compared with control group. It has been demonstrated previously that CYP2B gene expression is regulated by pregnane X receptor and constitutive androstane receptor (CAR) (Hiroi et al. 1998; Willson and Kliwer 2002). Human and rat possess in a high ratio of nuclear receptor amino acid identity. hPXR and rat PXR share 76% amino acid identity and transcription of human and rat drug metabolizing genes are regulated by same nuclear receptor; rat CYP2B1 (CAR/PXR) - hCYP2B6 (CAR/PXR), (Tolson and Wang 2010). Results of our study indicate that EH may disrupt the CAR and PXR ligand binding domain, which resulted with inhibition of CAR and PXR transcriptional activity and the suppression of CYP2B and CYP3A genes mRNA expressions. Moreover, It was demonstrated that overexpression of miR-148a induce CYP2B6 expression (2-fold) (Nakajima and Yokoi 2011).

The present work showed that CYP2E1 protein and mRNA levels were decreased 2.2-fold and 1.49-fold, respectively upon injection of plant extract to rats. The transcription of CYP2E1 in mammalian cells starts a few hours after birth and reach to its maximum in one day (Umeno et al. 1988). Transcription of CYP2E1 mRNA level was controlled by hepatic nuclear factors (HNF-1) (Cheung et al. 2003; Matsunaga et al. 2008). It was shown that transcription of CYP2E1 significantly decreased in HNF-1 knockout mice (Matsunaga et al. 2008). Methylation of CYP2E1 gene was shown to inhibit the enzyme in prenatal period (Vieira et al. 1998). On the other hand, in adult tissues, the methylation pattern of the CYP2E1 gene varies between various tissue types such as lung, kidney, placenta, liver, and skin, indicating that DNA methylation results in tissue-specific regulation (Botto et al. 1994b; Vieira et al. 1996). For instance; low expression of CYP2E1 in lung tumor tissues was found to be associated with hypomethylation of that gene (Botto et al. 1994a). Beside the methylation and receptor mediated control of CYP2E1, recent studies demonstrated that miR-378 is involved in the post-transcriptional regulation of CYP2E1 and overexpression of miR-378 significantly decreased CYP2E1 protein levels and enzyme activity (Mohri et al. 2010).

The most abundant P450 in human liver is CYP3A4 which is responsible for the metabolism of more than 50% of prescription drugs and endogenous compounds such as steroids. Intraperitoneal administration of EH caused a significant decrease in protein (7.27-fold) and mRNA (12.5-fold) expressions of CYP3A4. Transcriptional regulation of CYP3A genes is mainly controlled by pregnane X receptor (PXR). Amino acid analyses of hCYP3A4 (PXR/CAR) and rat PXR CYP3A2 (PXR) showed that they has over than 76 % identity (Tolson and Wang 2010). Up to now, just miR-27b has been described to regulate directly expression of CYP3A4 (Pan et al. 2009). On the other hand, regulation
of PXR expression by miR-148a leads its regulatory role on CYP3A4 (Takagi et al. 2008).

In addition to CYP proteins, protein and mRNA expressions of phase II and antioxidant systems were also analyzed. Main substrates of GST have a common degree of hydrophobicity and possess electrophilic centers which can attack cellular nucleophiles such as DNA, causing genotoxicity. Moreover, deletion of the genes in this super family may therefore result in an increased susceptibility to chemical damage and both GSTM1 and GSTT1 have been correlated with higher risk of cancer (Fryer et al. 1993; Strange et al. 1991). In the present study, nine days i.p administration of water extract of EH decreased protein (9.5-fold) and mRNA (11.1-fold) expressions of GST Mu (p<0.0001) and mRNA expression of GSTT1 (3.57-fold). Down-regulation of protein and mRNA expressions of GSTMu leads aromatic amines go in an alternative pathway, hydroxylation, which resulted with formation of unstable chemical species which can react with DNA as mutagens or cause direct damage to other cellular components such as proteins. Like CYP2B1 and CYP3A4, receptor mediated regulation of GST is controlled by PXR (Falkner et al. 2001). Overexpression of that gene increased GST expression 2-fold compared to control (Gong et al. 2006). An inverse correlation between GST gene expression and DNA methylation was demonstrated in murine prostate (Mavis et al. 2009). On the other hand, there is still no data for miRNA regulation of GST in literatures.

One of the important phase II enzyme known as NQO1 was also studied in terms of protein and mRNA levels after EH treatment of the rats. Both protein (2.97-fold) and mRNA (4.97-fold) expressions were significantly increased (p<0.0001) when compared with untreated group. As many phase II enzymes, regulation of the transcription of NQO1 is done by nuclear transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2) (Dhakshinamoorthy and Jaiswal 2000). In the case of oxidative stress, NRF2 binds to antioxidant response element (ARE) in the promoter region and increased NQO1 transcription (Nitoi et al. 2003). Not only the oxidative stress causes NQO1 transcription but presence of quinone also stimulates NQO1 transcription. In Parkinson’s and Alzheimer’s diseases, NQO1 expression has been reported to be elevated as an adaptive response against dopamine quinone metabolites’ potential accumulation (Raina et al. 1999; van Muiswinkel et al. 2004). Moreover, an Nrf2 mRNA level was found to be reversibly correlated with miR-28 expression (Yang et al. 2011). Therefore, miR-28 may down-regulate transcription of NQO1 gene.

In the present study, in vivo effect of EH on antioxidant enzyme; GPx was also investigated at the level of protein and mRNA. The data showed that administration of EH significantly increased GPx protein (1.93-fold) and mRNA (3.5-fold) expressions (p<0.0001). GPx is the enzyme reducing H2O2 and organic ROOH to water and alcohols (ROH). Increased level of GPx activity may enhance the resistance against reactive oxygen species. GPx1-overexpressing mice were found to be more resistant to paraquat-induced lethality than GPx1 knockout mice (Cheng et al. 1998). Beside these, increased level of GPx is also protective against cardiovascular diseases since reactive oxygen species such as superoxide radicals or hydrogen peroxides causes any changes in the vascular tone and structure (Battin and Brumaghim 2009). As a result, combining
induction of GPx together with NQO1 and SOD by the $EH$ treatment could be a reasonable significant enhancement against the toxicity of various agents.
CONCLUSION

Increasing interest of medicinal plants and their remedies lead the investigators to investigate the actions of phytochemicals on xenobiotic metabolism and their antioxidant, antitumour, anticarcinogenic and antimutagenic effects. One of these medicinal plants is *Epilobium hirsutum* L. known as its analgesic, anti-microbial and anti-proliferative activity. On the other hand, molecular mechanism of its action has not clarified yet. In our study, we have investigated not only chemical and molecular properties of that plant extract but also in vivo therapeutic and toxic effects of *EH* were studied by determining the mRNA and protein expressions, as well as the activities of rat enzymes involved in the metabolism of exogenous and endogenous molecules. *EH* has a significant inhibitory effect on cytochrome P450 (CYP) dependent enzymes. Those enzymes have a function on drug metabolism and metabolism of several chemicals. Several procarcinogens require metabolic activation of CYP enzymes in order to show their toxic and carcinogenic effect. Therefore, inhibition of that enzyme system prevents formation of hazardous metabolites. Besides, inhibition of CYP enzymes affects metabolism of drugs. Depending on the matter of drug, pro-drug or active drug, consumption of *EH* may modulate metabolism and toxicity of the drugs. Therefore *EH* should be used with caution in patients who use drugs metabolized by especially CYP2B6 and CYP3A4. Additionally, level of ROS may also decrease after consumption of *EH* which indirectly prevent formation of ROS by inhibiting ROS producing CYP enzymes. One of the Phase II enzymes is GST which has many functions such as detoxification of electrophilic compounds, activation of pro-carcinogenic compounds or increase resistance of many cancer types against chemotherapeutic drugs. Although, many attempts have been made to develop GST inhibitors many of those inhibitors such as synthetic analogues and glutathione conjugates are either too toxic to be used in vivo or are effective only in vitro. Furthermore, lacking stability and selectivity, and having a poor pharmacological profile, synthetic analogues cause problems when used. Due to its inhibitory effect on GST enzyme activity, *EH* can be accepted as a new natural GST inhibitor. Besides its inhibitory effects, *EH* was significantly increased other Phase II enzyme, NQO1 and antioxidant enzymes, GPx and SOD.

The presented evidences point out that *EH* alters the activity and expression of enzymes involved in xenobiotics activation/detoxification pathways. Therefore, modulatory effect of *EH* on these enzymes suggests an inherent chemopreventive action against a group of chemicals including carcinogens and drugs. However, necessary precautions such as medical advice should be taken regarding the usage of this plant in replacement treatments since it reveals possible interactions with drugs and dietary foods.
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complete amino acid sequence, and gene regulation. J. Biol. Chem. 261, 15794-15799.


APPENDIX

A-APPROVALS OBTAINED FROM ETHICAL COMMITTEE

ORTA DOĞU TEKNIK ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURUL KARARLARI

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Sayın:
Prof. Dr. Alaattin SEN
Fen Edebiyat Fakültesi
Biyoloji Bölümü
Öğretim Üyesi

İlgi: 22.01.2009 tarihli başvuruınız.

"Yakı Otunun Ksenobiyotik Metabolizmasını Üzerine Etkilerinin ve İlaç-Diyet Etkileşim Potansiyelinin Proteinik ve Moleküler Yaklaşımlar ile Aydınlatılması" konulu PAUHDEK-2009/007 no’lu çalışmınız 11.02.2009 tarih ve 02 sayılı toplantımızda görüşülmüş olup,

Çalışmanın yapılması için Hayvan Deneyleri Etği açıından uygun olduguna oy çokluğu ile karar verilmiştir.

Gereğini bilgilerinize rica ederim.

Doç. Dr. Vural KÜÇÜKATAY
Başkan

Not: Çok brave imzalamadığım belge fişe çektili ile gönderilecek.
T.C. 
PAMUKKALE ÜNİVERSİTESİ 
HAYVAN DENEYLERİ ETİK KURUL BAŞKANLIĞI 
TOPLANTI TUTANAGI 

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KARAR 3- Fen Edebiyat Fakültesi Biyoloji Bölümü Öğretim Üyesi Prof. Dr. Alattin ŞEN’in yürütücüsü olduğu “Yakıtı Otunun K$hene$byotik Metabolizması Üzerine Etkilerinin ve İlaç-Diyet Etkileşim Potansiyelinin Proteomik ve Moleküler Yaklaşımlar ile Aydınlatılması” konulu PAUHDEFK-2009/007 no’lu çalışması görüşülmesi octup, 

Çalışmanın yapılmasının Hayvan Deneysileri Etiği açılarından uygun olduğuna oy çoğunluğu ile karar verilmiştir.

Yrd.Doç.Dr. Murat KARATEPE

<table>
<thead>
<tr>
<th>Yrd.Doç.Dr. Viral KÇOKKATAY</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Doç. Dr. Çağrı ERGİN</th>
<th>Yrd.Doç.Dr. Mustafa KARATEPE</th>
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<tr>
<th>Doç. Dr. Yakup KASKA</th>
<th>Doç. Dr. Burhan KABAY</th>
</tr>
</thead>
</table>

|-------------------------------------|-----------------------------|

| Dr.Berra TURGUT | Şemsettin ÖZMEN |
# CURRICULUM VITAE

**Surname Name**: KARAKURT, Serdar  
**Academic Title**: Research Assistant

**Birthplace / Birthdate**: Gaziantep / 30.06.1980  
**Nationality**: Turkish  
**Work Tel**: (312)2105161  
**E-mail**: kserdar1@yahoo.com

**Appointed to**
- **Academic Unit**: Natural and Applied Sciences  
- **Department**: Biochemistry

## Education

### BACHELOR
- **University**: Ege University  
- **Academic Unit**: Faculty of Sciences  
- **Programme/Department**: Biology  
- **Country**: Turkey  
- **Graduation Year**: 2003

### MASTER
- **University**: Middle East Technical University  
- **Graduate School**: Natural and Applied Sciences  
- **Field of Study**: Biochemistry  
- **Title of Thesis**: The Effects of Phenolic Compound Tannic Acid on Phase II and Cytochrome P450 Dependent Enzymes in Rabbit Liver and Kidney  
- **Country**: Turkey  
- **Graduation Year**: 2008

### DOCTORATE
- **University**: Middle East Technical University  
- **Graduate School**: Natural and Applied Sciences  
- **Field of Study**: Biochemistry  
- **Title of Thesis**: Investigation of the Effects of Folk Medicinal Plant *Epilobium hirsutum* L. on Cytochrome P450 Dependent and Antioxidant Enzymes: A Molecular Approach  
- **Country**: Turkey  
- **Graduation Year**: 2013
### Foreign Languages

<table>
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<tr>
<th>Language</th>
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<tbody>
<tr>
<td>English</td>
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<tr>
<td>Finnish</td>
<td>Moderate</td>
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### Academic Titles

<table>
<thead>
<tr>
<th>University / Institution</th>
<th>Country</th>
<th>City</th>
<th>Department</th>
<th>Duty</th>
<th>Years</th>
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</thead>
<tbody>
<tr>
<td>Middle East Technical University</td>
<td>Turkey</td>
<td>Ankara</td>
<td>Biochemistry</td>
<td>Research Assistant</td>
<td>2005-2013</td>
</tr>
<tr>
<td>Institute of Biomedical Technology - University of Tampere</td>
<td>Finlandiya</td>
<td>Tampere</td>
<td>Molecular Biology of Prostate Cancer</td>
<td>Research Assistant</td>
<td>2011-2012</td>
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### Research Projects & Reports

**Duties in Research projects supported by national organizations**

<table>
<thead>
<tr>
<th>Project Type</th>
<th>Duty</th>
<th>Title</th>
<th>Year</th>
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<tbody>
<tr>
<td>TUBITAK</td>
<td>Project Manager</td>
<td>Effects of Plant Phenolic compounds Tannic Acid and Resveratrol on Prostate Cancer Metastasis and Chemotherapeutic Drug Metabolizing Phase I, Phase II Enzymes.</td>
<td>2013</td>
</tr>
<tr>
<td>BAP-01-08-2013-001</td>
<td>Researcher</td>
<td>Doğal olarak bulunulan ellajik asit, kuersetin, naringenin, resveratrol, rutin ve hesperidin gibi polifenolik bileşiklerin ilaç metabolizmasında rol oynayan enzim aktiviteleri üzerine etkileri</td>
<td>2013</td>
</tr>
<tr>
<td>TUBITAK</td>
<td>Scholarship</td>
<td>Yakıtotunun Ksenobiyotik Metabolizması Üzerine Etkilerinin ve İzla-Diyet Etkileşim Potansiyelinin Proteomik ve Moleküler Yaklaşılardı ile Aydınlatılması</td>
<td>2009-2012</td>
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<tr>
<td>ODTU-BAP-01-08-2011-002</td>
<td>Researcher</td>
<td>Tıbbi Bitki Epilobium hirsutum' un Sulu Ekstresinin Siçan Karaciğer Mikroozomal Flavin Monooksijenaz 3 (FMO3) Protein Ekspresyonu Üzerine Etkisinin Incelenmesi</td>
<td>2011</td>
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<tr>
<td>ODTU-BAP-01-08-2010-01</td>
<td>Researcher</td>
<td>Tıbbi Bitki Epilobium hirsutum' un Sulu Ekstresinin Siçan Karaciğer Mikroozomal Flavin Monooksijenaz Enzimi Üzerine Etkisi</td>
<td>2010</td>
</tr>
<tr>
<td>ODTU-BAP-01-08-2009-03</td>
<td>Researcher</td>
<td>Fenolik Bileşik Tannik Asitin Tavşan Karaciğer ve Böbreğinde Sitokrom P450 Bağımlı Anilin 4-Hidroksilaz ve Etilmorfin N-Demetilaz Enzim Aktiviteleri Üzerine Etkisi</td>
<td>2009</td>
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</table>
### Areas of Expertise

1. Biochemistry
2. Molecular Pharmacology and Toxicology
3. Molecular Biology

### Articles

**Article excluding technical notes, letter to editor, discussions, event presentations or abstracts published in international periodicals covered by SCI (Science Citation Index), SSCI (Social Science Citation Index) or AHCI (Arts and Humanities Citation Index)**

<table>
<thead>
<tr>
<th>#</th>
<th>Authors</th>
<th>Title</th>
<th>Journal</th>
<th>Year</th>
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</table>

**Technical notes, letter to editor, discussions, event presentations or abstracts published in international periodicals covered by SCI, SSCI or AHCI.**

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<th>Authors</th>
<th>Title</th>
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<th>Year</th>
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**Papers**

Paper, poster or display presented at international congress, conference or symposium and published as summary in proceedings.

<table>
<thead>
<tr>
<th>Year</th>
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<tbody>
<tr>
<td>Year</td>
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<tr>
<td>------</td>
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<tr>
<td>2009</td>
<td>Correlation Between Spatial Learning and The Expression of Different Nitric Oxide Synthase (NOS) Isoforms in Rat Hippocampus.</td>
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<tr>
<td>2008</td>
<td>Inhibitory Effect of Tannic Acid on Glutathione S-Transferase Activity in Rabbit liver and kidney.</td>
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<tr>
<td>2010</td>
<td>The effects of Ellagic acid on Phase I enzyme.</td>
</tr>
<tr>
<td>2010</td>
<td>In vitro Effects of Tannic Acid on Liver FMO, Cytochrome P450 and Antioxidant Enzyme Activities.</td>
</tr>
<tr>
<td>2009</td>
<td>The role of Tannic Acid on the Activities of Rabbit Liver Drug and Xenobiotics Metabolizing CYP2E1, CYP2B6 and CYP3A4 dependent enzymes.</td>
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Oral Presentation at international congress, conference or symposium and published as summary in proceedings.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors</th>
<th>Title</th>
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Oral Presentation at national congress, conference or symposium and published as summary in proceedings.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors</th>
<th>Title</th>
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</thead>
</table>

Çelebioglu H. U., KARAKURT S., Adali O. In Vitro Effects of Mercury and Silver on Rat Liver NAD(P)H:Quinone Oxidoreductase I. *Turk J Bioch.* 36(1), 78, XXIII. National Biochemistry meeting, Adana, TURKEY.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors</th>
<th>Title</th>
</tr>
</thead>
</table>

KARAKURT S., Adali O. Inhibition of Rabbit Liver and Kidney NQO1 Enzyme Activity by Tannic Acid. *Turk J Bioch.* 33 (S1), 99-100, Biochemistry Congress, Kapadokya, Nevsehir, TURKEY.

Scholarships and Honors

Scholarships awarded by international organizations for scientific research or art productions.

<table>
<thead>
<tr>
<th>Year</th>
<th>Details</th>
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<tbody>
<tr>
<td>2012</td>
<td>01 April 2012-31 October 2012. Sigrid Juselius Foundation. Foreign Scientists Grants, FINLAND</td>
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<tr>
<td>2012</td>
<td>04-09 September 2012. “22nd IUBMB &amp; 37th FEBS Congress”, Seville, SPAIN. Travel Grant</td>
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<tr>
<td>2008</td>
<td>17-21 May 2008. “10th European ISSX Meeting”, Vienna, AUSTRALIA. Travel Grant</td>
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<tr>
<td>2008</td>
<td>20-22 February EMBO Young Scientists Forum, Istanbul, TURKEY. Travel Grant</td>
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Scholarships awarded by national organizations for scientific research or art productions.

<table>
<thead>
<tr>
<th>Year</th>
<th>Details</th>
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<tbody>
<tr>
<td>2011</td>
<td>Best poster Award: XXIII National Biochemical Congress, TURKEY</td>
</tr>
<tr>
<td>2010</td>
<td>27-30 October XXI I National Biochemistry Meeting, Travel Grant</td>
</tr>
<tr>
<td>2009</td>
<td>28-31 October XXI National Biochemistry Meeting, Travel Grant</td>
</tr>
<tr>
<td>2008</td>
<td>29 October-01 November XX National Biochemistry Meeting, Travel Grant</td>
</tr>
<tr>
<td></td>
<td>Top student in the department (METU, ANKARA)</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td>6</td>
<td>High Performance award in MS (METU, ANKARA)</td>
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<tr>
<td>7</td>
<td>High Performance award in BS (The Turkish Education Foundation)</td>
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<tr>
<td>8</td>
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<td>9</td>
<td>High Performance award in BS (The Turkish Education Foundation)</td>
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### Membership of Scientific / Professional Organizations

<table>
<thead>
<tr>
<th>Organization</th>
<th>Dates</th>
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<tbody>
<tr>
<td>1 AAAS-American Association for the Advancement of Science</td>
<td>01.2010&lt;&gt;---</td>
</tr>
<tr>
<td>2 FEBS-Federation of the Societies of Biochemistry and Molecular Biology, TBD-Turkish Biochemical Society</td>
<td>01.2009&lt;&gt;---</td>
</tr>
<tr>
<td>3 International Society for the Study of Xenobiotics (ISSX)</td>
<td>01.2008&lt;&gt;---</td>
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### Certification Program

<table>
<thead>
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<tbody>
<tr>
<td>1 Techniques in Animal Cell Culture and Stem Cell, Ege University, İZMİR</td>
<td>2009</td>
</tr>
<tr>
<td>2 Animal use in Experimental Research, GATA, ANKARA</td>
<td>2008</td>
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<tr>
<td>3 DNA Damage and Repair Mechanisms, Dokuz Eylül University, İZMİR</td>
<td>2007</td>
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### Miscellaneous

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<td>Reviewer Human and Experimental Toxicolog</td>
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<tr>
<td>Reviewer Pharmaceutical Biology</td>
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