EFFECT OF MEDICINAL PLANTS *EPILOBIUM HIRSUTUM* L. AND *VISCUM ALBUM* L. ON RAT LIVER FLAVIN-CONTAINING MONOOXYGENASE ACTIVITY AND EXPRESSION

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ABSTRACT

EFFECT OF MEDICINAL PLANTS *EPILOBIUM HIRSUTUM* L. AND *VISCUM ALBUM* L. ON RAT LIVER FLAVIN-CONTAINING MONOOXYGENASE ACTIVITY AND EXPRESSION

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Epilobium hirsutum L. (Onagraceae), a medicinal plant known as hairy willow herb, has been used by people all around the world for treatment or prevention of inflammation, adenoma, rectal bleeding, menstrual disorders, constipates, and prostate. It contains polyphenolics including steroids, tannins such as gallic, ellagic, and p-coumaric acids and flavonoids such as myricetin, isomyricetin, and quercetin. Polyphenols have been known for their multiple biological health benefits, including antioxidant activities.

Viscum album L. (Loranthaceae), a species of mistletoe, contains lectins, polypeptides, mucilage, sugar alcohols, flavonoids, lignans, triterpenes, and phenylallyl alcohols. The leaves and twigs of *Viscum album* L., taken as tea, have been traditionally used for hypertension, stomachache, diarrhea, diabetes, dysuria and also as analgesic and cardiotonic agent in Anatolia, Turkey. In addition, in Europe, sterile extracts of *Viscum album* L. are among the most common herbal extracts applied in cancer treatment and have been used as prescription drugs, while in US, considered as dietary supplement.

Flavin-containing monooxygenases are FAD-containing phase I enzymes responsible for the oxidation of wide-range of nucleophilic nitrogen, sulfur, phosphorus, and selenium heteroatom-containing drugs such as tamoxifen,

methimazole and imipramine, pesticides, neurotoxins, and other chemicals using NADPH as cofactor.

The aim of this study was to determine the *in vivo* effects of *Epilobium hirsutum* L. and *Viscum album* L. (subspecies growing on pine trees-subsp. *austriacum* (Wiesb.) Vollmann) on FMO activity, mRNA and protein expressions in rat liver. The water extracts of *Epilobium hirsutum* L. (37.5 mg/kg body weight) and *Viscum album* L. (10 mg/kg body weight) were injected intraperitonally (i.p) into Wistar albino rats for 9 consecutive days. Following the decapitation, the livers were removed and microsomal fractions were prepared by differential centrifugation. Rat liver microsomal FMO activity using methimazole as substrate, mRNA expression by quantitative Real-Time PCR, and protein expression by Western Blot were determined.

The results showed that water extract of *Epilobium hirsutum* L. has no significant effect on FMO activity; however, it decreased significantly ($p<0.05$) FMO3 protein and mRNA expression 27.71% and 1.41 fold, respectively, compared as controls. Water extract of *Viscum album* L. decreased mRNA (2.56 fold), and protein expressions (27.66%) as well as enzyme activity (19%) of FMO with respect to controls. In conclusion, our current data suggest that the metabolism of xenobiotics including drug molecules by FMO-catalyzed reactions may be altered due to the changes in FMO expression and activity by medicinal plants *Epilobium hirsutum* L. and *Viscum album* L.

Key words: *Epilobium hirsutum* L., *Viscum album* L., Flavin-containing monooxygenase, FMO, mRNA and protein expression, Rat liver

ÖZ

TIBBİ BİTKİLER *EPILOBIUM HIRSUTUM* L. VE *VISCUM ALBUM* L.'NİN SIÇAN KARACİĞER MİKROZOMAL FLAVİN MONOOKSİJENAZ (FMO) AKTİVİTESİ VE EKSPRESYONU ÜZERİNE ETKİLERİ

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Tıbbi bitkilerden sayılan ve insanlar tarafından tüylü yakı otu olarak bilinen *Epilobium hirsutum* L. (Onagraceae) yüzyıllardır çeşitli hastalıklar ve semptomların tedavisinde ya da önlenmesinde kullanılmaktadır. Bunlar arasında iltihaplanmalar, adenom, rektal kanamalar, menstruasyon ile ilgili düzensizlikler, konstipasyon ve prostat yer almaktadır. İçerdiği polifenoller sayesinde bitkinin bu tür hastalıklara karşı etkili olduğu düşünülmektedir. Polifenoller bitkilerde doğal olarak bulunan ve antioksidan gibi çeşitli biyolojik etkilere sahip moleküllerdir.

Aynı şekilde ökse otu olarak bilinen *Viscum album* L. (Loranthaceae) tıbbi bitkisi de çeşitli hastalıklar için kullanılmaktadır. Türkiye'de *Viscum album* L. hipertansiyon, baş ağrısı, diyare ve diyabet için, aynı zamanda analjezik ve kardiyotonik olarak kullanılmaktadır. Ayrıca Avrupa'da steril ekstraktları kanser tedavisinde en çok kullanılan bitkisel ekstraktları arasındadır ve Amerika'da ise besin takviyesi olarak kullanılmaktadır.

Flavin monooksijenazlar (FMO) FAD içeren faz I enzimlerindendir ve azot, sülfür, fosfor ve selenyum heteroatom içeren tamoxifen, methimazole ve imipramine gibi çeşitli ilaçların ve pestisitlerin, nörotoksinlerin ve diğer ksenobiyotiklerin oksidasyonunda görev almaktadır. Kofaktör olarak NADPH kullanır ve moleküler oksijen yardımıyla substratı okside eder.

Bu çalışmada *Epilobium hirsutum* L. ve *Viscum album* L. (çam ağaçları üzerinde yetişen alt türü - subsp. *austriacum* (Wiesb.) Vollmann) bitkilerinin sıçan karaciğer FMO aktivitesi, mRNA ve protein ekspresyonları üzerine *in vivo* etkileri araştırılmıştır. Bitkilerin sulu ekstraktları Wistar albino sıçanlara 9 gün boyunca intraperitoneal enjeksiyon olarak uygulanmıştır. 11 tane sıçan ise kontrol olarak kullanılmıştır. Daha sonra, sıçanlar dekapite edilerek karaciğerleri çıkarılmış ve mikrozomal fraksiyonlar elde edilmiştir. FMO aktivitesi, substrat olarak methimazole kullanılarak tayin edilmiştir. mRNA ekspresyonu tayini, kantitatif gerçek zamanlı PZR kullanılarak, protein ekspresyonu tayini ise "Western blot" tekniği ile elde edilmiştir.

Deneyler sonucunda *Epilobium hirsutum* L. bitkisinin sulu ekstraktının FMO aktivitesi üzerine anlamlı herhangi bir etkisi olduğu bulunmamış, fakat mRNA ve protein ekspresyonlarını kontrollere göre sırasıyla %27.71 ve 1.41 kat oranında istatistiksel olarak anlamlı bir şekilde azalttığı ortaya çıkarılmıştır ($p<0.05$). Aynı şekilde *Viscum album* L. bitkisinin sulu ekstraktının FMO mRNA ve protein ekspresyonlarını istatistiksel olarak anlamlı bir şekilde sırasıyla 2.56 kat ve %27.66 oranında azalttığı ortaya çıkarılmıştır ($p<0.05$). Bunların yanı sıra *Viscum album* L. bitkisinin sulu ekstraktı FMO aktivitesini de kontrollere göre %19 oranında istatistiksel olarak anlamlı bir şekilde azaltmıştır ($p<0.05$).

Bu sonuçlar göstermektedir ki *Epilobium hirsutum* L. ve *Viscum album* L. tıbbi bitkileri FMO ekspresyonunu değiştirebilmekte ve böylelikle FMO tarafından katalizlenen ksenobiyotik metabolizmasını etkilemektedir.

Anahtar kelimeler: *Epilobium hirsutum* L., *Viscum album* L., Flavin monooksijenaz, FMO, mRNA ve protein ekspresyonu, Sıçan karaciğeri

Dedicated to my beloved wife

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APS	Ammonium per sulfate
BCIP	5-bromo 4-chloro 3-indoyl phosphate
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
dNTP	Deoxynucleoside triphosphate
DTNB	5,5-bithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
ε-ACA	ε-Amino caproic acid
EDTA	Ethylenediaminetetraacetic acid
EH	Epoxide hydrolase
ERB	Electrophoretic running buffer
FAD	Flavin adenine dinucleotide
FMO	Flavin-containing monooxygenase
GAE	Gallic acid equivalents
GST	Glutathione S-transferase
HNF1α	Hepatic nuclear factor 1 alpha
HNF4α	Hepatic nuclear factor 4 alpha
IL	Interleukin
kDa	kilo Dalton
$NADP^+$	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitrotetrazolium blue chloride
NK cell	Natural killer cell
OD	Optical density
PCR	Polymerase chain reaction

SDB	Sample dilution buffer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SULT	Sulphotransferase
TBST	Tris-buffered saline and Tween 20
TEMED	Tetramethylethylenediamine
TNB	Nitro-5-thiobenzoate
TNF-α	Tumor necrosis factor-alpha
UGT	Glucuronosyl transferase
UV	Ultra violet
$\mathbf{V_{max}}$	Maximum velocity

CHAPTER 1

INTRODUCTION

Since the beginning of the time, humankind has been using the plants as many domains, including flavoring, nutrition, beverages, dyeing, cosmetics and medicine. Some plants are known for their health benefits; some are yet to be known and their possible health benefits should become clear. Use of plants as alternative medicine is increasing each year. The herbs are thought as safe, while the confidence to the synthetics has been decreasing. People who could not get their expectations from the synthetic drugs have been turning to the herbs and natural products. Emphasis on the medicinal plants have been increased because human population increases, supply of drugs are inadequate, treatments are highly cost, there are side effects of several allopathic drugs and resistance to currently used drugs are developed in infectious diseases. Many plant species have been studied and showed to be beneficial for human health; for example antioxidant activities, digestive stimulation action, anti-inflammatory, antimicrobial, anti-mutagenic effects and anti-carcinogenic potential of some species have been determined. Thus, people who consume diet rich in fruits and vegetables are likely to have less risk for developing diseases like cancers (Stich & Rosin, 1984; Stoner & Mukhtar, 1995). However, increased use of medicinal plants by human also increased the reports for suspected toxicity and adverse effects of them. The safety of the herbal medicines has gained importance since serious adverse effects such as hepatotoxicity, renal failure and allergic reactions have been reported (Debbie *et al.*, 2012).

Increasing consumption of plant ingredients worldwide especially in US and Europe take a serious attention and more studies are demanded (Gruenwald *et al.*,

2000). Such plants possess thousands of phenolic and polyphenolic compounds that have important roles in human nutrition. These chemicals can modulate enzymes responsible for the metabolism of xenobiotic and endogenous compounds. One group of these enzymes is Flavin-containing monooxygenases. Flavin-containing monooxygenases (FMOs, EC 1.14.13.8) are FAD-containing Phase I detoxifying enzymes. They are responsible for catalysis of oxidation of a wide range of xenobiotics, including drugs, such as morphine, chlorpromazine, imipramine, tamoxifen and ranitidine, dietary constituents, environmental toxicants, and pesticides (Attar *et al.*, 2003; Bhamre *et al.*, 1995; Halpert et al., 1998; Hodgson *et al*., 1995; Kupfer & Dehal, 1996; Störmer *et al.,* 2000). Their substrates are soft nucleophiles that contain nitrogen, sulfur, phosphorous or selenium heteroatoms. FMO was first purified from pig liver microsomes in 1972 by Ziegler and Mitchell, but we know now they exist in many species and in more than one organ. They were purified from liver microsomes of rat (Kimura *et al.*, 1983), mouse (Sabourin & Hodgson, 1984; Sabourin *et al.*, 1984), rabbit (Tynes *et al.*, 1985), guinea pig (Yuno *et al.*, 1990), from pulmonary (Tynes *et al.*, 1985) and renal (Venkates *et al.*, 1991) microsomes of mouse, and from brain microsomes of rat (Kawaji *et al.*, 1995) and human (Bhagwat *et al.*, 1996). FMOs are bound to membranes of smooth endoplasmic reticulum and nuclear envelope of the cell, while cytosolic forms predominate in bacteria and unicellular organisms (Schlenk, 1998; Sum & Kasper, 1982). FMO activity, protein or mRNA has also been observed in several invertebrates, fish, reptile, avian and amphibian species (Schlenk, 1998). Although their main responsibility is xenobiotic metabolism, the plants also can express FMOs for the catalysis of specific steps in the biosynthesis of auxin or in the metabolism of glucosinolates, and, furthermore, for pathogen defense (Schlaich, 2007).

Although FMOs are responsible for the metabolism of xenobiotics such as drugs, pesticides, dietary constituents, and environmental toxicants, they can be modulated by some of these xenobiotics (Adali *et al.*, 1998; Can Demirdögen & Adali, 2005). Because they are prominent enzymes for the metabolism and detoxication of drugs and chemicals, understanding the effects of medicinal plants used for their possible health benefits on FMO activity and expression would

provide insight into features which would expedite drug discovery and development.

1.1 *Epilobium hirsutum* L.

Epilobium hirsutum L. is a flowering plant belonging to the Onagraceae family. It is known as hairy willow herb, hairy willow-weed, or willow herb in English and “tüylü yakı otu” in Turkish. Its habitat is moist waste ground of the Mediterranean region, Europe, Asia, and Africa. In Turkey, its distribution is very wide, almost everywhere in Turkey. *Epilobium hirsutum* L. is a perennial, flowering between July-September and it spreads by seeds (Figure 1.1). It is a semi-aquatic, softly-hairy herb that ranges in height from 30 cm to 2 meter tall. The overall plant is covered with fine soft hairs; the leaf arrangement is mostly opposite, and the toothed sessile leaves are lanceolate. Flowers are approximately 1.9 cm across (Davis, 1972; Shamsi & Whitehead, 1974).

Figure 1. 1 *Epilobium hirsutum* L.

The species has wind-dispersed seeds, (Salisbury, 1961) and the seeds seem to be potentially very mobile in respect to dispersal. After germination, the upper lateral roots of the primary root system of *Epilobium hirsutum* develop faster and they have equal importance with the primary axis. Then, adventitious roots arise from the hypocotyl, and on the basal parts of the stem. The plant has pink flowers and capsules in flowers having length of 4 to 10 cm contain seeds 1-1.5 mm in diameter (Shamsi & Whitehead, 1974).

Epilobium hirsutum L. contains polyphenolics including steroids (especially β-sitosterol and its esters), tannins (Gallic, protocatechuic, ellagic and p-coumaric acids) and flavonoids (in particular myricetin, isomyricetin, quercetin, and

quercetin-3-O-β-d-glucuronide) (Barakat *et al.*, 1997; Gruenwald *et al*., 2000; Ivancheva *et al.*, 1992).

Polyphenolic acids are typically found in both edible and inedible plants. These polyphenolics have been studied for their multiple biological benefits, including antioxidant activities (Kähkönen *et al*., 1999). They are a major class of phenolic compounds, characterized by the presence of large multiples of phenol structural units. Wojdylo *et al.* (2007) have found that *Epilobium hirsutum* has total phenolic content of 4.03 ± 0.12 mg of GAE/100 g of dry weight (dw). They also demonstrated that *Epilobium hirsutum* has phenolic acids, 23.1 ± 0.03 mg/100 g dw of caffeic acid, 38.3 ± 0.09 mg/100 g dw of p-coumaric acid, and 10.9 ± 0.29 mg/100 g dw of ferulic acid; flavonoids, 214 ± 0.03 mg/100 g dw of quercetin and 191 ± 0.24 mg/100 g dw of myricetin.

Epilobium hirsutum L. has been reported to be used for treatment of inflammation, adenoma and prostate tumors in European and Egyptian folkmedicine (Barakat *et al*., 1997). *Epilobium* sp. was also used for preventing rectal bleeding by Native Americans and for treating menstrual disorders by Chinese people (Gruenwald *et al*., 2000). For treating constipate and prostate, the leaves and roots of *Epilobium* sp. have been used in Anatolia. Moreover, they are used as antifebrile drug (Everest & Ozturk, 2005; Tita *et al.*, 2001).

Water extract of *Epilobium* sp. revealed significant inhibitory effects on edema of rat paws, although methanol extraction had weaker result (Gruenwald *et al.*, 2000). Antimicrobial effects of the plant have also been observed, inhibiting growth of *Pseudomonas pyocyanea*, *Candida albicans*, *Staphylococcus albus* and *Staphylococcus aureus*. The studies showed that *Epilobium* sp. can have tumor-inhibiting effects on transplanted tumors of mice and rats. It can be helpful for treating benign prostate hyperplasia and some urination disorders. However; the investigation about possible health or side effects of *Epilobium* sp. for humans is limited (Gruenwald *et al.*, 2000; Vitalone *et al.*, 2001).

1.2 *Viscum album* L.

The genus *Viscum* of Loranthaceae family has approximately 100 species distributed mostly in temperate or tropical regions of Europe, Africa, Madagascar and some in Asia (Davis, 1982; Zuber, 2004). *Viscum album* L. is the only species of this genus growing in Turkey and it is a perennial evergreen hemi-parasitic shrub, whose globe diameter reaching up to 150 cm. It is known as European mistletoe in English and "ökse otu" in Turkish. Its oppositely arranged leaves are obovate-oblong, obtuse in shape and 3-5 parallel veined. Its flowers are small, yellowish-green, inconspicuous, and sessile in 3-5 flowered fascicles (Figure 1.2). *Viscum album* L. is dioecious, which means its sexual organs male and female flowers are separated. It is hemi-parasitic, so the plant can synthesize it own chlorophyll but must obtain the water and other mineral supply from the host plant. Its sexual reproduction starts after 4 to 5 years of age. Since it is dioecious, it is impossible to fertilize itself. Therefore, external fertilizers such as honeybees, bumblebees, flies as well as wind are required. After a dormancy of 5–6 months, the seeds can germinate with help of light and temperature on a suitable host plant (Davis, 1982; Zuber, 2004). In Turkey, *Viscum album* is represented by three subspecies namely, subsp. *album*, subsp. *abietis* (Wiesb.) Abromeit and subsp. *austriacum* (Wiesb.) Vollmann. These taxa have different hosts -various Dicotyledonous trees (subsp. *album*), fir trees (subsp. *abietis*) and pine trees (subsp. *austriacum*)- and therefore be generally identified if these are known (Davis, 1982).

All over the world, from northern countries of Europe to Asian countries, *Viscum album* L. has been used traditionally as sedative, analgesic, anti-spasmolytic, cardiotonic, and anticancer agent, to treat epilepsy, hypertension, exhaustion, arthritis, anxiety, vertigo, degenerative inflammation of the joints, and epilepsy (Poppenga & Abvt, 2002). Moreover, the decoction of *Viscum* plant is used for enlarged spleen. In Turkey, the plant is used for cardiac disorders and hypertension in the Middle, East and Southeast Anatolia and in diabetes by the inhabitants of Beypazarı, Ankara, Turkey *(*Baytop, 1999; Gençler-Özkan and Koyuncu, 2005; Yücecan *et. al*, 1988; Simsek *et. al.*, 2004;Gupta *et al., 2012)*. In

addition, the preparations of *Viscum album* L. have been used clinically in adjuvant cancer therapy in Europe and therefore various studies have been conducted on the plant since 1926 (Yesilada *et al.*, 1998).

Figure 1. 2 *Viscum album* L.

Viscum album L. possess various biologically active compounds; flavonoids (Wollenweber *et al.*, 2000), lectins (Peumans & Van Damme, 1996; Stirpe *et al.*, 1980), viscotoxins (Orrù *et al.*, 1997; Samuelsson & Jayawardene, 1974), alkaloids, terpenoids (Deliorman *et al.*, 2001), polysaccharides, saponins, tannins, vitamins, phytosterols, hydrocarbons and long-chain fatty acids (Gupta *et al.*, 2012; Radenkovic *et al.*, 2006).

The mistletoe lectins, glycoproteins with carbohydrate proportion, have characteristics to agglutinate cells, recognize and bind some sugars. They consist of two chains; A-chain with toxic, enzymatic properties and B-chain binding to carbohydrate; these chains are bound each other by a disulphide bridge (Bar-Sela, 2011). They have two effects directly and indirectly. The direct effect of the lectins is damage to tumor cells through the inhibition of protein synthesis and induction of apoptosis (Büssing *et al*., 1996). The indirect effect is the stimulation of immunological processes (Wiedłocha *et al.*, 1991).

Viscotoxins of *Viscum album* L. are basic polypeptides having low molecular weight approximately 46 amino acid-length, connected by three disulphide bridges. They belong to thionine group since they have high cysteine content (Pal *et al*., 2008). Although the effects of viscotoxins are not studied well, like lectins, they also have roles in immunogenic responses such as induction of antibodies, activation of cytotoxic T-cells and so that they can easily destroy bacteria and also tumor cells, inhibit protein, RNA, and DNA syntheses and induction of NK-cell-mediated cytotoxicity against tumor cells (Mueller *et al.*, 1989; Schaller & Urech, 1996).

Viscum album L. has a various flavonoids within the leaves and stems. The researches have showed that these flavonoids have anti-nociceptive (pain caused by stimulation of peripheral nerves which respond to stimuli approaching or exceeding harmful intensity-nociceptors) and anti-inflammatory activities towards rats having p-benzoquinone-induced writhing and edema, respectively (Orhan *et al.*, 2006).

Table 1.1 summarizes some of the substances that *Viscum album* L. extract contains and their biological effects on tumor and immune cells.

Table 1. 1 Constituents of Viscum album L. (Adapted from Bar-Sela, 2011).

Macromolecule	Substance	Effect on tumor cell	Effect on immune cell
Flavonoids	Quercetin-derivatives	Induction of Apoptosis	Anti-Oxidative and Protective Effects
Glycoproteins	Mistletoe lectins I, II, and III	Cytotoxicity via Inhibition of Protein synthesis and Induction of Apoptosis	Release of TNF-α, IL1, IL2, IL6; Enhancement of NK-cell and Phagocytic Activities
	Visalb CBA	Mild Cytotoxicity	Stimulation of Lymphocyte
Polypeptides	Viscotoxins A1–A3, B, 1-Ps, U-PS	Cytotoxicity via Lysis of Cell Membrane	Activation of Macrophage and Enhancement of Phagocytic Activity of Granulocytes
Peptides	Peptide 5000 Da (Kuttan, Vasudevan, & Kuttan, 1992)	Cytotoxicity and Tumor Inhibition in Animal Studies	Activation of Macrophage and Enhancement of Cytotoxic Activity
Oligo- and Polysaccharides	Arabinogalactans, galacturonans	Tumor Inhibition in Animal Studies	Stimulation of T Helper-cells; Enhancement of NK cell activity and Release of Interferon- γ

1.3 Xenobiotic Metabolizing Enzymes

When administrated, a xenobiotic molecule faces with the metabolic pathway in a series of sequential reactions. At the end of this metabolic pathway, the molecule administrated is converted to metabolite which is either more active or less active. These reactions were classified into two phases: Phase I and Phase II Xenobiotic Metabolism.

Phase I reactions are responsible for the conversion of a molecule to more water-soluble (hydrophilic) metabolite by means of oxidation, reduction and hydrolysis (Nassar, 2009). If a lipophilic molecule enters to body, it can be trapped within the body because of the lipophilic characteristics of the membranes unless it is changed to hydrophilic metabolite. Therefore, in order to have hydrophilic properties, phase I reactions introduce a polar group to the xenobiotic molecule, or they unmask a functional polar group by hydrolysis, such as hydroxyl (-OH), amino (-NH-), and carboxylic acid (-COOH). Phase I xenobiotic metabolizing enzymes are known as Cytochrome P450-dependent enzymes (CYPs) and Flavin-containing monooxygenases (FMOs) (Coleman, 2005; Nassar, 2009).

Phase II reactions are conjugation reactions, in which glucuronidation, acetylation, methylation, glutathione conjugation, sulfonation, and glycine/glutamine conjugation occur by conjugation enzymes glucuronosyl transferases (UGTs), acetyl transferases, N-, O-, S-methyl transferases, glutathione S-transferases (GSTs), sulphotransferases (SULTs), and epoxide hydrolases (EHs) (Coleman, 2005). They are substitution reactions, conjugating a group to parent molecule (xenobiotic or endogenous compound) or the metabolite resulted from phase I reactions. Sulfonation, glucuronidation, and amino acid conjugation reactions lead to increase in polarity of the molecule; acetylation and methylation reactions lead to termination of therapeutic or toxic activities; glutathione conjugation reaction leads to protection against reactive metabolites (Nassar, 2009).

1.3.1 Phase I Xenobiotic Metabolizing Enzymes

Cytochrome P450 superfamily (CYPs), large and diverse group of phase I enzymes found in all living organisms from bacteria to mammals, is responsible for catalysis of most xenobiotic oxidation reactions (Meunier *et al.*, 2004; Nassar, 2009).

Although CYPs and FMOs both are phase I metabolizing enzymes and share some common features, they have distinct properties both in action mechanism and substrate specificity. The main distinct between two groups of enzymes is that FMOs have a unique action mechanism enzymes wherein a potentially reactive hydroperoxyflavin intermediate is formed in the presence of oxygen and NADPH, but in the absence of substrate (Poulsen & Ziegler, 1995). Unlike CYPs, the first step of catalysis mechanism of FMO is the reduction of FAD by NADPH. Then, molecular oxygen is added to FAD reduced and the enzyme becomes active before the substrate binding (Ziegler, 2002). Table 1.2 summarizes the similarities and differences between CYPs and FMOs. For example, FMOs are heat unstable so that without NADPH at 50ºC they lose their catalytic activity but in similar conditions CYPs retain about 85% of their activity in the microsomes. Moreover, high concentrations of some detergents like Emulgen can reduce the CYP activities but not significantly FMO activity (Cashman, 2005). CYPs and FMOs share some overlapping substrates and form similar products, but the catalytic mechanisms are quite different.

Table 1. 2 Comparison of FMO and CYP Enzymes (Cashman, 2005; Coleman, 2005; Ingelman-Sundberg, 2004).

Feature	FMO	CYP
Substrate	Soft nucleophiles containing N, S, P or Se heteroatoms	A very wide range of organics containing or not containing N, S, P, Se heteroatoms
Co-substrate	NADPH, O_2	NADPH, O_2
Localization in cell	Smooth endoplasmic reticulum and nuclear envelope of the cell	Endoplasmic reticulum and cytosol
Protein Structure	Tightly bound FAD	Contain heme cofactor
Co-enzyme	None	NADPH-CYP reductase
Isoform	5 in humans and pseudogenes	More than 3000 currently
Regulation	Various physiological factors such as cofactor supply and diet, tissue and species dependence	Many of inducers and inhibitors, based on competition or mechanism
Genetic Variation	Genomic DNA variants and SNPs	All CYPs are polymorphic

1.3.1.1 Flavin-containing monooxygenases

Flavin-containing monooxygenases (FMOs, EC 1.14.13.8) are distinct monooxygenases having roles in the metabolism of different xenobiotics from drugs to insecticides and pesticides (Krueger & Williams, 2005; Ziegler, 1993).

FMOs exist in five forms, FMO1 to FMO5; with six pseudogenes. The genes are grouped into a family if they share 82% or higher identity of nucleotide sequence (Lawton *et al.*, 1994). They show tissue- and temporal-dependent expression pattern. FMO1 has the broadest substrate range; FMO2 is the narrowest because its active site is restricted; FMO3 has the highest activity and also high expression; FMO4 shows the lowest expression-with its low activity considered insignificant with respect to drug metabolism; and FMO5 has significant expression, but has relatively low activity and very narrow substrate range (Nassar, 2009). Another important characteristic of FMOs is their thermal instability, which means that FMOs are unstable unless the optimum temperatures are met. In addition, FMO activity is dependent on pH and expression can be modulated by dietary component (Can Demirdögen & Adali, 2005; Nassar, 2009).

FMOs are highly polymorphic enzymes; there are significant variation in the frequencies of single- and multiple-site alleles, haplotypes and genotypes of FMO observed in DNA from healthy individuals (Cashman, 2004). FMO1 has 34, FMO2 has 57, FMO3 has 40, FMO4 has 30, and FMO5 has 40 genomic DNA variants found so far (Cashman, 2002). These variants can change the enzyme activity so that either decreased or increased metabolism of xenobiotics by FMOs can be observed. Loss-of-function mutations in FMO3 can lead to a disease state called Fish odor syndrome or trimethylaminuria. The main characteristic symptom of trimethylaminuria is the smell of body like rotting fish; there is no other physical symptom; the patients seem to be healthy. The impaired metabolic oxidation of trimethylamine, simple tertiary aliphatic amine derived from dietary precursors, such as choline and lecithin, via the action of bacteria in the intestine, leads to the excretion of excessive amounts so that sweat, urine, reproductive fluids, and breath of the patients smell like strong odor (Mitchell & Smith, 2001).

1.3.1.1.1 Action Mechanism of FMO

FMOs have ordered sequential catalytic cycle where before the addition of substrate to the enzyme, NADPH and molecular oxygen are added. $NADP^+$ and water both are released from the enzyme after the release of oxygenated substrate. Binding of NADPH and molecular oxygen to the enzyme forms a very reactive intermediate called 4a-hydroperoxyflavin, that can easily oxidize any soft nucleophile that contacts this form (Ziegler, 1993, 2002).

Figure 1.3 shows general action mechanism cycle for FMOs. In step 1, NADPH binds to enzyme and FAD in the structure undergoes two-electron reduction. Just after that, in step 2, reduced FAD interacts with molecular oxygen to form relatively stable 4a-hydroperoxyflavin intermediate. This intermediate can be produced due to two reasons. Firstly, 4a-hydroperoxyflavin intermediate is resistant to decomposition so that it lives remarkable long. This indicates there are some non-nucleophilic amino acid residues at the active site in order to create a lipophilic environment so this environment can preserve the highly reactive 4a-hydroperoxyflavin intermediate. Secondly, unlike CYPs which have only oxidizing agents after substrate binding, this form of FMO has a ready position for substrate and it is the predominant in the cell so that it can react with a suitable substrate (Krueger & Williams, 2005). Therefore, the energy which is needed for the reaction is already acquired before the substrate binding (Ziegler, 2002). In step 3, the soft nucleophilic substrate binds to 4a-hydroperoxyflavin intermediate, which results in transfer of one oxygen atom to substrate and one to form water, thought as the rate-limiting step of the reaction (step 4). The last step in the cycle is the release of $NADP^+$, which is also slow reaction (step 5). Thus, the addition of substrate has no effect on the V_{max}. If a substrate is not bound to 4a-hydroperoxyflavin intermediate at the step 3, the intermediate breaks down and returns to original form (step 6) (Krueger & Williams, 2005).

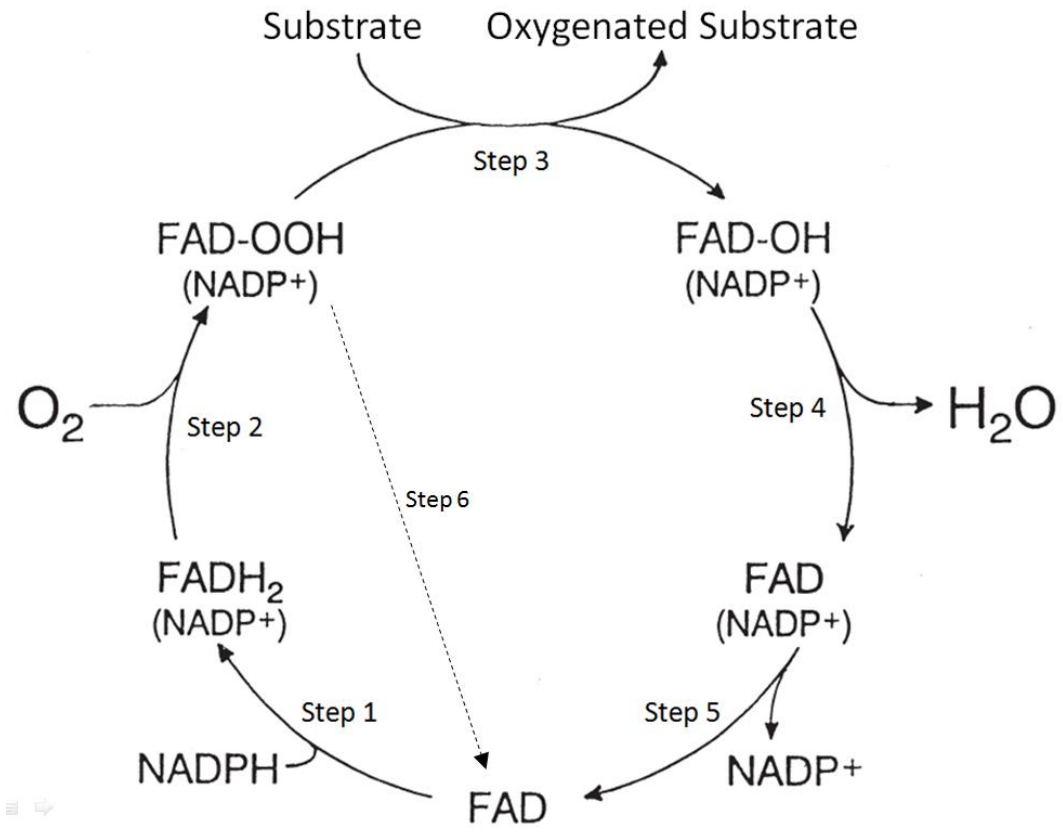


Figure 1. 3 Catalytic Cycle of FMO (Adapted from Ziegler, 2002).

Prosthetic group Flavin Adenine Dinucleotide (FAD) of FMO and NADPH are the main sites where catalytic activity of FMO is carried out. The study conducted with FMO protein from *S. pombe* by Eswaramoorthy *et al.* (2006) showed the sites of the protein where catalytic mechanism occurs. In this study, they showed that flavin binds to the enzyme strongly, while NADPH more loosely.

1.3.1.1.2 Isoforms of FMO

In human, there are five FMO families having functional roles in addition to pseudogenes. If the FMOs have greater than 40% identity, they are classified into same family (Lawton *et al*., 1994). The genes of FMO are located on long arm of chromosome 1, while another cluster of FMO genes can be found in another region of chromosome 1 but they are pseudogenes (Cashman *et al.*, 2001). It is thought that FMO genes arose by duplication of ancestral gene or through a series of events of independent gene duplications (Hernandez *et al.* , 2004).

FMO proteins have 532 to 558 amino acids with some specific conserved amino acid residues in all species. These conserved residues are particularly NADPH- and FAD- binding domains. The molecular masses of FMOs are between 60 to 63 kDa (Cashman *et al.*, 1995).

FMO1 is 532-amino acid in length with molecular mass of 60.3 kDa and has neutral pI of 6.9 (Phillips *et al*., 1995). It is the most expressed form in the adult human kidney and this expression is 10 to 14-fold higher than that in the fetal liver and small intestine. In other organs such as lung and brain, its expression is very low compared to kidney (Cashman & Zhang, 2006).

FMO2 has 535 amino acids with a molecular weight of 60.9 kDa and has a basic pI of 8.9 (Phillips *et al*., 1995). Its expression is predominant in the adult human lung, about 7-fold higher than FMO2 expression in the kidney. Liver and brain have very low expressions of FMO2 (Cashman & Zhang, 2006).

FMO3 has molecular mass of 60.1 kDa with 532 amino acids and has basic pI value of 8.3 (Phillips *et al*., 1995). It is the dominant form of FMO in adult human liver, while its expression in lung, kidney, fetal liver, and small intestine, is present 4.5%, 3.7%, 2.1%, and 1% of the amount of adult liver, respectively (Cashman & Zhang, 2006). Adult human brain tissues express less than 1% of adult liver FMO3 with no change during the development (Cashman & Zhang, 2006). Figure 1.4 shows the amino acid sequence alignment of FMO3 of human and rat.

Figure 1. 4 Amino Acid Sequence Alignment of Human and Rat FMO3 with the score of 80.0 (ClustalW2-Multiple Sequence Alignment). Asterisk (*) indicates entirely conserved residues, colon (:) indicates residues have roughly the same size and same hydropathy, and dot (.) indicates residues whose size and hydropathy have been preserved in the course of evolution. (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

FMO4 has 558 amino acid, molecular weight of 63.3 kDa, and pI value of 9.1 (Phillips *et al*., 1995). It has the most expression in adult liver and kidney, while its expressions are 10.9%, 10.8%, and 7.0% of FMO4 of adult liver in fetal liver, small intestine, and lung, respectively. Adult brain has the least expression for FMO4, less than 1% of the amount of FMO4 present in the liver and is not changed as a function of age (Cashman & Zhang, 2006).

FMO5 is 533-amino acid length, 60.2 kDa, and has pI value of 8.6. Although FMO5 enzyme activity has not been clearly determined, it is the most expressed FMO form (almost same as or greater than FMO3) in human adult liver, whereas its expressions in fetal liver, small intestine, kidney, and lung are at considerable levels. In brain, there is very low expression of FMO5, less than 1% that of adult liver and the amount does not change during development (Cashman & Zhang, 2006). It has very little or no activity towards methimazole and other common FMO substrates like ranitidine and cimetidine and therefore it is thought that FMO5, as well as FMO4, have very restricted substrate specificity and no considerable contribution to drug metabolism in human (Krueger & Williams, 2005).

FMOs have higher expressions in adult liver than fetal liver, except FMO1, which indicates that FMOs show tissue-specific and developmental stage-specific expressions as shown in Table 1.3 (Cashman & Zhang, 2006).

Table 1.4 shows the comparison between human and rat FMOs, with their amino acid length, the main organ found, and the evidence of expression (transcript or protein).

Table 1. 3 Expressions of FMOs in human tissues, as copies per ng of RNA (taken from Cashman & Zhang, 2006).

	FMO1	FMO2	FMO3	FMO4	FMO5
Fetal liver	945.7	93.1	445.6	488.3	4406.8
Adult liver	96.0	988.7	23,088	4881.7	26,539.5
Fetal brain	56.4	17.6	5.6	14.6	21.0
Adult brain	3.1	140.9	10.7	19.6	56.5
Adult kidney	6198.2	4682.7	530.9	2509.9	1628.3
Adult lung	595.7	115,895	2223.9	738.1	2274.9
Adult small intestine	522.9	928.7	74.2	403.0	2586.3

Table 1. 4 Comparison of FMO1 to FMO5 of Human and Rat. The information is obtained from UniProtKB Protein knowledgebase, with query numbers of Q01740 for human FMO1, Q99518 for human FMO2, P31513 for human FMO3, P31512 for human FMO4, P49326 for human FMO5; P36365 for rat FMO1, Q6IRI9 for rat FMO2, Q9EQ76 for rat FMO3, Q8K4B7 for rat FMO4, and Q8K4C0 for rat FMO5. (http://www.uniprot.org/uniprot/).

	FMO1	**FMO2**	**FMO3**	**FMO4**	**FMO5**
Human	532 a.a	471 a.a	532 a.a	558 a.a	533 a.a
	Kidney	Lung	Liver	Kidney and liver	Liver
	Transcript level	Protein level	Protein level	Transcript level	Transcript level
Rat	532 a.a	535 a.a	531 a.a	560 a.a	533 a.a
	Liver	Lung and kidney (Lattard *et al*, 2002)	Kidney and Liver	Kidney (Novick *et al.*, 2009)	Liver
	Protein level	Transcript level	Protein level	Transcript level	Protein level

1.3.1.1.3 FMO-catalyzed Metabolism

FMOs are known for their catalysis of oxidation of various xenobiotic compounds including sulfur-, nitrogen-, selenium-, and phosphorous-containing heteroatoms. In addition to a wide range of xenobiotics; a limited numbers of endogenous compounds can be metabolized by FMOs (Elfarra, 1995; Krueger & Williams, 2005). Therefore, the substrates metabolized by FMOs can be classified into two groups as endogenous and xenobiotic substrates, although the number of xenobiotic substrates is much higher than the endogenous substrates.

FMOs catalyze some sulfur- and nitrogen-containing endogenous compounds such as cysteamine, lipoic acid, methionine, and trimethylamine (Elfarra *et al.*, 1994; Krueger & Williams, 2005).

Cysteamine is a sulfur-containing substrate for FMOs and it is metabolized through S-oxygenation to its disulfide form, but the physiological importance of this metabolism has not been investigated yet (Krueger & Williams, 2005). Another example of sulfur-containing endogenous substrate for FMO is lipoic acid, which has role as cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Krueger & Williams, 2005); it is also available in antioxidant dietary sources (Bustamante *et al.*, 1998) and can be used as therapeutic drug (Smith *et al.*, 2004). FMOs metabolize disulfide lipoic acid and lipoamide S-oxygenation (Krueger & Williams, 2005).

Methionine, an endogenous substrate for FMOs (Figure 1.5), was metabolized through S-oxidation catalyzed by FMOs in mammalian liver and kidney (Elfarra, 1995).

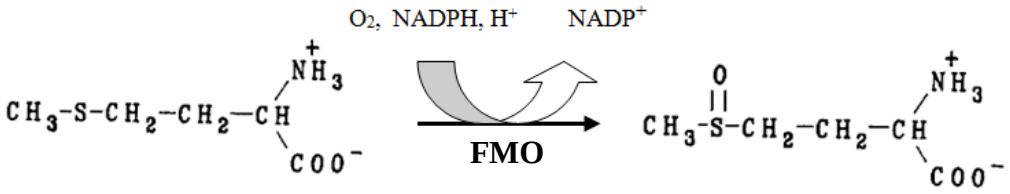


Figure 1. 5 Metabolism of Methionine by FMO-catalyzed S-oxygenation (taken from Elfarra, 1995).

Another example of endogenous compounds that are metabolized by FMO is trimethylamine, simple tertiary aliphatic amine also taken from the diet (Krueger & Williams, 2005). Trimethylamine is naturally converted from choline and carnitine found in egg yolk, liver, kidney, soybeans, peas, and salt-water fish, or by reduction of trimethylamine N-oxide in the gut by bacterial activities (Lang *et al.*, 1998; Rehman, 1999). After absorption through the gut, trimethylamine is N-oxygenated to the odorless metabolite, trimethylamine N-oxide by FMO in liver as shown in Figure 1.6 (Mitchell & Smith, 2001). Trimethylamine smells very odorously like rotting fish. If FMOs fail to catalyze the N-oxygenation of trimethylamine to its non-odorous metabolite, the genetic disease known as trimethylaminuria or "fish odor syndrome" is developed (Krueger & Williams, 2005). The syndrome is characterized by the body odor of rotting fish due to excess excretion of trimethylamine in the breath, sweat, urine and as well as other body secretions (Mitchell & Smith, 2001). The main isoform that catalyzes the N-oxygenation of trimethylamine is FMO3 and some polymorphisms of FMO3 can lead to inactivation of FMO3 catalytic activity so that metabolism of trimethylamine is impaired.

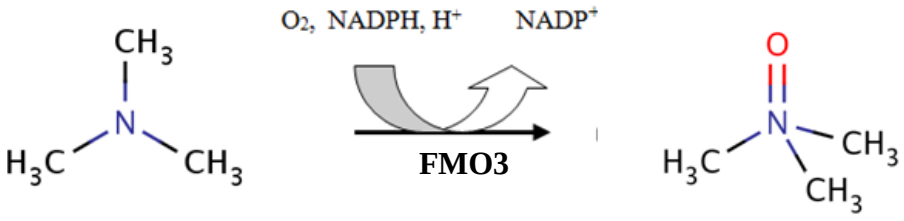


Figure 1. 6 N-Oxygenation of Trimethylamine by FMO3.

Unlike endogenous substrates, there is a wide variety of exogenous substrates for FMOs, from inorganic compounds to drugs such as tamoxifen, methimazole and imipramine, pesticides, neurotoxins, and other xenobiotics (Ziegler, 1993). Some of the xenobiotic substrates of FMOs are listed in Table 1.5.

Table 1. 5 Xenobiotics Metabolized by FMOs (adapted from Hodgson *et al*., 1995; Ziegler, 1988; Smart & Hodgson, 2008).

Substrate	Example
Inorganics	
HS^-, S_8, I^-, I^2	
Nitrogen-Containing	
Primary Amines	Tyramine, n-Octylamine
Secondary Amines	N-methylaniline, Perazine
Tertiary Amines	Nicotine, Imipramine, Tamoxifen
Heterocyclic Amines	Xanomeline, Clozapine
Hydrazines	Phenylhydrazine
Sulfur-Containing	
Sulfides	Dimethylsulfide
Disulfides	Butyl disulfide acid
Thioamides	Thioacetamide, Thionicotinamide
Thiocarbamides	Methimazole, Thiourea
Thiocyanates	Phenylisothiocyanate
Sulfoxides	Thiobenzamide sulfoxide
Sulfenic Acids	N-methylimidazole-2-sulfenic acid
Carbodithioic Acids	Phenylcarbodithionic
Phosphorous-Containing	
Phosphines	Diethylphenylphosphine
Phosphothioates	Fonofos
Selenium-Containing	
2-selenylbenzanilide	
Boronic Acid	

Nitrogen-containing heteroatoms have been known to be metabolized by FMOs. Primary, secondary, tertiary and heterocyclic amines as well as hydrazines can be oxidized in reactions catalyzed by FMOs (Cashman, 2000). Some examples are listed in Table 1.5.

Human liver FMO3 catalyzes the metabolism of biogenic amines such as tyramine, nicotine, and tamoxifen. Tyramine is metabolized to trans oxime in human liver; also it is N-oxygenated to hydroxylamine (Cashman, 2002). (S)-nicotine is N-oxygenated by FMO3 to form nicotine N-1'-oxide (Figure 1.7). (S)-nicotine has two heteroatom-containing chemical taken from the smoke and FMO3 catalyzes its metabolism efficiently to relatively stable and chemically well-characterized N-oxide metabolite (Cashman *et al*., 1995). Tamoxifen, anti-estrogenic drug widely used for breast cancer treatment, have been reported that it is N-oxidized mouse FMO1 and human FMO3 as shown in Figure 1.8 (Hodgson *et al.*, 2000).

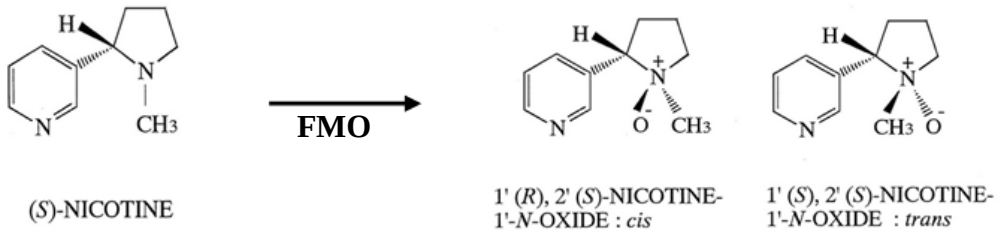


Figure 1. 7 N-Oxygenation of (S)-Nicotine catalyzed by FMO (adapted from Cashman et al., 1995).

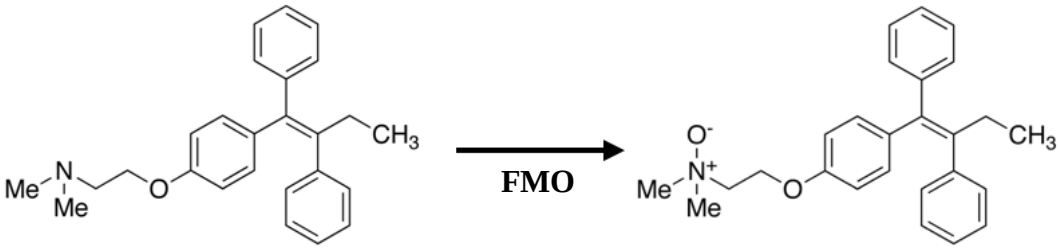


Figure 1. 8 N-Oxygenation of Tamoxifen catalyzed by FMO.

Another group of nitrogen-containing xenobiotic substrates for FMOs is nucleophilic cyclic tertiary amines. Due to the presence of enhanced nucleophilic nitrogen atom in the cyclic amine, they are good substrates of FMOs. Clozapine is an example of this group, showed to be N-oxygenated by human FMO3. In addition, caffeine has been reported for the substrate of human FMO3, but not considered as a good substrate (Cashman, 2000).

FMOs show high degree of specificity for the sulfur-containing compounds so enzyme discriminates different sulfur compounds. As shown in Table 1.5, FMOs can S-oxygenate organic sulfur-containing compounds such as sulfides, disulfides, thioamides, thiocarbamides, thiocyanides, sulfoxides, sulfenic acids, and carbodithioic acids (Smart & Hodgson, 2008). Methimazole is very good example for S-containing exogenous substrates of FMOs (Cashman *et al*., 1995). It is anti-thyroid drug used to treat hyperthyroidism, a condition that usually occurs when the thyroid gland produces too much thyroid hormone. Methimazole is S-oxygenated through the reaction catalyzed by FMOs.

FMOs also catalyze the metabolism of phosphines and phosphothioates (Table 1.5). One of them is Fonofos, very toxic insecticide; its metabolism is catalyzed by both FMOs and CYPs to form Fonofos oxon (Smyser & Hodgson, 1985).

1.4 Aim of the Study

Medicinal plants have been used for both treatment and prevention of diseases since the beginning of history of human. Two of them are known as *Epilobium hirsutum* L. (hairy willow herb) and *Viscum album* L. (European mistletoe). *Epilobium hirsutum* L. has been used for treatment of inflammation, adenoma menstrual disorders, constipate, and prostate, as well as prevention of rectal bleeding. *Viscum album* L. has been traditionally used as sedative, analgesic, anti-spasmolytic, cardiotonic, and anticancer agent, for treatment of epilepsy, hypertension, exhaustion, arthritis, diabetes, anxiety, vertigo, and degenerative inflammation of the joints. Both of these plants contain many biologically active compounds, including polyphenols and flavonoids. Such compounds have been known for their biological effects such as anti-oxidant activities of polyphenols, anti-nociceptive and anti-inflammatory activities of flavonoids. In addition, they can affect the activities and expressions of some important xenobiotic metabolizing enzymes such as CYPs and FMOs. However, there is no data available in the literature for the possible effects of *Epilobium hirsutum* L. and *Viscum album* L. on xenobiotic and endogen metabolizing flavin-containing monooxygenase enzymes (FMOs). Therefore, this study was aimed to elucidate *in vivo* effects of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. for their possible abilities to modulate rat liver flavin-containing monooxygenases, involved in the metabolism of a wide range of xenobiotic compounds including drugs such as tamoxifen, methimazole and imipramine, pesticides, neurotoxins, and other chemicals.

In order to achieve these purposes, firstly the plants were collected and their water extracts were prepared. Then, the extracts were injected intraperitonally to the albino Wistar rats (*Rattus norvegicus*) for nine consecutive days. After decapitation of the animals, their livers were removed and microsomal fractions were prepared by homogenization and differential centrifugation. The rat liver microsomal fractions were used for determination of FMO activities and FMO protein expressions by spectrophotometry and Western blot, respectively. For

determination of mRNA expressions, quantitative real-time PCR was carried out by using cDNA synthesized from total RNA of rat livers.

To our knowledge, this study is the first concerning the effects of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. on rat liver FMO activity and expression.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; A894987 605), dipotassium phosphate (K_2HPO_4; A611101 528), Folin-phenol Reagent (1.09001.0500), hydrochloric acid (HCl; 1.00314), magnesium chloride ($MgCl_2$; Art.5833), potassium chloride (KCl; 4935), potassium dihydrogen phosphate (KH_2PO_4; A319173-204), sodium carbonate (Na_2CO_3; 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), Triton X-100 (11869.1), zinc chloride ($ZnCl_2$; 108815) were purchased from Merck KGaA, Darmstadt, Germany.

Acrylamide (A-8887), ammonium per sulfate (APS; A-3678), N'-N'-Bis-methylene-acrylamide (M7256), bovine serum albumin (BSA; A788), bromophenol blue (B8026), diethanolamine (D-2286), diethylpyrocarbonate (DEPC; D5758), N-N-dimethylformamide (D-8654), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB; D-8130), dithiothreitol (DTT; D0632), Ficoll (F2637), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), methanol (34885), Methimazole (M-8506), phenazine methosulfate (P9625), sodium dodecyl sulfate (SDS; L4390), sodium-potassium tartarate (S-2377), Trisma base (T1503), Tween 20 (P1379), xylene cyanol (X4126) were the products of Sigma-Aldrich, Germany.

Ethylenediaminetetraacetic acid (EDTA; A5097), nicotinamide adenine dinucleotide phosphate (NADPH; A1395), and nitrotetrazolium blue chloride (NBT; A1243) were taken from AppliChem GmbH, Darmstadt, Germany.

5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821), dichlorodiphenyl trichloroethane (DDT; R0861), Light Cycler-Fast Start DNA MasterPlus SYBR Green I (FE-K0252), and M-MuLV Reverse Transcriptase (K1622) were obtained from Fermentas, USA.

Non-fat dry milk (170-6404) and tetramethylethylenediamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, CA, USA.

FMO3 primary antibody (sc-51288) was taken from Santa Cruz Biotechnology, CA, USA. Anti-mouse secondary antibody conjugated alkaline phosphatase (A3562) was taken from Sigma-Aldrich, Germany.

TRIzol® (12183-555) was purchased from Invitrogen; isopropanol (AS040-L50) from Atabay; and absolute ethanol (32221) from Riedel.

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

2.2 Methods

2.2.1 Plant Collection and Extractions

Flowering aerial parts of hairy willow-herb were collected from Gölyaka, Düzce, Turkey, at an altitude of 563 m., in June 2009. The plant was identified as *Epilobium hirsutum* L. (Davis, 1972) and a voucher specimen (No: AEF 25812) was deposited in Herbarium of Faculty of Pharmacy, Ankara University (AEF). European mistletoe samples growing on pine trees (*Pinus sylvestris* L.) were collected from Melikgazi village of Pınarbaşı district (Kayseri, Turkey) in August

2008 at an altitude of 1800 m. and identified as *Viscum album* L. subsp. *austriacum* (Wiesb.) Vollmann according to Flora of Turkey and the East Aegean Islands (Davis, 1982). Voucher specimen (No: AEF 25945) was deposited in AEF for future reference. *Viscum album* L. subsp. *austriacum* (Wiesb.) Vollmann was chosen among the three subspecies growing in Turkey for this study, because in Turkish Folk Medicine it is generally thought to be more effective than the other two subspecies known as subsp. a*lbum* and subsp. *abietis* (Wiesb.) Abromeit (Personal field work observation).

Air dried and powdered 50 g of plant material for each taxon has been subjected to active maceration in sterile distilled water for 8 hours by using mechanical shaker (Heidolph Instruments) at 300 rpm at room temperature. The extract obtained was filtered through filter paper, dried by lyophilizator (Christ Gamma 2-16 LSC), and weighed. Finally, after yield calculations (15% for *Epilobium hirsutum* L; 19% for *Viscum album* L.: weight/weight), the plant extracts were stored at -20ºC without any oxygen interaction until using for the experiments. This part of the experiments was performed with the collaboration of Pharmaceutical Botany Department, Faculty of Pharmacy at Ankara University.

2.2.2 Animal Studies

Male Wistar Albino rats (12 weeks old) weighing 200-250 g were purchased from Experimental Research Department of Pamukkale University, Denizli. They were housed University Animal House in standard conditions and fed with standard diet with water and libitum. All experimental procedures in animals such as administration on substances i.p., collection of blood and tissue etc. are performed to the national standards under appropriate regimes with Veterinary services and in accordance with the Declaration of Helsinki (ethical committee reports are at Appendix 1). The water extracts of *Epilobium hirsutum* L. and *Viscum album* L. samples were injected into Wistar albino rats (*Rattus norvegicus*) intraperitonally (i.p). Rats were randomly assorted into the following three groups:

C: Control group with no injection

EHT: *Epilobium hirsutum* L.-treated group

VAT: *Viscum album* L.-treated group

For *Epilobium hirsutum* L. injection, 37.5 mg of plant extract per kg body weight was injected to 26 rats for consecutive 9 days. For *Viscum album* L. injection, 10 mg of plant extract per kg body weight was injected to 15 animals for consecutive 9 days. On the other hand, 11 rats were not treated and used as controls. At the end of the experimental period and following 16 h of fasting, the animals were sacrificed. The livers were isolated and stored at -80°C until used for preparation of microsomes. This part of experiments was carried out with the collaboration of Biology Department, Pamukkale University.

2.2.3 Preparation of Microsomal Fractions

The livers were removed immediately after killing the animals by decapitation. In order to prevent release of the contents of gall bladders which could have inhibitory effects to monooxygenase activities, they were removed from the livers. The connective and fat tissues were removed, followed by washing with cold distilled water and then with 1.15% KCl solution several time to remove the excess blood. All subsequent steps were performed at 0-4ºC.

Rat liver microsomes were prepared according to method of Schenkman and Cinti (1978) and optimizations of Sen and Kirikbakan (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 that contains 3 mM EDTA pH 7.8, 50 mM Tris-HCl, pH7.5, 0.3 mM ε-ACA,0.5 mM PMSF, 0.15mM 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 were centrifuged at 1500x g for 10 minutes. The supernatant was recentrifuged at 12000x g for 25 minutes. The firmly packed microsomal pellet was suspended in homogenization solution and resedimented at 32000x g for 20 minutes. Supernatant was discarded and pellet was resuspended in a solution containing 10% glycerol and 2 mM EDTA at a volume of 0.5 ml for each gram of liver tissue. The microsomal suspensions were gassed with nitrogen and stored at -80 ºC for enzymatic assays.

2.2.4 Determination of Protein Concentrations

Protein concentrations of microsomal fractions obtained from rat livers were determined according to method of Lowry *et al*. (1951) by using crystalline bovine serum albumin as standard.

The principle of protein concentration determination by the Lowry method is based on the reaction between peptide nitrogen and the copper ions in the alkaline medium, while Folin reagent is subsequently reduced to heteropolymolbdenum blue by the copper-catalyzed oxidation of aromatic amino acids (Lowry *et al.*, 1951).

Reagents:

Reagent 1: 2% $CuSO_4.5H_2O$, Copper Source

Reagent 2: 2% Na-K Tartarate, to prevent Cu^{2+} in alkaline

Reagent A**:** 2% Na_2CO_3 in 0.1 N NaOH

Lowry ACR Reagent: Reagent A, 1, and 2 were mixed as ratio 100:1:1

Folin-Phenol Reagent: (light sensitive) 2 N stock (commercial) was diluted as 1:1 with distilled water

Bovine Serum Albumin (BSA) Protein Standard: 0.02, 0.05, 0.10, 0.15, 0.20 mg/mL

10 mL of 1mg/mL BSA Stock solution was prepared by dissolving 10 mg BSA in 10 mL distilled water. Then, serial dilution was done to prepare other concentrations of BSA as shown in the Figure 2.1.

Protein Sample: Rat liver microsomes were diluted 200 times (1:200)

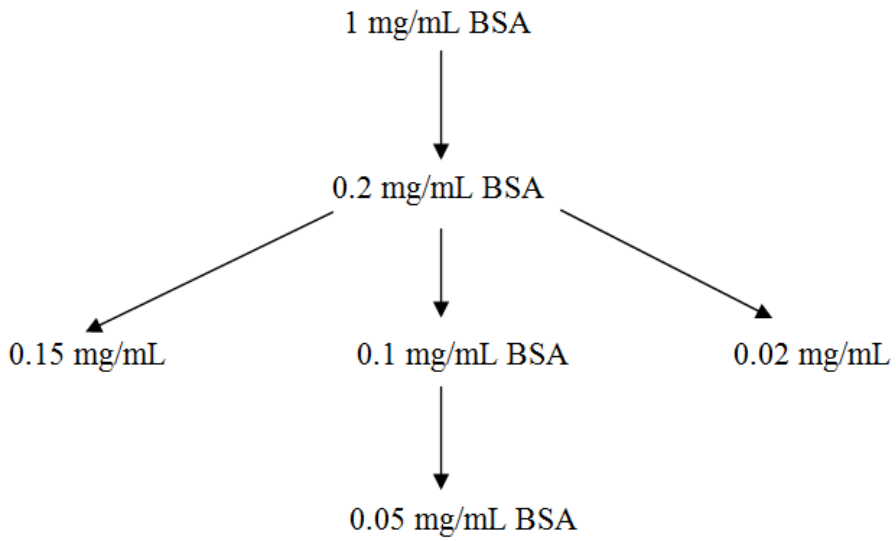


Figure 2. 1 Serial dilution of Bovine Serum Albumin (BSA) used as standards.

The standards, distilled water, and samples were put into the tubes as given in Table 2.1. 2.5 mL of Lowry ACR was added to each tube and mix. The tubes were incubated for 10 minutes at room temperature. 0.25 mL of Folin reagent was added and the tubes were mixed in 8 seconds. The tubes were further incubated for 30 minutes at room temperature. Finally, the absorbances of each tube were read at 660 nm by using spectrophotometer, followed by the construction of the calibration curve with BSA standards and calculation of protein concentrations of the microsomal fractions by using this calibration curve.

Table 2. 1 Content of tubes prepared for determination of protein concentrations.

Tube Number	**Standards (mL)**	**dH_2O (mL)**	**Sample (mL)**	**Lowry ACR (mL)**	**Folin (mL)**
1-1' (Blank)	---	0.5	---	2.5	0.25
2-2' (0.02)	0.5	---	---	2.5	0.25
3-3' (0.05)	0.5	---	---	2.5	0.25
4-4' (0.10)	0.5	---	---	2.5	0.25
5-5' (0.15)	0.5	---	---	2.5	0.25
6-6' (0.20)	0.5	---	---	2.5	0.25
7-7'	---	0.4	0.1	2.5	0.25
8-8'	---	0.25	0.25	2.5	0.25
9-9'	---	---	0.5	2.5	0.25

2.2.5 Determination of FMO Activity

FMO enzyme activities of microsomal fractions obtained from rat livers were determined according to methods of Dixit and Roche (1984), developed with minor modifications by Demirdöğen and Adalı (2005). In this method, methimazole was used as a substrate for determination of FMO activity. Methimazole is very specific substrate for FMOs and oxidized in the presence of NADPH and molecular oxygen as shown in Figure 2.2.

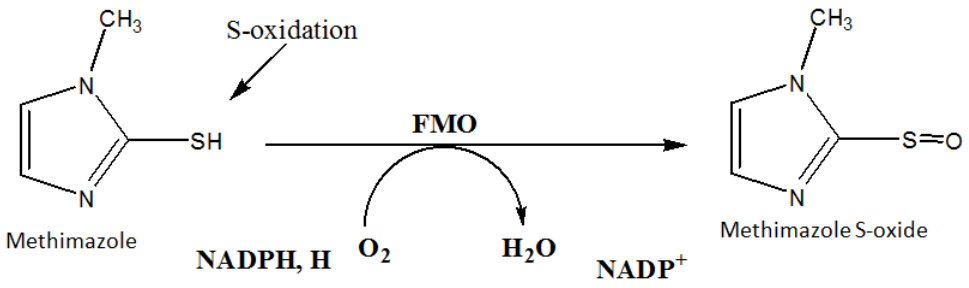


Figure 2. 2 Methimazole S-oxidation reaction catalyzed by FMO.

Methimazole cannot reduce 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), but its conjugate disulfide is a strong oxidant that rapidly and completely oxidizes nitro-5-thiobenzoate (TNB) to DTNB. TNB used in the assay is produced from the reaction of DTNB with dithiothreitol (DTT). TNB has a yellowish color with absorbance about 0.6 A at 412 nm. The oxidation of TNB results in the colorless product DTNB. The measured reaction results in the oxidation of two molecules of TNB per catalytic turnover as a result of the reactions shown in Figure 2.3. Therefore, FMO activity is measured by the decrease in amount of TNB monitored by spectrophotometer at 412 nm.

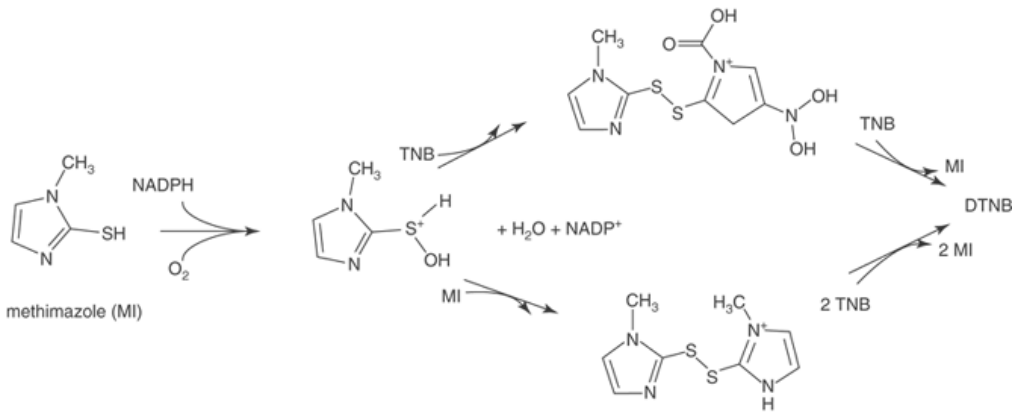


Figure 2. 3 Oxidation of methimazole and reaction of the oxidized product with TNB to generate DTNB (modified from Dixit and Roche, 1984).

Reagents:

Tris-HCl Buffer: 0.5 M, pH 8.0 (Stock)

12.11 g of Tris was dissolved in 150 mL distilled water and pH was adjusted to 8.0 with HCl and then the volume was completed to 200 mL.

KPO_4: 0.1 M, pH 8.0 (Stock)

1.74 g of K_2HPO_4 was dissolved in 100 mL distilled water to obtain 0.1 M of K_2HPO_4.

1.36 g of KH_2OPO_4 was dissolved in 100 mL distilled water to obtain 0.1 M of KH_2OPO_4.

pH of 0.1 M of K_2HPO_4 was adjusted with 0.1 M of KH_2OPO_4 to prepare 0.1 M of KPO_4.

DTNB: 1 mM in 0.1 mM KPO_4 (Fresh, Keep in ice)

3.96 mg of DTNB was dissolved in 10 mL of 0.1 M KPO_4, pH 8.0

DTT: 2 mM (Fresh, Keep in ice)

3 mg of DTT was dissolved in 10 mL distilled water

NADPH: 10 mM (Fresh, Keep in ice)

4.17 mg of NADPH was dissolved in 500 µL distilled water

Methimazole: 100 mM (Fresh, Room Temperature, Light Sensitive)

5.71 mg of methimazole was dissolved in 500 µL distilled water

Triton X-100: 10%

Microsomal Protein: diluted 2 times

The enzyme reaction should be performed at 37°C, so water and Tris-HCl buffer, pH 8.0 used in assay were kept at 37ºC. The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, 0.06 mM DTNB in KPO_4 buffer, 0.02 mM DTT, 0.1 mM NADPH, 2-times diluted microsomal fraction of the sample, and 0.1% Triton X-100 in final concentrations (Table 2.2). The endogenous absorbances before addition of the substrate methimazole were recorded at 412 nm. Then, the reaction

was started with addition of methimazole (1 mM final concentration) at 37°C. The decrease in absorbance was measured at 412 nm for 3 minutes against blank cuvette containing 0.5 M of Tris-HCl buffer by using double beam spectrophotometer (Hitachi Ltd., Japan). FMO enzyme activities were calculated from the difference between the rates obtained with and without substrate (ΔOD/min) and using an extinction coefficient of 28.2 mM^{-1} $.cm^{-1}$ at pH 8.0 by the formula given below.

$$FMO\ Activity = \frac{\Delta OD_{412}}{28.2} \times \frac{1000}{Protein\ Amount\ (\mu L)} \times \frac{1}{Protein\ Amount\ (mg)} \times Dilution\ Factor$$

Table 2. 2 The constituents of reaction mixture for FMO Activity.

Stock Solutions	Final Concentrations
Tris-HCl Buffer	50 mM
DTNB in KPO_4 Buffer	0.06 mM
DTT	0.02 mM
NADPH	0.1 mM
Microsomal Protein	2-times diluted
Triton X-100	0.1%
Methimazole	1 mM

2.2.6 Determination of FMO3 Protein Expression by Western Blot

FMO3 protein expressions were determined using the techniques of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4% stacking gel and 7.5% separating gel in a discontinuous buffer system as described by Laemmli (1970) and Western Blot analysis described by Towbin *et al.* (1979).

Reagents:

Gel Solution**:** Acrylamide and N'-N'-Bis-Methylene-Acrylamide (Stock)

14.6 g of Acrylamide and 0.4 g of N'-N'-Bis-Methylene-Acrylamide were dissolved separately, and then mixed, followed by filtering through filter paper. The volume was completed to 50 ml with distilled water.

Separating Buffer: 1.5 M Tris-HCl, pH 8.8 (Stock)

18.15 g of Tris-base was dissolved with 50 mL water, and then titrated with 10 M HCl till pH 8.8. The volume was completed to 100 mL with distilled water. The pH of the solution was controlled at the end.

Stacking Buffer: 0.5 M Tris-HCl, pH 6.8 (Stock)

6 g of Tris-base was dissolved with 60 mL distilled water, and then titrated with 10 M HCl till pH 6.8. The volume was completed to 100 mL with distilled water. The pH was controlled at the end.

Sodium Dodecyl Sulfate SDS: 10% (Stock)

1 g of SDS in water, and then complete the volume to 10 mL.

Ammonium per sulfate APS: 10% (Fresh)

12.5 g of APS per gel was dissolved in 125 µL of distilled water for each.

Tetramethylethylenediamine (TEMED): (Commercial)

Sample Dilution Buffer (SDB): 4x (stock)

2.5 mL of 1 M Tris-HCl buffer (pH 6.8) and 4 mL of Glycerol were taken; 0.8 g of SDS was added, 2 mL of ß-Mercaptoethanol and 0.001 g Bromophenol Blue were added. The volume was completed to 10 mL with distilled water. After several uses, 1 mL of Mercaptoethanol was added.

Electrophoretic Running Buffer (ERB): 0.25 M Tris, 1.92 M Glycine (10x Stock, diluted to 1x before use by adding 0.1% SDS)

15 g of Tris-Base was dissolved in 350 mL distilled water, and then 72 g of Glycine was added. They were mixed well, followed by completing the volume to 500 mL with distilled water.

1 g of SDS was added per liter of 1x buffer before use.

Transfer Buffer: 25 mM Tris, 192 mM Glycine (Stock)

3.03 g of Trisma-base and 14.4 g of Glycine were dissolved in 200 mL of Methanol (20% v/v), and the volume was completed to 1 L with distilled water.

Tris-Buffered Saline and Tween 20 (TBST): 20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 0.05% Tween 20 (Fresh)

9.5 g of NaCl was dissolved in some water; 6.5 mL of 1 M Tris-HCl Buffer pH 7.4 was added. 165 µL of Tween 20 was added, and finally the volume was completed to 350 mL with distilled water.

Blocking Solution: 5% Non-Fat Dry Milk (Stock)

5 g Non-Fat Dry Milk was dissolved in 100 mL Tris-buffered saline (TBS)

Primary Antibody: 1/500 dilution in blocking solution

Secondary Antibody**:** 1/5000 dilution in blocking solution

Substrate Solution: (Fresh, Light Sensitive)

Solution A: 2.67 mL of 1.5 M Tris-HCl Buffer (pH 8.8), 4 mL of 1 M NaCl, 96 µL of Diethanolamine, 820 µL of 100 mM $MgCl_2$, 40 µL of 100 mM $ZnCl_2$, and 12.2 mg of Nitrotetrazolium Blue Chloride (NBT) were mixed and titrated with saturated Tris to pH 9.55. Then the volume was completed to 40 mL with distilled water. The pH of the solution was controlled at the end.

Solution B: 2 mg of Phenazine Methosulfate was dissolved in 1 mL of distilled water.

Solution C: 5.44 mg of 5-bromo 4-chloro 3-indoyl phosphate (BCIP) was dissolved in 136 µL of N-N-dimethylformamide.

To prepare the substrate solution, 20 mL of Solution A, 68 µL of Solution C, and 134 µL of Solution B were mixed for each membrane and used immediately.

Mini-PROTEAN Tetra cell 165-8033 equipment (Bio-Rad Laboratories, Richmond, CA, USA) was used for gel preparation and electrophoresis. The separating (7.5%) and stacking gel (4%) solutions were prepared following a procedure below in a given order (Table 2.3).

Table 2. 3 Content of separating and stacking gel solutions.

	Separating Gel Solution	**Stacking Gel Solution**
Monomer Concentration	7.5%	4%
Gel Solution	7500 µL	1300 µL
dH_2O	14530 µL	6100 µL
Separating Buffer	7500 µL	---
Stacking Buffer	---	2500 µL
10% SDS	300 µL	100 µL
10%APS	150 µL	50 µL
TEMED	30 µL	20 µL
Total Volume	*30 mL*	*10 mL*

The gel sandwich made between two glass plates was used to prepare polyacrylamide slab gels. First, separating gel solution was added to the gel sandwich till the desired height of the solution was obtained between glass plates. Top of the polymerizing gel was covered with a layer of isobutanol to obtain smooth gel surface. Then, the gel was incubated for polymerization for about 30 min at room temperature. After polymerization, the alcohol layer was poured totally. The stacking gel polymerization solution was prepared and added to the gel sandwich till the sandwich was filled completely. Following stacking gel addition, a 1.0 mm Teflon comb containing 15 wells was inserted into the layer of polymerizing stacking gel solution. The attention was given not to get any bubbles at the edges of the comb during placing the comb. Polymerization was completed in about 30 min at room temperature. The Teflon comb was carefully removed without damaging the wells. Then, wells were filled with electrode running buffer

(25 mM Tris, 192 mM glycine and 0.1% SDS) with a fine needle syringe for removal of any formed air bubbles or unpolymerized chain particles. Finally, the gel running module was filled with a sufficient volume of electrode running buffer.

The protein samples were diluted with 4x sample dilution buffer containing 0.25 M of Tris-HCL, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol and 0.01% bromophenol blue to obtain 2 mg/mL in distilled water and boiled in water bath for 1.5 minutes. Then, 30 µg of sample and 3 µL of commercially available protein molecular weight marker (Fermentas, SM0671) were applied on different wells by Hamilton syringe. Gel-running module was placed in the main buffer tank filled with electrode running buffer. The electrophoresis unit was connected to the power supply to run electrophoresis at 150 V and 15 mA for 40 minutes in stacking gel and 300 V and 30 mA for 2 hours in separating gel.

After electrophoresis was finished gels were removed from set-up for western blotting. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 15 min with constant shaking to adjust the final size of gel and remove the buffer salts and SDS coming from SDS-PAGE step. Nitrocellulose membrane was prepared by cutting 1 cm larger than the dimension of the gel and two pieces of Whatman paper were cut in dimensions little bit larger than the membrane.

Nitrocellulose membrane, two filter papers and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Western blot sandwich was prepared as shown in Figure 2.4. A test tube was used to remove any air bubbles between the layers by gently rolling over the sandwich. This was the very critical step since any air bubbles formed between layers block the transfer of proteins. Then, the sandwich was put into the Mini Trans-Blot module 165-8033 (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with cold transfer buffer. Voltage and current were set to 90 V and 400 mA, respectively. The transfer was carried out 90 minutes with Bio-Rad PowerPac basic power supply (Bio-Rad Laboratories, Richmond, CA, USA).

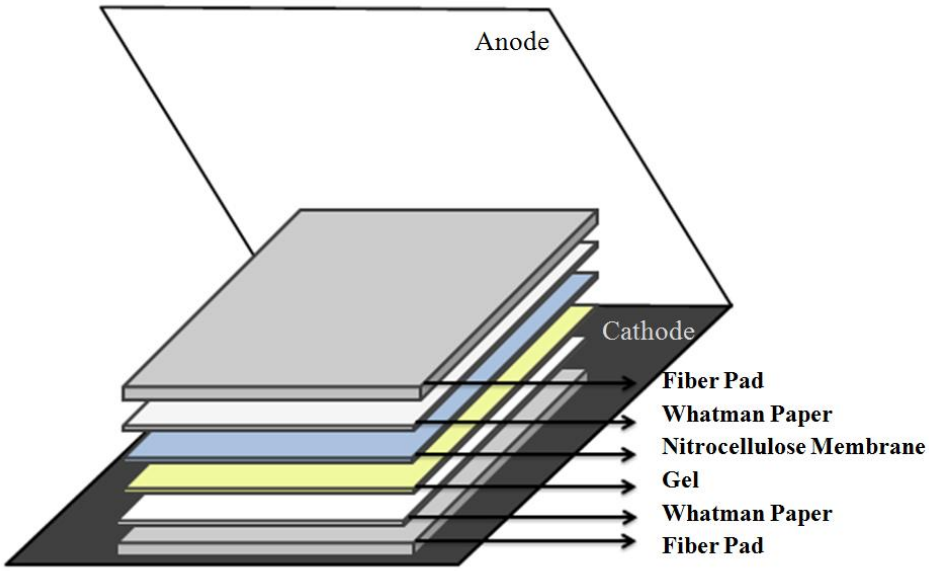


Figure 2. 4 Preparation of Western blot sandwich.

At the end of this process, the nitrocellulose membrane carrying the transferred proteins, i.e. “blot” was obtained and removed from the module. Then, membrane was transferred to a plastic dish as protein side facing upwards and washed with TBST (Tris Buffered Saline plus Tween 20: 20 mM Tris-HCL, pH 7.4, 0.5 M NaCl and 0.05 % Tween 20) for 10 min. This washing step removes the salts and buffers from transfer medium. Then, the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBST) for 60 min so that empty spaces between transferred proteins were filled. This filling inhibits the non-specific binding of antibodies to the membrane. After that, the blot was incubated with primary antibody (500 times diluted goat polyclonal FMO3 antibody in Tris-buffer saline with 5% non-fat dry milk) for 2 hours. Following this step, the blot was washed three times with 50 ml of TBST for 5 min each time to remove excess, unbound antibody. The blot was then incubated with 5000-times diluted secondary antibody (alkaline phosphatase conjugated mouse anti-goat IgG) for 1

h. The blot was washed 3 times with 50 ml TBST for 5 min each to remove excess antibody. The complete removal of TBST between each washing steps were extremely important since non-specifically bound regions can give reaction with substrate solution.

Finally, substrate solution was prepared while the membranes were being washed with TBST and then the membranes were incubated with the substrate solution as described by Ey and Ashman (1986) to visualize the specifically bound antibodies. The final images were photographed by using computer-based gel-imaging instrument, Infinity 3000 (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) by using Infinity-Capt Version 12.9 software. Immunoreactive protein bands were then quantified by densitometric scanning method using an Image J software package program developed by NIH.

2.2.7 Determination of FMO3 mRNA Expression by qRT-PCR

Quantification of mRNA expression by conventional methods, such as Nothern blotting and ribonuclease protection assays, require large amounts of RNA and are not always feasible when the transcripts of interest display low expression levels and/or sample size may be limiting. On the other hand, qRT-PCR has many advantages in terms of accuracy, sensitivity, dynamic range, high-throughput capacity, and absence of post–PCR manipulation. To quantitatively analyze the mRNA expression, total RNA was isolated from rat livers; cDNA from total RNA was synthesized; and finally qRT-PCR was carried out by using forward and reverse primers specific to FMO3 cDNA sequence.

2.2.7.1 Total RNA Isolation from Rat Liver

Total RNA is isolated by using the method of TRIzol® without degrading (Chomczynski & Sacchi, 1987). This method is based on single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture. But TRIzol® is a commercial product which makes the isolation easier.

Reagents:

Diethylpyrocarbonate (DEPC) Water: %0.1 (v/v)

1 mL of DEPC was mixed with 1 L of distilled water.

TRIzol®: It is the brand name of the product from Invitrogen, and the brand name from MRC.

Chloroform: Stock, Stored at -20°C

Isopropanol: Stock, Stored at -20°C

75% Ethanol: Stock, Stored at -20°C

75 mL of ethanol was mixed with 25 mL of distilled water.

Before the experiment, all equipments were treated with DEPC water in order to inhibit RNase activity. Excess DEPC was evaporated under hood for several hours and autoclaved. The distilled water used for the solutions was also treated with DEPC and autoclaved.

70 mg of rat tissue was minced and homogenized with 700 µL of TRIzol® in a glass-Teflon homogenizer for 15 seconds 2-3 times. With extra each mg of tissue, extra 10 µL of TRIzol® was added. The homogenized tissue was transferred into an eppendorf tube and frozen with liquid nitrogen. Before complete melting, 100 µL of chloroform was mixed with homogenate. The mixture, then, was vortexed for one minute, followed by incubation at room temperature for 5 minutes. Further incubation in ice was carried out for 5 minutes. After that, the homogenate was centrifuged at 13000 rpm for 15 minutes at 4°C. The uppermost of the three phases formed after centrifugation was taken and same volume of isopropanol was added with gently mix. The mixture was incubated at

room temperature for 10 minutes. After further centrifugation at 13000 rpm for 5 minutes at 4^{o}C, the supernatant was discarded and the pellet was mixed with 1 mL of 75% ethanol. The mixture was centrifuged again at 7500 rpm for 5 minutes at 4°C; the pellet was taken and dried in hood. Finally, RNA was dissolved in 75 µL of Nuclease-free distilled water and used for further studies (keeping at -80°C).

2.2.7.2 Determination of RNA Concentration and Qualification of RNA by Agarose Gel Electrophoresis

Because RNA molecule gives maximum absorbance at 260 nm, reading at this wavelength was used to calculate the concentration of nucleic acid in the sample. Moreover the purity of RNA can be estimated using the ratio between OD values at 260 nm and 280 nm (OD260/ OD280). The presence of RNA isolated from the rat livers should be checked by using agarose gel electrophoresis.

Reagents:

Tris-EDTA (TE) Buffer: 100 mM Tris, 10 mM EDTA, pH 8.0

Tris-Borate-EDTA (TBE) Buffer: 0.5 x, pH 8.3

Loading Buffer: 0.25% bromophenol blue, 025 % xylene cyanol FF, 15% Ficoll

Therefore, 7 µl of RNA solution was mixed with 693 µl of TE buffer in quartz cuvettes and the absorbances at 260 and 280 nm were measured spectrophotometrically. The ratio of OD260/ OD280 should be between 1.8 and 2.2. The optical density of 1.0 is corresponded to 40 µg/ml of RNA so RNA concentration was calculated according to the formula:

$$\mathrm{RNA}(\mu\,\mathrm{g/mL}) = \mathrm{OD}_{260} \times \mathrm{DF} \times 40\ \mu\,\mathrm{g/mL\ RNA\ (1\ OD}_{260}\mathrm{unit)}$$

Presence 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 0.5X TBE buffer. The agarose was dissolved in microwave oven. The solution was cooled approximately 60°C and ethidium bromide solution (10 mg/ml) was added to final concentration of 0.5 mg/ml and the solution was mixed thoroughly. Agarose gel solution was poured into electrophoresis tray and comb was placed. After solidification, gel tank was filled with 300 ml of 0.5 X TBE buffer. After the comb was removed, 10 µl of RNA solution was mixed with 2 µl of loading buffer and 2 µL of glycerol; the mixture was loaded into the wells. Electrophoresis was carried out at 100 V and 500 mA for 45 minutes. The gel was analyzed under UV light and photographed.

2.2.7.3 cDNA Synthesis and Quantitative Real-Time PCR

Reagents:

Reaction Buffer: 250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DDT

M-MuLV-RT: Reverse Transcriptase

cDNA is synthesized from RNA by using reverse transcription. For that, 2.5-5 µg of total RNA isolated from rat liver was mixed with 0.5 µg (1 µl from 500 µg/ml stock) of oligo dT primers. Final volume was completed to 12 µl with DEPC-treated water because volume of RNA is variable due to different concentrations. Then, mixture was incubated at 70°C for 5 minutes; 4 µl of 5x reaction buffer was added. Then, 1 µl of Ribolock and 2 µl of 10 mM dNTP mixture were added with gently mix and collecting the drops by microfuge. The sample was incubated at 37°C for 5 minutes. After incubation, 1 µl of M-MuLV-RT was added and further incubated at 42°C for 1 hour. Finally, the reaction was stopped by heating to 70°C for 10 minutes, followed by chilling on ice. The synthesized cDNA was stored at -20°C for further use.

Quantitative real-time PCR assays for rat mRNA expressions were carried out using Light Cycler 1.5. Reactions were performed in 20 μL volume using Light Cycler-Fast Start DNA Master^{Plus} SYBR Green I. Briefly, 5 μL of cDNA was added to a total reaction volume of 20 μL, containing 9 μL of PCR-grade water, 2 μL of FMO3 forward and reverse primers-shown in Table 2.4, and 4 μL of master mix containing Fast Start Taq DNA Polymerase, $MgCl_2$, SYBR Green I dye and dNTP mix. PCR-grade water and a sample in which reverse transcriptase is omitted during reverse transcription, are included in every PCR-run as negative controls to confirm that there is no genomic DNA contamination in the cDNA samples. The following Light Cycler run protocol was used: Pre-incubation program is at 95°C for 10 minutes in order to activate Taq polymerase and denature DNA; amplification and quantification program repeated 45 times (95°C for 20 seconds, annealing at 47°C for 20 seconds and extension at 72°C for 30 seconds with a single fluorescence measurement); melting curve program 65-95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement; finally a cooling step to 40°C for 30 seconds (Table 2.5). Melting curve analysis of the amplification product was performed at the end of each amplification reaction in order to confirm whether a single PCR product was detected or not. Quantities of specific mRNAs in the sample was measured according to the corresponding gene specific relative standard curve derived from dilution series of a control cDNA (1:10, 1:100, 1:500, 1:000, and 1:5000) and expression level of the target genes was measured relative to housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Light Cycler quantification software was used to draw the standard curve from the dilution series of a control cDNA.

Table 2. 4 FMO3 and GAPDH forward and reverse primers used for qRT-PCR.

Gene	Primer Sequences (5'→ 3')	Annealing Temperature (C°)	Product Length (bp)
FMO3	**F:** GTGTTTTCCAGACTTCCC **R:** GTACCACCAGTCAGAGAT	47 C°	568
GAPDH	**F:** TGATGACATCAAGAAGGTGGTGAAG **R:** TCCTTGGAGGCCATGTGGGCCAT	60 C°	240

F: Forward Primer
R: Reverse Primer

Table 2. 5 Quantitative Real-Time PCR conditions.

Pre-incubation		95°C	10 min
Cycling (45 repeats)	Denaturation	95°C	20 sec
	Annealing	47°C	20 sec
	Extension	72°C	30 sec
Melting		65-95°C heating rate of 0.1°C/sec	30 sec
Cooling		40°C	30 sec

2.2.8 Statistical Analysis

Statistical analysis was performed by using GraphPad Prism ver. 5 statistical software package for Windows. All results were expressed as means with their Standard Deviation (SD). Unpaired, two-tailed student's *t*-test was used for statistical analysis and $p < 0.05$ was taken as the minimum level of significance.

CHAPTER 3

RESULTS

3.1 Protein Concentrations of Rat Liver Microsomal Fractions

In this study, rats were assorted into three groups; Control group (C) with no injection, *Epilobium*- treated group (EHT) i.p. injected with 37.5 mg water extract of *Epilobium hirsutum* L./kg of body weight, and *Viscum*-treated group (VAT) injected with 10 mg water extract of *Viscum album* L./kg of body weight. Control, EHT and VAT groups composed of 11, 26 and 15 animals, respectively. The microsomal fractions were prepared by differential centrifugation after homogenization of the livers. Average protein concentrations of control and extract treated rat liver microsomes were determined and results are listed in Table 3.1.

Table 3. 1 Average protein concentrations of rat liver microsomes.

Group	Average Protein Concentration (mg/mL)
Control (N=11)	20.47±6.14
EHT (N=26)	20.52±4.96
VAT (N=15)	27.36±5.73

3.2 Effect of Water Extract of *Epilobium hirsutum* L. on Rat Liver FMO Activity and Expression

Influence of medicinal plant *Epilobium hirsutum* L. on rat liver microsomal Flavin-containing monooxygenase activity and protein and mRNA expressions were studied with the injection of aqueous extract of this plant to albino Wistar rats.

3.2.1 Effect of *Epilobium hirsutum* L. on Rat Liver FMO Activity

Rat liver microsomal FMO activity was determined using the substrate methimazole coupled with the oxidation of TNB to DTNB; the amount of oxidized TNB was determined spectrophotometrically at 412 nm in 3 minute-time scale. Molar absorbance coefficient of 28,200 $M^{-1}cm^{-1}$ was used to calculate rat liver FMO activity. The liver microsomal samples from 11 control and 26 *Epilobium hirsutum* L.-treated (EHT) animals were assayed in duplicate or triplicate for the determination of FMO specific activities as nmol/min/mg. Results were given as the mean values of these duplicate or triplicate determinations of enzyme specific activities.

Table 3.2 and Figure 3.1 show the rat liver microsomal FMO enzyme activities of control (N=11) and *Epilobium hirsutum* L. treated (N=26) animals. As shown in the figure, methimazole oxidation rate did not change significantly between the control and EHT groups. The average of FMO activities for control group was calculated as 5.11±0.92 nmol/min/mg, while that for EHT was 5.31±1.35 nmol/min/mg. There was a slight change in specific activity of rat liver microsomal FMO in EHT with insignificant p value (unpaired, two-tailed student's *t*-test).

Table 3. 2 FMO activities of rat liver microsomal samples from control and EHT groups.

Control (nmol/min/mg)		EHT (nmol/min/mg)	
C1	6.22	**EHT1**	6.00
C2	5.52	**EHT2**	6.20
C3	4.90	**EHT3**	3.50
C4	4.86	**EHT4**	4.86
C5	4.27	**EHT6**	5.55
C6	4.63	**EHT8**	6.50
C9	4.00	**EHT9**	5.40
C10	4.29	**EHT10**	6.95
C11	6.30	**EHT11**	6.84
C12	6.64	**EHT12**	4.63
C13	4.55	**EHT13**	4.98
Mean ± SD	5.11 ± 0.92	**EHT14**	4.32
		EHT15	4.53
		EHT16	3.85
		EHT17	5.61
		EHT18	5.16
		EHT19	7.68
		EHT20	6.11
		EHT21	4.81
		EHT22	7.83
		EHT23	7.12
		EHT24	5.82
		EHT27	3.43
		EHT28	3.41
		EHT29	3.86
		EHT30	3.19
		Mean ± SD	5.31 ± 1.35

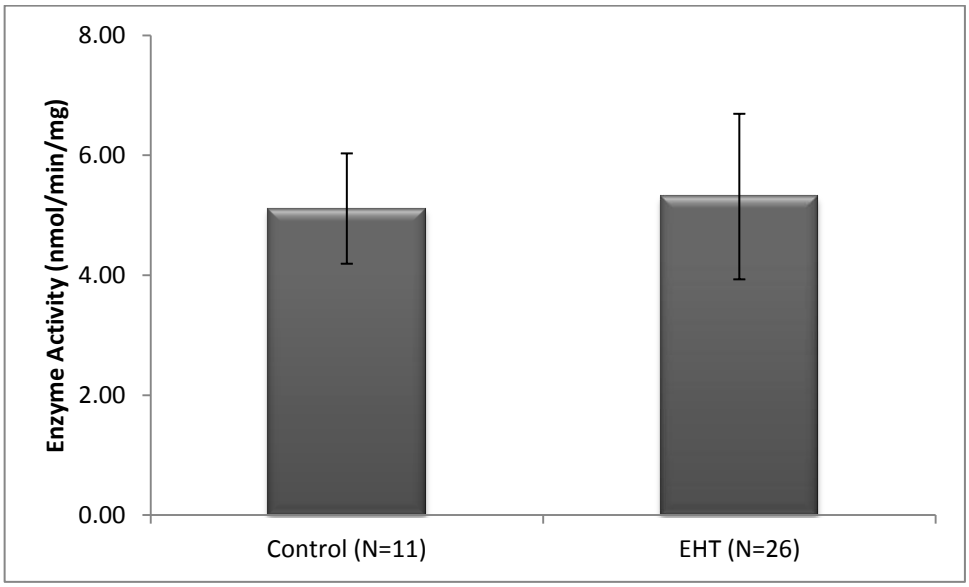


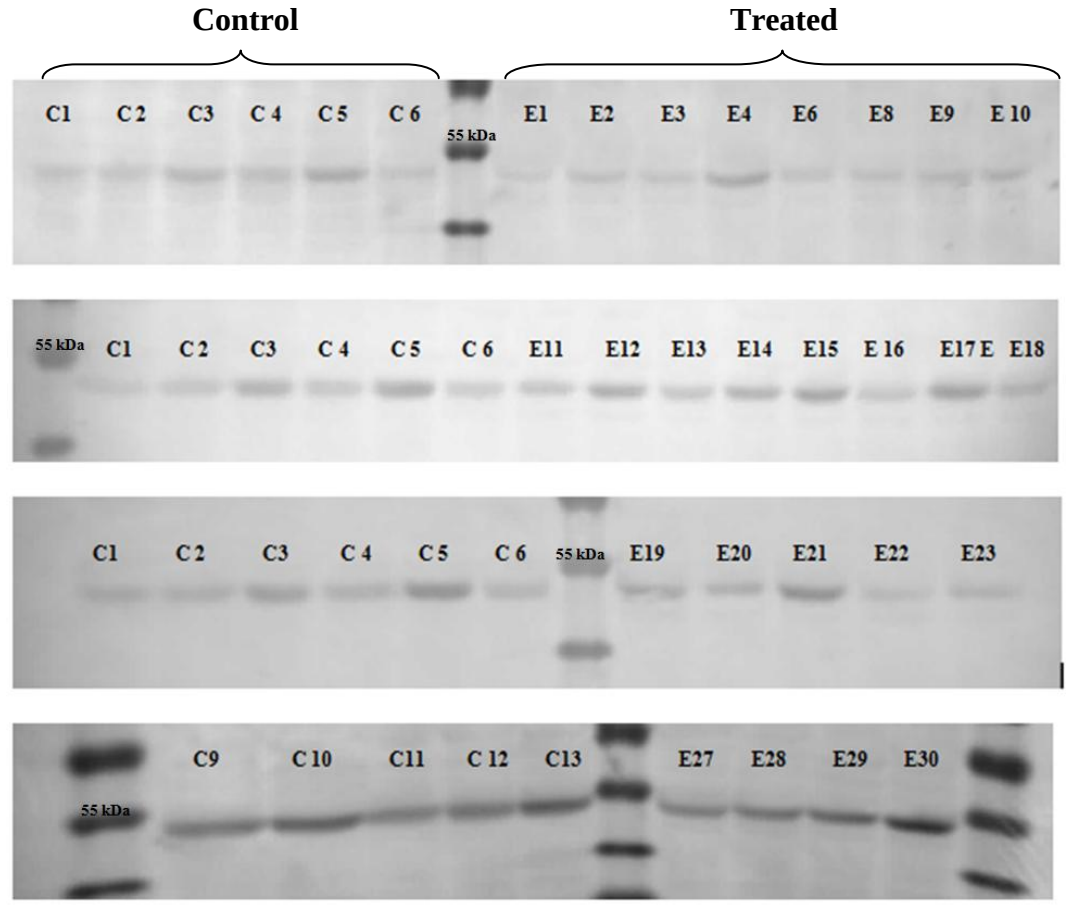
Figure 3. 1 Effect of water extract of *Epilobium hirsutum* L. on rat liver FMO Activity. The reaction mixture contained standard assay constituents described in the "Method". The reactions were started with 1mM methimazole and recorded at 412 nm for 3 minutes. The mean specific activity of duplicate or triplicate measurements of total 11 controls was calculated as 5.11±0.92 nmol/min/mg. The mean specific activity of duplicate or triplicate measurements of total 26 EHT was calculated as 5.31±1.35 nmol/min/mg. The p value obtained from unpaired, two-tailed student's *t*-test was 0.65.

3.2.2 Effect of *Epilobium hirsutum L.* on Rat Liver FMO3 Protein Expression

Rat liver microsomal FMO3 protein expressions of control and *Epilobium hirsutum* L- treated groups were determined using Western blot procedure following the separation of proteins by SDS-PAGE. Immunochemical detection of liver microsomal FMO3 protein was performed by incubating the nitrocellulose in diluted goat polyclonal FMO3 primary antibody and then secondary antibody, alkaline phosphatase conjugated mouse anti-goat IgG.

Monomer molecular weight of FMO3 is known as 58 kDa and Figure 3.2 shows the exact position of FMO3 on nitrocellulose membrane. ß-actin was used as the internal loading control (MW of 42 kDa). The intensity of immunoreactive protein bands were analyzed by using ImageJ visualization software. The results illustrated in Figure 3.3 show the relative protein expressions where mean of protein expressions of control group (N=11) was taken as 100 (SD 27.8) and then mean protein expression of treated group (N=25) was calculated as 72.3 (SD 25.7). As shown in Figure 3.3, rat liver microsomal FMO3 protein expression was decreased in EHT group compared to control group (unpaired, two-tailed student's *t*-test, $p<0.05$). The mean FMO3 protein expression of treated group was found as 72.29% of that of control group.

A (FMO3)



B (β-Actin)

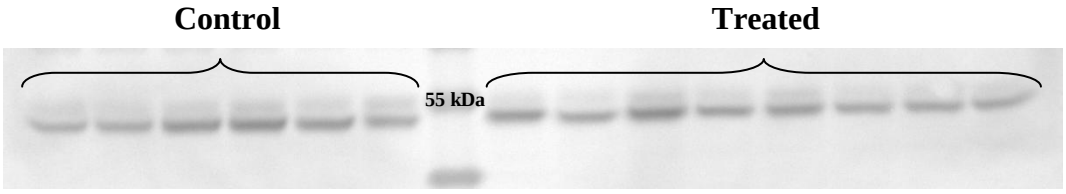


Figure 3. 2 **(A)** Immunoreactive protein bands of control (N=11) and *Epilobium hirsutum* L.-treated (N=25) groups representing FMO3 (58 kDa). **(B)** ß-actin (42 kDa) was used as the internal control for each membrane.

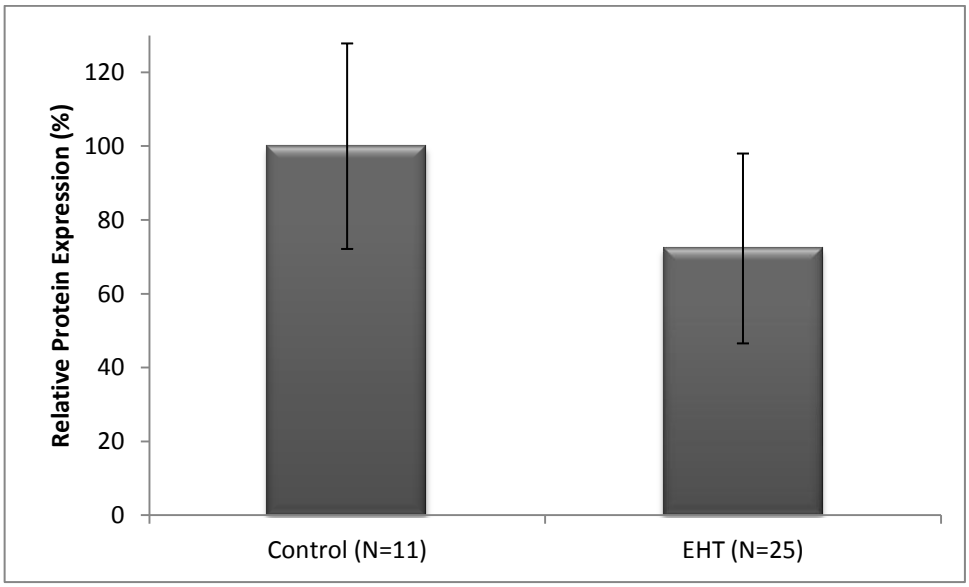


Figure 3. 3 Effect of water extract of *Epilobium hirsutum* L. on rat liver FMO3 protein expression. The band quantifications are expressed as mean ± SD of the relative intensity. The mean relative protein expression of *Epilobium hirsutum* L-treated group is 72.29% of that of control group. The p value obtained from unpaired, two-tailed student's *t*-test was 0.009.

3.2.3 Effect of *Epilobium hirsutum* L. on Rat Liver FMO3 mRNA Expression

Total cellular RNAs were isolated from rat livers of both control and treated groups, as explained in Materials and Methods. RNA isolation was critical step in this part of the study because DNA contamination could interfere with the subsequent qRT-PCR. Purity and quantity of RNA isolated were assessed by $OD_{260/280}$ ratio, followed by agarose gel electrophoresis. The RNA samples having $OD_{260/280}$ ratio between 1.7 and 2.0 were taken for the further experiments. Figure 3.5 shows representative agarose gel electrophoresis of rat liver total RNA.

mRNA expressions of FMO3 were determined using qRT-PCR and relative expressions of FMO3 of control and treated animals were calculated using GAPDH as internal control (housekeeping gene). Figure 3.6 and 3.7 show standard curve of diluted cDNAs used for quantification of the samples and melting curve to confirm that single PCR product was detected, respectively. In this study, the standard cDNA was diluted 1:10, 1:100, 1:500, 1:000, and 1:5000.

Figure 3.8 and 3.9 represents the PCR products of FMO3 and GAPDH, respectively. The band patterns in figures confirm that both FMO3 and GAPDH products are at the expected position of 568 and 240 bp, respectively. Therefore, these patterns suggested that qRT-PCR protocol used in this study produced the cDNAs of FMO3 and GAPDH. The results obtained using Rotor-Gene 1.7.87 quantitation software were normalized with GAPDH (housekeeping gene) and the Livak method (Livak & Schmittgen, 2001) was used to determine relative FMO3 mRNA expression change when animals injected by plant extract. Table 3.3 gives the Livak ($2^{-\Delta\Delta ct}$) method for calculation of relative mRNA expression using Ct values.

Table 3. 3 The Livak ($2^{-\Delta\Delta ct}$) method for calculation of relative mRNA expression using Ct values.

	Control	**EHT**			
Ct_{FMO3}	17.86	19.16			
Ct_{GAPDH}	18.15	18.96			
ΔCt_{EHT} =	**Ct_{GAPDH}**	**-**	**Ct_{FMO3}**		
$\Delta Ct_{reference}$ =	**Ct_{GAPDH}**	**-**	**Ct_{FMO3}**		
ΔCt_{EHT} =	18.96	-	19.16	=	-0.2
$\Delta Ct_{reference}$ =	18.15	-	17.86	=	0.29
	$\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{EHT}$			**=**	**0.49**
$2^{-\Delta\Delta ct}$	**=**	**0.71**			

qRT-PCR results regarding the effect of water extract of *Epilobium hirsutum* L. on rat liver FMO3 mRNA expression are represented in Figure 3.10. After standardization with internal control GAPDH, relative mRNA expression level of FMO3 was found to be decreased 1.41 fold in EHT group (N=26) with respect to control (N=11) group ($p<0.05$, unpaired, two-tailed student's *t*-test).

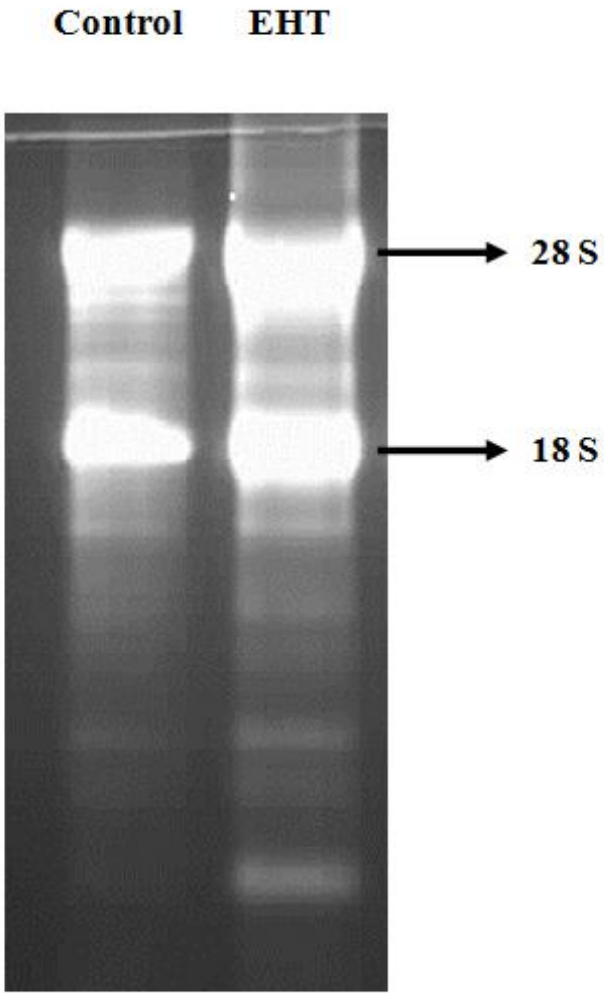


Figure 3. 4 Agarose gel electrophoresis pattern of total RNA isolated from livers of control and EHT rat groups. Lanes contain 5 µL of total RNA.

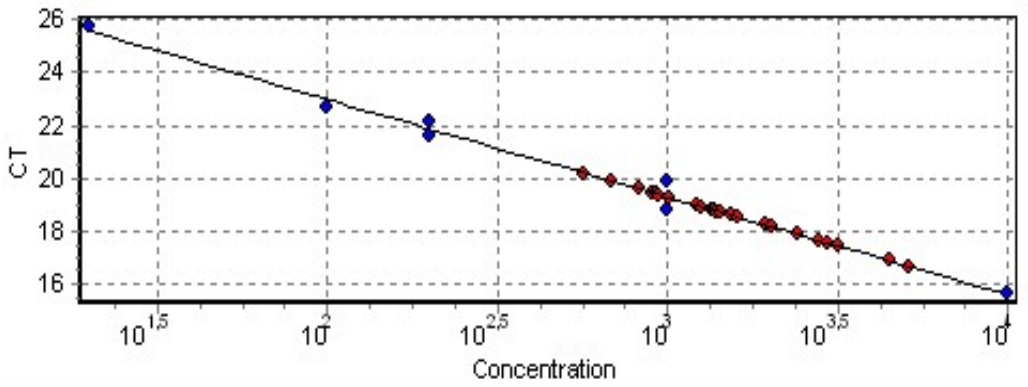


Figure 3. 5 Standard curve derived from dilution series of a control cDNA to measure quantities of specific mRNAs in the sample.

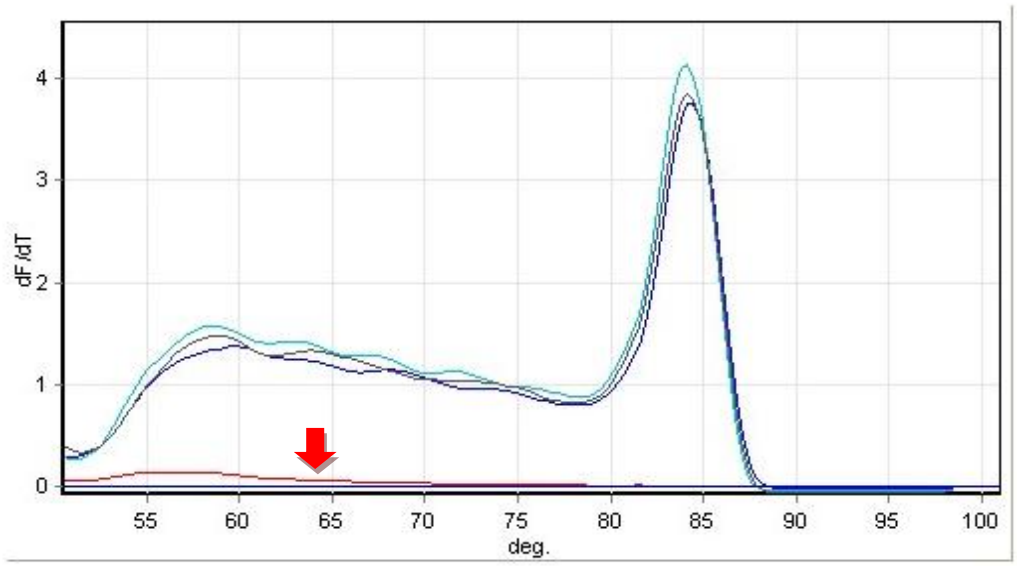


Figure 3. 6 Melting curve showing the fluorescence of SYBR Green I dye versus the temperature. One peak in the melting curve confirms the detection of single PCR product. Red arrow points the non-template control and confirms there was no contamination coming from water used for the reaction.

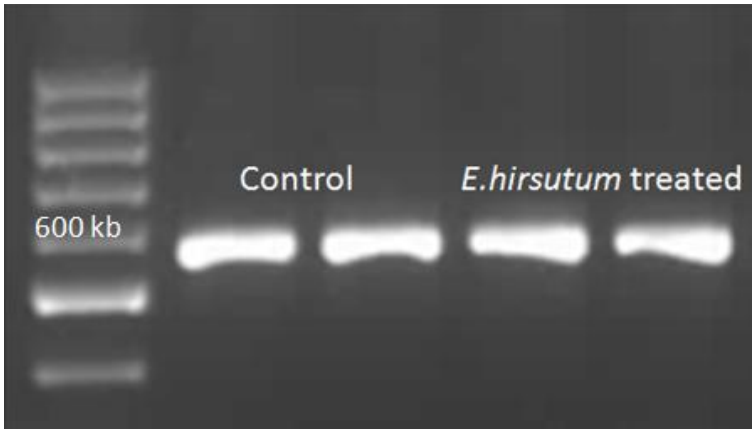


Figure 3. 7 qRT-PCR product of FMO3 cDNA (568 bp). Each lane contains 10 µl of qRT-PCR product.

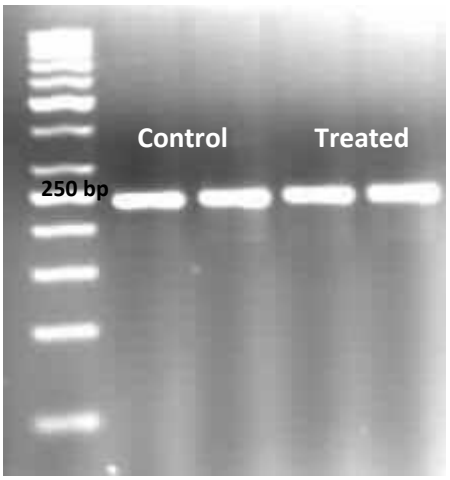


Figure 3. 8 qRT-PCR product of GAPDH cDNA (240 bp). Each lane contains 10 µl of qRT-PCR product.

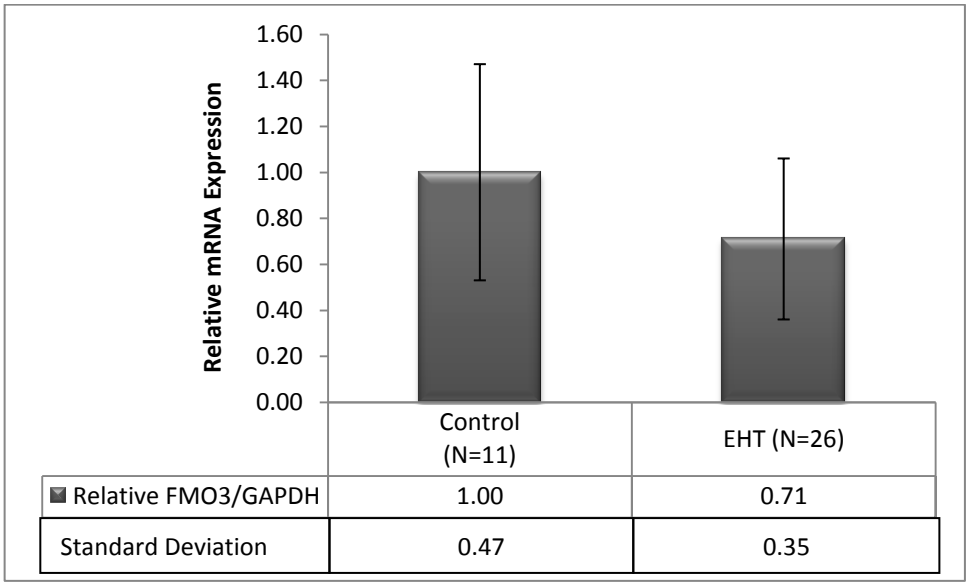


Figure 3. 9 Effect of water extract of *Epilobium hirsutum* L. on rat liver FMO3 mRNA expression. The quantifications are expressed as mean ± SD of the relative expression. Relative mRNA expression of *Epilobium hirsutum* L-treated group is 1.41 fold lower than that of control group. The p value obtained from unpaired, two-tailed student's *t*-test was 0.034.

3.3 Effect of Water Extract of *Viscum album* L. on Rat Liver FMO Activity and Expression

Influence of medicinal plant *Viscum album* L. on rat liver microsomal Flavin-containing monooxygenase activity and protein and mRNA expressions were studied with the injection of aqueous extract of this plant to albino Wistar rats with the concentration of 10 mg/kg of body weight.

3.3.1 Effect of Water Extract of *Viscum album* L. on Rat Liver FMO Activity

Rat liver microsomal FMO activity was determined using the substrate methimazole coupled with the oxidation of TNB to DTNB. The liver microsomal samples from 11 control and 15 *Viscum album* L.-treated (VAT) animals were assayed in duplicate or triplicate for the determination of FMO specific activities as nmol/min/mg. Results were given as the mean values of these duplicate or triplicate determinations of enzyme specific activities.

Table 3.3 and Figure 3.11 show the rat liver microsomal FMO enzyme activities of control (N=11) and *Viscum album* L. treated (N=15) animals. The average of FMO activities for control group was calculated as 5.11±0.92 nmol/min/mg, while that for VAT was 4.15±1.08 nmol/min/mg. There was a significant decrease in specific activity of rat liver microsomal FMO in VAT with p value of 0.026 (unpaired, two-tailed student's *t*-test).

Table 3. 4 FMO activities of rat liver microsomal samples from control and VAT groups.

Control (nmol/min/mg)		VAT (nmol/min/mg)	
C1	6.22	**VAT1**	2.40
C2	5.52	**VAT2**	4.12
C3	4.90	**VAT3**	6.20
C4	4.86	**VAT4**	4.61
C5	4.27	**VAT5**	4.92
C6	4.63	**VAT6**	5.36
C9	4.00	**VAT7**	4.07
C10	4.29	**VAT8**	5.10
C11	6.30	**VAT9**	5.02
C12	6.64	**VAT10**	4.46
C13	4.55	**VAT11**	3.57
Mean ± SD	5.11 ±0.92	**VAT12**	3.48
		VAT13	3.38
		VAT14	2.87
		VAT15	2.72
		Mean ± SD	4.15 ± 1.08

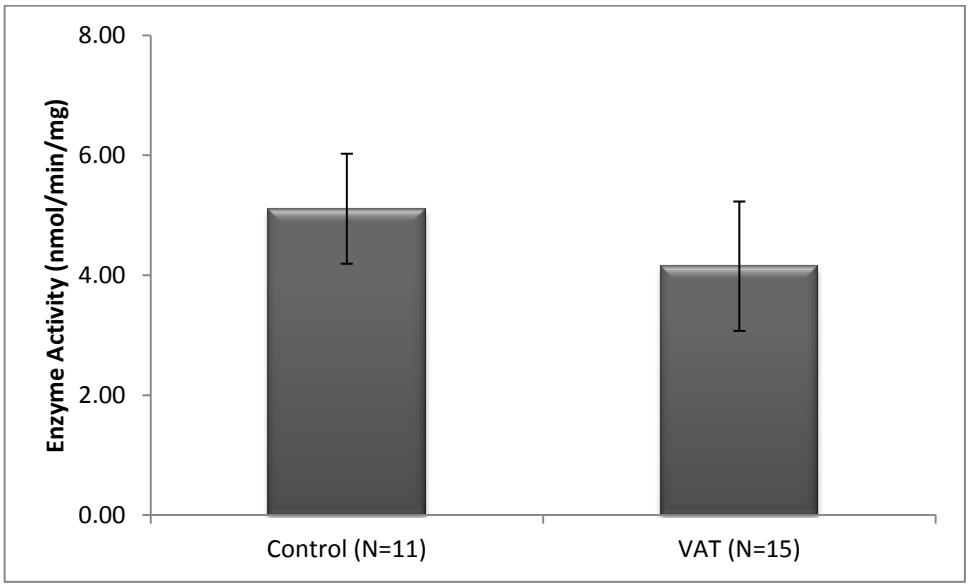
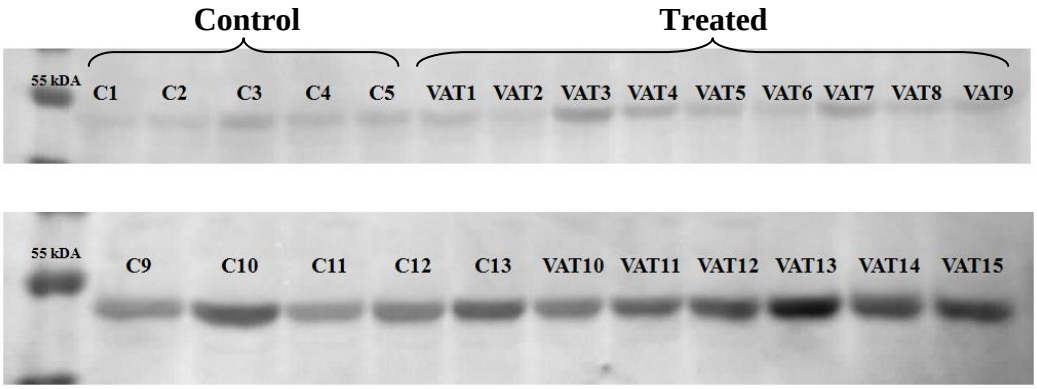


Figure 3. 10 Effect of water extract of *Viscum album* L. on rat liver FMO Activity. The reaction mixture contained standard assay constituents described in the "Method". The reactions were started with 1mM methimazole and recorded at 412 nm for 3 minutes. The mean specific activity of duplicate or triplicate measurements of total 11 controls was calculated as 5.11±0.92 nmol/min/mg. The mean specific activity of duplicate or triplicate measurements of total 15 VAT was calculated as 4.15±1.08 nmol/min/mg. The p value obtained from unpaired, two-tailed student's *t*-test was 0.026.

3.3.2 Effect of Water Extract of *Viscum album* L. on Rat Liver FMO3 Protein Expression

Rat liver microsomal FMO3 protein expressions of control and *Viscum album* L-treated groups were determined using Western blot procedure following the separation of proteins by SDS-PAGE. Figure 3.11 shows the position of FMO3 (monomer MW of 58 kDa) on nitrocellulose membrane. ß-actin was used as the internal loading control (MW of 42 kDa). The intensity of immunoreactive protein bands were analyzed by using ImageJ visualization software and the results illustrated in Figure 3.12 show the relative protein expressions where mean of protein expressions of control group was taken as 100 with SD of 25.0 and then mean protein expression of VAT group was calculated as 72.34 with SD of 24.9. As shown in Figure 3.12, rat liver microsomal FMO3 protein expression was decreased in VAT group compared as control group (unpaired, two-tailed student's *t*-test, $p<0.05$). The mean FMO3 protein expression of VAT group was found as 72.34% of that of control group.

A (FMO3)



B (β-Actin)

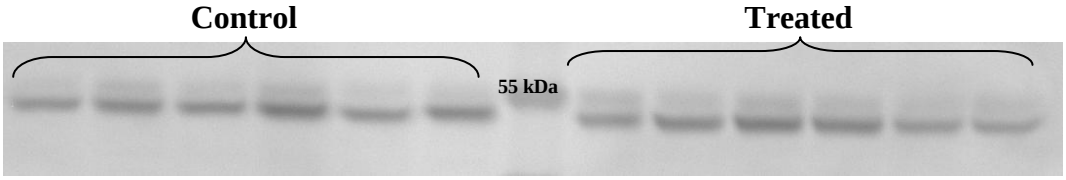


Figure 3. 11 **(A)** Immunoreactive protein bands of control (N=10) and *Viscum album* L.-treated (N=15) groups representing FMO3 (58 kDa). **(B)** ß-actin (42 kDa) was used as the internal control for each membrane.

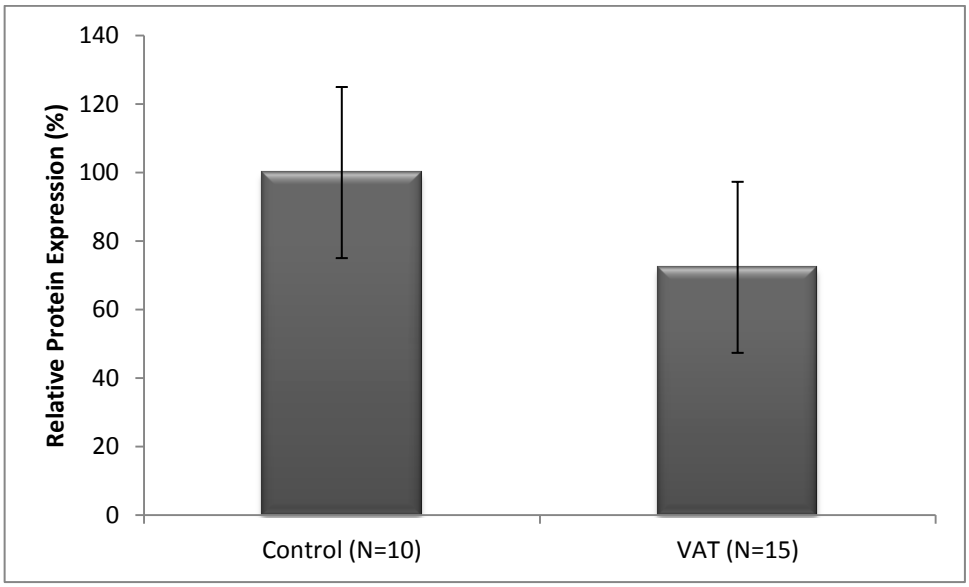


Figure 3. 12 Effect of water extract of *Viscum album* L. on rat liver FMO3 protein expression. The band quantifications are expressed as mean ± SD of the relative intensity. The mean relative protein expression of VAT group is 72.34% of that of control group. The p value obtained from unpaired, two-tailed student's *t*-test was 0.036.

3.3.3 Effect of Water Extract of *Viscum album* L. on Rat Liver FMO3 mRNA Expression

Because DNA contamination could interfere with the subsequent qRT-PCR used for cDNA synthesis, purity and quantity of RNA isolated were assessed by $OD_{260/280}$ ratio, followed by agarose gel electrophoresis. The samples having $OD_{260/280}$ ratio between 1.7 and 2.0 were taken for the further experiments. Figure 3.13 shows representative agarose gel electrophoresis of rat liver total RNA of control and VAT groups.

mRNA expressions of FMO3 were determined using qRT-PCR and relative expressions of FMO3 of control and treated animals were calculated using GAPDH as internal control (housekeeping gene). Figure 3.14 and 3.15 show standard curve of diluted cDNAs used for quantification of the samples and melting curve to confirm that single PCR product was detected, respectively. In this study, the standard cDNA was diluted 1:10, 1:100, 1:500, 1:000, and 1:5000.

Figure 3.16 and 3.17 represents the PCR products of FMO3 and GAPDH, respectively. The band patterns in figures confirm that both FMO3 and GAPDH products are at the expected position of 568 and 240 bp, respectively. Therefore, these patterns suggested that qRT-PCR protocol used in this study produced the cDNAs of FMO3 and GAPDH. The results obtained using Rotor-Gene 1.7.87 quantitation software were normalized with GAPDH (housekeeping gene) and the Livak method (Livak & Schmittgen, 2001) was used to determine relative FMO3 mRNA expression change when animals injected by plant extract. Table 3.5 gives the Livak ($2^{-\Delta\Delta ct}$) method for calculation of relative mRNA expression using Ct values.

Table 3. 5 The Livak ($2^{-\Delta\Delta ct}$) method for calculation of relative mRNA expression using Ct values.

	Control	**VAT**			
Ct_{FMO3}	23.95	26.7			
Ct_{GAPDH}	17.39	18.77			
ΔCt_{VAT} =	**Ct_{GAPDH}**	**-**	**Ct_{FMO3}**		
$\Delta Ct_{reference}$ =	**Ct_{GAPDH}**	**-**	**Ct_{FMO3}**		
ΔCt_{VAT} =	18.8	-	26.7	=	-7.9
$\Delta Ct_{reference}$ =	17.4	-	23.95	=	-6.55
	$\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{VAT}$			**=**	**1.35**
$2^{-\Delta\Delta ct}$	**=**	**0.39**			

qRT-PCR results regarding the effect of water extract of *Viscum album* L. on rat liver FMO3 mRNA expression are represented in Figure 3.18. After standardization with internal control GAPDH, relative mRNA expression level of FMO3 was found to be decreased 2.56 fold in VAT group (N=15) with respect to control (N=11) group ($p<0.0001$, unpaired, two-tailed student's *t*-test).

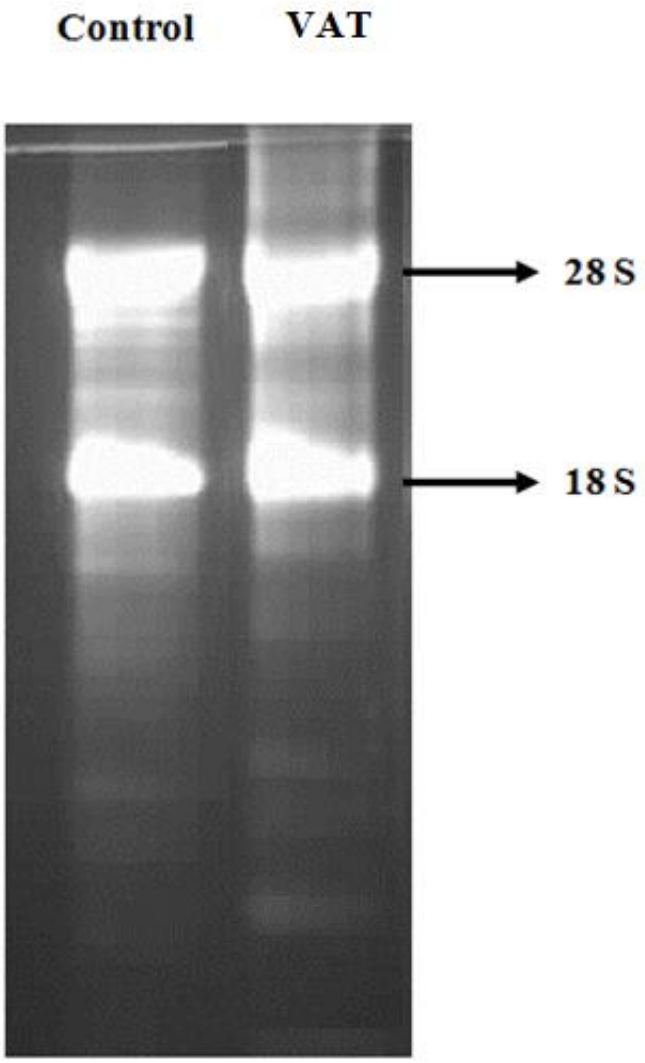


Figure 3. 13 Agarose gel electrophoresis pattern of total RNA isolated from rat livers of control and VAT groups. Lanes contain 5 µL of total RNA.

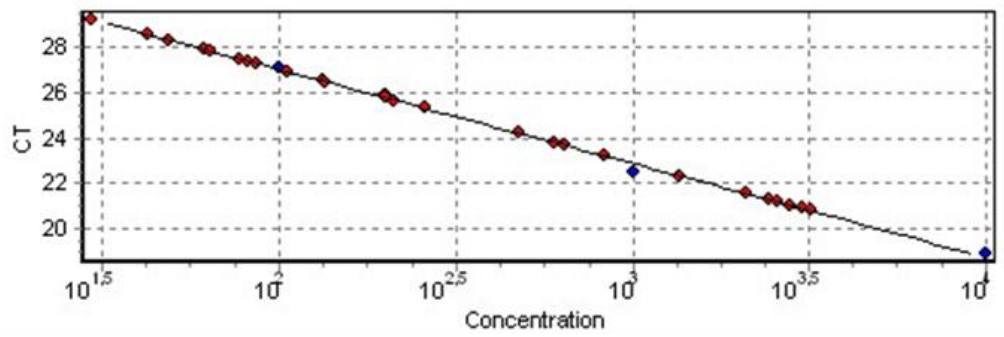


Figure 3. 14 Standard curve derived from dilution series of a control cDNA to measure quantities of specific mRNAs in the sample.

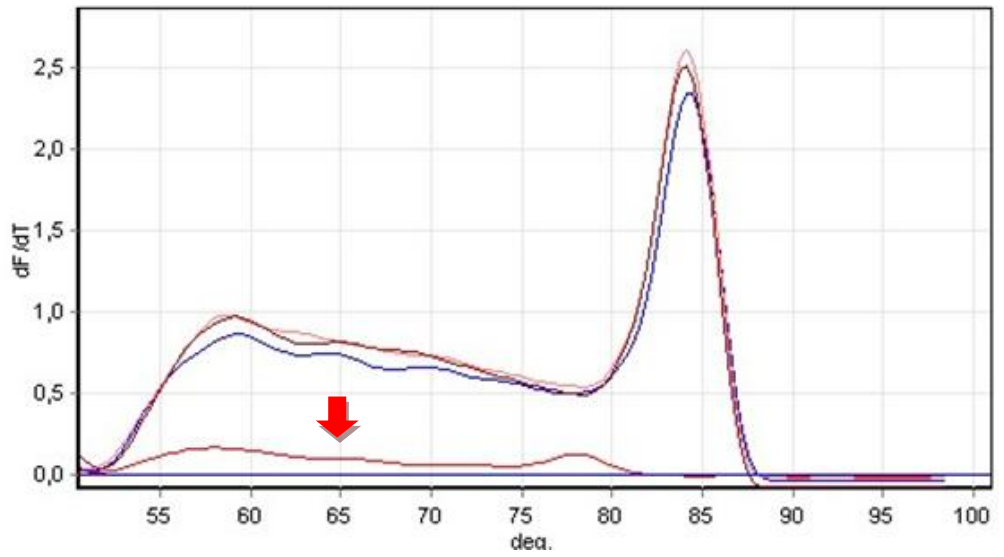


Figure 3. 15 Melting curve showing the fluorescence of SYBR Green I dye versus the temperature. One peak in the melting curve confirms the detection of single PCR product. Red arrow points the non-template control and confirms there was no contamination coming from water used for the reaction.

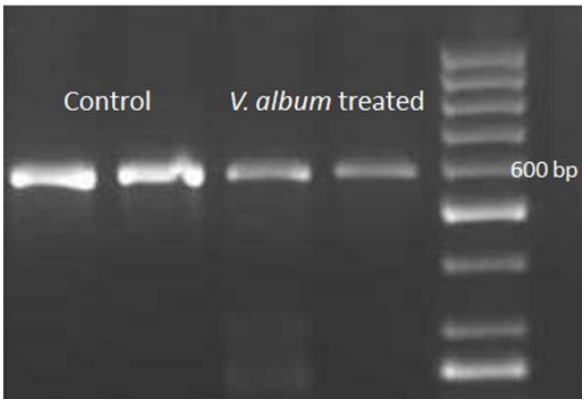


Figure 3. 16 qRT-PCR product of FMO3 cDNA (568 bp). Each lane contains 10 µl of qRT-PCR product.

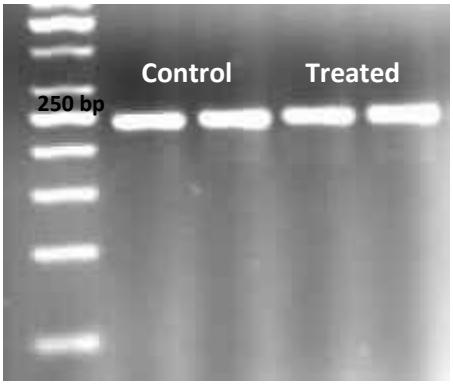


Figure 3. 17 qRT-PCR product of GAPDH cDNA (240 bp). Each lane contains 10 µl of qRT-PCR product.

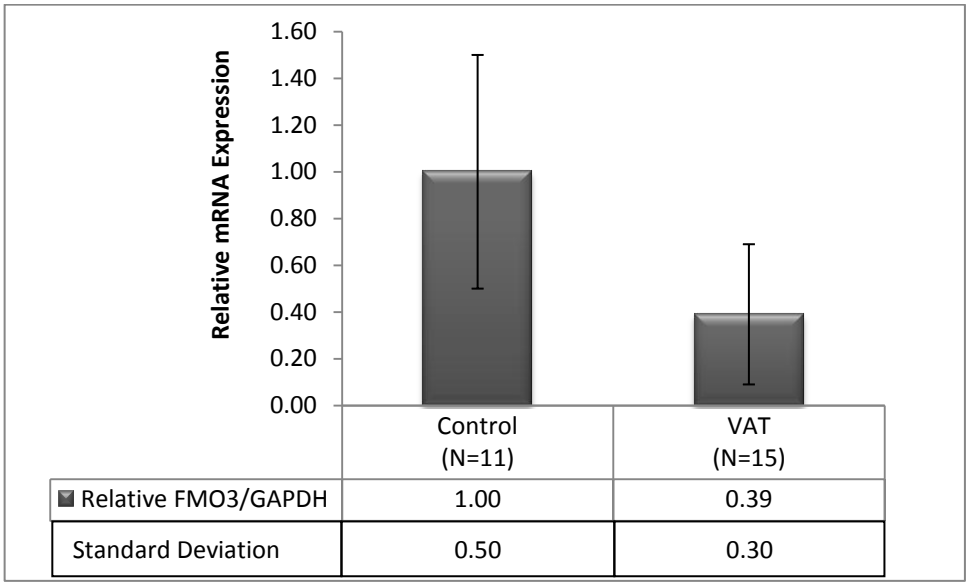


Figure 3. 18 Effect of water extract of *Viscum album* L. on rat liver FMO3 mRNA expression. The quantifications are expressed as mean ± SD of the relative expression. Relative mRNA expression of *Viscum album* L-treated group is 2.56 fold lower than that of control group. The p value obtained from unpaired, two-tailed student's *t*-test was less than 0.0001.

CHAPTER 4

DISCUSSION

Plants have an important potential for health benefits because they contain many biological active compounds that may have positive effects on human health. A few examples of these biological active compounds include phenolic compounds, flavonoids, tannins, and glycosides. For example, tannins are polyphenolic compounds having a wide range of chemical diversity. They are found in fruits, grains, and beverages and have beneficial effects (Okuda, 2005). p-Coumaric acid is one of the tannins present in citrus fruits, grapes and wines, legumes, and cereals (Clifford, 1999). It is known for antioxidant benefits, radical scavenging activities, as well as their ability to reduce low-density lipoprotein cholesterol oxidation (Rice-Evans *et al.*, 1997; Zang *et al*., 2000).

Flavin monooxygenase (FMO) forms a stable 4α-hydroperoxy flavin enzyme intermediate which is dependent on NAD(P)H and oxygen in the absence of suitable substrate. It seems that access to this stabilized intermediate can control substrate specificity from which metabolism of a wide range of xenobiotics by this enzyme results. Substrates of FMO include tertiary and secondary alkyl- and arylamines, thioamides, thiocarbamides, hydrazines, thiols, sulfides, disulfides, and other soft nucleophilic compounds. Thus, FMOs are generally considered as mammalian detoxification enzyme system, involving the conversion of nucleophilic nitrogen, sulfur, phosphorus, and selenium heteroatom-containing chemicals into more polar and readily excreted metabolites. This property makes FMOs have roles in detoxication of dietary compounds and other xenobiotics which otherwise can be converted into more active compounds by other enzyme systems. However, there are some exceptions

that FMOs metabolize to more active compounds. For example, mercaptopyrimidines and thiocarbamides seem to be converted to more active compounds by FMOs. Therefore, FMOs can have crucial roles in some steps of the toxicity (Hines *et al.*, 1994).

FMOs can be modulated and regulated at several levels such as enzyme expression by physiological factors, cofactor supply, and diet (Cashman & Zhang, 2006). The important sources of diet include a variety of plants. Among them, medicinal plants have special importance and have been used for both treatment and prevention of diseases. Medicinal plant, *Epilobium hirsutum* L. has been used for treatment of inflammation, adenoma menstrual disorders, constipate, and prostate, as well as prevention of rectal bleeding. *Viscum album* L. has been traditionally used as sedative, analgesic, anti-spasmolytic, cardiotonic, and anticancer agent, for treatment of epilepsy, hypertension, exhaustion, arthritis, anxiety, vertigo, and degenerative inflammation of the joints. Both of these plants contain many biologically active compounds, including polyphenols and flavonoids. Such compounds have been known for their biological effects such as anti-oxidant activities of polyphenols, anti-nociceptive and anti-inflammatory activities of flavonoids. In addition, they can affect the activities and expressions of some important xenobiotic metabolizing enzymes such as CYPs and FMOs. However, there is no data available in the literature for the possible effects of *Epilobium hirsutum* L. and *Viscum album* L. on xenobiotic and endogen metabolizing flavin-containing monooxygenase enzymes (FMOs). Therefore, this study was aimed to elucidate *in vivo* effects of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. for their possible abilities to modulate rat liver flavin-containing monooxygenases, involved in the metabolism of a wide range of xenobiotic compounds including drugs such as tamoxifen, methimazole and imipramine, pesticides, neurotoxins, and other chemicals.

To our knowledge, this is the first study that investigates *in vivo* effects of *Epilobium hirsutum* L. and *Viscum album* L. on rat liver microsomal phase I xenobiotic-metabolizing FMO activity and expression. Our findings have shown that water extracts of medicinal plants *Epilobium hirsutum* L. and *Viscum album*

L. had an effect on rat liver FMO activity and mRNA and protein expression. In this work, 37.5 mg/kg of body weight of *Epilobium hirsutum* L. and 10 mg/kg of body weight of *Viscum album* L. were injected to 30 and 15 Wistar albino rats, respectively, for 9 consecutive days. Then, animals were sacrificed by decapitation and their livers were removed. After preparation of microsomal fractions, protein concentrations, FMO activities, protein-mRNA expressions were determined. Our data suggested that these plants may have roles in modulating rat liver FMO enzyme system at both transcription and translation levels. Table 4.1 summarizes the results obtained from *in vivo* experiments.

Table 4. 1 Effects of water extracts of *Epilobium hirsutum* L. and *Viscum album* L. on FMO activity, protein and mRNA expressions.

	Activity (nmol/min/mg)	**Protein Expression (%)**	**mRNA Expression (%)**
Control	5.11±0.92	100	100
EHT	5.31±1.35	72.29	71
VAT	4.15±1.08	72.34	39

In this study, level of mRNA was determined using quantitative Real-Time PCR, as described in "Methods". Using specific primers of FMO3, the mRNA levels of EHT and VAT treated groups were expressed as relative to control groups and normalized to GAPDH mRNA expression. The results showed that water extracts of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. decreased the mRNA level of FMO3, 1.41 and 2.56 folds, respectively, as compared to controls. The decrease in mRNA expression suggests transcriptional changes because the constituents in the plants probably can affect transcription factors responsible for FMO3 mRNA expression. Although molecular mechanisms of expression regulation of FMOs are not clear, a study conducted by Luo and Hines (2001) suggested that *FMO* gene expression can be regulated by hepatocyte nuclear factors 1α, 4α (HNF1α and HNF4α), and Yin Yang 1 (YY1) transcription factors.

HNF4α is a highly conserved nuclear receptor that is ligand dependent transcription factor. It is major transcription factor specific for liver gene expression. Protein phosphorylation of HNF4α and binding of fatty acyl CoA thioesters, the endogenous ligand, can modulate transcriptional activity of HNF4α (Hertz *et al.*, 1998; Jiang *et al.*, 1997). Moreover, environmental factors and diet can change the expression of HNF4α metabolism (Hwang-Verslues & Sladek, 2010). For example, a study conducted by Burke et al. (1994) showed that HNF4 level fell in response to injury in mice, as well as HNF1, so that the genes regulated by this factor can have altered expression. In addition, its mRNA and protein expressions can be upregulated during fasting (Xie *et al*., 2009) and can be altered by bile acids (products of dietary metabolism) so that affecting the transcription of drug-metabolizing genes (Zhang *et al*., 2010). Other studies showed that FMO3 mRNA expression may depend aryl hydrocarbon receptor, AHR (Celius *et al*., 2008, 2010).

Although there is not much known about mRNA stability of FMOs or selective stimulation for translation of mRNA transcripts, a recent study showed that mRNA levels of FMO1 decreased in cultured rat hepatocytes through cyclic guanosine monophosphate (cGMP)-independent destabilizing effect of nitric

oxide by decreasing the half-life of mRNA when the cells were administrated with lipopolysaccharides and pro-inflammatory cytokines (Ryu *et al.*, 2004).

In the present study, determination of protein expression of rat liver FMO3 was conducted by using Western blot followed SDS-PAGE, as described in "Method". Specific diluted goat polyclonal FMO3 antibody was used to detect FMO3 protein having molecular weight of 58 kDa. ß-Actin was used as internal protein loading control for each sample in the experiments. Our data showed that water extracts of both plants *Epilobium hirsutum* L. and *Viscum album* L. decreased the protein expression of FMO3 27.71% and 27.66%, respectively. This is probably due to the decrease in FMO3 mRNA expression in both EHT and VAT groups. In addition to this possibility suggested in the current work, a study showed that a toxic lectin isolated from *Viscum album* inhibited protein synthesis in lysate of rabbit reticulocytes (Stirpe *et al*., 1980). The lectins found in the *Viscum album* L. could lead to reduction in protein synthesis so that FMO3 protein level might also be reduced when rats treated with the water extract of this plant.

Rat liver microsomal FMO activities were determined using the method described by Dixit and Roche (1984) with minor modifications of Can Demirdögen and Adali (2005) as explained in detail in "Methods". Methimazole was used as specific substrate for FMO activity and liver microsomal fractions from both control and treated groups were assayed. Our results showed that water extract of *Viscum album* L. caused a decrease in the FMO activity towards methimazole from 5.11 nmol/min/mg to 4.15 nmol/min/mg (19% decrease with respect to controls) . This decreased activity of FMO is probably due to the reduced expressions of mRNA and protein of FMO3.

In contrast to the findings of FMO3 mRNA and protein expressions, water extract of *Epilobium hirsutum* L. did not change significantly FMO activity towards methimazole. While the control mean activity of FMO was found to be 5.11 nmol/min/mg, the mean activity of treated group was found as 5.31

nmol/min/mg. This might be due to possible post-translational modifications of FMO3 protein caused by the constituents of *Epilobium hirsutum* L.

Studies showed that multiple post-translational modifications on FMO proteins can affect the enzyme stereoselectivity (Lawton *et al.*, 1991; Lomri *et al.*, 1993). For example, N-acetylation can occur at N-terminal amino acid of pig liver FMO. In addition, studies revealed that there is a covalently attached carbohydrate present in the pig liver FMO (Lomri *et al*., 1993). Another site of post-translation in FMO1 is Asn 120 which is N-glycosylated, determined by mass spectrometry (Korsmeyer *et al*., 1998). Because this site is highly conserved, this region of the protein could play important roles for functional or structural properties in the enzyme activity. However, when cDNA of FMOs was expressed in bacterial systems by Brunelle *et al*. (1997), the enzyme activity did not need this modification.

The decrease in activity and expression levels of FMO by *Viscum album* L. treatment of can result in increased efficacy of some drugs used for cancer treatment. For example, epidemiological studies show that non-steroidal anti-inflammatory agents have roles in chemoprevention of colorectal cancers (Thun *et al.*, 2002). One example of these agents is sulindac, whose mechanism seems to be via inhibition of cyclooxygenase-2 which is overexpressed in colorectal carcinomas and adenomas. It is a pro-drug which contains racemic S-oxide form reduced to sulfide (Duggan *et al.*, 1977). The reduced form sulindac sulfide is active form and metabolized by FMO3 through S-oxygenation to sulfoxide and then sulfone (Hamman *et al.*, 2000). A study showed that FMO3 polymorphisms resulting in reduced FMO activity (at E158K and E308G loci) can decrease the metabolism of active form sulindac sulfide to inactive form sulfoxide, so increase the effects of sulindac (Hisamuddin *et al.*, 2004). Therefore, *Viscum album* extract decreasing the FMO activity and expression, in some cases, can lead to increase the effectiveness of the drugs which are metabolized and inactivated by FMO.

In contrast, reduced activity and mRNA and protein expressions of FMO can have negative effects on the rate of metabolism and hence the detoxication of

some xenobiotics. When exposed to toxicants such as pesticides and insecticides, the decreased FMO activity due to *Viscum album* L. taken can lead to altered metabolism of these toxicants and more severe adverse effects could be observed.

Drug adverse effects could also be observed when drug is taken with plant extract. For example, cimetidine is widely used for treatment of peptic ulcer and gastric hyper-secretion syndromes and known as inhibitor of CYPs and cimetidine-mediated inhibition of CYPs can lead to clinical importance for drug interactions (Cashman *et al*., 1995). It was also proposed that FMO3 is the principle enzyme that is responsible for metabolism of cimetidine through S-oxygenation (Cashman *et al.*, 1995). In addition, some commonly used insecticides for various crops such as fenthion can be metabolized by CYPs and FMOs. While CYP-contributed biotransformation of fenthion is thought as bioactivation, at high concentrations FMOs contribute to detoxication of fenthion by metabolizing it to sulfoxide form (Leoni *et al.*, 2008). Thus, in the case of acute poison by such insecticides coming from the corps, the reduced activity of FMOs resulting from the plant extract can lead to inability to detoxify the xenobiotics; besides, it can cause CYPs to produce more toxic form.

As mentioned before, there are few cases in which FMOs catalyze the bioactivation of some drugs or other xenobiotics to more reactive metabolites such as N-alkylaryl amines N-oxygenized by FMOs subsequently gaining carcinogenic characteristics (Ziegler *et al.*, 1988), amphetamine and methamphetamine metabolized by FMO3 to corresponding hydroxylamines so that becoming potential toxicants (Cashman *et al*., 1999). Therefore, decreased expression of FMO3 protein and mRNA by *Viscum album* L. treatment may reduce the toxic effects of metabolites produced from FMO-catalyzed reactions of such drugs and xenobiotics.

Although mRNA and protein expressions of rat liver FMO3 were significantly reduced by *Epilobium hirsutum* L. treatment, the FMO activity was preserved due to possible post-translational modifications. There are some other studies suggesting the similar conclusion in which the overall activity of FMO

was changed slightly, while the mRNA and protein expressions were significantly changed. As an example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) highly induced mRNA and protein expression levels of FMO2 and FMO3, but overall FMO catalytic activity was increased only slightly (Celius *et al*., 2008, 2010).

In recent years, regarding the drug development and metabolism including drug-drug interactions and adverse effects, the studies on FMOs and their roles in metabolism have gained importance. Initially, FMOs were thought not to be modulated by xenobiotics or dietary compounds, but then recently, it has been found that there are numerous modulators for FMO activity and expression (Adali *et al.* 1998; Cashman *et al.*, 1999).

In the present study, only one subspecies of *Viscum album*, *Viscum album* L. subsp. *austriacum* (Wiesb.) Vollmann, was used as plant material. However, the other two *Viscum* subspecies also merit further studies for the evaluation of the comparative effects of these subspecies.

In conclusion, the results of this study showed that water extracts of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. can modulate the xenobiotic metabolism via altering the activity and expression of protein and mRNA of FMO. However, FMOs are highly polymorphic and have species-, tissue-, and gender-dependent expressions; therefore, further experiments using different laboratory animals and tissues of them and then human are required for investigation of effects of *Epilobium hirsutum* L. and *Viscum album* L. on FMO activity and expression. Besides, the major forms of FMOs in livers of human and rats are different, therefore the effects of the medicinal plants on human FMOs should be studied.

CHAPTER 5

CONCLUSION

Epilobium hirsutum L., also known as hairy willow herb as a medicinal plant, has been used for treatment or prevention of inflammation, adenoma, rectal bleeding, menstrual disorders, constipates, and prostate. It contains mainly polyphenolics and flavonoids. It is common knowledge that polyphenols have biological health benefits, including antioxidant activities.

Viscum album L., also known as European mistletoe, contains lectins, polypeptides, mucilage, sugar alcohols, flavonoids, lignans, triterpenes, and phenylallyl alcohols. The leaves and twigs of *Viscum album* L., taken as tea, have been traditionally used for hypertension, stomachache, diarrhea, dysuria and also as analgesic and cardiotonic agent in Turkey. Moreover, in Europe, sterile extracts of *Viscum album* L. are among the most common herbal extracts applied in cancer treatment and have been used as prescription drugs, while in US, considered as dietary supplement.

FMOs are FAD-containing phase I enzymes and responsible for the oxidation of wide-range of drugs such as tamoxifen, methimazole and imipramine, pesticides, neurotoxins, and other chemicals by using NADPH as cofactor.

To our knowledge, this is the first study investigating the *in vivo* influence of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. on enzyme activity and protein and mRNA expression of rat hepatic FMO. The water extracts of *Epilobium* hirsutum L. (37.5 mg/kg body weight) and Viscum *album* L.

(subspecies growing on pine trees-subsp. *austriacum* (Wiesb.) Vollmann) (10 mg/kg body weight) were injected intraperitonally (i.p) into Wistar albino rats for 9 consecutive days. Following the decapitation, the livers were removed and microsomal fractions were prepared by differential centrifugation. Rat liver microsomal FMO activity using methimazole as substrate, mRNA expression by quantitative Real-Time PCR, and protein expression by Western Blot were determined.

The results showed that water extract of *Epilobium hirsutum* L. has no significant effect on FMO activity; however, it decreased significantly ($p<0.05$) FMO3 protein and mRNA expression 27.71% and 1.41 fold, respectively, compared as controls. Water extract of *Viscum album* L. decreased mRNA (2.56 fold), and protein expression (27.66%) as well as enzyme activity (19%) of FMO with respect to controls.

In conclusion, the metabolism of xenobiotics including drug molecules by FMO-catalyzed reactions may be altered due to the changes in FMO expression and activity by medicinal plants *Epilobium hirsutum* L. and *Viscum album* L.

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APPENDIX 1

T.C
PAMUKKALE ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURULU BAŞKANLIĞI

Sayı :B.30.2.PAÜ.0.01.00.00.400-1/ 08 17.02.2009
Konu :Çalışma Başvurusu

Sayın;
Prof. Dr. Alaattin ŞEN
Fen Edebiyat Fakültesi
Biyoloji Bölümü
Öğretim Üyesi

İlgi : 22.01.2009 tarihli başvurunuz.

"Yakı Otunun Ksenobiyotik Metabolizması Üzerine Etkilerinin ve İlaç-Diyet Etkileşim Potansiyelinin Proteomik ve Moleküler Yaklaşımlar ile Aydınlatılması" konulu PAUHDEK-2009/007 no'lu çalışmanız 11.02.2009 tarih ve 02 sayılı toplantımızda görüşülmüş olup,

Çalışmanın yapılmasının **Hayvan Deneyleri Etiği açısından uygun olduğuna** oy çokluğu ile karar verilmiştir.

Gereğini bilgilerinize rica ederim.

Doç. Dr. Vural KÜÇÜKATAY
Başkan

Not: Gıdılu imzalı belge Proje çıktısı ile gönderilecek.
2/3/09

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T.C. PAMUKKALE ÜNİVERSİTESİ HAYVAN DENEYLERİ ETİK KURUL BAŞKANLIĞI TOPLANTI TUTANAĞI			
Toplantı Tarihi	11.02.2009	**Karar**	3
Toplantı Sayısı	2	**Toplantı Saati**	15:00
Toplantı Günü	Çarşamba	**Toplantı Yeri**	Dekanlık

Karar 3- Fen Edebiyat Fakültesi Biyoloji Bölümü Öğretim üyesi Prof. Dr. Alaattin ŞEN'in yürütücüsü olduğu "Yakı Otunun Ksenobiyotik Metabolizması Üzerine Etkilerinin ve İlaç-Diyet Etkileşim Potansiyelinin Proteomik ve Moleküler Yaklaşımlar ile Aydınlatılması" konulu PAUHDEK-2009/007 no'lu çalışması görüşülmüş olup,

Çalışmanın yapılmasının **Hayvan Deneyleri Etiği** açısından **uygun olduğuna** oy çokluğu ile karar verilmiştir.

Yrd.Doç.Dr.Mustafa KARATEPE	Doç. Dr. Vural KÜÇÜKATAY Başkan		
Doç.Dr.Çağrı ERGİN		Yrd.Doç.Dr.Mustafa KARATEPE	
Doç.Dr.Yakup KASKA		Doç.Dr.Burhan KABAY	**KATILMADI**
Yrd.Doç.Dr.Funda BÖLÜKBAŞI HATİP		Uzm.Vet.Hek.Barbaros ŞAHİN	
Dr.Berna TURGUT		Şemsettin ÖZMEN	

T.C.
PAMUKKALE ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURULU BAŞKANLIĞI

SAYI :B.30.2.PAÜ.0.01.00.00.400-1/23 16.05.2008
KONU :Çalışma Başvurusu

İLGİLİ MAKAMA

İlgi: Prof.Dr.Alaattin ŞEN'in 30.04.2008 tarihli başvurusu.

İlgi dilekçe ile başvuran Prof.Dr.Alaattin ŞEN'in "Ülkemizde Tıbbi Bitki Olarak Kullanılan Epilobium hırsitum ve Viscum album Bitkileirnin Tedavi Edivi ve zararlı Etkileirnin ve Etki Mekanizmalarının Moleküler Yaklaşımlar ile Aydınlatılması" konulu PAUHDEK-2008/012 no'lu çalışması 16.05.2008 tarih ve 06 sayılı toplantımızda görüşülmüş olup,

Çalışmanın yapılmasının **Hayvan Deneyleri Etiği açısından uygun olduğuna** oy birliği ile karar verilmiştir.

İş bu belge kişinin isteği üzerine verilmiştir.

Doç. Dr. Vural KÜÇÜKATAY
Başkan

ORTA DOĞU TEKNİK ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURUL KARARLARI

KARAR SAYISI : Etik-2009/16	**KARAR TARİHİ: 27.08.2009**

O.D.T.Ü. Biyoloji Bölümü Öğretim Üyesi Prof. Dr. Orhan ADALI'nın 2009/16 no'lu **"Yakıotunun Ksenobiyotik Metabolizması Üzerine Etkilerinin ve İlaç-Diyet Etkileşim Potansiyelinin Proteomik ve Moleküler Yaklaşımlar ile Aydınlatılması"** başlıklı araştırma projesinin etik başvurusu değerlendirilmiştir.

Etik Kurul'un 27.08.2009 tarihinde yapılan toplantısında, yukarıda adı geçen projenin araştırmadaki deney hayvanlarının tür, sayı ve kullanım amaçlarının ve projenin deney hayvanlarına ilişkin yönlerinin O.D.T.Ü. Hayvan Deneyleri Yerel Etik Kurul Yönergesinde belirtilen "Hayvan Deneyleri ile İlgili Etik İlkeler" dikkate alınarak hazırlandığı saptanmıştır. Bu değerlendirme sonucu çalışmanın 60 adet RAT ile yapılmasının hayvan etiği açısından uygun olduğu oy çokluğu ile onaylanmıştır.

Doç. Dr. Ewa (Havva) Doğru Başkan	Jale Esin Sivil Üye, Başkan Vekili
KATILMADI Prof. Dr. Orhan Adalı Üye	Prof. Dr. Gülay Özcengiz Üye
Prof. Dr. İnci Togan Üye	Prof. Dr. Vasıf Hasırcı Üye
KATILMADI Prof. Dr. Feza Korkusuz Tıp Hekim, Üye	KATILMADI Y. Doç. Dr. Barış Parkan Üye
Dr. Uğur Göğüş Vet. Hekim, Üye	Dr. Seçil Cabuk Vet. Hekim, Üye
KATILMADI Av. Neşide Öncül Kurum İçinden Seçilen Hukuk Müşaviri	Nilgün Egemen Sivil Toplum Kuruluşu Dernek Üyesi