IN VIVO EFFECT OF *EPILOBIUM HIRSUTUM* L. AND *VISCUM ALBUM* L. ON PROTEIN AND mRNA EXPRESSIONS OF RAT LIVER VITAMIN D_3 METABOLIZING CYP24A1 AND CYP27B1 ENZYMES

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submitted by **Melike SEVER** in partial fulfillment of the requirements for the degree of the degree of **Master of Science in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN   _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Candan GÜRAKAN   _____
Head of Department, **Biochemistry**

Prof. Dr. Orhan ADALI   _____
Supervisor, **Biology Dept., METU**

Prof. Dr. Alaattin ŞEN   _____
Co-Supervisor, **Biology Dept. Pamukkale University**

Examining Committee Members:

Prof. Dr. Musa DOĞAN   _____
Biology Dept., METU

Prof. Dr. Orhan ADALI   _____
Biology Dept., METU

Prof. Dr. Alaattin ŞEN   _____
Biology Dept., Pamukkale University

Prof. Dr. İrfan KANDEMİR   _____
Biology Dept., Ankara University

Assoc. Prof. Dr. Ewa JAKUBOWSKA-DOĞRU   _____
Biology Dept., METU

Date: 13.09.2012

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Name, Last Name: Melike Sever

Signature:

ABSTRACT

IN VIVO EFFECT OF *EPILOBIUM HIRSUTUM* L. AND *VISCUM ALBUM* L. ON PROTEIN AND mRNA EXPRESSIONS OF RAT LIVER VITAMIN D$_3$ METABOLIZING CYP24A1 AND CYP27B1 ENZYMES

Sever, Melike

M.Sc., Department of Biochemistry

Supervisor: Prof. Dr. Orhan Adalı

Co-Supervisor: Prof. Dr. Alaattin Şen

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Epilobium hirsutum L. (Onagraceae) is a flowering, tall and perennial plant and native to Eurasia. It shows analgesic, anti-microbial and anti-proliferative activity, and it is used in our country as an alternative medicine. The pharmacological effect of *Epilobium hirsutum* L. could be explained by the presence of polyphenolics including steroids, tannins and flavonoids in the aerial parts. *Viscum album* L. (Loranthaceae) is a shrub that grows as an epiphyte on the branches of deciduous trees. It involves in the enhancement of macrophage phagocytic and cytotoxic mediated abilities as well as the strengthening the immune system.

CYP24A1 and CYP27B1 are members of cytochrome P450 superfamily and the most important enzymes involved in the metabolism of vitamin D$_3$. CYP27B1 and CYP24A1 are mitochondrial enzymes and also known as 25-hydroxyvitamin D$_3$ 1alpha-hydroxylase and 24-hydroxylase, respectively. CYP24A1 involves in 24-hydroxylation of 25-OH-D$_3$ and 1,25-(OH)$_2$D$_3$ which is required for the catabolism of vitamin D$_3$

compounds while CYP27B1 involves in 1α-hydroxylation of 25-OH-D$_3$ into 1,25-(OH)$_2$D$_3$.

In this study, *in vivo* effects of *Epilobium hirsutum* and *Viscum album* (subspecies growing on pine-trees-subsp. *austriacum* (Wiesb.) Vollmann) on rat liver CYP24A1 and CYP27B1 mRNA and protein expressions were investigated. To achieve this goal, 37.5 mg water extract of *Epilobium hirsutum* L./kg body weight/day was intraperitoneally injected to male rats for 9 days. To study the effect of *Viscum album* L., 10 mg water extract of *Viscum album* L./kg body weight/day was injected with the same conditions. After decapitation, livers were removed and S1.5 fractions were prepared. Effects of *Epilobium hirsutum* L. and *Viscum album* L. on rat liver mRNA and protein expressions were analyzed by qRT-PCR and western blotting, respectively.

Epilobium hirsutum L. extract caused 31% and 18% decrease in rat liver CYP24A1 ($p < 0.0001$) and CYP27B1 ($p < 0.05$) protein expressions, respectively. The effect of *Epilobium hirsutum* L. on mRNA expression of CYP24A1 could not be observed, because CYP24A1 mRNA was almost undetectable in liver. Injection of *Epilobium hirsutum* L. to rats caused 2.7 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) expression as an internal reference ($p < 0.005$).

Viscum album L. caused 17% decrease in CYP24A1 protein expression ($p < 0.05$). When rats injected with plant extract of *Viscum album* L., 18% decrease in CYP27B1 protein expression was observed ($p < 0.05$). The effect of *Viscum album* L. on mRNA expression of CYP24A1 could not be observed since CYP24A1 mRNA was almost undetectable in liver. Injection of *Viscum album* L. to rats caused 3.8 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) expression as an internal reference ($p < 0.005$).

In conclusion, vitamin D_3 metabolism may be affected by medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. due to the changes in mRNA and protein expressions of CYP24A1 and CYP27B1 enzymes.

Key words: *Epilobium hirsutum* L., *Viscum album* L., Vitamin D_3, CYP24A1, CYP27B1, mRNA and protein expression, rat liver

ÖZ

EPILOBIUM HIRSUTUM L. VE *VISCUM ALBUM* L.'NİN SIÇAN KARACİĞER VİTAMİN D₃ METABOLİZMASINDA ROL ALAN CYP24A1 VE CYP27B1 ENZİMLERİNİN PROTEİN VE mRNA EKSPRESYONU ÜZERİNDEKİ *İN VİVO* ETKİLERİ

Sever, Melike

Yüksek Lisans, Biyokimya Bölümü

Tez Yöneticisi: Prof. Dr. Orhan Adalı

Ortak Tez Yöneticisi: Prof. Dr. Alaattin Şen

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Epilobium hirsutum L. Avrasya'ya özgü, tıbbi bitki olarak kabul edilen ve Türkiye'de tüylü yakı otu olarak bilinen çok yıllık bir bitkidir. Analjezik, anti-mikrobiyal ve antiproliferatif etkilere sahiptir. İçerdiği polifenoller sayesinde çeşitli farmakolojik etkilere sahip olduğu için birçok hastalığın tedavisinde ve önlenmesinde etkili olduğu düşünülmektedir. *Viscum album* L. ise aynı şekilde alternatif tıpta kullanılan ve Türkiye'de ökse otu olarak bilinen çalı türüdür. Makrofajların fagositik ve sitotoksik etkilerinin genişletilmesinde etkili olduğu gibi bağışıklık sisteminin güçlendirilmesinde de rol almaktadır. Ayrıca, steril ekstraktları kanser tedavisinde kullanılmaktadır.

CYP24A1 ve CYP27B1, sitokrom P450 enzimleri arasında yer alıp vitamin D₃ metabolizmasında görev yapan en önemli enzimlerdendir. CYP24A1 ve CYP27B1

enzimlerinin ikisi de mitokondriyal enzimler olup birçok organda bulunmaktadırlar. CYP24A1 enzimi vitamin D bileşiklerinin inaktivasyonunda yer alır. 25-OH-D$_3$ ve 1,25-(OH)$_2$D$_3$ moleküllerinin 24-hidroksilasyonunu katalizlediği için 24-hidroksilaz enzimi olarak da bilinir. CYP27B1 ise esas olarak vitamin D aktivasyonunda görev yapar. 25-OH-D$_3$ molekülünün 1α-hidroksilasyonunu katalizlediği için 1α-hidroksilaz enzimi olarak da bilinir.

Bu çalışmada *Epilobium hirsutum* L. ve *Viscum album* L. (çam ağaçları üzerinde yetişen alt türü-subsp. *austriacum* (Wiesb.) Vollmann) bitkilerinin sıçan karaciğer CYP24A1 ve CYP27B1 mRNA ve protein ekspresyonları üzerine *in vivo* etkileri araştırılmıştır. Bunun için, *Epilobium hirsutum*'un 18.75mg sulu ekstraktı wistar albino sıçanlara 9 gün boyunca intraperitonal olarak enjekte edilmiştir. *Viscum album* içinse bu miktar 10 mg olarak belirlenmiştir. 11 tane sıçan ise kontrol olarak kullanılmıştır. Daha sonra, sıçanlar dekapite edilerek karaciğerleri çıkarılmış ve S1.5 fraksiyonlar elde 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 CYP24A1 protein ekspresyonunu %31 azalttığı gözlemlenmiştir ($p<0.0001$). Ayrıca, *Epilobium hirsutum* L., CYP27B1 protein ekspresyonunu %18 azaltmıştır ($p<0.05$). CYP24A1 mRNA ekspresyonunun karaciğerde saptanması zor olduğu için, bu bitkinin CYP24A1 mRNA ekspresyonu üzerine etkileri deneysel olarak gösterilemezken, *Epilobium hirsutum* L.'in CYP27B1 mRNA ekspresyonunu kontrollere göre 2.7 kat arttırtığı bulunmuştur ($p<0.005$).

Aynı şekilde *Viscum album* L. bitkisinin sulu ekstraktı CYP24A1 ve CYP27B1 protein ve mRNA ekspresyonlarını etkilemiştir. *Viscum album* L. CYP24A1 protein ekspresyonunu %17 azaltmıştır ($p<0.05$). Ayrıca, *Viscum album* L.'in CYP27B1 protein ekspresyonunu %18 azalttığı bulunmuştur ($p<0.05$). CYP24A1 mRNA ekspresyonunun karaciğerde saptanması zor olduğu için, bu bitkinin CYP24A1 mRNA ekspresyonu

üzerine etkileri deneysel olarak gösterilemezken, *Viscum album* L. CYP27B1 mRNA ekspresyonunu kontrollere göre 3.8 kat arttırdığı gözlemlenmiştir (p<0.005).

Sonuç olarak, *Epilobium hirsutum* L. ve *Viscum album* L. tıbbi bitkilerinin CYP24A1 ve CYP27B1 ekspresyonunu değiştirebilmekte ve dolayısıyla vitamin D_3 metabolizmasını etkilemektedir.

Anahtar kelimeler: *Epilobium hirsutum* L., *Viscum album* L., vitamin D_3, CYP24A1, CYP27B1, mRNA and protein ekspresyonu, sıçan karaciğeri

To My Family,

For their endless support and love

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TABLE OF CONTENT

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

APS	Ammonium per sulfate
BCIP	5-bromo 4-chloro 3-indoyl phosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CaR	Calcium-sensing receptor
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold cycle
CRE	cAMP response element
CYP	Cytochrome P450
DBP	Vitamin D binding protein
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ε-ACA	ε-Amino caproic acid
EDTA	Ethylenediaminetetraacetic acid
ERB	Electrophoretic running buffer
FAD	Flavin adenine dinucleotide
FGF-23	Fibroblast growth factor 23
FMN	Flavin mononucleotide
GAE	Gallic acid equivalents
i.p.	intraperitoneal
kDa	kilo Dalton
Km	Michaelis constant
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form

NBT   Nitrotetrazolium blue chloride

PCR   Polymerase chain reaction

PIP$_2$ Phosphatidylinositol 4,5-bisphosphate

PTH   Parathyroid hormone

rpm   Revolutions per minute

RNA   Ribonucleic acid

RXR   Retinoic X receptor

SDB   Sample dilution buffer

SDS   Sodium dodecyl sulfate

SDS-PAGE   Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBST   Tris-buffered saline and Tween 20

TEMED   Tetramethylethylenediamine

VDR   Vitamin D receptor

VDRE   Vitamin D response element

UV   Ultraviolet

CHAPTER 1

INTRODUCTION

Plants have always been used in medicine throughout the history. At the beginning, plants were used as medicine in their raw forms such as teas, powders, poultices and other several herbal forms (Balick & Cox, 1996). The methods and formulations of these plants were transmitted orally, but then the informations were recorded. In recent history, the active compounds of these plants began to be isolated. Today, characterization of these active compounds in medicinal plants still continue. There are several important examples for the usage of these medicinal plants in drug discovery, which are introduced to the markets after important clinical trials. These plants have many serious properties such as antioxidant, anti-inflammatory, antimicrobial, anti-mutagenic characteristics. For instance, *Viscum album* L. and *Epilobium hirsutum* L. are known as important medicinal plants used in alternative medicine. *Epilobium hirsutum* L. is used as anti-inflammatory and edema preventer (Kiss *et al.*, 2011) while *Viscum album* L. has important role in the treatment of some cancers as well as cardiovascular diseases (Gupta *et al.*, 2012).

Medicinal plants have numerous phenolic and polyphenolic compounds which display important role in human health. These compounds may have effects on the modulation of enzymes responsible for the xenobiotic or endogen metabolism. Vitamin D_3, one of the endogens metabolized in the body, mainly involves in maintenance of calcium and phosphate homeostasis. Vitamin D_3 functions in the keratinocytes of skin, parathyroid glands, macrophages and T-cells of the immune system, ovarian cells and islet cells. 25-

hydroxyvitamin D_3 1alpha-hydroxylase (CYP27B1) and 1alpha,25-dihydroxyvitamin D3 24-hydroxylase (CYP24A1) are members of the cytochrome P450 superfamily, and key enzymes of vitamin D_3 metabolism for the activation and inactivation of vitamin D_3, respectively (Sakaki *et al.*, 2005).

Since these CYPs are important enzymes in vitamin D_3 metabolism, understanding the possible effects of *Epilobium hirsutum* and *Viscum album*, as medicinal plants used in the treatment of several disorders, on CYP24A1 and CYP27B1 expressions may provide insight about the role of these plants in drug discovery and development.

1.1. *Epilobium hirsutum* L.

Epilobium hirsutum L. is a tall, perennial and flowering plant between July-September with branched robust stems and numerous hairs (Figure 1.1). It is also called as hairy willow herb, great willow herb or great hairy willow herb and it is known as "Tüylü yakı otu" in Turkish (Baytop, 1999). *Epilobium hirsutum* is a member of the Onagraceae family. *Epilobium hirsutum* L. is a characteristic plant in Eurasia. It is found in moist waste, marshy ground of the Mediterranean region, Europe, Asia, and Africa. It forms large, long-lived colonies in England, Wales, and Ireland. Its height ranges from 30 cm to 2 meter tall. It has bright, pink-purple flowers with white centers and notched petals. Leaves are positioned oppositely and they attach directly on the stem (Stuckey, 1970).

Epilobium hirsutum L. is a semi-aquatic plant, so it prefers a wide range of moist soils, including wetlands, ditch and stream banks, low fields, pastures, and meadows to live. pH of 5.5 or higher is required by hairy willow-herb for the germination of seeds. Seeds and rhizomes are important for spreading of this plant. *Epilobium hirsutum* L. reproduces by wind dispersed. Also, vegetative reproduction occurs by thick rhizomes. Flower buds develop after 10 to 12 weeks of growth (Stuckey, 1970)

Figure 1. 1 *Epilobium hirsutum* L.

The composition of *Epilobium hirsutum* L. is complex, because it has rich content including flavonoids (kaempferol, myricetin, quercetin etc.), free sterols (β-sitosterol and ester forms of β-sitosterol), tannins (Gallic, ellagic acid, protocatechuic and p-coumaric acids), polyphenols and ellagitannines (Ivancheva *et al.*, 1992; Barakat *et al.*, 1997; Gruenwald, 2000).

The total flavonoid content of *Epilobium hirsutum* L. is about 5.8% which provides intense phytotherapeutical effects (Roman *et al.*, 2010). Flavonoids have antioxidant properties of cleaning and buffering free radicals. Polyphenolic compounds are present

3

in plants which are medicinally used. They display many important biological effects such as antioxidant properties (Kähkönen *et al.*, 1999). Total phenolic content of *Epilobium hirsutum* L. is 4.03 ± 0.12 mg of gallic acid equivalents (GAE)/100 g of dry weight (dw). *Epilobium hirsutum* L. has the phenolic acids and flavonoids indicated below:

Phenolic acids:

- Caffeic acid: 23.1 ± 0.03 mg/100 g dw
- p-coumaric acid: 38.3 ± 0.09 mg/100 g dw
- Ferulic acid: 10.9 ± 0.29 mg/100 g dw

Flavonoids:

- Quercetin: 214 ± 0.03 mg/100 g dw
- Myricetin: 191 ± 0.24 mg/100 g dw (Wojdylo *et al.*, 2007)

It is used in folk medicine of Europe and Egypt for the treatment of prostate tumours, inflammation, and adenoma (Barakat *et al.*, 1997).

Internal usage of willow herb is thought to be beneficial for mucous membrane lesions in mouth, for micturition problems coming from prostatic hyperplasia (Stages I to II) and for gastrointestinal disorders. It is used for rectal bleeding by Native Americans; while it is used for menstrual disorders by Chinese people. External use of water extract of willow herb is for the improvement of the healing of wounds (Gruenwald, 2000). The body of *Epilobium hirsutum* L. is used as anti-inflammatory and edema preventer (Kiss *et al.*, 2011). The leaves and roots are used as anti-febrile drug and it is also important in the treatment of constipate and prostate (Barakat *et al.*, 1997). Also, *in vitro* studies showed that ethanolic extracts of *Epilobium hirsutum* L. displayed antimicrobial activity on gram-positive and gram-negative bacteria, fungi and yeasts (Battinelli *et al.*, 2001).

1.2. *Viscum album* L.

Viscum album L. is a shrub which is widely used in folk medicine. It involves in the family of *Loranthaceae*. European Mistletoe is the common name of *Viscum album* L.(Barlow, 1987; Kuijt, 1990). It is also known as "ökseotu" in Turkish. It is the only species of this genus growing in Turkey. White berries with small and sticky shape are the characteristic for this plant. *Viscum album* L. also has long green, oppositely arranged leaves and small flowers which cannot be easily detected (Figure 1.2) (Tompkins, 2009). It has faint odor and bitter taste. *Viscum album* L. has a wide distribution all over the world especially in Europe, Northern Asia and Africa (*Committee for Veterinary Medicinal Products Viscum album Summary Report*, 2000). In Turkey, there are three subspecies of *Viscum album* L. 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).

Figure 1. 2 *Viscum album* L.

Viscum album L. is a multi-component plant. The active chemical constituents in *Viscum album* L. are composed of;

- Glycoproteins:

High-molecular-weight glycoproteins composed of mistletoe lectins I, II, and III are the main constituents of *Viscum album* L. They are cytotoxic compounds with two chain structures; Chain A and Chain B. Chain A causes the inhibition of protein synthesis on the ribosomal stage. Chain B involves in the activation of macrophages and forces lymphocytes for releasing lymphokines. For mistletoe lectin I, Chain A together chain B prevent the release of histamine from leukocytes due to allergen induction. Both of these

chains also suppress release of serotonin from platelets caused by collagen induction (Franz, 1986).

The quantity and the degree of biological activity of this component depend on harvest time, manufacturing process and type of host tree (Büssing & Schietzel, 1999).

- Proteins:

Viscotoxin is a peptide which is composed of 46 amino acids and it is characteristic for *Viscum album* L. It is involved in cell membrane damage as well as enhancement of cytotoxic T-cells and granulocytes activity against bacteria and tumour cells.

- Polysaccharides:

Galacturonan and arabinogalactan are primary polysaccharides in *Viscum album* L. Esterified galacturonan is present in the leaves and stems of *Viscum album* L., while arabinogalactan primarily exists in the berries (Jordan & Wagner, 1986). In immune system, they function in the stimulation of T-helper cells, increase in NK-cell activity and interferon ɣ release.

- Alkaloids:

Alkaloids are nitrogenous compounds of *Viscum album* L. They are thought to be involved in cytotoxic property of mistletoe (Franz, 1986).

The other constituents involved in *Viscum album* L. are flavonoids, sulphurous components, caffeic acids, phenylpropane derivatives, mucilages, phytosterols, stigmasterol, cyclitols, amines including histamine and acetylcholine, triterpenoids and phytosterols (*Committee for Veterinary Medicinal Products Viscum album Summary Report*, 2000; Tompkins, 2009).

Viscum album L. is used extensively in alternative medicine to prevent or treat several diseases and disorders. 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; Gencler-Ozkan & Koyuncu, 2005; Yucecan *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).

The pharmacological studies which have been documented up to now have focused on immune-stimulant, cardiotoxic and cytotoxic abilities of *Viscum album* L. (Gupta *et al.*, 2012). The active role of *Viscum album* L. in nervous disorders, joint and bone disorders such as arthritis, cardiovascular diseases including atherosclerosis and hypertension, was demonstrated with several studies (Wichtl & Bisset, 1994; Bartram, 1998; Murray, 1995; Newall *et al.*, 1996).

The studies of Kienle (2010) and Mitchell (2003) showed that *Viscum album* L. has role in the enhancement of macrophage ability in phagocytosis. The active components of *Viscum album* L., lectins and viscotoxins, exhibit growth inhibition and cytotoxic effects. *Viscum album* L. involves in stimulation of thymus glands and improves the activation of T-cells, granulocytes, monocytes, natural killer cells and dendritic cells for strengthening the immune system. It also has DNA stabilizing properties (Tompkins, 2009).

Moreover, in cardiovascular system, *Viscum album* L. directly affects the cholinergic pathways which are important in decreasing the blood pressure (Wagner & Jordan, 1986). *Viscum album* L. has reputation due to showing anti-mycobacterial (Deliorman *et al.*, 2001a, b), anticancer (Burger *et al.*, 2001), anti-inflammatory (Hegde *et al.*, 2011), hypotensive (Ofem *et al.*, 2007), antioxidant and hypoglycemic (Orhan *et al.*, 2005) properties.

1.3. Vitamin D$_3$ and its metabolism

1.3.1. Introduction

Vitamin is defined as an organic compound which is not synthesized in the body even it is essential for the organism and it must be acquired from the diet. According to this definition, vitamin D is not a vitamin at all. More than this classical definition, vitamin D is described as a prohormone, so it must be metabolized to its biologically active form so that it can function as a steroid hormone (Omdahl *et al.*, 2002; Sutton & MacDonald, 2003). Vitamin D is the general name for a group of chemically related compounds. Vitamin D$_2$ and vitamin D$_3$ are the most important members of this group (Figure 1.3). Vitamin D$_2$ also called as ergocalciferol is derived from ergosterol, which is a plant steroid. Vitamin D$_3$ is also known as cholecalciferol and it is synthesized from 7-dehydrocholesterol through UV irradiation (Figure 1.4). Besides the biosynthesis, both vitamin D$_2$ and vitamin D$_3$ can be taken from the dietary sources.
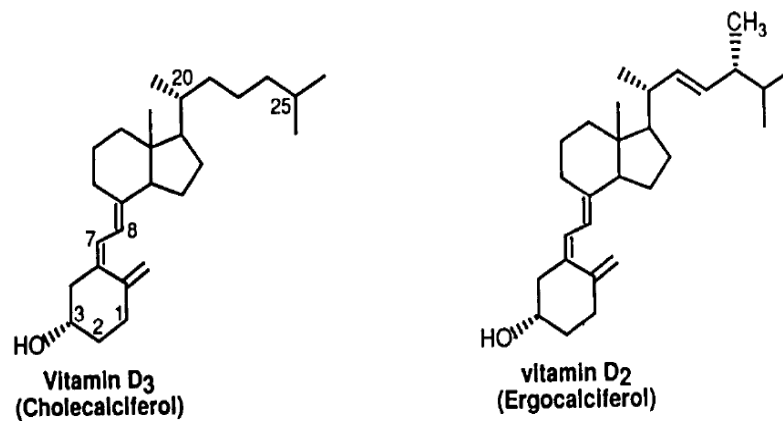
Figure 1. 3 Nutritional forms of vitamin D.

Vitamin D_3 contains three intact rings with three double bonds in its structure. It is formed by irradiation of 7-dehydrocholesterol (Figure 1.4). Normally, Vitamin D_3 is unstable in light and it undergoes oxidation reaction if it is exposed to air at 24 ^{0}C for 72 hours. Best storage temperature for vitamin D_3 is 0 ^{0}C (Norman & Henry, 2007).
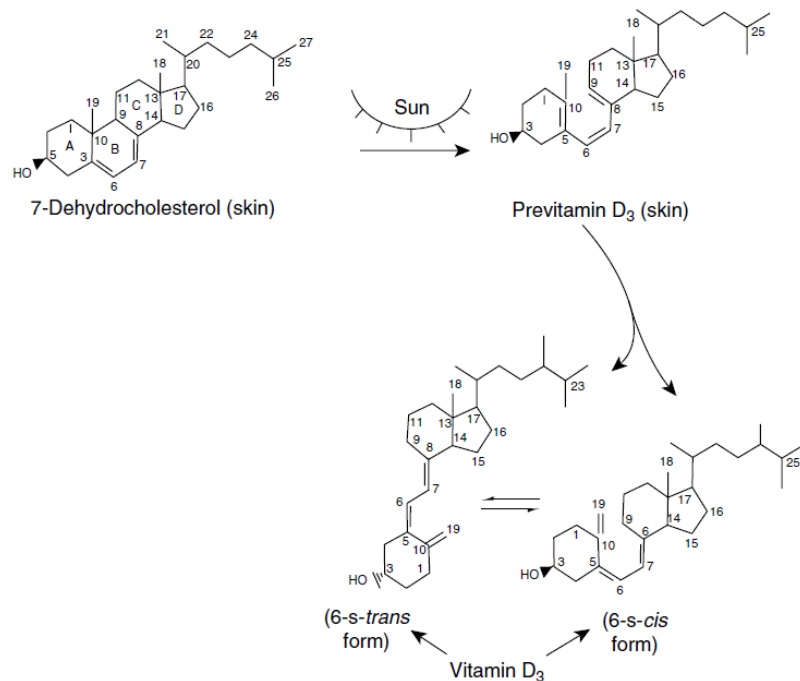
Figure 1. 4 Chemistry and irradiation pathway for production of vitamin D_3.

1.3.2. Vitamin D_3 metabolism

When skin is exposed to ultraviolet light (270-300 nm), 7-dehydrocholesterol in the skin is converted to vitamin D, in the form of vitamin D_3. Dietary intake is the other source for the uptake of vitamin D_3. Vitamin D_3 circulation in the bloodstream does not take place for very long time. It can be stored in the adipose tissues or further metabolized in the liver. Vitamin D_3 can be stored for months or years in humans; however finally it undergoes metabolic reactions (Figure 1.5). The first step for the activation of vitamin D_3 after UV irradiation is 25-hydroxylation reaction which takes place mainly in the

liver. A hydroxyl group is added to 25-carbon to generate 25(OH)-D_3. Vitamin D_3 25-hydroxylase enzyme catalyzes the formation of 25(OH)-D_3. After 25-hydroxylation in liver, 25(OH)D_3 again comes to circulatory system. In the circulatory system, vitamin D binding protein (DBP) transports 25(OH)D_3 to the kidney for the addition of another hydroxyl group at the C-1 position into the A ring (Norman & Henry, 2007). This reaction is catalyzed by 1α-hydroxylase. As a result of 1α-hydroxylation, 1,25(OH)$_2D_3$ is formed as an active form of vitamin D_3. Although there are other tissues for this reaction such as liver, 1α-hydroxylation mainly takes place in kidney.
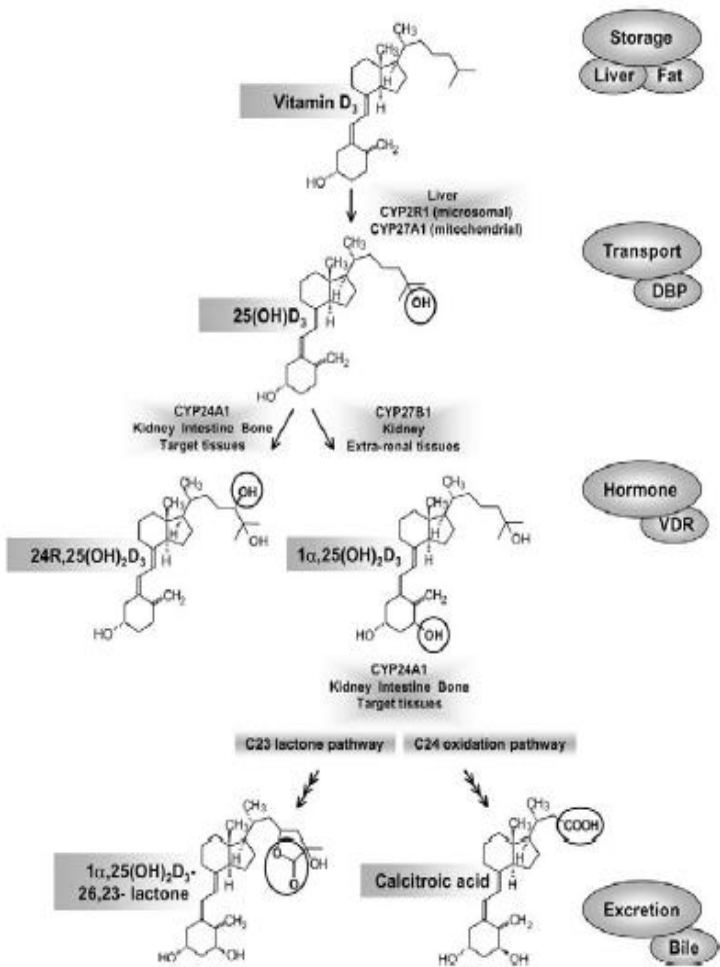
Figure 1. 5 Pathways of vitamin D activation and inactivation (St-Arnaud, 2011).

12

The catabolism of vitamin D_3 compounds which are $25(OH)D_3$ and $1,25(OH)_2D_3$ is carried out by 24-hydroxylation. This reaction is catalyzed by 24-hydroxylase and it is also known as CYP24A1. C-24 oxidation pathway primarily takes place in kidney even there are other sites in the body for this reaction such as liver.

1.3.2.1.   25-hydroxylation

25-hydroxylation is the first reaction for the activation of vitamin D_3. This reaction is catalyzed by 25-hydroxylase enzyme which is primarily found in liver. Besides liver, in intestinal, skin and renal extracts, 25-hydroxylase activity was found (Tucker *et al.*, 1973). The product of this reaction is $25(OH)D_3$ which is the main circulating form of vitamin D_3 due to the stability coming from the binding to vitamin D-binding protein (DBP) (Cooke & Haddad, 1989; Cooke *et al.*, 1991). Serum level of $25(OH)D_3$ is used as an indicator for vitamin D status, since its level is directly affected by increased uptake of vitamin D. The following metabolism of $25(OH)D_3$ depends on the calcium level in the animals. According to requirements or abundance of calcium, it undergoes further activation or inactivation, respectively. Requirement of calcium causes 1α-hydroxylation of $25(OH)D_3$; however excess amount of calcium leads to 24-hydroxylation of $25(OH)D_3$.

The subcellular locations of 25-hydroxylation are mitochondria and microsomes (Norman & Henry, 2007). In humans, there are two types of 25-hydroxylase enzyme; CYP2R1 and CYP27A1. CYP2R1 is a microsomal 25-hydroxylase with high specificity and low capacity. Unlike CYP2R1, CYP27A1 is a mitochondrial 25-hydroxylase with low affinity, but high capacity.

1.3.2.2. 1α-hydroxylation

After 25-hydroxylation, the following step for the activation of vitamin D_3 is 1α-hydroxylation which converts $25(OH)D_3$ to $1,25(OH)_2D_3$ –the active form of vitamin D_3. A second hydroxyl group is introduced into A ring at 1α position by 1α-hydroxylase which is also known as CYP27B1. Kidney is the major organ for this reaction; however recent studies showed that CYP27B1 expression was detected in non-renal tissues such as skin, intestine, liver, bone, brain, macrophages and testis (Zehnder *et al.*, 2001). CYP27B1 protein and mRNA expression are under tight regulation by several factors. Parathyroid hormone (PTH) potentially stimulates the transcription and activity of CYP27B1 in the kidney, and this stimulation is mediated through cAMP/phosphatidylinositol 4,5-bisphosphate (PIP_2) signal transduction pathway at the molecular level (Henry & Luntao, 1989). Calcitonin also involves in upregulation of CYP27B1 (Shinki *et al.*, 1999). Unlike PTH and calcitonin, $1,25(OH)_2D_3$ down-regulates the PTH-stimulated CYP27B1 gene activation (Brenza & DeLuca, 2000).

1.3.2.3. 24-hydroxylation

Further metabolism of $1,25-(OH)_2D_3$ is the 24-hydroxylation. This reaction is catalyzed by 24-hydroxylase enzyme and it is also known as CYP24A1. CYP24A1 is found in mitochondrial inner membrane and mainly functions in kidney. The initial product of this reaction is short-lived $1,24,25(OH)_3D_3$. $25(OH)D_3$ is also substrate of CYP24A1 and this reaction causes the formation of $24,25(OH)_2D_3$ which is circulating in bloodstream.

$1,25(OH)_2D_3$ stimulates the expression of *CYP24A1* gene in target tissues of vitamin D_3, especially in intestine and kidney. Unlike $1,25(OH)_2D_3$, PTH represses the CYP24A1 activity in kidney.(Reinhardt & Horst, 1990; Shinki *et al.*, 1992; Zierold *et al.*, 2000) In kidney, suppressed CYP24A1 by PTH may cause an increase in $1,25(OH)_2D_3$ and circulating calcium level. (Bai *et al.*, 2003; Inoue *et al.*, 2005; Larsson *et al.*, 2004; Perwad *et al.*, 2007).

1.3.3. Factors affecting the vitamin D_3 metabolism

PTH is one of the factors that affect the vitamin D_3 metabolism. In case of low circulating calcium level, PTH works as a potent stimulator for the production of $1,25(OH)_2D_3$. PTH leads to upregulation of CYP27B1 mRNA expression (St-Arnaud *et al.*, 1997) as well as enzymatic activity (Garabedian *et al.*, 1972) in the kidney. This regulation was shown with the studies on parathyroidectomised animals. CYP27B1 enzyme activity was stimulated in parathyroidectomised animals when they were treated with PTH (Lobaugh *et al.*, 1993; Weisinger *et al.*, 1989). The induction of $1,25(OH)_2D_3$ by PTH occurs through receptor-mediated $cAMP/PIP_2$ signal transduction mechanism (Henry *et al.*, 1992). Unlike the effect of PTH on CYP27B1 activity, PTH represses the CYP24A1 activity in the kidney (Shinki *et al.*, 1992). This negative effect is not present in other tissues such as intestine due to the lack of PTH receptors (Abou-Samra *et al.*, 1994).

The other factor affecting the vitamin D_3 metabolism is calcium. The interaction of calcium with calcium-sensing receptors (CaR) present in the parathyroid gland indirectly affects the $1,25(OH)_2D_3$ production in the kidney. Low level of calcium stimulates the parathyroid cells to produce PTH resulting in increased production of $1,25(OH)_2D_3$. There is also direct regulation of $1,25(OH)_2D_3$ by calcium in the kidney. In PTH-repleted rats, it was shown that increase in calcium level causes decrease in circulating $1,25(OH)_2D_3$ level (Matsumoto *et al.*, 1987; Weisinger *et al.*, 1989). It was suggested that this regulation is mediated through CaR.

Calcitonin also involves in regulation of vitamin D_3 metabolism. Unlike PTH-mediated stimulation under hypocalcemia, calcitonin particularly involves in the stimulation of CYP27B1 mRNA expression in the kidney when circulating calcium level is normal (Shinki *et al.*, 1999). In the promoter region of CYP27B1 gene, there is a positive regulatory site for calcitonin.

$1,25(OH)_2D_3$ leads to upregulation of CYP24A1 activity. When $1,25(OH)_2D_3$ binds to VDR, they directly induce the mRNA expression of CYP24A1 due to the interaction with VDREs present on the promoter region of CYP24A1 gene (Hahn *et al.*, 1994; Kerry *et al.*, 1996). On the other hand, $1,25(OH)_2D_3$ suppresses the CYP27B1 activity in the kidney. Since no VDREs have been found in the proximal promoter region of CYP27B1 gene, probably the mechanism of suppression by $1,25(OH)_2D_3$ is indirect and requires other factors such as PTH or calcium (Brenza & DeLuca, 2000).

1.3.4. Biological actions of $1,25(OH)_2D_3$

There is coordination between kidney, bone, intestine and parathyroid gland for the maintenance of calcium homeostasis. This coordinated mechanism provides enough calcium to achieve muscle and nerve function as well as maintenance of bone mineralization. Although the exact role of $1,25(OH)_2D_3$ is still under question, it has recently found that there is an association between $1,25(OH)_2D_3$ and immune system, pancreas, ovarian cells and some neuronal tissues.

The main role of $1,25(OH)_2D_3$ in small intestine is the stimulation of calcium absorption. This stimulation is carried out through VDR-mediated transcription regulation of the genes specific to calcium absorption. In particularly, calbindin-D9k is a cytosolic calcium-binding protein important in calcium translocation across the enterocyte. $1,25(OH)_2D_3$ leads to up-regulation of calbindin-D9k expression together with VDRE which is found to be proximal region of gene promoter (Darwish & DeLuca, 1996). Besides this mode of action of $1,25(OH)_2D_3$, the direct role of $1,25(OH)_2D_3$ for the regulation of calbindin-D9k was shown in VDR knockout mice. Both protein and mRNA expression were decreased when the intestinal VDR was knocked-out (Li *et al.*, 1998).

In vitamin D-deficient and VDR-knocked out animals, normal bone mineralization was detected when they were fed high level calcium (Weinstein *et al.*, 1984). It suggests that $1,25(OH)_2D_3$ does not have a key role in bone mineralization. The main point in defective mineralization may be the defective intestinal absorption which affects the availability of phosphorus and calcium. However, *in vitro* studies showed that $1,25(OH)_2D_3$ is involved in regulation of mineralization, transcription and differentiation of osteoblast gene (Matsumoto *et al.*, 1991; Rickard *et al.*, 1995). Osteocalcin and osteopontin are the bone matrix proteins having VDRE in their promoter sites. $1,25(OH)_2D_3$ induces the transcription of the genes which encodes for these proteins. $1,25(OH)_2D_3$ also function in stimulation of osteoclast development indirectly by generating osteoblast-derived factor which then affects the osteoclastogenesis (Abe *et al.*, 1981; Suda *et al.*, 1995; Tanaka *et al.*, 1982).

PTH is involved in stimulation of $1,25(OH)_2D_3$ production in kidney as a result of hypocalcemia, so $1,25(OH)_2D_3$ provides negative feedback regulation by suppressing PTH secretion and synthesis from parathyroid glands (Cantley *et al.*, 1985; Chan *et al.*, 1986) as well as by growth control of parathyroid cells (Szabo *et al.*, 1989). There is VDRE in the promoter site of PTH gene, so it is under direct regulation by $1,25(OH)_2D_3$-bound VDR-RXR heterodimer (Liu *et al.*, 1996).

$1,25(OH)_2D_3$ shows negative feedback regulation for its own synthesis by suppressing CYP27B1 and stimulating CYP24A1 in kidney. Also, $1,25(OH)_2D_3$ stimulates the expression of calbindin-28k which functions in calcium transport in kidney (Bar *et al.*, 1990; Varghese *et al.*, 1988). $1,25(OH)_2D_3$ has also been associated with facilitation of calcium reabsorption and calcium transport regulated by PTH in kidney (Friedman & Gesek, 1993; Yamamoto *et al.*, 1984).

17

1.4. Cytochrome P450s

1.4.1. Introduction

Cytochrome P450 (CYP) is the general name of a large family of different enzymes metabolizing most of the drugs and chemicals. The term P450 refers to a pigment that absorbs light at 450 nm when reduced form is exposed to carbon monoxide (Omura and Sato, 1964). Besides xenobiotic metabolism, many CYP enzymes have important roles in different physiological processes. Cholesterol and steroid biosynthesis, metabolism of fatty acids and calcium homoeostasis and its maintenance are the most important physiological processes in which CYP enzymes involve (Mckinnon *et al.*, 2008).

1.4.2. Nomenclature

The nomenclature of a cytochrome P450 gene includes (Figure 1.6):

- The abbreviation CYP for cytochrome P450;
- Following number indicating for the CYP family;
- Capital letter for the subfamily; and
- Final numeral representing for the individual gene (Nebert *et al.*, 1987).
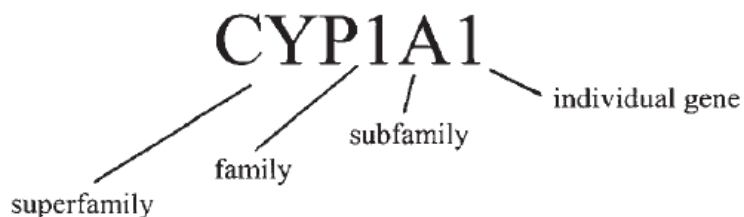
Figure 1. 6 Nomenclature of cytochromes P450.

For describing a CYP gene, italic letters and numerals are used. For the CYP enzymes, same nomenclature in non-italic form is used.

It is thought that divergence of CYP families from each other took place about 1.2 billion years ago. Therefore, at least 40% amino acid sequence homology is required to be involved in the same family. Divergence of the subfamilies is estimated to occur 400 million years ago. For being in the same subfamily, greater than 55% identity is prerequisite (Coleman, 2005).

1.4.3. Mechanism

CYPs can be classified into four groups according to delivery of electrons from NAD(P)H to the catalytic sites;

- CYPs with iron sulfur redoxin and FAD-containing reductase;
- CYPs with FAD/FMN-containing P450 reductase;
- Self-sufficient CYPs with no requirement for electron donor; and

19

- CYPs with direct electron transfer from NAD(P)H (Werck-Reichhart & Feyereisen, 2000).

Despite the electron delivery differences, all CYP enzymes share a common catalytic mechanism for the oxygenation of a substrate (Figure 1.7).
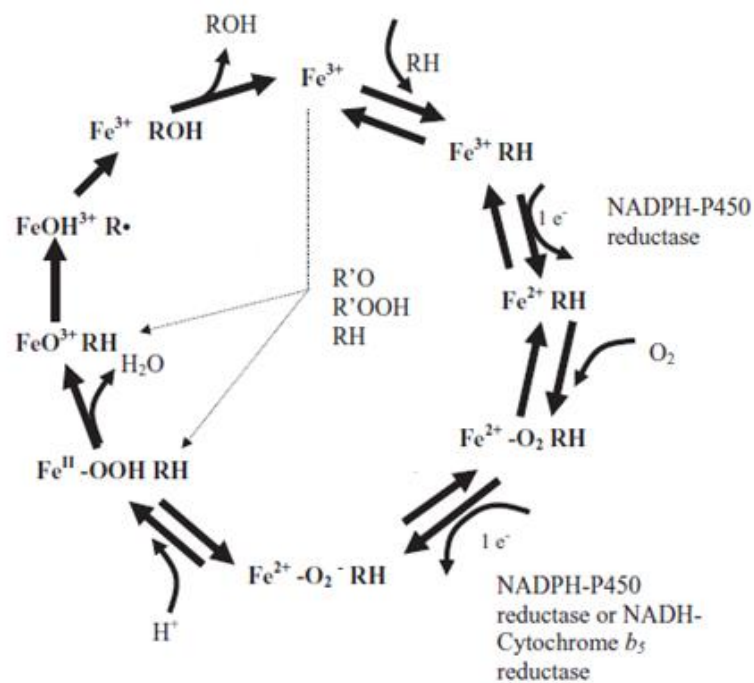
Figure 1. 7 Generalized cytochrome P450 catalytic cycle for the oxidation of substrates (Guengerich, 2001).

Binding of substrate (RH) to the ferric (Fe^{3+}) form of CYP enzyme initiates the cycle. After the initiation of the cycle, electron transfer from NAD(P)H is carried out and this transfer reduces the iron atom and changes it into ferrous state (Fe^{2+}). Then, O_2 binds to ferrous form of CYP enzyme producing an unstable structure, and it leads to generation of superoxide anion and ferric iron. After this step, NADPH-P450 reductase or NADH-cytochrome b_5 reductase inserts a further electron and a proton (H^+) into the cycle. H_2O is then released and high-valent FeO^{3+} complex formed after the breakage of Fe O-O bond. Direct interaction of FeO^{3+} with the substrate leaves an electron or hydrogen atom; therefore the product dissociates from the CYP enzyme and the cycle is completed (Zeldin, 2008).

1.4.4. Tissue distribution and function

Generally, CYP enzymes are found most abundantly in liver, where most of the drugs and endogenous substances are metabolized. Besides liver, several isoforms of CYP enzymes are found in extrahepatic tissues. CYP expression also takes place in kidney, heart, lung, brain, olfactory mucosa and gastrointestinal tract (Zeldin, 2008; Adali & Arinc, 1990; Arinc et al., 1995; Adali et al., 1996; Arinc et al., 2000).

CYP enzymes show differences in terms of function (Table 1.1). They can participate in xenobiotic metabolism as well as metabolism of endogeneous compounds.

Table 1. 1 Human cytochrome P450 genes (Nelson, 2009).

Human P450 families	Functional members	Main functions
CYP1 (3 subfamilies)	1A1, 1A2, 1B1	Drug/xenobiotic metabolism
CYP2 (13 subfamilies)	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18,2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1	Drug/xenobiotic metabolism and steroid metabolism
CYP3 (1 subfamily)	3A4, 3A5, 3A7, 3A43	Drug/xenobiotic metabolism
CYP4 (6 subfamilies)	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Arachadonic acid and fatty acid metabolism
CYP5 (1 subfamily)	5A1	Thromboxane A_2 synthesis
CYP7 (2 subfamilies)	7A1, 7B1	Rate-limiting step of bile acid biosynthesis (cholesterol elimination)
CYP8 (2 subfamilies)	8A1, 8B1	Prostacyclin and bile acid biosynthesis
CYP11 (2 subfamilies)	11A1, 11B1, 11B2	Key steps in steroid biosynthesis
CYP17 (1 subfamily)	17A1	Testosterone and oestrogen biosynthesis
CYP19 (1 subfamily)	19A1	Oestrogen biosynthesis (aromatase)
CYP20 (1 subfamily)	20A1	Unknown
CYP21 (1 subfamily)	21A2	Steroid biosynthesis
CYP24 (1 subfamily)	24A1	Vitamin D metabolism/inactivation
CYP26 (3 subfamilies)	26A1, 26B1, 26C1	Retinoic acid metabolism/inactivation
CYP27 (3 subfamilies)	27A1, 27B1, 27C1	Bile acid biosynthesis, vitamin D activation
CYP39 (1 subfamily)	39A1	Cholesterol metabolism
CYP46 (1 subfamily)	46A1	Cholesterol metabolism
CYP51 (1 subfamily)	51A1	Cholesterol biosynthesis

Besides these functional human P450 genes, there are 58 pseudogenes being determined (Nelson, 2009).

There are two types of cytochrome P450 enzymes which are type I and type II forms. Type I form of CYP enzymes are the mitochondrial enzymes while type II forms are found in the endoplasmic reticulum. Most of the vitamin D_3 metabolizing enzymes are involved in type I forms CYP enzymes. They function as oxidases and use electrons coming from molecular oxygen and NADPH (Black & Coon, 1987; Nebert, 1987).

Among these CYP enzymes, CYP24A1 and CYP27B1 enzymes are two most important enzymes involved in Vitamin D_3 metabolism.

1.4.5. Cytochrome P45024A1 (CYP24A1)

CYP24A1 (EC.1.14.13.126) is the member of cytochrome P450 enzymes which is also known as 25-hydroxyvitamin D_3-24-hydroxylase. It is a mitochondrial inner membrane enzyme with 65 kDa molecular weight and composed of 514 amino acids. The crystal structure of CYP24A1 is given in Figure 1.8. It catalyzes the conversion of 25-hydroxyvitamin D_3 (25-OH-D_3) and 1,25-dihydroxyvitamin D_3 (1,25-(OH)$_2$D$_3$) into the corresponding 24-hydroxylated products. Therefore, it has important role in the degradation of vitamin D_3 molecules.

For the degradation process, CYP24A1 catalyzes the 24-hydroxylation reaction of both 25-OH-D_3 and 1,25-(OH)$_2$D$_3$. *Km* value for 25-OH-D_3 is ten times higher than *Km* value for 1,25-(OH)$_2$D$_3$, meaning that 1,25-(OH)$_2$D$_3$ is more preferable substrate of CYP24A1. CYP24A1 is mainly responsible for the 24-hydroxylation of 1,25-(OH)$_2$D$_3$, which results in the production of calcitroic acid, a biliary catabolite (Makin *et al.*, 1989; Reddy & Tserng, 1989). Also, CYP24A1 catalyzes the 23-hydroxylation reaction with the end-product, 1,25-(OH)$_2$D$_3$-26,23-lactone (Sakaki *et al.*, 2000; Yamada *et al.*, 1984).

CYP24A1 gene expression is strongly stimulated by 1,25-$(OH)_2D_3$ while PTH involves in the suppression of 24-hydroxylase activity in the kidney.
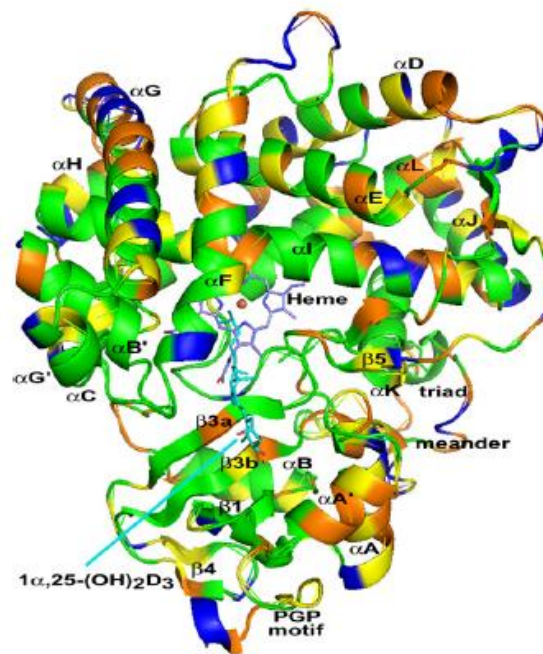
Figure 1. 8 Crystal Structure of rat CYP24A1 with 1,25-$(OH)_2D_3$ (Annalora *et al.*, 2010).

CYP24A1 gene is not only expressed in kidney, but also expressed in many vitamin D target tissues which contain vitamin D receptor (VDR) such as bone, intestine and liver (Jones *et al.*, 1998). According to promoter analysis of *CYP24A1* gene in human and rats, two vitamin D responsive elements (VDREs) are required for the induction by 1,25-$(OH)_2D_3$ via vitamin D receptor (VDR). One of them is present around -150 bp (proximal) and the other is localized to -250 bp (distal) (Hahn *et al.*, 1994; Kerry *et al.*, 1996; Ohyama *et al.*, 1994, 1996; Zierold *et al.*, 1994, 1995).

Besides the catabolism of vitamin D_3 compounds, CYP24A1 also functions in removal of several vitamin D analogs such as the ones used in treatment of chronic kidney diseases in the world: 22-oxa-1,25-$(OH)_2D_2$ (maxacalcitol), 1α-OH-D_2 (doxercalciferol) and 19-nor-1,25-$(OH)_2D_2$ (paricalcitol) (Masuda *et al.*, 1994; Masuda *et al.*, 2006; Shankar *et al.*, 2001). All these analogs undergo C-23 or/and C-24 metabolism by CYP24A1.

Since the increase in CYP24A1 expression may cause the vitamin D deficiency, it was suggested that CYP24A1 may be used as a potential therapeutic target and the inhibitors of CYP24A1 enzyme can be used in the treatment of enhanced vitamin D catabolism-related diseases. These CYP24A1 inhibitors include;

- Genistein, which is a natural product found in soya products (Cross *et al.*, 2004; Farhan & Cross, 2002)
- General azole-based inhibitors, which functions by binding to the heme region at the catalytic core of CYP24A1 enzyme (Ly *et al.*, 1999; Muindi *et al.*, 2010)
- A family of imidazole derivatives, which involves in increasing the selectivity of CYP24A1 and vitamin D signaling (Schuster *et al.*, 2001)
- Highly-CYP24A1 specific inhibitors, which are potent CYP24A1 inhibitor and highly-selective (Posner *et al.*, 2010)

1.4.6. Cytochrome P45027B1 (CYP27B1)

CYP27B1 (EC.1.14.13.13) is the member of cytochrome P450 enzymes and it involves in the XXVIIB subfamily of the cytochrome P450 family. It is also known 1α-hydroxylase. CYP27B1 is a mitochondrial inner membrane enzyme with 55 kDa molecular weight and composed of 508 amino acids. The three dimensional structure model is given in Figure 1.9. It catalyzes 1α-hydroxylation of 25-OH-D_3 to form 1,25-$(OH)_2D_3$ which is the active form of vitamin D_3.
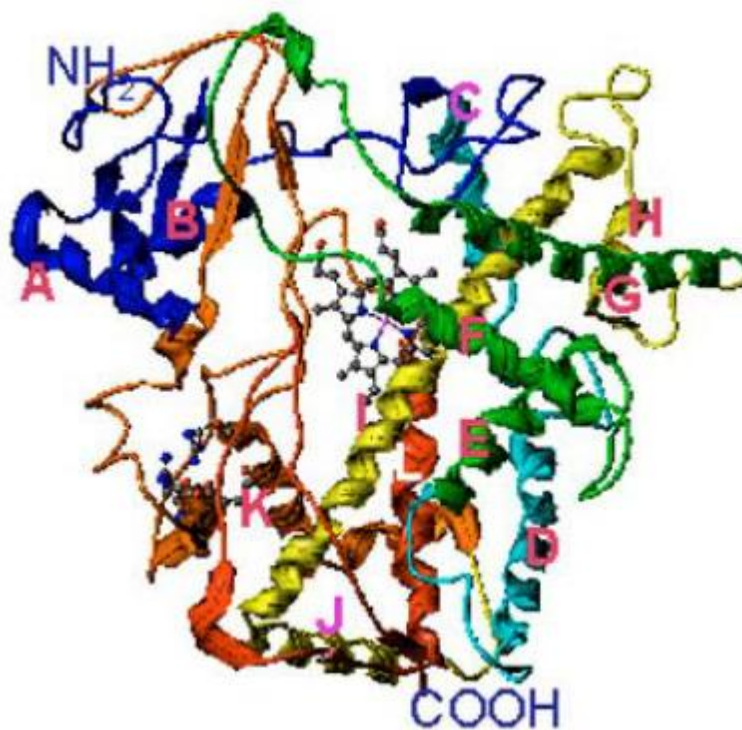
Figure 1. 9 Three-dimensional structure model of CYP27B1. The structure was constructed by homology modeling technique (Sakaki *et al.*, 2005).

CYP27B1 gene expression is tightly regulated by $1,25\text{-}(OH)_2D_3$, PTH and calcitonin (Sutton & MacDonald, 2003). Calcitonin and PTH have role in the upregulation of CYP27B1 gene expression (Shinki *et al.*, 1999). On the other hand, $1,25\text{-}(OH)_2D_3$ which is the end-product of the reaction catalyzed by CYP27B1 is the down-regulator of gene expression of CYP27B1 at the transcriptional level (Murayama *et al.*, 1998).

Vitamin D-dependent ricket type I which is also known as pseudo-vitamin D deficiency rickets is a kind of autosomal recessive inherited disorder. Secondary hyperparathyroidism, low serum level of calcium and low levels of circulating $1,25\text{-}(OH)_2D_3$ are the characteristics of this disorder. Genome analysis of the patients showed some missense mutations, insertions and deletions in *CYP27B1* gene (Fu *et al.*, 1997; Kitanaka *et al.*, 2002; Wang *et al.*, 1998; Yoshida *et al.*, 1998). Besides the genome analysis, *Cyp27b1* knockout mouse studies revealed compatible results with vitamin D-dependent ricket type I and $1,25\text{-}(OH)_2D_3$ was not detected in circulation (Dardenne *et al.*, 2001; Panda *et al.*, 2001). These studies showed that CYP27B1 is the only enzyme for the 1α-hydroxylation of $25\text{-}OH\text{-}D_3$ to synthesize $1,25\text{-}(OH)_2D_3$.

1.5. Aim of the study

Plants have always played important roles in human life and they are also used in medicine throughout the history. They have been traditionally used for both treatment and prevention of diseases. Many of the modern medicines are produced indirectly from medicinal plants. *Epilobium hirsutum* L. (hairy willow herb) and *Viscum album* L. (European mistletoe) are the two medicinally important plants which are used in the treatment of several diseases and disorders. *Epilobium hirsutum* L. is used in treatment of inflammation, menstrual disorders, prevention of rectal bleeding and treatment of constipate and prostate. *Viscum album* L. has also been reputed against cardiovascular diseases (hypertension and atherosclerosis), various bone and joint disorders including periarthritis and arthritis. These plants contain many biologically active compounds such

acid phenolic acids and flavonoids, and these compounds provide some important biological activities including anti-inflammatory and antioxidant actions. Besides these properties, these plants may have effects on enzymes involved in physiological processes such as vitamin D_3 metabolizing enzymes, CYP24A1 and CYP27B1 which are responsible for the inactivation and activation of vitamin D3, respectively. Nevertheless, there is no data available in the literature for the possible effects of *Epilobium hirsutum* L. and *Viscum album* L. on vitamin D_3 metabolizing CYP24A1 and CYP27B1 enzymes. Therefore, in the present study, it was aimed to show the possible *in vivo* effects of the medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. on vitamin D_3 metabolizing CYP24A1 and CYP27B1 enzymes.

For this purpose, these medicinal plants were collected from unpolluted stream beds and their water extracts were obtained. Then, the extracts were injected to the albino wistar rats (*Rattus norvegicus*) intraperitoneally for nine consecutive days. After decapitation of the animals, their livers were removed and S1.5 fractions were prepared by homogenization and centrifugation. The rat liver S1.5 fractions were used for determination of CYP24A1 and CYP27B1 protein expressions by Western blot technique. Quantitative real-time PCR was carried out by using cDNA synthesized from total RNA of rat livers to determine the mRNA expressions.

So far, there has not been such a study about the effects of these plants on vitamin D3 metabolizing enzymes. This study is the first one concerning the effects of medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. on rat liver CYP24A1 and CYP27B1 protein and mRNA expression.

CHAPTER 2

MATERIALS & METHODS

2.1. Materials

Boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; A894987 605), folin-phenol reagent (1.09001.0500), magnesium chloride ($MgCl_2$; Art.5833), potassium chloride (KCl; 4935), sodium carbonate (Na_2CO_3; 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), zinc chloride ($ZnCl_2$; 108815) were purchased from E. Merck, Darmstadt, Germany.

Acrylamide (A-8887), ammonium per sulfate (APS; A-3678), bovine serum albumin (BSA; A788), bromophenol blue (B5525), diethanolamine (D-2286), diethylpyrocarbonate (DEPC; D5758), ficoll (F2637), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), methanol (34885), N'-N'-bis-methylene-acrylamide (M7256), N-N-dimethylformamide (D-8654), phenazine methosulfate (P9625), secondary antibody AP mouse (A3562), secondary antibody AP rabbit (A3687), sodium dodecyl sulfate (SDS; L4390), sodium-potassium (Na-K) tartarate (S-2377), tween 20 (P1379), xylene cyanol (X4126) were the products of Sigma, Aldrich, Germany.

5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821), dithiothreitol (DDT; R0861), gene rulerTM 50 bp DNA ladder (SM0371), light cycler-fast start DNA Master^{Plus} SYBR green I (K0252), Maloney murine leukemia virus reverse transcriptase (M-MuLu-RT;

K1622), pre-stained protein ladder (SM0671) were purchased from MBI Fermentas, USA.

Primary antibody 24A1 (sc-66851) and primary antibody 27B1 (sc-67260) were purchased from Santa Cruz Biotechnology, Inc. USA.

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

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

Ethanol (32221) was obtained from Riedel de-Haen Chemical Company, Germany. Isopropanol (AS040-L50) was the product of Atabay, İstanbul, Turkey. TRIzol® (12183-555) was purchased from Invitrogen.

2.2. Methods

2.2.1. Collection of the plants and extraction procedure

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. From among the three subspecies

growing in Turkey, *Viscum album* L. subsp. *austriacum* (Wiesb.) Vollmann was chosen for this study, because in Turkish Folk Medicine it is generally thought to be more effective than the rest (Personal field work observation).

50 g of crushed and powdered plant tissue for each taxon was subjected to active maceration in sterile ddH$_2$O by using Heidolph mechanic shaker at 300 rpm/minute at room temperature for 8 hours. Obtained extract was filtered from whatman filter paper and dried in the freeze drying machine (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 study was carried out with the collaboration of Ankara University, Faculty of Pharmacy.

2.2.2. Animal Studies

Male Wistar Albino rats (*Rattus norvegicus*) which were 12 weeks old and 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 of substances by i.p., collection of blood and tissues etc. were performed according to national standards under appropriate regimes with Veterinary services and in accordance with the Declaration of Helsinki (ethical committee reports are at Appendix A).

For the experiment, rats were randomly assorted into the following three groups:

C: Control group with water injection

EHT: *Epilobium hirsutum* L.-treated group

VAT: *Viscum album* L.- treated group

37.5 mg water extract of *Epilobium hirsutum* L. /kg body weight/day was intraperitoneally (i.p) injected to 25 rats for 9 consecutive days. For *Viscum album-* treated group, 10 mg water extract of *Viscum album* L./kg body weight/day was intraperitoneally (i.p) injected to 15 animals for 9 days. 11 rats were used as a control group and no treatment was done. 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 ^{0}C until used for preparation of S1.5 fractions. This part of the study was carried out with the collaboration of Pamukkale University, Biology Department.

2.2.3. Preparation of Rat Liver S1.5 Fraction

Rat liver S1.5 fraction was prepared by the method of Schenkman and Cinti (1978) as optimized by 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 containing 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 2 600 rpm. Ten passes were made for the homogenization of liver tissue. The resulting liver homogenate was centrifuged at 1500 x g for 10 minutes to obtain S1.5 fraction. The supernatant fraction (S1.5 fraction) was removed and stored at -80 ^{0}C until use in determination of protein expressions of the mitochondrial enzymes.

2.2.4. Determination of Protein Concentrations

Protein concentrations of S1.5 fractions were determined according to the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

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

Reagents:

Reagent I: 2% (w/v) $CuSO_4.5H_2O$

Reagent II: 2% (w/v) Na-K tartarate ($NaKC_4H_4O_6$)

Reagent A: 2% (w/v) Na_2CO_3 in 0.1 N NaOH

Lowry Alkaline C upper Reagent (ACR): Reagent I, Reagent II and Reagent A are mixed in the same order with the ratio of 1:1:100.

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

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

Protein Sample: Rat liver S1.5 fractions

Rat liver S1.5 fractions were initially diluted 200 times. After initial dilutions, dilutions within test tubes were performed by taking aliquots of 0.1 mL, 0.25 mL, and 0.5 mL from the initially diluted samples into the test tubes. Their volumes were then completed to a final volume of 0.5 mL with distilled water. 10 mL of 1mg/mL BSA stock solution was prepared by dissolving 10 mg BSA in 10 mL distilled water. Serial dilutions were done to prepare other concentrations of BSA. The BSA standards with 5 different

concentrations, distilled water as a blank, and samples were put into the tubes. 2.5 mL of Lowry ACR was added to each tube and mix. The tubes were incubated for 10 minutes at room temperature for copper reaction in alkaline medium. 0.25 mL of Folin reagent was added and the tubes were mixed during 8 seconds by vortex. Reaction mixture for each tube was indicated in Table 2.1. The tubes were further incubated for 30 minutes at room temperature. After the incubation, the absorbance of each tube was read at 660 nm by using spectrophotometer. A standard curve was drawn by using absorbance readings of standards and slope of this curve was used for the calculation of protein amounts in the samples.

Protein concentration will be calculated by the following formula:

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

Table 2. 1 The reaction mixture of tubes prepared for determination of protein concentrations.

Tube Number	Standards (mL)	dH$_2$O (mL)	Sample (mL)	Lowry ACR (mL)	Folin (mL)
Blank	---	0.5	---	2.5	0.25
Standard 1 (0.02 mg/mL)	0.5	---	---	2.5	0.25
Standard 2 (0.05 mg/mL)	0.5	---	---	2.5	0.25
Standard 3 (0.10 mg/mL)	0.5	---	---	2.5	0.25
Standard 4 (0.15 mg/mL)	0.5	---	---	2.5	0.25
Standard 5 (0.20 mg/mL)	0.5	---	---	2.5	0.25
Sample	---	0.4	0.1	2.5	0.25
Sample	---	0.25	0.25	2.5	0.25
Sample	---	---	0.5	2.5	0.25

2.2.5. Determination of protein expression

2.2.5.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE):

Western blot analysis described by Towbin *et al.* (1979) was used for the analysis of protein expression levels of control and plant treated rat liver enzymes. Before blotting, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4% stacking gel and 7.5% separating gel in a discontinuous buffer system as described by Laemmli (1970). Separating and stacking gel solutions were prepared according to Table 2.2 as indicated order.

Table 2. 2 Constituents of separating and stacking gel solutions.

Constituents	Separating Gel Solution	Stacking Gel Solution
Monomer Concentration	7,5 %	4%
Gel solution (mL)	7.5	1.3
dH$_2$O (mL)	14.53	6.1
Separating gel buffer (mL)	7.5	--------
Stacking gel buffer (mL)	--------	2.5
10 % SDS (mL)	0.3	0.1
10% ammonium per sulfate (mL)	0.15	0.05
TEMED (mL)	0.03	0.02
Total volume (mL)	**30**	**10**

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, mixed and filtered through filter paper. The volume was completed to 50 mL with distilled water.

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

18.15 g of Tris-base was dissolved with distilled water and titrated with 10 M HCl to pH 8.8. The volume was completed to 100 mL with distilled water. The pH of the solution was checked at the end whether it is stable at pH 8.8.

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

6 g of Tris-base was dissolved with distilled water and titrated with 10 M HCl to pH 6.8. The volume was completed to 100 mL with distilled water. The pH of the solution was checked at the end whether it is stable at pH 6.8.

Sodium Dodecyl Sulfate SDS (10%)

1 g of SDS was dissolved in distilled water. The volume was completed to 10 mL.

Ammonium per sulfate APS: (10%, Fresh)

20 g of APS per gel was dissolved in 200 µL of distilled water for each.

Tetramethylethylenediamine (TEMED) (Commercial)

Sample Dilution Buffer (SDB) (4x)

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

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

After dissolving of 15 g of Tris-Base in 350 mL distilled water, 72 g of Glycine was added. They were mixed and the volume was completed to 500 mL with distilled water.

It was prepared as 10x stock solution and it was diluted to 1x and 1 g of SDS was added per liter of 1x buffer before use.

Vertical slab gel electrophoresis was performed by using Scie-plasV10-CDC vertical electrophoresis unit (Southam, England). Sandwich unit of module was set up by using two glass plates. Before use, acetone was used for cleaning. Just after preparation of separating gel solution indicated in Table 1, 4250 µL of separating gel solution was poured into the sandwich unit, and 750 µL of alcohol was added at the top of separating gel. The alcohol provides smooth gel surface and fast polymerization since it prevents the contact of the gel with air. It also removes the air bubbles. After the polymerization of separating gel, the alcohol was removed and the stacking gel solution was poured and the comb was placed. The combs were removed without giving damage to the gel after the polymerization of stacking gel. The wells were filled out with 1x ERB and cleaned up by a syringe to remove air bubbles and remaining gel particles which were not polymerized properly.

To get the 2 mg/mL concentration, the proteins were diluted with distilled water according to the following formula:

$$V = \frac{[protein\ concentration]}{2.666} \times 20 - 20$$

V is the volume of distilled water to be added to dissolve 20 µL of sample.

After mixing 25 µL of 4x sample dilution buffer with 75 µL diluted sample, the samples were boiled in water bath for 1.5 minutes. Then, the samples were taken from water bath and each sample was loaded on different well by using Hamilton syringe. 3 µL of protein ladder was loaded as marker. The buffer tank was filled with electrode running buffer and gel-running module was placed in it. The tank was connected to the Bio-Rad power supply and electrophoresis was run at 150 V and 15 mA for 40 minutes for stacking and 300 V and 30 mA for 2 hours in separating of the proteins.

2.2.5.2.   Western blotting:

<u>Reagents:</u>

<u>Transfer Buffer</u> 25 mM Tris, 192 mM Glycine

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.

<u>Tris-Buffered Saline and Tween 20 (TBST):</u> 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.

<u>Blocking Solution:</u> 5% Non-Fat Dry Milk (Stock)

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

<u>Primary Antibody:</u> 1/1000 dilution in blocking solution

<u>Secondary Antibody</u>**:** 1/2000 dilution in blocking solution

<u>Substrate Solution:</u> (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.

To remove the buffer salts coming from electrophoretic running buffer and SDS as well as the preparation of the gels for transfer system, the gels were placed into transfer buffer for 10 minutes after SDS PAGE. After 10 minutes, the gels were removed from glasses. Nitrocellulose membrane, two Whatman papers, gel, and two fiber pads were placed in transfer sandwich as shown in Figure 2.1. A test tube was used to remove any air bubbles between the layers by gently rolling over the sandwich. The transfer sandwich was placed into Mini Trans-Blot module 165-8033 (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with transfer buffer. The transfer was carried out at 90 volt and 400 mA for 90 minutes. After transfer, the nitrocellulose membranes were washed with TBST for 10 minutes to get rid of the buffer salts. Then, the nitrocellulose membrane was incubated with blocking solution in room temperature for an hour.
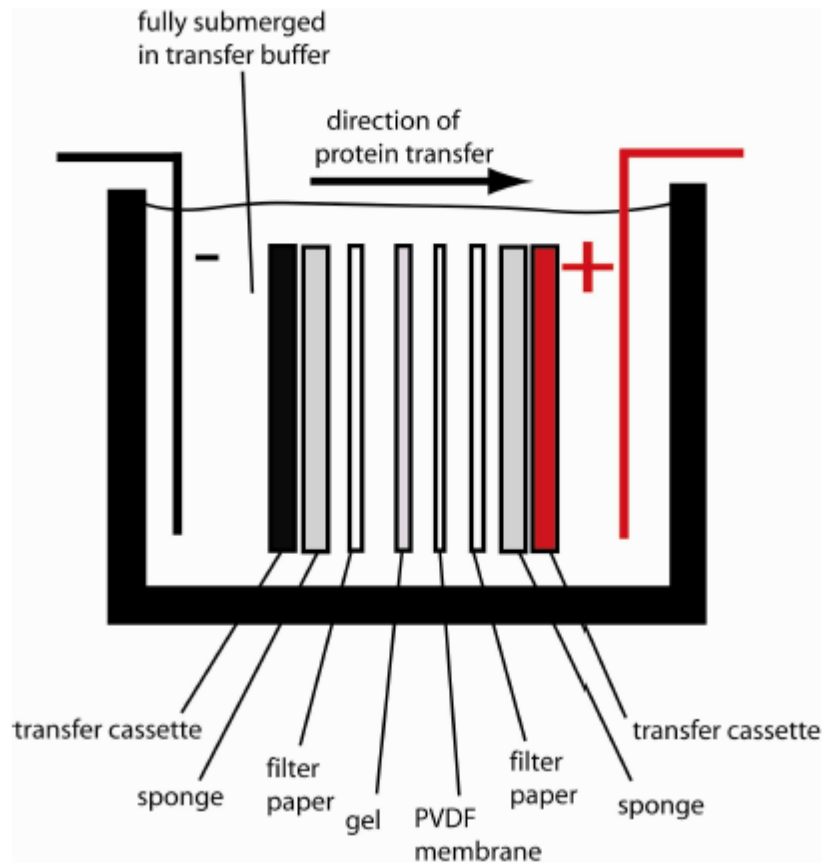
Figure 2. 1 Western Blot sandwich.

After that, immunochemical staining of the separated proteins on nitrocellulose membrane was done by incubating in primary antibody which is diluted 1000 times in blocking solution for 2 hours by shaking. The membranes were further incubated with TBST three times each of which is 10 minutes to remove excess, unbound primary antibody. After removal of excess primary antibody, the membranes were incubated

with secondary antibody which is 2000 times diluted alkaline phosphatase conjugated mouse anti-goat IgG for 1 hour. After 1 hour, the membrane was washed again with TBST. The final step was the preparation of the substrate solution. As soon as it was prepared as described by Ey and Ashman (1986) to visualize the specifically bound antibodies (Figure 2.2), the membrane was incubated with the substrate solution. The final images were scanned and ImageJ visualization software developed by NIH was used to quantitatively analyze the final images.
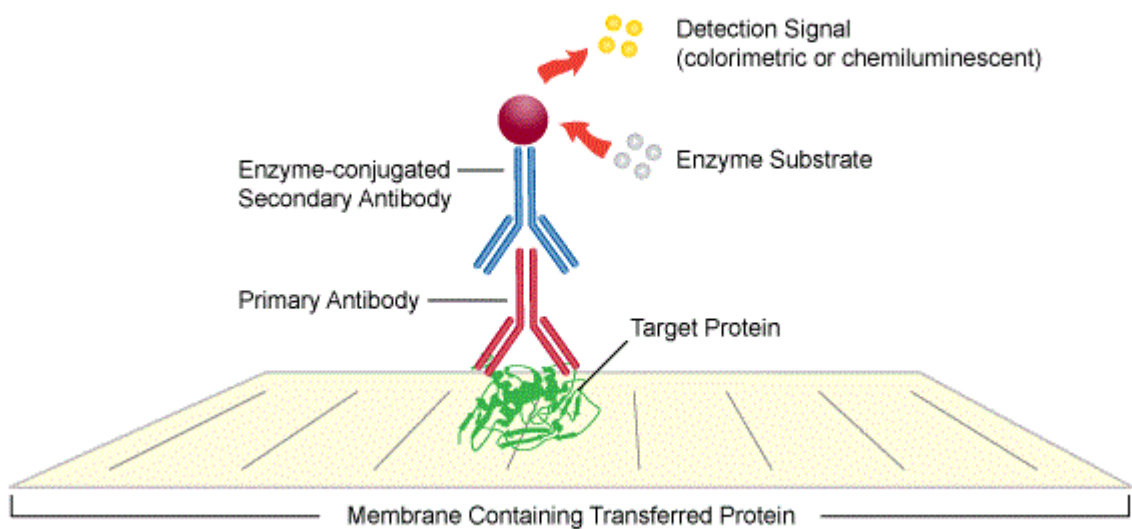
Figure 2. 2 Detection of immunoreactive bands in Western blots.

2.2.6. Determination of mRNA expression

2.2.6.1. Isolation of total RNA from rat liver tissues

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

At the beginning of the experiment, all equipments were treated with Diethylpyrocarbonate (DEPC) water in order to inhibit RNase activity. After the evaporation of excess DEPC, they were autoclaved.

50 mg of rat tissue was minced and homogenate with 500 µL of TRIzol® in a glass-Teflon homogenizer for 15 seconds 2-3 times. Extra 10 µL of TRIzol® was added for further 1 mg of tissue. The homogenate tissue was transferred into an eppendorf tube and put into liquid nitrogen to make it frozen. Before complete melting, 100 µL of chloroform was added and vortexed for one minute and incubated at room temperature for 5 minutes. Then, it was incubated in ice for 5 minutes. After the incubation periods, the homogenate was centrifuged at 13000 rpm for 15 minutes at 4 °C. The uppermost layer of the three phases formed after centrifugation was taken and same volume of

isopropanol was added. It was gently mixed. The mixture was incubated at room temperature for 10 minutes. It is centrifuged at 13000 rpm for 5 minutes at 4 $^{\circ}$C again, 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 $^{\circ}$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. It was kept at -80 °C before using.

2.2.6.2. Determination of RNA concentration

Reagents:

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

RNA molecules give maximum absorbance at 260 nm, so reading absorbance at 260 nm was used to calculate the concentration of nucleic acid in the sample. Also, the ratio between OD values at 260 nm and 280 nm (OD_{260}/ OD_{280}) was used to estimate the purity of RNA molecules. 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 OD_{260}/ OD_{280} must be between 1.8 and 2.2. The optical density of 1.0 corresponded to 40 µg/mL for RNA, so RNA concentration will be calculated according to the following formula:

RNA (µg/ml)=OD_{260} x DF x 40 µg RNA /ml (1 OD_{260} unit)

2.2.6.3. Qualification of RNA molecules by agarose gel electrophoresis

Reagents:

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

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

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 melting 1 g of agarose within 100 mL 0.5 X TBE buffer in microwave oven. The solution was cooled approximately 60 °C. 7 µL of ethidium bromide solution (10 mg/ml) was added and the solution was mixed well. Agarose gel solution was poured into electrophoresis tray and comb was placed to form wells. After the polymerization, gel tank was filled with 300 mL of 0.5 X TBE buffer and 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 observed and the photographed under UV light.

2.2.6.4. cDNA synthesis

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

Reverse transcription is the process by which cDNA is synthesized from RNA template. For cDNA synthesis, 2.5-5 µg of total RNA isolated from rat liver and 0.5 µg (1 µL

from 500 µg/mL stock) of oligo dT primers will be mixed in an eppendorf tube. Due to the variation of RNA concentrations, volume of RNA is variable, too. Therefore, final volume will be completed 12 µL with DEPC treated water. The solution was mixed gently and spinned down in microfuge. Mixture was incubated at 70 °C for 5 minutes, then it was chilled on ice and the drops were collected by microfuge. After that, 4 µL of 5X reaction buffer, 1 µL Ribolock and 2 µL of 10 mM dNTP were added successively. The tube was mixed gently and the drops again were collected by microfuge. It was incubated at 37 °C for 5 minutes. After incubation, 1 µL of M-MuLu-RT was added and incubated at 42 °C for 1 hour. Finally, the reaction was stopped by keeping at 70 °C for 10 min and chilled on ice. cDNA was stored at -20 °C for further use.

2.2.6.5.   Quantitative real-time PCR

Quantitative real-time PCR assays for rat mRNA expressions were carried out using Light Cycler 1.5. Reactions took place in reaction mixture by using Light Cycler-Fast Start DNA Master[Plus] SYBR Green I.

20 µL reaction mixture was composed of 7.5 µL of PCR-grade water, 0.5 µL of forward and 0.5 µL reverse primers (Table 2.3) whose sequences are indicated in table, and 12.5 µL of master mix containing Fast Start Taq DNA Polymerase, $MgCl_2$, SYBR Green I dye and dNTP mix. 5 µL of cDNA was added to a total reaction mixture of 20 µL. No template control which contains PCR-grade water instead of cDNA sample was used in every RT-PCR run to check whether there is contamination in the components of the reaction mixture.

The following Light Cycler run protocol was used:

- Preincubation program at 95 °C for 10 minutes in order to activate Taq polymerase and denature DNA
- Amplification and quantification program repeated 45 times

47

- 95 °C for 20 seconds

- 54-57 °C annealing (depending on the gene) for 30 seconds

- 72 °C extension 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

- Cooling step to 40 °C for 30 seconds.

Melting curve analysis of the amplification product was done at the end of each amplification reaction to confirm the detection of a single PCR product. Quantities of specific mRNAs in the sample were 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. 3 Primer sequences, annealing temperatures and product sizes.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Annealing Temperature (°C)	Product Size (bp)
CYP27B1	GAGATCACAGGCGCTGTGAAC	TCCAACATCAACACTTCTTTGATCA	55	107
CYP24A1*	TGGATGAGCTGTGCGATGA	TGCTTTCAAAGGACCACTTGTTC	59	75
CYP24A1*	TTGAAAGCATCTGCCTTGTGT	GTCACCATCATCTTCCCAAAC	57	130
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT	55	240

*Two different sets of primers were used for CYP24A1 gene.

2.2.7. Statistical Analysis

Statistical analyses were performed by using GraphPad Prism version 5 statistical software package for Windows. All results were expressed as means with their Standard Deviation (SD) or Standard Error Mean (SEM). Unpaired, two-tailed student's t-test and $p < 0.05$ were chosen as the level for significance.

CHAPTER 3

RESULTS

3.1. Protein concentrations of rat liver S1.5 fractions

The animals used in this study were divided into three groups: Control (C) group with no injection, *Epilobium hirsutum*-treated (EHT) and *Viscum album*-treated (VAT) groups. EHT and VAT groups were injected with 37.5 mg water extract of *Epilobium hirsutum* L./kg body weight/day and 10 mg water extract of *Viscum album* L. /kg body weight/day, respectively. Control, EHT and VAT groups were composed of 11, 25 and 15 animals, respectively. The S1.5 fractions were prepared by centrifugation after homogenization of the livers. Average protein concentrations of control and extract-treated rat liver S1.5 fractions were determined and they are listed in Table 3.1.

Table 3. 1 Average protein concentrations of rat liver S1.5 fractions.

Group	Average Protein Concentration (mg/mL)
Control **(N=11)**	36.15±5.56
Epilobium *hirsutum*-**treated** **(N=25)**	33.89±9.19
Viscum album-**treated** **(N=15)**	46.19±15.07

3.2. Effects of *Epilobium hirsutum* L. on CYP24A1 and CYP27B1 protein expression

Rat liver mitochondrial CYP24A1 and CYP27B1 protein expressions of control and *Epilobium hirsutum*-treated groups were determined by using Western blotting technique following the separation of proteins in S1.5 fractions by SDS-PAGE. Immunochemical detection of liver mitochondrial CYP24A1 and CYP27B1 proteins were performed by incubating the nitrocellulose membrane in diluted rabbit polyclonal primary antibodies and then secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG.

3.2.1. Effect of *Epilobium hirsutum* L. on CYP24A1 protein expression

Monomer molecular weight of CYP24A1 is known as 65 kDa and Figure 3.1 shows the exact position of CYP24A1 on nitrocellulose membrane. β-actin with molecular weight of 42 kDa is used as an internal standard and protein loading control. The intensity of immunoreactive protein bands of both control and EHT animals were analyzed by using ImageJ visualization software. Figure 3.2 shows the relative protein expressions where mean of protein expressions of control group was taken as 100 ± 8.37 (N=11) and then mean protein expression of *Epilobium-hirsutum*-treated group was calculated as 69 ± 10.29 (N=25). As illustrated in Figure 3.2, rat liver mitochondrial CYP24A1 protein expression decreased in EHT group compared to control group (unpaired, two-tailed student's *t*-test, p<0.0001). The mean CYP24A1 protein expression of EHT group was found as 69% of the mean of control group.
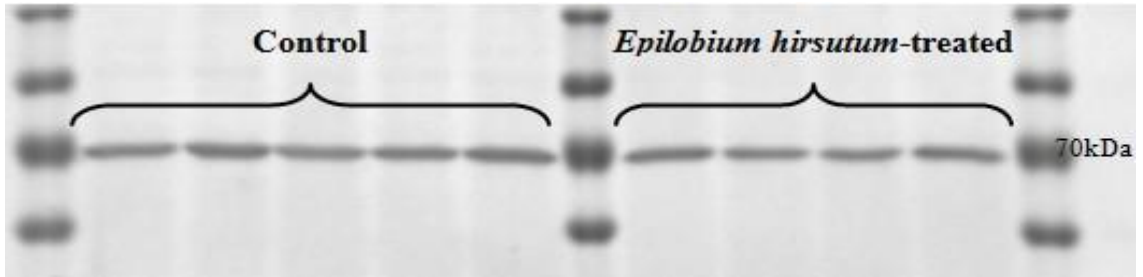
A. CYP24A1

B. β-actin

Figure 3. 1 (A) Immunoreactive protein bands of control (N=11) and *Epilobium hirsutum* L.-treated (N=25) groups representing CYP24A1 (MW of 65 kDa). **(B)** β-actin (MW of 42 kDa) was used as internal control for each membrane.
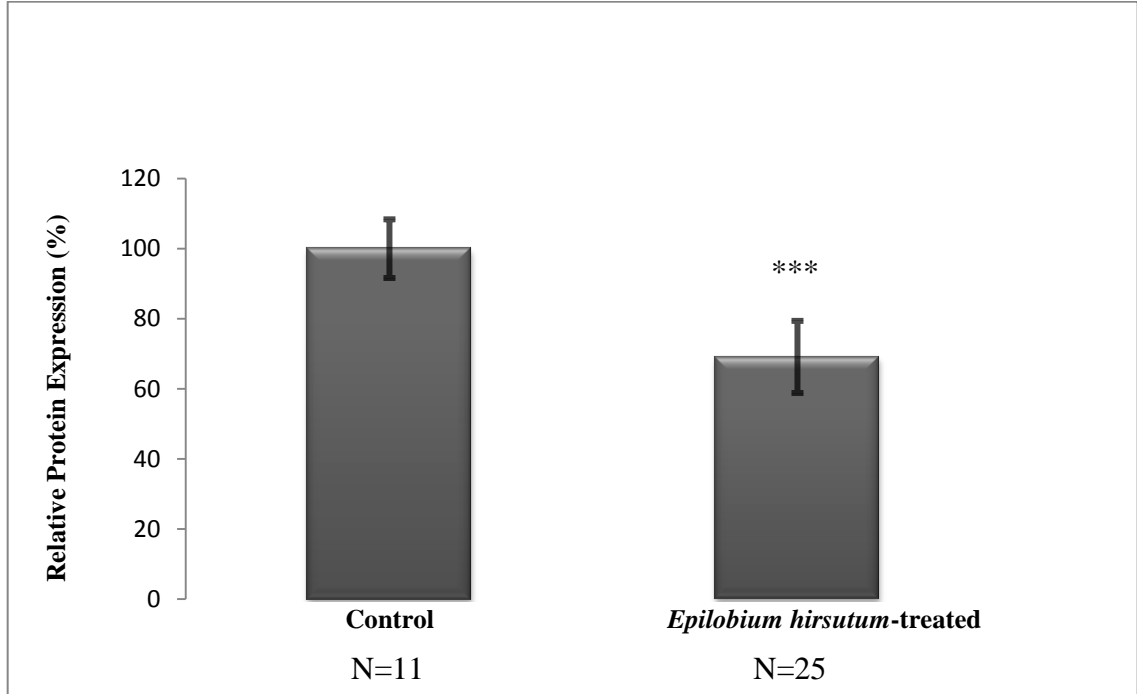
Figure 3. 2 *In-vivo* effect of *Epilobium hirsutum* L. on CYP24A1 protein expression. The band quantifications are expressed as mean ±SD of the relative intensity. The mean relative protein expression of *Epilobium hirsutum* L.-treated group is 69% of the mean of control group. The *p* value obtained from unpaired, two-tailed student's *t*-test is less than 0.0001.
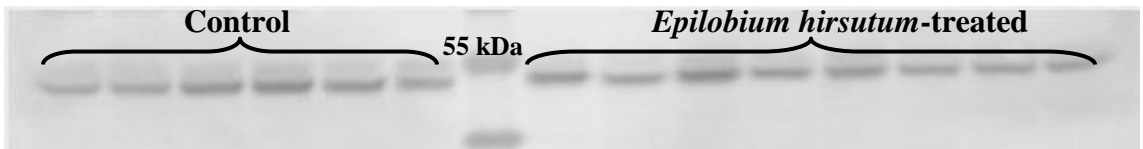
3.2.2. Effect of *Epilobium hirsutum* L. on CYP27B1 protein expression

Monomer molecular weight of CYP27B1 is known as 54 kDa and Figure 3.3 shows the exact position of CYP27B1 on nitrocellulose membrane. β-actin is used as internal standard (Molecular weight of β-actin is 42 kDa). The intensity of immunoreactive

protein bands of control and EHT animals were analyzed by using ImageJ visualization software. Figure 3.4 shows the relative protein expressions where mean of protein expressions of control group was taken as 100 ± 19.32 (N=11) and then mean protein expression of *Epilobium-hirsutum*-treated group was calculated as 82 ± 12.78 (N=25). As illustrated in Figure 3.4, rat liver mitochondrial CYP27B1 protein expression decreased in EHT group compared to control group (unpaired, two-tailed student's *t*-test, $p<0.05$). The mean CYP27B1 protein expression of EHT group was found as 82% of the mean of control group.
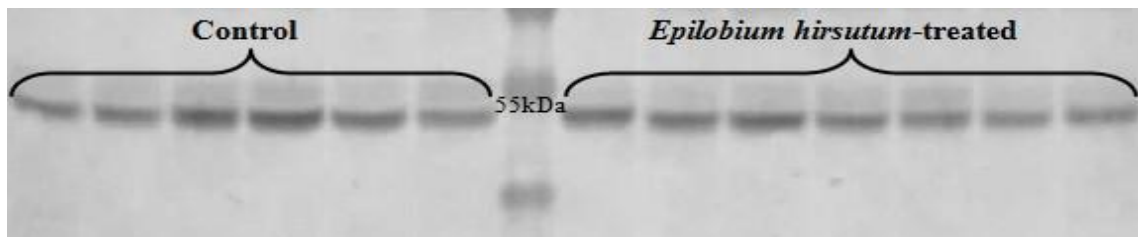
A. CYP27B1

B. β-actin
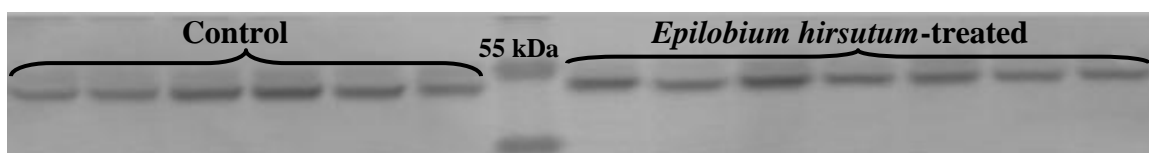
Figure 3. 3 (A) Immunoreactive protein bands of control (N=11) and *Epilobium hirsutum* L.-treated (N=25) groups representing CYP27B1 (MW of 54 kDa). **(B)** β-actin (MW of 42 kDa) was used as internal control for each membrane.
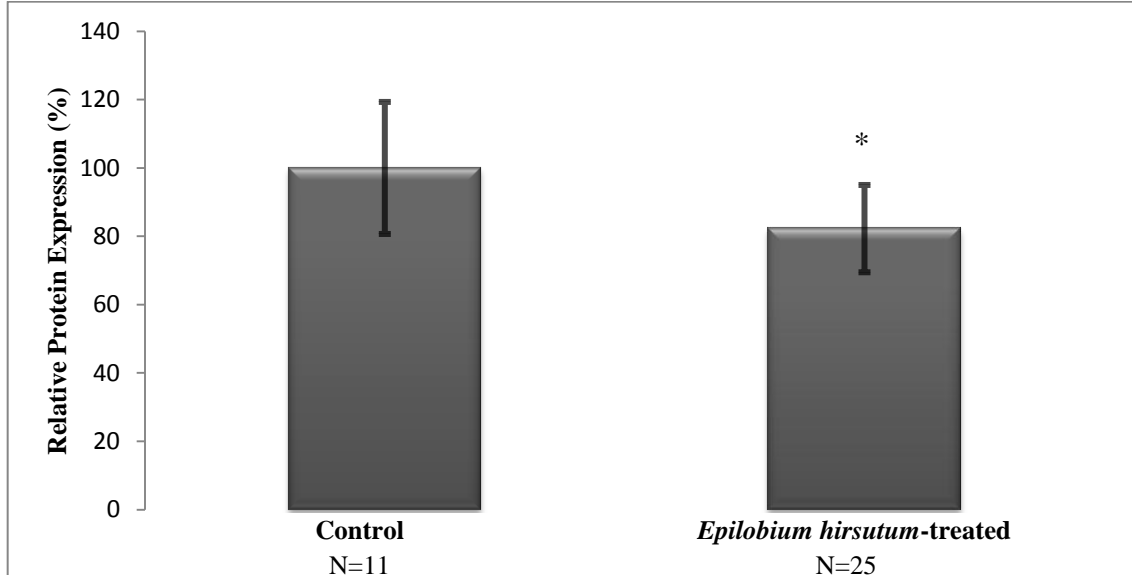
Figure 3. 4 *In-vivo* effect of *Epilobium hirsutum* L. on CYP27B1 protein expression. The band quantifications are expressed as mean ±SD of the relative intensity. The mean relative protein expression of *Epilobium hirsutum* L.-treated group is 82% of the mean of control group. The *p* value obtained from unpaired, two-tailed student's *t*-test is less than 0.028.

3.3. Effect of *Epilobium hirsutum* L. on CYP24A1 and CYP27B1 mRNA expression

3.3.1. Quality control of RNA molecules by agarose gel electrophoresis

Total RNA was isolated from rat liver tissue as described in Materials & Methods part. Due to the risk of DNA contamination, RNA isolation was one of the most critical parts

of the experiments. Qualities of RNA molecules were checked with OD_{260}/OD_{280} ratio which must be between 1.8 and 2.2. OD_{260}/OD_{280} ratio of the samples was good enough to be used in cDNA synthesis and real-time PCR studies. After that, 28S and 18S RNA bands were checked with agarose gel electrophoresis. RNA molecules having OD_{260}/OD_{280} between 1.8 and 2.2 showed intact and well-separated 28S and 18S RNA bands after agarose gel electrophoresis as shown in Figure 3.5.

Figure 3. 5 Agarose gel electrophoresis pattern of RNA samples isolated from rat liver tissues.

3.3.2. Effect of *Epilobium hirsutum* L. on CYP24A1 mRNA expression

qRT-PCR technique was used to determine the mRNA expression of CY24A1. For the beginning, serial optimization was done with samples from the control group. For the optimization part, different primer pairs and cDNA concentrations were used. For the annealing, the temperatures between 45 ^{0}C and 60 ^{0}C were tested. Also, qRT-PCR of GAPDH used as internal control (housekeeping gene) was done to eliminate the possible risks of contamination which may come from the cDNA samples, SYBR-green and water. To eliminate the contamination risk of primers, no template control which included water instead of cDNA samples was used. These optimization steps were done at different annealing temperatures with different cycle points. Also, two different primer sets specific for *CYP24A1* gene were tested. However, no consistent results were obtained probably due to the undetectable properties of CYP24A1 mRNA in the liver. Two of the melting curves generated from the optimization experiments are shown in Figures 3.6 and 3.7. In Figure 3.6, 55 ^{0}C was used as annealing temperature, but it caused the formation of primer dimer. To get rid of primer dimer, annealing temperature was increased, but still no meaningful results were obtained as represented in Figure 3.7.
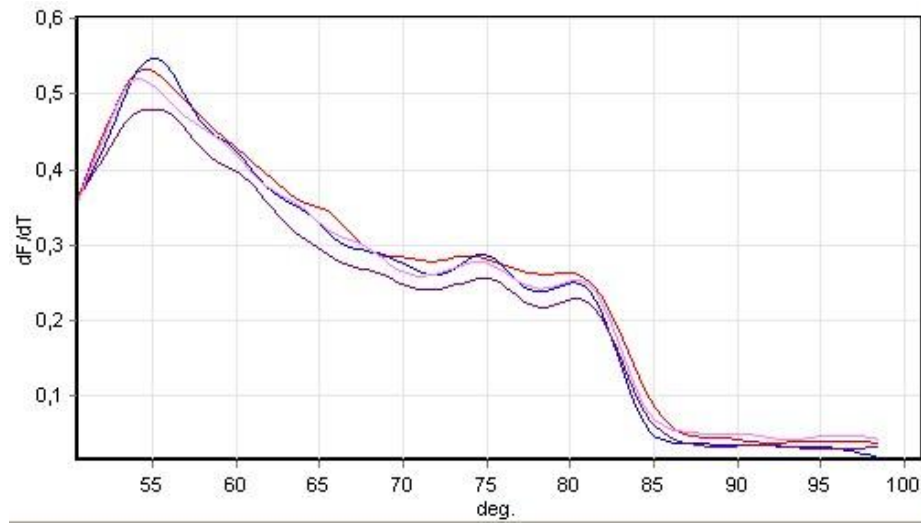
Figure 3. 6 Melting curve showing the fluorescence of SYBR green dye I versus temperature. 55 ^{0}C was used as annealing temperature.
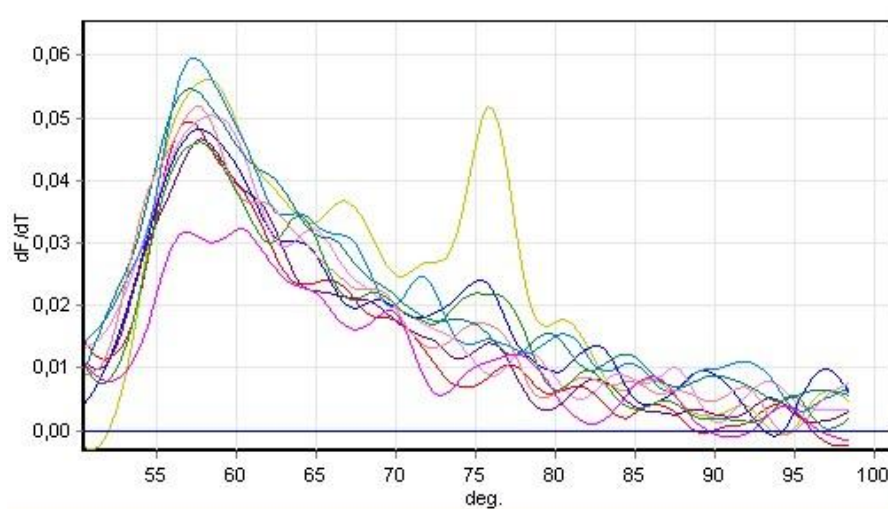
Figure 3. 7 Melting curve showing the fluorescence of SYBR green dye I versus temperature. 60 ^{0}C was used as annealing temperature.

3.3.3. Effect of *Epilobium hirsutum* L. on CYP27B1 mRNA expression

qRT-PCR technique was used to determine the mRNA expression of CYP27B1. GAPDH was used as internal control to calculate the relative mRNA expression of *Epilobium hirsutum*-treated CYP27B1. The specific annealing temperatures of the primers of CYP27B1 and GAPDH were used in qRT-PCR.

mRNA expressions of CYP27B1 were determined by using qRT-PCR and relative expressions of CYP27B1 of control and treated animals were calculated using GAPDH as internal control (housekeeping gene). Figure 3.8 shows the standard curve which was generated from 1:10, 1:100, 1:500, 1:1000, and 1:5000 diluted cDNAs used for quantifications of the samples. Figure 3.9 illustrates the amplification plot showing the changes in fluorescence of SYBR green dye I versus cycle number of rat liver CYP27B1 gene of control and *Epilobium hirsutum*-treated samples. In Figure 3.10, melting curve with one peak is represented for the detection of single PCR product.
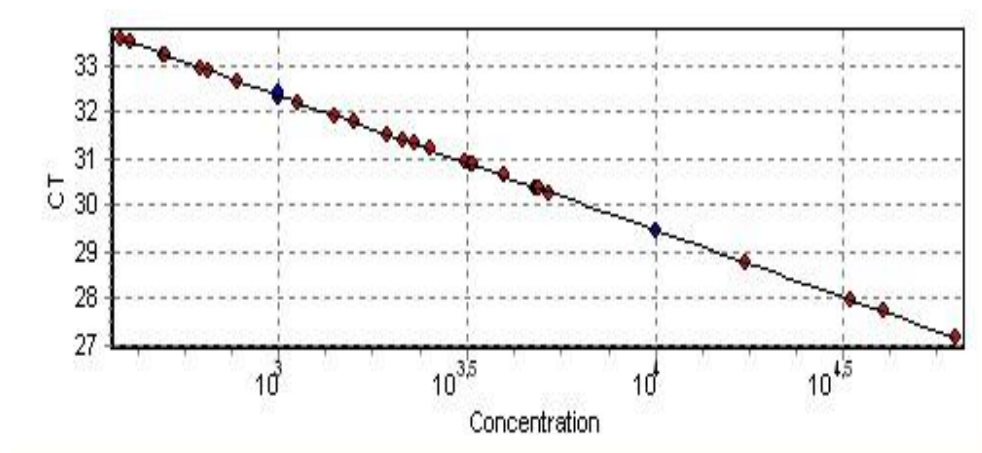
Figure 3. 8 Standard curve derived from dilution series of a control cDNA to measure quantities of specific CYP27B1 mRNAs in the sample.
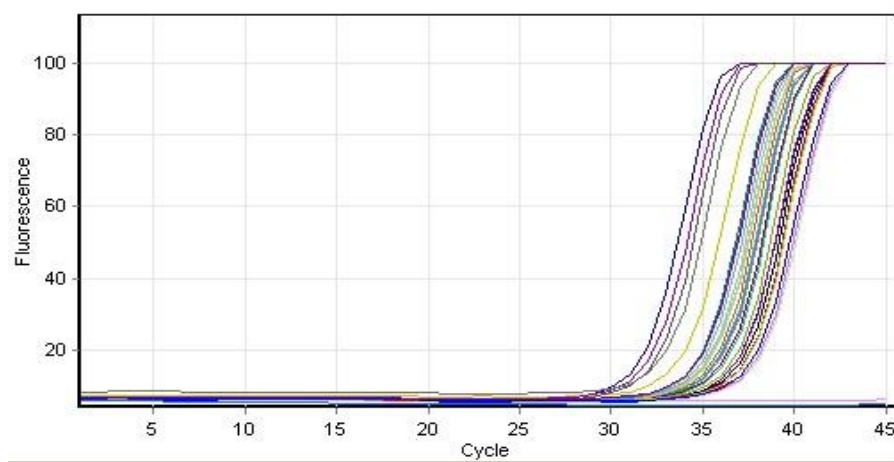
Figure 3. 9 Amplification plot showing the changes in fluorescence of SYBR green dye I versus cycle number of rat liver CYP27B1 gene of control and *Epilobium hirsutum*-treated samples.
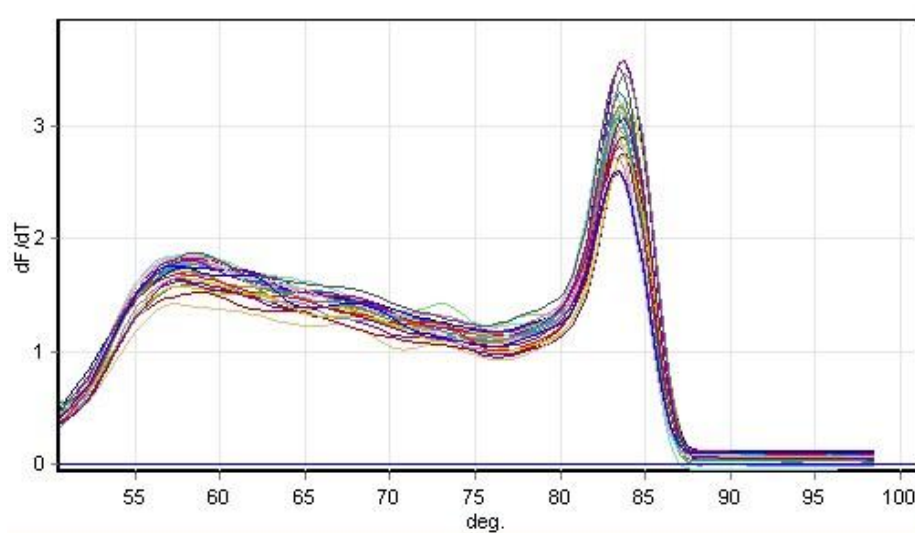
Figure 3. 10 Melting curve showing the fluorescence of SYBR green dye I versus temperature. One peak in the melting curve confirms the detection of single PCR product.

In Figures 3.11 and 3.12, the PCR products of CYP27B1 and GAPDH are represented, respectively. The band patterns in gels confirm the exact position of CYP27B1 and GAPDH products produced in qRT-PCR which are 107 and 240 bp, respectively. 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 CYP27B1 mRNA expression change when animals injected by plant extract. Table 3.2 gives the Livak ($2^{-\Delta\Delta ct}$) method for calculation of relative mRNA expression using Ct values.
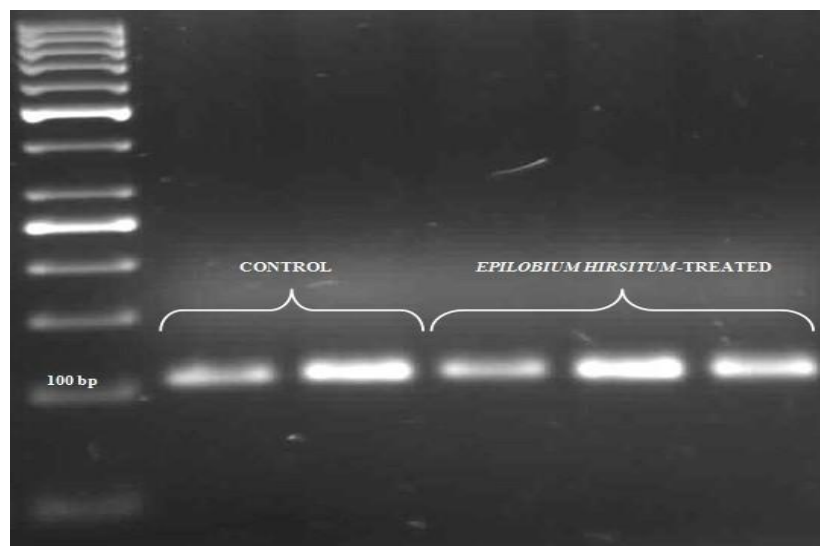
Figure 3. 11 qRT-PCR product of liver CYP27B1 cDNA (107 bp) of control and *Epilobium hirsutum*-treated animals. Each lane contains 10 µL of qRT-PCR Product.
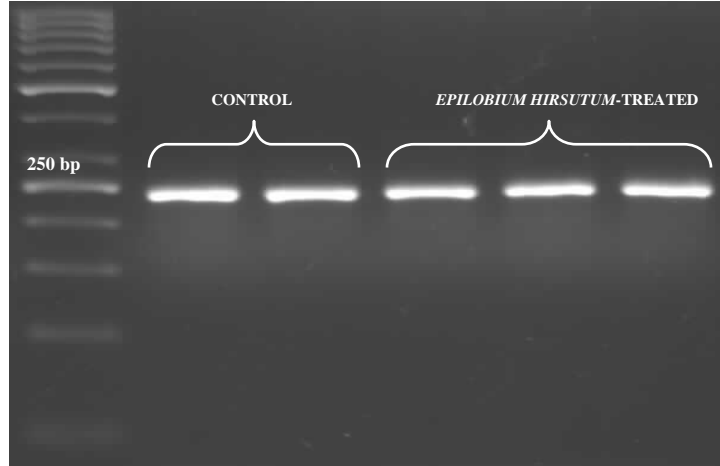
Figure 3. 12 qRT-PCR product of liver GAPDH cDNA (240 bp) of control and *Epilobium hirsutum*-treated animals. Each lane contains 10 µL of qRT-PCR Product.

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

	Control	EHT			
Ct$_{CYP27B1}$	32.03	31.31			
Ct$_{GAPDH}$	18.25	18.97			
ΔCt$_{EHT}$ =	**Ct$_{GAPDH}$**	**-**	**Ct$_{CYP27B1}$**		
ΔCt$_{reference}$ =	**Ct$_{GAPDH}$**	**-**	**Ct$_{CYP27B1}$**		
ΔCt$_{EHT}$ =	18.97	-	31.31	=	-12.34
ΔCt$_{reference}$ =	18.25	-	32.03	=	-13.78
	ΔΔCt = ΔCt$_{reference}$ - ΔCt$_{EHT}$			**=**	**-1.44**
$2^{-\Delta\Delta ct}$	**=**	**2.7**			

The normalized average liver CYP27B1 mRNA level of control animals was found to be 1.45 ± 0.27 (mean ± SEM, N=11). The average CYP27B1 mRNA amount was calculated as 3.91 ± 0.49 (mean ± SEM, N=25) for liver tissue of *Epilobium hirsutum*-treated animals. As shown in Figure 3.13, *Epilobium hirsutum* treatment of animals caused 2.7 fold increase in mRNA expression with respect to control group ($p < 0.005$, unpaired, two-tailed student's *t*-test).

	control N=11	treated N=25
Relative CYP27B1/GAPDH	1.00	2.70
Standard Error Mean	0.19	0.34
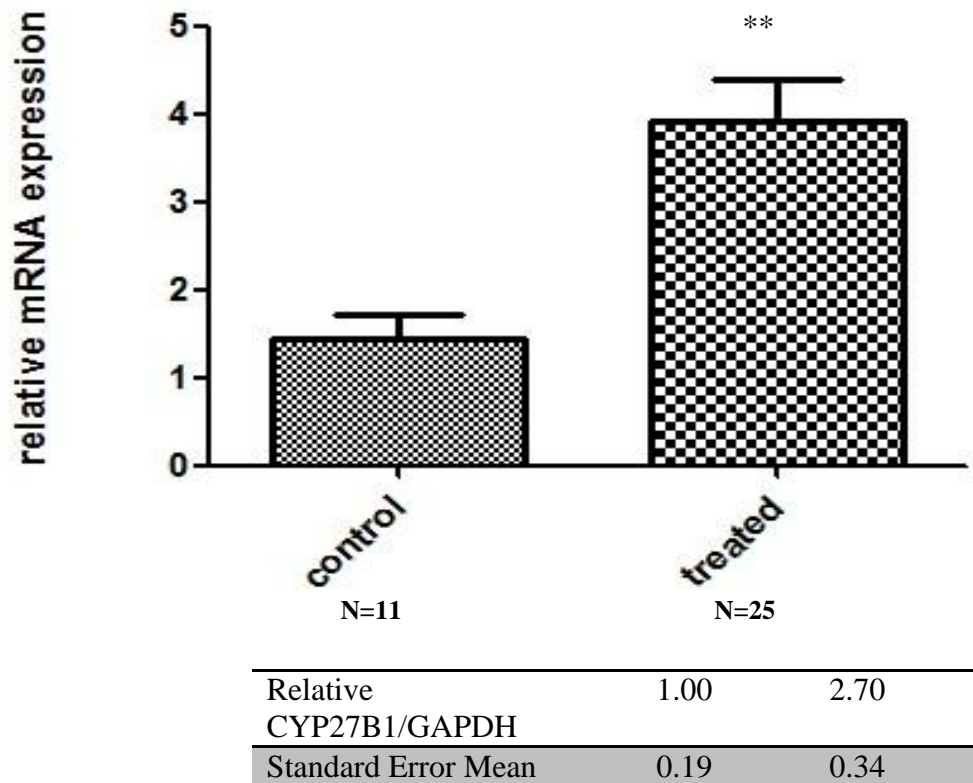
Figure 3. 13 *In vivo* effect of *Epilobium hirsutum* L. on rat liver CYP27B1 mRNA expression. The quantities are expressed as mean ±SEM of the relative expression. Relative mRNA expression of *Epilobium hirsutum* L-treated group is 2.7 fold higher than that of control group. The *p* value obtained from unpaired, two-tailed student's *t*-test was 0.0017.

65

3.4.Effect of *Viscum album* L. on CYP24A1 and CYP27B1 protein expression

Rat liver mitochondrial CYP24A1 and CYP27B1 protein expressions of control and *Viscum album*-treated groups were determined by using Western blotting technique following the separation of proteins in S1.5 fractions by SDS-PAGE. Immunochemical detection of liver mitochondrial CYP24A1 and CYP27B1 proteins were performed by incubating the nitrocellulose membrane in diluted rabbit polyclonal primary antibodies and then secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG.

3.4.1. Effect of *Viscum album* L. on CYP24A1 protein expression

Monomer molecular weight of CYP24A1 is known as 65 kDa and Figure 3.14 shows the exact position of CYP24A1 on nitrocellulose membrane. β-actin with a molecular weight of 42 kDa is used as an internal standard and protein loading control. The intensity of immunoreactive protein bands of both control and EHT animals were analyzed by using ImageJ visualization software. Figure 3.15 shows the relative protein expressions where mean of protein expressions of control group was found as 100 ± 16.93 (N=11) and then mean protein expression of *Viscum album*-treated group was calculated as 83 ± 13.49 (N=15). As illustrated in Figure 3.6, rat liver mitochondrial CYP24A1 protein expression decreased in EHT group compared to control group (unpaired, two-tailed student's *t*-test, $p<0.05$). The mean CYP24A1 protein expression of EHT group was found as 83% of the mean of control group.

A. CYP24A1

B. β-actin
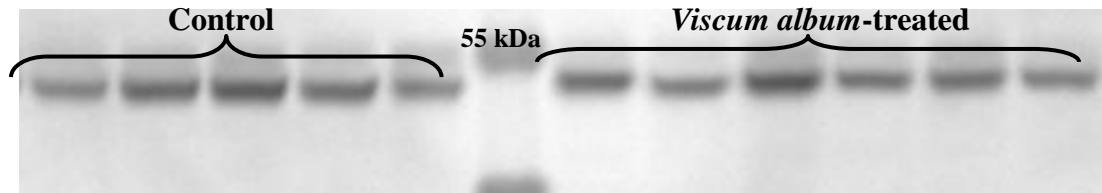
Figure 3. 14 (A) Immunoreactive protein bands of control (N=11) and *Viscum album* L.-treated (N=15) groups representing CYP24A1 (MW of 65 kDa). **(B)** β-actin (MW of 42 kDa) was used as internal control for each membrane.
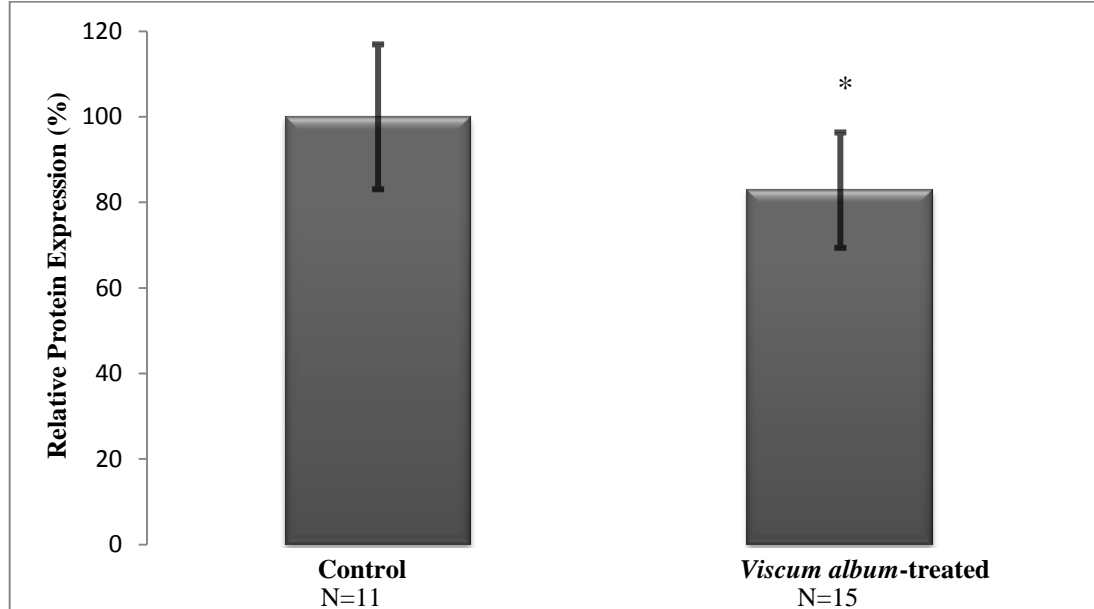
Figure 3. 15 *In-vivo* effect of *Viscum album* L. on CYP24A1 protein expression. The band quantifications are expressed as mean ±SD of the relative intensity. The mean relative protein expression of *Viscum album* L.-treated group is 83% of the mean of control group. The *p* value obtained from unpaired, two-tailed student's *t*-test is less than 0.014.

3.4.2. Effect of *Viscum album* L. on CYP27B1 protein expression

Monomer molecular weight of CYP27B1 is known as 54 kDa and Figure 3.16 shows the exact position of CYP27B1 on nitrocellulose membrane. β-actin is used as internal standard (Molecular weight of β-actin is 42 kDa). The intensity of immunoreactive protein bands of control and EHT animals were analyzed by using ImageJ visualization software. Figure 3.17 shows the relative protein expressions where mean of protein expressions of control group was found as 100±15.19 (N=11) and then mean protein expression of *Viscum album*-treated group was calculated as 82±15.57 (N=15). As

illustrated in Figure 3.8, rat liver mitochondrial CYP27B1 protein expression decreased in EHT group compared to control group (unpaired, two-tailed student's *t*-test, p<0.05). The mean CYP27B1 protein expression of EHT group was found as 82% of the mean of control group.
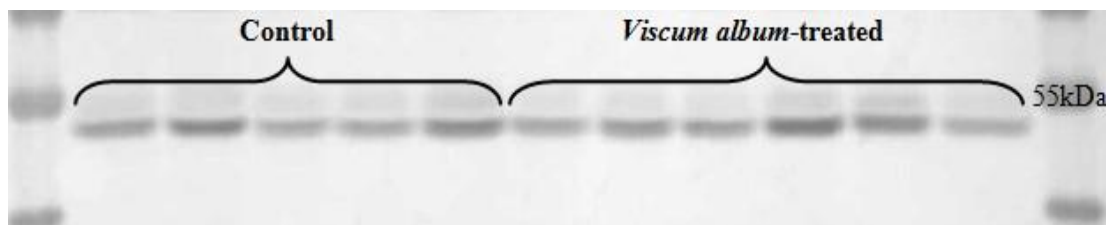
A. CYP27B1

B. β-actin
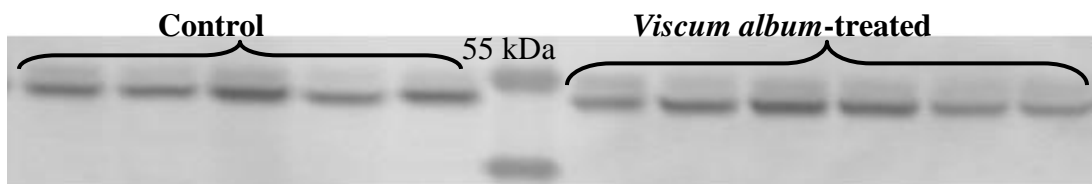
Figure 3. 16 (A) Immunoreactive protein bands of control (N=11) and *Viscum album* L.-treated (N=15) groups representing CYP27B1 (MW of 54 kDa). **(B)** β-actin (MW of 42 kDa) was used as internal control for each membrane**.**
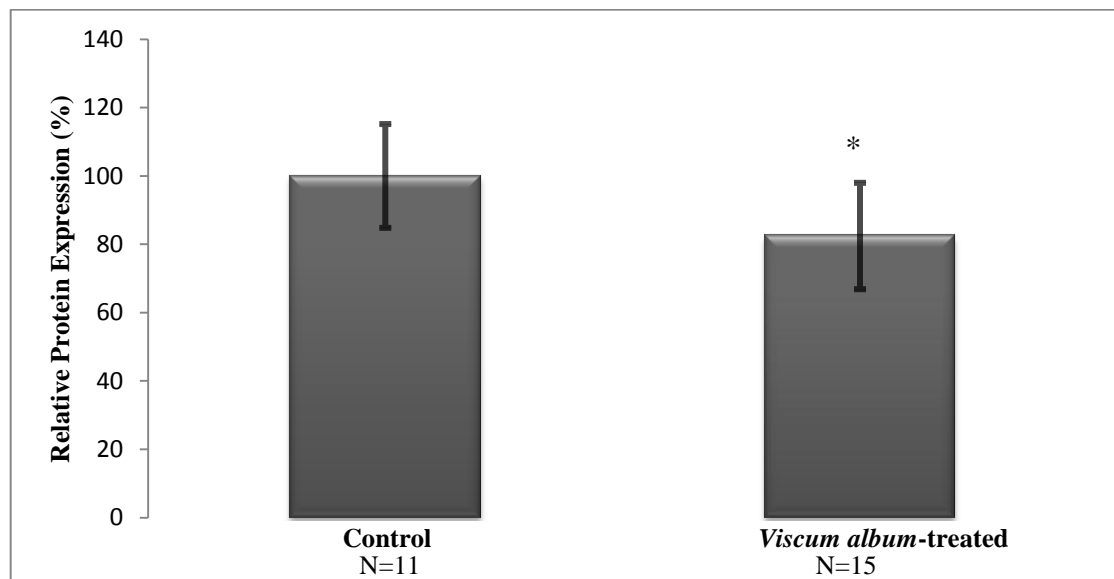
Figure 3. 17 *In-vivo* effect of *Viscum album* L. on CYP27B1 protein expression. The band quantifications are expressed as mean ±SD of the relative intensity. The mean relative protein expression of *Viscum album* L.-treated group is 82% of the mean of control group. The *p* value obtained from unpaired, two-tailed student's *t*-test is less than 0.013.
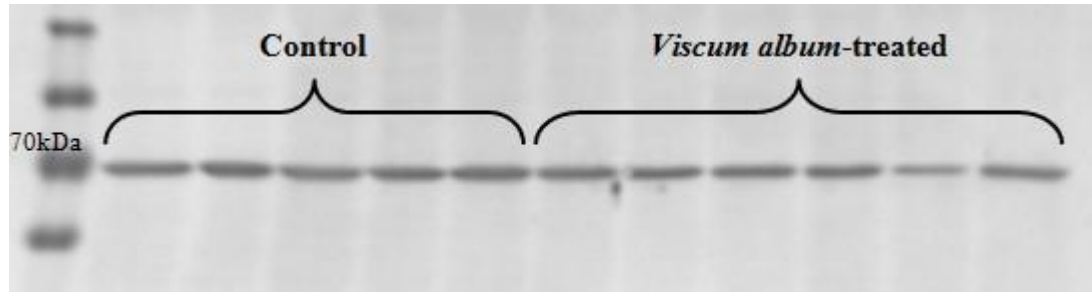
3.5. Effect of *Viscum album* L. on CYP24A1 and CYP27B1 mRNA expression

3.5.1. Quality control of RNA molecules by agarose gel electrophoresis

Total RNA was isolated from rat liver tissue as described in Materials & Methods part. Due to the risk of DNA contamination, RNA isolation was one of the most critical parts of the experiments. Qualities of RNA molecules were checked with OD_{260}/OD_{280} ratio which must be between 1.8 and 2.2. OD_{260}/OD_{280} ratio of the samples was good enough to be used in cDNA synthesis and real-time PCR studies. After that, 28S and 18S RNA bands were checked with agarose gel electrophoresis. RNA molecules having

OD_{260}/OD_{280} between 1.8 and 2.2 showed intact and well-separated 28S and 18S RNA bands after agarose gel electrophoresis as shown in Figure 3.18.

Figure 3. 18 Agarose gel electrophoresis pattern of RNA samples isolated from rat liver tissues.

3.5.2. Effect of *Viscum album* L. on CYP24A1 mRNA expression

qRT-PCR technique was used to determine the mRNA expression of CY24A1. For the beginning, serial optimization was done with samples from the control group. For the optimization part, different primer pairs and cDNA concentrations were used. For the annealing, the temperatures between 45 ^{0}C and 60 ^{0}C were tested. Also, qRT-PCR of GAPDH used as internal control (housekeeping gene) was done to eliminate the possible risks of contamination which may come from the cDNA samples, SYBR-green and

water. To eliminate the contamination risk of primers, no template control which included water instead of cDNA samples was used. These optimization steps were done at different annealing temperatures with different cycle points. Also, two different primer sets specific for *CYP24A1* gene were tested. However, no consistent results were obtained probably due to the undetectable properties of CYP24A1 mRNA in the liver. Two of the melting curves generated from the optimization experiments are shown in Figures 3.19 and 3.20.
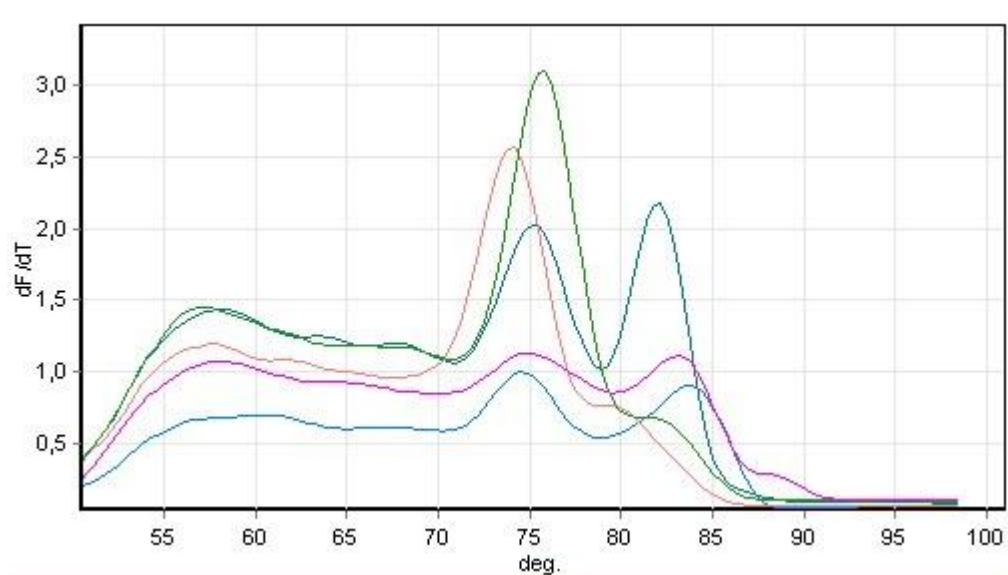
Figure 3. 19 Melting curve showing the fluorescence of SYBR green dye I versus temperature. 50 ^{0}C was used as annealing temperature.
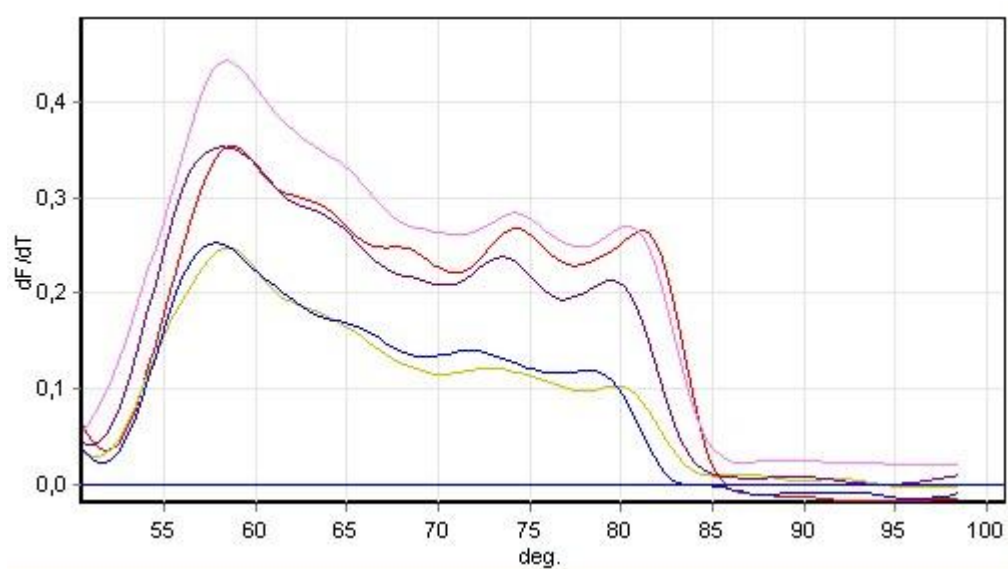
Figure 3. 20 Melting curve showing the fluorescence of SYBR green dye I versus temperature. 60 ^{0}C was used as annealing temperature.

3.5.3. Effect of *Viscum album* L. on CYP27B1 mRNA expression

qRT-PCR technique was used to determine the mRNA expression of CYP27B1. GAPDH was used as internal standard to calculate the relative mRNA expression of *Viscum album*-treated CYP27B1. The specific annealing temperatures of the primers of CYP27B1 and GAPDH were used in qRT-PCR.

Figure 3.21 shows the standard curve which was generated from 1:10, 1:100, 1:500, 1:000, and 1:5000 diluted cDNAs used for quantifications of the samples. Figure 3.22 illustrates the amplification plot showing the changes in fluorescence of SYBR green dye I versus cycle number of rat liver CYP27B1 gene of control and *Viscum album*-treated samples. In Figure 3.23, melting curve with one peak is represented for the detection of single PCR product.
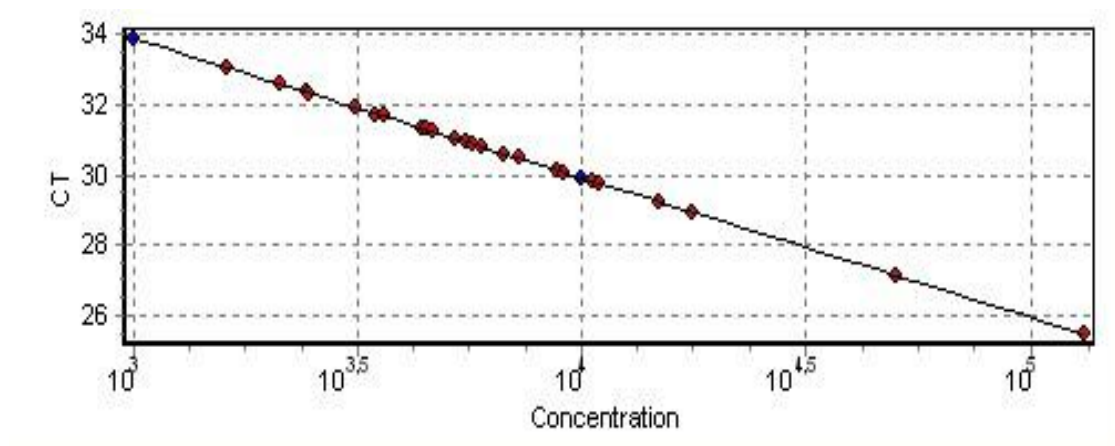
Figure 3. 21 Standard curve derived from dilution series of a control cDNA to measure quantities of specific CYP27B1 mRNAs in the sample.
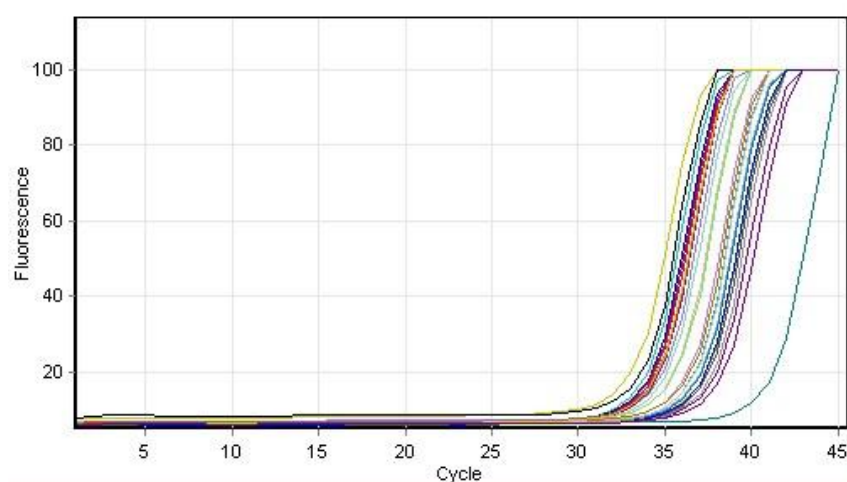
Figure 3. 22 Amplification plot showing the changes in fluorescence of SYBR green dye I versus cycle number of control and *Viscum album*-treated rat livers' CYP27B1 gene.
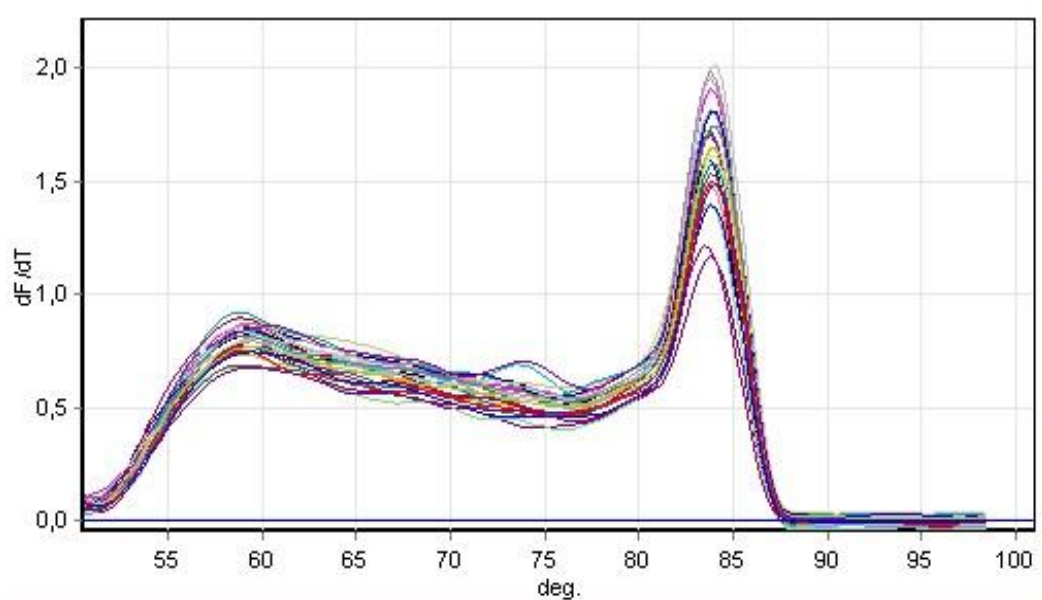
Figure 3. 23 Melting curve showing the fluorescence of SYBR green dye I versus temperature with one peak meaning single PCR product detection. One peak in the melting curve confirms the detection of single PCR product.

In Figures 3.24 and 3.25, the PCR products of CYP27B1 and GAPDH are represented, respectively. The figures confirm the exact position of CYP27B1 and GAPDH products produced in qRT-PCR.

mRNA expressions CYP27B1 were determined by using qRT-PCR and relative expressions of CYP27B1 of control and treated animals were calculated using GAPDH as internal control (housekeeping gene). Figure 3.21 shows the standard curve which was generated from 1:10, 1:100, 1:500, 1:1000, and 1:5000 diluted cDNAs used for quantifications of the samples. Figure 3.22 illustrates the amplification plot showing the changes in fluorescence of SYBR green dye I versus cycle number of control and

75

Viscum album-treated rat livers' CYP27B1 gene. In Figure 3.23, melting curve with one peak is represented for the detection of single PCR product.

In Figures 3.24 and 3.25, the PCR products of CYP27B1 and GAPDH are represented, respectively. The band patterns of the gels confirm the exact position of CYP27B1 and GAPDH products produced in qRT-PCR which are 107 and 240 bp, respectively. 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 CYP27B1 mRNA expression change when animals injected by plant extract. Table 3.2 gives the Livak ($2^{-\Delta\Delta ct}$) method for calculation of relative mRNA expression using Ct values.
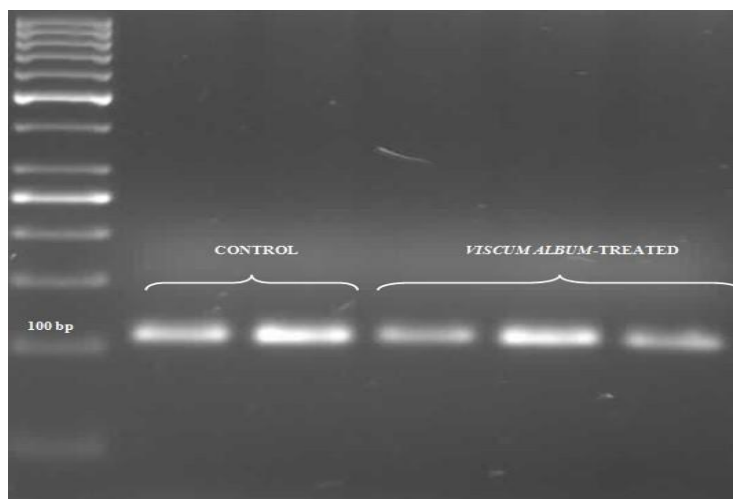
Figure 3. 24 qRT-PCR product of liver CYP27B1 cDNA (107bp) of control and *Viscum album*-treated animals. Each lane contains 10 µL of qRT-PCR Product.
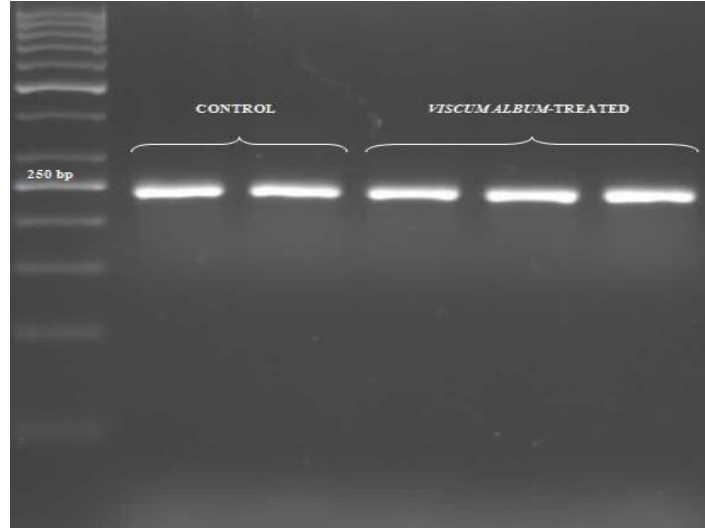
Figure 3. 25 qRT-PCR product of liver GAPDH cDNA (240 bp) of control and *Viscum album*-treated animals. Each lane contains 10 µL of qRT-PCR Product.

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

		Control	**VAT**			
Ct$_{CYP27B1}$		31.41	30.56			
Ct$_{GAPDH}$		17.45	18.52			
ΔCt$_{VAT}$	=	**Ct$_{GAPDH}$**	-	**Ct$_{CYP27B1}$**		
ΔCt$_{reference}$	=	**Ct$_{GAPDH}$**	-	**Ct$_{CYP27B1}$**		
ΔCt$_{VAT}$	=	18.52	-	30.56	=	-12.04
ΔCt$_{reference}$	=	17.45	-	31.41	=	-13.96
		ΔΔCt = ΔCt$_{reference}$ - ΔCt$_{VAT}$			=	**-1.92**
$2^{-\Delta\Delta ct}$		=	**3.8**			

The normalized average liver CYP27B1 mRNA level of control animals was found to be 1.44 ± 0.23 (mean ± SEM, N=11). The average CYP27B1 mRNA amount was calculated as 5.42 ± 0.95 (mean ± SEM, N=15) for liver tissue of *Viscum album*-treated animals. As shown in Figure 3.26, *Viscum album* treatment of animals caused 3.8 fold increase in mRNA expression with respect to control group (p<0.005, unpaired, two-tailed student's *t*-test).

	control N=11	treated N=15
Relative CYP27B1/GAPDH	1.00	3.80
Standard Error Mean	0.22	0.66
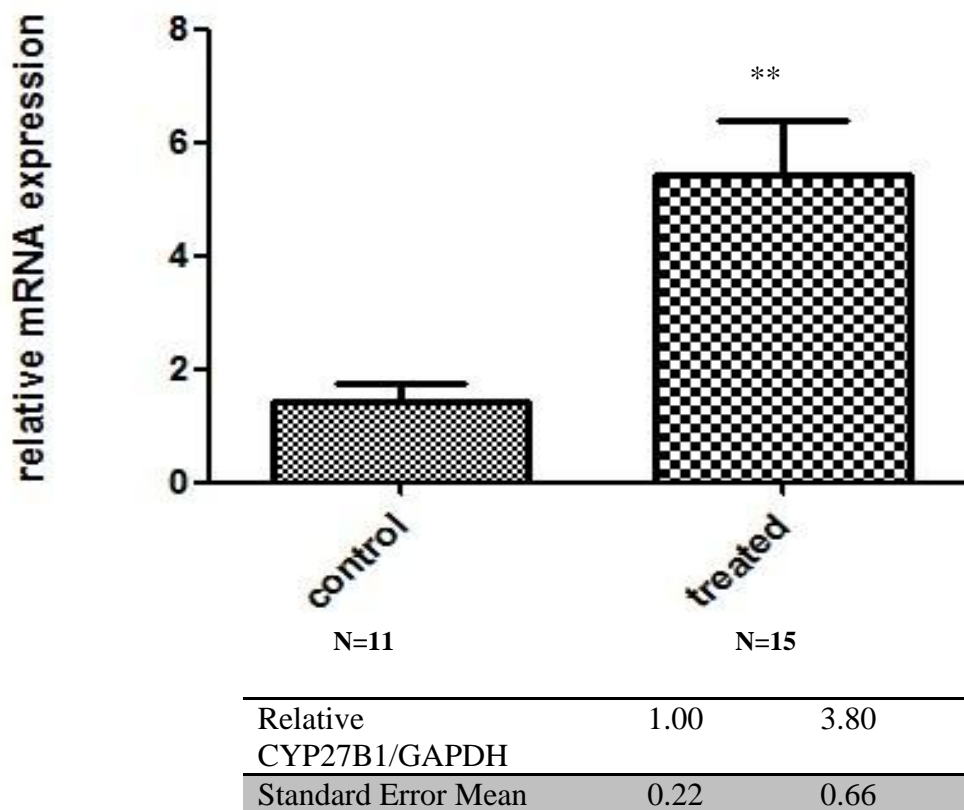
Figure 3. 26 *In vivo* effect of *Viscum album* L. on rat liver CYP27B1 mRNA expression. The quantifications are expressed as mean ±SEM of the relative expression. Relative mRNA expression of *Viscum album* L-treated group is 3.8 fold higher than that of control group. The *p* value obtained from unpaired, two-tailed student's *t*-test was 0.0018.

CHAPTER 4

DISCUSSION

Plants possess primary importance in medicinal applications since they have numerous phytochemicals which are composed of a diverse range of biologically active compounds. These active compounds are the secondary plant metabolites showing pharmacological or toxicological effects in animals. Among these biologically active compounds; terpenoids, polyphenolics, glucosinolates, phytosterols and thiosulfanates are the most important phytochemicals. Phytochemicals display some beneficial health promoting impacts as antioxidants, blood pressure or blood sugar influencing substances, or agents with anti-inflammatory, anticarcinogenic, antithrombotic, immunity-supporting, cholesterol-lowering, antifungal, antibacterial or antiviral effects (Schreiner & Huyskens-Keil, 2006). For instance, ellagic acid is a natural polyphenol found in many plant species and has antimutagenic, antioxidant and anti-inflammatory activity in bacterial and mammalian systems (Vattem & Shetty, 2005).

Vitamin D_3, also known as cholecalciferol, is an important compound which has essential biologic functions especially on calcium and bone metabolism. It is formed from 7-dehydrocholesterol by UV irradiation. To get the active form of vitamin D_3, it has to be subjected to two successive hydroxylation reactions. First hydroxylation reaction is catalyzed by CYP27A1 while the latter is catalyzed by CYP27B1. On the other hand, CYP24A1 functions for the inactivation of vitamin D_3 compounds (Sakaki *et al.*, 2005).

CYP24A1 and CYP27B1 are the members of cytochrome P450 super-family and key enzymes involved in the metabolism of Vitamin D_3. CYP24A1 and CYP27B1 are mitochondrial enzymes and also known as 24-hydroxylase and 25-hydroxyvitamin D_3 1alpha-hydroxylase, respectively. CYP27B1 involves in 1α-hydroxylation of 25-OH-D3 into 1,25-$(OH)_2D_3$ while CYP24A1 involves in 24-hydroxylation of 25-OH-D_3 and 1,25-$(OH)_2D_3$ which is required for the catabolism of vitamin D_3 compounds (Ohyama & Yamasaki, 2004; Prosser & Jones, 2004).

It is well known that both protein and mRNA expressions of P450 enzymes can be affected by physiological and pathophysiological conditions as well as diet (Arinc *et al*, 2007). For instance, CYP27B1 is under tight regulation of 1,25-$(OH)_2D_3$, PTH and calcitonin. While 1,25-$(OH)_2D_3$ down-regulates the gene expression of CYP27B1, PTH and calcitonin are the upregulators of CYP27B1 expression (Murayama *et al.*, 1998; Shinki *et al.*, 1999).

In this study, *in vivo* effects of water extracts of the medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. on 24-hydroxylase (CYP24A1) and 1α-hydroxylase (CYP27B1) protein and mRNA expressions were analyzed and demonstrated for the first time. For this purpose, 37.5 mg water extract of *Epilobium hirsutum* L./kg of body weight and 10 mg water extract of *Viscum album* L. /kg of body weight were injected to 30 and 15 Wistar albino rats, respectively, for 9 consecutive days. Then, decapitation of the animals was done and their livers were removed. After preparation of S1.5 fractions including mitochondrial enzymes, protein and mRNA expression analysis of hepatic CYP24A1 and CYP27B1 were performed to show the effects of these plants at both translational and transcriptional levels (Table 4.1).

Table 4. 1 Summary of results of protein and mRNA expression analysis of hepatic CYP24A1 and CYP27B1 from control, EHT and VAT groups.

	CYP24A1		CYP27B1	
	Protein expression (% of control)	mRNA expression (% of control)	Protein expression (% of control)	mRNA expression (% of control)
EHT	69	not detected	82	270
VAT	83	not detected	82	380

The results have shown that the water extract of the medicinal plant *Epilobium hirsutum* L. caused 31% and 18% decrease in CYP24A1 and CYP27B1 protein expressions, respectively. As indicated before, CYP24A1 works for inactivation of vitamin D_3 compounds while CYP27B1 involves in the activation of vitamin D. Treatment of *Epilobium hirsutum* L. caused the same effect on these reverse acting enzymes. It can be explained by the presence of several phytochemicals each of which can interfere with different metabolic pathways. These phytochemicals include steroids (β-sitosterol and its esters), tannins (gallic, protocatechuic, ellagic and p-coumaric acids) and flavonoids (in particular myricetin, isomyricetin, and quercetin). To detect the specific active metabolite which modulates the protein expression of CYP24A1 or CYP27B1, isolation and characterization of these pharmacologically active compounds of *Epilobium hirsitum* L. must be done.

Epilobium hirsitum L. caused 2.7 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH (Glyceraldehyde 3-phosphate

dehydrogenase) expression as an internal reference. It appears that the attempted increase of CYP27B1 mRNA expression does not translate into an appropriate increase of CYP27B1 protein levels. Post-transcriptional regulations generally cause low correlation between protein and mRNA level. For most of the eukaryotic organisms, variations in mRNA level explain only half or less of variation of protein abundance. Transcription, decay of mRNA, translation and degradation of the proteins are the main points which determine the steady state protein concentration even their relative contributions for the final protein amount in eukaryotes are mostly unknown (Vogel *et al.*, 2010). The negative correlation between mRNA and protein level is currently unknown, but there are some possible explanations. It might be due to possible microRNA (miRNA) regulation or reduced translation efficiency, probably through a block of the protein translation machinery. miRNAs are small, noncoding RNA molecules which involves in regulation of gene expression especially in animals. It is predicted that miRNAs control more than 60% of protein coding gene activity without affecting the mRNA stability (Brodersen *et al.*, 2008; *Friedman et al.*, 2009; Lanet *et al.*, 2009). miRNA can function either in inhibition of target mRNA translation or deadenylation and degradation of target mRNAs (Fabian *et al.*, 2010). Since mRNA expression of CYP27B1 was detected by qRT-PCR technique as described in Materials & Methods part of the present study, miRNA would probably act through the inhibition of mRNA translation without degradation. This inhibition occurs through either initiation block or post-initiation block as described by Fabian *et al.*, 2010 and illustrated in Figure 4.1. In initiation blockage, miRNA induced silencing complex (miRISC) can inhibit translation by acting as a repressor of cap recognition and prevent binding of 40S subunit or act as 60S antagonist and prevent joining of 60S subunit. For post-initiation blockage, miRISC inhibits ribosomal elongation by making ribosome to drop-off. Also, it can cause proteolysis of the newly formed polypeptides (Fabian *et al.*, 2010). Also, protein degradation process affects the protein abundance. Ubiquitination and then degradation of target proteins involve in regulation of protein abundance. E3 ubiquitin ligases recognize the signals on target proteins and provide attachment of poly-ubiquitin

chain. Then, proteasomes involve in degradation of these ubiquitinated proteins (Vogel *et al.*, 2010).
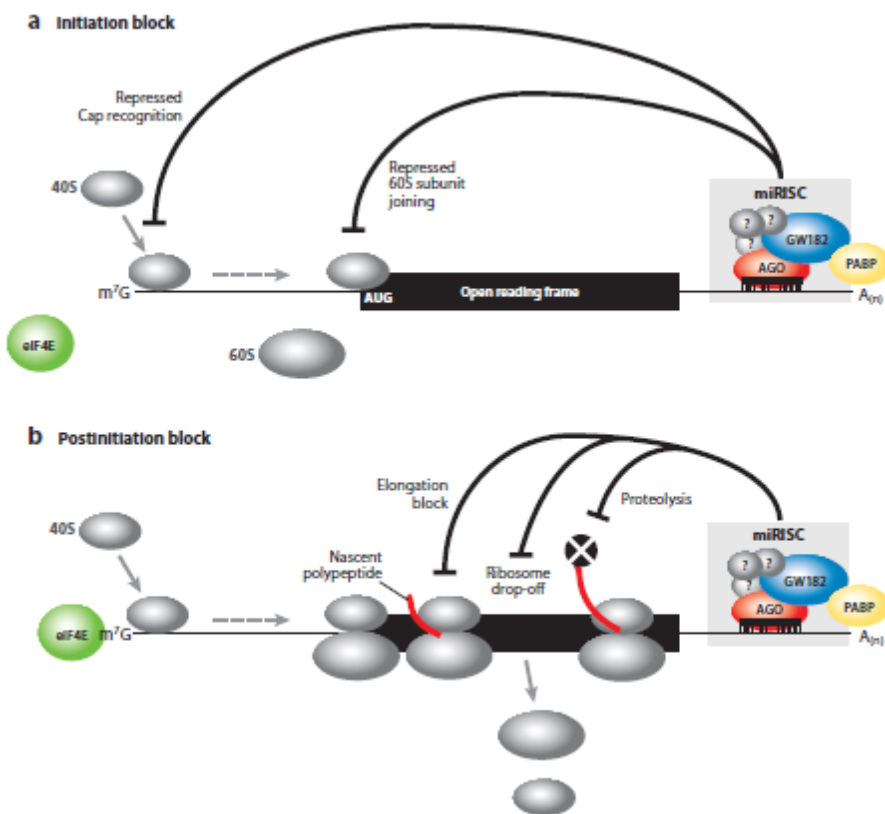
Figure 4. 1 Schematic diagram of miRNA-mediated translational repression (Fabian *et al.*, 2010).

The effect of *Epilobium hirsutum* L. as well as *Viscum album* L. on mRNA expression of CYP24A1 couldn't be observed, because CYP24A1 mRNA was almost undetectable in liver. These negative mRNA expression findings may propose the followings. mRNA of CYP24A1 can be degraded too fast to reliably be tracked due to extremely short half-life or extracellular stimuli and viral infections and proteins found in the liver can have much longer life spans. It was shown that human CYP24A1 mRNA is under post-transcriptional miRNA regulation by miR-125b (Komagata *et al.*, 2009). This miRNA regulation may occur through the degradation of target mRNAs, so we couldn't observe the mRNA of CYP24A1 in the liver. However, further studies must be carried out to understand the exact mechanism of miR-125b. Undetectable level of CYP24A1 mRNA level can also be explained by the presence of mRNA instability determinants. Some sequence elements found in mRNA decide the half-life of mRNA. While some of these determinants promote stabilization of mRNA, the other can involve in rapid mRNA decay. All of these determinants can be present in the 5'- untranslated region (5'-UTR), the coding region, and the 3'- untranslated region (3'-UTR) which require RNA-binding proteins or small non-coding RNAs to exert their effects.

Experimental results have demonstrated that *Viscum album* L. caused 17% and 18% decrease in CYP24A1 and CYP27B1 protein expressions, respectively. As indicated before, CYP24A1 and CYP27B1 work for inactivation and activation of vitamin D, respectively. Treatment of *Viscum album* L. caused the same effect on these reverse acting enzymes. It can be explained by the presence of several phytochemicals each of which can interfere with different metabolic pathways. These phytochemicals include phenylpropanes, caffeic acids, flavonoids, alkaloids, triterpenes, stigmasterol, lectins and viscotoxins. To detect the specific active metabolites which modulate the protein expression of CYP24A1 or CYP27B1, isolation and characterization of these pharmacologically active compounds from *Viscum album* L. must be done.

Viscum album L. also caused 3.8 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) expression as an internal reference. Increased CYP27B1 mRNA level is

uncoupled from protein synthesis and that CYP27B1 protein level in the liver is in fact reduced by the treatment of *Viscum album* L. This inverse relationship between mRNA and protein level might be probably due to miRNA regulation or reduced translation efficiency, as described previously in the effect of *Epilobium hirsutum* L. treatment.

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 vitamin D_3 metabolism by changing the expression of protein and mRNA of CYP24A1 and CYP27B1. However, both CYP24A1 and CYP27B1 enzymes have different expression levels on different tissues and different species, further studies are required to determine the effects of these plants on different organs as well as different animal models and humans. Also, the isolation and characterization of pharmacologically active compounds from these medicinal plants should be performed to verify the specific compound responsible for the change in protein expression of CYP24A1 as well as both mRNA and protein expression of CYP27B1 enzyme.

CHAPTER 5

CONCLUSION

In this present study, *in vivo* effects of medicinal plants *Epilobium hirsutum* L. (Onagraceae, hairy willow herb) and *Viscum album* L. (Loranthaceae, European mistletoe) on rat liver mitochondrial 24-hydroxylase (CYP24A1) and 1α-hydroxylase (CYP27B1) protein and mRNA expressions were demonstrated for the first time.

These medicinal plants were collected from unpolluted stream beds and their water extracts were obtained. 37.5 mg *Epilobium hirsutum* L./kg body weight/day was intraperitoneally (i.p) injected to 25 Wistar albino rats (*Rattus norvegicus*) for 9 days; 10 mg *Viscum album* L. (subspecies growing on pine trees-subsp. *austriacum* (Wiesb.) Vollmann) /kg body weight/day was injected i.p to 15 rats for 9 days; and 11 animals were used as control group.

Epilobium hirsutum L. injection to rats caused 31% decrease in liver mitochondrial CYP24A1 protein expression ($p < 0.0001$). In addition, 18% decrease in liver CYP27B1 protein expression ($p < 0.05$) was observed when animals treated with the same dose of water extract of epilobium. *Epilobium hirsutum* L. caused 2.7 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) expression as an internal reference ($p < 0.005$). The effect of *Epilobium hirsutum* L. on mRNA expression of CYP24A1 could not be observed, because CYP24A1 mRNA was almost undetectable in liver.

Viscum album L. caused 17% decrease in rat liver mitochondrial CYP24A1 protein expression ($p < 0.05$). Injection of water extract of *Viscum album* L. to rats led to 18% decrease in CYP27B1 protein expression ($p < 0.05$). *Viscum album* L. caused 3.8 fold increase in mRNA expression of CYP27B1 with respect to controls and normalized with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) expression as an internal reference ($p < 0.005$). However, the effect of *Viscum album* L. on mRNA expression of CYP24A1 could not be observed since CYP24A1 mRNA was almost undetectable in liver.

The results of the present study showed that the medicinal plants *Epilobium hirsutum* L. and *Viscum album* L. can have regulatory effects on CYP24A1 and CYP27B1 protein and mRNA expressions. Since both CYP24A1 and CYP27B1 enzymes have different expression levels on different tissues and different species, further studies are required to determine the effects of these plants on different organs as well as different animal models and humans. In addition, these two medicinal plants have several phytochemicals and therefore each of these active compounds can modulate CYP24A1 and CYP27B1 expressions differently. For future perspective, isolation and characterization of pharmacologically active compounds from these medicinal plants should be performed. Therefore, the specific compounds which modulate the protein and mRNA expressions of CYP24A1 and CYP27B1 enzymes can be investigated. Furthermore, effects of these medicinal plants can also be studied in disease states related with vitamin D metabolism.

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APPENDIX A

APPROVALS OBTAINED FROM ETHICAL COMMITEE

T.C
PAMUKKALE ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURULU BAŞKANLIĞI

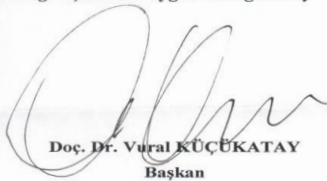Sayı   :B.30.2.PAÜ.0.01.00.00.400-1/08   17.02.2009
Konu :Çalışma Başvurusu

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

106

	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	

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

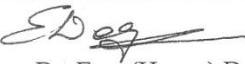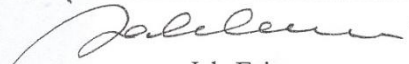**İlgi:** Prof.Dr.Alaattin ŞEN'in 30.04.2008 tarihli başvurusu.

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

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