

THE EFFECTS OF FOLK MEDICINAL PLANTS *ViScUM ALBUM L.* AND
EPILOBIUM HIRSUTUM L. ON PROTEIN AND mRNA EXPRESSIONS OF RAT
LIVER BILE ACID SYNTHESIZING CYPs

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LIVER BILE ACID SYNTHESIZING CYPs**

submitted by **TUBA ÇULCU** in partial fulfillment of the requirements for the degree of the degree of **Doctor of Philosophy in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan Adalı
Head of Department, **Biochemistry**

Prof. Dr. Orhan Adalı
Supervisor, **Biology Dept., METU**

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

Examining Committee Members:

Prof. Dr. N.Tülün Güray
Biology Dept., METU

Prof. Dr. Orhan Adalı
Biology Dept., METU

Prof. Dr. Mümtaz İşcan
Faculty of Pharmacy, Ankara University

Assoc. Prof. Dr. Çağdaş Devrim Son
Biology Dept., METU

Assoc. Prof. Dr. Birsen Can Demirdöğen
Biomedical Engineering Dept., TOBB

Date: 03.09.2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Tuba ÇULCU

Signature :

ABSTRACT

THE EFFECTS OF FOLK MEDICINAL PLANTS *VISCUM ALBUM L.* AND *EPILOBIUM HIRSUTUM L.* ON PROTEIN AND mRNA EXPRESSIONS OF RAT LIVER BILE ACID SYNTHESIZING CYPs

Çulcu, Tuba

Ph. D., Department of Biochemistry

Supervisor : Prof. Dr. Orhan Adalı

Co-supervisor : Prof. Dr. Alaattin Şen

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Plants include many phenolic and polyphenolic compounds that are important in medicine, cosmetics and nutrition. *Viscum album L.* is a bi-perennial shrub and an evergreen semi-parasitic plant widely distributed in Europe. *Epilobium hirsutum L.*, great willow-herb, grows in Eurasia, North Africa, Europe, Southern Australia and United States. Due to having a huge phenolic profiles, *Viscum album L.* and *Epilobium hirsutum L.* have many different biological functions including anti-tumor, anti-oxidant and anti-cancer. Thus, *Viscum album L.* and *Epilobium hirsutum L.* have been used traditionally for prevention and treatment of some diseases since the throughout the history of human. In mammals, excessive cholesterol is removed mainly through conversion to bile acids and cytochromes P450s initiate all quantitatively significant pathways of cholesterol metabolism and bile acid biosynthesis. The bile acid pathway is very important in maintaining normal cholesterol levels. There are two pathways namely the classical pathway and the acidic pathway involved in bile acid synthesis catalyzed by CYP7A1 and CYP27A1, respectively. These two pathways

produce cholic acid (CA) and chenodeoxycholic acid (CDCA) roughly equal by CYP8B1 and CYP7B1.

This study is the first about the possible potency of medicinal plants *Viscum album L.* and *Epilobium hirsutum L.* extracts and their major polyphenolic ingredients, *o*-coumaric acid and ellagic acid on rat liver cholesterol and bile acid metabolizing enzymes. In addition to molecular studies, bioactive compounds of *Viscum album L.* and *Epilobium hirsutum L.* extracts were identified using Liquid Chromatograph-Mass Spectrometry (LC-MS) technique. The water extracts of *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid were injected intraperitoneally as 10 mg/kg, 37.5 mg/kg, 30 mg/kg and 20 mg/kg for 9 days, respectively. Then, *in vivo* effects of these plants and their major phenolic ingredients on rat liver cholesterol and bile acid metabolizing CYPs were analyzed by determining protein and mRNA expression levels using western blotting and qRT-PCR techniques, respectively. The results showed that *Viscum album L.* caused a 0.9 fold decrease in protein expression of CYP7A1 and CYP7B1, but 1.4 and 1.3 fold increases in protein expression of CYP27A1 and CYP8B1, respectively. *Viscum album L.* caused 9, 1.3 and 2.4 fold decrease in mRNA expression of CYP7A1, CYP27A1, CYP7B1, respectively, however, 4.2 fold increase in mRNA expression of CYP8B1. Injection of *o*-coumaric acid to rats resulted 0.8 fold decrease in protein expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1. However, *o*-coumaric acid caused 1.8, 1.3, 2.3 and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1, respectively. *Epilobium hirsutum L.* caused 0.6, 0.8 and 0.6 fold decrease in protein expression of CYP7A1, CYP8B1 and CYP7B1, respectively, whereas 1.3 fold increase in protein expression of CYP27A1. *Epilobium hirsutum L.* caused 2.1, 1.4 and 4.1 fold decrease in mRNA expression of CYP7A1, CYP27A1, CYP7B1, respectively, however, 2.3 fold increase in mRNA expression of CYP8B1. Injection of ellagic acid to rats resulted 0.7, 0.8 and 0.8 fold decrease in protein

expression of CYP27A1, CYP8B1, CYP7B1, respectively, but one fold increase of protein expression of CYP7A1. Ellagic acid caused 2.6, 1.7, 1.8 and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1, respectively. In addition, the CYP7A1 enzyme activity was determined by an Isocratic High-Performance Liquid Chromatography Assay using a calibration curve obtained by different cholesterol concentrations. According to these results, even though *Viscum album L.* and *o*-coumaric acid caused fold change in protein and mRNA expressions of bile acid metabolizing CYPs, serum cholesterol levels remained unchanged. *Epilobium hirsutum L.* and ellagic acid-treated animals showed a significant decrease in total cholesterol level compared to control animals. Therefore, bases on doses and time point that applied in this study, the medicinal plant *Epilobium hirsutum L.* and its polyphenolic compound, ellagic acid, may have regulatory effects on bile acid metabolizing enzymes. In conclusion, the synthesis of bile acids from cholesterol by CYP-catalyzed reactions may be altered due to the changes in CYP protein and mRNA expressions which may be resulted by the treatment of animals with *Epilobium hirsutum L.* and ellagic acid.

Key words: *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid, ellagic acid, CYP7A1, CYP27A1, CYP8B1, CYP7B1, bile acids, cholesterol, protein expression, mRNA expression, rat liver.

ÖZ

TIBBİ BİTKİ *VISCUM ALBUM L.* VE *EPİLOBİUM HİRSUTUM L.* 'NİN SIÇAN KARACİĞER SAFRA ASİT METABOLİZMASINDA ROL OYNAYAN CYP ENZİMLERİNİN PROTEİN VE mRNA EKSPRESYONLARI ÜZERİNDEKİ ETKİLERİ

Çulcu, Tuba

Doktora, Biyokimya Bölümü

Tez Yöneticisi: Prof. Dr. Orhan Adalı

Ortak Tez Yöneticisi: Prof. Dr. Alaattin Şen

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Bitkiler tıp, kozmetik ve beslenme sektöründe önemli rol oynayan çok fazla sayıda fenolik ve polifenolik maddeler içerirler. *Viscum album L.* Avrupa'da yaygın olarak dağılan ve iki yıllık yapraklarını dökmeyen yarı parazit bir bitkidir. *Epilobium hirsutum L.*, yakı otu, Avrasya, Kuzey Afrika, Avrupa, Güney Avustralya ve Amerika Birleşik Devletleri'nde yetişir. Önemli fenolik profillerine sahip olan *Viscum album L.* ve *Epilobium hirsutum L.* anti-tümör, anti-oksidan ve anti-kanser gibi çok farklı biyolojik işlevlere sahiptir. Bu nedenle *Viscum album L.* ve *Epilobium hirsutum L.* insanlık tarihi boyunca bazı hastalıkların önlenmesinde ve tedavi edilmesinde geleneksel olarak kullanılmıştır. Memelilerde fazla kolesterol genellikle safra asitlerine dönüştürülerek vücuttan uzaklaştırılır. Siokrom P450 enzimleri kolesterol ve safra asit metabolizmasının önemli basamaklarını başlatır. Safra asit yolağı kolesterol dengesinin korunmasında çok önemlidir. Safra asit sentezinde klasik ve asidik olmak üzere iki yolak vardır

ve bu yolaklar sırasıyla CYP7A1 ve CYP27A1 enzimleri tarafından katalizlenir. Ayrıca bu iki yolak CYP8B1 ve CYP7B1 enzimleri vasıtasıyla kolik asit ve kenodoksikolik asit üretir.

Bu çalışma tıbbi bitkiler *Viscum album L.* ve *Epilobium hirsutum L.* ve onların polifenolik içerikleri olan *o*-kumarik asit ve elajik asit „in sıçan karaçiğer kolesterol ve safra asitlerini metabolize eden enzimler üzerine etkilerini araştırılmasında bir ilktir. Moleküler çalışmalara ek olarak, *Viscum album L.* ve *Epilobium hirsutum L.* özütlerinin biyolojik olarak aktif bileşenleri LC-MS yöntemi ile belirlenmiştir. *Viscum album L.* (10 mg/kg), *Epilobium hirsutum L.* (37.5 mg/kg), *o*-kumarik asit (30 mg/kg) ve elajik asit (20 mg/kg) „in sulu ekstraları wistar albino sıçanlara 9 gün boyunca intraperitoneal olarak enjekte edilmiştir. Daha sonra, bu bitkilerin ve fenolik bileşiklerinin kolesterol ve safra asitleri metabolizmasında rol oynayan CYP“ler üzerine *in vivo* etkileri protein ve mRNA seviyesinde western bolt ve qRT-PCR tekniği kullanılarak analiz edilmiştir. Sonuçlar *Viscum album“*un CYP7A1 ve CYP7B1 enzimlerini protein ekspresyonlarını 0.9 kat azalttığını fakat CYP27A1 ve CYP8B1 enzimlerinin protein ekspresyonlarını sırasıyla 1.4 ve 1.3 kat arttırdığını göstermiştir. *Viscum album L.* CYP7A1, CYP27A1, CYP7B1 enzimlerinin protein ekspresyonlarını sırasıyla 9, 1.3 ve 2.4 kat azaltırken, CYP8B1“ in ekspresyonunun 4.2 kat artmasına neden olmuştur. *o*-Kumarik asit tüm enzimlerin protein ekspresyonunu 0.8 kat azaltırken, CYP7A1, CYP27A1, CYP8B1 ve CYP7B1 enzimlerinin mRNA ekspresyonlarını sırasıyla 1.8, 1.3, 2.3 ve 2.3 kat arttırmıştır. *Epilobium hirsutum L.* CYP7A1, CYP8B1, CYP7B1 enzimlerinin protein ekspresyonlarını sırasıyla 0.6, 0.8 ve 0.6 kat azaltırken, CYP27A1 enziminin protein ekspresyonunu 1.3 kat arttırmıştır. Ayrıca *Epilobium hirsutum L.* CYP7A1, CYP27A1, CYP7B1 enzimlerinin mRNA ekspresyonlarının sırasıyla 2.1, 1.4 ve 4.1 kat azalmasına neden olurken, CYP8B1 enziminin mRNA ekspresyonunun 2.3 kat artmasına neden olmuştur. Elajik asit CYP27A1, CYP8B1, CYP7B1 enzimlerinin protein

ekspresyonlarını sırasıyla 0.7, 0.8 ve 0.8 kat azaltırken, CYP7A1 enziminin protein ekspresyonunu 1 kat arttırmıştır. Ayrıca elajik asit CYP7A1, CYP27A1, CYP8B1 ve CYP7B1 enzimlerinin mRNA ekspresyonlarını sırasıyla 2.6, 1.7, 1.8 ve 2.3 kat arttırmıştır. Ayrıca CYP7A1 enzim aktivitesi farklı kolesterol konsantrasyonları kullanılarak standart grafik çizilerek HPLC yöntemi ile belirlenmiştir. Tüm bu sonuçlara göre, *Viscum album L.* ve *o*-kumarik asit özütleri ile muamele edilmiş hayvanlarda safra asit sentezinde rol oynayan enzimlerin protein ve mRNA ekspresyonlarında değişim belirlenmesine rağmen, serum kolesterol seviyesinde değişim belirlenmemiştir. *Epilobium hirsutum L.* ve elajik asit özütleri muamele edilmiş hayvanların serum kolesterol seviyesi kontrol örnekleri ile karşılaştırıldığında önemli bir azalma tespit edilmiştir. Bu çalışmada uygulanan dozlar ve dozların uygulanma zamanı dikkate alındığında tıbbi bitki *Epilobium hirsutum L.* ve onun fenolik bileşeni olan elajik asitin safra asit metabolizmasındaki enzimler üzerinde düzenleyici etkileri olduğu gösterilmiştir. Sonuç olarak, *Epilobium hirsutum L.* ve elajik asit safra asit sentezini katalizleyen CYP enzimlerinin mRNA ve protein ekspresyonunu değiştirerek safra asit sentezini etkileyebilir.

Anahtar kelimeler: *Viscum album L.*, *Epilobium hirsutum L.*, *o*-kumarik asit, elajik asit, CYP7A1, CYP27A1, CYP8B1, CYP7B1, safra asitleri, kolesterol, protein ekspresyonu, mRNA ekspresyonu, sıçan karaciğeri.

To My Family,

For their endless love and support

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LIST OF SYMBOLS AND ABBREVIATIONS

APS	Ammonium per sulfate
ARE	Antioxidant response element
BCIP	5-bromo 4-chloro 3-indoyl phosphate
BSA	Bovine serum albumin
CA	Cholic acid
CDCA	Chenodeoxycholic acid
cDNA	Complementary DNA
CO	Carbon monoxide
Ct	Threshold cycle
CYP	Cytochrome P450
DCA	Deoxycholic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
ERB	Electronic running buffer
FBS	Fetal bovine serum
HPLC	High-Performance Liquid Chromatography

kDa	kilo Dalton
K_m	Michaelis constant
LCA	Lithocholic acid
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrotetrazolium blue chloride
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
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

CHAPTER 1

INTRODUCTION

Plants include many phenolic and polyphenolic compounds that are important in medicine, cosmetics and nutrition. The phytochemical compounds are used mostly in medicine as alternative medicine and many of them have been reported to have prominent impacts on health, especially as anti-oxidant, anti-inflammatory, anti-microbial, anti-carcinogenic and anti-mutagenic activity.

In mammals, excessive cholesterol is removed mainly through conversion to bile acids, and only a small portion is utilized for production of steroid hormones and other endogenous compounds. Enzymes called cytochromes P450 (P450s or CYPs) initiate all quantitatively significant pathways of cholesterol metabolism and bile acid biosynthesis.

Viscum album L., *Epilobium hirsutum L.* and their major polyphenolic ingredients, *o*-coumaric acid (2-hydroxycinnamic acid) and ellagic acid have been used as CAM (Complementary and Alternative Medicine). Recent studies have only evaluated the anti-oxidant effect of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients. Since CYPs are important for cholesterol metabolism, it is important to understand the possible impacts of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients, *o*-coumaric acid and ellagic acid, on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein and mRNA expression. Thus, the idea can be taken about the role of these plants and their major polyphenolic ingredients in drug discovery and development.

1.1 Cytochrome P450 monooxygenase system

Enzymes are the catalysts for nearly all biochemical reactions in the body. Biotransformation is the process where a substance is changed from one form to another by a chemical reaction within the body and enzymes are essential for this process. Biotransformation is an enzyme-catalyzed process in which hydrophobic molecules are converted to hydrophilic, water-soluble molecules, which, usually, are excreted from the body in urine and/or bile. This process is divided into two major biochemical reaction classes: Phase I and Phase II reactions.

Cytochrome P450 (P450s or CYPs) enzyme system is the most important and the best-researched enzyme family involved in Phase I. CYPs belong to super-family of heme-thiolate-monooxygenases. Cytochrome P450 enzymes have a single heme group and give a Soret peak at 450 nm when reduced iron forms and bound to CO (carbon monoxide) (Nebert & Russell 2002; Coon 2005; Pikuleva 2006b; Petterson 2009; Pikuleva & Waterman 2013).

Cytochrome P450 enzyme is expressed in nearly all live forms such as animals, plants, fish, yeasts and bacteria (Nelson *et al.* 1999; Hukkanen 2000; Pikuleva 2006b; Wauthier *et al.* 2007). So far, 18 families and 43 subfamilies of cytochrome P540 have been identified in human (<http://dnelson.utmem.edu>).

CYP families and subfamilies are categorized depending on their resemblance of amino acid sequence. CYP nomenclature is designated by the letters "CYP" (family), a letter (subfamily) and an individual number (gene). Same enzyme families indicate 40 % similarity of amino acid sequence (e.g. CYP7A), and members of same subfamily share 55 % similarity (e.g. CYP7A1) (Nelson 1993; Glue & Clement 1999; Nebert &

Russell 2002; Al-anizy 2005; Pikuleva 2006b; Tang 2007; Lamprecht 2009; Pettersson 2009).

CYPs, known as heme proteins, are distributed in hepatic and extra-hepatic tissues such as kidney, lung, adrenal, and brain. P450s are membrane bound enzymes located generally in endoplasmic reticulum and inner mitochondrial membrane (Russell 2003). However, some data showed that cytochromes P450 also were detected in peroxisomes, different golgi compartments, the outer nuclear membrane and the plasma membrane (Seliskar & Rozman 2006).

CYPs play a key role in biological and clinical processes because they possess numerous substrates both endogenous compounds including steroids and fatty acids, and exogenous compounds including drugs, environmental chemicals and pollutants (Hollenberg & Hager 1973; Arınç & Philpot 1976; Nebert & Russell 2002; Graham & Peterson 1999; Graham & Peterson 1999; Schenkman *et al.* 2003; Coon 2005; Nebert & Dalton 2006; Omura 2006; Estabrook 2003; Wauthier *et al.* 2007; Petterson 2009; Zhang *et al.* 2013).

Different CYPs present in the human enzyme families of 1-3 metabolize different exogenous chemical substances as given in Table 1.1. However, we know that other human CYP families have central biological function because they metabolize physiologically important endogenous compounds such as the cholesterol, bile acids, steroids, eicosanoids, vitamin D3 and fatty acids (Nebert & Russell 2002; Shinki *et al.* 1997; Hukkanen 2000; Hedlund *et al.* 2001; Coon 2005; Pikuleva 2006b; Estabrook 2003; Wauthier *et al.* 2007; Pettersson 2009).

Table 1.1 Human CYP families and their main functions (Hukkanen2000).

Human CYP Families	Function
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic and arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid metabolism
CYP5	Thromboxane synthesis
CYP7	Bile acid synthesis
CYP8	Prostacyclin and bile acid synthesis
CYP11	Steroid synthesis
CYP17	Estrogen and testosterone synthesis
CYP19	Estrogen hormone synthesis
CYP20	Drug metabolism and cholesterol synthesis
CYP21	Steroid synthesis
CYP24	Vitamin D degradation
CYP26	Retinoic acid metabolism
CYP27	Bile acid synthesis and vitamin D ₃ activation
CYP39	Cholesterol synthesis
CYP46	Cholesterol synthesis
CYP51	Cholesterol synthesis

1.1.1 Mechanism of cytochrome P450-dependent hydroxylation

While system of the microsomal cytochrome P450 comprise of cytochrome P450 and NADPH-cytochrome P450 reductase, the mitochondrial system involves three components, cytochrome P450, adrenodoxin and NADPH-adrenodoxin reductase (Figure 1.1). Both microsomal and mitochondrial cytochrome P450 systems are dependent on NADPH (Adalı & Arınç 1990; Arınç 1993; Arınç 1995; Norlin 2000; Omura 2006).

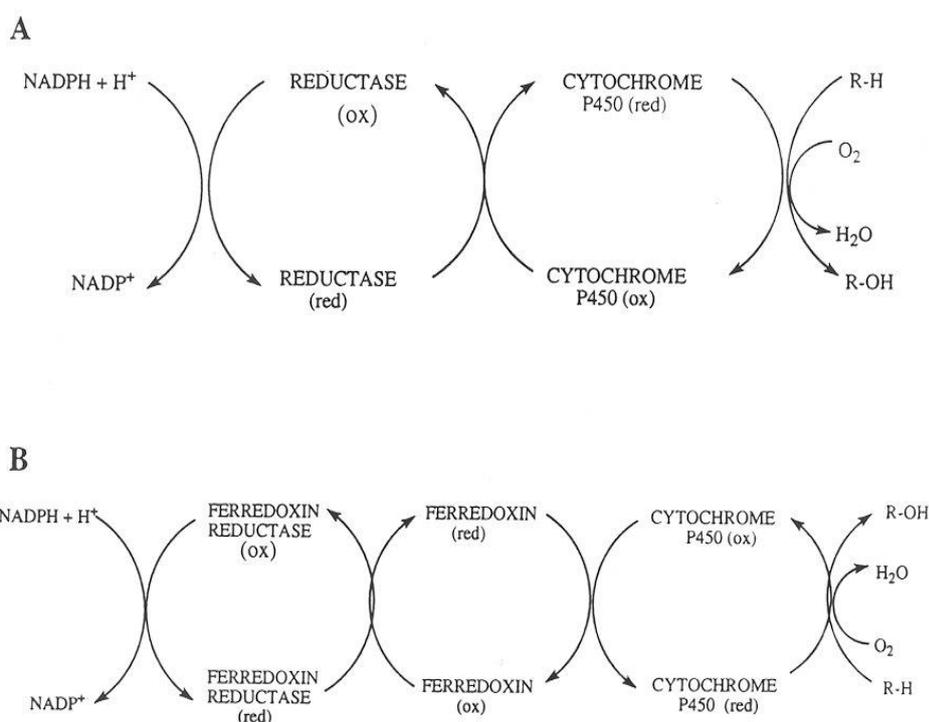
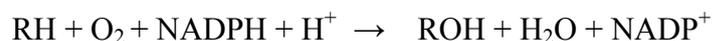


Figure 1. 1 Mechanism of hydroxylation reaction of (A) microsomal and (B) mitochondrial cytochrome P450 (Norlin 2000).

The general catalytic reaction of cytochrome P450 is mainly monooxygenation. The overall reaction is:



RH symbolizes substrate such as drug or endogenous compounds, and ROH is the product. The enzyme activates one atom of molecular oxygen to introduce into the substrate and reduces the other to water.

Figure 1.2 represents catalytic cycle of cytochrome P450. RH associates with the active site of oxidized form of CYP, and yields a ferric-hem substrate complex. The formed cytochrome P450-substrate complex is then reduced by an electron transferred from NADPH via NADPH-cytochrome P450 reductase (microsomal system) or via adrenodoxin and adrenodoxin reductase (mitochondrial system) (Figure 1.1). Then, the first electron donation of iron (Fe^{3+}) is reduced to ferrous state (Fe^{2+}). Next, oxygen is inserted into substrate-enzyme complex after which a second electron is transferred from NADPH via the microsomal or mitochondrial electron transferring components. Substrate is oxidized and a water molecule is produced. CYPs transform generally hydrophobic substrate to a hydrophilic form, more water soluble to become easier to excrete from the body or to facilitate metabolism of other enzymes (Guengerich 1991; Shet *et al.* 1993; Omura 1999; Estabrook 2003; Lamprecht 2009).

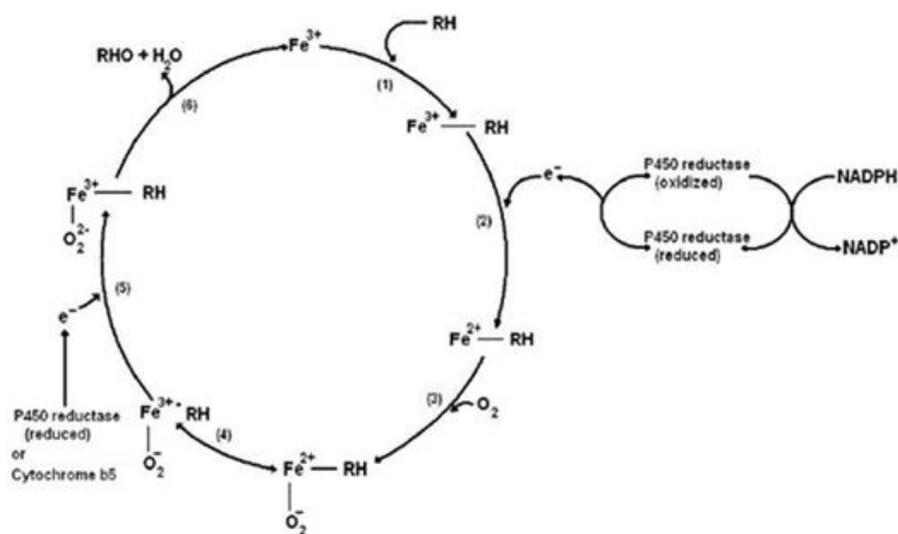


Figure 1. 2 Catalytic cycle of cytochrome P450 reactions. (Fe^{3+}) = oxidized CYP450 enzyme, Fe^{2+} = reduced CYP450 enzyme, Cytochrome b5 = co-enzyme for CYP450, RH = substrate, ROH = product and e^- = electron (Lamprecht 2009).

Mitochondrial P450s are integral membrane proteins of the inner mitochondrial membrane, and receive electrons for the catalysis of monooxygenation reactions from NADPH via NADPH-adrenodoxin reductase and adrenodoxin, which are both soluble matrix proteins. Adrenodoxin (Adrenal ferredoxin, Adx) is a ferredoxin type [2Fe-2S] iron-sulfur protein. Because ferredoxins are low molecular weight (6-25 kDa), soluble and acidic proteins, they include only one [2Fe-2S] iron-sulfur cluster in a molecule as prosthetic group. They act as electron carrier proteins (Grinberg *et al.* 2000; Pechurskaya *et al.* 2007; Ewen 2011). Adrenodoxin reductase (AdR, NADPH-adrenal ferredoxin oxidoreductase) is a FAD-containing flavoprotein. Adrenodoxin reductase, a monomeric kidney-shaped avoenzyme, gets electrons from NADPH and delivers

electrons by adrenodoxin to mitochondrial CYPs. Adrenodoxin and adrenodoxin reductase are joined with the mitochondrial membrane, by ionic interactions (Ziegler *et al.* 1999; Grinberg *et al.* 2000; Hanukoğlu 2000; Beilke *et al.* 2002; Omura 2006; Pechurskaya *et al.* 2006; Ewen *et al.* 2011).

Microsomal P450s are synthesized by the membrane-bound ribosomes of rough ER in the cytoplasm, and co-translationally incorporated into the ER membrane to become integral membrane proteins. On the other hand, free polysomes in the cytoplasm synthesize mitochondrial P450s as precursor peptides with a cleavable presequence at the amino-terminus, released into the cytoplasm, and selectively targeted to mitochondria. The human genome contains 7 genes to code mitochondrial P450s (Table 1.2).

Table 1. 2 Physiological functions of mitochondrial P450s (Omura 2006).

CYP Families	Physiological functions	Tissue distribution
CYP11 A	Side chain cleavage of cholesterol to form pregnenolone	Adrenal cortex, gonad
CYP11 B1	11- β hydroxylation of 11-deoxycortisol to form cortisol	Adrenal cortex
CYP11 B2	Synthesis of aldosterone from 11- deoxy-corticosterone	Adrenal cortex
CYP24 A	24-hydroxylation of 25-hydroxyvitamin D ₃	Kidney
CYP27 A	27-hydroxylation of cholesterol in bile acid biosynthesis, 25- hydroxylation of Vitamin D ₃	Liver, Kidney
CYP27 B	1 α -hydroxylation of 25-hydroxyvitamin D ₃	Kidney
CYP27 C	Unknown	

1.2 Cholesterol

Cholesterol is composed of 27 carbon atom and it has four carbon rings forming steroid structure (Figure 1.3). Both hydrophobic and insoluble cholesterol is lightly amphipathic. Cholesterol presents in all cells of the body and is the most plentiful sterol (a combination of steroid and alcohol) in tissues. Cholesterol is a structural component of cellular membranes, causes cells to become less permeable to water-soluble molecules and balances temperature of the cells. Cholesterol is a main component of the brain, the peripheral nerves and spinal cord. There is cholesterol in myelin sheet in the nervous system, which protects the nerves from the surrounding tissues. Cholesterol has also been found to be connected with gene transcription, protein degradation, enzyme activity, programmed cell death and signal transduction (Sabine 1977; Liscum & Dahl 1992; Estabrook 2003; Pikuleva 2006a).

In addition to function in cellular membranes, cholesterol serves as a parent compound for synthesis of bile acids, steroid hormones (progesterone, estrogen, testosterone, glucocorticoids, and mineralocorticoids) and vitamin D₃ (Sabine 1977; Liscum & Dahl 1992, Maccarrone *et al.* 1998; Incardona & Eaton 2000).

Liver controls the homeostasis of cholesterol in the body. Cholesterol has two sources, both are synthesized from acetyl-coenzyme A (acetyl-CoA) and are taken daily from diet. In human, daily cholesterol input is approximately 1200 mg. 1200 mg of this total is 300-500 mg from diet and 600-900 mg from *de novo* synthesis. 400-600 mg of cholesterol is converted to bile acids, 600 mg is secreted in bile, 85 mg is lost by sloughing of cells, and 50 mg is used for biosynthesis of steroid hormones (Repa & Mangelsdorf 2000; Pikuleva 2006a).

Under normal conditions, cholesterol output is equal to input and cholesterol homeostasis is tightly regulated. Unbalance of cholesterol homeostasis may cause increased cholesterol levels and development of cardiovascular disease (Russell 2003).

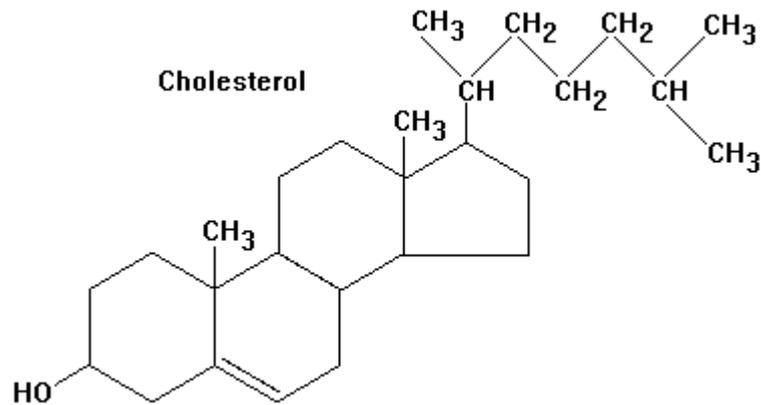


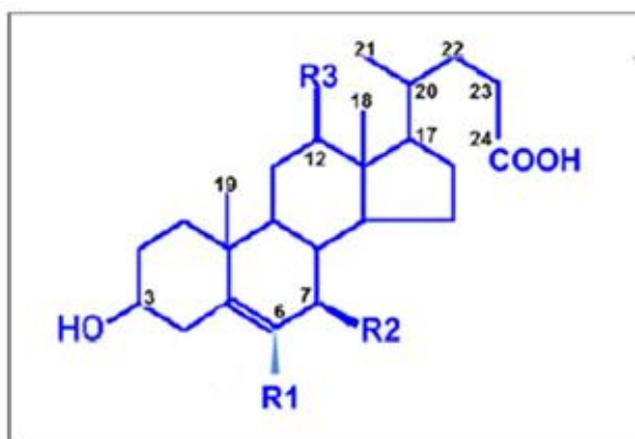
Figure 1. 3 Structure of cholesterol.

1.3 Bile acids

Bile acids are main natural final water-soluble product of cholesterol elimination from body in mammals. This metabolic process has extremely important role in support of balance of cholesterol in the body. Bile acids have 24 carbon atoms, include the steroid nucleus and consist of three six-member rings (A, B and C) and a five-member ring (D), and one hydroxyl group at the 3-carbon position of the steroid nucleus (Figure 1.4) (Nebert & Russell 2002; Estabrook 2003; Pikuleva 2006a; Monte *et al.* 2009).

Bile acids aid absorption of dietary cholesterol and fat-soluble vitamins since they are potent detergents. Besides, bile acids serve as signaling molecules that operate the metabolic pathways. They activate the nuclear receptors (e.g. FXR, PXR) by binding to regulate important genes in drug, vitamin D and lipid metabolism (Russell 2003; Pikuleva 2006a).

Bile acids are divided into two groups, primary and secondary. The primary bile acids (BAs) in humans and rats, synthesized in hepatocytes, are cholic acid (CA) and chenodeoxycholic acid (CDCA). Primary bile acids are precursor for secondary bile acids, deoxycholic acid (DCA), and lithocholic acid (LCA) in human (Figure 1.4). In intestine, deoxycholic acids (DCA) are formed from cholic acids (CA), whereas lithocholic acids (LCA) are formed from chenodeoxycholic acids (CDCA) by bacterial enzyme through deconjugation and dehydroxylation at position-7. The secondary bile acids are less water-soluble and more toxic than the primary bile acids (Salvioli *et al.* 1982; Botham & Boyd 1983; Lida *et al.* 1989; Miki *et al.* 1993; Elias 1999, Eyssen *et al.* 1999; Duarte *et al.* 2009).



Bile acids	R1	R2	R3
Cholic acid	H	OH	OH
Chenodeoxycholic acid	H	OH	H
Deoxycholic acid	H	H	OH
Lithocholic acid	H	H	H

Figure 1. 4 Structure of the most common bile acids (Eyssen *et al.* 1999; Fujino *et al.* 2004; Thomas *et al.* 2008).

Bile acids have a different structure in the orientation of the additional hydroxyl groups. According to Figure 1.4, cholic acid includes two hydroxyl groups at the 7 and 12 carbon positions; while chenodeoxycholic acid and deoxycholic acid include one hydroxyl group at 7 and 12 carbon positions, respectively. But, lithocholic acid does not include any hydroxyl groups in addition to the 3 α -OH group (Radomska *et al.* 1993; Fujino *et al.* 2004; Thomas *et al.* 2008).

1.3.1 Bile acid biosynthesis

Bile acid synthesis initiated by one of different ways, catabolized by cholesterol 7 α -hydroxylase (CYP7A1), cholesterol 24-hydroxylase (CYP46A1), cholesterol 25-hydroxylase (CH25H) and sterol 27-hydroxylase (CYP27A1) (Russell 2003; Pikuleva 2006a). There are two major pathways of bile acid synthesis, termed as classic (neutral) and alternative (acidic), beginning with CYP7A1 and CYP27A1, respectively (Figure 1.5) (Nnamani 2009). These two pathways involved in bile acid biosynthesis produce cholic and chenodeoxycholic acids.

The classic pathway is the most crucial pathway for the biosynthesis of bile acids from cholesterol, and if classic pathway has fallen down under a normal condition, alternative pathway may keep up the bile acid biosynthesis (Pettersson 2009).

The enzymes get involved in two pathways located in different compartments such as endoplasmic reticulum, mitochondria, cytosol and peroxisomes.

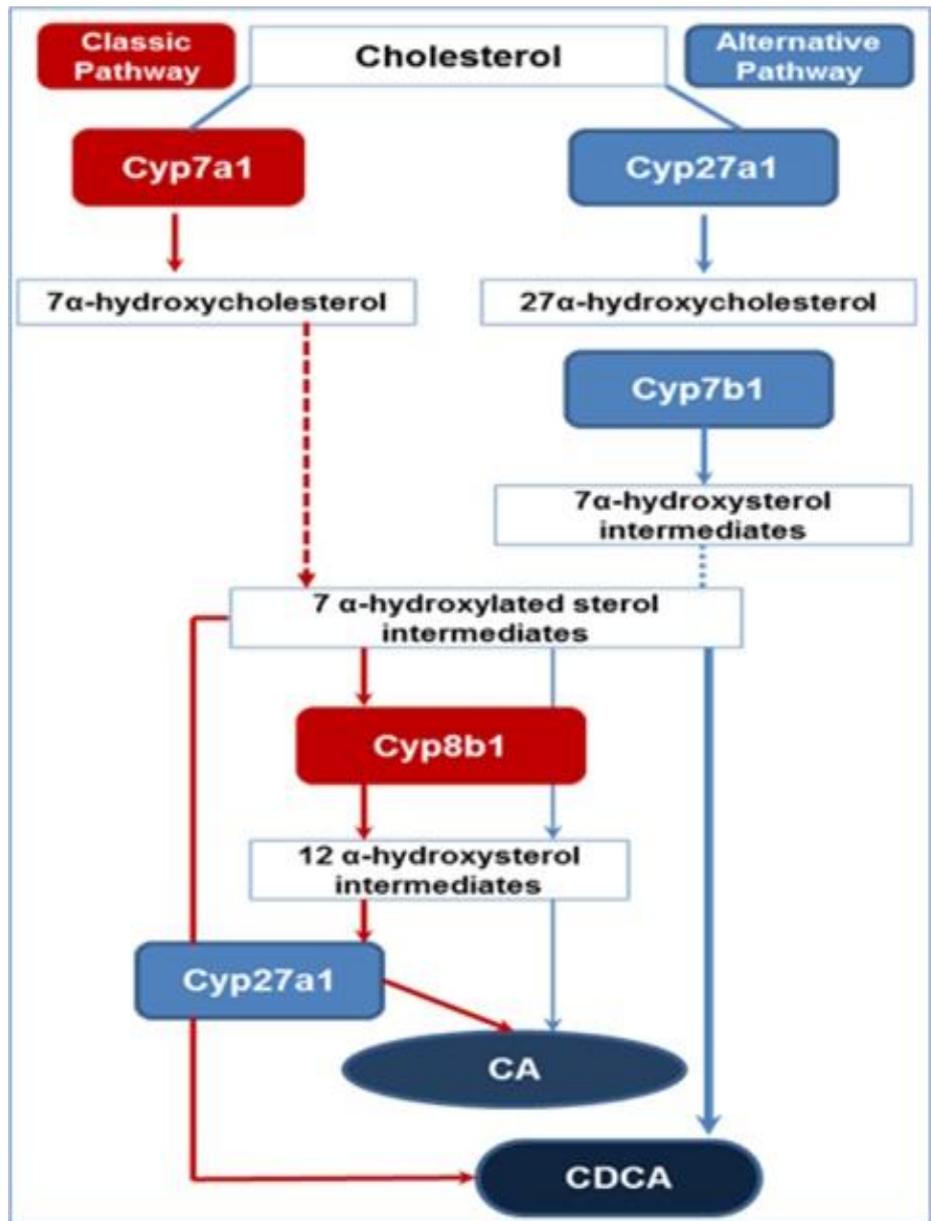


Figure 1. 5 Major bile acid biosynthesis pathways: The classic (red arrows) and the alternative pathways (blue arrows) (Song 2010).

1.3.1.1 The classic or neutral pathway, Cholesterol 7 α -hydroxylase Mediated Pathway

The first and most intermediates of the classic pathway are neutral sterols, for this reason classic pathway is also termed as the neutral pathway (Chiang 2002). It has been described that neutral pathway is responsible approximately for 50 % and 90 % synthesis of total bile acid in rats and humans, respectively (Pikuleva 2006a).

The first and rate limiting enzyme of neutral pathway is cholesterol 7 α -hydroxylase (CYP7A1). Microsomal cholesterol 7 α -hydroxylase is a hepatic enzyme; therefore, this pathway takes place solely in liver. Cholesterol (5-cholesten-3 β ol) is hydroxylated at position-7 of cholesterol, and this way it is converted into 7 α -hydroxycholesterol (5-cholesten-3 β -7 α diol) by CYP7A1 (cholesterol 7-alpha monooxygenase) (Russell 2003; Abrahamson 2005; Pikuleva 2006a,b; Li *et al.* 2006; Estabrook 2003; Song 2010). In the proceeding reaction, 7-hydroxy-4-cholestene-3-one is produced from 7 α -hydroxycholesterol by microsomal 3-hydroxy-C27-steroid dehydrogenase/isomerase (3-HSD). This intermediate product follows two different ways to produce cholic acid (CA) and chenodeoxycholic acid (CDCA). Cholic acid and chenodeoxycholic acids are formed nearly equal in human classic pathway (Chiang 2002).

1.3.1.2 The alternative or acidic pathway, Sterol 27-hydroxylase Mediated Pathway

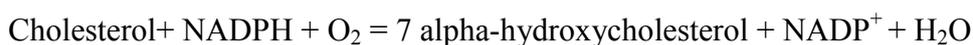
Alternative pathway is also named as acidic pathway, which yields acidic sterols. Alternative pathway is initiated by mitochondrial CYP27A1 (sterol 27-hydroxylase). Unlike CYP7A1; CYP27A1 is a multi-functional mitochondrial enzyme, expressed in many tissues such as lung, kidney, liver, prostate, brain, small intestine, arterial endothelium, skin, fibroblast and macrophages. In contradistinction to neutral pathway, acidic pathway may start in extra-hepatic tissues. The alternative pathway produces mainly CDCA (Russell 2003; Pikuleva 2006a; Pettersson 2009).

Under normal conditions, less than 18 % of total hepatic bile acid synthesis in human is produced by an alternative pathway (Duane & Javitt 1999). This pathway catalyzes the conversion of cholesterol into more polar products in peripheral tissues, and it is important since it disposes of cholesterol in peripheral tissues (Russell 2003).

1.3.1.2.1 Cholesterol 7 alpha-hydroxylase (CYP7A1)

There are four major steps for the synthesis of primary bile acids; initiation, ring structure modification, shortening and conjugation. These 4 steps include 17 enzymes and the synthesis of bile acids starts with one of the several routes. In the classical pathway, cholesterol 7 alpha-hydroxylase (CYP7A1) converts cholesterol (5 α -cholesten-3 β -ol) into 7 α -hydroxycholesterol (5 α -cholesten-3 β -7 α -diol). CYP7A1 is found only in liver and has a low turnover number and prefers cholesterol as substrate.

Cholesterol tightly binds to the active site of CYP7A1 which needs NADPH and molecular oxygen for hydroxylation as given below (Russell 2003; Guengerich 2005).



Cholesterol 7 α -hydroxylase (CYP7A1) is a regulatory enzyme to maintain cellular balance of cholesterol by dominating under normal conditions for a daily degradation of cholesterol (400-600 mg). Gene expression and enzyme activity of cholesterol 7 α -hydroxylase are highly regulated by bile acids and other factors (Nebert *et al.* 2013). Lipid malabsorption, vitamin deficiency and postnatal lethality due to liver failure are related with deficiency of CYP7A1 in mice. In these animals with deficiency of CYP7A1 there occurred 75 % reduction in bile acid biosynthesis, and the reduction was not compensated by other enzymes in bile acid biosynthesis (Pikuleva 2006a, Nebert *et al.* 2013).

Intestinal cholesterol absorption is 50 % less in mutant CYP7A1 mice than a normal mouse, thus cholesterol synthesis increases 200 % in liver to support the maintenance of cholesterol homeostasis. Only 10 % of CYP7A1 deficient mice live after birth (Russell 2003). Medical studies showed that individuals with homozygous a null mutation in CYP7A1 have higher total and LDL cholesterol levels than normal individuals because of the lack of CYP7A1 enzyme activity. Thus, cholesterol accumulates in the liver and excretion of bile acid decreases. Heterozygote mutation in CYP7A1 can also cause hypercholesterolemia due to resistance to cholesterol lowering statins (Nebert & Russell 2002; Nebert *et al.* 2013).

Enzyme activity of CYP7A1 is also influenced by some drugs and hormones, diet, obesity, age and diurnal rhythm (Pikuleva 2006a). It is believed that the activity of CYP7A1 is controlled by transcriptional regulation but, changes of mRNA expression are not usually relevant with the activity of enzyme. It is suggested that the activity of CYP7A1 is regulated by post-transcriptional modification such as cytosolic factors, cholesterol availability, disulfide bonds in the structure of enzyme and phosphorylation.

1.3.1.2.2 Sterol 27-hydroxylase (CYP27A1)

CYP27A1 is called as sterol 27-hydroxylase, P450c27 and vitamin D₃ 25-hydroxylase. CYP27A1 is found in many tissues such as the liver, kidney, lung, brain, prostate, small intestines, arterial endothelium, fibroblasts and macrophages in the body (von-Bahr 2002; Tang 2007; Pettersson 2009; Nebert *et al.* 2013).

Sterol 27-hydroxylase is responsible for the metabolism of Vitamin D₃ in kidney and balance of cholesterol, such as 27-hydroxylation of intermediates in classic pathway and 27-hydroxylation of cholesterol in acidic pathway of bile acid synthesis in liver (von-Bahr 2002; Araya *et al.* 2003; Li *et al.* 2006; Pikuleva 2006a,b; Pettersson 2009). The most plentiful oxysterol in plasma of human and mouse is 27-hydroxycholesterol, and it is produced by sterol 27-hydroxylase from cholesterol (Russell 2003). It has been identified that CYP27A1 has a broad substrate specification but it shows more interest in polar substrates than unpolar substrates (Guengerich 2005).

Inactivation of gene of CYP27A1 in mice leads to a serious impact on biosynthesis of bile acids, which brings about the reduction of intestinal cholesterol absorption and increment of cholesterol synthesis in hepatic and peripheral tissues (Russell 2003).

It has been revealed that more than 50 different mutations in CYP27A1 related with the cerebrotendinous xanthomatosis (CTX), is an uncommonly inherited autosomal recessive multisymptom disorder (Russell 2003; Pettersson 2009). CTX has some distinguishing features such as, decreased primary bile acid synthesis (especially chenodeoxycholic acid) increased excretion of bile salt, low levels of cholesterol in plasma and accumulation of cholesterol and cholestenol, a cholesterol derivative (5 α reduced derivative) which is neurotoxic, in most tissues especially in brain and tendon. This disease causes neurological abnormalities (Russell 2003; Chiang 2004; Pettersson 2009; Pikuleva & Waterman 2013). Also, cholic acid can be used for the treatment of deficiency of CYP27A1 to prevent the synthesis of toxic sterol intermediates in the bile acid biosynthesis (Russell 2003).

In cholesterol homeostasis, CYP27A1 has an important role because 27-hydroxycholesterol formed from CYP27A1 suppresses HMG-CoA reductase, one of enzymes in cholesterol synthesis (Pettersson 2009). Furthermore, bile acids down-regulate mRNA and enzyme activities of CYP27A1. According to some research, there is a huge difference between expression of mRNA and enzyme activity of CYP27A1. Researchers suggested that the reason may be post-transcriptional regulation but mechanism hasn't been known yet (Pikuleva 2006a).

1.3.1.2.3 Sterol 12 α -hydroxylase (CYP8B1)

Sterol 12 α -hydroxylase, microsomal cytochrome P450 (CYP8B1), is essential for production of cholic acid and balance of ratio between cholic acid and chenodeoxycholic acid (Russell 2003; Guengerich 2005; Pikuleva 2006a). It is a liver specific enzyme and has broad substrate specificity. CYP8B1 gene is different and unique from other CYPs due to the lack of intron in its gene sequence (Guengerich 2005; Estabrook 2003; Nebert *et al.* 2013).

7 α -hydroxylated sterol intermediates produced from both neutral and acidic pathway of the bile acid biosynthesis can be hydroxylated by CYP8B1 through the addition of hydroxyl group to the 12 position, which produces cholic acid (CA). If there was no CYP8B1, all intermediates would be converted to chenodeoxycholic acid (CDCA) (Nebert & Russell 2002; Russell 2003). Until now, no disease has been linked with CYP8B1 mutations (Nebert *et al.* 2013).

According to some studies cholic acid arranges feedback regulation of the bile acids biosynthesis. Loss of the CYP8B1 enzyme activity leads to increase of the bile acid biosynthesis by re-suppression of cholesterol 7 α -hydroxylase (Russell 2003).

Destruction of balance of cholic acid and chenodeoxycholic acid brings about hepatic toxicity by reason of high level of chenodeoxycholic acid (CDCA). The increased level of CDCA causes the suppression of CYP7A1 resulting in a decrease of the bile acid biosynthesis and an increase of LDL-cholesterol level (Pikuleva 2006a). The activity of sterol 12 α -hydroxylase is regulated at transcriptional level, and insulin, thyroid hormone and cholesterol reduce mRNA expression of CYP8B1 (Pikuleva 2006a, b). To decrease cholesterol, CYP8B1 can be used because cholic acid produced

from CYP8B1 is more effective than chenodeoxycholic acid to excite intestinal cholesterol absorption (Pikuleva 2006a, b).

1.3.1.2.4 Oxysterol 7 α -hydroxylase (CYP7B1)

CYP7B1, oxysterol 7 α -hydroxylase, is present in a lot of tissues including brain, lung, liver, prostate and kidney. Hydroxylation reaction catalyzed by CYP7B1 can occur either in carbon 7- or carbon 6- area. CYP7B1 participates in 7- α -hydroxylation of 25- and 27-hydroxycholesterol in an alternative pathway of bile acid synthesis (Estabrook 2003; Li-Hawkins 2007; Pikuleva & Waterman 2013; Nebert *et al.* 2013).

The oxysterol 7 α -hydroxylase enzyme activity and expression of protein and mRNA are not changed by cholesterol in rodents, but are increased in rats (Pikuleva 2006a,b). Also, a mutation related with CYP7B1 gene leads to intense hyperoxysterolemia, very high level oxysterol in serum in rats, and neonatal cholestasis in only new born male mice (Estabrook 2003; Li-Hawkins 2007).

CYP7B1, microsomal P450, has an important role in metabolism of oxysterol, neurosteroids and sex hormones. This enzyme is also related with immunosystem, brain function, and cholesterol homeostasis. CYP7B1 is nearly 40 % similar to CYP7A1 (Nebert *et al.* 2013).

1.4 Phytochemicals

Ethno-botanicals, herbs, and plants include nutrients and non-nutrient molecules. Phytochemicals known as non-nutritive active compounds are found naturally in plants and more than a thousand of them are known. They are not fundamental for human life, but recent research showed that they have preventive and curative properties. They act as mimicking hormones and antioxidants and stimulating enzymes, they can also destroy bacteria, enhance the immune system and detoxify carcinogens by activating CYP and phase II enzymes. While some phytochemicals work with other nutrients such as vitamins, some others work alone.

1.4.1 *Viscum album L. (Loranthaceae)*

European mistletoe is named as *Viscum album* in Latin because it has white, viscous and sticky fruit. Its Turkish name is „Ökse otu“ (Ergun and Deliorman 1995). *Viscum album L.* is a bi-perennial shrub and an evergreen semi-parasitic plant widely distributed in Europe. *Viscum album L.* had been placed in Viscaceae family (Loranthaceae) but according to recent research it has been classified a member of Santalaceae family (APG 2003). This folkloric and medicinal plant grows as an epiphyte on the branches of deciduous and dicotyledonous trees such as pine, poplar, apple and locust. It is photosynthetic but this hemi-parasite and epiphyte plant takes minerals and water from its hosts' tissues through a complex endophyte system (Figure 1.6). The phytochemical content of white-berried mistletoe depends on not only the host trees but also its growth area and season (Onay-Ucar *et al.* 2006; Alpsoy *et al.* 2010; Vicaş *et al.* 2011a; Klingbeil *et al.* 2013;

Yusuf *et al.* 2013; Amoako-Attah 2014; Pietrzak 2014; Poruthukaren *et al.* 2014).



Figure 1. 6 *Viscum album L.*

Viscum album L. includes many active ingredients such as viscotoxins, lectins (ML-1, ML-2, and ML-3), glycoproteins, oligo- and polysaccharides, alkaloids, flavonoids (quercetin and its methyl derivatives, kaempferol and some of its methyl derivatives, flavanone and naringenin and phenolic acids (digallic and *o*-coumaric acid) (Luczkiewicz *et al.* 2001; Haas *et al.* 2003; Vicaş *et al.* 2011a,b; Pietrzak 2014). Due to having a huge phenolic profile, mistletoe has many different biological functions. These include anti-tumor, apoptosis-inducing, anti-oxidant, anti-mitotic, anti-bacterial, anti-viral, anti-diabetic, anti-epileptic, anti-cancer and immunomodulatory activities (Hajtó *et al.* 2005; Orhan *et al.* 2005; Onay-Ucar *et al.* 2006; Cetin & Ozcelik 2007; Oluwaseun & Ganiyu 2008; Choudhary *et al.* 2010; Vicaş *et al.* 2011a,b; Lee *et al.* 2014).

Based on the pharmacological research documented until now about these biological functions, *Viscum album L.* (VA) preparations are extensively used as a complementary therapy of cancer. According to a study to understand the mechanism of anti-tumor activity of *Viscum album*, it displays anti-tumor activity by inducing the apoptosis and inhibiting the angiogenesis and several other immuno-modulatory mechanisms (Hedge *et al.* 2011).

Vicaş *et al.* (2011a) showed antioxidant activity of mistletoe by scavenging free-radicals. In that study, *Viscum album L.* increased activity of Phase II enzymes, glutathione S-transferase (GST) and quinone oxidoreductase (QR), and inhibited ovarian tumor cells of humans.

Karagöz *et al.* (2003) showed that *Viscum album L. ssp. album* growing on lime trees has a potent anti-parainfluenza virus activity (Karagöz *et al.* 2003). Moreover, Kim *et al.* (2014) reported that Korean mistletoe extract (KME) has anti-diabetic effect by increasing insulin secretion from the pancreatic β -cell without any effects of cytotoxicity. They suggested that KME may be used for type I diabetic patients by reducing level of blood glucose. (Kim *et al.* 2014).

Alpsoy *et al.* (2010) researched anti-oxidant and anti-mutagenic activities of *Viscum album L.* Since trichloroethylene (TCE) induces oxidative and genotoxic damage, *Viscum album L.* extract was applied on TCE- treated lymphocyte cultures. *Viscum album L.* on TCE treated lymphocyte cultures showed protective properties against especially lipid peroxidation and induction properties to DNA repair mechanism, and the treated-lymphocyte cultures also exhibited anti-oxidant and anti-mutagenic activities (Alpsoy *et al.* 2010).

The effect of an aqueous extract prepared from the leaves of *Viscum album* (Mistletoe) on plasma cholesterol and albumin levels were studied (Ben *et al.* 2006). Whole blood was collected from rats for analysis and results

showed that while the level of total cholesterol (TC) and high density lipoproteins (HDL) significantly increased, the LDL, the total protein and albumin levels did not show any significant change. Thus, Ben *et al.* (2006) suggested that the crude aqueous extract from mistletoe leaf may be relatively safe for therapeutic use as it neither predisposes cardiovascular risk nor adversely affects protein metabolism following a prolonged period of administration.

On account of these biologically important functions, since ancient times mistletoe has been used to treat several illnesses including hypertension, cardiovascular disease, arthritis, diabetes, cancer, some cerebral, congestive and menopausal dysfunctions and epilepsy against the hypotension (Didier *et al.* 2009; Jäger *et al.* 2011; Hong and Lyu 2012).

1.4.1.1 Trans-2-Hydroxycinnamic acid (*o*-coumaric acid)

Phenolic acids can be categorized in two groups: hydroxycinnamic acids (C3-C6 compounds; caffeic acid, *o*-coumaric acid and chlorogenic acid) and hydroxybenzoic acids (C1-C6 compounds; gallic acid, *p*-hydroxybenzoic acid and protocatechuic acid) (Shahidi & Chandrasekara 2010).

Hydroxycinnamic acid also known as phenylpropanoid, is secondary metabolite of herbs. Hydroxycinnamic acid and its derivatives (eg. coumaric acid) are important therapeutic agents for preventing of high blood pressure and stroke, and they also have anti-tumor activity (Svoboda *et al.* 2003; Shahidi & Chandrasekara 2010). Coumaric acid has three isomers based on position of the hydroxyl group on the phenyl group: *p*-, *o*- and *m*-coumaric acid (Figure 1.7). *o*-Coumaric acid, which also named as *trans-o*-hydroxycinnamic acid, is found in many plant products, such as vinegar, coffee, tea, beer, oats and peanuts.

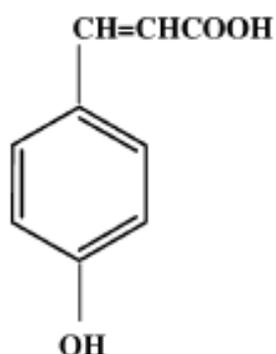


Figure 1. 7 Structure of *o*-coumaric acid (Medina *et al.* 2007).

Researches have evaluated that *o*-coumaric acid has many biological activities, such as anti-lipidemic, anti-obesity, anti-oxidant, and anti-carcinogenic activities (Yeh *et al.* 2005; Hsu *et al.* 2009). Based on its broad range of activities many investigations have been made. Medina *et al.* (2007) investigated anti-oxidant role of hydrocinnamic acids and catechin in fish muscle. Chlorogenic and caffeic acids showed a powerful anti-oxidant (chelating) capacity when compared to *o*-coumaric and ferulic acids based on the number of hydroxylic groups in ortho positions (Medina *et al.* 2007). Moreover, Hsu *et al.* (2009) looked into anti-obesity effect of *o*-coumaric acid and rutin in obese rats. Tissues weight and serum profile of lipid, insulin and leptin, and hepatic cholesterol and triacylglycerol were compared between control and obese rats. Research parameters showed a decrease in *o*-coumaric acid and rutin treated obese-rats compare to non-treated obese rats. They suggested intake of *o*-coumaric acid (OCA) and rutin may be important for repression of oxidative stress, hepatosteatosis and dyslipidemia in rats (Hsu *et al.* 2009).

Sen *et al.* (2013) investigated anti-cancer activity of *o*-coumaric acid in human breast cancer cells. Since *o*-coumaric acid stimulates apoptosis and tumor suppression, they suggested that *o*-coumaric acid can be used as an alternative agent for curing of breast cancer (Sen *et al.* 2013). Afterward, Sen *et al.* (2015) continued to research anti-cancer activity of *o*-coumaric acid and studied the impact of *o*-coumaric acid (OCA) on mRNA and protein expression of CYPs in human hepatocarcinoma cell line. Sen *et al.* (2015) suggested that *o*-coumaric acid induces expression of CYP2E1 and CYP1A2 which metabolize some toxic chemicals such as benzene and pyridine. Thus, it has been offered *o*-coumaric acid may be used for treatment of some hepatic diseases (Sen *et al.* 2015).

In addition to all these biological functions, it is shown that *o*-coumaric acid is important in the field of agriculture. For example, *Eupatorium adenophorum* is known as injurious weed because it creates huge economic loss in agriculture. *Eupatorium adenophorum* possesses large phenolic content and according to many studies, phenolic content of *Eupatorium adenophorum* acts as phytotoxin. To investigate secondary metabolites of *Eupatorium adenophorum* and ecological effects of these secondary metabolites, Zheng *et al.* (2012) isolated 23 compounds including *o*-coumaric acid from *Eupatorium adenophorum*. It has been proposed that 2-hydroxycoumaric acid (*o*-coumaric acid) is the richest compound in *Eupatorium adenophorum* exhibited a functional role in the plant's invasion by suppressing of seed germination and triggering of root tips cell death (Zheng *et al.* 2012). Furthermore, in another research, derivatives of trans-cinnamic acids in grape can be used for making wine to protect unpleasant taste and smell of wine (Söylemezoğlu 2003).

1.4.2 *Epilobium hirsutum* L. (Onagraceae)

Epilobium is one of the genus in Onagraceae family. Genus of *Epilobium* includes more than 200 species; especially it's most known are *Epilobium hirsutum*, *Epilobium angustifolium*, and *Epilobium parviflorum* (Battinelli *et al.* 2001; Onar *et al.* 2012).

Epilobium hirsutum L. is also commonly called as greathairy willow herb, codlins and cream, European fireweed, apple-pie and cherry-pie. *Epilobium hirsutum* L., great willow-herb, grows in Eurasia, North Africa, Europe, Southern Australia and United States. *Epilobium hirsutum* distributed in restricted geographical area since it does not show tolerance to extreme conditions. It specifically needs nutrition and moisture, so native habitats of great willow-herb are ditches, stream or river beds, pastures, and meadows (Shamsi & Whitehead 1974; Pakvaran *et al.* 2012).

Epilobium hirsutum L. is an angiosperm, semi-aquatic and herbaceous perennial plant. It is softly-hairy herb because fine soft hairs cover all herbs. Between 3 to 6 feet tall flowering plant has rose-purple colored flowers, each flower includes four notched petals and sepals and eight stamens and lanceolate, while shaped leaf is arranged oppositely (Figure 1.8). Herb is flowering in mid-summer (July and August) (Bakarat 1997).



Figure 1. 8 *Epilobium hirsutum* L.

Bakarat *et al.* (1997) used HPLC to analyze phenolic profile of *Epilobium hirsutum* as a fingerprint, they found 30 polyphenolics including ellagic, gallic, protocatechuic, valoneic dilactone and *p*-coumaric acids, methyl gallate, *p*-methoxy gallic acid methyl ester, 6-O-galloylglucose, 1,6-di-O-galloylglucose, 2,3'-di-O-galloylglucose, 1,2,6-tri-O-galloylglucose and the 3'-O-glucuronides, 3'-O-arabinosides and 3'-O-rhamnosides of kaempferol, quercetin and myricetin, their free aglycones 3'-O-galactoside (Bakarat *et al.* 1997; Baldwin 1999).

Epilobium hirsutum was used traditionally to treat gastrointestinal and prostate diseases, menstrual disorder, stomach ulceration, gastritis and sleeping disorders (Melchior 1972; Hiermann *et al.* 1986; Battinelli *et al.* 2001; Štajner *et al.* 2007). *Epilobium hirsutum* also has anti-bacterial, anti-microbial, anti-exudative, anti-nociceptive, anti-septic and anti-phlogistic effects (Gruenwald *et al.* 1998; Štajner *et al.* 2007; Ebrahimzadeh *et al.* 2010; Pakvaran *et al.* 2012).

There are many medical studies to investigate of these biological impacts of *Epilobium hirsutum*. Wojdylo *et al.* (2007) studied 32 Polish herbs which have rich phenolic content including *Epilobium hirsutum*. After DPPH and ABTS radical cation assays, the highest antioxidant activity was taken from *Epilobium hirsutum*. That is a good relationship between total phenolic content and the antioxidant activity. Researchers suggested some plants have rich phenolic content like *Epilobium hirsutum* could be used a natural antioxidant source (Wojdo *et al.* 2007). Furthermore, to evaluate anti-tumor action of *Epilobium hirsutum* L. (Onagraceae) and *Maclura aurantiaca* (Moraceae), Wojdo *et al.* 2007 treated mice with these two plants extract. *Epilobium* treated mice showed prolonged the life time and the highest antioxidant activity (Wojdo *et al.* 2007). Moreover, because phenolic compound can be used as free radical scavengers, Ebrahimzadeh *et al.* (2008) detected a linear relationship between phenolic content and chelatory

activity. In that research, *Epilobium hirsutum* and *Mentha arvensis* showed a good chelator activity (Ebrahimzadeh *et al.* 2008). Also to evaluate antibacterial effect of *Epilobium hirsutum*, *Cyprinus carpio* was fed different concentrations of the great willow-herb. Whereas there was no significant difference of between control and treated group from growth performance and survival patterns, there was a significant difference *Aeromonas hydrophila* challenge. *Aeromonas hydrophila*, a gram negative pathogen, is found generally in the gastrointestinal tract of fish. According to the research, ethanol extract of great willow herb inhibits the growth of *Aeromonas hydrophila* (Pakvaran *et al.* 2012).

Above all Ghaderian and Ravandi (2012) collected 146 plants from Sarcheshmeh copper mining region whose soil is rich from copper (Cu) in order to check copper concentration of these plants. Only *Epilobium hirsutum* and *Piptostigma fugax* showed highest concentration of copper. In the light of results Ghaderian and Ravandi offered these two plants can be used as phytoremediation to dispose contamination of copper (Ghaderian & Ravandi 2012).

Lipid oxidation and protein oxidation threat quality of meat. Phytochemicals known as natural antioxidants are important tools to inhibit oxidation reaction. In a recent investigation, Cando *et al.* (2014) examined *Epilobium hirsutum* treated cooked beef. It had been found that the great willow-herb especially inhibits lipid oxidation (Cando *et al.* 2014).

1.4.2.1 Ellagic acid

2,3,7,8-tetrahydroxy-chromeno [5,4,3-cde] chromene-5,10-dione, ellagic acid, is a dimeric derivative of gallic acid (Figure 1.9) (Landete 2011; Sepulveda *et al.* 2011). Ellagic acid, a dilactone of hexahydroxydephenolic acid and hydrolysable tannins, has four rings that exhibit lipophilic domain and four phenolic groups and two lactones that exhibit hydrophilic domain. So, it is unaffected by high temperatures (Ascacio-Valdes *et al.* 2010; Sepulveda *et al.* 2011).

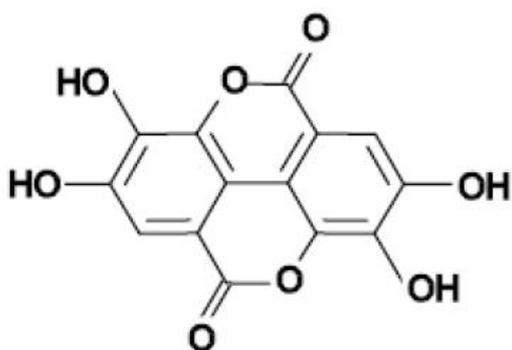


Figure 1. 9 Structure of ellagic acid.

Ellagic acid is therapeutic agent for many diseases and it is found in pomegranate, strawberries, cloudberry, blueberry, blackberry, grapes, raspberries, cranberries, pecans, walnuts and black currants (Bala *et al.* 2006). Ellagic acid in foodstuff is found either generally conjugated with a glycoside moiety or part of ellagitannins (Seeram *et al.* 2004).

It has been showed that ellagic acid has activity of anti-cancer, anti-inflammatory, antioxidant, anti-nociceptive, anti-atherosclerotic, anti-

hyperlipidemic, oxygen-scavenging, vasorelaxing, antiperoxidating, estrogenic and/or anti-estrogenic and anti-viral (Hassoun *et al.* 2004; Landete 2011). The researchers suggested that active metabolites of ellagic acid, especially Uro-A and Uro-B, have an important role on these therapeutic uses (Usta *et al.* 2013).

For the first time, Tell (1986) proposed that ellagic acid has a potent activity of anti-carcinogenic and anti-mutagenic by making DNA-adduct, which ellagic acid masks binding side of DNA in order to protect engagement of carcinogen or mutagen agents (Tell 1986).

Furthermore, it has been showed that pomegranate exhibits anti-oxidant activity owing to its phytochemical content (Yu *et al.* 2005). Yu *et al.* (2005) indicated that pomegranate extract has good effects in atherosclerotic animals due to presence of ellagic acid (Yu *et al.* 2005; Bell & Hawthorne 2008). Pomegrate juice includes not only ellagic acid both also pro-estrogenic and estrogenic compounds that they have effects on spread and growth of prostate tumors. So, it is important to create a treatment establish on one compound such as ellagic acid for hormone dependent disorder (Pantuck *et al.* 2006). Also, phenolic compounds including ellagic acid can help cell to show anti-oxidant defense by inhibiting the expression of CYP and NADPH oxidase (Mazumder *et al.* 1997). Larrosa *et al.* (2006) researched apoptotic effect of ellagic acid in colon cancer. It has been found that ellagic acid induces apoptosis in colon cancer cells (Larrosa *et al.* 2006). According to results taken from studies of human bladder cancer, ellagic acid has effects on subcellular signaling by inducing p53 and p21, and decreasing COX-2 cycling D1 and nuclear factor kappa (NF κ B) (Li *et al.* 2005). Moreover, in another investigation, to study anti-inflammatory and anti-nociceptive activity of ellagic acid (EA), it was been suggested ellagic acid has a beneficial effect on inflammatory pathway by inhibiting COX (cyclooxygenase), Interleukin (IL) 1-Beta, mutagen activated protein

kinases and tumor necrosis factor alpha induced activation of activator protein 1, by inducing iNOS (nitric oxide synthetase), COX2 (Afaq *et al.* 2005; Umeselma & Sudhandrian 2010).

1.5 Scope of the Study

In mammals, excessive cholesterol is removed mainly through conversion to bile acids. Cytochromes P450s initiate all quantitatively significant pathways of cholesterol metabolism and bile acid biosynthesis. The bile acid pathway is very important in maintaining normal cholesterol levels. If cholesterol homeostasis is changed, some conditions such as atherosclerosis, cholestasis and neurological diseases can be seen because of accumulation of cholesterol in one or more compartment in the body.

There is a great promise towards traditional medicine such as herbal remedies or dietary supplements. Mistletoe (*Viscum album L.*) and willow herb (*Epilobium hirsutum L.*) have been used traditionally for prevention and treatment of some diseases since the throughout the history of human. These two plants include phytochemicals such as phenolic acids (*o*-coumaric acid and ellagic acid). Phytochemicals have important role in human health as substrates, co-factors and inhibitors for biochemical reaction; scavengers of toxic compounds and complexes improve the soaking up of essential nutrients. Phytochemicals have also biological activities such as anti-oxidant, anti-cancer and anti-bacterial.

So far, it has been generally studied anti-oxidant activity of herbs. This study is the first about the possible potency of medicinal plants *Viscum album L.* and *Epilobium hirsutum L.* extracts and their major polyphenolic ingredients, *o*-coumaric acid (2-hydroxycinnamic acid) and ellagic acid on

rat liver cholesterol and bile acid metabolizing CYP7A1, CYP27A1, CYP8B1 and CYP7B1. The water extracts of *Viscum album L.*, *Epilobium hirsutum L.* plants and also *o*-coumaric acid and ellagic acid phenolic compounds were injected intraperitoneally as 10 mg/kg, 37.5 mg/kg, 30 mg/kg and 20 mg/kg for 9 days, respectively. Then, *in vivo* effects of *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid on rat liver cholesterol and bile acid metabolizing CYPs were analyzed by determining protein and mRNA expression levels using western blotting and qPCR techniques, respectively.

It has been very popular to research effects of total plant extract, but there is a big dilemma about using total plant extract because plants include many different polyphenolic compounds. To solve this problem, researchers began to prefer investigate polyphenolic compounds which are isolated from plants rather than use of total plant extracts.

The overall scope of the present study was to investigate the relevance of the biosynthesis of bile acids from cholesterol and phytochemicals. The specific aims were:

- to research and compare possible effects of *Viscum album L.* and its major ingredient (*o*-coumaric acid) on rat liver cholesterol and bile acid metabolizing CYPs.

- to research and compare possible effects of *Epilobium hirsutum L.* and its major ingredient (ellagic acid) on rat liver cholesterol and bile acid metabolizing CYPs.

Therefore, in order to accomplish these; we analyzed that phenolic profile (LC-MS analysis), phenolic and flavonoid content of these plants. Also, we studied the expression of protein and mRNA of cholesterol and bile acid metabolizing CYP7A1, CYP27A1, CYP8B1 and CYP7B1 in control and

treated groups of animals. Besides, we analyzed CYP7A1 enzyme activity and total cholesterol levels in serum.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; A894987 605), dipotassium phosphate (K_2HPO_4 ; A611101 528), ethylene diamine tetra acetic acid disodium salt (EDTA; 08421), folin-phenol reagent (1.09001.0500), glacial acetic acid (0056), glycerol (04093), hydrochloric acid (HCl; 1.00314), magnesium chloride (MgCl_2 ; Art.5833), methanol (02500), potassium chloride (KCl; 4935), potassium dihydrogen phosphate (KH_2PO_4 ; A319173-204), sodium carbonate (Na_2CO_3 ; 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), Triton X-100 (11869.1), zinc chloride (ZnCl_2 ; 108815) were purchased from Merck KGaA, Darmstadt, Germany.

Acrylamide (A-8887), ammonium acetate (A7672), ammonium per sulfate (APS; A-3678), bovine serum albumin (BSA; A788), bromophenol blue (B8026), cholesterol (C-8667), diethanolamine (D-2286), diethylpyrocarbonate (DEPC; D5758), N^ε-N^ε-Bis-methylene-acrylamide (M7256), N-N-dimethylformamide (D-8654), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB; D-8130), dithiothreitol (DTT; D0632), glycine (G-7126), β-mercaptoethanol (M6250), phenazine methosulfate (P9625), secondary antibody AP mouse (A3562), secondary antibody AP rabbit (A3687), sodium dodecyl sulfate (SDS; L4390), sodium-potassium tartarate (S-2377),

trisma base (T1503), tween 20 (P1379), xylene cyanol (X4126) were the products of Sigma-Aldrich, Germany.

Nitrotetrazolium blue chloride (NBT; A1243) was obtained from AppliChem GmbH, Darmstadt, Germany.

5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821), dichlorodiphenyl trichloroethane (DDT; R0861), gene ruler™ 50 bp DNA ladder (SM0371) and pre-stained protein ladder (SM0671) were taken from Fermentas, USA.

Primary antibody CYP7A1 (sc-25536), primary antibody CYP27A1 (sc-14835), Primary antibody CYP8B1 (sc-23515) and Primary antibody CYP7B1 (sc-26087) were purchased from Santa Cruz Biotechnology, Texas, USA.

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

Ethanol (32221) and acetyl acetone (33005) were obtained from Riedel de-Haen Chemical Company, Germany.

Fast Start Universal SYBR Green Master (ROX) (04913850001) was obtained from Roche Applied Science, Basel, Switzerland.

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

2.2 Methods

2.2.1 Collection of *Viscum album L.* and *Epilobium hirsutum L.*

This part of the study was carried out with the collaboration of Ankara University, Faculty of Pharmacy, Pharmaceutic Botany Department. The location, habitat, distribution, and blooming time of *Viscum album L.* and *Epilobium hirsutum L.* were identified based on publishes of Davis (1978 and 1984). These plants were collected from unpolluted field and their locations were described below;

Viscum album L.: Turkey, Kayseri, Pınarbaşı, Melikgazi Village, 1800 m. altitude, in August (2008). Samples of *Viscum album* growing on pine trees (*Pinus sylvestris L.*) identified as *Viscum album L. sub austriacum* (Wiesb.)

Epilobium hirsutum L.: Turkey, Düzce, Gölyaka, 563 m. altitude, in June (2009).

Voucher specimens (No: AEF 25945; No: AEF 25812) of *Viscum album* and *Epilobium hirsutum* were deposited in the Herbarium of Faculty of Pharmacy at Ankara University, respectively.

2.2.2 Extraction of *Viscum album L.* and *Epilobium hirsutum L.*

Before losing the morphology, collected herbs were pressed depending on the herbarium rules and plants stored in the herbarium of Ankara University (AEF). Samples dried with press and lyophilized to use in the laboratory.

Air dried and powdered 50 g plant samples for each taxon were subjected to maceration in sterile ddH₂O by using Heidolph mechanic shaker at 300 rpm/minute at RT (room temperature) for 8 hours. Plant extracts obtained were filtered through Whatman filter paper, dried in freeze dryer (Christ Gamma 2-16 LSC), and weighed. Then, yield calculations were made (19 % for *Viscum album L.*; 15 % for *Epilobium hirsutum L.*: weight/weight), the extracts of plants were gassed with nitrogen to prevent oxygen interaction, and held in at -20°C until use for the assays.

2.2.3 Phenolic Profiles of Plants

2.2.3.1 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of *Viscum album L.* and *Epilobium hirsutum L.* Water Extracts

Identification and quantification of the bioactive compounds of *Viscum album L.* and *Epilobium hirsutum L.* were carried out by Liquid Chromatography Mass Spectrometry (LC-MS) (AGILENT 6460 Triple Quadrupole System (ESI+Agilent Jet Stream) coupled with AGILENT 1200 Series HPLC"). The analysis was performed by METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey. Zorbax SB-C18 (2.1 x 50mm x 1.8 μ) colon was used in Liquid Chromatography for reverse phase

chromatographic separation of all samples while Proshell 120 EC-C18 (3.0 x 50 mm x 2.7 μ) colon was used for ellagic acid. Mobile phase was prepared using 2 solvent systems; Solvent 1: 0.05 % Formic acid + 5 mM ammonium formate (10 mM Ammonium acetate for ellagic acid) and Solvent 2: Methanol (MS grade). The spectra of eluted samples were monitored between 200 and 500 nm. Flow rate was arranged as 0,3 ml/min., analysis length was 13 minutes with gradual mobile phase flow and injection volume was 5 μ L. Standard curve was drawn based on the serial dilution of samples (0,15-0,31-0,625-1,25-2,5-5-10 ppm). Mass Spectrometry was carried out in Agilent 6460 LC-MS. In this procedure, ESI+Agilent Jet Stream, Agilent Bin Pump-SL (G1312B9), Agilent h-ALS-SL+ (G1367D) and UHPLCMS 30 were used as ionization source, pump, automatic sampler, and nitrogen sampler, respectively. After choosing MRM (Multiple reaction monitoring) as an analysis mode, gas temperature, sheath gas temperature, gas flow rate, nozzle voltage and capillary voltage were arranged to 350°C, 350°C, 10 ml/ml, 500 V and 4000V, respectively. The following pure standards were used for analysis of phenolic content of *Viscum album L.* extract; chlorogenic acid, gallic acid, caffeic acid, quercetin dehydrate and *o*-coumaric acid. For analysis of bioactive compounds of *Epilobium hirsutum L.* extract; ellagic acid, gallic acid, caffeic acid, *p*-coumaric acid and quercetin dehydrate were selected as standards based on the literature. Agilent G3793AA Mass Hunter Optimizer software was used for analysis.

2.2.3.2 Determination of Total Phenolic Content

Total phenolic content was determined by the Folin-Ciocalteu method (Singleton *et al.* 1999). This method is based on the reduction of phosphotungstate-phosphomolybdate complex by phenolic compounds. The

reaction product gives blue color and its absorbance can be measured at 765 nm spectrophotometrically. *Viscum album L.* extracts were diluted in dH₂O with serial dilutions as 1 mg/ml and 2 mg/ml whereas *Epilobium hirsutum L.* extracts were diluted as 0,2 mg/ml and 0,6 mg/ml. 23 µl of samples was mixed with 1817 µl distilled water, 115 µl Folin-Ciocalteu reagent (dilution 1:10, v/v) and 345 µl of 15% Na₂CO₃ solution. Then, the mixture incubated at room temperature for 2 hours in the dark. The absorbance was measured at 765 nm using a spectrophotometer (Shimadzu). The calibration curve was drawn using range of concentrations between 25-200 µg/ml gallic acids. The results were expressed in mg gallic acid equivalents (GAE)/g fresh matter. The experiments were carried out duplicates and repeated three times.

2.2.3.3. Determination of Total Flavonoid Content

Determination of total flavonoid content was performed by colorimetric method of Chang *et al.* (2002) in which sodium nitrite reacts with aluminum chloride and flavonoids and generates the red color. *Viscum album L.* extracts were diluted in dH₂O with serial dilutions as 1 mg/ml and 2 mg/ml whereas *Epilobium hirsutum L.* extracts were diluted as 1 mg/ml and 1,5 mg/ml. 10 % (w/v) sample in methanol was mixed with 1.5 mL of methanol, 0.1 mL of 10 % aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, and incubated at room temperature for 30 min. The absorbance of the mixture was measured at 510 nm using a spectrophotometer (Shimadzu). Total flavonoid content was calculated based on the calibration curve prepared by using catechin solutions at concentrations from 50 to 300 µg/ml. The experiments were carried out duplicates and repeated three times.

2.2.4 Animal Studies

This part of the study was carried out with the collaboration of Pamukkale University, Biology Department. Permissions of Ethical Committee were taken from Pamukkale University Experimental Animal Ethics Committees and METU.

12 weeks old healthy male Wistar Albino rats (*Rattus norvegicus*) which 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 were carried out based on national standards under fitting regimes with Veterinary services and in accordance with the Declaration of Helsinki.

The water extracts of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients (*o*-coumaric acid and ellagic acid) were intraperitoneally (i.p.) injected to the rats. For the experiment, animals were divided into 5 groups:

C: Control (no injection): Rats in this group never made any application but they were kept under the same conditions with other groups during the experiment.

VA: The water extract of *Viscum album L.* was injected intraperitoneally as 10 mg/kg for 9 days.

EH: The water extract of *Epilobium hirsutum L.* was injected intraperitoneally as 37.5 mg/kg for 9 days.

CA: *o*-Coumaric acid was injected intraperitoneally as 30 mg/kg for 9 days.

EA: Ellagic acid was injected intraperitoneally as 20 mg/kg for 9 days.

At the end of the treatment protocol given above and after 16 h of fasting, the rats were euthanized with decapitation. Blood was collected by heart puncture and the livers were isolated and stored at -80 °C till preparation of microsomes and S1.5 fractions.

2.2.5 Preparation of Rat Liver Microsomes and S1.5

All steps were performed at 0-4° C. The fat and connective tissues were removed from livers, and to remove the excess blood, the livers washed with cold distilled water and 1.15 % KCl solution several times. Serum samples and livers were stored at - 80°C. Microsomal and S1.5 fractions of the rat livers were prepared based on Schenkman and Cinti method (1978) as optimized by Şen and Kırıkbakan (2004). The livers were weighed and chopped with scissors. The minced livers were homogenized in homogenization solution containing 50 mM Tris-HCl, pH 7.5, 3 mM EDTA pH 7.8, 0.5 mM PMSF, 0.3 mM ε-ACA, 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 glass homogenizer (Potter-Elvehjem) coupled with a motor-driven teflon pestle at 2600 rpm. Ten passes were applied to liver tissues for the homogenization. The homogenates were centrifuged at 1500 x g for 10 minutes to obtain S1.5 fraction. The pellets were re-centrifuged at 12 000 x g for 25 minutes and the supernatant fraction containing endoplasmic reticulum and soluble fraction of the cell was filtered by cheese cloth. The supernatants were mixed cold 0.16 M CaCl₂ and centrifuged at 32 000 x g for 25 minutes. The microsomal pellets were suspended in homogenization solution and then re-sedimented at 32 000 x g for 20 minutes. Supernatants were removed and pellets were re-suspended in re-suspension solution of 10 % glycerol containing 2 mM EDTA at a volume of 0.5 ml for each gram of liver tissues. The microsomal suspensions were stored at - 80 °C for assays.

2.2.6 Protein Determination

The protein concentrations of microsomes and S1.5 were determined according to the method of Lowry *et al.* (1951) using BSA (Crystalline bovine serum albumin) as the standard. Initial dilutions of microsomal and S1.5 fractions were made and for each tube dilution was done by adding 0.1 to 0.5 ml of initially diluted samples into each tube and completed it to final volume of 0.5 ml with distilled water. Then, 2.5 ml of Lowry alkaline copper reagent (including 2 % copper sulphate, 2 % sodium potassium tartarate and 0.1 N NaOH containing 20 % sodium carbonate in a ratio of 1:1:100 in respectively) was added to each test tube. After mixed tubes, tubes stored at RT (room temperature) for 10 minutes for occurring copper reaction in alkaline medium. Next, 0.25 ml of folin reagent (2N) diluted 1:1 ratio using distilled water was added to the each test tube and mixed within 8 second by vortex. Absorbance of samples was measured at 660 nm after all tubes were incubated at RT (room temperature) for 30 minutes. Five different protein concentrations of standard tubes (20, 50, 100, 150, 200 µg BSA / ml) were prepared from BSA. Using absorbance of standards, a standard curve was drawn and protein concentration of samples was calculated by the following formula:

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

2.2.7 Protein Expression Analysis by Western Blotting Technique

The impacts of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients (*o*-coumaric acid and ellagic acid) on protein expressions of cholesterol and bile acid metabolizing CYP7A1, CYP27A1, CYP8B1 and CYP7B1 were determined according to the method of Towbin *et al.* (1979) with some optimizations. Before application of Western Blot immunodetection technique, proteins were separated using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) method as reported by Laemmi *et al.* (1970).

In the SDS-PAGE method, proteins were separated in 4 % stacking gel and 8.5 % separating gel through a discontinuous buffer system. Preparation of stacking and separating gel solutions were given in Table 2.1.

Mini Protean-tetra cell mini trans blot module (165-8033; Bio-Rad, Richmond, CA, USA) was used to carry out vertical slab gel electrophoresis. The gel sandwich unit was set up between two glass plates with one cm space. Firstly, prepared separating gel as described in Table 2.1 was transferred into sandwich unit. Not only to provide smooth gel layer but also to make fast polymerization isopropanol alcohol was added on top of the separating gel solution since it protects the gel to contact with the air and take away air bubbles. Isopropanol was poured after polymerization of separating gel. Then, stacking gel solution was prepared as described in Table 2.1. Fresh prepared stacking gel solution was transferred into the sandwich and a comb was immediately inserted into stacking solution. The comb was removed without giving any damage the wells after polymerization of stacking gel solution. Afterward, electrode running buffer (ERB; 25 mM Tris, 192 mM glycine and 0.1 % SDS) was poured to fill

wells and a syringe was used to remove un-polymerized chain particles and air bubbles into the wells.

Table 2. 1 Constituents of stacking and seperating gel solutions.

Components	Stacking Gel (4 %) (0.125 M Tris, pH 6.8)	Separating Gel (8.5 %) (0.375 M Tris, pH 8.8)
Gel Solution (ml)	1.3	8.5
Distilled water (ml)	6.1	13.52
Separating gel buffer (ml)	-	7.5
Stacking gel buffer (ml)	2.5	-
10 % SDS (ml)	0.1	0.3
10 % Ammonium persulfate (ml)	0.05	0.15
TEMED (ml)	0.02	0.03
Total volume (ml)	10	30

Protein samples were diluted with 4X sample dilution buffer in a ratio 1:3 (1 part buffer and 3 parts sample) to obtain 2 mg/ml of protein. After dilution with 4X sample dilution buffer (0.25 M Tris HCl pH6.8; 40 % glycerol; 8 % SDS; 20 % β -mercaptoethanol and 0.01 % bromophenol blue), diluted samples were boiled for 120 second in water bath. After the running module was filled with ERB, 20 μ g each of samples and protein molecular weight marker (Fermentas; SM0671) were applied to different wells by using Hamilton syringe. Before the electrophoresis unit was connected with power supply (Bio Rad Laboratories, CA, USA) to run at 15 mA-150 V for stacking gel and 30 mA-300 V for separating gel, gel running module was put in running tank.

When electrophoresis was completed, gel was removed for western blot. Gel was transferred into transfer buffer (TB; 25 mM Tris, 192 mM glycine and 20 % methanol) for 15 minutes in order to equilibrate the gel and remove SDS and buffer salt coming from the SDS-PAGE method. Transfer sandwich was set up as shown in Figure 2.1.

The Western Blot sandwich was replaced into Mini Trans Blot module (165-8033; Bio-Rad Laboratories, Richmond, Ca, USA) after sandwich saturated with transfer buffer. Samples transferred from gel to PVDF membrane was carried out at 400 mA-90 V for 90 minutes in cold room. End of this process, „blot“, a membrane including transferred proteins, was obtained. Later, in order to remove transfer medium buffer and salts the membrane washed with TBST (Tris Buffer Saline- Tween 20; 20 mM Tris-HCl pH 7.4, 0.5 M NaCl and 0.05 % Tween 20) for 10 minutes. After washing step, the „blot“ was incubated with blocking solution (5 % nonfat dry milk in TBST) for one night. Blocking process helps to cover empty spaces of protein to prevent the non-specific binding.

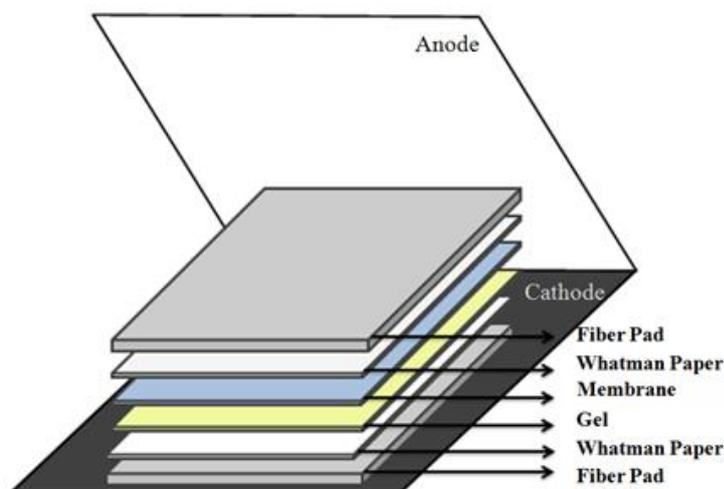


Figure 2.1 Westren Blot sandwich preparation.

The blot was incubated with primary antibody for 2 hours. Antibodies used in the experiments, their brands and the optimum dilutions for Western Blot Analysis were given in Table 2.2. Before incubation with secondary antibody, the blot was washed with TBST 3 times to remove excessive and nonbonding antibody. After 1 hour incubation with secondary antibody, the blot was washed again with TBST three times for 5 minutes each. Washing step is very important to remove excessive antibody because it can give non-specific reaction with substrate solution. At the end, the blot was incubated with substrate solution (alkaline phosphate substrate solution) prepared as given in Table 2.3. Finally, the image was photographed by a gel imaging instrument which based on a computer, (Infinity 3000, Vilber Lourmat, Marne-la-Vallee Cedex1, France) through Infinity-Capt Version 12.9 software. The density of protein bands were quantified using Image J visualization software (Scion Image Version Beta 4.0.2).

Table 2.2 Antibodies used in experiments, their brands and the optimum dilutions for Western Blot Analysis.

	Primary	Primary	Brand	Secondary	Secondary	Brand
	AB	AB		AB	AB	
		Dilution			Dilution	
CYP7A1	Rabbit Anti- CYP7A1	200	Santa Cruz	Anti-Rabbit IgG AP Conj.	2000	Santa Cruz
CYP27A1	Goat Anti- CYP27A1	1000	Santa Cruz	Anti- Mouse IgG AP Conj.	2000	Santa Cruz
CYP8B1	Goat Anti- CYP8B1	1000	Santa Cruz	Anti- Mouse IgG AP Conj	1000	Santa Cruz
CYP7B1	Goat Anti- CYP7B1	200	Santa Cruz	Anti- Mouse IgG AP Conj	1000	Santa Cruz

Table 2. 3 Preparation of substrate solution for immunodetection (Ey & Ashman 1986).

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

2.2.8 Gene Expression Analysis by Quantitative Real time PCR

Conventional methods need broad amount of RNA in order to quantification of mRNA expression. It is not desirable since sample may not be enough and expression level may be low. Although reverse transcription – polymerase chain reaction (RT-PCR) can get over some of these type problems, it needs remove wasting much time standardization procedure and post-PCR uses. On the other hand, real time PCR has many advantages

in terms of preciseness, sensitiveness, high-throughput potential, and lack of post – PCR manipulation.

2.2.8.1 Total RNA Isolation from Rat Liver

All equipments for RNA isolation were treated with distilled water with 0.1 % (v/v) diethylpyrocarbonate (DEPC) to inhibit RNAase activity. Excess DEPC was evaporated under hood for overnight and DEPC water was autoclaved before use. Total RNA was isolated from rat liver tissue using TRIzol reagent.

80 mg of rat liver tissue was minced and homogenized in 800 μ l of TRIzol reagent and also for each mg of liver tissue was added 10 μ l of TRIzol reagent. Homogenization process is made for 15 seconds 3 times using a glass-teflon-homogenizer. After transferring the homogenate into an eppendorf tube, liquid nitrogen was added and the mixture was incubated for 5 minutes at RT (room temperature). Then, 100 μ l of chloroform was added and vortexed for 1 minutes, and incubated in room temperature and in ice for 5 minutes each of them, respectively. Later, the homogenate mixture was centrifuged at 1300 x g for 15 minutes at 4 °C. After centrifugation process, three phases were seen in centrifugation tube. Starting from bottom, these three layers were phenol-chloroform phase, inter-phase and colorless upper phase, respectively. The upper phase including RNA was transferred clean tube, added same volume isopropanol, mixed gently and incubated for 10 minutes at RT (room temperature). After 10 minutes incubation, mixture was centrifuged 1300 x g at for 5 minutes at 4 °C. The pellet was mixed with 1ml of 75 % ethanol. After centrifugation 7500 x g for 10 minutes at 4°C, the pellet was dried in hood for 45 minutes. Lastly, after RNA was dissolved in 75 μ l Nuclease (RNAase/DNAase) free water by carefully

pipetting, it was incubated at 50°C for 12 minutes and stored at - 80 °C for further investigations.

2.2.8.2 Determination of RNA Concentration

RNA concentration was determined by reading the absorbance at 260 nm, because RNA molecule gives maximum absorbance at this wavelength, and also purity of RNA determined the ratio between absorbance at 260 nm and 280 nm (OD_{260}/OD_{280}). The ratio of OD_{260}/OD_{280} of pure RNA must be between 1.8 and 2.2. While below values of 1.8 indicate the DNA contamination, higher values of 2.2 indicate the protein or phenol contamination.

The optical density of 1.0 corresponded to 40 µg/ml for RNA therefore; RNA concentration was determined using NanoDrop™ 2000 (Thermo Scientific) and calculated according to the following formula:

$$\text{RNA } (\mu\text{g/ml}) = OD_{260} \times DF \times 40 \mu\text{g RNA /ml (1 } OD_{260} \text{ unit)}$$

2.2.8.3 Qualification of RNA by Agarose Gel Electrophoresis

0.4 g of agarose was mixed with 40 ml 0.5X TBE (Tris-Borate-EDTA) buffer, pH 8,3 in order to prepare 1 % (w/v) agarose gel. The agarose was dissolved in microwave oven. After the agarose solution was cooled enough, 7 µL of ethidium bromide solution was added to make final

concentration 0.5 µg/ml and the solution was mixed immediately. Agarose gel solution was poured into electrophoresis before tray and comb was placed for well formation. After solidification of gel, tank was filled with 0.5X TBE buffer till buffer covered the all gel surface. 10 µl of RNA solution was mixed with 2 µl of loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 15 % Ficoll) and the mixture was loaded into the wells. Electrophoresis was performed at 70 V for 1 hour. The gel was observed under UV light and photographed.

2.2.8.4 cDNA Synthesis

cDNA is synthesized from RNA by reverse transcription process using Fermentas RevertAid™ First Strand cDNA synthesis Kit (K1631). According to manufacturer's instructions, 4 µg of total RNA and 0.5 µg oligo dT primers were mixed in the eppendorf tube. After complete to 12 ml with RNAase free water, the mixture was spin down and incubated at 70°C for 5 minutes. Then, mixture was cooled in ice, 4 µL of 5X reaction buffer, 1 µL of Ribolock and 2 µL of 10 mM dNTP were added into the mixture. After incubation at 37°C for 5 minutes, 1µl of M-MuLV-RT (Moloney-Murine Leukemia Virus Reverse Transcriptase) was added and incubated at 42°C for 1 hour. In the end, the reaction was stopped through heating 70°C for 10 minutes and chilled on ice. cDNA was hold on at -20°C for further use.

2.2.8.5 Quantitative Real-Time PCR

The impacts of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients on CYP7A1, CYP27A1, CYP8B1 and CYP7B1

gene expressions were analyzed by quantitative Real Time PCR (qRT-PCR) using Corbett Rotor Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137). Quantitative Real Time PCR was carried out by using Light Cycler FastStart DNA Master^{Plus} SYBR Green I (Roche Applied Science, Basel, Switzerland). Assay was performed manufacturer's protocols.

5 µl PCR-grade water and a sample in which reverse transcriptase was omitted during reverse transcription, were included in every PCR-run as negative controls to confirm that there is no genomic DNA contamination in the cDNA samples.

After addition of 3 µl of cDNA, 2 µl gene-specific primer for CYPs and 12.5 µl Fast Start Universal SYBR Green Master (ROX), reaction volume was completed to 25 µl with RNase free distilled water. All samples were run in triplicates, no template control (NTC) was used in order to determine contamination. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), housekeeping gene, was used as an internal standard. The primers were designed using NCBI and the blasts were confirmed the based on *Rottus norvegicus*. Primer sequences, annealing temperatures and product sizes for gene expression analysis by real-time PCR were given in Table 2.4.

Table 2. 4 Primer sequences, annealing temperatures and product sizes for gene expression analysis.

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Annealing Temperature (°C)	Product Size (bp)
CYP7A1	TGCCCTCTGTACCGAGTGATGTT	ACCGGCAGGTCATTCAGTTGCACT	60	518
CYP27A1	ATG TGG CCA ATC TTC TCT ACC	GGG AAG GAA AGT GAC ATA GAC	55	163
CYP8B1	CAGGTTGGAAGCCGAGACAT	TGCCCACTGGCCATCTTTAG	60	158
CYP7B1	CCTTCTCTTTGCCGGTCACCT	TTTCAGGGCCCATGCCAAGAT	06	121
GAPDH	GATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT	06	240

According to Lightcycler run protocol; after preincubation at 95°C for 15 minutes to denature DNA and activate Taq polymerase, amplification and quantification repeated 45 times (95°C for 20 second, 55-60 °C annealing (depending on the researched genes) for 30 second, 72°C extension for 30 second with a single fluorescence measurement). Next, melting curve program at 65-95°C with a heating rate of 0.1 °C/s and continuous fluorescence measurement were carried out. After melting, cooling step was done at 40°C for 30 second. Melting curve analysis of the amplification product was carried out at the end of the each amplification reaction in order to confirm a single PCR product. Quantity of specific mRNA in the sample was measured based on corresponding gene specific relative standard curve obtained from serial dilution of control sample and expression level of the target gene was measured relative housekeeping gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Light cycler quantification software (Version 3.5) was used to draw the standard curve from serial dilution of control cDNA and to compare the amplification between treated and control groups.

2.2.9 Determination of Total Cholesterol

Total cholesterol was determined in the Düzen Clinical Laboratory, Ankara. Cholesterol is found in two forms in the blood as free form or esterified form. In this method, enzymes (cholesterol esterase and cholesterol oxidase) are very specific to determine the forms of cholesterol. Cholesterol esterase converted the cholesterol esters into the cholesterol. Next, the cholesterol was oxidized by cholesterol oxidase to produce ketone cholest-4-en-3-one and hydrogen peroxide. After reaction between horseradish peroxidase and hydrogen peroxide, a colored compound obtained gave an absorbance at

540-570 nm. The principle of total cholesterol determination was illustrated in Figure 2.2.

Different concentrations of standard (cholesterol) were prepared. After 45 minutes incubation of samples with enzymes, samples were compared to known concentrations of cholesterol.

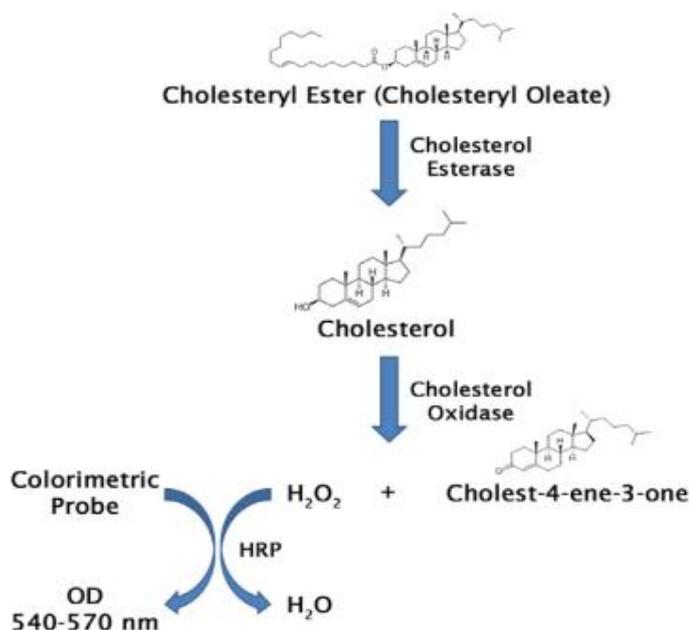


Figure 2. 2 The principle of total cholesterol determination.

2.2.10. CYP7A1 Enzyme Activity Determination

The CYP7A1 - catalyzed cholesterol 7 α -hydroxylation was determined using an Isocratic High-Performance Liquid Chromatography Assay (Waxman & Chang 2002). This method is based on the conversion of the primary cytochrome P450 metabolite, 7 α -hydroxycholesterol, into 7 α -

hydroxy-4-cholesten-3-one in a reaction catalyzed by exogenous cholesterol oxidase, followed by chromatographic separation with monitoring at 212 nm. This technique is known to be applicable to enzymatic studies for determination of cholesterol 7 α -hydroxylation activity catalyzed by cDNA-expressed CYP7A1 and animal or human liver microsomes.

CYP7A1 is catalytically active in cholesterol 7 α -hydroxylation and immunoinhibition experiments with anti-human CYP7A1 antibodies have shown that CYP7A1 accounts for most the cholesterol 7 α -hydroxylation activity in human liver microsomes. Thus, microsomal cholesterol 7 α -hydroxylation activity can be used as a marker for human liver CYP7A1. Several analytical methods have been developed to quantify hepatic microsomal cholesterol 7 α -hydroxylation, including reverse-phase high-performance liquid chromatography (HPLC), isotope dilution-mass spectrometry, and thin-layer chromatography. The method used in the present study describes a normal-phase, isocratic HPLC assay for determination of cholesterol 7 α -hydroxylation activity based on the conversion by cholesterol oxidase of the primary cytochrome P450 (P450) metabolite 7 α -hydroxycholesterol into 7 α -hydroxy-4-cholesten-3-one, which can be detected at 212 nm (Waxman & Chang 2002).

Reagents:

-Substrate: 1 mM cholesterol dissolved in toluene.

-Specialty reagents: 7 α -hydroxycholesterol; cholesterol oxidase. Reconstitute to give a stock solution of 12.5 U/mL in 10 mM potassium phosphate buffer, pH 7.4; 1 mM dithiothreitol and 20 % (v/v) glycerol.

-Metabolite standard: 7 α -hydroxy-4-cholesten-3-one (enzymatically produced from 7 α -hydroxycholesterol by cholesterol oxidase).

- Assay buffer: 100 mM HEPES, pH 7.4, containing 0.025 mM ethylenediamine tetraacetic acid (EDTA), and 50 mM NaF.
- Cofactor: prepare a 10 mM (8.3 mg/mL) stock solution of nicotinamide adenine dinucleotide phosphate (NADPH) and keep on ice.
- Enzymes: e.g., cDNA-expressed CYP7A1 or human liver microsomes. Dilute in assay buffer to a working concentration of 10 mg of protein/mL and keep on ice.
- 5 % (v/v) Sodium cholate.
- Deproteinizing agent: methanol.
- Extraction reagent: petroleum ether.

High-Performance Liquid Chromatography

- Mobile phase: hexane/2-propanol (95:5)
- Alltech silica column, 4.6 × 250 mm, 5- μ m particle size,
- Ultraviolet (UV) detector at 212 nm.

50 μ L of assay buffer, 10 μ L of 1 mM sonicated aqueous cholesterol suspension and 20 μ L of P450-containing enzyme sample were added to each tube and total incubation volume was completed to 100 μ L. After prewarming the incubation tubes to 37°C, 20 μ L of 10 mM NADPH (1 mM final concentration) was added to initiate the enzymatic reaction. Later, the samples were shaken at 37°C for 20-30 min in a water bath. 150 μ L of distilled water, 30 μ L of 5 % (v/v) sodium cholate, and 20 μ L of cholesterol oxidase were added to each tubes and were incubated at 37°C for 10 min. 300 μ L of ice-cold methanol was added to stop the enzymatic reaction and

then all tubes were placed on ice. Next, they were extracted with 3 mL of petroleum ether and centrifuged at 5000 g for 5 min. Then, the extract was transferred to a clean test tube and evaporated under a gentle stream of nitrogen. The residue was reconstituted with 100 μ L of hexane/2-propanol (95:5) and injected 20-40 μ L/100 μ L of reconstituted sample onto an HPLC column. Finally the column was run with a mobile phase of hexane/2-propanol (95:5) at a flow rate of 1 mL/min and the eluent was monitored at 212 nm.

Under these conditions, the retention times were 2.7 min for cholesterol and 6.4 min for 7 α -hydroxy-4-cholesten-3-one. Next, the background activity was measured in blank incubation tubes containing the complete incubation mixture but with heat-inactivated enzymes. Standards were prepared by adding a known amount of authentic 7 α -hydroxycholesterol metabolite to tubes containing the complete incubation mixture but with heat-inactivated enzymes. The amount of product formation was determined by comparison to a standard curve derived from 7 α -hydroxy-4-cholesten-3-one. Finally, cholesterol 7 α -hydroxylation activity was calculated and it expressed as nanomoles of product formed (minute-milligrams of microsomal protein) or as nanomoles of product formed (minute-nanomoles of total P450).

2.2.11 Statistical analysis

Statistical analysis was performed by student t-test using GraphPad Prism 6.0 statistical software package for Windows. Results were expressed as means with their Standard derivation (SD) and $p < 0.05$ were chosen as level for significance.

CHAPTER 3

RESULTS

3.1 LC-MS Analysis of *Viscum album L.* and *Epilobium hirsutum L.*

Recently, Liquid chromatography-Mass Spectrometry (LC-MS) has attained popularity owing to its specificity and sensitivity. This has made it an essential tool for analyzing the pharmacological profiles of herbs. LC-MS analysis technique includes liquid chromatography for separation, coupled to mass spectrometry for mass detection. The analysis in this study was carried out using AGILENT 1200 HPLC Series coupled with AGILENT 6460 LC-MS spectrometer.

LC-MS was applied to analyze the phenolic profiles of *Viscum album L.* and *Epilobium hirsutum L.* All samples and standards were dissolved in distilled water. In this study, we determined and quantified 5 phenolic compounds from *Viscum album L.* (Table 3.1) including chlorogenic acid, gallic acid, caffeic acid, quercetin dehydrate, and *o*-coumaric acid. LC-MS chromatogram of phenolic compounds of *Viscum album L.* is presented in Figure 3.1. The chromatogram was used as a fingerprint of *Viscum album L.* and to quantify its phenolic compounds.

According to the results of quantitative LC-MS analysis, chlorogenic acid (1.0528 ppm) was the dominant compound among the standard phenolics used in this study in *Viscum album L.* Gallic acid and quercetin dehydrate

amounts were less than 0.31 ppm. Caffeic acid and *o*-coumaric acid values were calculated as less than 0.15 ppm and 0.27 ppm, respectively.

Table 3. 1 Quantitative LC-MS analysis of *Viscum album L.* extract.

Sample	Gallic acid (ppm)	Caffeic acid (ppm)	Quercetin dehydrate (ppm)	Chlorogenic acid (ppm)	<i>o</i> -coumaric acid (ppm)
<i>Viscum album L.</i>	≤ 0.31 ppm	≤ 0.15 ppm	≤ 0.31 ppm	1.0528±0.0453	≤ 0.27 ppm

Epilobium hirsutum L. extract was analyzed and quantified for the presence of 5 phenolic compounds (Table 3.2), including ellagic acid, gallic acid, caffeic acid, *p*-coumaric acid, and quercetin dehydrate. LC-MS chromatogram of the *Epilobium hirsutum L.* extract and 4 phenolic compounds are presented in Figure 3.2, while the chromatogram of ellagic acid is shown in Figure 3.3 since it was analyzed using different column and solvent system.

According to quantitative LC-MS analysis, gallic acid (11.906 ppm) was the major compound among the standard phenolics used in this study in *Epilobium hirsutum L.* *p*-coumaric acid amount was 1.0642 ppm, while quercetin dehydrate was less than 0.625 ppm, and the ellagic acid value was calculated as less than 0.31 ppm.

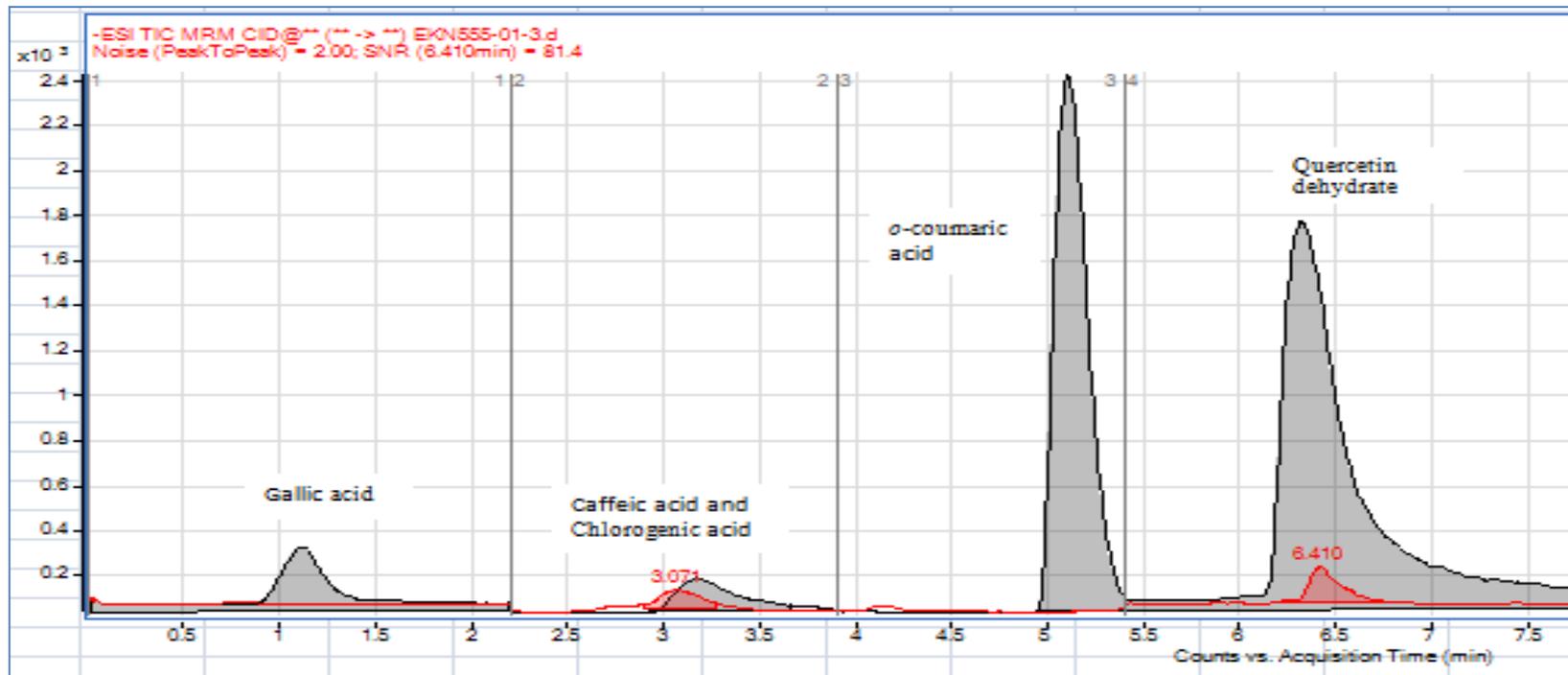


Figure 3. 1 LC-MS chromatogram of *Viscum album L.* extract. Grey line shows mixture of standards (total 1.25 ppm). Red line shows *Viscum album L.* extract (10 mg/ml).

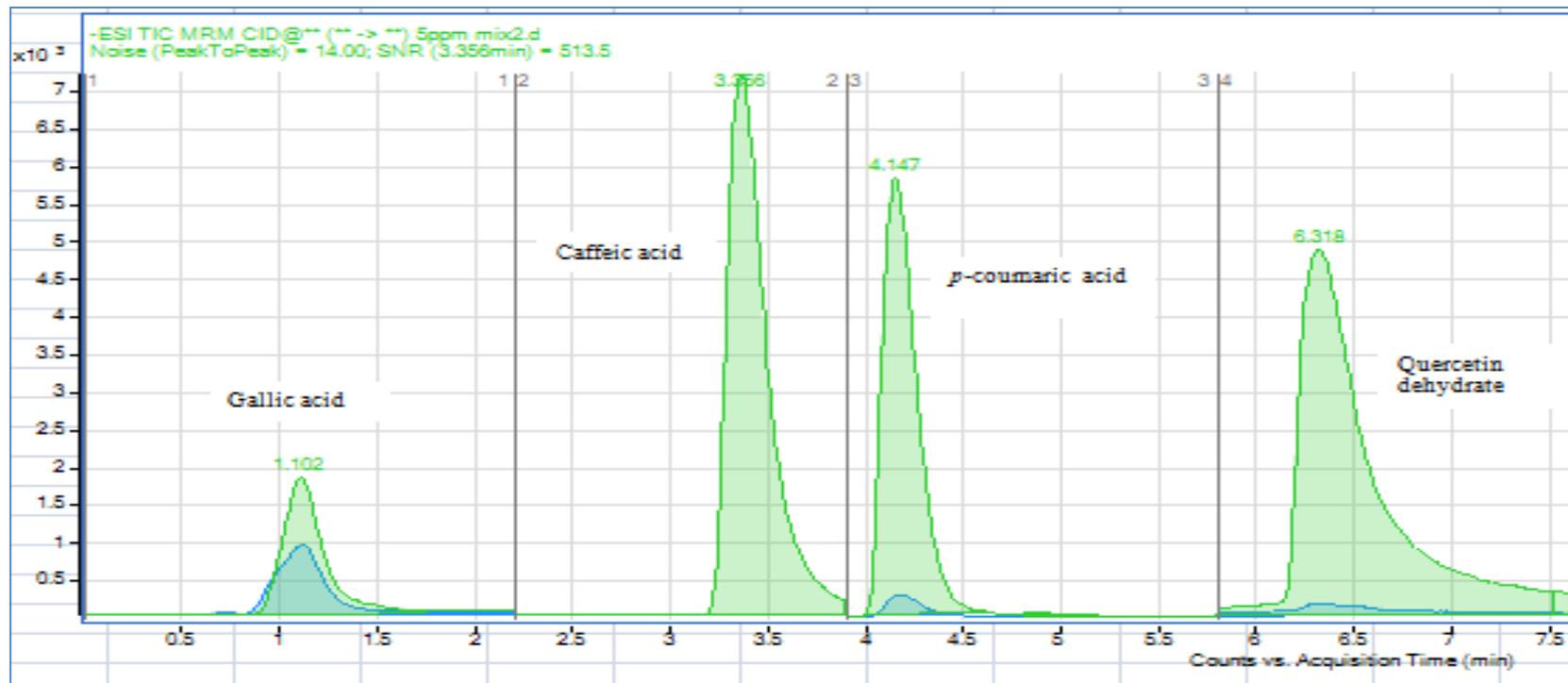


Figure 3. 2 LC-MS chromatogram of *Epilobium hirsutum L.* extract. Bright green line shows mixture of standards (total 5 ppm). Dark green line shows *Epilobium hirsutum L.* extract (10 mg/ml).

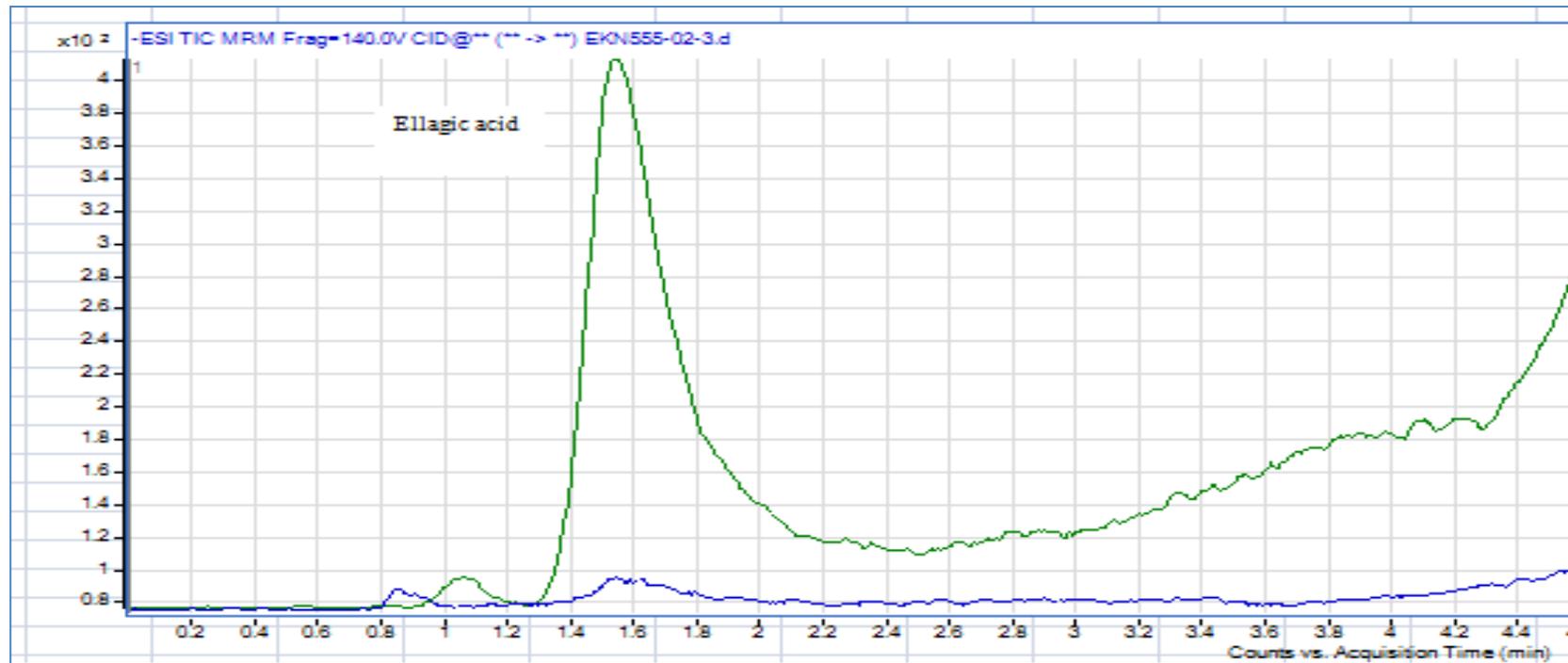


Figure 3. 3 LC-MS chromatogram of *Epilobium hirsutum L.* extract. Grey line shows 2.5 ppm ellagic acid. Blue line shows *Epilobium hirsutum L.* extract (10 mg/ml).

Table 3. 2 Quantitative LC-MS results of *Epilobium hirsutum L.* extract.

Sample	Galic acid (ppm)	Caffeic acid (ppm)	<i>p</i> -coumaric acid (ppm)	Quercetin dehydrate (ppm)	Ellagic acid (ppm)
<i>Epilobium hirsutum L.</i>	11.906 ± 0.1810	≤ 0.31 ppm	1.0642±0.0027	≤ 0.625ppm	≤ 0.31 ppm

3.2 Total Phenolic Content of *Viscum album L.* and *Epilobium hirsutum L.*

The total phenolic content of *Viscum album L.* and *Epilobium hirsutum L.* was determined by the Folin-Ciocalteu method (Singleton *et al.* 1999) as defined in Materials and Methods. Folin-Ciocalteu reagent includes phosphomolybdic acid and phosphotungstic acid. This reagent oxidizes the phenolic groups of samples to give a green-blue complex at 750 nm. Gallic acid was selected as a standard due to its high solubility in Folin-Ciocalteu reagent. The calibration curve was drawn using the absorbance values of gallic acid obtained by different range of concentrations between 25-200 µg/ml. The calibration curve is shown in Figure 3.4, and the linear equation of this curve was calculated as $y = 0.106x$ with an $R^2 = 0.985$. The phenolic content of samples was calculated with respect to gallic acid equivalent value (GAE) in gram of the sample extracts by using the equation and the plot and the results were given in Table 3.3.

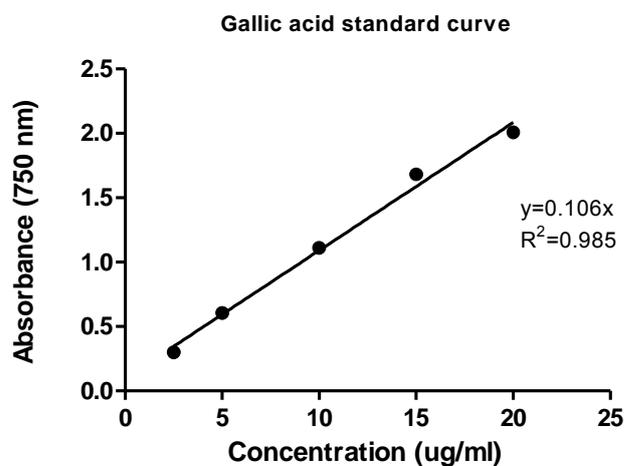


Figure 3. 4 Gallic acid standard curve for total phenolic content determination.

The data represented in Table 3.3 reveals that *Epilobium hirsutum L.* has a higher phenolic content compared to *Viscum album L.* The total phenolic content of *Epilobium hirsutum L.* was 225 ± 2.123 mg GAE/g dried extract while that of *Viscum album L.* was 57 ± 1.756 mg GAE/g dried extract.

Table 3. 3 Total phenolic content of *Viscum album L.* and *Epilobium hirsutum L.*

Extracts	Total Phenol GAE (mg/g) \pm SD
<i>Viscum album L.</i>	57 ± 1.756
<i>Epilobium hirsutum L.</i>	225 ± 2.123

3.3 Total Flavonoid Content of *Viscum album L.* and *Epilobium hirsutum L.*

Total flavonoid content was determined by the colorimetric method outlined in Chang *et al.* (2002), with slight modifications as described in Materials and Methods. In this method, aluminum chloride reacts with flavonoid parts of the samples in the presence of sodium nitrite, forming a red color complex. Catechin was chosen as a standard to determine the total flavonoid content. The calibration curve of catechin is displayed in Figure 3.5, the slope was calculated as $y=0.002x$ with an $R^2=0.992$. The flavonoid content of samples was determined as catechin equivalent value (CE) in gram of the sample extracts by using the equation and the plot, and the result were given in Table 3.4.

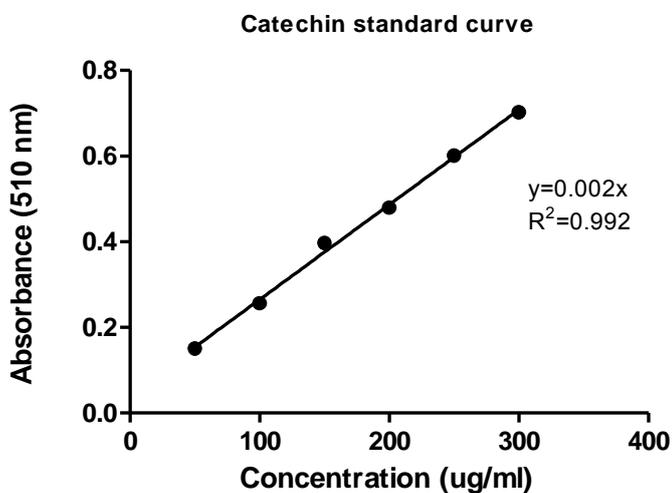


Figure 3. 5 Catechin standard curve for total flavonoid content determination.

The data presented in Table 3.4 shows that the total flavonoid content of *Viscum album L.* and *Epilobium hirsutum L.* (19 mg CE/ g dried extract) was found similar.

Table 3. 4 Total flavonoid content of *Viscum album L.* and *Epilobium hirsutum L.*

Extracts	Total Flavonoid CE (mg/g)±SD
<i>Viscum album L.</i>	19.0278 ±1.2615
<i>Epilobium hirsutum L.</i>	19.2593±0.8225

3.4 Effects of *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid, and Ellagic acid on Protein Expression of Cholesterol and Bile Acid Metabolizing CYPs

The effects of water extracts of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients (*o*-coumaric acid and ellagic acid) on rat liver bile acid metabolizing CYPs (CYP7A1, CYP27A1, CYP8B1, and CYP7B1) were analyzed using the western blot technique. Western-blot analysis was carried out to determine the differences between control and treated animal groups in terms of the expressions of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 enzymes under specific conditions. All western-blotting protocol parameters, including primary antibody concentration, secondary antibody concentration, and total protein amounts were optimized. Primary antibodies differ generally in concentration, quality, and

affinity. We tried different dilutions based on the manufacturer datasheets. The primary antibody dilutions were chosen to observe the best band image with minimum antibody consumption, as given in Table 2.2 in the section of Materials and Methods. Protein bands were quantified by using Image J.

We investigated the changes in the CYP27A1 protein levels in rat liver S1.5 fraction, and CYP7A1, CYP8B1, and CYP7B1 protein levels in rat liver microsomes following treatment of animals with *Viscum album L.*, *Epilobium hirsutum L.*, and their major polyphenolic ingredients (*o*-coumaric acid and ellagic acid) according to the protocols given in Materials and Methods. The student's t-test was used to evaluate alterations in measured parameters between normal and treated animals. Data was expressed as mean \pm standard deviation (SD). The correlation between treated and control animals was tested, and a possibility of 0.05 and 0.005 was stated depending on the statistical significance level. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for the western blot analysis.

3.4.1 Effect of *Viscum album L.* on Rat Liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 Protein Levels

The effect of *Viscum album L.* on rat liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) protein expression was measured via western-blot by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) as an internal control. Protein levels were compared relative to GAPDH expression. Figure 3.6-A illustrated the results of Western-blot analysis of CYP proteins with GAPDH co-immunostaining.

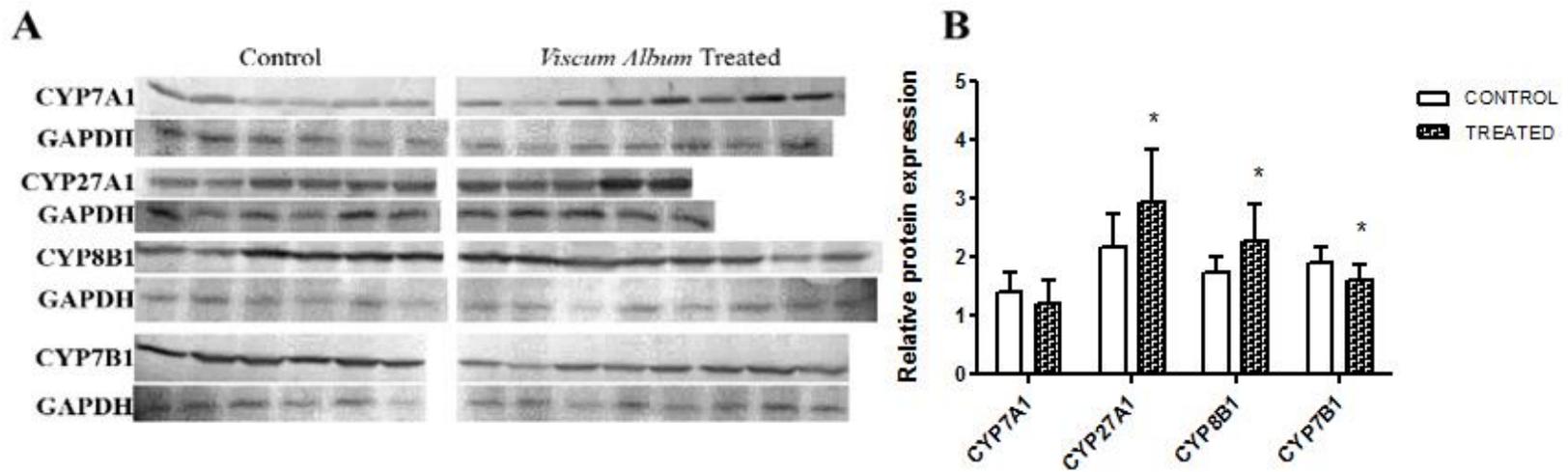


Figure 3. 6(A) Representative immunoblot of liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) proteins in experimental control and *Viscum album L.* treated animals. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) was used as an internal standard for each membrane. B) Comparison of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 protein expressions of the control (n=11) and *Viscum album L.* treated (n=11) groups. Band quantification was expressed as the mean \pm SD of the relative intensity with respect to that of the internal control GAPDH. *Significantly different from the respective

The intensity of each band was analyzed as an arbitrary unit, relative peak area (RPA), with the help of the Image J software program. Relative expressions obtained from the densitometric values of the proteins of control and *Viscum album L.* treated rats are illustrated in Figure 3.7. The protein expressions of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 in *Viscum album*-treated rats were significantly different from control rats ($p < 0.005$). *Viscum album L.* caused a 0.9 fold decrease in protein expression of CYP7A1 and CYP7B1, but a 1.4 and 1.3 fold increase in protein expressions of CYP27A1 and CYP8B1, respectively (Figure 3.6 -B).

3.4.1.1 Effects of *o*-coumaric acid on Rat Liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 Protein Levels

The effects of *o*-coumaric acid on rat liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) protein expression was analyzed via western-blot, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) as an internal control. Figure 3.8 shows the results of the western-blot analysis of the CYP proteins with GAPDH co-immunostaining.

Intensity of each band was determined with the Image J software program. Relative expressions of *o*-coumaric acid with respect to housekeeping GAPDH protein are demonstrated in Figure 3.9. Protein expression of CYP7A1, CYP8B1, and CYP7B1 in *o*-coumaric acid treated rats was significantly decreased compared to control ($p < 0.05$). *o*-coumaric acid caused a 0.8 fold decrease in the protein expressions of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 (Figure 3.9).

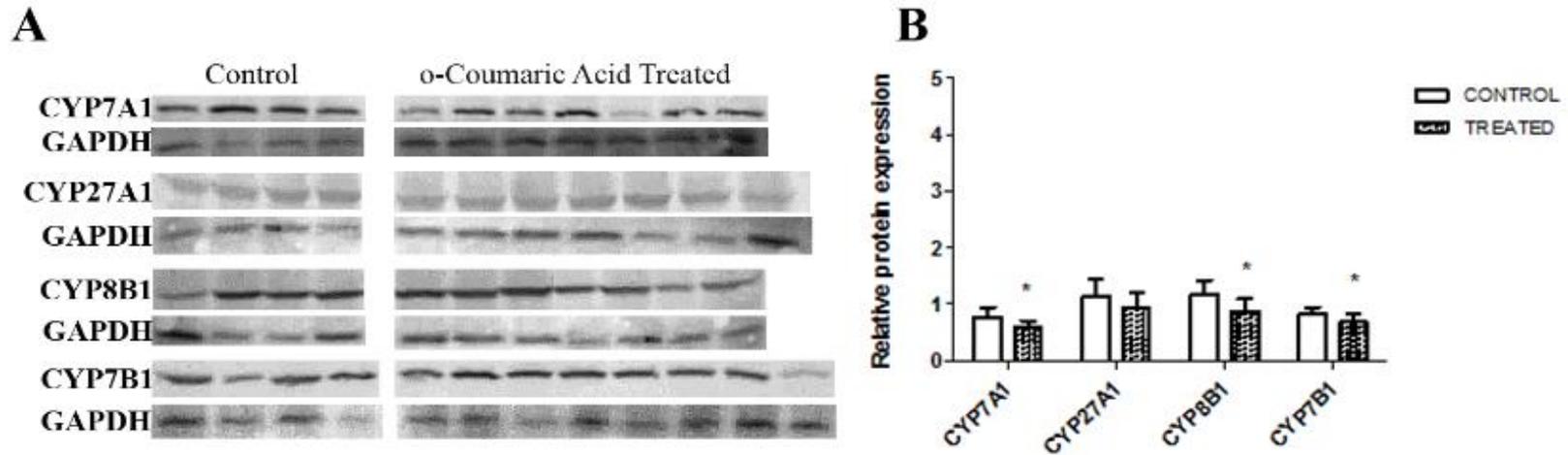


Figure 3. 7 (A) Representative immunoblot of liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) proteins in experimental control and *o*-coumaric acid treatment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) was used as an internal standard for each membrane. B) Comparison of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 protein expression of the control (n=6) and *o*-coumaric acid treated (n=9) groups. Band quantification was expressed as the mean \pm SD of the relative intensity with respect to that of GAPDH, used as the internal control. *Significantly different from the respective control value *p<0.05.

3.4.2 Effects of *Epilobium hirsutum L.* on Rat Liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 Protein Expression

The impact of *Epilobium hirsutum L.* on rat liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) protein expression were determined via western-blot, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37kDa) as an internal control. Figure 3.10 shows the results of western-blot analysis of the CYP proteins and GAPDH, with co-immunostaining to compare the relative expressions.

Intensity of immunoreactive protein bands of *Epilobium hirsutum L.* and control were evaluated with Image J. Relative expressions of *Epilobium hirsutum L.* with reference to housekeeping GAPDH protein are illustrated in Figure 3.11. The protein expressions of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 in *Epilobium hirsutum*-treated rats were significantly different from control rats. *Epilobium hirsutum L.* caused a 0.6, 0.8, and 0.6 fold decrease in protein expression of CYP7A1, CYP8B1, and CYP7B1 respectively, and a 1.3 fold increase in CYP27A1 (**p<0,0001) (Figure 3.11).

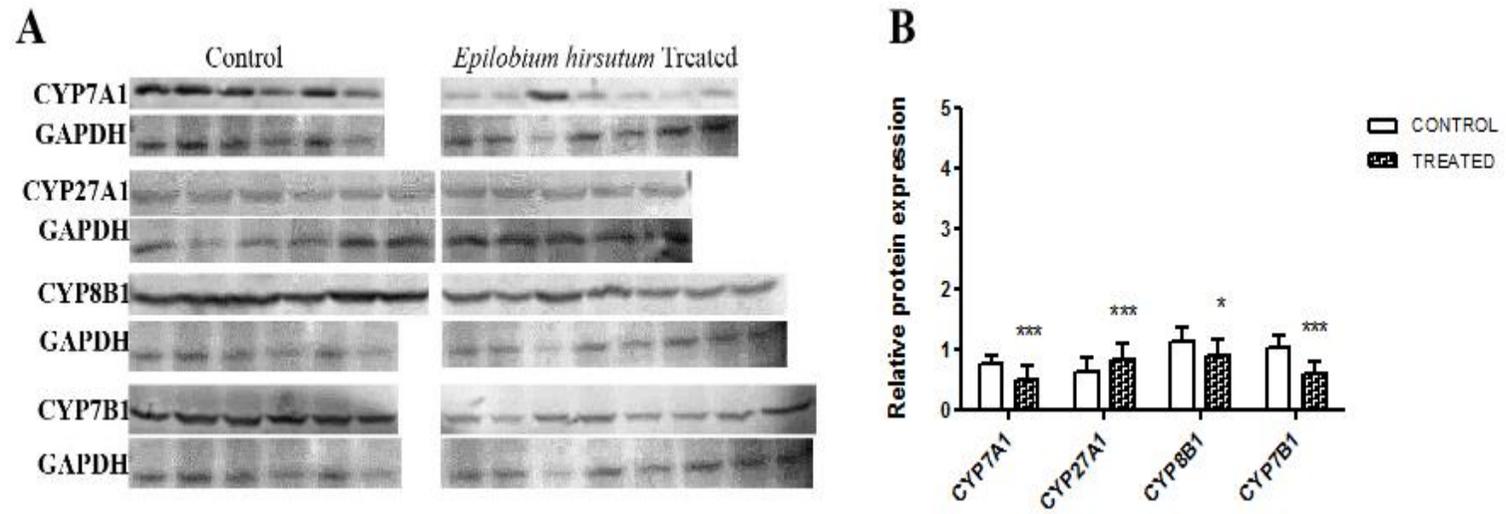


Figure 3. 8 (A) Representative immunoblot of liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) proteins in experimental control and *Epilobium hirsutum L.* Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) was used as an internal standard for each membrane. B) Comparison of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 protein expression of the control (n=21) and *Epilobium hirsutum L.* treated (n=16) groups. Band quantification was expressed as the mean \pm SD of the relative intensity with respect to that of GAPDH, used as the internal control. Asterisks denote the level of significance: ***p<0, 0001.

3.4.2.1 Effects of Ellagic Acid on Rat Liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 Protein Levels

Western blot analysis was carried out to estimate the impact of ellagic acid on rat liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) protein expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH-37 kDa) used as an internal control. Results were expressed relative to GAPDH levels. Figure 3.12 shows the Western-blot bands with GAPDH co-immunostaining.

Band intensity was estimated using the Image J software program. Relative expressions of CYP proteins with respect to the housekeeping GAPDH are displayed in Figure 3.13. The expression levels of CYP27A1 and CYP8B1 in ellagic acid-treated rats were significantly different from control rats. The densitometric analysis of the liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 displayed in Figure 3.13 shows that ellagic acid caused a 0.7, 0.8 and 0.8 fold decrease in protein expression of CYP27A1, CYP8B1, and CYP7B1 respectively, but a 1 fold increase of CYP7A1.

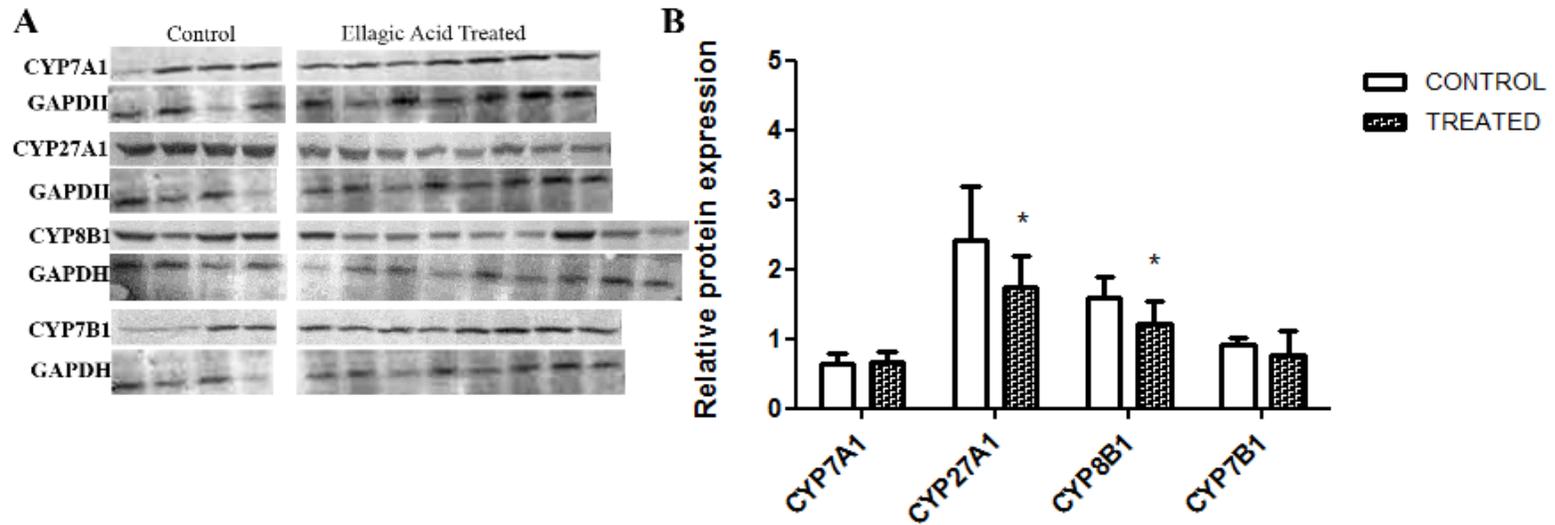


Figure 3. 9 (A) Representative immunoblot of liver CYP7A1 (58 kDa), CYP27A1 (60 kDa), CYP8B1 (57 kDa), and CYP7B1 (58 kDa) proteins in experimental control and ellagic acid. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) was used as an internal standard for each membrane. B) Comparison of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 protein expression of the control (n=6) and ellagic acid treated (n=6) groups. Band quantification was expressed as the mean \pm SD of the relative intensity with respect to that of GAPDH, used as the internal control. *Significantly different from the respective control value $p < 0.005$.

3.5 Effects of *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid, and Ellagic Acid on mRNA Expression of Cholesterol and Bile Acid Metabolizing CYPs

3.5.1 Quality control of RNA molecules by agarose gel electrophoresis

Total cellular RNAs were isolated from rat liver as described in the Materials and Methods. RNA isolation was critical in this part of the study as DNA contamination interferes with the subsequent Real-Time PCR.

Figure 3.10 shows a representative agarose gel electrophoresis pattern of liver total RNA samples. Majority of the RNA preparations had an OD260/280 ratio between 1.8 -2.0 and produced intact and well-separated 28S and 18S RNA bands after gel electrophoresis.

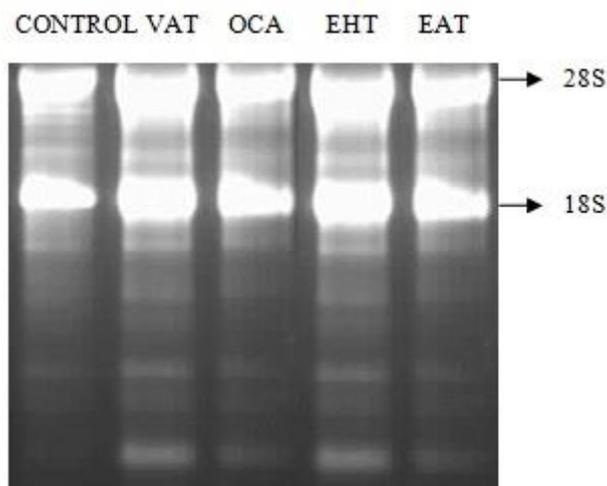


Figure 3. 10 Agarose gel electrophoresis pattern of RNA isolated from rat liver tissue of control, *Viscum album* (VAT), *o*-coumaric acid (OCA), *Epilobium hirsutum* (EHT), and ellagic acid (EAT). All lanes contain 5.0 μ l of total RNA, with different concentrations.

3.5.2 Effects of *Viscum album L.* on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA Expression

The effect of *Viscum album L.* on the mRNA expression of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 was evaluated using quantitative real time PCR (qRT-PCR). Relative mRNA expressions of *Viscum album*-treated and control animals were calculated using GAPDH as the internal control. Figure 3.11 displays the standard, amplification, and melting curves of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 genes of *Viscum album*-treated and control samples. The standard curve was generated using different diluted cDNAs of control samples. The amplification curve shows the change of fluorescence dye versus the cycle number, and the melting curve confirmed that there was a single PCR product.

Real time-PCR results of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA levels are shown in Figure 3.12. GAPDH was used to normalize the results. The Livak method (Livak & Schmittgen 2001) was applied to determine the mRNA expression changes of CYPs. *Viscum album L.* treatment caused significant changes in the mRNA expressions of CYP7A1, CYP8B1, and CYP7B1. *Viscum album L.* caused 9, 1.3, and 2.4 fold reductions in mRNA expression of CYP7A1, CYP27A1, and CYP7B1 respectively, with respect to controls, and a 4.2 fold increase the expression of CYP8B1 ($p < 0.05$). In Figure 3.15, the real time-PCR products for all of the genes for both *Viscum album L.* and *o*-coumaric acid-treated samples are presented. The band patterns confirm that the qRT-PCR products are indeed CYP7A1, CYP27A1, CYP8B1, and CYP7B1, which are 518, 163, 158 and 121 bp, respectively.

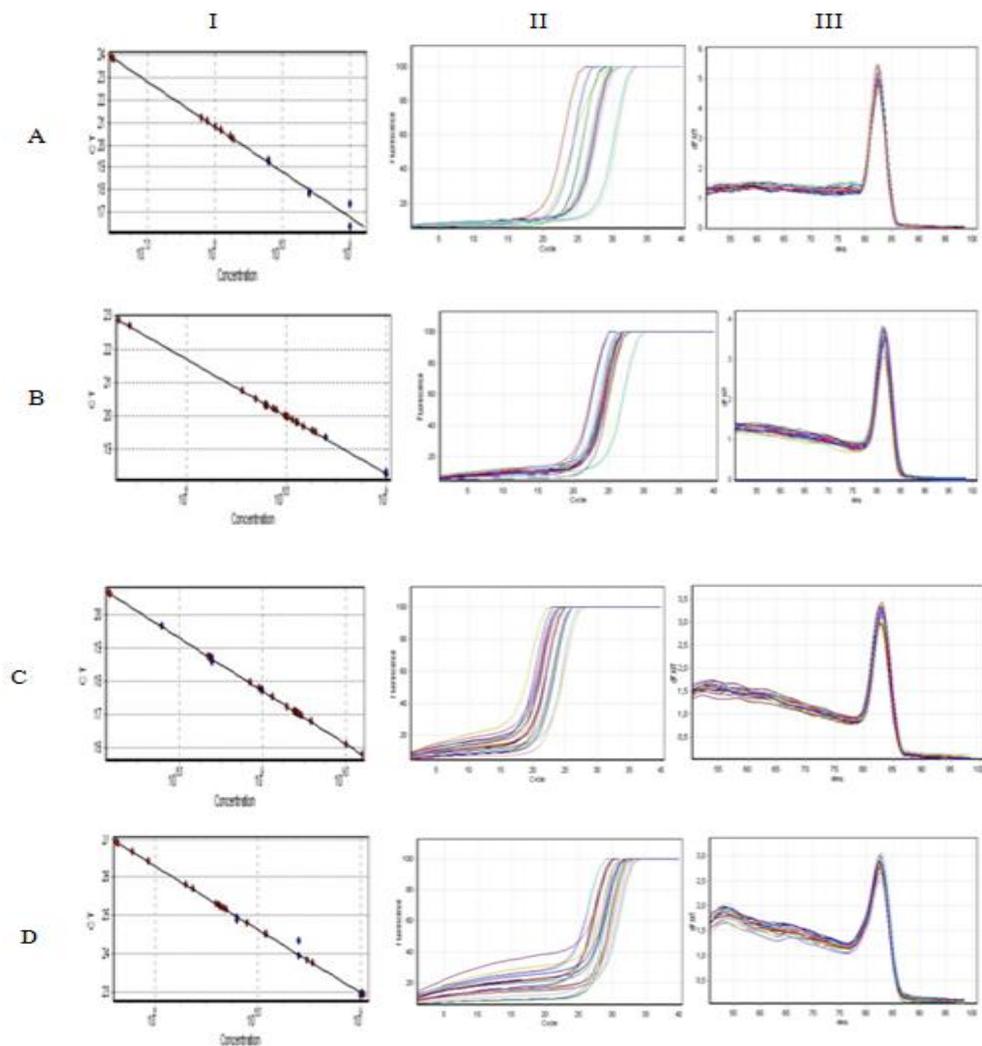


Figure 3. 11 I: Standard curves generated from serial dilutions of a control cDNA sample to calculate mRNA quantities of each gene in *Viscum album L.* treated groups. II: Amplification curves showing the accumulation of fluorescence emission versus cycle number of rat liver bile acid metabolizing genes of control and *Viscum album*-treated samples. III: Melting curves showing the fluorescence of SYBR green dye I versus temperature. A single peak in the melting curves confirmed the detection of single PCR products. A, B, C, and D show CYP7A1, CYP27A1, CYP8B1, and CYP7B1, respectively.

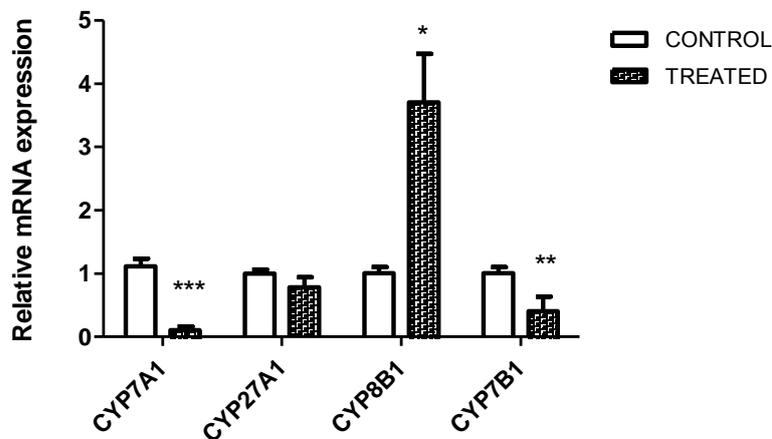


Figure 3. 12 Comparison of *in vivo* effects of *Viscum album L.* on rat liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA expressions. The quantities are expressed as mean \pm SD of the relative expression.

3.5.2.1 Effects of *o*-coumaric acid on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA Expression

mRNA expression of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 was determined by using qRT-PCR. Relative mRNA expressions of *o*-coumaric acid-treated animals were calculated using GAPDH as an internal control. Figure 3.13 shows the standard, amplification, and melting curves of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 in *o*-coumaric acid-treated and control samples.

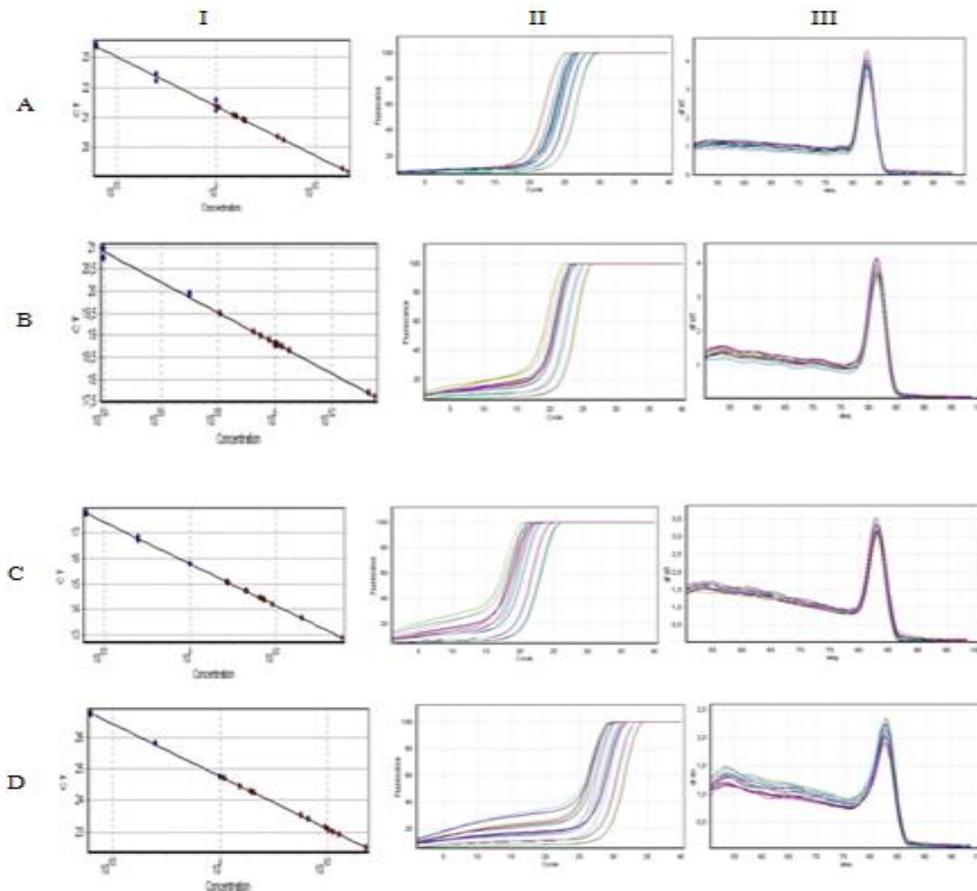


Figure 3.13 I: Standard curves generated from serial dilutions of a control cDNA to calculate mRNA quantities of each gene in *o*-coumaric acid treated groups. II: Amplification curves showing the accumulation of fluorescence emission versus cycle number of rat liver bile acid metabolizing genes of control and *o*-coumaric acid-treated samples. III: Melting curves showing the fluorescence of SYBR green dye I versus temperature. A single peak in the melting curves confirmed the detection of single PCR products. A, B, C, and D show CYP7A1, CYP27A1, CYP8B1, and CYP7B1, respectively.

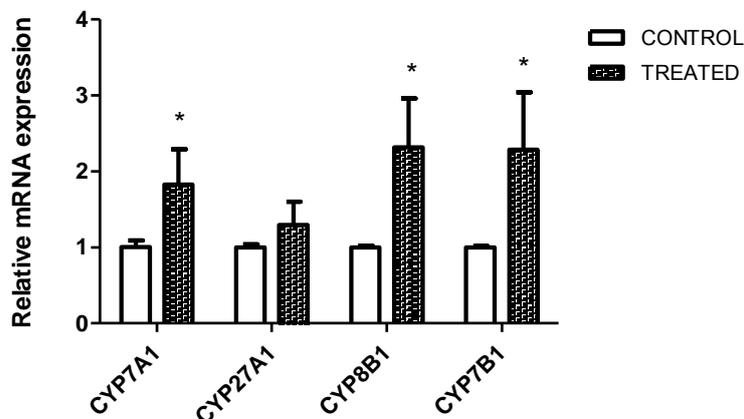


Figure 3. 14 Comparison of *in vivo* effects of *o*-coumaric acid on rat liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA expression. The quantities are expressed as mean \pm SD of the relative expression.

Relative mRNA expression of CYP7A1, CYP8B1, and CYP7B1 in *o*-coumaric acid-treated rats was significantly increased compared to control animals ($p < 0.05$) (Figure 3.14). *o*-Coumaric acid treatment induced a 1.8, 1.3, 2.3, and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 respectively, compared to controls and normalized with GAPDH expression as an internal reference.

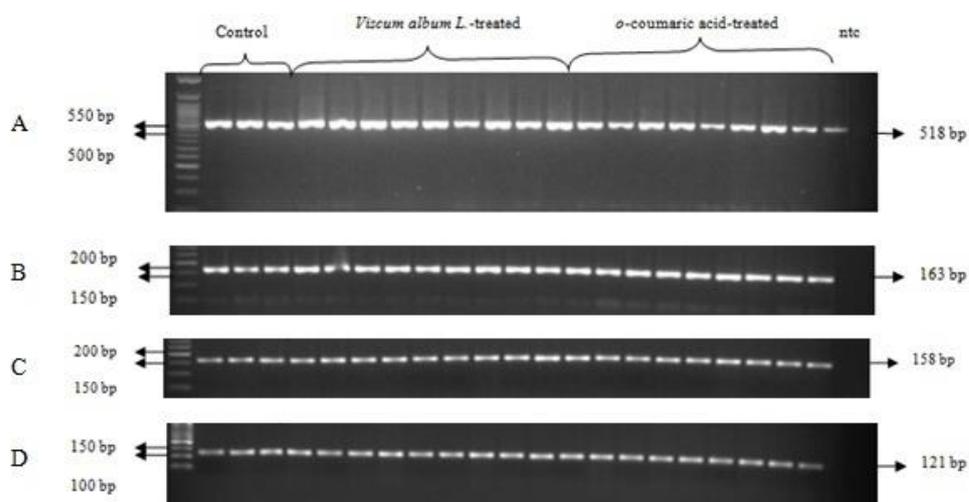


Figure 3. 15 qRT-PCR products of liver CYP7A1 (518 bp), CYP27A1 (163 bp), CYP8B1 (158 bp), and CYP7B1 (121bp) enzymes in control, *Viscum album L.*-treated, and *o*-coumaric acid-treated animals, and NTC (no template control). Each lane contains 10 μ l qRT-PCR product. A, B, C, and D show CYP7A1, CYP27A1, CYP8B1, and CYP7B1, respectively.

3.5.3 Effects of *Epilobium hirsutum L.* on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA Expression

Changes in mRNA expression of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 due to treatment of *Epilobium hirsutum L.* were measured through quantitative real time PCR (qRT-PCR). Figure 3.16 shows the standard, amplification, and melting curves of the CYP7A1, CYP27A1, CYP8B1, and CYP7B1 genes of *Epilobium hirsutum L.*-treated and control samples.

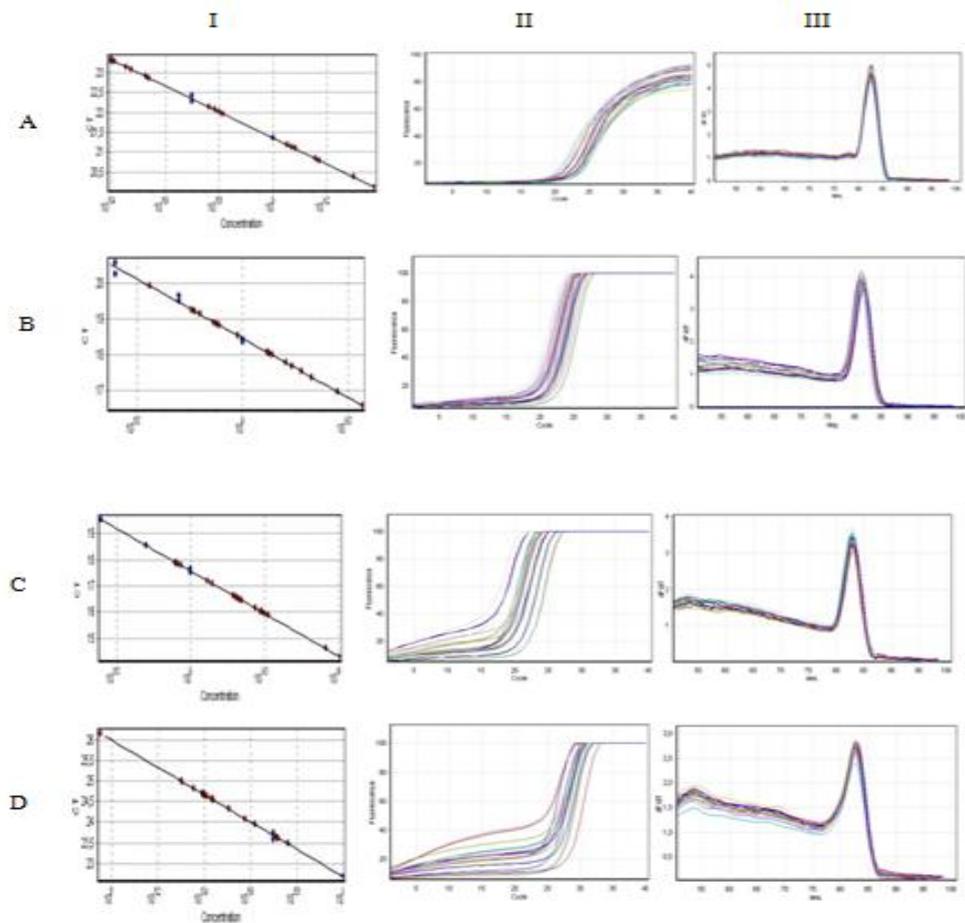


Figure 3. 16 I: Standard curves generated from serial dilutions of a control cDNA to calculate mRNA quantities of each gene in *Epilobium hirsutum L.* treated groups. II: Amplification curves showing the accumulation of fluorescence emission versus cycle number of rat liver bile acid metabolizing genes of control and *Epilobium hirsutum*-treated samples. III: Melting curves showing the fluorescence of SYBR green dye I versus temperature. A single peak in the melting curves confirmed the detection of single PCR products. A, B, C, and D show CYP7A1, CYP27A1, CYP8B1, and CYP7B1, respectively.

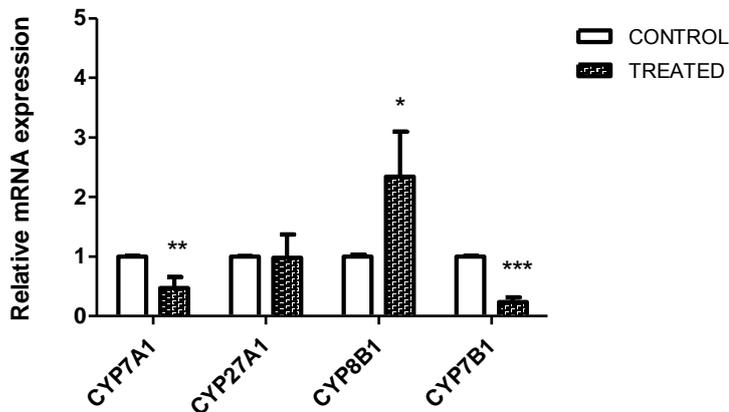


Figure 3. 17 Comparison of *in vivo* effects of *Epilobium hirsutum L.* on rat liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA expression. The quantities are expressed as mean \pm SD of the relative expression.

Real time-PCR results of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA levels are presented in Figure 3.17. GAPDH was used to normalize the results. Livak method (Livak & Schmittgen 2001) was applied to determine the mRNA expression changes of CYPs. *Epilobium hirsutum L.* treatment led to significant changes in mRNA expression of CYP7A1, CYP8B1, and CYP7B1. *Epilobium hirsutum L.* promoted a 2.1, 1.4 and 4.1 fold decrease in mRNA expression of CYP7A1, CYP27A1, and CYP7B1 respectively, with respect to controls and normalized with GAPDH expression as an internal reference, and a 2.3 fold increase in mRNA expression of CYP8B1. In Figure 3.20, the real time-PCR products of both *Epilobium hirsutum L.* and ellagic acid-treated groups are presented. The band patterns confirm that the products are indeed CYP7A1, CYP27A1, CYP8B1, and CYP7B1, which are 518, 163, 158 and 121 bp, respectively.

3.5.3.1 Effects of Ellagic acid on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA Expression

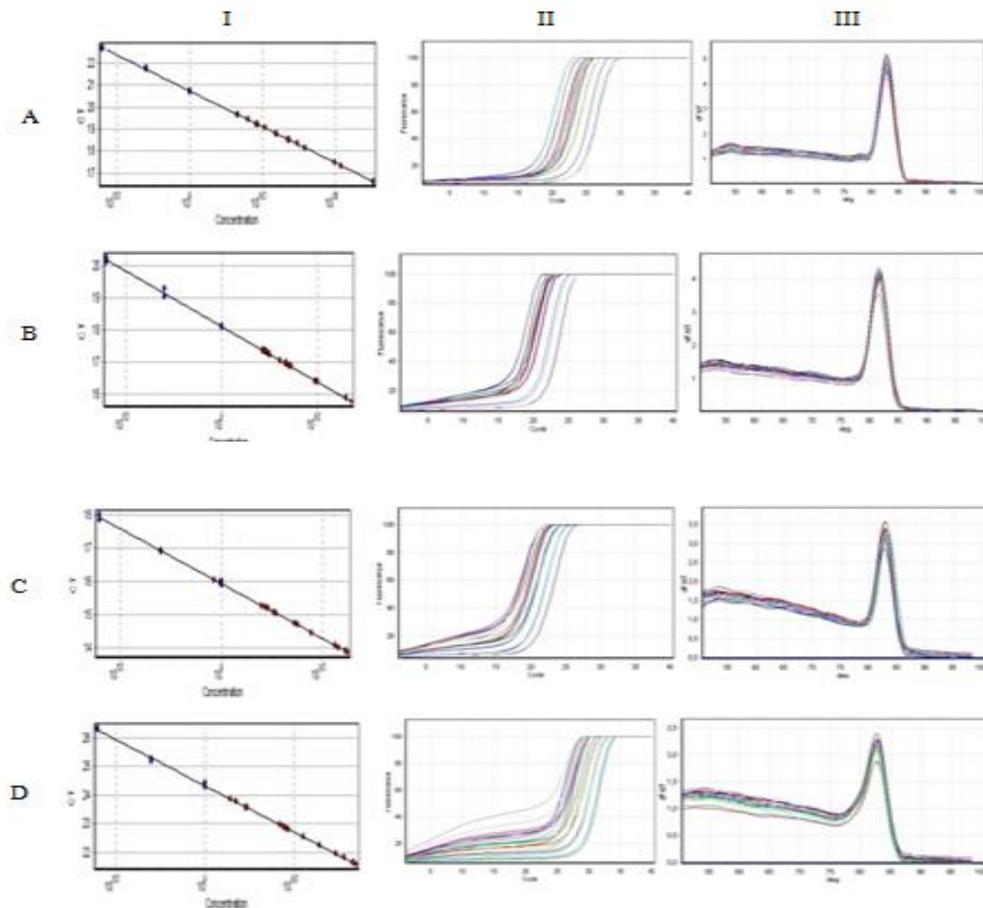


Figure 3. 18 I: Standard curves generated from serial dilutions of a control cDNA to calculate quantities of each gene in ellagic acid. II: Amplification curves showing the accumulation of fluorescence emission versus cycle number of rat liver bile acid metabolizing genes of control and ellagic acid-treated samples. III: Melting curves showing the fluorescence of SYBR green dye I versus temperature. A single peak in the melting curves confirmed the detection of single PCR

products. A, B, C, and D show CYP7A1, CYP27A1, CYP8B1, and CYP7B1, respectively.

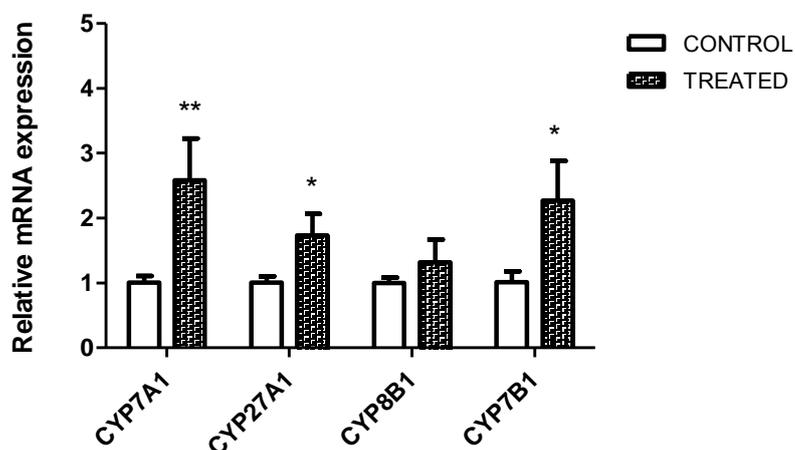


Figure 3. 19 Comparison of *in vivo* effects of ellagic acid on rat liver CYP7A1, CYP27A1, CYP8B1, and CYP7B1 mRNA expressions. The quantities were expressed as mean \pm SD of the relative expression.

mRNA expression of ellagic acid treated CYP7A1, CYP27A1, CYP8B1, and CYP7B1 was determined using qRT-PCR, with relative expression based on GAPDH as internal control. Figure 3.18 shows the standard, amplification, and melting curves of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 genes of ellagic acid-treated and control samples.

qRT-PCR data for the effects of ellagic acid on CYP7A1, CYP27A1, CYP8B1, and CYP7B1 expression is presented in Figure 3.19. The relative mRNA expression of CYP7A1, CYP8B1, and CYP7B1 in ellagic acid-treated rats was increased significantly compared to control animals. Ellagic acid treatment induced a 1.8, 1.3, 2.3, and 2.3 fold increase in mRNA expression

of CYP7A1, CYP27A1, CYP8B1, and CYP7B1 respectively, with respect to controls and normalized with GAPDH expression as an internal reference. In Figure 3.21, the PCR products for GAPDH are presented. The band patterns confirm the correct qRT-PCR product, which is 240 bp.

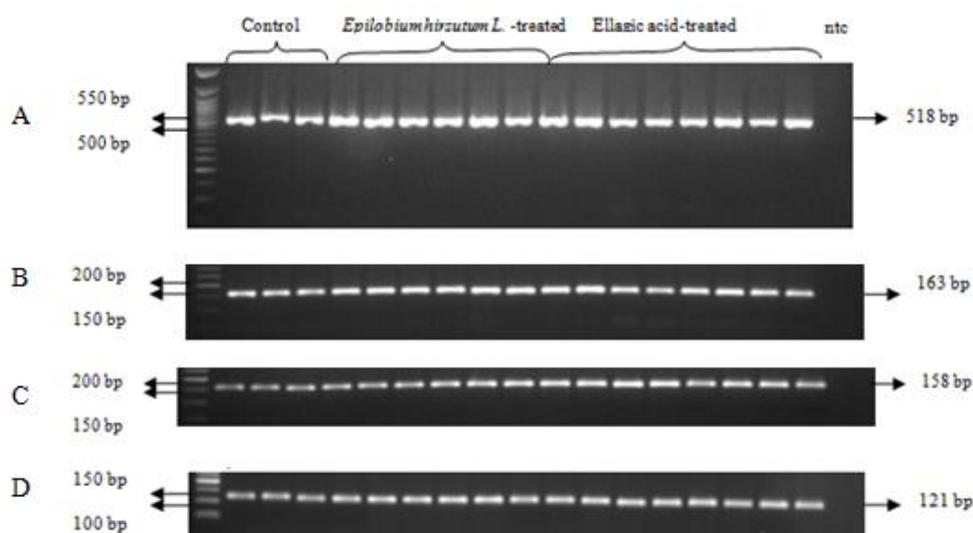


Figure 3. 20 qRT-PCR products of liver CYP7A1 (518 bp), CYP27A1 (163 bp), CYP8B1 (158 bp), and CYP7B1 (121 bp) for control, *Epilobium hirsutum*-treated, ellagic acid-treated animals and NTC (no template control). Each lane contains 10 μ l of qRT-PCR product. A, B, C, and D show CYP7A1, CYP27A1, CYP8B1, and CYP7B1, respectively.

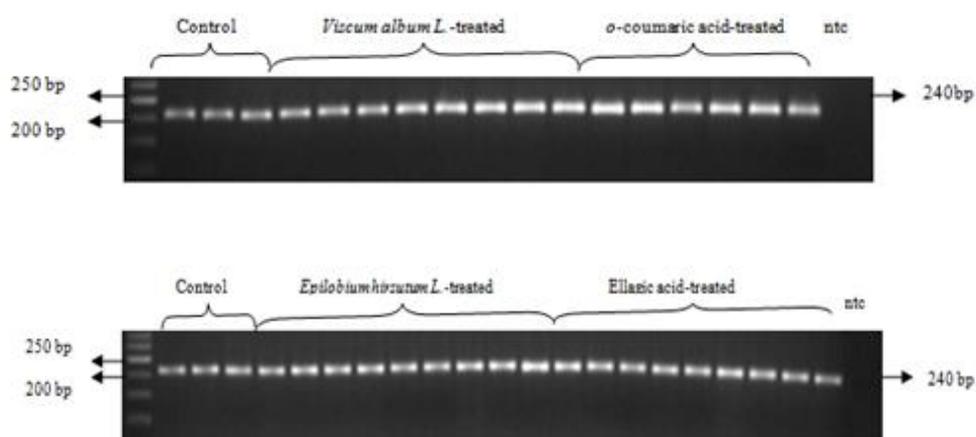


Figure 3. 21 qRT-PCR products of liver GAPDH cDNA (240 bp) for control, *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid, and ellagic acid -treated animals. Each lane contains 10 μ l of qRT-PCR product.

3.6 Blood Serum Total Cholesterol Levels in Control and Treated Rats

Rat serum total cholesterol levels were determined by enzymatic and spectrophotometric techniques as described in the Materials and Methods section. Serum samples obtained from *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid treated rats were determined and results were given in Table 3.5. For calculation of total cholesterol concentration of samples, a standard curve obtained by different concentrations of cholesterol was used. When cholesterol concentration of serum samples from *Viscum album L.* and *o*-coumaric acid treated rats were compared to control animals, no significant change was found. However, *Epilobium hirsutum L.* and ellagic acid-treated animals showed a significant

decrease in total cholesterol level compared to control animals (Figure 3.26).

Table 3. 5 Blood serum total cholesterol values of control and *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid treated animals.

Sample	Total cholesterol (mg/dL)
Control	70.5±1.905
<i>Viscum album L.</i>	61.25±2.333
<i>o</i> -coumaric acid	68±2.793
<i>Epilobium hirsutum L.</i>	46.25±2.306
Ellagic acid	46±3.572

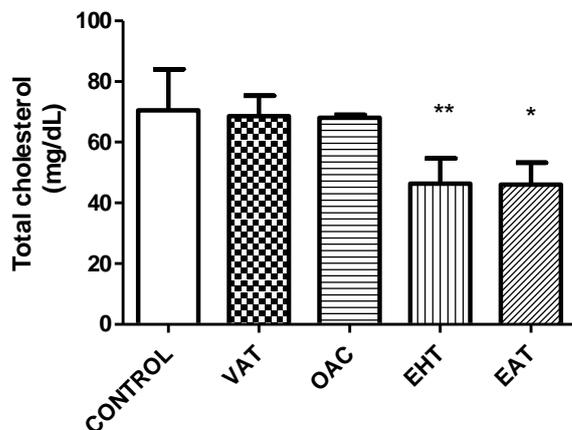


Figure 3. 22 Comparison of blood serum total cholesterol levels between *Viscum album L.* (VAT), *Epilobium hirsutum L.* (EHT), *o*-coumaric acid (OAC), ellagic acid (EAT) treated, and control animals. *Significantly different from the respective control value $p < 0.05$.

3.7 CYP7A1 Enzyme Activity

Cholesterol 7 α -hydroxylase has important function in the regulation of bile acid biosynthesis. However, our knowledge about this enzyme is already not enough due to the limitations in the assay procedure. Different methods were used to determine the 7 α -hydroxycholesterol enzyme activity. According to literature, the method described in this study “Materials and Methods” is sensitive, rapid, useful and more accurate than radioactive and GS-MS methods. The assay for 7 α -hydroxylase activity was determined according to the method Waxman & Chang (2002) with a slight modification. The enzyme reactions were analyzed by HPLC equipped with

an Ultraviolet (UV)-spectrometer; column was Altech silica column (4.6 x 250 mm, 5 μ m particle size). The solvent system were isopropanol:n-hexane mixture in a ratio of 5:95. Chromatographic separation was carried out at 30°C and the effluents were monitored at 212 nm. The areas of peaks of compounds were calculated by a computer equipped in HPLC. We prepared 50 μ M, 100 μ M and 1 mM cholesterol dissolved in hexane. Under these conditions, the retention time was 2.7 min for cholesterol. Calibration curve of cholesterol was shown in Figure 3.27. Also Figure 3.28, Figure 3.29 and Figure 3.30 show the elution profile of 50 μ M, 100 μ M and 1 mM cholesterol from HPLC, respectively.

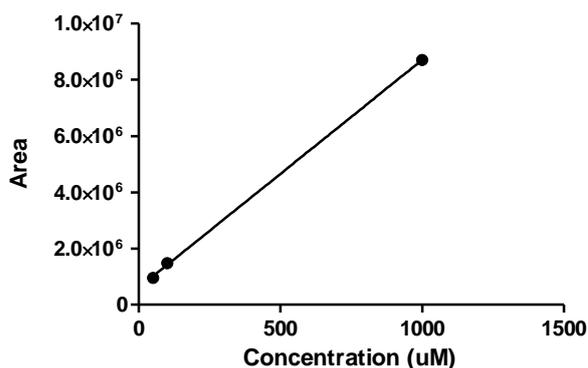


Figure 3.23 Calibration curve for cholesterol.

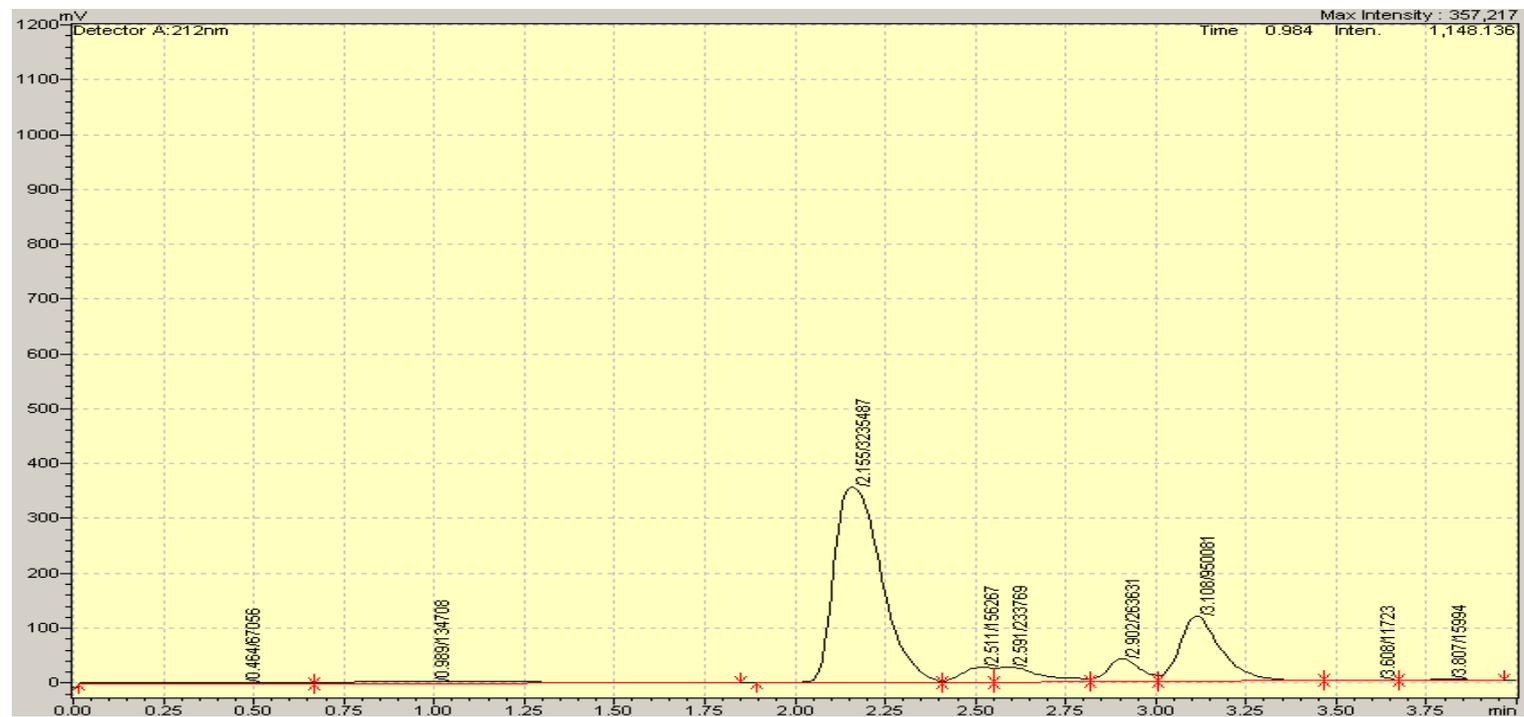


Figure 3.24 High performance liquid chromatogram of 50 μ M cholesterol.

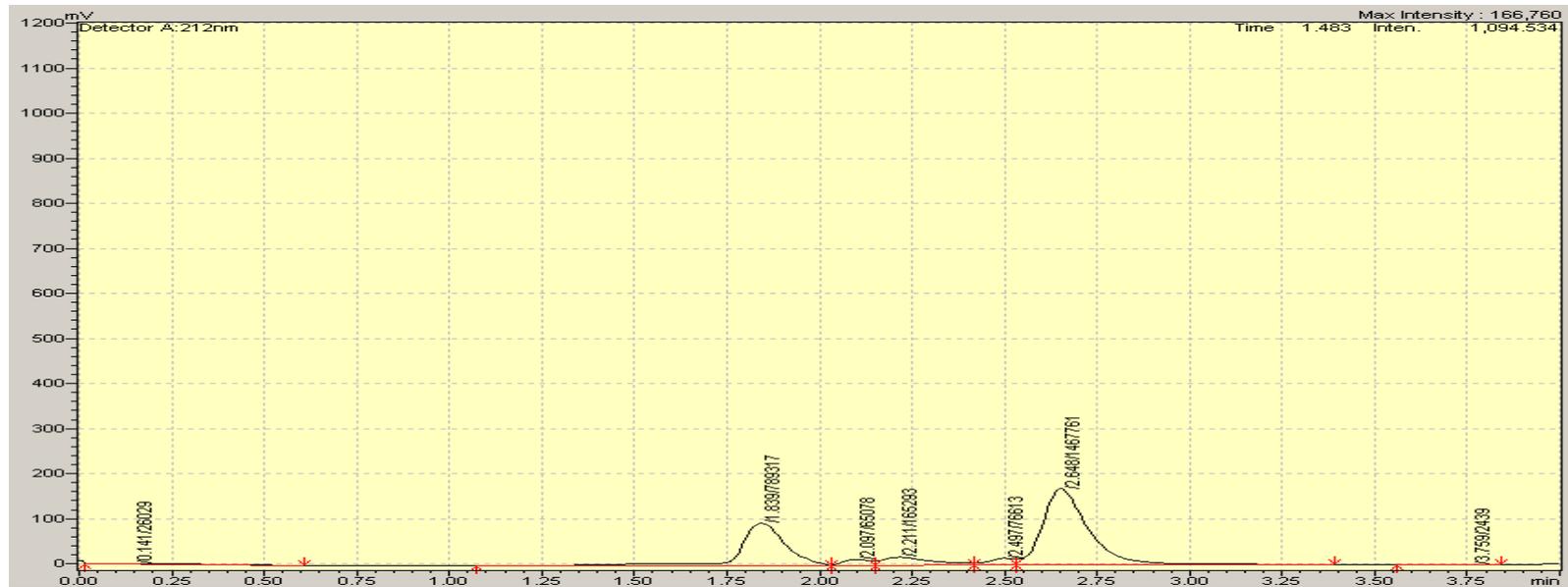


Figure 3.25 High performance liquid chromatogram of 100 μ M cholesterol.

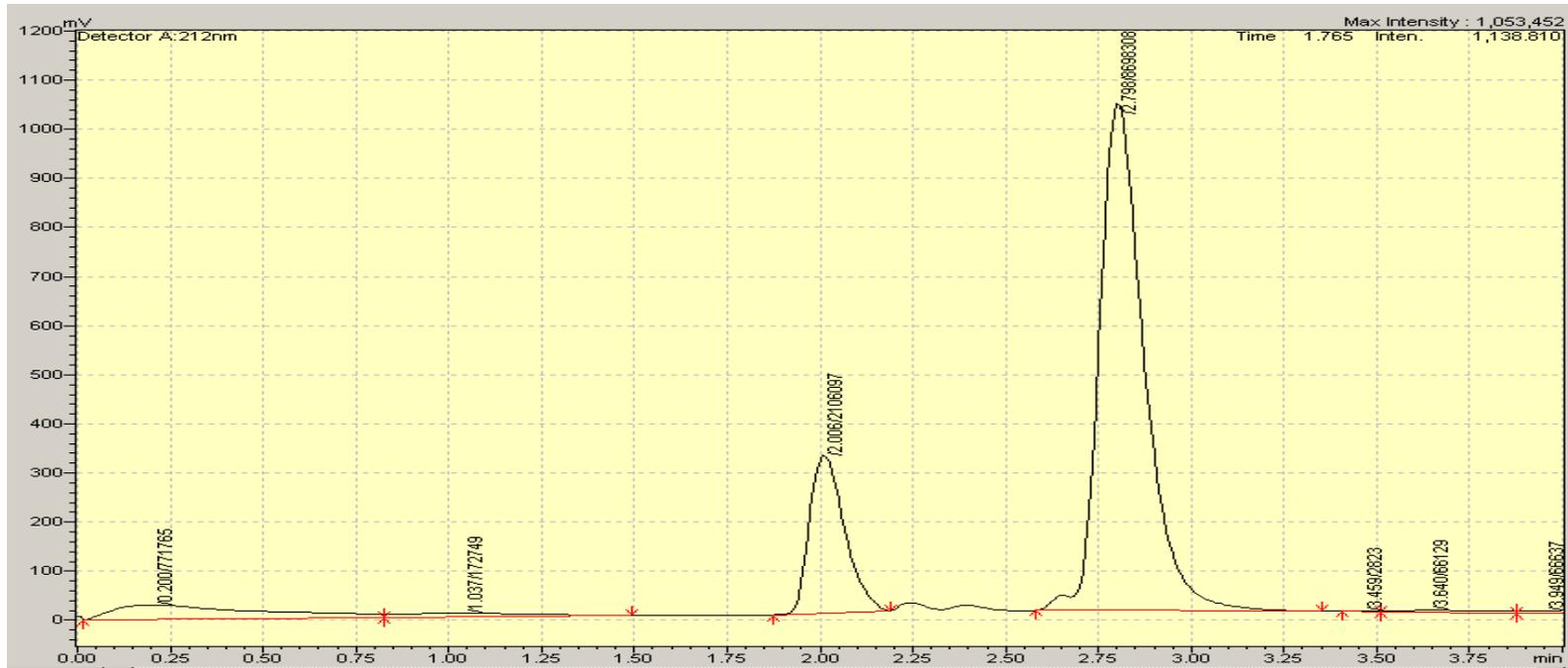


Figure 3.26 High performance liquid chromatogram of 1 mM cholesterol.

We used different solvents to solve 7 α -hydroxycholesterol such as isopropanol, hexane, ether, ethyl acetate, benzene, chloroform. But, so far, we could not find the solvent most efficiently to solve 7 α -hydroxycholesterol. We are still working on to solve this problem.

CHAPTER 4

DISCUSSION

Until now, two-thirds of the plants identified have medicinal importance and they have been used as a medicine for human health and a supplement to maintenance of a healthy lifestyle for centuries. Many species of plants have been documented to have medicinal properties and beneficial impacts on health, especially as antioxidant activity, anti-carcinogenic potential, anti-microbial, anti-inflammatory, anti-mutagenic effects and digestive stimulation action (Kliebenstein & Osbourn 2012). Several phytochemicals founded in plants have been elucidated of therapeutical importance. Thousands of secondary metabolites (phenolics and terpenoids etc.), bioactive compounds, in plants have shown promising capacity to prevent and/or cure of diseases (Kliebenstein & Osbourn 2012; Bonilla *et al.* 2015; Kasote *et al.* 2015).

CYPs (Cytochromes P450 enzymes) play biological and clinical key role because they possess numerous substrates both endogenous compounds including steroids and fatty acids, and exogenous compounds including drugs, environmental chemicals and pollutants (Arınç *et al.*, 1991; Nebert & Russell 2002; Graham & Peterson 1999; Arınç *et al.* 2000a, 2000b; Estabrook 2003; Schenkman *et al.* 2003; Coon 2005; Nebert & Dalton 2006; Omura 2006; Wauthier *et al.* 2007; Petterson 2009). In mammals, excessive cholesterol is removed mainly through conversion to bile acids, and only a small portion is utilized for production of steroid hormones and other endogenous compounds. Bile acids are the products of cholesterol catabolism in the liver and function as signaling molecules that support cholesterol homeostasis and detergent in intestine to facilitate absorption

and digestion of fat. Cytochromes P450 (P450s or CYPs) initiate all quantitatively significant pathways of cholesterol metabolism and bile acid biosynthesis. It has been reported that herbs pharmaco-kinetically affect mRNA and protein expressions and activities of especially cytochrome P450 enzymes. Also CYPs are influenced by diet as well as pathophysiological and physiological situations (Arınç *et al.* 2007; Sen *et al.* 2015).

This is the first study to demonstrate the *in vivo* effects of medicinal plants *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients, *o*-coumaric acid (2-hydroxycinnamic acid) and ellagic acid on rat liver bile acid metabolizing CYPs (CYP7A1, CYP27A1, CYP8B1 and CYP7B1). The water extracts of *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid were injected i.p. as 10 mg/kg, 37.5 mg/kg, 30 mg/kg, 20 mg/kg and for 9 days, respectively. *In vivo* effects of *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid on rat liver bile acid metabolizing CYPs were analyzed by determining protein and mRNA expression levels using western blotting and qRT-PCR techniques, respectively.

4.1 Analysis of *Viscum album L.* and *o*-Coumaric Acid Protein and mRNA Expression Results

European mistletoe, *Viscum album L.*, includes many bioactive compounds. Its composition bases on not only the harvest season and the host trees but also the manufacturing process. This folkloric and medicinal plant grows different trees and our sample *Viscum album ssp. austriacum* (Wiesb) Volim grows mainly on *Pinus sylvestris L.* In Table 4.1 was shown the determined bioactive compounds in *Viscum album L.* (Barney *et al.* 1998; Nazaruk & Orlikowsk 2015).

Table 4.1 The chemical profile of *Viscum album L.* (Barney *et al.* 1998; Nazaruk & Orlikowsk 2015).

Chemical class	Name of the Compound
Lectins	Mistletoe lectins ML I, ML II, ML III
Viscotoxins	Viscotoxins A2, A3, B
Alkoloids	4,5,40-trihydroxy-3,30-iminodibenzoic acid, 4,5,40,50-tetrahydroxy-3,30- iminodibenzoic acid
Phenolic acids	protocatechuic, phydroxybenzoic, caffeic, salicylic, ferulic, sinapic, <i>o</i> -coumaric, digallic acids
Flavonoids	5,7-dimethoxy-flavanone-4''-O-glucoside; 2''-hydroxy-4'',6''-dimethoxychalcone-4-O-glucoside; 5,7-dimethoxyflavanone-4''-O-[2''-O-(5''''-O-trans-cinnamoyl)-apiosyl]-glucoside); 2''-hydroxy-4'',6''-dimethoxychalcone-4-O-[2''-O-(5''''-O-trans-cinnamoyl)-apiosyl]-glucoside; 2''-hydroxy-3,4'',6''-trimethoxychalcone-4-O-glucoside, 2''-hydroxy-4'',6''-dimethoxychalcone-4-O [apiosyl(1→2)]glucoside; 5,7-dimethoxyflavanone-4''-O-[apiosyl-(1→2)]-glucoside; (2S)-3'',5,7-trimethoxyflavanone-4''-O-glucoside, quercetin, kaempferol
Phenylpropanoids	coniferyl alcohol 4-O-b- D-glucoside (coniferin), syringenin 4-O-b-D-glucoside (syringin), coniferyl alcohol- and syringenin 4-O-b-D-apiosyl(1→2)-b-D-glucoside, lignans – syringaresinol 40,400-di-O-glucoside (eleutheroside E), syringaresinol-O-glucoside
Triterpenes	β-amyrin acetate, oleanolic acid, betulinic acid
Carbohydrates	1→α 4 galacturonic acid methyl ester, highly methylated homogalacturonan, pectin, arabinogalactan

Until now, it has been proved that *Viscum album L.* has important pharmacological impacts. Since ancient times this plant has been used to cure of different diseases such as diabetes mellitus, dizziness, nervous tachycardia, hysteria, hypertensive headache, atherosclerosis, stroke, chorea, peri-arthritis, and arthritis (Murray 1995; Newall *et al.* 1996; Ohiri *et al.* 2003). Also, since 1927, the plant has been used as a supportive element especially oak mistletoe for cancer treatment since it stimulates immune response-system (Ramm 2015). According to clinical experiments, total mistletoe extracts and its isolated polyphenolic compounds have essential effects in treatment of cancer. For example, lectins may play a critical role in process of apoptosis on cancer cells in order to inhibit the growth of tumor cells (Ribe'reau-Gayon *et al.* 1996). Based on this reason, Delebinski *et al.* (2012) researched the effects of *Viscum album L.* on acute lymphoblastic leukaemia. They treated cell lines with enhanced triterpene and lectin-containing extracts. These extracts lead to apoptosis induction in lymphoblastic leukaemia (ALL). Following a study researched by Kienle *et al.* (2009) revealed that mistletoe extracts display therapeutic effect in breast cancer by improving life quality and relieving side effects. Besides, Hong and Lyu (2012) determined galactose- and N-acetyl-D-galactosamine-specific lectin founded in mistletoe have anti-cancer activity. Also, Schaller *et al.* (1996) demonstrated viscotoxins (especially viscotoxin-3) have cytotoxic activity. Thus, Hong and Lyu (2012) proposed that viscotoxins and flavonoids isolated from *Viscum album L.* have a beneficial effect at hypertension (Hong & Lyu 2012). In other study, isolated phenylpropanoids and flavonoids from mistletoe caused vascular relaxation by lowering the blood pressure. Most importantly, Deliorman *et al.* (2000) suggested that all above discussed these bioactive ingredients inhibit (cAMP)-phosphodiesterase (Deliorman *et al.* 2000).

As mentioned above, *Viscum album* includes many bioactive compounds and we chose to work with *o*-coumaric acid. *o*-Coumaric acid, which also

named as trans-*o*-hydroxycinnamic acid, is found in many plant products, such as vinegar, coffee, tea, beer, oats and peanuts. Researches evaluated that *o*-coumaric acid has many biological activities, such as anti-lipidemic, anti-obesity, anti-oxidant, and anti-carcinogenic activities (Yeh *et al.* 2005; Hsu *et al.* 2009).

According to quantitative LC-MS analysis carried out in the present study, chlorogenic acid (1.0528 ppm) was the dominant compound in the *Viscum album L.* Gallic acid and quercetin dehydrate amounts were less than 0.31 ppm. Caffeic acid and *o*-coumaric acid values were calculated less than 0.15 ppm and 0.27 ppm, respectively (Table 4.2). Also, the data represented in Table 4.2 revealed that the amount of total phenolic content of *Viscum album L.* was 57 ± 1.756 mg GAE/g dried extract and, the result of total flavonoid of *Viscum album L.* was 19.0278 ± 1.2615 mg CE/ g dried extract.

Vicaş *et al.* (2011b) worked with *Viscum album* from different host trees (*Acer campestre*, *Fraxinus excelsior*, *Populus nigra*, *Malus domestica*, and *Robinia pseudoacacia*). Gallic acid, a phenolic acid, was only found in *Viscum album* hosted by *Robinia pseudoacacia*. The mistletoe hosted by *Fraxinus excelsior* contains very high concentration of caffeic acid in leaves (13.98 µg/g dry matter) and stems (15.86 µg/g dry matter). Trans-cinnamic acid (*o*-coumaric acid), a phenolic compound, was identified in stem of *Viscum album* hosted by *Acer campestre*. Moreover, Haas *et al.* (2003) and Materska (2008) showed that quercetin, a flavanol, and its methyl derivatives founded in mistletoe, have strong antioxidant activity. Also, Condrat *et al.* (2009) determined that quercetin was found very low concentration in mistletoe extracts (0.20 µmol/g dry matter). Luczkiewicz *et al.* (2001), analyzed the *Viscum album* hosted by *Populus nigra*, they displayed the main component of mistletoe was chlorogenic acid (12.34 mg %).

Table 4. 2 Quantitative LC-MS analysis of selected bioactive compounds from *Viscum album L.* and *Epilobium hirsutum L.*; and total phenolic and flavonoid content of these plants.

Bioactive compound	<i>Viscum album L.</i>	<i>Epilobium hirsutum L.</i>
Gallic acid	≤ 0,31 ppm	11,906 ± 0,1810
Cafeic acid	≤ 0,15 ppm	≤ 0,31 ppm
Quercetin dhydrate	≤ 0,31 ppm	≤ 0,625ppm
Chlorogenic acid	1,0528±0,0453	-
<i>o</i> -coumaric acid	≤ 0,27 ppm	-
<i>p</i> -coumaric acid	-	1,0642±0,0027
Ellagic acid	-	≤ 0,31 ppm
Total phenolic content	57 ±1,756 (mgGAE/g dried extract±SD)	225±2, 123 (mgGAE/g dried extract±SD)
Total flavonoid content	19,0278 ±1,2615 (mg CE/g dried extract±SD)	19,2593±0,8225 (mg CE/g dried extract±SD)

In the same research, Vicaş *et al.* (2011b) identified the highest total phenolic content of mistletoe hosted by *Robinia pseudoacacia* was 200.51 ± 0.00 mg GAE/g fresh matter, while the lowest value of mistletoe hosted by *Malus domestica* was 176.87 ± 0.003 mg GAE/g fresh matter. The highest total flavonoid content in *Viscum album* by hosted *Robinia pseudoacacia* was determined as 21.86 mg CE/ g fresh matter whereas the lowest total flavonoid content in *Viscum album* by hosted *Malus domestica* was 0.36 mg CE/ g fresh matter. Sengül *et al.* (2009) determined of total phenolic content of *Viscum album* as 42.29 mgGAE/g dry extract. Also, Pietrzak *et al.* (2014) analyzed total phenolic and flavonoid content of *Viscum album* subsp *abietis* as 37.152 ± 1.895 mgGAE/g dry extract and 9.955 ± 0.482 mgCE/g dry extract, respectively. As a result of these experiments total phenolic and flavonoid content are correlated with LC-MS result and also our results are very similar with literature for *Viscum album* extract.

Guillermo *et al.* (1999) worked with Argentinean mistletoe to investigate the effect of blood fluidity through the hemorheological behavior on treated wistar rats. The red blood cell membrane composition was changed by blood cholesterol content. Argentinean mistletoe-treatment decreased plasma cholesterol levels and increased the viscosity and reduced deformation of red blood cell. Cholesterol excretion (bile acids) and cholesterol biliary secretion also lead to reduction of plasma cholesterol level. Increment of bile acid excretion was same with bile flow increment (Guillermo *et al.* 1999). Later, Poruthukaren *et al.* (2014) evaluated the anti-hypertensive effect of *Viscum album*. *Viscum album* was given patients newly diagnosed and had not used any drug for hypertension. After 12 weeks administration of mistletoe, serum total cholesterol and serum triglyceride levels was statistically evaluated. There was not any significant change of serum cholesterol level while serum triglyceride was significantly decreased (Poruthukaren *et al.* 2014). Another research about anti-hypertensive activity elucidated that *Viscum album* decreased the blood

pressure in hypertensive rats (Radenkovic *et al.* 2009). Also, Avci *et al.* (2006) chose five plants including *Viscum album* from Turkish flora to research anti-hypercholesterolemic and antioxidant activity of these five plants. They investigated the impacts of water and ethanol extracts of plants on serum total cholesterol, triglyceride, HDL-C, LDL-C, glucose, AST and ALT concentrations in mice fed with cholesterol-rich diet. The ethanol extracts of *Viscum album* decreased the total serum cholesterol level while water extract did not affect significantly the total serum cholesterol concentration. Level of HDL was increased and, meanwhile LDL level was decreased by the ethanolic extract of *Viscum album*. Under the light of the data they suggested that ethanolic extract of *Viscum album* has a beneficial effect in the deal with atherosclerosis and cardiovascular diseases.

Hsu *et al.* (2009) looked into anti-obesity impacts of *o*-coumaric acid and rutin in obese rats. Rats separated into 2 groups as obese and control. *o*-Coumaric acid and rutin were administrated 100 mg/kg and 50 mg/kg respectively. Tissues weight and serum profile of lipid, insulin, leptin, hepatic cholesterol and triacylglycerol were compared between control and obese rats. Research parameters showed a decrease in *o*-coumaric acid and rutin treated obese-rats compare to non-treated obese rats. Especially, hepatic cholesterol and triacylglycerol levels were drastically diminished. They determined the total cholesterol level as 53 ± 5.0 mg/dl in normal rats, 76.2 ± 13.0 mg/dl in fed with high fat diet. After administration of low and high doses of *o*-coumaric acid, total cholesterol levels were determined as 67.3 ± 11 and 65.2 ± 7.0 respectively. According to these results, they suggested that intake of *o*-coumaric acid and rutin may be important agent for repression of oxidative stress, hepatosteatosi and dyslipidemia in rats, and also *o*-coumaric acid can be promising parameter for the treatment of obesity (Hsu *et al.* 2009).

Determination of serum total cholesterol level is one of the important biomarker for especially to identify of cardiovascular diseases. When we compared the total cholesterol level of the water extracts of *Viscum album L.* (61.25 ± 2.333) and *o*-coumaric acid (68 ± 2.793) treated samples to control (70.5 ± 1.905) in this study there was no significant change in total cholesterol levels and also our results are very similar with literature for *Viscum album* extract.

In this study, protein and mRNA expressions of bile acid metabolizing enzymes were analyzed in *Viscum album* and *o*-coumaric acid-treated samples. According to results, *Viscum album L.* caused 0.9 fold a decrease in protein expression of CYP7A1 and CYP7B1, but 1.4 and 1.3 fold increase in protein expression of CYP27A1 and CYP8B1, respectively. *Viscum album L.* caused 9, 1.3 and 2.4 fold decrease in mRNA expression of CYP7A1, CYP27A1, CYP7B1, respectively, with respect to controls and normalized with GAPDH expression as an internal reference ,however, 4.2 fold increase in mRNA expression of CYP8B1. It indicates that the decrease of CYP27A1 mRNA expression does not interpret with a proper decrease of CYP27A1 protein levels.

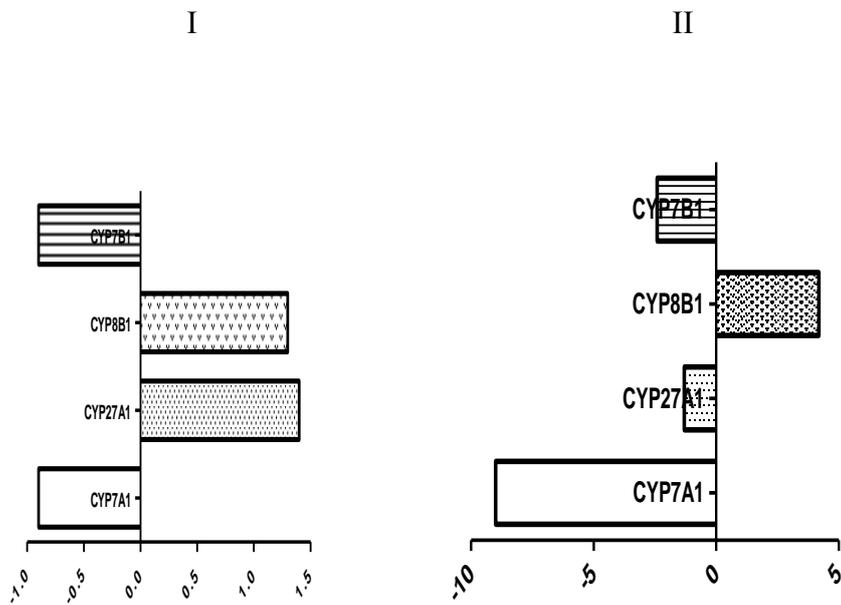


Figure 4. 1 Effects of *Viscum album L.* on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions. I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.

In present study, *o*-coumaric acid caused 0.8 fold decrease in protein expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1. *o*-Coumaric acid caused 1.8, 1.3, 2.3 and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 respectively, with respect to controls and normalized with GAPDH expression as an internal reference. There is no correlation between relative mRNA and protein expressions of *o*-coumaric acid treated CYP7A1, CYP27A1, CYP8B1 and CYP7B1 as shown Figure 4.2.

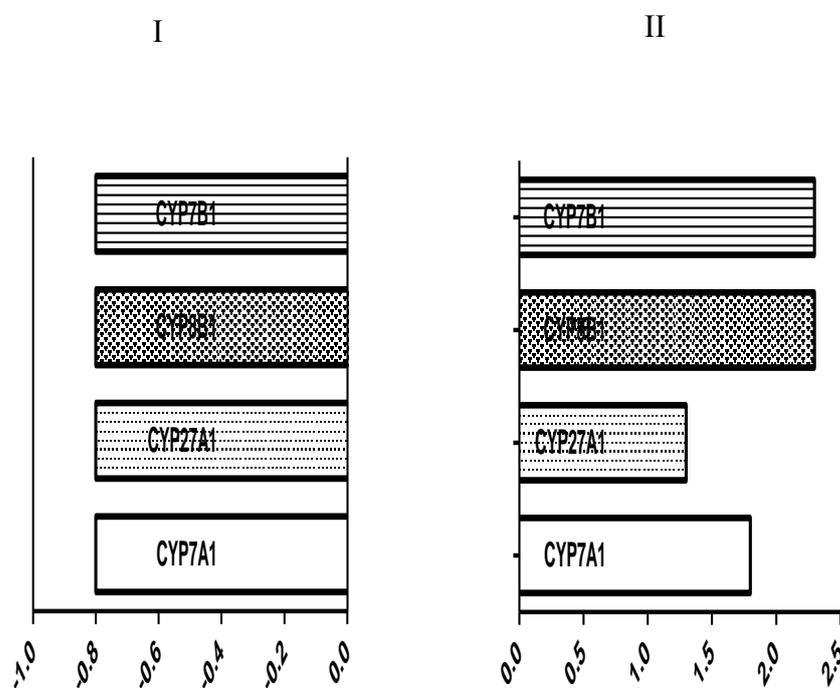


Figure 4. 2 Effects of *o*-coumaric acid on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions. I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.

There is a correlation relative to protein and mRNA expression of CYP7A1, CYP8B1 and CYP7B1 in *Viscum album*-treated samples. While according to all western-blot and qRT-PCR results clearly not corroborated for *o*-coumaric acid-treated samples. Bussing *et al.* (1997) theorized that whole plant extract of *Viscum album* is more efficient than purified phenolic and flavonoid compounds. According to our data (Figure 4.3), we obtained same conclusion. When we compared *Viscum album* and its phenolic compound (*o*-coumaric acid), *Viscum album* is more effective than *o*-coumaric acid. European mistletoe extracts are still provoking intense discussion, hence research should be conducted with a lot of caution.

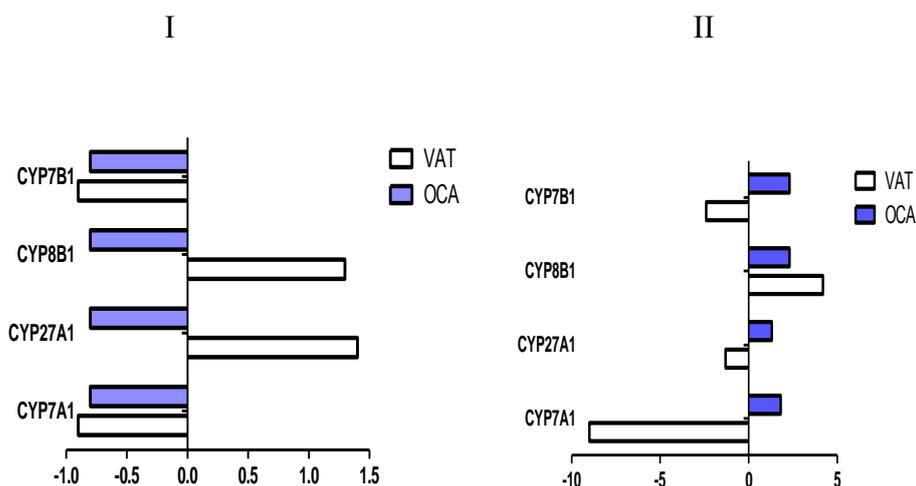


Figure 4. 3 Effects of *Viscum album L.* and *o*-coumaric acid on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions. I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.

4.2 Analysis of *Epilobium hirsutum* L. and Ellagic Acid Protein and mRNA Expression Results

Epilobium hirsutum L. is also commonly called as great-hairy willow herb, codlins and cream, European fireweed, apple-pie and cherry-pie. *Epilobium hirsutum* L., great willow-herb, grows in Eurasia, North Africa, Europe, Southern Australia and United States. *Epilobium hirsutum* L. was used traditionally to treat gastrointestinal and prostate diseases, menstrual disorder, stomach ulceration, gastritis and sleeping disorders (Melchior 1972; Hiermann *et al.* 1986; Battinelli *et al.* 2001; Štajner *et al.* 2007). *Epilobium hirsutum* L. also has anti-bacterial, anti-microbial, anti-exudative, anti-nociceptive, anti-septic and anti-phlogistic effects (Gruenwald *et al.* 1998; Štajner *et al.* 2007; Ebrahimzadeh *et al.* 2010; Pakvaran *et al.* 2012). In Table 4.3 was shown the determined bioactive compounds in *Epilobium hirsutum* L. (Toth *et al.* 2009).

Table 4. 3 The chemical profile of *Epilobium hirsutum* L. (Toth *et al.*2009).

Chemical class	Name of the Compound
Steroids	β -sitosterol and its esters
Tannins	Valoneic acid dilactone, elagintannin
Phenolic acids	Caffeic acid, p-coumaric acid, ferulic acid , gallic acid, protocatechuic acid, ellagic acid
Flavonoids	myricetin, quercetin, kaempferol

Ellagic acid is one of the most studied phenolic acids and *Epilobium hirsutum* includes it. Ellagic acid is a therapeutic agent for many diseases and it is found in pomegranate, strawberries, cloudberry, blueberry, blackberry, grapes, raspberries, cranberries, pecans, walnuts and black currants (Bala *et al.* 2006). Ellagic acid in foodstuff is found either generally conjugated with a glycoside moiety or part of ellagitannins (Seeram *et al.* 2004).

Çelik *et al.* (2013) evaluated effects of different doses of ellagic acid on rat liver enzymes. 10 mg/kg ellagic acid (lower dose) was not enough to be effective while 30 mg/kg ellagic acid (high dose) was effective. They found that high dose ellagic acid suppressed CYP1A, CYP2E, and CYP19 and induced GSTs, NQO1, GPX, and CAT enzymes expression. They suggested that ellagic acid may play an important chemo-preventive role (Çelik *et al.* 2013). Later, Çelik *et al.* (2014) researched inhibitory effects of *Epilobium hirsutum* and its major polyphenolic content (ellagic acid) on drug metabolizing enzymes. Erythromycin N-demethylase, benzphetamine N-demethylase and 7-benzyloxyresorufin-o-debenzylase activities were decreased after treatment of *Epilobium hirsutum* and ellagic acid. Protein expressions of CYP2B1, CYP2C6, CYP2D2, and CYP3A1 also decreased on *Epilobium hirsutum* and ellagic acid treated rats. qRT-PCR analyses showed same results. They concluded that inhibition of drug clearances by CYP enzymes can cause drug toxicity in the people used *Epilobium hirsutum* as a complementary supplement. These situations can be occurred by ellagic acid content of *Epilobium hirsutum* (Çelik *et al.* 2014). Moreover, Meyer *et al.* (1998) investigated that inhibition of copper-catalyzed human LDL oxidation was occurred by five plant phenols including ellagic acid had a similar o-dihydroxy moiety. The order of anti-oxidant activity was catechin, cyanidin, caffeic acid, quercetin and ellagic acid, respectively. In that study, ellagic acid was not effective in LDL oxidation. They explained that the reason of different inhibition of LDL oxidation was in their different structural properties. Catechin, cyanidin and

quercetin 3,5,7,3',4' penta-hydroxy phenols possessing same features of hydroxyl groups in their A and B rings. Also, caffeic acid much stronger antioxidant than ellagic acid since ellagic acid has more hydroxyl groups. Besides, Laranjinha *et al.* (1994) suggested that ellagic acid shows low antioxidant activity than caffeic acid owing to internal hydrogen bonding in ellagic acid between a proximate phenolic OH group and the oxygen atom in (one of) the lactonic ring(s). Apparently, hydrogen atom-donating antioxidant mechanism, a chain-breaking, such hydrogen bonding would reduce the ability of ellagic acid to give a phenolic hydrogen atom to a peroxy radical (Laranjinha *et al.* 1994). Later, Seeram *et al.* (2005) researched the antioxidant and anti-atherosclerotic effects of pomegranate and its polyphenols including punicalagin, ellagitannin, and ellagic acid. They founded pomegranate has more antioxidant activity than its polyphenolic compound. Taken results showed that punicalagin, ellagic acid and ellagitannin decreased tumor cells of human oral, colon and prostate while pure pomegranate showed highest activity compared with its phenolic compounds. It is concluded that though the purified polyphenols exhibited important anti-proliferative, apoptotic and antioxidant effects alone, the excellent activity of pomegranate proposes multifactorial effects and chemical synergy of the action of multiple compounds compared to single purified active ingredients (Seeram *et al.* 2005).

According to quantitative LC-MS analysis carried out in the present study, gallic acid (1.0528 ppm) was the dominant compound in the *Epilobium hirsutum L.* *p*-coumaric acid amount was 1.0642 ppm, while quercetin dehydrate was less than 0.625 ppm, and the ellagic acid value was calculated as less than 0.31 ppm (Table 4.2). Also, the data represented in Table 4.2 revealed that *Epilobium hirsutum L.* has higher phenolic content when compared with *Viscum album L.* The amount of total phenolic content of *Epilobium hirsutum L.* was 225 ± 2.123 mg GAE/g dried extract and the

result of total flavonoid of *Epilobium hirsutum L.* was 19.2593 ± 0.8225 CE/g dried extract.

Wajdylo *et al.* (2007) identified total phenolic content of *Epilobium hirsutum L.* as 4.03 ± 0.12 mg of gallic acid equivalents (GAE)/ 100g of dry weight. As a result of these experiments total phenolic and flavonoid content was correlated with LC-MS result. But our total phenolic content result of *Epilobium hirsutum* was higher than literature.

Total cholesterol levels were determined by enzymatic and spectrophotometric technique. When we compared the total cholesterol level of *Epilobium hirsutum L.* (46.25 ± 2.306) and ellagic acid (46 ± 3.572) treated animals to control (70.5 ± 1.905) in this study, they showed a significant decrease in total cholesterol levels.

In this study, protein and mRNA expressions of bile acid metabolizing enzymes were analyzed in *Epilobium hirsutum L.* and ellagic acid-treated samples. According to results, *Epilobium hirsutum L.* caused 0.6, 0.8 and 0.6 fold decrease in protein expression of CYP7A1, CYP8B1, CYP7B1, respectively, on the contrary 1.3 fold increase in protein expression of CYP27A1. *Epilobium hirsutum L.* caused 2.1, 1.4 and 4.1 fold decrease in mRNA expression of CYP7A1, CYP27A1, CYP7B1, respectively, with respect to controls and normalized with GAPDH expression as an internal reference, however, 2.3 fold increase in mRNA expression of CYP8B1. Figure 4.4 indicates that the decrease of CYP27A1 mRNA expression does not interpret with a proper decrease of CYP27A1 protein levels. Moreover, the increase of CYP8B1 mRNA expression does not correlate with a fitting increase of CYP8B1 protein expression.

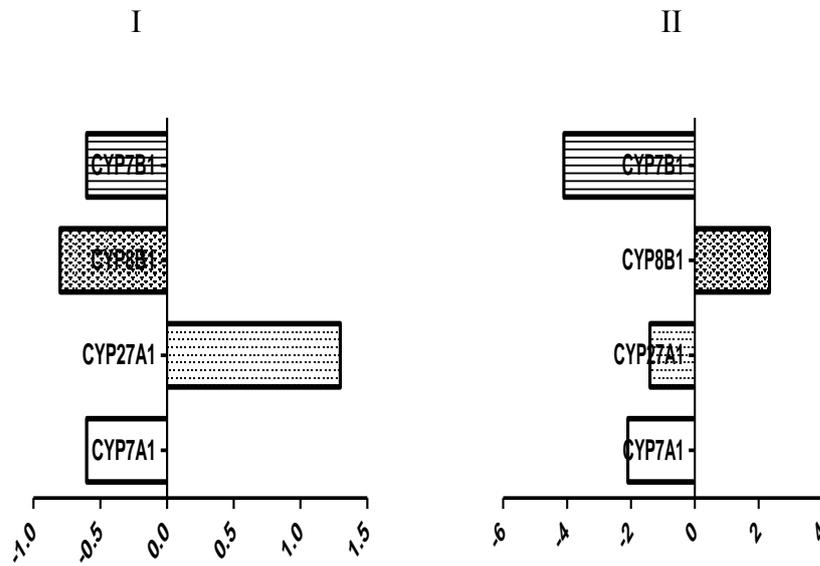


Figure 4. 4 Effects of *Epilobium hirsutum* L. on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions. I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.

According to results, ellagic acid caused 0.7, 0.8 and 0.8 decrease in protein expression of CYP27A1, CYP8B1, CYP7B1, respectively, but one fold increase of protein expression of CYP7A1. Ellagic acid caused 2.6, 1.7, 1.8 and 2.3 fold increase in mRNA expression of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 respectively, with respect to controls and normalized with GAPDH expression as an internal reference. There is only a correlation between relative mRNA and protein expressions of ellagic acid-treated CYP7A1 enzyme as shown Figure 4.5.

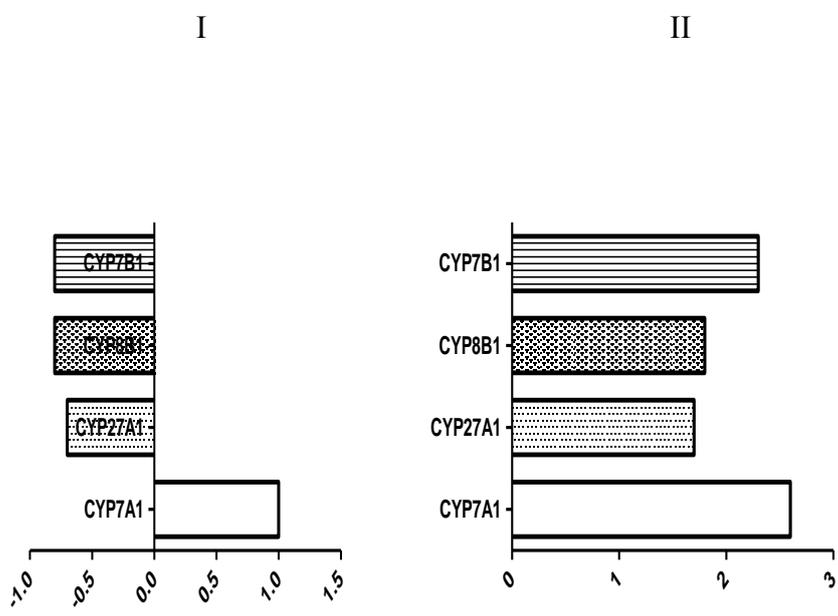


Figure 4. 5 Effects of ellagic acid on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions. I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.

According to all western-blot and qRT-PCR results clearly not corroborated the enzyme expressions. There is a correlation relative to protein and mRNA expression of CYP7A1 and CYP7B1 in *Epilobium hirsutum*-treated samples and CYP7A1 in ellagic acid-treated samples.

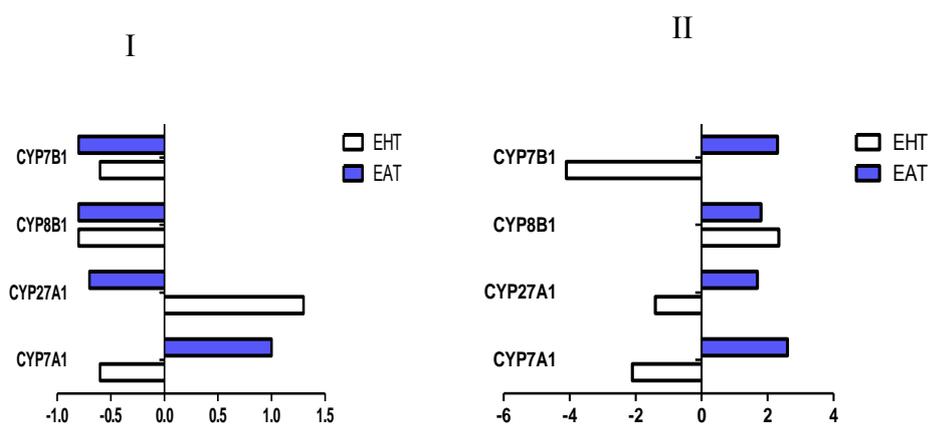


Figure 4. 6 Effects of *Epilobium hirsutum* L. and ellagic acid on CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein (I) and mRNA (II) expressions. I: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 protein expression. II: Fold change of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 mRNA expression.

CYPs selectively chose their substrates. Different endogenous and exogenous compounds induce and/or inhibit CYPs genes. Mechanism of induction and inhibition is very important to adjust according to the needs of organism. Herbs are one of the members of the inducers and/or inhibitors. It appears that in Figure 4.3 and Figure 4.6 when mRNA and protein expression of CYPs were compared, there was no significant correlation. The expressions of CYPs gene is regulated transcriptionally, post-transcriptionally, translationally and post-translationally (Lang & Mathiev 2000; Plotkin 2010). These mechanisms are important for steady-state protein concentration. There are some reasons for these poor correlation or non-correlation. Translation and protein degradation are as important as mRNA transcription and stability of the final product of protein. Transcription is first and major target for gene regulation. But transcriptional regulation is not enough for describe protein abundance. One of the explanation is micro RNA (miRNAs), 21 nucleotide long non-coding RNA, mediates gene expressions. Generally miRNAs repress the protein synthesis by inhibiting translation and/or via facilitating deadenylation and following degradation of mRNA targets (Fabian *et al.* 2010). Initiation process of translation is the rate limiting step for transcriptional control. It has been determined that miRNAs repress the translation at initiation step either by targeting the cap recognition step or by inhibiting ribosome 80S complex formatting. Also, miRNAs may inhibit translation at postinitiation steps by repressing ribosome elongation, inducing ribosome drop-off, or facilitating proteolysis (Abaza & Gebaer 2008; Fabian *et al.* 2010; Vogel *et al.* 2010; Li *et al.* 2013). In same manner, protein degradation influence protein concentration. Ubiquitin-proteosome mediated proteolysis is regulator for protein degradation and protein abundance (Vogel *et al.* 2010). Other reason is proteins have different half lives in their *in vivo* conditions. So, there are varied protein synthesis and degradation (Greenbaum *et al.* 2003).

4.3 Regulation of Bile Acid Metabolizing Enzymes

The first and rate limiting enzyme of neutral pathway is cholesterol 7 α -hydroxylase (CYP7A1). Bile acids can regulate cholesterol homeostasis via bile acid synthesis pathway. Bile acid synthesis is regulated by many factors, including bile acids, nutrients, diets, and hormones, especially through regulating transcription of CYP7A1 gene. Besides, many liver specific transcription factors, commonly nuclear receptors have an important role in cholesterol and bile acid metabolism (Chiang 2002; Davis *et al.* 2002; Fuchs 2003; Li & Chiang 2012).

Toth *et al.* (2009) suggested that *Epilobium* species showed different flavonoid composition and antioxidant activity. Thus, *Epilobium* species show multidirectional effect (Toth *et al.* 2009). During recent years, *Epilobium* species (especially *Epilobium angustifolium* L.) were used to treatment for Benign Prostatic Hyperplasia (BPH). Kujawski *et al.* (2009) researched the impacts of water extract of *Epilobium angustifolium* L. on CYP3A1 and CYP7A1 since they are important in steroid metabolism. The results showed that the plant has strong inhibitory effect on CYP3A1/2 and slight induction effect on CYP7A1 mRNA expression in rat liver. They suggested that the plant extract may regulate transcriptional level of different CYP genes in different ways (Kujawski *et al.* 2009).

In addition, there are some other researches related with bile acid metabolizing enzymes. Procyanidins are members of proanthocyanidins, which are polyphenols. Proanthocyanidins are founded in apples, grape seeds, cocoa beans and cinnamon etc. Proanthocyanidins founded in grape seed have many biological activities such as anti-atherosclerosis, anti-carcinogenesis, anti-hyperglycemic and anti-oxidant. Jiao *et al.* (2010) suggested that grape seed proanthocyanidins can decrease plasma total cholesterol and triacylglycerol level while it can increase protein and mRNA

expression of cholesterol-7 α -hydroxylase (CYP7A1). They informed that hypocholesterolemic activity of grape seed proanthocyanidins has important to increase of bile acid elimination via induction of CYP7A1 production (Jiao *et al.* 2010). In another research, red wine decreases the fatality from cardiovascular diseases. The polyphenols founded in red wine especially procyanidins exhibit anti-atherogenic actions. Bas *et al.* (2005) showed that procyanidins lowered plasma triglyceride, free fatty acids and LDL-cholesterol and slightly increased HDL-cholesterol. mRNA level of cholesterol 7 α -hydroxylase (CYP7A1), small heterodimer partner (SHP) and cholesterol biosynthetic enzymes increased. They concluded that procyanidins induces in the liver the over expression of CYP7A1 (suggesting an increase of cholesterol elimination via bile acids) and SHP (a nuclear receptor emerging as a key role of lipid homeostasis at the transcriptional level). Thus, using moderate red wine has beneficial long-term effects (Bas *et al.* 2005). Also, Dolara *et al.* (2005) researched the effects of polyphenols from red wine on colon cancer. They administered red wine polyphenols (50 mg/kg) with rats. They founded that polyphenol-treated animals showed reduced tumor production compared to controls; and also polyphenol up-regulated the CYP7A1 gene. They concluded that polyphenols founded red-wine prevent movement of colon carcinogenesis in rats (Dolara *et al.* 2005). Moreover, recent studies demonstrated apple polyphenols mainly procyanidins developed the elimination of bile acids via feces. Apple polyphenols significantly increased the CYP7A1 enzyme activity. Catechins and procyanidins affected micellar solubility by inhibiting the intestinal cholesterol absorption and increasing the elimination lipid and cholesterol in feces (Koutsos *et al.* 2015).

In another research about CYP7A1 are made by Tong *et al.* (2015). Barley is a member of grass family and it includes highly soluble β -glucan. Tong *et al.* (2015) investigated hypercholesterolemic effects of barley β -glucan. They treated barley β -glucan to hamsters fed with the hypercholesterolemic

diet. Barley β -glucan decreased the plasma LDL-cholesterol level and increased elimination of total cholesterol in feces. Also, CYP7A1 activity was increased. They suggested that barley β -glucan decreased plasma cholesterol level by increasing elimination of fecal lipids and regulating CYP7A1 activity (Tong *et al.* 2015). In another study related with CYP7A1 gene expression, curcumin (*Curcuma longa* L.) was used as a protective supplement for cardiovascular disease since it diminishes the total cholesterol and LDL-cholesterol levels (Kim & Kim 2010; Rodrigues *et al.* 2015). Kim and Kim (2010) investigated the hypercholesterolemic effects of curcumin in serum triglyceride on rats fed with a high fat diet. They founded that curcumin decreased the total cholesterol, and LDL-cholesterol levels while up-regulated the gene expression of CYP7A1 (Kim & Kim 2010).

Furthermore, recent studies demonstrated that interleukin-1b and tumor necrosis factor influence the cholesterol metabolism via increasing cholesterol levels. Interleukin-1b and tumor necrosis factor induced gene expression of HMGCoA reductase, and significantly repressed gene expression of CYP7A1 (Karthikesan & Pari 2008). Too much alcohol consumption induces liver disease via fat accumulation in the liver. Ethanol raises the fatty acids uptake and restricts lipoprotein export. Fat accumulation in the liver trigger the hepatitis since fat makes the liver more sensitive the inflammation. 3, 4-dihydroxycinnamic acid, caffeic acid, is a member of hydrocinnamic acid. It is mostly founded in tea, cinnamon, apricot and red wine. Caffeic acid is powerful antioxidant agent. Karthikesan and Pari (2008) investigated effects of caffeic acid on alcohol induced liver injury. Caffeic acid (12 mg /kg/bw) was administrated orally to alcohol fed rats. Caffeic acid reduced the tissue and serum lipid levels compared to control. They suggested that caffeic acid can reduce the hypercholesterolemia because of its anti-inflammatory characteristic (Karthikesan & Pari 2008). Additionally, Norlin (2002) investigated

developmental differences of CYP7A1, CYP7B1, CYP27A1 and 3 beta-hydroxy-delta 5-C27-steroid dehydrogenase in different ages pigs. He suggested activity of CYP27A1 and 3 beta-hydroxy-delta 5-C27-steroid dehydrogenase were nearly similar while activity of CYP7A1 increased in livers of newborn and 6 month old pigs. Level of CYP7B1 in liver increased with age in but decreased in the kidney. He suggested CYP7B1 has a tissue-specific and age-dependent regulation because of hormonal factors.

According to our protein and mRNA expression results, *Viscum album* and *Epilobium hirsutum* repressed the CYP7A1 transcription while ellagic acid induced it. As mentioned above, regulating transcription of CYP7A1 gene is very important bile acid synthesis. The CYP7A1 promoter includes two bile acid response elements (BAREI and BARE-II). BAREs contain many AGGTCA-like repeating sequences. BAREs identified in rat and human CYP7A1, rat and human CYP8B1 and human CYP27A1 exhibiting nearly similar characteristic. BAREI, a DR4 element, is found in rat and mouse CYP7A1 gene, not found human promoter. BAREI contains the binding site for the liver X receptor /retinoid X receptor (LXR/RXR) heterodimer. LXR (Liver orphan receptors) was activated by oxysterols in order to stimulate CYP7A1 transcription and ATP-binding cassette family of transporters, which drive the transfer of cholesterol from the peripheral tissues to liver for bile acid degradation. BARE II contains a conserved DR1 element for hepatocyte nuclear factor-4 (HNF4) binding and a liver receptor homolog response element for LRH-1 binding. Binding of these two factors is essential for liver-specific basal expression of CYP7A1. Bile acid stimulates the FXR (farnesoid X receptor) by inhibiting the CYP7A1 enzyme production and increasing the bile acid elimination and transportation. Thus, it is a bile acid sensor because FXR protects the liver accumulation of toxic bile acids and xenobiotics. However, CYP7A1 promoter does not contain any FXR binding sites. Bile acids mediate their repression on CYP7A1 through an indirect mechanism. FXR binds to the IR1 element in the small

heterodimer partner (SHP) promoter and in response to bile acids SHP transcription is increased. Increased SHP, a non DNA binding protein, associates with the liver receptor homolog (LRH-1), an obligate factor required for transcription of CYP7A1. This interaction represses the transcription activation by LRH-1, so CYP7A1 expression is decreased (Figure 4.7, and Table 4.4)

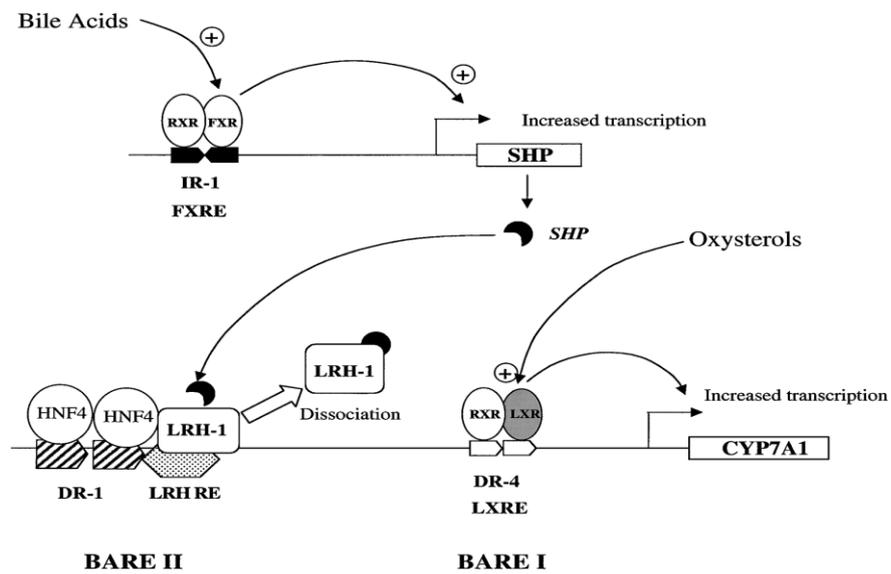


Figure 4. 7 Regulation of CYP7A1 gene transcription (Chiang 2002).

Table 4. 4 Nuclear receptors and target genes related with cholesterol and bile acid homeostasis (Chiang 2002).

Rceptor	Target genes	Functions
RAR/RXR	↑CYP7A1(rat)	Bile acid biosynthesis
LXR	↑CYP7A1(rat, mouse) ↑LXR	Bile acid biosynthesis Cholesterol sensor
FXR	↓CYP7A1,CYP8B1, CYP27A1 ↑SHP	Bile acid biosynthesis Nuclear receptor inhibitor
PPAR α	↓CYP7A1 ↑ CYP8B1	Bile acid biosynthesis Bile acid biosynthesis
HNF4 α	↑CYP7A1,CYP8B1,CYP27A1 ↑FTF	Bile acid biosynthesis Liver gene expression
FTF	↑CYP7A1 (mouse) ↓CYP7A1 (human) ↑CYP8B1 (rat) ↓CYP8B1 (rat) ↑SHP ↑HNF4 α	Bile acid biosynthesis Bile acid biosynthesis Bile acid biosynthesis Bile acid biosynthesis Nuclear receptor inhibitor Lipid metabolism
SHP	↓ CYP7A1,CYP8B1,CYP27A1	Bile acid biosynthesis
PXR	↓ CYP7A1	Bile acid biosynthesis

As a result, LXR and FXR play a crucial role coordinately way on bile acid synthesis, transport and absorption in liver and intestine, and cholesterol metabolism in the liver and peripheral tissues. When cholesterol increases in liver, oxysterols activate LXR α , which stimulates conversion of cholesterol to bile acids by inducing CYP7A1 transcription. Increased bile acid synthesis and pool stimulate FXR, which inhibits CYP7A1 to decrease bile acid synthesis but stimulates BSEP (bile salt export pump) expression to excrete bile acids into bile. Thus, bile acid- regulated genes and nuclear receptors are promising target for drug development for decreasing the serum cholesterol and triacylglycerol levels, and liver and cardiovascular diseases (Chiang 2002; Davis *et al.* 2002).

As mentioned Table 4.4 , nuclear receptors involved in bile acid and cholesterol metabolism (RXR, LXR, FXR, PPAR α , HNF4 α , FTF, SHP and PXR) can regulate not only the main transcription but also feedback regulation of CYP7A1 gene and other bile acid metabolizing related enzymes (Chiang 2002). For example, peroxisomal proliferator activator receptors (PPARs) are activated by polyunsaturated fatty acids. PPARs show a dual role in the regulating cholesterol metabolism as well as regulating of fatty acid metabolism. PPAR α stimulates LXR, so LXR promotes reverse cholesterol transport pathway to liver. In the liver, PPAR α induces the expression of CYP8B1 and represses the expression CYP7A1 enzyme.

In addition to nuclear receptors, there are other factors to regulate the CYP7A1 expression. Rodent model studies have established that CYP7A1 is highly regulated by physiological factors that influence hepatic bile acid biosynthesis, including cholesterol feeding, diurnal factors, and bile acids, which feedback inhibit the overall biosynthetic pathway in large part at the level of CYP7A1 gene expression. CYP7A1 is down-regulated via chenodeoxycholic acid and induced via dietary cholesterol. For example,

when food consumed, bile acid synthesis and CYP7A1 expression show a circadian cycle in which CYP7A1 expression is very high level. Also, bile acid (end product) shows negative feedback on regulation of CYP7A1 to prevent accumulation of bile acids. Besides, feedback regulation, post-transcriptional regulation of CYP7A1 expression (mRNA stability) is very important. 3' unregulated region of CYP7A1 mRNA level has AUUUA elements, which proposes the regulation and stability of mRNA level. The rate of CYP7A1 turnover number is unusually rapid (<5 min). Also, AUUUA elements causes of rapid degradation of chimeric mRNA.

Moreover, the disruption of CYP7A1 gene leads to reduce of bile acid biosynthesis, so CYP7A1 gene is upregulated to compensation of bile acid synthesis. As a same way, the CYP27A1 gene expression is upregulated via cholesterol and down regulated via bile acids. Also, CYP8B1 gene is inhibited at the pretranslational level by thyroid hormone and insulin, and induced by bile acids, subsequent CYP7A1 mRNA increases.

Decreased cholesterol level leads to repression of CYP7A1 enzyme production. *Epilobium hirsutum*-treated animals showed a significant decrease in total cholesterol levels. *Epilobium hirsutum*-treated animals also showed a decrease in protein and mRNA expression of CYP7A1 enzyme, since decreased cholesterol suppresses the CYP7A1 enzyme transcription. To create the bile acids that are needed by the body *Epilobium hirsutum L.* activates the alternative pathway inducing CYP27A1. However, ellagic acid induces production of CYP7A1 which is the rate limiting enzyme of classic pathway. Although fold change of protein and mRNA expressions of bile acid metabolizing CYPs is different, serum cholesterol levels of *Viscum album L.* and *o*-coumaric acid treated animals remained unchanged (Table 4.5).

	CYP7A1		CYP27A1		CYP8B1		CYP7B1		Serum Cholesterol Level
	Protein Expression	mRNA Expression	Protein Expression	mRNA Expression	Protein Expression	mRNA Expression	Protein Expression	mRNA Expression	
VA	0.9↓	9↓	1.4↑	1.3↓	1.3↑	4.2↑	0.9↓	2.4↓	NSC
OCA	0.8↓	1.8↑	0.8↓	1.3↑	0.8↓	2.3↑	0.8↓	2.3↑	NSC
EHT	0.6↓	2.1↓	1.3↑	1.4↓	0.8↓	2.3↑	0.6↓	4.1↓	1.5↓
EAT	1↑	2.6↑	0.7↓	1.7↑	0.8↓	1.8↑	0.8↓	2.3↑	1.5↓

Table 4. 5 Fold change in protein and mRNA expressions of *Viscum album L.*, *o*-coumaric acid, *Epilobium hirsutum L.* and ellagic acid-treated CYP7A1, CYP27A1, CYP8B1 and CYP7B1 (NSC: no significant change).

CHAPTER 5

CONCLUSION

Cholesterol homeostasis is essential for the living organisms. If cholesterol levels increase in blood, dangerous consequences can be occurred. In mammals, excessive cholesterol is removed mainly through conversion to bile acids, and only a small portion is utilized for production of steroid hormones and other endogenous compounds. Cytochromes P450 (P450s or CYPs) play an important role all quantitatively significant pathways of cholesterol metabolism and bile acid biosynthesis.

This study is the first *in vivo* study that investigates the possible effects of *Viscum album L.*, *Epilobium hirsutum L.* and their major polyphenolic ingredients, *o*-coumaric acid and ellagic acid on bile acid metabolizing Cytochrome P450 enzymes. Therefore, in order to accomplish these; we analyzed that phenolic profile (LC-MS analysis), phenolic and flavonoid content of these plants. Also, we studied the expression of protein and mRNA of cholesterol and bile acid metabolizing CYP7A1, CYP27A1, CYP8B1 and CYP7B1 in control and treated groups of animals. Besides, we analyzed the CYP7A1 enzyme activity.

According to these results, even though *Viscum album L.* and *o*-coumaric acid caused fold change in protein and mRNA expression of bile acid metabolizing CYPs, serum cholesterol levels remained unchanged (Figure 5.1). *Epilobium hirsutum* and ellagic acid-treated animals showed a significant decrease in total cholesterol levels. Also, *Epilobium hirsutum*-treated animals showed a decrease in protein and mRNA expression of

CYP7A1 enzyme, since decreased cholesterol suppresses the CYP7A1 enzyme transcription. To create the bile acids that are needed by the body *Epilobium hirsutum* L. activates the alternative pathway inducing CYP27A1. However, ellagic acid induces production of CYP7A1 which is the rate limiting enzyme of classic pathway (Figure 5.1). Therefore, based on doses and time point that applied in this study, the medicinal plant *Epilobium hirsutum* L. and its polyphenolic compound, ellagic acid, may have regulatory effects on bile acid metabolizing enzymes. In conclusion, the synthesis of bile acids from cholesterol by CYP-catalyzed reactions may be altered due to the changes in CYP protein and mRNA expressions which may be resulted by the treatment of animals with *Epilobium hirsutum* L. and ellagic acid. For future perspective, activity assays of CYP7A1, CYP27A1, CYP8B1 and CYP7B1 should be carried out. In addition, other bile acid metabolizing related enzymes should be investigated.

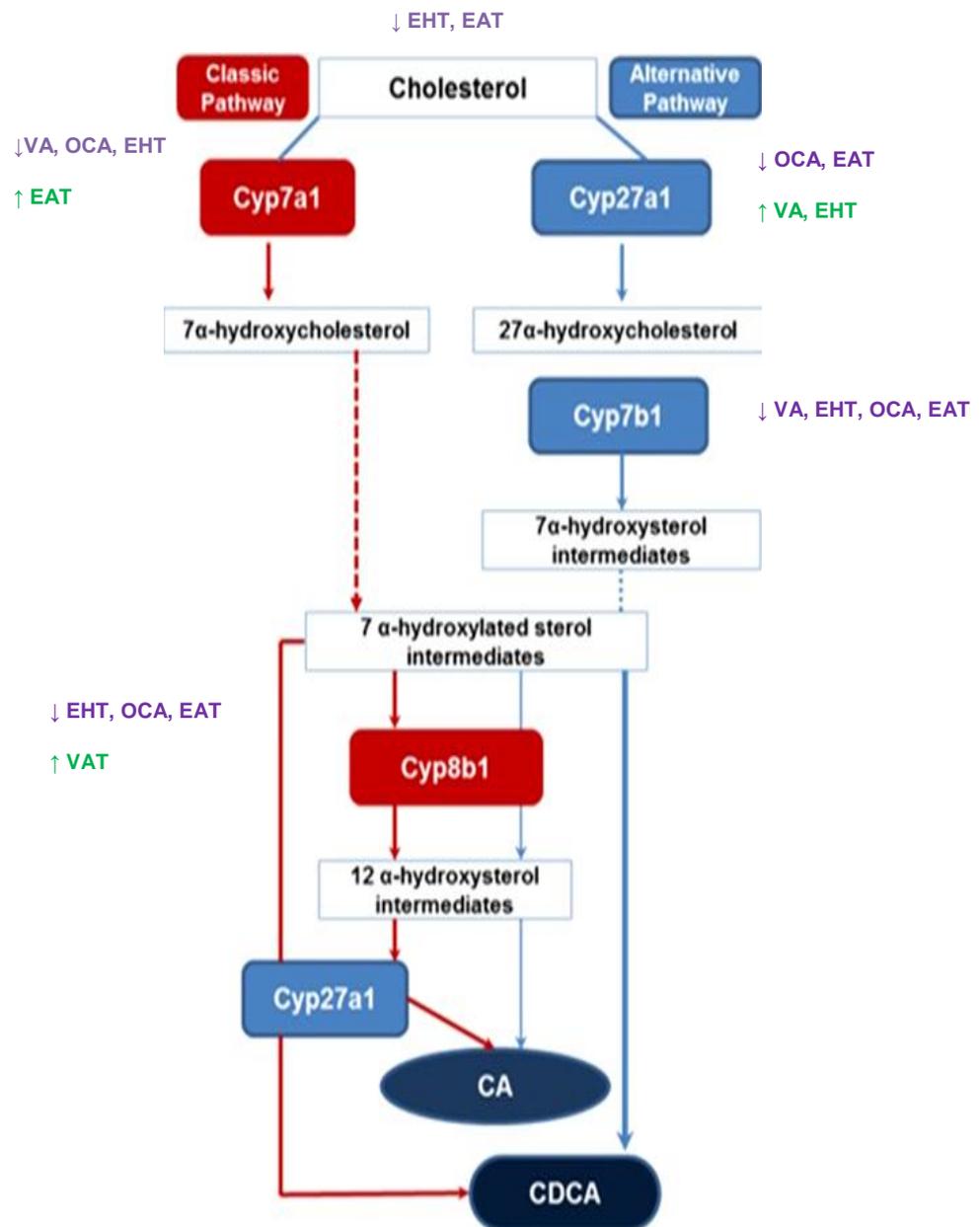


Figure 5. 1 The possible effects *Viscum album L.*, *Epilobium hirsutum L.*, *o*-coumaric acid and ellagic acid on bile acid metabolizing Cytochrome P450.

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CURRICULUM VITAE

PERSONAL INFORMATION

Name : Tuba Çulcu
Address : Middle East Technical University, Department of Biological Sciences
Telephone : +903122105161
Fax : +903122107976
E-mail : ctuba@metu.edu.tr/tubaculcu@gmail.com
Nationality : Republic of Turkey
Date of birth : 28.11.1981

EDUCATION AND TRAINING

PhD (2009-2015): Middle East Technical University, Department of Biochemistry.

1 March – 30 November 2013: Visiting Scholar at University of Michigan Medical School, Pharmacology, under the supervision of Prof. Dr. Paul F. Hollenberg and Dr. Haoming Zhang.

Biochemistry Cognitive Preparedness Program (2008-2009) Middle East Technical University, Department of Biochemistry.

M.S (2004-2007): Anadolu University, Molecular Biology and Genetics Department.

B.S (2000-2004): Selçuk University, Department of Biology.

WORK EXPERIENCE

Research Assistant (2007-2015) : Middle East Technical University,
Department of Biological Sciences.

Visiting Scholar (2013) : Michigan Medical School,
Pharmacology.

FOREIGN LANGUAGES

Advanced English

MEMBERSHIPS

Turkish Biochemical Society Turkish

International Society for the Study of Xenobiotics (ISSX)

CERTIFICATE

- Required Comprehensive Laboratory Safety Training, 2013, University of Michigan, Michigan, USA.
- Required Centrifuge Training, 2013, University of Michigan, Michigan, USA.
- Online Required Bloodborne Pathogens Training, 2013, University of Michigan, Michigan, USA.
- Statistical Analysis Course, 28-29 March 2012, İstanbul, Turkey.
- Experimental Animal User Training Program Certificate, 3-14 May 2011, Ankara, Turkey.
- Job Security Certificate, 2011, Ankara, Turkey.

Publications:

Culcu T., Sözen E., Tüylü A. B., (2010), Determination Of Genotoxicants induced DNA Damage By Using RAPD-PCR in Human Peripheral Blood Lymphocytes. Fresenius Environmental Bulletin, Vol.19, No: 10, 2005-2009.

Poster Presentations in National and International Congresses:

- **Culcu, T.** Gençler Özkan A. M., Şen A., Adalı O. Medicinal Plants *Epilobium irsutum L.* and *Viscum Album L.* Alter Protein and mRNA Expressions of Rat Liver Bile Acid Synthesizing Cyp7a1, 13th European ISSX Meeting, 22-25 June 2015, Scotland, Glasgow.
- Zhang, H., **Culcu, T.**, Hollenberg, P. High Throughput Screening of an NIH Clinical Collection Library for Specific Inhibitors of Cytochromes P450 2E1, 19A1 and 17A1, MDO Congress, 18-22 May 2014, Stuttgart, Germany.
- **Çulcu T.**, Gençler Özkan A. M., Şen A., Adalı O. The Effects of Folk Medicinal Plant *Viscum Album L.* on Protein And mRNA Expressions Of Cyp27a1 in Rat Liver IUBMB&FEBS Congress, 4 - 9 September 2012, Sevilla, Spain.
- **Culcu T.**, Tüylü A. B., Sözen E. The investment of DNA damages and mutations in human cells by using RAPD technique. 21. National Biochemistry Congress, 28-31 October 2009, İstanbul, Turkey.

Participation in National and International Congress:

- 13th European ISSX Meeting, 22-25 June 2015, Glasgow, Scotland.
- 20th International on Microsomes and Drug Oxidations, 18-22 May 2014, Stuttgart, Germany.
- Great Lakes Drug Metabolism and Discussion Group Meeting, 9-10 May 2013, Toledo, Ohio, USA.
- IUBMB&FEBS Congress, 4-9 September 2012, Sevilla, Spain.
- Embo young scientists forum, 14-16 June 2012, İstanbul, Turkey.
- Statistical Analysis Course, 28-29 March 2012, İstanbul, Turkey.
- Experimental Animal User Training Program Certificate, 3-14 May 2011, Ankara, Turkey.
- 3rd Microarray Training and Data Analysis Course, 20-25 December 2010, METU, Ankara, Turkey.
- 20. National Biology Congress, 21-25 June 2010, Denizli, Turkey.
- 21. National Biochemistry Congress, 28-21 October 2009, İstanbul, Turkey.