EFFECTS OF RESVERATROL AND QUERCETIN ON VITAMIN D
METABOLIZING CYTOCHROME P450 ENZYMES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY
MERVE AKKULAK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCES
IN
BIOCHEMISTRY

SEPTEMBER 2016

Approval of the Thesis:

EFFECTS OF RESVERATROL AND QUERCETIN ON VITAMIN D METABOLIZING CYTOCHROME P450 ENZYMES

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ABSTRACT

EFFECTS OF RESVERATROL AND QUERCETIN ON VITAMIN D METABOLIZING CYTOCHROME P450 ENZYMES

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September 2016, 148 pages

Vitamin D has long been known to function in the calcium homeostasis. Besides, it has widespread pleiotropic hormone actions. Therefore, studies on metabolism of vitamin D have attracted much attention in recent years. Vitamin D metabolism is regulated by cytochrome P450 enzymes (CYPs) such as CYP2R1, CYP27A1, CYP27B1 and CYP24A1. Phenolic compounds including quercetin and resveratrol are widely studied due to their important roles in human health as activators or inhibitors for enzymes catalyzing biochemical reactions. Although the interaction between these phenolic compounds and CYPs has been reported widely so far, there is no reported study about the role of quercetin and resveratrol on vitamin D metabolizing CYPs.

This study was aimed to investigate the effects of quercetin and resveratrol on vitamin D metabolizing CYPs in human embryonic kidney cell line (HEK-293) and human hepatocellular carcinoma cell line (HUH-7). For this purpose, IC50 values of quercetin and resveratrol for HEK-293 and HUH-7

cells were determined by Alamar Blue assay. While IC50 values of quercetin were determined as 60.72 and 185.31 μM for HEK-293 and HUH7 cells, IC50 values of resveratrol were determined as 34.36 and 131.93 μM for HEK-293 and HUH-7 cells, respectively. Furthermore, the effects of those phenolic compounds on protein and mRNA expressions of Vitamin D metabolizing CYPs were investigated by Western Blotting and q-RT-PCR, respectively.

The results showed that quercetin treatment caused an increase in mRNA expression of CYP2R1 and CYP27A1 in HEK-293 cells. However, protein expressions of CYP2R1 and CYP27A1 in HEK-293 cells were decreased by quercetin treatment. In addition, CYP24A1 protein expression was increased in response to quercetin treatment for the same cell line. Resveratrol resulted an increase in mRNA expression of CYP27A1, CYP27B1 and CYP24A1 in HEK-293 cells. However, protein expressions of CYP27A1 and CYP27B1 in HEK-293 cells were decreased by resveratrol treatment. In addition, CY24A1 protein expression was upregulated with resveratrol treatment in HEK-293 cells. In HUH-7 cells, quercetin treatment caused an increase in mRNA expression of CYP2R1, CYP27B1 and CYP24A1. However, it did not significantly affect the protein expressions of CYP27B1 and CYP24A1 in HUH-7 cells. Resveratrol treatment caused an increase in mRNA expression of CYP2R1 and CYP24A1, whereas it caused decrease in mRNA expression of CYP27A1 and CYP27B1 in HUH-7 cells. Furthermore, resveratrol resulted a decrease in protein expression of CYP24A1. In this study, no CYP27A1 and CYP2R1 protein expressions were observed in HUH-7 cells. All these results show that there is no significant correlation between mRNA transcript and protein levels. In conclusion, protein and mRNA expressions

of vitamin D metabolizing CYPs were modulated differently depending on the type of phenolic compound and cell line.

ÖZ

RESVERATROL VE KUERSETİNİN VİTAMİN D METABOLİZMASINDA ROL ALAN SİTOKROM P450 ENZİMLERİNE ETKİLERİ

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Vitamin D'nin kalsiyum homeostasisinde rol aldığı uzun süredir bilinmektedir. Ayrıca, Vitamin D yaygın görülen, çok yönlü hormon etkilerine de sahiptir. Bu nedenle, Vitamin D metabolizması üzerine yapılan çalışmalar son yıllarda büyük ilgi çekmektedir. Vitamin D metabolizması CYP2R1, CYP27A1, CYP27B1 ve CYP24A1 gibi sitokrom P450 enzimleri tarafından regüle edilmektedir. Fenolik bileşikler örneğin kuersetin ve resveratrol, biyokimyasal reaksiyonları katalize eden enzimlerin aktivatörü ya da inhibitörü olarak insan sağlığı üzerindeki önemli etkileri nedeniyle fazlaca çalışılmaktadır. Bu fenolik bileşikler ve sitokrom P450 enzimleri arasındaki etkileşimi gösteren fazlaca yayın olmasına rağmen, kuersetin ve resveratrolün vitamin D metabolizmasında görevli sitokrom P450 enzimleri üzerine etkilerini gösteren bir çalışma bulunmamaktadır.

Bu çalışmada, quercetin ve resveratrolün HEK293 insan embriyonik böbrek ve HUH7 insan hepatoselüler karsinoma hücre hattında vitamin D metabolizmasında rol olan sitokrom P450 enzimleri üzerine etkilerini araştırmak amaçlanmıştır. Bu amaçla, kuersetin ve resveratrolün HEK-293 ve HUH-7 hücre hatlarındaki IC50 değerleri alamar blue tahlili ile belirlenmiştir. Kuersetinin IC50 değerleri HEK-293 ve HUH-7 hücre hatları için sırasıyla 60.72 µM and 185.31 µM belirlenirken, bu değerler resveratrol için aynı hücre hatlarında 34.36 µM ve 131.93 µM olarak belirlenmiştir. Ayrıca bu fenolik bileşiklerin protein ve mRNA ekspresyonları üzerine etkileri sırasıyla Western Blot ve q-RT-PCR teknikleri ile incelenmiştir.

Bu çalışmanın sonuçlarına göre, kuersetin muamelesi HEK293 hücre hattında CYP2R1 ve CYP27A1 mRNA ekspresyonlarında artışa sebep olmuştur. Fakat kuersetin muamelesiyle HEK293 hücre attında CYP2R1 ve CYP27A1 protein ekspresyonları azalmıştır. Ayrıca, CYP24A1 protein ekspresyonu, kuersetin muamelesine rağmen aynı hücre hattında artış göstermiştir. Resveratrol CYP27A1, CYP27B1 and CYP24A1 mRNA ekspresyonlarında HEK-293 hücre hattında artışa sebep olmuştur. Buna karşın CYP27A1 ve CYP27B1 protein ekspresyonları resveratrol muamelesiyle HEK-293 hücre hattında azalmıştır. Ayrıca, resveratrol muamelesiyle HEK-293 hücre hattında, CYP24A1 protein ekspresyonu artmıştır. HUH7 hücre hattında, kuersetin muamelesi CYP2R1, CYP27B1 ve CYP24A1 mRNA ekpresyonlarında artışa sebep olmuştur. Ancak, CYP27B1 ve CYP24A1 protein ekspresyonunu aynı hücrede önemli ölçüde etkilememiştir. Resveratrol muamelesi HUH-7 hücre hattında CYP2R1 ve CYP24A1 mRNA ekspresyonunda artışa sebep olurken, CYP27A1 ve CYP27B1 mRNA ekspresyonunda azalışa neden olmuştur. Ayrıca, resveratrol CYP24A1 protein ekspresyonunda azalışa sebep olmuştur. Bu çalışmada, CYP27A1 ve CYP2R1 protein ekspresyonları HUH-7 hücre hattında gözlemlenememiştir.

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Tüm bu sonuçlar mRNA transkripti ve protein ekspresyonları arasında anlamlı bir korelasyon olmadığını göstermektedir. Sonuç olarak, vitamin D metabolizmasında rol alan sitokromların mRNA ve protein ekspresyonları fenolik bileşik çeşidi ve hücre tipine bağlı olarak farklı modüle edilmiştir.

Anahtar kelimeler: Vitamin D, Kuersetin, Resveratrol, Sitokrom P450, HEK-293, HUH-7, Western Blot, qRT-PCR.

To My Family,

For their endless support and love

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

xvi

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
APS	Ammonium per sulfate
ATP	Adenosine triphosphate
BCIP	5-bromo 4-chloro 3-indoyl phosphate
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
cDNA	Complementary DNA
cAMP	Cyclic adenosine monophosphate
Ct	Threshold cycle
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ERB	Electronic running buffer
FBS	Fetal bovine serum
FGF 23	Fibroblast like growth factor 23
GSH	Glutathione
GST	Glutathione S-transferase
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
H_2O_2	Hydrogen Peroxide
IC50	Half maximal inhibitory concentration
kDa	kilo Dalton

miRNA	micro RNA
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrotetrazolium blue chloride
NQO1	NAD(P)H: Quinone Oxidoreductase I
O_2^-	Superoxide anion
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen-Strep	Penicillin-Streptomycin
PMSF	Phenylmethylsulfonyl flüoride
PTH	Parathyroid hormone
PXR	Pregnane X receptor
rpm	Revolutions per minute
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SD	Standard deviation
SDB	Sample dilution buffer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SUMO	Small ubiquitin related modifier
TBST	Tris-buffered saline and Tween 20
TEMED	Tetramethylethylenediamine
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element

CHAPTER 1

INTRODUCTION

1.1 Polyphenols

Polyphenols, strong antioxidants in the diet, are secondary plant metabolites and mainly synthesized to protect themselves against UV-radiation and pathogens. These naturally existing compounds found widely in fruits, vegetables and beverages particularly grapes, berries, onions, tea and red wine. Phenolic compounds provide the characteristic features of foods including odor, flavor and color (Pandey and Rizvi, 2009). The studies on polyphenols have been increasing day by day because of their abundant consumption with a diet, strong antioxidant properties and reported health benefits against some diseases such as cancer, diabetes, cardiovascular and neurodegenerative diseases (Middleton *et al.,* 2000; Wang *et al,* 2014; Tangney and Rasmussen, 2013).

Polyphenols are classified into four main groups based on the number of phenolic rings and the structural components linking these rings: phenolic acids, flavonoids, stilbenes and lignans (Manach *et al.,* 2004). The chemical structures of these four classes of polyphenols are shown in Figure 1.1. The present study will center on main dietary flavonoid, quercetin and stilbene, resveratrol.
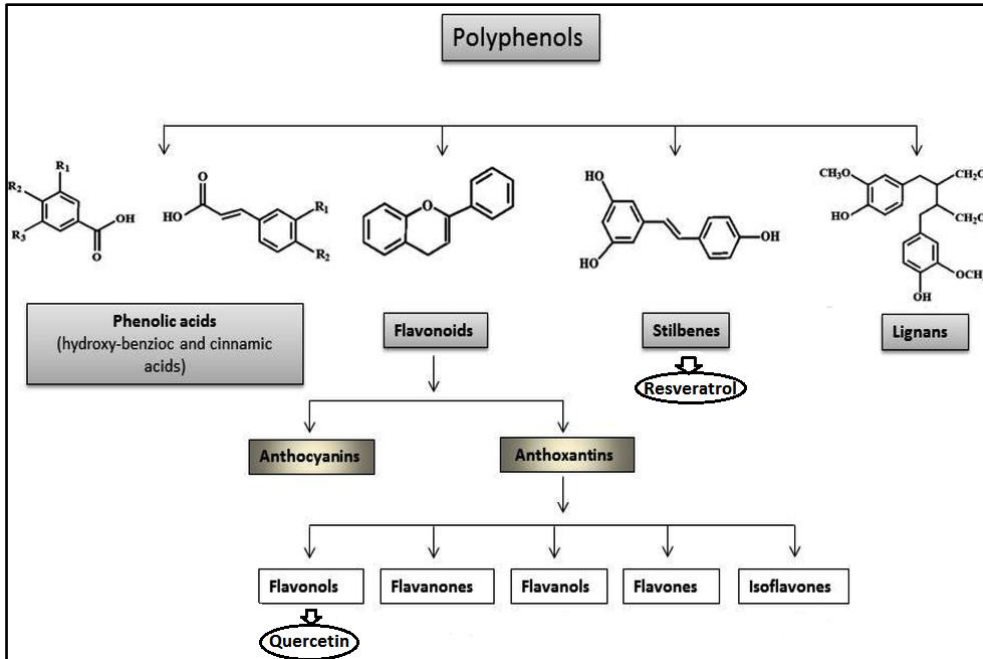
Figure 1.1 Classification and structure of the main polyphenols (Câmara et al., 2013).

1.1.1 Quercetin

Quercetin is a polyphenol belonging the class of flavonoids and categorized as a flavonol with 3-hydroxyflavone backbone (D'Andrea, 2015). Figure 1.2 shows the chemical structure of quercetin.
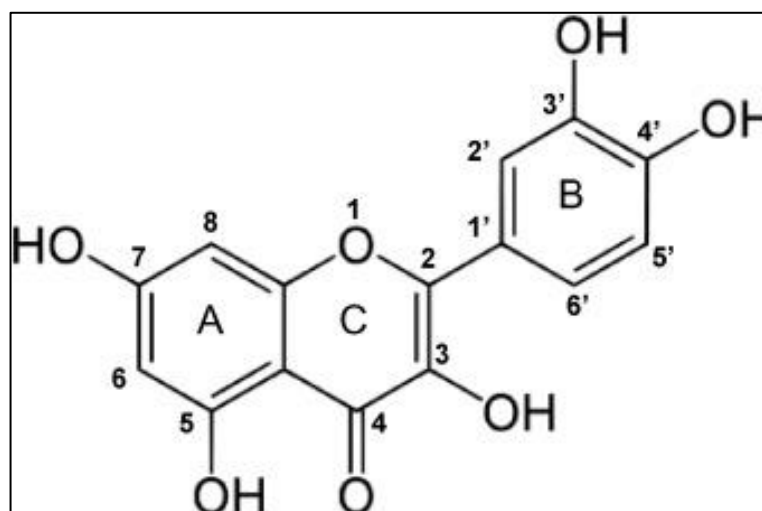
Figure 1.2 The chemical structure of quercetin (D'Andrea, 2015).

Quercetin is the main and widely consumed flavonol in human diet (Hollman *et al*., 1995). It is present in different types of fruits, vegetables and beverages including onions, apples, berries, nuts, tea and wine. It was estimated that the daily intake of quercetin is reached to 30 mg per day (median of 10 mg) in a Western diet (D'Andrea, 2015; Egert *et al.,* 2008).

Quercetin is commonly found linked to a sugar moiety as glycoside forms in nature (Hollman *et al.,* 1997; Rice-Evans, 2001). These sugar parts increase the hydrophilicity, thus absorption of glycoside form is less than aglycone (sugar-free) form. Aglycones can adequately pass through the gut wall. However, quercetin glycosides are hydrolyzed through small intestine or in the colon and aglycone form of quercetin occurs (Murota and Terao, 2003; Hollman *et al.,* 1997). The resulting lipophilic aglycone is absorbed in the

3

large intestine and metabolized with phase II conjugation enzymes to glucuronidated, sulfated and methylated metabolites of quercetin in liver (D'Andrea, 2015). Conjugated quercetin metabolites are bound to serum albumin and circulate in the blood stream (Murota *et al*., 2007). Thus, conjugated metabolites of quercetin accumulate mainly in plasma (D'Andrea, 2015).

Quercetin was reported to possess antioxidative, anticancer and anti-inflammatory activities (Chopra *et al.,* 2000; Granado-Serrano *et al.,* 2010). Antioxidative activity is associated with free radical scavenging and transition metal ion binding characteristics of quercetin with the aid of phenolic hydroxyl groups at the B-ring and the 3-position of quercetin (De Souza and De Giovani, 2004; Heijnen *et al.,* 2002). These abilities also provide to inhibit lipid peroxidation (Hollman and Katan, 1997; Afanas'ev *et al.,* 1989). Antioxidative property of quercetin is known to be associated with phase II enzymes. For example, NQO1 possess ability to hinder the formation of reactive oxygen species. Quercetin provided to increase in NQO1 expression in HepG2 cells and also provided to increase in mRNA expression, protein expression and enzyme activity of NQO1 in MCF-7 cells (Tanigawa *et al.,* 2007; Valerio *et al.,* 2001). In addition, different studies demonstrated that quercetin increases both GSH concentration and GST activity (Granado-Serrano *et al.,* 2012; Meyers *et al.,* 2008). The increase in GSH concentration and GST activity can inhibit oxidative-stress induced damage. However, quercetin is considered as not only anti-oxidant but also pro-oxidant. The reason can be explained that quercetin is converted to o-quinone and o-semiquinone radicals with a reactive intermediate. These radicals react with oxygen molecules and cause to produce superoxide anion (O_2^{-}) and hydrogen peroxide (H_2O_2). Thus, it can

induce cellular oxidative damage (Kawanishi *et al.,* 2005). It is believed that these biphasic effects depend on quercetin concentrations, stress conditions and cell types (Choi *et al.,* 2012; Wätjen *et al.,* 2005; Robaszkiewicz *et al,* 2007; Van Der Woude *et al.,* 2003).

Recently, there are a few studies about interaction between vitamin D receptor (VDR) and quercetin. In one study, it has been shown that quercetin increases transactivation of VDR target gene in Caco-2 cell line (Inoue *et al.,* 2010). In another study, the interaction between the VDR and quercetin was reported at molecular level, resulting that VDR activation by quercetin is exerted by direct interaction (Lee *et al*., 2016). Taken together, quercetin may affect the expression of VDR target genes.

1.1.2 Resveratrol

Resveratrol (3,5,4′-trihydroxystilbene) is categorized as stilbene. It is generated with certain plants in response to environmental stressors such as fungal pathogens, UV irradiation and water deprivation (Morelli *et al.,* 2006); (Langcake and Pryce, 1977). Resveratrol has cis and trans isoforms and Figure 1.3 represents the structure of trans-resveratrol. Trans-resveratrol is reported as more biologically effective than its cis isoform (Mukherjee *et al.,* 2010).
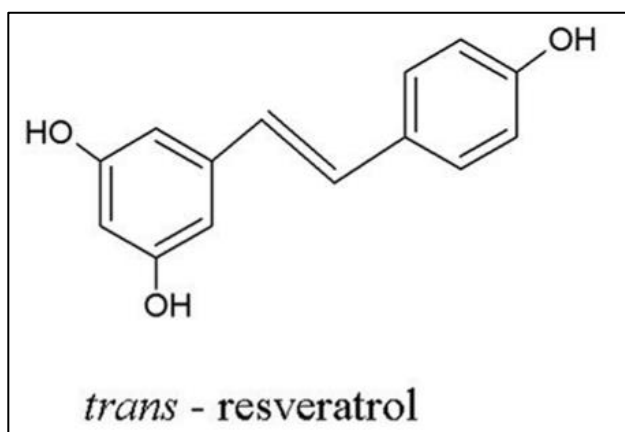
Figure 1.3 The chemical structure of trans-resveratrol (Mukherjee et al., 2010).

Resveratrol was first found in the roots of white hellebore in 1940 (Takaoka, 1940) and has since been found in many plant species including grapes, mulberries blueberries, peanuts and many others (Baur and Sinclair, 2006). Red wine is also one of the main sources of resveratrol. It has been reported that consumption of red wine is associated with the decrease in occurrence of cardiovascular problem. For example, in France, the occurrence of heart disease is approximately 40% less than the other European countries even though their diet is rich in saturated fat (Renaud and Lorgeril, 1992). This is called as the 'French Paradox' (Renaud and Lorgeril, 1992; Kopp, 1998). According to some researchers, antioxidative, anti-inflammation and inhibition of platelet aggregation features of resveratrol can support to protect the cardiovascular system (Hao and He, 2004; Fauconneau *et al*., 1997; C. J. Chen *et al.,* 2009; Birrell *et al.,* 2005; Pace-Asciak *et al.,* 1996).

The concentrations of resveratrol used in studies change depending on cell and tissue types (Baur and Sinclair, 2006). According to studies, resveratrol dosage seems likely be less than 1.0 g/day to prevent side effects of it. Even though resveratrol is adequately absorbed after oral administration, but fast phase II metabolism, conjugation to glucuronic acid and/or sulfates, decreases its plasma bioavailability mainly (Patel *et al.,* 2011). When 1.0 g of resveratrol was consumed, only ~0.6 µM plasma resveratrol concentrations can be obtained in humans. In order to reflect the clinical scenario accurately, resveratrol concentrations used in vitro studies should not exceed about 1 µM (Brown *et al.,* 2010). However, resveratrol concentrations using in most of the studies are more than this value. Therefore, there are doubts whether the effects observed in preclinical studies can be translated to humans. However, resveratrol is a lipophilic compound, so it forms a good mixture with lipids such as membranes and lipoproteins (Timmers *et al*., 2012). Thus, the resveratrol levels in tissues may be higher than plasma levels.

Furthermore, it has been shown that resveratrol has also various effects rather than cardiovascular protection such as anti-carcinogen, anti-diabetes, anti-obesity and anti-aging effects (Athar *et al.,* 2009; 2007; Szkudelska and Szkudelski, 2010; Udenigwe *et al.,* 2008; Kasiotis *et al.,* 2013). Resveratrol increases AMP/ADP ratio by inhibiting F1F0-ATPase activity, which provides the activation of AMP protein kinase (AMPK). This AMPK activation by resveratrol affects various cellular processes such as mitochondrial biogenesis, autophagy, lipolysis, and stress responses (Madrigal-Perez, 2016). In 2003, resveratrol was found as activator of sirtuin 1 (SIRT1), which uses (NAD+) for deacetylation of metabolic enzymes important in glycolysis and Krebs cycle (Howitz *et al*., 2003; Borra *et al.,* 2005). It also activates PGC-1α, master regulator of

mitochondrial biogenesis (Lagouge *et al.,* 2006). Therefore, they have an essential role in lipid and glucose homeostasis. These bring idea that resveratrol can be important in the treatment of metabolic diseases including obesity, diabetes by targeting SIRT1 and AMPK. AMPK has also related with regulation of cytochrome P450 (CYP) enzymes. It has been reported that AMPK activators induce CYP2B6 gene expression in human hepatocytes (Rencurel *et al.,* 2005). Also, it has been shown that AMPK activation is necessary for phenobarbital to promote expression CYP2B6 (Rencurel *et al.,* 2005; 2006). Resveratrol can induce CYP enzymes by activating AMPK via mitochondrial ATP synthesis inhibition and can provide to increase a response for harmful xenobiotics like phenobarbital.

Resveratrol has interactions with enzymes, drugs, chemicals and foods. Resveratrol modulates the expression and activity of CYP1A1, CYP1A2, CYP1B1, CYP3A4 and CYP2E1 (Ciolino and Yeh, 1999; Ciolino *et al.,* 1998; Chen *et al.,* 2004; Mollerup *et al.,* 2001; Piver *et al.,* 2001; Revel *et al.,* 2003). Resveratrol antagonizes the transactivation of genes mediated by aryl hydrocarbon receptor (AhR) interactions (Casper *et al.,* 1999). In addition, resveratrol inhibits CYP3A4 activity in healthy volunteers (Chow *et al.,* 2010). So, taking high amount of resveratrol and using drugs which have extensive first-pass metabolism by CYP3A4 such as HMG-CoA reductase inhibitors, calcium channel antagonists may increase the drug toxicity. Resveratrol has been shown to induce Phase II detoxification and antioxidant enzymes such as UDP-glucuronosyltransferase (UGT), glutathione S-transferase (GST), and quinone reductase activity, which helps to suppress free radical formation (Szaefer *et al.,* 2009; Gerhäuser *et al.,* 2003; Chow *et al.,* 2010).

Resveratrol and known VDR ligands have general structural symmetry and similar configuration. These bring forward an idea that resveratrol can be taken role in the regulation of VDR and VDR target genes. There are some results to support this idea. It has been shown that resveratrol induces VDR mediated transcription in kidney, colon and myoblast cells. Also, increase in the transcriptional activity has been observed with combine treatment containing both resveratrol and 1,25D. Resveratrol has induced heterodimerization of VDR with RXR more than 1,25D treatment alone (Dampf Stone *et al.,* 2015). These elucidate that resveratrol can be affected in the regulation of vitamin D and its target genes.

1.2 Vitamin D and its Metabolism

Vitamin D, first recognized as fat- soluble vitamin, is now known as a pro-hormone belongs to the steroid hormones. Vitamin D, consists of fat-soluble seco-sterols, have two forms: Vitamin D3 (cholecalciferol) and Vitamin D2 (ergocalciferol) (Fig. 1.4) (Norman, 2008; Jones *et al.,* 1998).
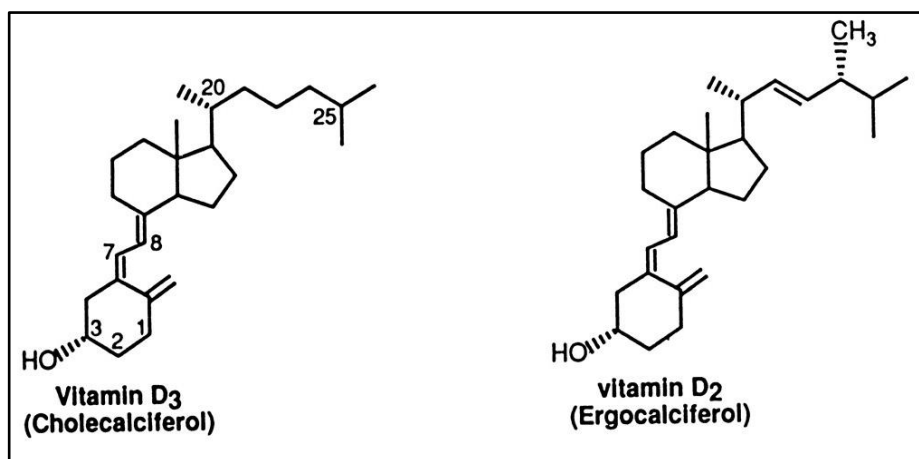
Figure 1.4 Two forms of Vitamin D (Jones et al., 1998).

9

Vitamin D_3 is mainly synthesized in the skin from 7-dehydrocholesterol with non-enzymatic processes and is taken in the diet slightly. Vitamin D_2 (ergocalciferol) is derived mainly from diet. Photochemical synthesis of vitamin D_3 begins with the absorption of UVB light radiation (spectrum 280-320 UVB) from the sun by 7-dehydrocholesterol. This causes formation of pre-vitamin D_3. Subsequently, previtamin D_3 isomerizes to the more thermodynamically stable form, cholecalciferol (vitamin D_3) as shown in Fig.1.5 (Holick *et al.,* 1981, 1995; Holick, 2004; Deluca, 2014). Since vitamin D is predominantly synthesized photochemically in humans, its production changes according to season, latitude, pigmentation of skin, sunscreen use and aging (Holick, 1987). For example, melanin prevents UVB photons to reach 7-dehydrocholesterol by absorbing them efficiently. Therefore, individuals having high skin melanin pigmentation need longer exposure period to sunlight for same amount of vitamin D production (Holick, 2004). In addition, it has been reported that obese people possess lower amount of vitamin D because fat sequesters vitamin D from circulation (Wortsman *et al.,* 2000). In order to have sufficient amount of vitamin D level for fair skinned people, it is enough thirty minutes three times per week exposure to midday sunlight in summer (Lips, 2006).
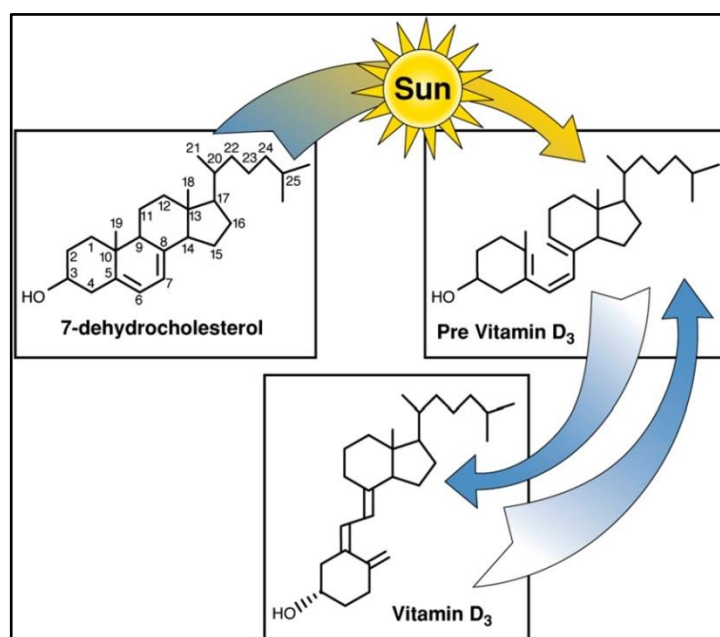
Figure 1.5 The conversion of 7-dehydrocholesterol to vitamin D3 (Deluca, 2014).

Both cholecalciferol and ergocalciferol are considered biologically inactive. In liver, vitamin D is hydroxylated at C-25 by some of cytochrome P450 enzymes such as CYP27A1, CYP2R1 and 25-hydroxyvitamin D (25(OH)D) is formed (Omdahl *et al.,* 2002; Prosser and Jones, 2004). Binding affinity of D_2 to vitamin D binding protein is less than D_3 due to structural difference. This less affinity causes faster clearance from the circulation, so inhibits its conversion to 25 hydroxyvitamin D (Hollis, 1984; Horst *et al.,* 1986; Houghton and Vieth, 2006). Therefore, If D_2 supplementation does not taken daily, 25(OH)D blood level does not increase sufficiently (Tripkovic *et al.,* 2012).

The resultant 25(OH)D is transferred to kidney with DBP. In kidney, 25(OH)D is hydroxylated at C-1 of A ring by 25-hydroxyvitamin D-1α-hydroxylase known as CYP27B1, and 1,25(OH)$_2$D (calcitriol), hormonally active form of vitamin D, is formed (Christakos *et al.,* 2010). Calcitriol is ligand for vitamin D receptor and responsible for biological actions of vitamin D. Calcitriol is destructed by 24-hydroxylase enzyme (CYP24A1) and turned into 1,24,25(OH)$_3$D (Fig.1.6). Calcitriol has strongest metabolic activity by stimulating CYP24A1 and inhibiting CYP27B1, which provides to control vitamin D level (Jones *et al.,* 1998).
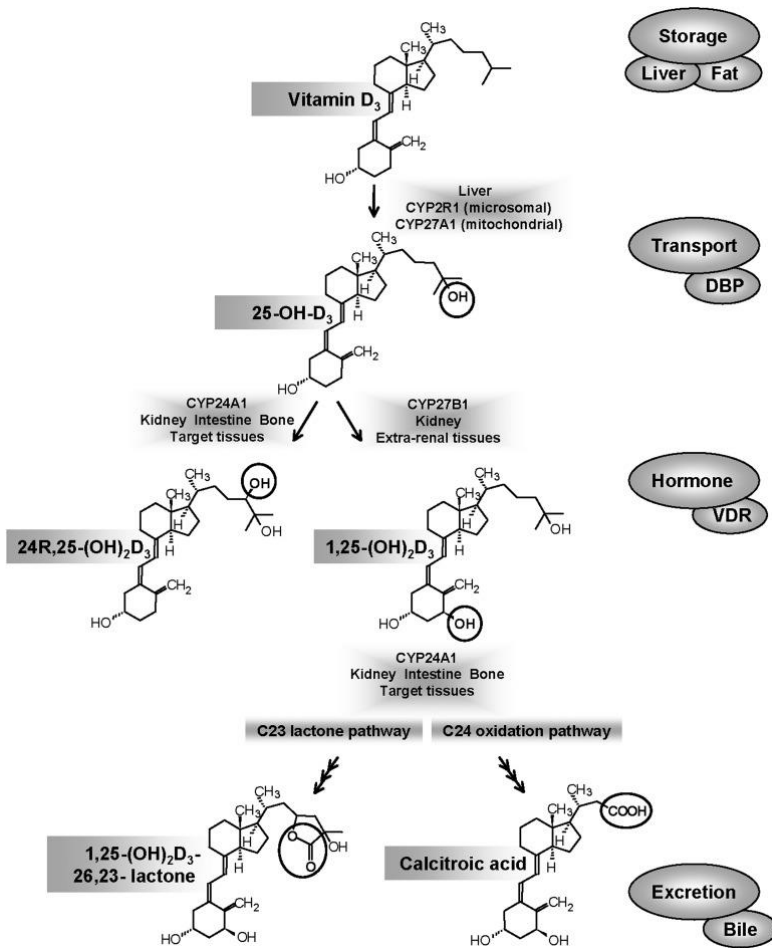
Figure 1.6 Vitamin D$_3$ Metabolism (Jones *et al.,* 2014).

1,25(OH)$_2$D binds to vitamin D receptor (VDR) to carry out biological activities (Mizwicki and Norman, 2009; Shin *et al*., 2010; Huhtakangas *et al.,* 2004).

After Vitamin D was discovered to treat rickets, over the 80 years, vitamin D has been known with classical actions take place in the kidney, liver, bone and intestine including calcium and phosphorous homeostasis and protection of bone health (Lips, 2006). With the detection of VDR for 1,25(OH)$_2$D in different tissues and cells, increased the awareness of importance of vitamin D and non-classical actions were appeared (Bikle, 2009). For example, it has been reported that 1,25(OH)$_2$D$_3$ causes apoptosis in breast cancer cells, which suggests that Vitamin D can be beneficial for human cancer. However, many of the patients react limited to Vitamin D$_3$ analog treatment. VDR expression may enhance the effect of vitamin D treatment on cancer. It was found that human VDR gene contains estrogen responsive promoter region. Both this promoter region and VDR protein expression were upregulated in ER dependent manner with phytochemicals, genistein and resveratrol. These results suggest that phytochemicals can increase the effectiveness of Vitamin D$_3$ based therapies by upregulating VDR (Wietzke and Welsh, 2003).

1.3   Cytochrome P450s

Cytochrome P450 (CYP) is a large class of heme-containing proteins that catalyze monooxygenase reactions as terminal oxidases in NADPH dependent electron transport pathways. The name "P450" was given in the beginning of 1960s with a discovery of a pigment having an absorption peak at 450 nm in reduced state when bound to carbon monoxide (Klingenberg, 1958; Omura and Sato, 1964). CYPs are involved in metabolism of drugs, chemicals and endogenous substrates including cholesterol, bile acid, steroid, arachidonic acid, eicosanoids and vitamin D (Hafner *et al.,* 2011).

CYPs are found as membrane bound and are categorized as microsomal or mitochondrial CYPs based on their localization. Both require NADPH and electron transfer chain. While microsomal CYP contains cytochrome P450 and NADPH-cytochrome P450 reductase, the mitochondrial cytochrome P450 contains cytochrome P450, ferredoxin and ferredoxin reductase (Peterson and Prough, 1986). Whether microsomal or mitochondrial, the cycle starts with the binding of substrate to oxidized form ($Fe^{3+,}$ ferric) of cytochrome P450. Then, while in microsomal system, NADPH cytochrome P450 reductase provides the transfer of electron from NADPH to cytochrome P450-substrate complex; in mitochondrial system, ferrodoxin reductase and ferrodoxin induces this electron transfer, so either way, Fe^{3+} is reduced to Fe^{2+} (ferrous). In the next step, molecular oxygen binds to this reduced complex and unstable Fe^{2+}-O_2 is occurred, which is then converted to Fe^{+3}-O_2^- . After that, a second reduction step occurs with the transfer of electron from NADPH via the microsomal or mitochondrial electron transferring components and molecular oxygen is reduced. O-O bond is broken and one molecule of oxygen is inserted into the substrate. Water is

formed and hydroxylated substrate is generated. Therefore, it reverts back to the oxidized state (Guengerich *et al.,* 1991; Norlin, 2000). Mitochondrial and microsomal P450 catalytic cycles are given in Figure 1.7 (Jones *et al.,* 2014).

CYPs, taken role in vitamin D metabolism, are both microsomal and mitochondrial and catalyze hydroxylation reactions on particular carbons of the vitamin D substrate by using heme-bound, oxygenated-iron (Fe-O) intermediate. It is known that these enzymes are affected by various substrates including polycyclic aromatics, drugs and foods.
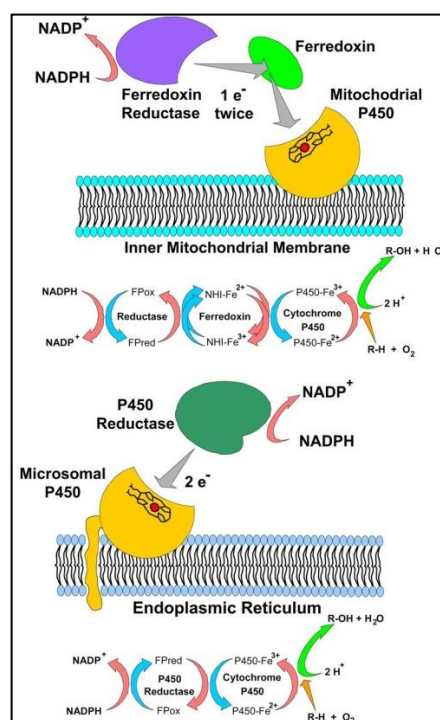
Figure 1.7 Mitochondrial and microsomal P450 catalytic cycles (Jones *et al.,* 2014)

1.3.1 CYP2R1

CYP2R1, one of the vitamin D 25-hydroxylases, was identified in 2003 by Cheng *et al*. CYP2R1 is microsomal enzyme and mainly expressed in liver (the main site of 25-hydroxylation) and testis. The gene of CYP2R1 is located on human chromosome 11p15 (Cheng *et al.,* 2003). It is highly conserved between species than other CYP2 family members (Nelson, 2003).

Studies showed that variants at the locus for CYP2R1 affect circulating 25-OH-D concentrations (Wang *et al.,* 2010). This implied that CYP2R1 is responsible for 25-hydroxylation. Genetically, CYP2R1 was proved as vitamin D 25-hydroxylase when a Nigerian individual having $25(OH)D_3$ deficiency and rickets was examined (Casella *et al.,* 1994; Cheng *et al.,* 2004). The difference of this patient from control people with similar origin was being homozygous for a transition mutation in the CYP2R1 gene. Because of this mutation, proline was replaced with leucine (L99P), which affects the 25-hydroxylase activity. The importance of this amino acid was understood detailed in 2008, when crystal structure of CYP2R1 was obtained (Fig.1.8) (Strushkevich *et al.,* 2008). Leu99 is located in the B-helix close to the heme binding loop and has hydrogen binding interaction with Arg445, which helps to form secondary structure. Substitution of proline destroys the helical structure and inhibits the vitamin D_3 25-hydroxylation activity of CYP2R1 (Cheng, 2004 *et al*; Strushkevich *et al.,* 2008).
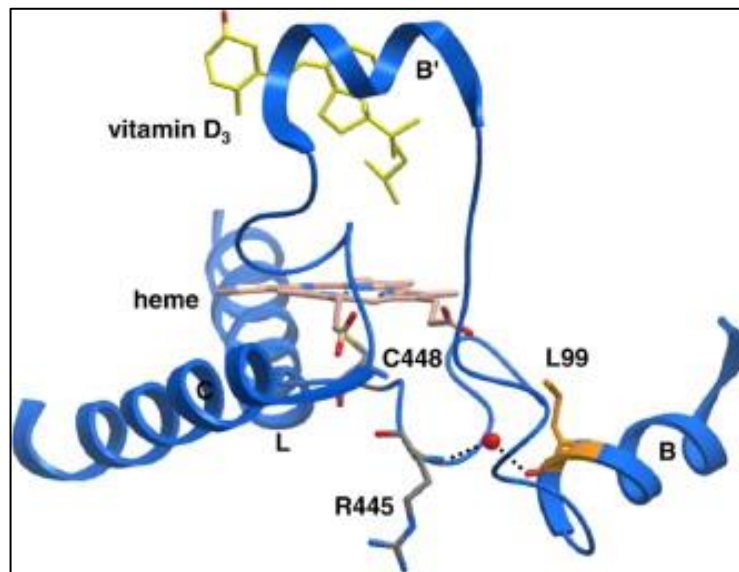
Figure 1.8 Crystal structure of CYP2R1 (Strushkevich *et al.,* 2008).

However, CYP2R1 is not the only enzyme having 25- hydroxylase activity, which was understood by using CYP2R1 deleted mice. In that mice, 25(OH)D blood level was decreased but not reached to zero (Zhu *et al.,* 2013). Thus, there are also other enzymes for this activity such as CYP27A1 and CYP3A4. CYP2R1 has high affinity for vitamin D and can activate the 25-hydroxylation of both vitamin D2 and vitamin D3, different than CYP27A1 (Shinkyo *et al.,* 2004; Guo *et al.,* 1993).

CYP2R1 can be modulated by agents that induce or suppress expression in a tissue specific way. In one study, phenobarbital, efavirenz and calcitriol suppressed the mRNA expression of CYP2R1 in fibroblasts but only calcitriol inhibited the mRNA expression of CYP2R1 in LNCaP cells.

The difference between tissues can be PXR, a nuclear receptor taken role in gene regulation by xenobiotics. While it is expressed in fibroblasts, it is not found in LNCaP cells (Ellfolk *et al.,* 2009).

1.3.2 CYP27A1

CYP27A1, the only mitochondrial 25-hydroxylase, was discovered by Cali *et al*. in the beginning of 1990s (Cali *et al.,* 1991). It is mainly expressed in liver and slightly expressed kidney, intestine, ovary, lung, macrophages, prostate and skin (Araya *et al.,* 2003; Gascon-Barré *et al*., 2001; Quinn *et al.,* 2005; Tang *et al.,* 2007). It has role in bioactivation of vitamin D_3 by catalyzing 25-hydroxylation. It does not 25-hydroxylate vitamin D_2 unlike CYP2R1 (Hosseinpour and Wikvall, 2000; Schuster, 2011).

CYP27A1 is involved in not only vitamin D metabolism but also cholesterol metabolism. In fact, CYP27A1 was initially identified as a sterol 27-hydroxylase involved in bile acid synthesis and cholesterol metabolism in 1970s (Björkhem and Gustafsson, 1973; Zhu and Deluca, 2012). However, both sterol 27-hydroxylase and vitamin D 25-hydroxylase were verified as the same enzyme with molecular cloning of cyp27a1 gene in rabbit, rat and human (Anderson *et al.,* 1989; Usui *et al.,* 1990; Su *et al.,* 1990; Cali *et al.,* 1991; Guo *et al.,* 1993). CYP27A1 has an essential role in cholesterol homeostasis. CYP27A1 provides the formation of 27-hydroxycholesterol, which suppresses HMG-CoA reductase (Chiang, 2002; Schroepfer, 2000). By inhibiting this enzyme, cholesterol serum level decreases. This is helpful for reduction the risk of having cardiovascular diseases.

The importance of CYP27A1 is shown in humans and mice by mutations in cyp27a1 gene (Cali *et al.,* 1991; Rosen *et al.,* 1998). When there is anomaly in CYP27A1 enzyme, cholesterol metabolism is directly affected but loss of CYP27A1 did not induce health defects seen in vitamin D deficiency such as rickets, bone lesions (Moghadasian, 2004). These results imply that CYP27A1 has less effect on vitamin D metabolism than other vitamin D 25-hydroxylases, which compensate for loss of CYP27A1 activity (Cheng *et al.,* 2003).

It has been reported that human transcriptional regulation and enzymatic activity of CYP27A1 are affected by hormones, such as growth hormone, glucocorticoids, endogenous sex hormones (Araya *et al.,* 2003). However the effect of these hormones depends on cell type. For example, while estrogen results in downregulation of CYP27A1 mRNA expression and enzyme activity in HEPG2 cells; it causes upregulation of CYP27A1 mRNA expression in LNCaP cells (Tang *et al.,* 2007).

1.3.3 CYP27B1

CYP27B1 is mitochondrial 25-hydroxyvitamin D-1-hydroxylase (1-hydroxylase) and metabolizes 25(OH)D to 1,25(OH)$_2$D, which is the biologically active form of vitamin D.

CYP27B1 gene locus was previously determined for vitamin D dependent rickets type 1(VDDRI) with linkage analysis and mutations in CYP27B1 gene results in VDDRI disease (Labuda *et al.,* 1990; Kitanaka *et al.,* 1998). In addition, 1α-hydroxylated vitamin D metabolites are not be observed in CYP27B1-null mice, which means that CYP27B1 is the only enzyme for 1α-hydroxylation.

CYP27B1 is mainly found in the proximal tubules of the kidney (Zehnder *et al.,* 1999; Brunette *et al.,* 1978). However, it is also expressed by extra-renal tissues such as gastrointestinal tract, pancreas, epidermis, brain, endothelial cells, adipose tissue, and placenta (Hewison *et al.,* 2000; 2004; Zehnder *et al.,* 2001). These extra renal expressions give support the nonclassical actions of vitamin D (Townsend *et al.,* 2005). The regulation of CYP27B1 can be different depend on tissues. In kidney, CYP27B1 is tightly regulated by several factors including parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), calcium and $1,25(OH)_2D$ (Fig. 1.9).
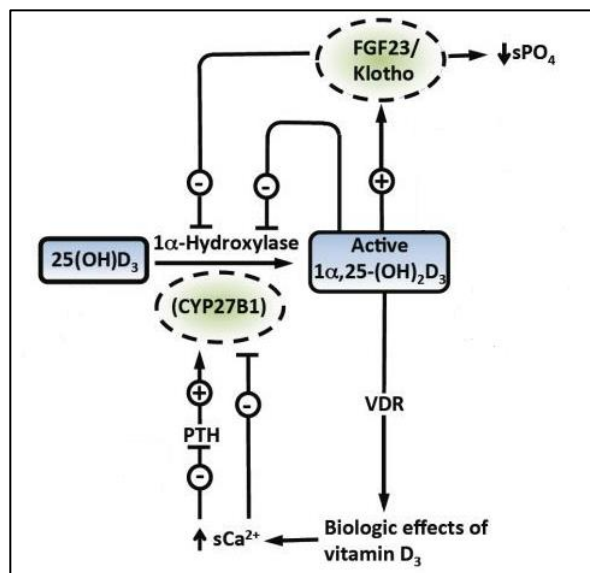
Figure 1.9 Regulation of CYP27B1 with several factors (Jones, 2012).

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PTH upregulates CYP27B1 via cAMP since proximal promoter of CYP27B1 gene has consensus cAMP response elements (Rost *et al.,* 1981). FGF23, $1,25(OH)_2D$, high calcium concentrations or high phosphate concentrations suppress CYP27B1 directly or indirectly (Urakawa *et al.,* 2006; Murayama *et al.,* 1998).

CYP27B1 has also epigenetic regulation. While DNA methylation causes the downregulation of CYP27B1 expression, demethylation enhances the CYP27B1 expression (Kouzmenko *et al.,* 2011; Jones *et al.,* 2014). CYP27B1 expression is suppressed in many cancer types such as breast, prostate, leukemia. The reason can be hypermethylation of CpG island in CYP27B1 (Khorchide *et al.,* 2005; Shi *et al.,* 2002; Wjst *et al.,* 2010; Fetahu *et al.,* 2014). With the suppression of CYP27B1 expression in cancer cells, local concentration of 1,25-D is reduced and this limits its activities.

1.3.4 CYP24A1

CYP24A1, located in the inner mitochondrial membrane, is the only determined 24-hydroxylase associated with vitamin D metabolism. CYP24A1 catabolizes the $1,25(OH)_2D$, hormonally active vitamin D metabolite, to inactive or less active metabolites. In addition, this enzyme can hydroxylase 25(OH)D, less preferable substrate than $1,25(OH)_2D$ (Jones *et al.,* 1998). It has not only 24-hydroxylase activity but also has 23-hydroxylase activity. While 24-hydroxylase pathway causes calcitroic acid production, 23-hydroxylase pathway produces 1,25-26,23 lactone at the end of pathway (Sakaki *et al.,* 2000).

CYP24A1 is expressed mainly in kidney and oocytes but also found in many cells having the vitamin D receptor (Jones *et al.,* 1998). This implies that CYP24A1 regulates both circulating concentrations of $1,25(OH)_2D$ and amount of $1,25(OH)_2D$ within the cell, causes a suitable cellular response. CYP24A1 provides to control the action of $1,25(OH)_2D$ with a negative feedback (Lohnes and Jones, 1992). It has been reported that $1,25(OH)_2D$ induces the expression of CYP24A1. In addition, vitamin D response element (VDRE) element is located in the upstream promoter of CYP24A1 gene which supports the regulation of CYP24A1 with $1,25-(OH)_2D$ (Ohyama *et al.,* 1993). Therefore, cells can be protected from excess activation of VDR. In addition to $1,25-(OH)_2D$, CYP24A1 expression can be regulated with some other factors such as PTH and FGF23. However, their effects can be changed depend on cell types (Zierold *et al.,* 2000; 2001; Armbrecht *et al.,* 1998; Perwad *et al.,* 2005).

It has been reported that a number of tumor types have high CYP24A1 expression and that increase in CYP24A1 expression causes to catabolize the $1,25(OH)_2D$, which suppresses the anti-tumorigenic effects such as antiproliferative, antiangiogenic and proapoptotic effects (Horvath *et al.,* 2010; Friedrich *et al.,* 2003; Mimori *et al.,* 2004; Parise *et al.,* 2006). In addition, it has been detected that high tumor CYP24A1 expression is associated with an inadequate prognosis. All of these suggest that CYP24A1 may be a candidate oncogene (Albertson *et al.,* 2000). Therefore, inhibitors of CYP24A1 can be important for the prevention and treatment of cancer and vitamin D catabolism related diseases. Up to now, some compounds have been found as CYP24A1 inhibitor such as ketoconazole, genistein but these are not only specific to CYP24A1 (Muindi *et al.,* 2010; Cross *et al.,* 2004). Finding very specific CYP24A1 inhibitors can be useful for very wide field of applications about vitamin D deficiency related diseases.

1.4 Aim of the Study

Vitamin D, a secosteroid hormone, has been widely known for its remarkable role in maintaining bone health by regulating calcium and phosphate homeostasis. In addition to this typical role, vitamin D has a much broader range of effects on human health than believed previously such as muscle function, cardiovascular homeostasis, nervous function, and the immune response. Vitamin D is metabolized by cytochrome P450 enzymes (CYPs). CYPs play a key role not only in vitamin D metabolism, but also metabolism of a wide range of endogenous compounds such as cholesterol, steroids, fatty acids, and bile acids, as well as exogenous compounds including drugs and dietary products. Because of the increase consumption of herbal products containing polyphenols, numerous studies on interaction between these compounds and CYPs have been documented so far. From these reports, it has been shown that these enzymes are regulated by plant phenolic compounds and these phenolics are indicated as the main inducers or inhibitors of cytochrome P450 enzymes. Among these phenolic compounds, resveratrol and quercetin were selected specifically for this thesis project. So far, accumulating data is available for the effect of resveratrol or quercetin on some of the CYP450 enzymes. Although few studies poorly revealed the interaction between vitamin D receptor (VDR) and these phenolic compounds, the role of resveratrol and quercetin on vitamin D metabolizing CYPs were not studied before. Therefore, the purpose of present study was to evaluate the potential effects of resveratrol and quercetin on vitamin D metabolizing CYP27A1, CYP2R1, CYP24A1 and CYP27B1 enzymes. This study is the first concerning individual effects of quercetin and resveratrol on vitamin D metabolizing CYPs.

In that purpose, human embryonic kidney cell line (HEK-293) and human hepatocellular carcinoma cell line (HUH-7) were treated with resveratrol and quercetin at half maximal inhibitory concentrations (IC50) to obtain protein and total RNA for in vitro investigation of the effects of resveratrol and quercetin on the protein and mRNA expressions of vitamin D metabolizing CYPs by using Western Blot technique and qRT-PCR method, respectively.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

In this study, the effects of quercetin and resveratrol on vitamin D metabolizing cytochrome P450 enzymes are studied on human embryonic kidney cell line HEK-293 and human hepatoma cell line HUH-7. Embryonic kidney cell line HEK-293 (ATCC® CRL-1573™) was a gift from Assoc. Prof. Dr. A. Elif Erson Bensan, Biology Department, Middle East Technical University. Hepatocellular carcinoma HUH-7 (JCRB0403) cancer cell line was a gift from Assoc. Prof. Dr. Rengül Çetin Atalay, Bioinformatics Institute, Middle East Technical University, Ankara, Turkey.

2.1.2 Chemicals and Materials

Resveratrol (R5010), Quercetin (Q4951), Bicinchoninic acid (D8284), ammonium acetate (A7672), bovine serum albumin (BSA; A7511), phenyl methane sulfonyl fluoride (PMSF; P7626), sodium potassium tartarate (Rochella salt; S2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T1378), acrylamide (A-8887), ammonium per sulfate (APS; A-3678), bromophenol blue (B5525), diethanolamine (D-2286), diethylpyrocarbonate (DEPC; D5758), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), methanol (34885), N'-N'-bis-methylene-acrylamide (M7256),

N-N-dimethylformamide (D-8654), phenazine methosulfate (P9625), sodium dodecyl sulfate (SDS; L4390), sodium-potassium (Na-K) tartarate (S-2377), tween 20 (P1379) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Magnesium chloride ($MgCl_2$; 05833), potassium chloride (KCl 104935), potassium dihydrogen phosphate (KH_2PO_4; 04871), di-potassium hydrogen phosphate (K_2HPO_4; 05101), sodium carbonate (06398), sodium hydroxide (06462), boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; A894987 605), folin-phenol reagent (1.09001.0500), sodium carbonate (Na_2CO_3; 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), zinc chloride ($ZnCl_2$; 108815) were the products of E. Merck, Darmstadt, Germany.

Absolute ethanol (32221) and acetyl acetone (33005) were obtained from Riedel de-Haen Chemical Company, Germany. Isopropanol (AS040-L50) was the product of Atabay, Istanbul, Turkey. QIAzol® (79306) was purchased from QIAGEN, Hilden, Germany.

5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821), dithiothreitol (DDT; R0861), gene rulerTM 50 bp DNA ladder (SM0371), light cycler-fast start DNA Master[Plus] SYBR green I (K0252), Maloney murine leukemia virus reverse transcriptase (M-MuLu-RT; K1622), pre-stained protein ladder (SM0671) were purchased from MBI Fermentas, USA.

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

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

The CYP24A1 (sc-66851), CYP27B1 (sc-67260), CYP2R1 (sc-48987), CYP27A1 (sc-14835), β-Actin (sc-47778) primary antibodies, donkey anti-goat IgG-AP (sc2022), goat anti-rabbit IgG-AP (sc-2034) and donkey anti-mouse IgG-AP (sc-2320) were purchased from Santa Cruz (Santa Cruz, CA). Primers were made by Alpha DNA, Montreal, Canada.

Dulbecco's Modified Eagle's Medium (DMEM; BE12-614F) was purchased from Lonza, Walkersville, MD, USA.
Pen-Strep solution (03-031-1B) and trypsin-EDTA solution (03-050-1B) were the products of the Biological Industries, Beit-Haemek, Israel. RIPA buffer (9806) was purchased from Cell Signaling Technology, Beverly, MA.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture Conditions

HEK-293 and HUH-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5g/L glucose, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine. Cells were incubated at 37°C with 5% carbon dioxide (CO_2) in a humidified EC 160 NÜVE incubator. NÜVE MN 090 Class II Safety Cabinet is used for cell culture studies. The culture mediums were renewed in 2-3 days for appropriate growth conditions.

2.2.1.2 Cell Thawing

Cells, which were in cryotubes, were defrosted at 37°C water bath after taken from liquid nitrogen. Then, they were immediately transferred to T25 cell culture flask involving 7 mL of pre-warmed growth medium and incubated in 37°C CO_2 incubator. After 24 hours, medium was changed with new medium in order to eliminate dimethylsulfoxide (DMSO).

2.2.1.3 Subculturing the Cell Lines

When cells were 80% confluent in the T25 flask, they were transferred to T75 flask. To transfer cells, medium was removed and cells were washed with pre-warmed 7 mL of 10 mM phosphate buffered saline (PBS) and then cells were detached with 1 ml trypsin and transferred to T75 flask by addding 6 ml growth medium. Then, cells again placed in CO_2 incubator. When cells were 80% confluent, they were routinely split in 1:4 in the T75 flasks as follows. The medium was removed and cells were washed with prewarmed 10 mL of 10 mM phosphate buffered saline (PBS). In order to detach cells, 1 ml trypsin was added to the flask and incubated at 37°C for 3-5 minutes. After detaching cells, 3 ml prewarmed growth medium was added and 1 ml of this mixture was transferred into new T75 flask containing 14 ml fresh culture medium. The culture was placed in CO_2 incubator at 37°C. This procedure was repeated in every 3-4 days.

2.2.1.4 Cell Freezing

When cells were 80% confluent in the T-75 flask, they can be stored in cryotubes for future studies. Cells were freezed by using following procudere. Initially, growth medium was removed and cells were washed

with 15 mL of pre-warmed 10 mM PBS and 1 mL of pre-warmed trypsin was added to T-75 flask and placed in 37°C CO_2 incubator for 3-5 minutes. Then, 4 mL of growth medium was added over the detached cells. Medium provides the inactivation of trypsin. This mixture was transferred into a 15 mL falcon tube and centrifuged at 400 x g for 5 minutes at room temperature. This supernatant was removed, 1 ml growth medium was added to resuspend pellet and pipetting was done. After that, the cell suspension was transferred to cryotube and 100 µL DMSO, cryoprotectant, was added. Cryotube was immediately placed in the -80°C freezer and after a week it was transferred to liquid nitrogen tank for longer term storage.

2.2.1.5 Polyphenol Treatments of Cells for IC50 Determination

HEK-293 and HUH-7 cells were seeded in 24 well plate with 500 µl DMEM media at a density of 9×10^4 per well and 6×10^4 per well, respectively. Cells were incubated 24 hours at 37˚C and 5 % CO_2. One day after seeding, as given in Table 2.1, cells were treated with a different range of resveratrol or quercetin concentrations to obtain a half maximal inhibitory concentration (IC50) for 48 hours incubation period and compared with vehicle (DMSO) control cells.

For treatment of HEK293 cells, a fresh solution of 2 mM resveratrol and 2 mM quercetin; for treatment of HUH-7 cells, a fresh solution of 4 mM resveratrol and 4 mM quercetin were prepared in DMEM growth medium containing 0,5 % and 0,35 % DMSO, respectively. These solutions were diluted to 200µM and 400µM for HEK293 and HUH7 cells, respectively. Concentration range of treatments according to the type of polyphenols and cell lines are given in Table 2.1. After 48 hours, Alamar Blue Assay was performed for cell proliferation and IC50 determination.

Table 2.1 Concentration range of treatments according to the type of polyphenols and cell lines.

	Resveratrol	Quercetin
HEK293	0-90 µM	0-140 µM
HUH7	0-200 µM	0-250 µM

2.2.1.6 Alamar Blue Assay for IC50 Determination

In this study, the effects of quercetin and resveratrol on vitamin D metabolizing cytochrome P450 enzymes were studied on human embryonic kidney cell line HEK-293 and human hepatoma cell line HUH-7. For that purpose, at the beginning of this study, the effects of polyphenols (quercetin and resveratrol) on HEK-293 and HUH-7 cells' viability and IC50 values were determined by Alamar Blue (AB) Assay.

Alamar Blue involves resazurin, which is weakly fluorescent blue indicator dye. This dye is cell permeable and non toxic to the cells. When this dye enters the cells, resazurin is reduced to resorufin with a reducing environment in the cytosol of living cells. Resorufin is pink and highly fluorescent. Viable cells increases the overall fluorescence and pink color of the media due to continuous convertion of resazurin to resorufin. Therefore, the intensity of fluorescence produced is proportional to the number of living cells.

For Alamar Blue Assay, cells were seeded into a 24 well plate and allowed to attach overnight. Attached cells were treated with a range of polyphenol concentrations for 48 hours as mentioned before. After 48 hours incubation time with polyphenols, Alamar Blue (AB) Assay was carried out. The medium was discarded and cells were washed with PBS. Then, cells were incubated 4 hours with medium containing Alamar Blue reagent at a final concentration of 10% and transferred to 96 well plate to read absorbance values by Multiskan™ FC Microplate Photometer (Thermo Scientific) at 570 and 600 nm. Percent reduction of cells was calculated by using the following formula;

$$\%\text{AB reduction} = \frac{(\varepsilon_{ox}\lambda_2)(A\lambda_1) - (\varepsilon_{ox}\lambda_1)(A\lambda_2)}{(\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1)} \times 100$$

In the formula, $\varepsilon\lambda_1$ and $\varepsilon\lambda_2$ are constants representing the molar extinction coefficient of AB at 570 and 600 nm, respectively, in the oxidized (ε_{ox}) and reduced (ε_{red}) forms. $A\lambda_1$ and $A\lambda_2$ represent the absorbance of test wells at 570 and 600 nm, respectively.

$A'\lambda_1$ and $A'\lambda_2$ represent absorbance of negative control wells at 570 and 600 nm, respectively. After obtaining %AB reduction, an inhibitory concentration of polyphenols at which 50% of cell growth was inhibited (IC50) was calculated by using GraphPad Prism statistical software.

2.2.2 Protein Extraction

Cells were seeded to 100 x 20mm tissue culture petri dishes at plating density of 2.700.000 cells/ petri dish (for HEK293 cells) and 1.800.000 cells/ petri dish (for HUH7 cells). Three groups were constructed in triplicate for each experiment. First group is used as a control, second group as a vehicle (DMSO) control, third group as a treated one. One day after seeding, while first group dishes were replaced with fresh growth medium as the control group; second group dishes were replaced with new medium containing DMSO as a vehicle control. At the same time, third group dishes treated with resveratrol or quercetin as determined IC50 concentrations. Thereafter, the same procedure is used for all groups. After 48 hours treatment incubation, growth medium was removed and the cells were washed three times by using cold (4 °C) PBS buffer. After the last washing, 2 ml PBS was added to the dishes. Dishes were incubated on ice for 5 minutes and the cells were scraped. Lysates were taken into eppendorf tubes and centrifuged at 14000 x g in a cold microfuge for 10 minutes. Supernatant was removed to get rid of PBS. Commercially available 10X RIPA buffer from Cell Signaling Technology was diluted to 1X with distilled water and then, 1mM phenylmethanesulfonyl fluoride (PMSF) was added as a protease inhibitor. 400 μL of prepared buffer was added into the cell pellet in each eppendorf to lyse the cells. These lysates mixed well with pipetting and incubated on ice for 5 minutes. Then, the lysates were sonicated for 4 minutes and centrifuged at 14000 x g in a cold microfuge for 10 minutes. Supernatants were taken and stored at -80 °C freezer.

2.2.3 Determination of Protein Concentration with BCA Method

Protein concentrations of cell culture lysates were determined by the BCA (Bicinchoninic Acid) method using crystalline bovine serum albumin as a standard (Smith *et al*., 1985).

The principle of this method is conversion of Cu^{2+} to Cu^{1+} under alkaline conditions and reaction of two BCA molecules with one Cu^{1+} ion. This reaction causes forming a purple color that absorbs light at a wavelength of 562 nm. The absorbance at this wavelength is directly proportional to the amount of protein concentration.

Reagents:

Reagent A:

0.4 g of $CuSO_4.5H_2O$ was dissolved in 10 mL dH_2O.

Reagent B:

8 g of $Na_2CO_3.H_2O$ and 1.6 g of $NaKC_4H_4O_6$ was dissolved with dH_2O and titrated with $NaHCO_3$ to pH 11.25 and the volume was completed to 100 mL with dH_2O. The pH of the solution was checked at the end.

Reagent C:

4 g of BCA was dissolved in 100 mL of dH_2O.

BCA Solution:

Reagent A, Reagent B and Reagent C were mixed in the same order with the ratio of 1:25:25.

Bovine Serum Albumin (BSA) Protein Standards:

0.025, 0.05, 0.075, 0.1, 0.15, 0.2 mg/mL

Protein Sample:

While control samples were diluted 100 times; treated samples were diluted 50 times.

100 µL of BSA standards and samples were mixed with 100 µL of BCA solution and incubated at 60°C for 15 minutes. Then, 100 µL of this mixture was added into the 96 well plate. The absorbances of samples were measured at 562 nm with Multiskan™ GO Microplate Spectrophotometer.

Protein concentration was calculated by the following formula;

$$\text{Protein Concentration (mg/ml)} = \frac{[OD_{562nm}]}{\text{Slope of standards}} \times \text{Dilution}$$

2.2.4 Determination of Protein Expression

2.2.4.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The effects of quercetin and resveratrol on protein expressions of vitamin D metabolizing CYP2R1, CYP27A1, CYP24A1 and CYP27B1 enzymes of HEK293 and HUH7 cell lines were analyzed by Western blot method as described by Towbin *et al*. (1979) with some modifications.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 4% stacking gel and 10% separating gel through a discontinuous buffer system as described by Laemmli (1970). Separating and stacking gel solutions were prepared freshly according to Table 2.2.

Table 2.2   Components of separating and stacking gel solutions.

Components	Separating Gel Solution	Stacking Gel Solution
Monomer Concentration	10 %	4%
Gel Solution	5 mL	650 µL
dH$_2$O	6,02 mL	3,05 mL
Separating Buffer	3,75 mL	---
Stacking Buffer	---	1,25 mL
10% SDS	150 µL	50 µL
10%APS	75 µL	25 µL
TEMED	15 µL	5 µL
Total Volume	15 mL	5 mL

Vertical slab gel electrophoresis was performed with Mini-PROTEAN tetra cell mini trans blot module (Bio-Rad, Richmond, CA). The gel sandwich unit was set up by using two glass plates with one cm space. 4,25 mL separating gel solution was put into the sandwich unit. Then, butanol was added at the top of the seperating gel to obtain smooth gel surface and to

provide fast polymerization of gel. When seperating gel was polymerized, the butanol was discarded. After that, stacking gel solution was added over the seperating gel and immediately the comb was inserted. After the stacking gel was polymerized, the comb was removed carefully. Then, 1X electroning running buffer was added to fill the wells. Wells were cleaned up by a syringe to remove air bubbles and remaining gel particles, which can inhibit the running of protein samples. Protein samples were diluted with dH$_2$O according to the following formula;

$$V = \frac{[\text{Conc. of Protein}]}{1.333} \times 20 - 20$$

V is the volume of dH$_2$O to be added to dissolve 20 µL of sample. This calculation provides to obtain 1 mg/ mL concentration after sample dilution buffer was added. Then, 45µl diluted sample was mixed with 15µl sample dilution buffer (4X) and diluted samples were incubated 1.5 minutes at 100ºC heat block. After that, 20 or 30 µg of each samples (depends on genes) and 4 µg of protein ladder as marker was loaded on different wells. After loading the samples, gel running module was placed in the main buffer tank filled with electronic running buffer (ERB). The tank was connected to the Bio-Rad power supply and electrophoresis was run at 20 mA–90 V in stacking gel and 30 mA–200 V in separating gel.

Reagents:

Gel Solution

14.6 g acrylamide and 0.4 g N'-N'-bis-methylene-acrylamide were dissolved separately with dH$_2$O then mixed and filtered through filter paper. The final volume was completed to 50 mL.

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

18.15 g of tris-base was dissolved with 50 mL dH_2O, and titrated with 10 M HCl to pH 8.8. The volume was completed to 100 mL. The pH was checked at the end.

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

6 g of tris-base was dissolved with 60 mL dH_2O, and titrated with 10 M HCl to pH 6.8. The volume was completed to 100 mL. The pH was checked at the end.

Sodium Dodecyl Sulfate - SDS (10%)

1 g of SDS was dissolved with dH_2O, and the volume was completed to 10 mL.

Ammonium Persulfate - APS (10%, Fresh)

40 mg of APS was dissolved in 400 µL distilled water.

Tetramethylethylenediamine - TEMED (Commercial)

Sample Dilution Buffer-SDB (4x)

2.5 mL of 1 M tris-HCl buffer (pH 6.8), 4 mL glycerol, 0.8 g SDS, 2 mL ß-mercaptoethanol and 0.001 g bromophenol blue were used and the volume was completed to 10 mL with dH_2O.

Electrophoretic Running Buffer - ERB:

0.25 M Tris, 1.92 M glycine (10x Stock, diluted to 1x before use by adding 0.1% SDS)

15 g tris-base was dissolved with 350 mL dH_2O, then 72 g glycine was added. The volume of the mixture was completed to 500 mL.

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

2.2.4.2 Western Blotting

After electrophoretic separation was finished, gel was taken and transferred into transfer buffer for 10 minutes in order to equilibrate the gel and to get rid of SDS. At the same time, the PVDF membrane was cut as equel size with the gel. Because PVDF membrane is hydrophobic, it was incubated with 100% methanol for 30 seconds. Then the membrane was equilibrated in transfer buffer for 5 minutes. After that, the gel, PVDF membrane, Whatman papers and two fiber pads were placed in transfer sandwich as shown in Figure 2.1. The transfer sandwich was placed into Mini Trans-Blot module (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with transfer buffer. The transfer was carried out at 90 volt and 400 mA for 90 minutes.
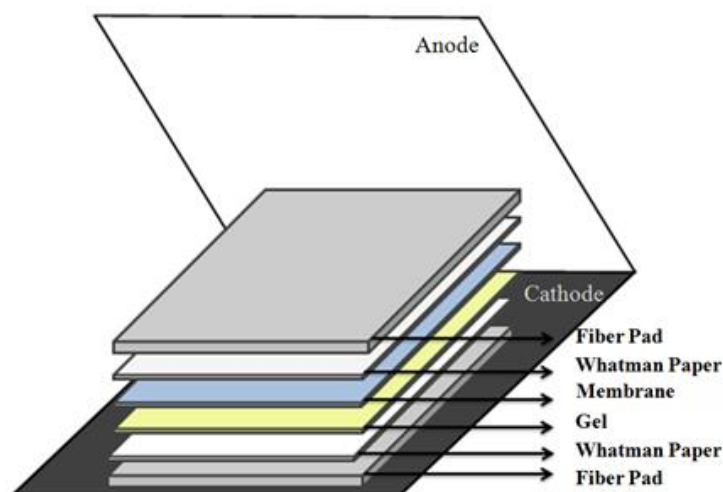
Figure 2.1 Western blot sandwich.

When tranfer was finished, the membrane was washed with TBST for 15 minutes to remove transfer buffer and salts. After that, membrane was incubated with blocking solution in room temperature for an hour, which prevents non-specific binding by covering empty spaces. After that, the membrane was incubated with primary antibodies for 2 hours at room temperature or overnight at 4˚C by shaking depends on types of antibodies. Antibodies used in the experiments and their optimized dilutions were given in Table 2.3. The membrane was washed with TBST for three times each of which is 10 minutes to remove the unbound primary antibody. Then, the membrane was incubated with alkaline phosphatase conjugated secondary antibody for an hour. After incubation with secondary antibody, the membrane was washed with TBST for three times each of which is 10 minutes to remove the unbound secondary antibody and to prevent non-

specific reaction. Finally, the blot was incubated with alkaline phosphate substrate solution. The band intensities were analyzed by Image J visualization software developed by NIH.

Table 2.3 Antibodies and the optimum dilutions for Western Blot Analysis.

	Primary antibody (dilution)	Secondary antibody (dilution)
CYP2R1	goat polyclonal anti-CY2R1 (1/250 dilution)	AP conjugated anti-goat IgG (1/1000)
CYP27A1	goat polyclonal anti-CY27A1 (1/250 dilution)	AP conjugated anti-goat IgG (1/2000)
CYP24A1	Rabbit polyclonal anti-CY24A1 (1/1000 dilution)	AP conjugated anti-rabbit IgG (1/2000)
CYP27B1	Rabbit polyclonal anti-CY27B1 (1/500 dilution)	AP conjugated anti-rabbit IgG (1/2000)

Reagents:

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

3.03 g trisma-base and 14.4 g glycine was dissolved in 200 mL methanol, and the volume was completed to 1 L with distilled water.

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

9.5 g of NaCl was dissolved in some water and 6.5 mL of 1 M tris-HCl buffer was added. Then pH of the solution was adjusted to 7.4. Finally, 165 µL tween 20 was added and volume was completed to 350 mL with distilled water.

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

5 g non-fat dry milk was dissolved in 100 mL TBS

Primary Antibody: 1/250 to 1/1000 dilution

Secondary Antibody: 1/1000 to 1/3000 dilution

Alkaline Phosphatase Substrate Solution:

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

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

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

To prepare the substrate solution, 40 mL of Solution A, 136 µL of Solution C, and 268 µL of Solution B were mixed for two membranes.

2.2.5 Determination of mRNA Expression

2.2.5.1 Isolation of Total RNA from Cell Lines

The important step in RNA isolation is the inhibition of RNase activity. For that reason, distilled water containing % 0.1 (v/v) diethylpyrocarbonate (DEPC) is used to treat all plastic and glass equipments used for total RNA. After the evaporation of excess DEPC, the equipments were autoclaved.

Cell lines were seeded into 6 well plate at plating density of 450.000 cells/well (for HEK-293 cells) and 300.000 cells/well (for HUH-7 cells) for RNA isolation. Three groups were constituted in the same way used during protein extraction. One day after seeding, while first group dishes were replaced with fresh growth medium as the control group, second group dishes were replaced with new medium containing DMSO as a vehicle control. At the same time, third group dishes treated with resveratrol or quercetin at IC50 concentrations. After 48 hours of treatment, growth medium in the wells was removed and the cells were washed three times by using cold PBS buffer. After PBS buffer was removed, 1 mL of QIAzol® was added into the wells and incubated for 5 minutes at room temperature in chemical hood. Then, pipetting was done to detach the cells and QIAzol® - cell lysate mixture was transferred into a 2 mL eppendorf tube. 200 µL of chloroform was added to tube and the tube was shaken vigorously. After shaking, the tube was incubated for 3 mimutes in room temperature. The tube was centrifuged at 12000 x g for 15 minutes at 4°C. After centrifugation, three layers were observed in the tube. Because, RNA was included in the upper aqueous phase, this upper part (~500 µl) was transferred into new eppendorf tube. After that, 500 µl cold isopropanol was added into the tube and the tube was shaken gently. It was incubated at room temperature for 10 minutes. Then, it was centrifuged at 12000 x g for

20 minutes at 4°C. After centifugation, the supernatant was removed and the pellet was mixed with 1 mL of 75% ethanol. The tube was centrifuged again at 7500 x g for 5 minutes at 4°C. The supernatant was removed without thouching pellet and excess amount of ethanol was evaporated in hood. Finally, RNA was dissolved in 20 μL of nuclease-free distilled water and stored at -80°C.

2.2.5.2 Determination of RNA Concentration

RNA concentration was determined by measuring the absorbance at 260 nm, at which RNA gives maximum absorbance. Purity of RNA was checked with the 260/280 nm ratio. The ratio of OD260/OD280 must be between 1.8 and 2.2. While, below 1.8 indicates the DNA contamination, above 2.2 indicates the protein or phenol contamination. The concentration and purity of the RNA were measured at NanoDrop™ 2000 (Thermo Scientific).

2.2.5.3 cDNA Synthesis

Reagents:

5X Reaction Buffer : 250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DDT

M-MuLV-RT: Moloney-Murine Leukemia Virus Reverse Transcriptase

Ribolock: RNase inhibitor

Oligo (dT)18 primer: a synthetic single-stranded 18-mer oligonucleotide with 5'- and 3'-hydroxyl ends

dNTP: Deoxyribonucleotide triphosphate (10mM)

cDNA is synthesized from RNA by reverse transcription process using Fermentas RevertAid^{TM} First Strand cDNA synthesis Kit (K1622). 1 μg of total RNA isolated from cell lines and 1 μL of oligo dT primer (Fermentas, Hanover, MD, USA) were mixed in an eppendorf tube. Then, the final volume of the mixture was completed to 10 μL with nuclease-free distilled water. The solution was mixed gently and spinned down by microfuge. Mixture was incubated at 70°C for 5 minutes. Then, it was cooled in ice. After that, 4 μL of 5X reaction buffer, 7 μL nuclease-free distilled water, 1 μL Ribolock, 1 μL M-MuLV-RT (Moloney-Murine Leukemia Virus Reverse Transcriptase) and 2 μL of 10 mM dNTP were added. The tube was mixed gently, spinned down by microfuge and incubated at 42°C for 1 hour. Finally, the reaction was stopped by heating at 70°C for 10 minutes and chilled on ice. cDNA synthesized was stored at -20°C.

2.2.5.4 Quantitative Real-Time PCR

The effects of quercetin and resveratrol on CYP2R1, CYP27A1, CYP24A1 and CYP27B1 gene expressions in HEK293 and HUH7 cell lines were analyzed by quantitative Real Time PCR (qRT-PCR) using Light Cycler FastStart DNA Master^{Plus} SYBR Green I (Roche Applied Science, Basel, Switzerland) and Corbett Rotor Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137).

The 20 μL of reaction mixture was prepared with 100 ng cDNA, 5 μM reverse and forward primers, 10 μL Fast Start Universal SYBR Green Master (ROX) and RNase free distilled water. No template control (NTC) was used in order to detect any contamination. RPL13A was used as an internal standard. Primer sequences for related genes, annealing temperatures and product sizes for gene expression analysis by real-time PCR were given in Table 2.4.

The following qRT-PCR run cycle protocol was used:

- initial melting at 95 °C for 10 minutes,
- amplification and quantification program repeated 45 times;

  - melting at 95 °C for 30 seconds,

  - annealing at 58-62 °C (depending on the gene) for 20 seconds,

  - extension at 72 °C for 20 seconds with a single fluorescent measurement.

- melting curve program 50-99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement.

At the end of each amplification reaction, melting curve analysis of the amplification product was done to confirm the detection of a PCR product. Quantities of specific mRNAs in the sample were measured according to corresponding gene specific relative standard curve obtained from serial dilution of control sample (1:5, 1:50, 1:500, 1:1000). Light cycler quantification software was used to draw the standard curve.

Table 2.4 Primer sequences, annealing temperatures and product sizes of the genes.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Annealing Temp (°C)	Product Size (bp)
RPL13A	CCGCCCTACGACAAGAAA	CAGGGTGGCTGTCACTGC	60	141
CYP27A1	CGCAGGATTGCAGAGCTGGAG	CTCATCTGAGACATGGCAGCAGC	62	247
CYP2R1	TTTATCGGCAACATCTATTCCCTGG	AGCCATTTAGAACCACAGTTGATATGC	60	133
CYP27B1	CTGGCAGAGCTTGAATTGCAAATG	TCCAGTTTGGTCAGATAGGCATTAGG	62	265
CYP24A1	CCAAGTGCAACAAAAGCTTCTTAAGG	TGTCTTCACTGGATCCCAACACCTG	60	245

2.2.6 Statistical Analysis

GraphPad Prism version 6 statistical software package for windows is used for statistical analysis. All results were expressed as means ± standard deviation (SD). Unpaired, two-tailed student's t test is used and $p<0.05$ were chosen as the level for significance.

CHAPTER 3

RESULTS

3.1 Cell culture

3.1.1 Cell Viability Assay by Alamar Blue and IC50 Determination

Alamar Blue (AB) assay was carried out to observe cell proliferation and to determine IC50 value. In this study, the effects of polyphenols; quercetin and resveratrol on HEK-293 and HUH-7 cells' viability and determination of IC50 values were carried out by Alamar Blue assay. Percent reduction of cells was calculated by a following formula;

$$\%\text{AB reduction} = \frac{(\varepsilon_{ox}\lambda_2)(A\lambda_1) - (\varepsilon_{ox}\lambda_1)(A\lambda_2)}{(\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1)} \times 100$$

The details of Alamar Blue protocol and calculation of IC50 were explained in method part.

3.1.1.1 IC50 Determination of Quercetin Treated HEK293 Cells

The effect of quercetin on viability of HEK-293 cells was examined using Alamar Blue assay. Percent survival values of quercetin treated HEK293

cells are given in Table 3.1 and Figure 3.1. Results showed that quercetin treatment ranging from 0 to 140 μM inhibited the proliferation of HEK-293 cells in a concentration dependent manner. Values reflecting the cell number are expressed as percentages of the control cells (vehicle DMSO control) and represented as mean ± standard deviation (SD). In order to determine IC50 value, quercetin concentrations were converted to their log value and cell viability graph was drawn (Figure 3.2). According to this analysis, IC50 value of quercetin was estimated to be 60,72 μM for HEK293 cells.

Table 3.1   Percent survival of HEK293 cells with quercetin treatment ranging from 0 to 140 μM.

Quercetin Conc. (μM)	Percent Survival
0	100,00 ± 6,13
20	80,68 ± 5,43
40	61,68 ± 1,48
60	48,79 ± 5,07
80	47,56 ± 6,64
100	41,03 ± 5,21
120	39,62 ± 1,41
140	2,24 ± 6,89
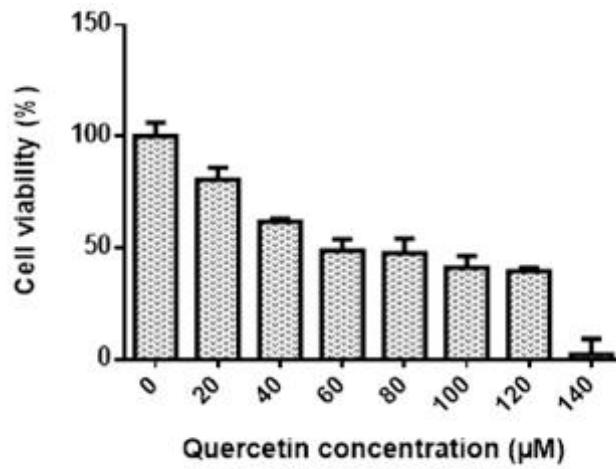
Figure 3.1 The cell viability graph of HEK-293 treated with quercetin ranging from 0 to 140 µM. Data presented as mean ± SD, n ≥3.

Y = -28.71*X + 101.2
$R^2 = 0,9778$
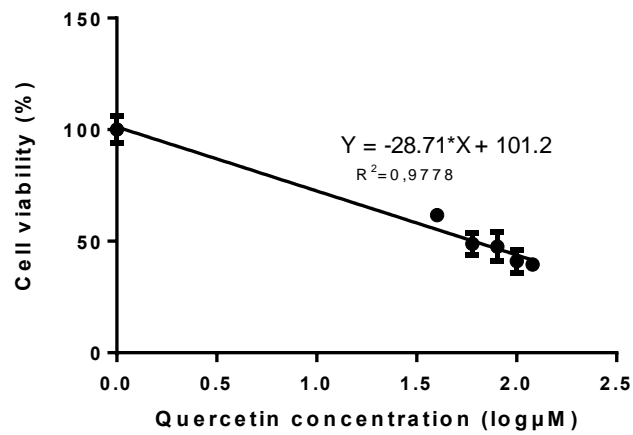
Figure 3.2 The cell viability curve of HEK-293 treated with quercetin. Data presented as mean ± SD, n ≥3.

3.1.1.2 IC50 Determination of Resveratrol Treated HEK293 Cells

The effect of resveratrol on viability of HEK-293 cells was observed with Alamar Blue assay. Percent survival values of resveratrol treated HEK293 cells are given in Table 3.2 and Figure 3.3. Results showed that resveratrol treatment ranging from 0 to 90 µM inhibited the proliferation of HEK-293 cells in a concentration dependent manner. Values reflecting the cell number are expressed as percentages of the control cells (vehicle DMSO control) and represented as mean ± standard deviation (SD). In order to determine IC50 value, quercetin concentrations were converted to their log value and cell viability graph was drawn (Figure 3.4). According to this analysis, IC50 value of resveratrol was estimated to be 34,36 µM for HEK293 cells.

Table 3.2 Percent survival of HEK293 cells with resveratrol treatment ranging from 0 to 90 µM.

Resveratrol Conc. (µM)	Percent Survival
0	100,00 ± 6,77
15	80,32 ± 3,98
30	47,29 ± 2,62
45	41,23 ± 3,64
60	39,06 ± 2,28
75	36,79 ± 3,76
90	14,48 ± 2,58
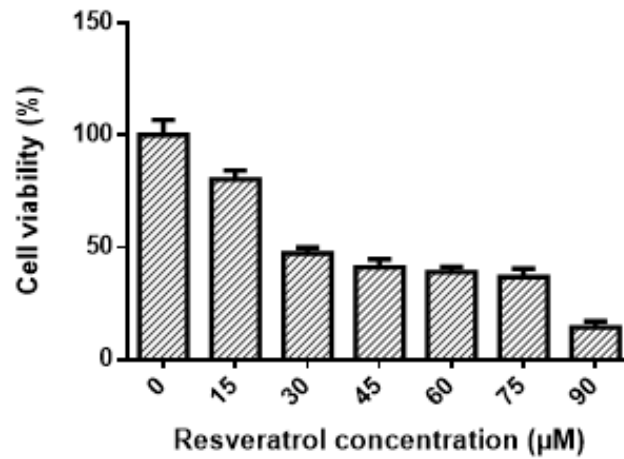
Figure 3.3   The cell viability graph of HEK-293 treated with resveratrol ranging from 0 to 90 µM. Data presented as mean ± SD, n ≥3.

$$Y = -35.74*X + 104.9$$
$$R^2 = 0.8906$$
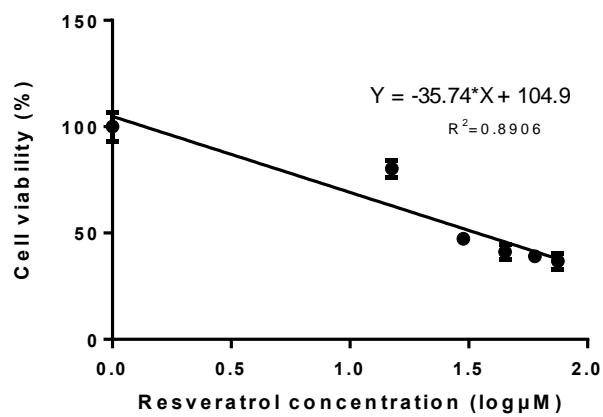
Figure 3.4   The cell viability curve of HEK-293 treated with resveratrol. Data presented as mean ± SD, n ≥3

3.1.1.3 IC50 Determination of Quercetin Treated HUH-7 Cells

The effect of quercetin on viability of HUH-7 cells was observed using Alamar Blue assay. Percent survival values of quercetin treated HUH-7 cells are given in Table 3.3 and Figure 3.5. Results showed that quercetin treatment ranging from 0 to 250 µM inhibited the proliferation of HUH-7 cells in a concentration dependent manner. Values reflecting the cell number are expressed as percentages of the control cells (vehicle DMSO control) and represented as mean ± standard deviation (SD). In order to determine IC50 value, cell viability graph was drawn (Figure 3.6). According to this analysis, IC50 value of quercetin was estimated to be 185,31 µM for HUH-7 cells.

Table 3.3 Percent survival of HUH7 cells with quercetin treatment ranging from 0 to 250 µM.

Quercetin Conc. (µM)	Percent Survival
0	100,00 ± 0,77
50	92,77 ± 4,79
100	87,50 ± 2,45
125	88,37 ± 2,79
150	70,62 ± 7,51
200	33,31 ± 2,72
250	24,38 ± 1,93
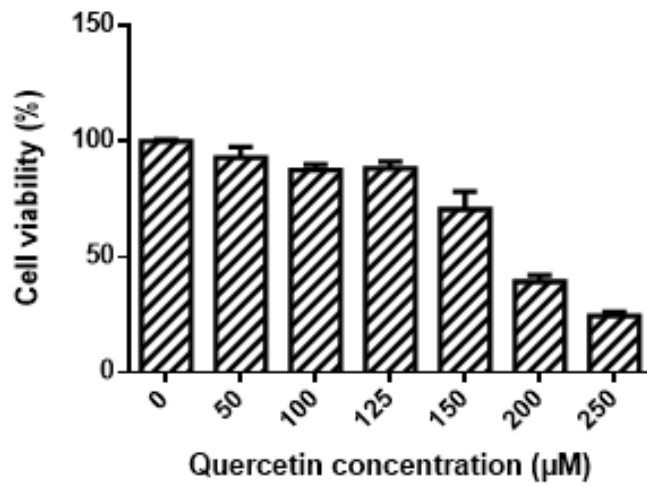
Figure 3.5   The cell viability graph of HUH-7 treated with quercetin ranging from 0 to 250 µM. Data presented as mean ± SD, n ≥3.

$$Y = -0.3173*X + 108.8$$

$$R^2 = 0,9289$$
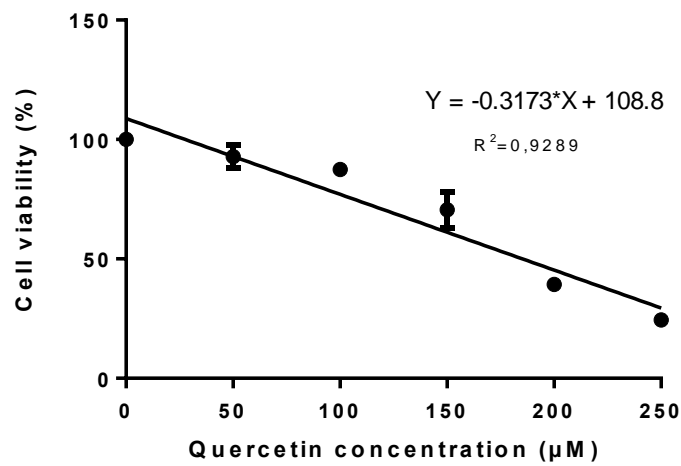
Figure 3.6   The cell viability curve of HUH-7 treated with quercetin. Data presented as mean ± SD, n ≥3.

3.1.1.4 IC50 Determination of Resveratrol Treated HUH-7 Cells

The effect of resveratrol on viability of HUH-7 cells was observed with Alamar Blue assay. Percent survival values of resveratrol treated HUH-7 cells are given in Table 3.4 and Figure 3.7. Results showed that resveratrol treatment ranging from 0 to 200 µM inhibited the proliferation of HUH-7 cells in a concentration dependent manner. Values reflecting the cell number are expressed as percentages of the control cells (vehicle DMSO control) and represented as mean ± standard deviation (SD). In order to determine IC50 value, cell viability graph was drawn (Figure 3.8). According to this analysis, IC50 value of resveratrol was estimated to be 131,93 µM for HUH-7 cells.

Table 3.4 Percent survival of HUH7 cells with resveratrol treatment ranging from 0 to 200 µM.

| Resveratrol Conc. (µM)	Percent Survival
0	100,00 ± 3,72
50	85,61 ± 8,66
100	62,65 ± 9,00
125	52,60 ± 7,01
150	43,62 ± 0,78
175	37,91 ± 3,56
200	17,15 ± 3,79
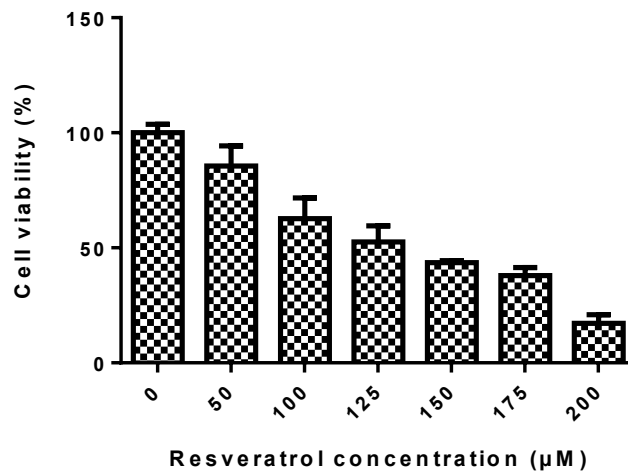
Figure 3.7   The cell viability graph of HUH-7 treated with resveratrol
ranging from 0 to 200 μM. Data presented as mean ± SD, n ≥3.
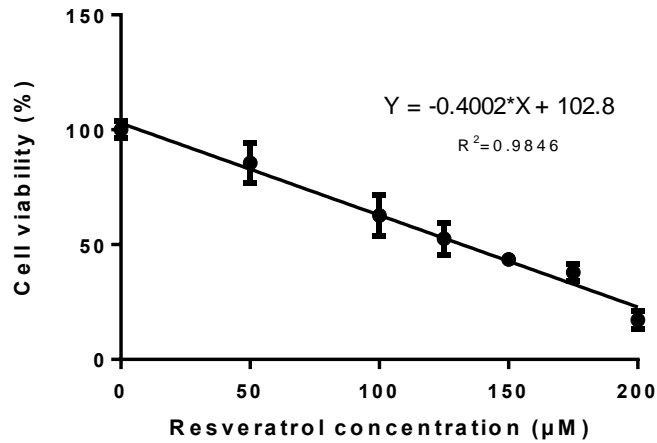
Figure 3.8   The cell viability curve of HUH-7 treated with resveratrol.
Data presented as mean ± SD, n ≥3.

3.1.1.5 Morphology of HEK293 and HUH7 Cells Treated with Quercetin and Resveratrol

HEK-293 and HUH-7 cells were treated with quercetin and resveratrol using IC50 concentrations determined in this study as given in Table 3.5. Morphological changes of HEK-293 and HUH-7 cells observed upon treatment with quercetin and resveratrol were shown in Figure 3.9 and Figure 3.10, respectively. Results showed that morphology of cells was changed depending on the type polyphenol used for treatment.

Table 3.5 IC50 values of quercetin and resveratrol for HEK293 and HUH7 cells.

	Quercetin	Resveratrol
HEK-293	60,72 µM	34,36 µM
HUH-7	185,31 µM	131,93 µM
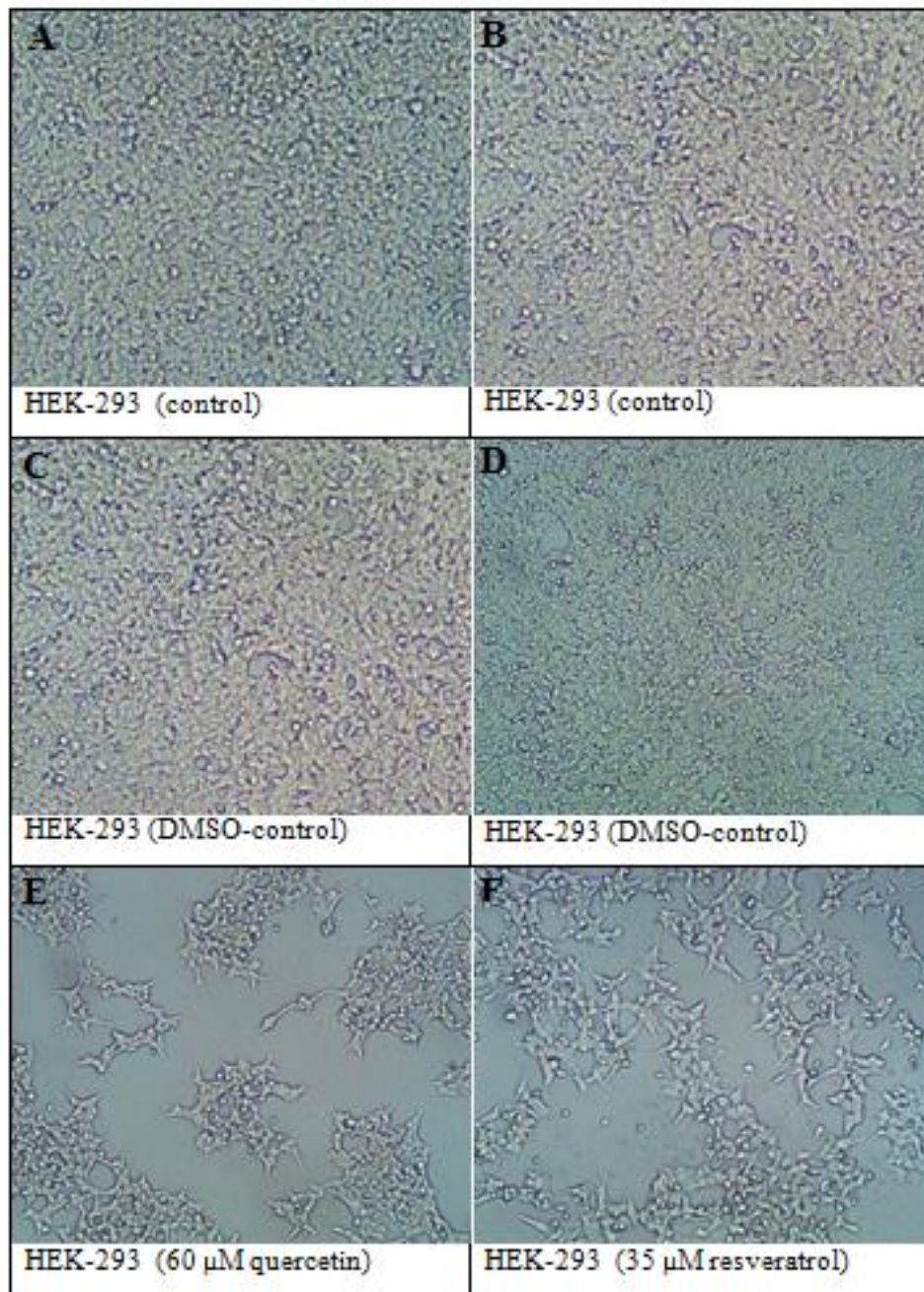
Figure 3.9 Effects of quercetin (60 µM) and resveratrol (35 µM) on cell morphology of HEK-293. Control cells (A, B); DMSO-Control cells (C, D); HEK-293 cells treated with 60 µM quercetin (E) or 35 µM resveratrol (F) for 48 hours. Images were observed under a BestScope inverted biological microscope (magnification: x10).
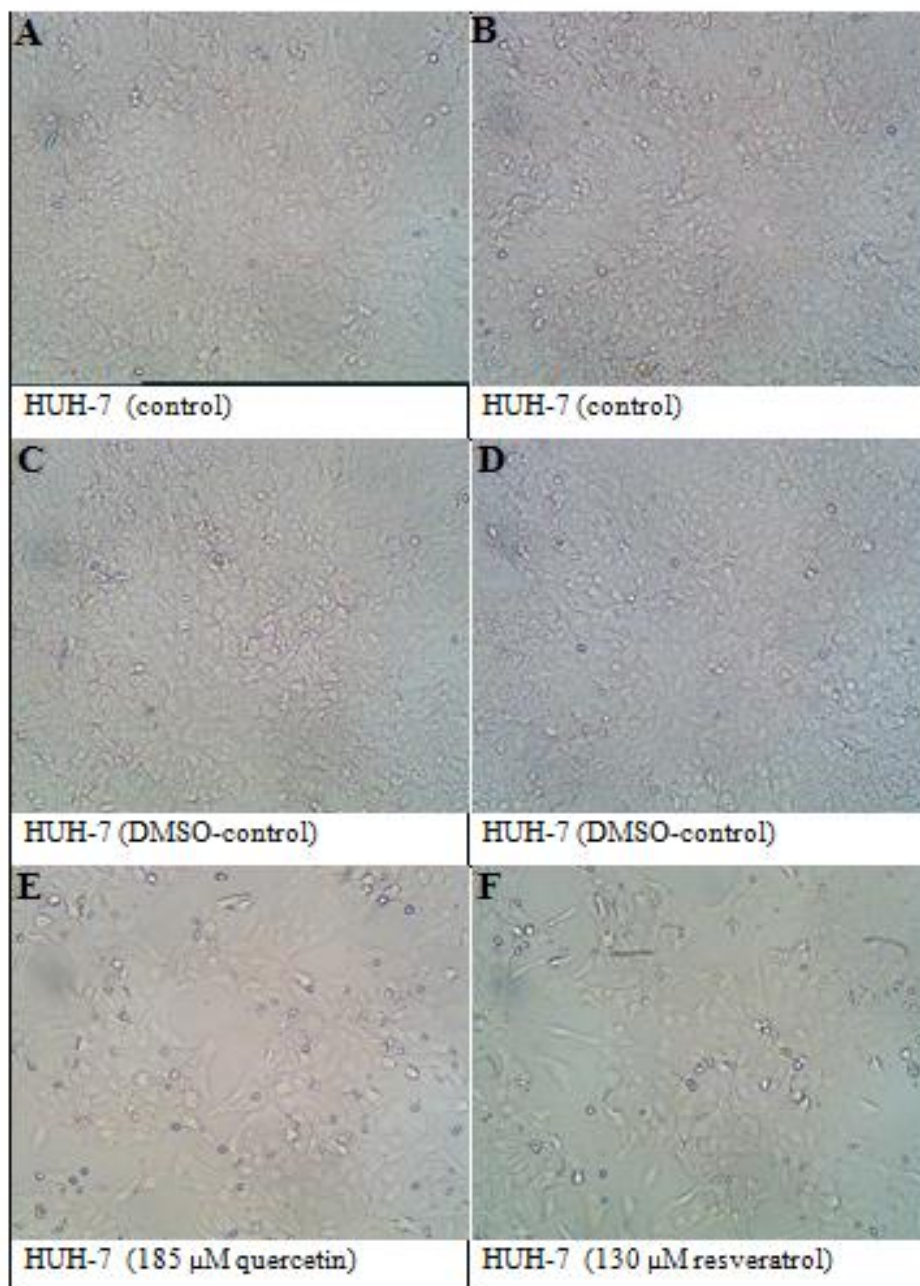
Figure 3.10 Effects of quercetin (185 µM) and resveratrol (130 µM) on cell morphology of HUH-7. Control cells (A,B); DMSO-Control cells (C,D); HUH-7 cells treated with 185 µM quercetin (E) or 130 µM resveratrol (F) for 48 hours. Images were observed under a BestScope inverted biological microscope (BS2090) (magnification: x10).

3.2 Effects of Quercetin and Resveratrol on Protein Expression of Vitamin D Metabolizing CYPs in HEK-293 and HUH-7 cell lines

The effects of quercetin and resveratrol on protein expression of CYP2R1, CYP27A1, CYP27B1, and CYP24A1 in HEK-293 and HUH-7 cell lines were determined by Western Blotting. Western blot experiments were carried out on total cellular extracts of the control, vehicle-DMSO control and polyphenol-treated HEK-293 and HUH-7 cells. β-actin (42 kDa) was used as internal standard. The intensity of each band was analyzed by Image J software program. Unpaired, two-tailed student's t-test was used for statistical analyze. p<0.05 was chosen as level for significance.

3.2.1 Effect of Quercetin on Protein Expression of Vitamin D Metabolizing CYPs in HEK293 Cells

3.2.1.1 CYP2R1 Protein Expression in Control and Quercetin Treated HEK293 Cells

Immunostaining results of CYP2R1 and β-actin proteins from control and quercetin treated HEK293 cells are given in Figure 3.11. Figure 3.12 shows the relative protein expressions. Quercetin treatment decreased the relative protein expression of CYP2R1 in HEK-293 significantly with respect to DMSO control (*P≤0.05).

61

Figure 3.11 Immunoreactive protein bands of control, DMSO control and quercetin treated HEK-293 cells representing CYP2R1(~57kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: quercetin treated HEK-293 cells.

Figure 3.12 Comparison of CYP2R1 protein expression of control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05; **P≤0.01.

3.2.1.2 CYP27A1 Protein Expression in Control and Quercetin Treated HEK293 Cells

Immunostaining results of CYP27A1 and β-actin proteins from control and quercetin treated HEK-293 cells are given in Figure 3.13. Figure 3.14 shows the relative protein expressions. Quercetin treatment decreased the relative protein expression of CYP27A1 in HEK-293 significantly with respect to DMSO control (***P≤0.001).

Figure 3.13 Immunoreactive protein bands of control, DMSO control and quercetin treated HEK-293 cells representing CYP27A1 (~60kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: quercetin treated HEK-293 cells.

Figure 3.14 Comparison of CYP27A1 protein expression of control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05; ***P≤0.001.

3.2.1.3 CYP27B1 Protein Expression in Control and Quercetin Treated HEK293 Cells

Immunostaining results of CYP27B1 and β-actin proteins from control and quercetin treated HEK-293 cells are given in Figure 3.15. Figure 3.16 shows the relative protein. Quercetin treatment did not cause a significant change in the relative protein expression of CYP27B1 in HEK-293 with respect to DMSO control.

Figure 3.15 Immunoreactive protein bands of control, DMSO control and quercetin treated HEK-293 cells representing CYP27B1 (~57 kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: quercetin treated HEK-293 cells.

Figure 3.16 Comparison of CYP27B1 protein expression of control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05.

3.2.1.4 CYP24A1 Protein Expression in Control and Quercetin Treated HEK293 Cells

Immunostaining results of CYP24A1 and β-actin proteins from control and quercetin treated HEK-293 cells are given in Figure 3.17. Figure 3.18 shows the relative protein. Quercetin treatment increased the relative protein expression of CYP24A1 in HEK-293 significantly with respect to DMSO control (*P≤0.05).
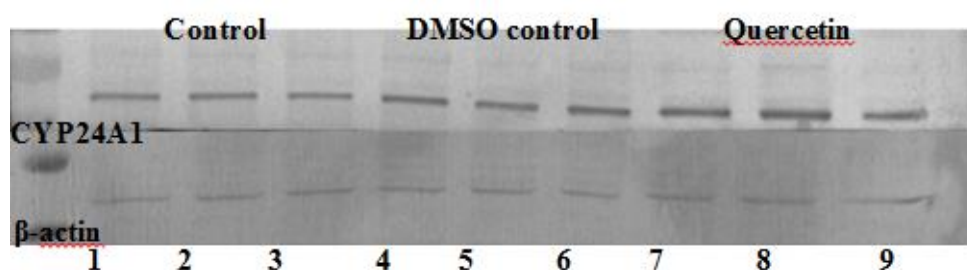
Figure 3.17   Immunoreactive protein bands of control, DMSO control and quercetin treated HEK-293 cells representing CYP24A1 (~59 kDa). β-actin (42 kDa) was used as internal standard.   Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: quercetin treated HEK-293 cells.

Figure 3.18 Comparison of CYP24A1 protein expression of control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05; **P≤0.01.

3.2.2 Effect of Resveratrol on Protein Expression of Vitamin D Metabolizing CYPs in HEK293 Cells

3.2.2.1 CYP2R1 Protein Expression in Control and Resveratrol Treated HEK293 Cells

Immunostaining results of CYP2R1 and β-actin proteins from control and resveratrol treated HEK-293 cells are given in Figure 3.19. Figure 3.20 shows the relative protein expressions. Resveratrol treatment did not cause a significant change in the relative protein expression of CYP2R1 in HEK-293 with respect to DMSO control.
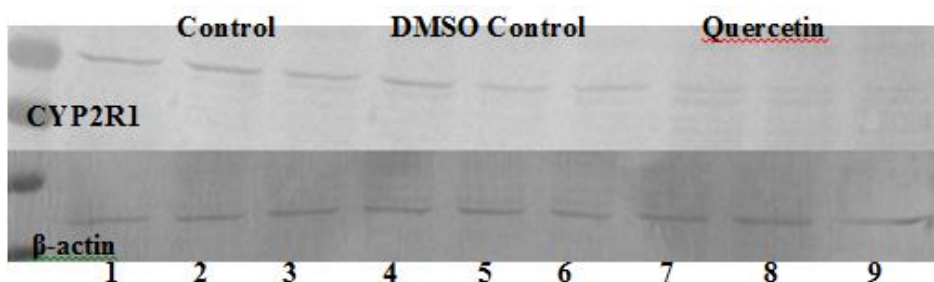

Figure 3.19 Immunoreactive protein bands of control, DMSO control and resveratrol treated HEK-293 cells representing CYP2R1 (~57kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: resveratrol treated HEK-293 cells.
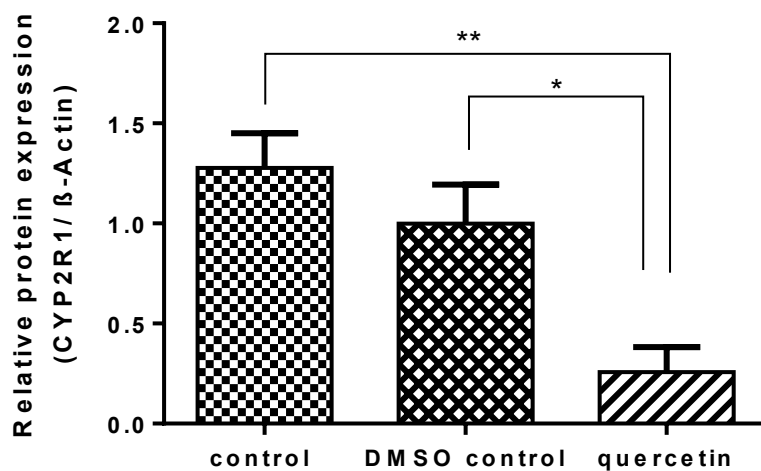
68

Figure 3.20 Comparison of CYP2R1 protein expression of control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. The data was statistically analyzed by unpaired, two-tailed student's t-test. *P≤0.05.

3.2.2.2 CYP27A1 Protein Expression in Control and Resveratrol Treated HEK293 Cells

Immunostaining results of CYP27A1 and β-actin proteins from control and resveratrol treated HEK-293 cells are given in Figure 3.21. Figure 3.22 shows the relative protein expressions. Resveratrol treatment decreased the relative protein expression of CYP27A1 in HEK-293 significantly with respect to DMSO control (* P≤0.05).
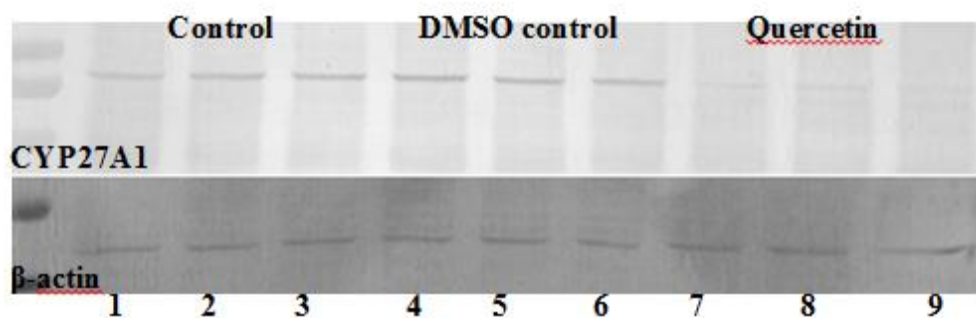
Figure 3.21 Immunoreactive protein bands of control, DMSO control and resveratrol treated HEK-293 cells representing CYP27A1 (~60kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: resveratrol treated HEK-293 cells.

Figure 3.22 Comparison of CYP27A1 protein expression of control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05.

70

3.2.2.3 CYP27B1 Protein Expression in Control and Resveratrol Treated HEK293 Cells

Immunostaining results of CYP27B1 and β-actin proteins from control and resveratrol treated HEK-293 cells are given in Figure 3.23. Figure 3.24 shows the relative protein expressions. Resveratrol treatment caused a decrease in the relative protein expression of CYP27B1 in HEK-293 significantly with respect to DMSO control (* P≤0.05).

Figure 3.23 Immunoreactive protein bands of control, DMSO control and resveratrol treated HEK-293 cells representing CYP27B1 (~57 kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: resveratrol treated HEK-293 cells.
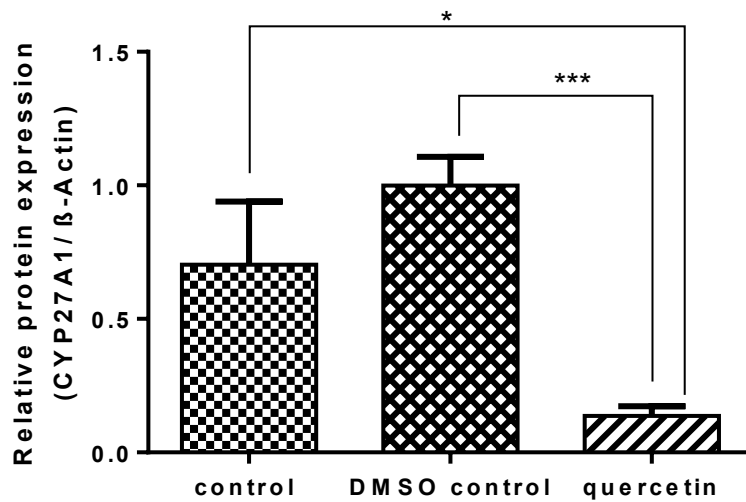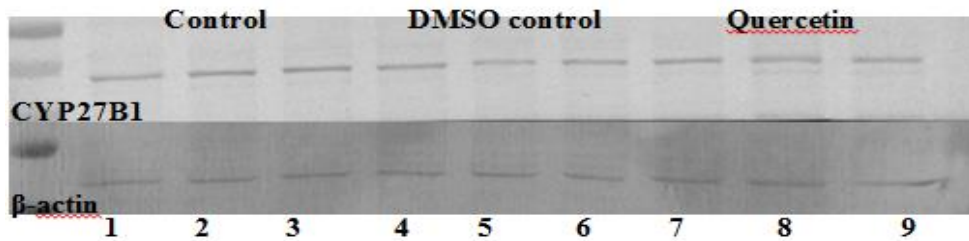
Figure 3.24 Comparison of CYP27B1 protein expression of control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05.

3.2.2.4 CYP24A1 Protein Expression in Control and Resveratrol Treated HEK293 Cells

Immunostaining results of CYP24A1 and β-actin proteins from control and resveratrol treated HEK-293 cells are given in Figure 3.25. Figure 3.26 shows the relative protein expressions. Resveratrol treatment increased the relative protein expression of CYP24A1 in HEK-293 significantly with respect to DMSO control (**P≤0.01).

Figure 3.25 Immunoreactive protein bands of control, DMSO control and resveratrol treated HEK-293 cells representing CYP24A1 (~59 kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: resveratrol treated HEK-293 cells.
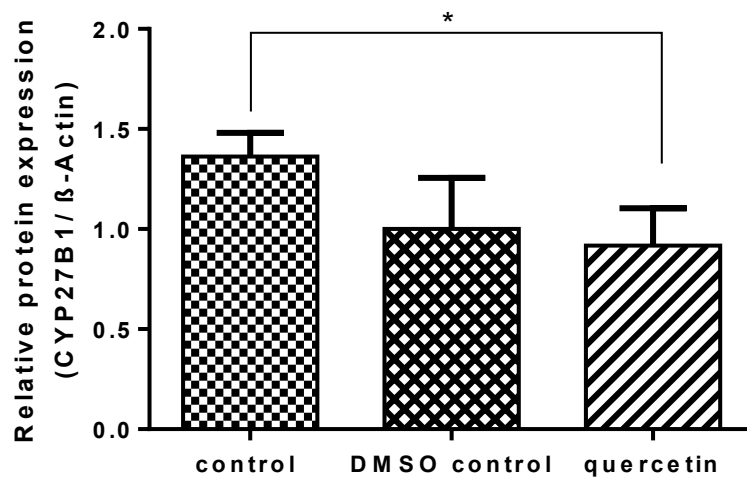

Figure 3.26 Comparison of CYP24A1 protein expression of control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05; **P≤0.01.

3.2.3 Effect of Quercetin on Protein Expression of Vitamin D Metabolizing CYPs in HUH-7 Cells
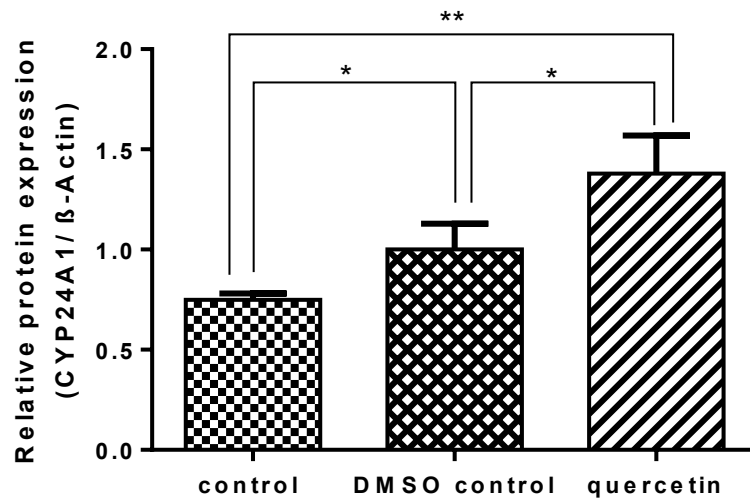
3.2.3.1 CYP2R1 Protein Expression in Control and Quercetin Treated HUH-7 Cells

Figure 3.27 shows the immunostaining results of CYP2R1 protein. The CYP2R1 expression was not observed in control, DMSO control and resveratrol treated HUH-7 cells.
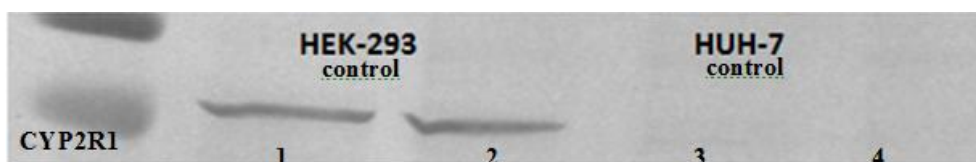
Figure 3.27 Immunoreactive protein bands of HEK-293 and HUH-7 cells representing CYP2R1(~57kDa). Lane 1-2: HEK-293 Control, Lane 3-4: HUH-7 Control.

3.2.3.2 CYP27A1 Protein Expression in Control and Quercetin Treated HUH-7 Cells

Figure 3.28 shows the immunostaining results of CYP27A1 protein. The CYP27A1 expression was not observed in control, DMSO control and resveratrol treated HUH-7 cells.
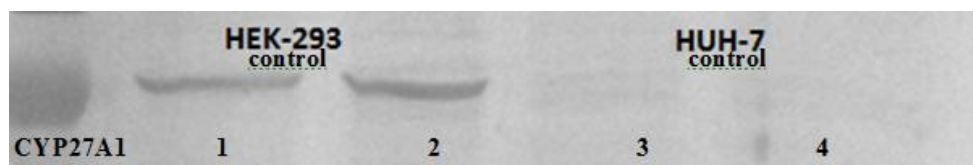
Figure 3.28 Immunoreactive protein bands of HEK-293 and HUH-7 cells representing CYP27A1(~60kDa). Lane 1-2: HEK-293 Control, Lane 3-4: HUH-7 Control.

3.2.3.3 CYP27B1 Protein Expression in Control and Quercetin Treated HUH-7 Cells

Immunostaining results of CYP27B1 and β-actin proteins from control and quercetin treated HUH-7 cells are given in Figure 3.29. Figure 3.30 shows the relative protein expressions. Quercetin treatment did not cause a significant change in the relative protein expression of CYP27B1 in HUH-7 with respect to DMSO control.
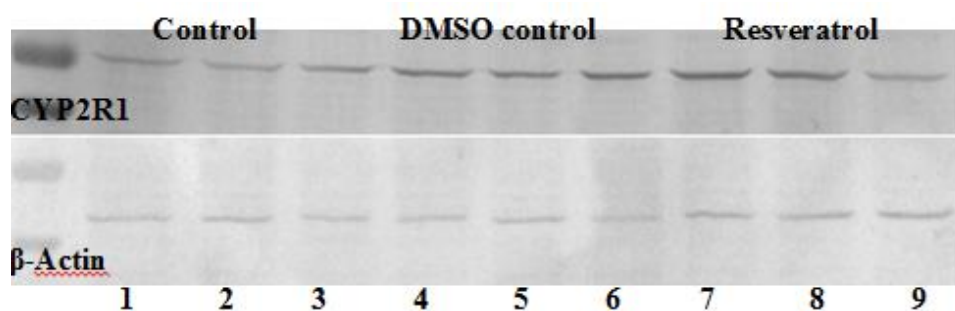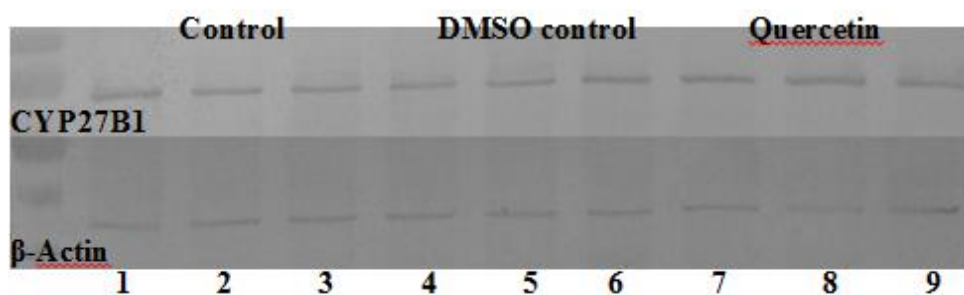

Figure 3.29 Immunoreactive protein bands of control, DMSO control and quercetin treated HUH-7 cells representing CYP27B1 (~57kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: quercetin treated HUH-7 cells.
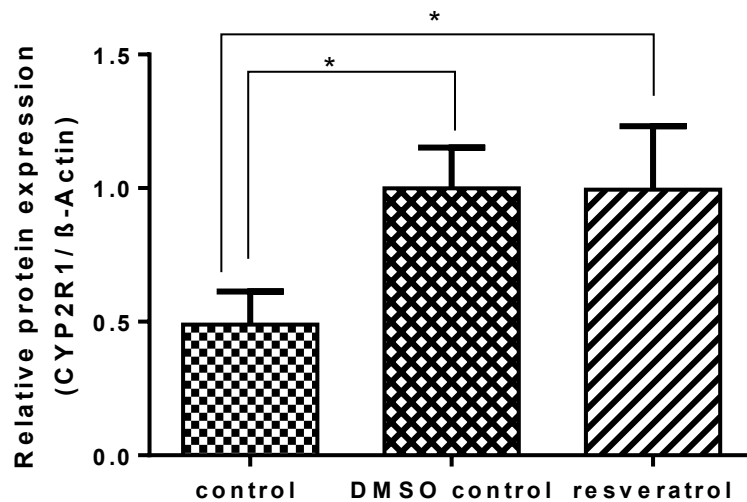
Figure 3.30 Comparison of CYP27B1 protein expression of control, DMSO control and quercetin treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments.

3.2.3.4 CYP24A1 Protein Expression in Control and Quercetin Treated HUH-7 Cells

Immunostaining results of CYP24A1 and β-actin proteins from control and quercetin treated HUH-7 cells are given in Figure 3.31. Figure 3.32 shows the relative protein expressions. Quercetin treatment was not led to significant change in the relative protein expression of CYP24A1 in HUH-7 with respect to DMSO control.
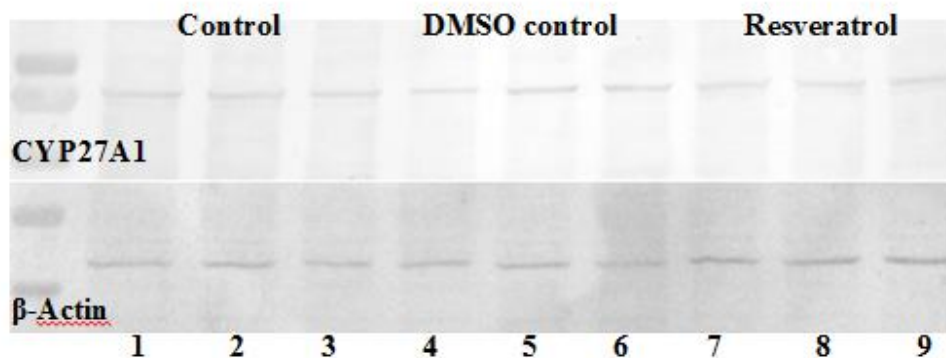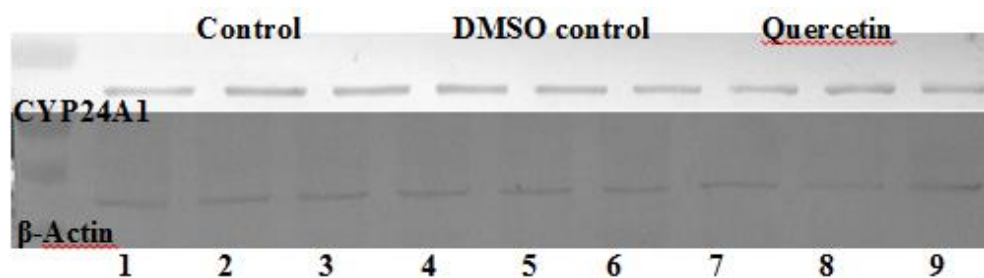
Figure 3.31 Immunoreactive protein bands of control, DMSO control and quercetin treated HUH-7 cells representing CYP24A1 (~59 kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: quercetin treated HUH-7 cells.
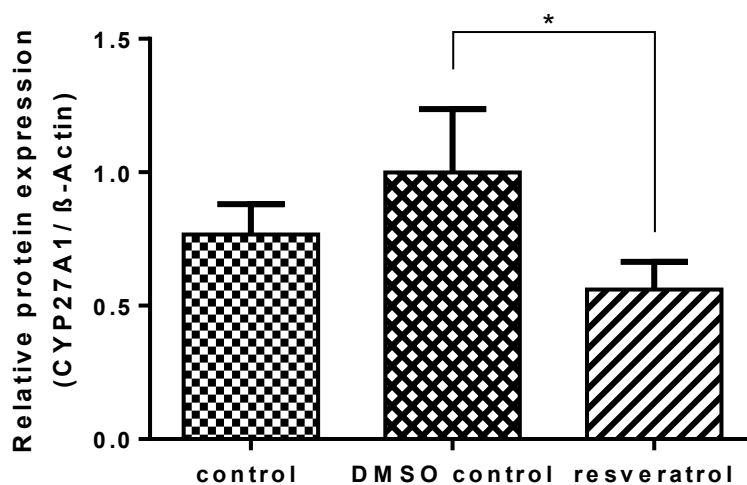

Figure 3.32 Comparison of CYP24A1 protein expression of control, DMSO control and quercetin treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. ** P≤0.01.

3.2.4 Effect of Resveratrol on Protein Expression of Vitamin D Metabolizing CYPs in HUH-7 Cells

3.2.4.1 CYP2R1 Protein Expression in Control and Resveratrol Treated HUH-7 Cells

Figure 3.33 shows the immunostaining results of CYP2R1 protein. The CYP2R1 expression was not observed in control, DMSO control and resveratrol treated HUH-7 cells.
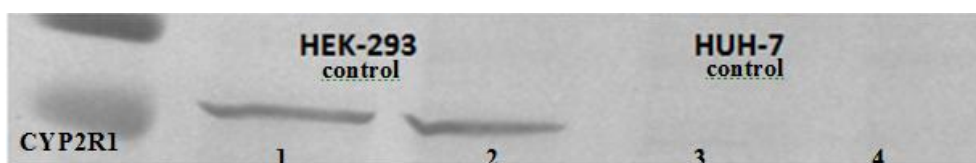
Figure 3.33 Immunoreactive protein bands of HEK-293 and HUH-7 cells representing CYP2R1(~57kDa). Lane 1-2: HEK-293 Control, Lane 3-4: HUH-7 Control.

3.2.4.2 CYP27A1 Protein Expression in Control and Resveratrol Treated HUH-7 Cells

Figure 3.34 shows the immunostaining results of CYP27A1 protein. The CYP27A1 expression was not observed in control, DMSO control and resveratrol treated HUH-7 cells.
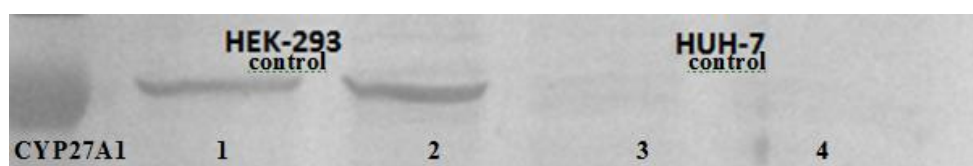
Figure 3.34 Immunoreactive protein bands of HEK-293 and HUH-7 cells representing CYP27A1(~60kDa). Lane 1-2: HEK-293 Control, Lane 3-4: HUH-7 Control.

3.2.4.3 CYP27B1 Protein Expression in Control and Resveratrol Treated HUH-7 Cells

Immunostaining results of CYP27B1 and β-actin proteins from control and resveratrol treated HUH-7 cells are given in Figure 3.35.. Figure 3.36 shows the relative protein expressions. Resveratrol treatment did not cause a significant change in the relative protein expression of CYP27B1 in HUH-7 with respect to DMSO control.
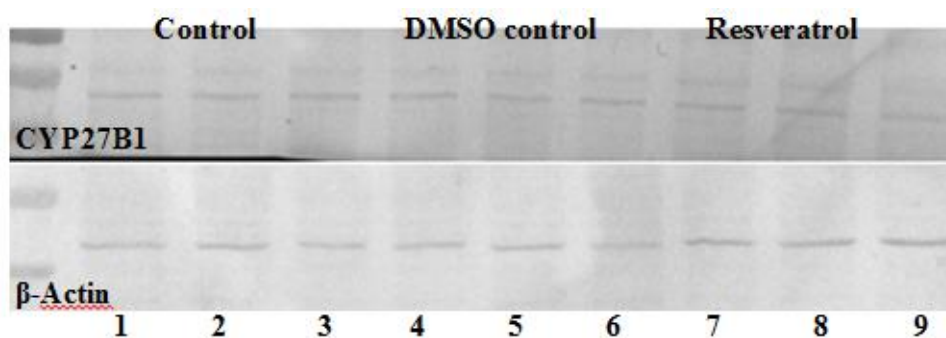

Figure 3.35 Immunoreactive protein bands of control, DMSO control and resveratrol treated HUH-7 cells representing CYP27B1 (~57kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: resveratrol treated HUH-7 cells.
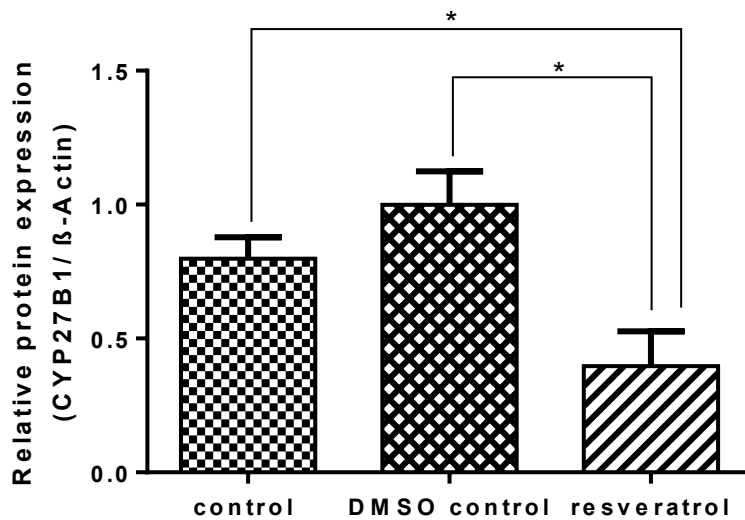
Figure 30.36 Comparison of CYP27B1 protein expression of control, DMSO control and resveratrol treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments.

3.2.4.4 CYP24A1 Protein Expression in Control and Resveratrol Treated HUH-7 Cells

Immunostaining results of CYP24A1 and β-actin proteins from control and resveratrol treated HUH-7 cells are given in Figure 3.37. Figure 3.38 shows the relative protein expressions. Resveratrol treatment caused a decrease in the relative protein expression of CYP24A1 in HUH-7 significantly with respect to DMSO control (****P≤0,0001).
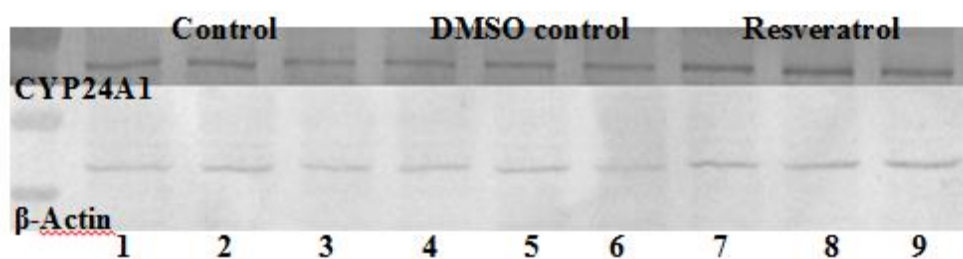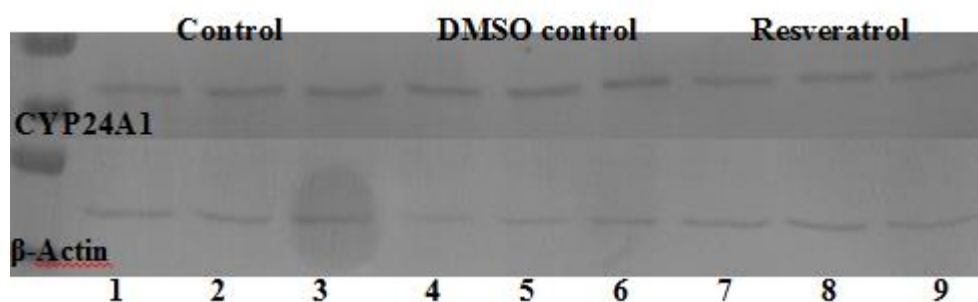
Figure 3.37   Immunoreactive protein bands of control, DMSO control and resveratrol treated HUH-7 cells representing CYP24A1 (~59 kDa). β-actin (42 kDa) was used as internal standard. Lane 1-3: Control, Lane 4-6: vehicle-DMSO control, Lane 7-9: resveratrol treated HUH-7 cells.
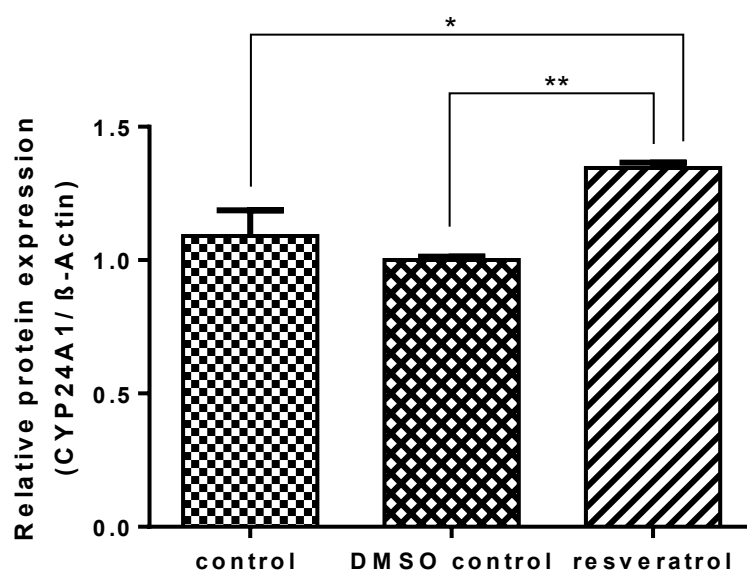

Figure 3.38   Comparison of CYP24A1 protein expression of control, DMSO control and resveratrol treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. ** $P \leq 0.01$; ****$P \leq 0,0001$.

3.3 The Effects of Quercetin and Resveratrol on mRNA Expression of Vitamin D Metabolizing CYPs in HEK-293 and HUH-7 cell lines

The effects of quercetin and resveratrol on the mRNA expression of CYP2R1, CYP27A1, CYP27B1, and CYP24A1 in HEK-293 and HUH-7 cell lines were determined using qRT-PCR. RPL13A was used as the internal control and standard curve was generated by using diluted cDNAs from HEK-293 or HUH-7 control cells.

Amplification and melting curves of CYP2R1, CYP27A1, CYP27B1, and CYP24A1 genes were given in Figure 3.39 and Figure 3.40, respectively. Livak method was applied to determine relative mRNA expressions. The Table 3.6 shows the formulation for Livak ($2^{-\Delta\Delta ct}$) method. For reference, Ct values of vehicle DMSO control was selected.

The data was statistically analyzed by student t-test and $p<0.05$ was chosen as level for significance.
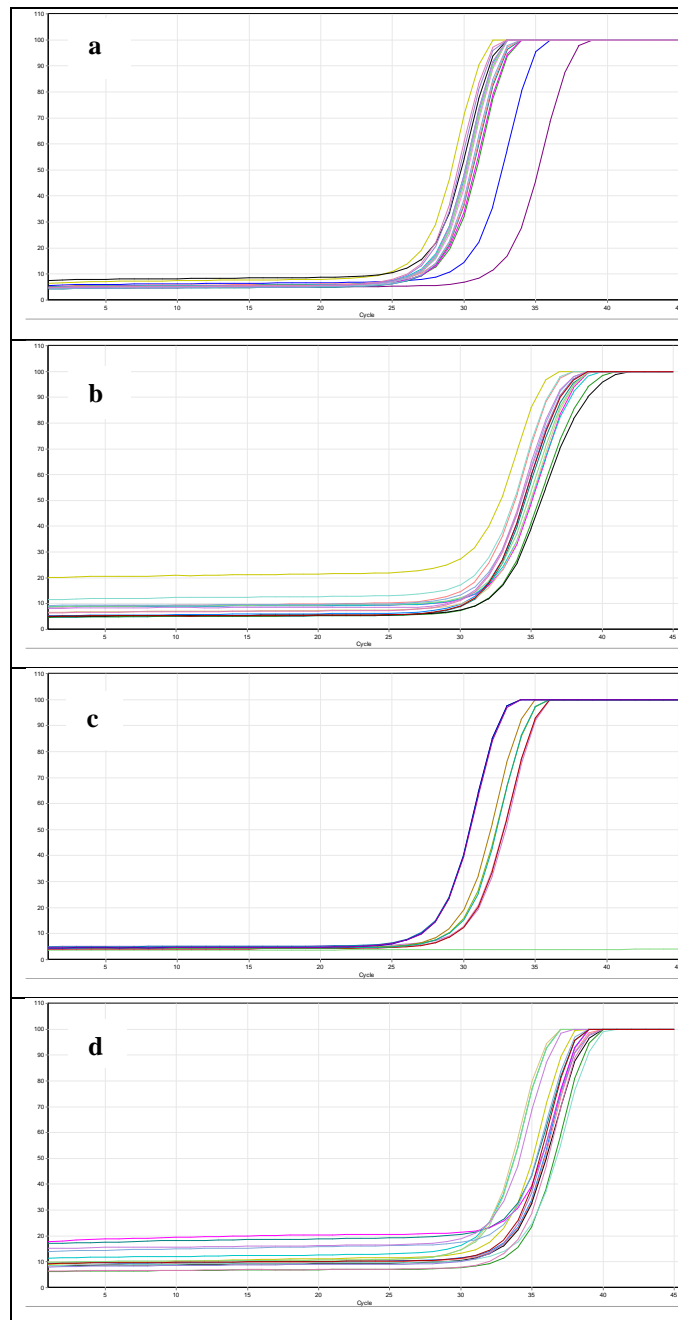
Figure 3.39 Amplification curves of CYP2R1 (a), CYP27A1 (b), CYP27B1 (c) and CYP24A1 (d).
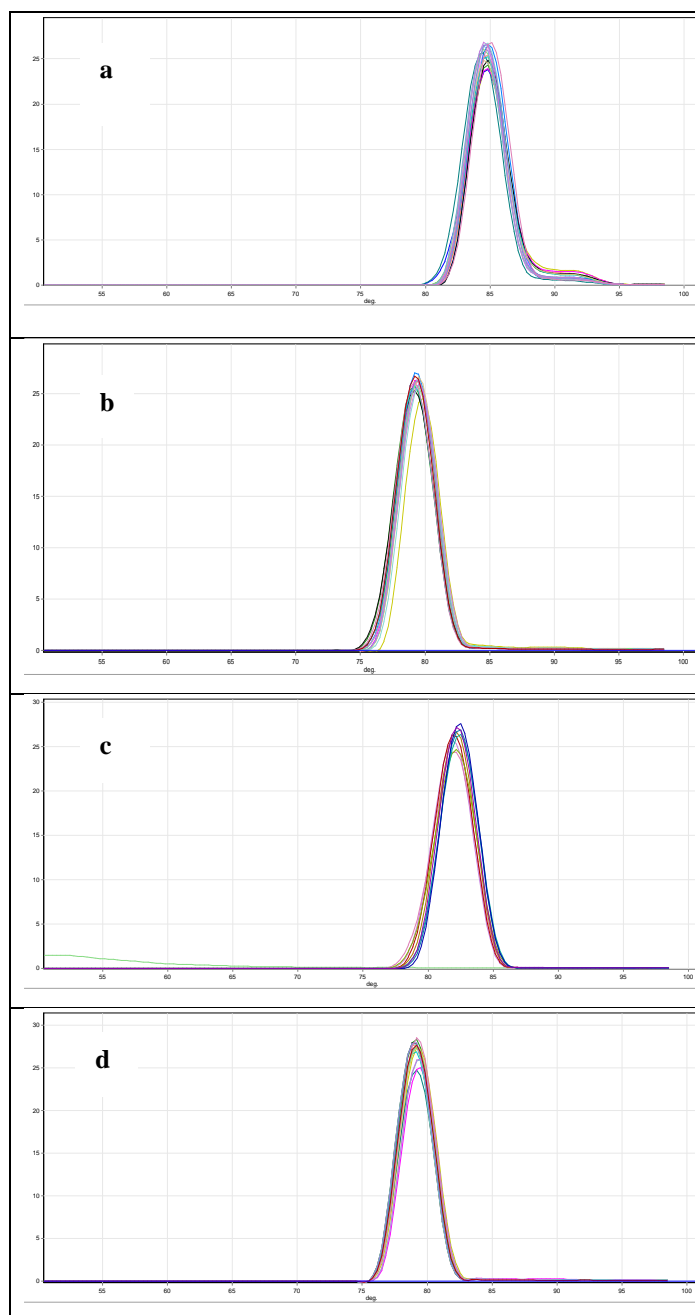
Figure 3. 40 Melting curves of CYP2R1 (a), CYP27A1 (b), CYP27B1 (c), and CYP24A1 (d).

Table 3.6   The Livak method for the calculation of relative mRNA expression.

	Control	DMSO Control	Treated
$\text{Ct}_{\text{CYP2R1}}$	21,16	21,42	20,89
$\text{Ct}_{\text{bactin}}$	14,80	15,17	15,14
$\Delta\text{Ct}_{\text{Cells}} =$	$\text{Ct}_{\text{RPL13A}}$	$- \text{Ct}_{\text{CYP27A1}}$	
$\Delta\text{Ct}_{\text{Reference}} =$	$\text{Ct}_{\text{RPL13A}}$	$- \text{Ct}_{\text{CYP27A1}}$	
$\Delta\text{Ct}_{\text{Cells}} =$	-6,36	-6,25	-5,76
$\Delta\Delta\text{Ct} =$	0,11	0	-0,49
$2^{-\Delta\Delta\text{Ct}}$	0,93	1	1,41

3.3.1 Effects of Quercetin on mRNA Expression of Vitamin D Metabolizing CYPs in HEK293 Cells

3.3.1.1 Effect of Quercetin on mRNA Expression of CYP2R1 in HEK293 Cells

Figure 3.41 shows the mean ± SD of the relative CYP2R1 mRNA expressions of control, vehicle DMSO-control and quercetin treated HEK-293 cells. Quercetin treatment increased the relative mRNA expression of CYP2R1 in HEK-293 cells significantly with respect to DMSO control (*P≤0.05).

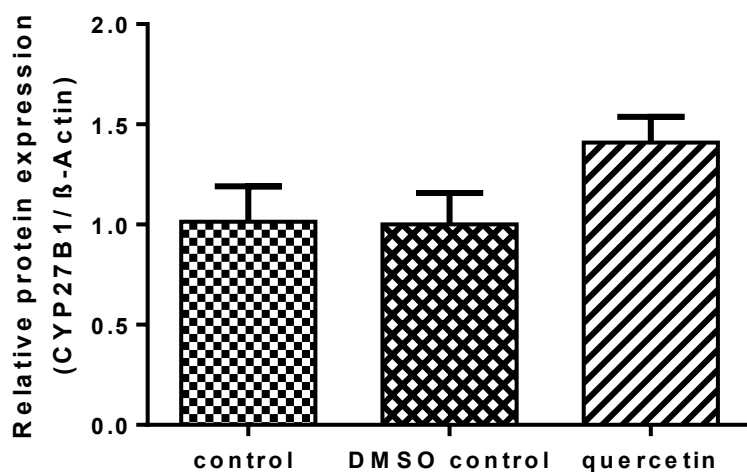**Figure 3.41** Comparison of CYP2R1 mRNA expressions between control, DMSO-control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05; ** P≤0.01.

3.3.1.2 Effect of Quercetin on mRNA Expression of CYP27A1 in HEK293 Cells

Figure 3.42 shows the mean ± SD of the relative CYP27A1 mRNA expressions of control, vehicle DMSO-control and quercetin treated HEK-293 cells. Quercetin treatment increased the relative mRNA expression of CYP27A1 in HEK-293 significantly with respect to DMSO control (*P≤0.05).

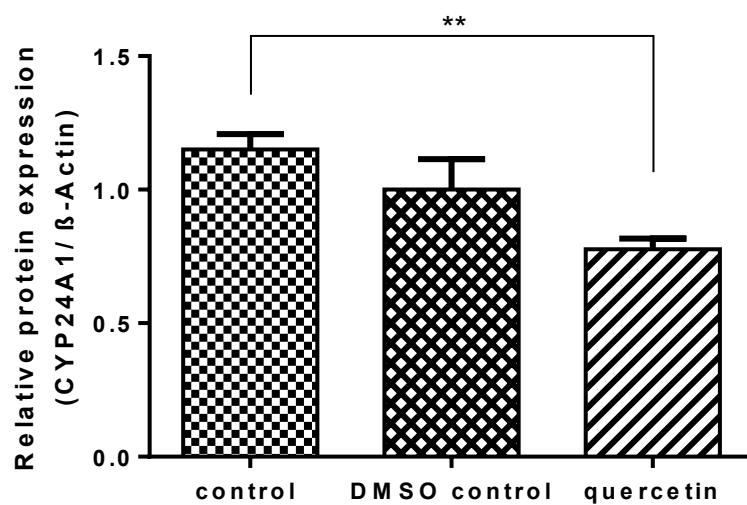**Figure 3.42** Comparison of CYP27A1 mRNA expressions between control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05; ** P≤0.01.

3.3.1.3 Effect of Quercetin on mRNA Expression of CYP27B1 in HEK293 Cells

Figure 3.43 shows the mean ± SD of the relative CYP27B1 mRNA expressions of control, vehicle DMSO-control and quercetin treated HEK-293 cells. Quercetin treatment was not led to significant change in the relative mRNA expression of CYP27B1 in HEK-293 with respect to DMSO control.

Figure 3.43 Comparison of CYP27B1 mRNA expressions between control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments.

3.3.1.4 Effect of Quercetin on mRNA Expression of CYP24A1 in HEK293 Cells

Figure 3.44 shows the mean ± SD of the relative CYP24A1 mRNA expressions of control, vehicle DMSO-control and quercetin treated HEK-293 cells. Quercetin treatment was not led to significant change in the relative mRNA expression of CYP24A1 in HEK-293 with respect to DMSO control.

Figure 3.44 Comparison of CYP24A1 mRNA expressions between control, DMSO control and quercetin treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments.

3.3.2 Effects of Resveratrol on mRNA Expression of Vitamin D Metabolizing CYPs in HEK293 Cells

3.3.2.1 Effect of Resveratrol on mRNA Expression of CYP2R1 in HEK293 Cells

Figure 3.45 shows the mean ± SD of the relative CYP2R1 mRNA expressions of control, vehicle DMSO-control and resveratrol treated HEK-293 cells. Resveratrol treatment was not led to significant change in the relative mRNA expression of CYP2R1 in HEK-293 with respect to DMSO control.
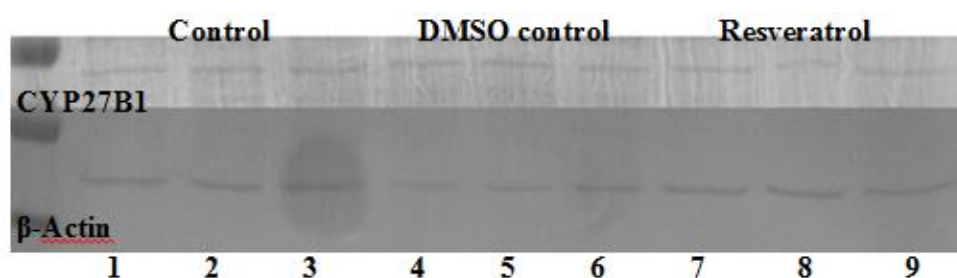
Figure 3.45 Comparison of CYP2R1 mRNA expressions between control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05.

3.3.2.2 Effect of Resveratrol on mRNA Expression of CYP27A1 in HEK293 Cells

Figure 3.46 shows the mean ± SD of the relative CYP27A1 mRNA expressions of control, vehicle DMSO-control and resveratrol treated HEK-293 cells. Resveratrol treatment increased the relative mRNA expression of CYP27A1 in HEK-293 significantly with respect to DMSO control (*P≤0.05).
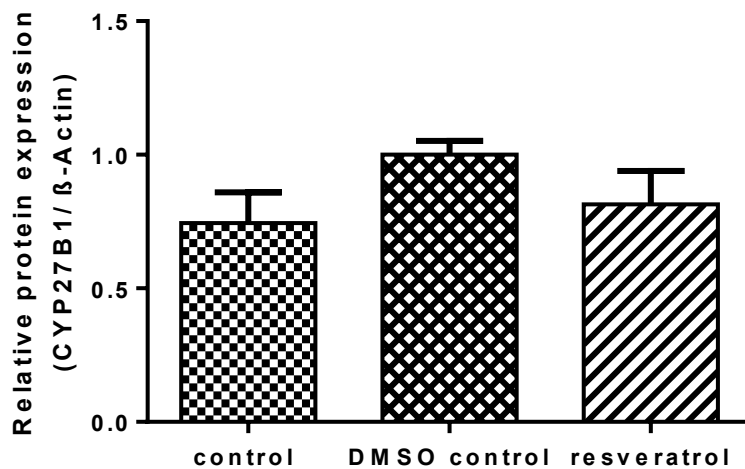
Figure 3.46 Comparison of CYP27A1 mRNA expressions between control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. *P≤0.05; ** P≤0.01.

3.3.2.3 Effect of Resveratrol on mRNA Expression of CYP27B1 in HEK293 Cells

Figure 3.47 shows the mean ± SD of the relative CYP27B1 mRNA expressions of control, vehicle DMSO-control and resveratrol treated HEK-293 cells. Resveratrol treatment increased the relative mRNA expression of CYP27B1 in HEK-293 significantly with respect to DMSO control (**P≤0.01).
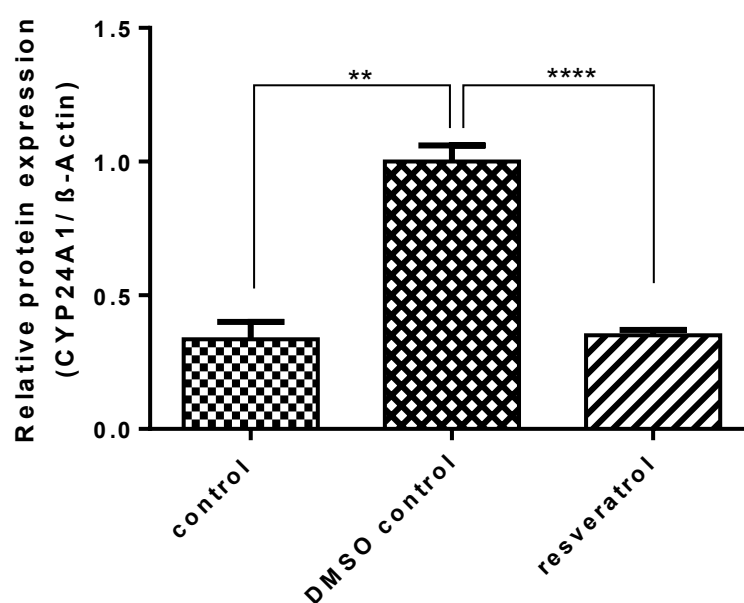
Figure 3.47 Comparison of CYP27B1 mRNA expressions between control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. ** P≤0.01.

3.3.2.4 Effect of Resveratrol on mRNA Expression of CYP24A1 in HEK293 Cells

Figure 3.48 shows the mean ± SD of the relative CYP24A1 mRNA expressions of control, vehicle DMSO-control and resveratrol treated HEK-293 cells. Resveratrol treatment increased the relative mRNA expression of CYP24A1 in HEK-293 significantly with respect to DMSO control (**P≤0.01).
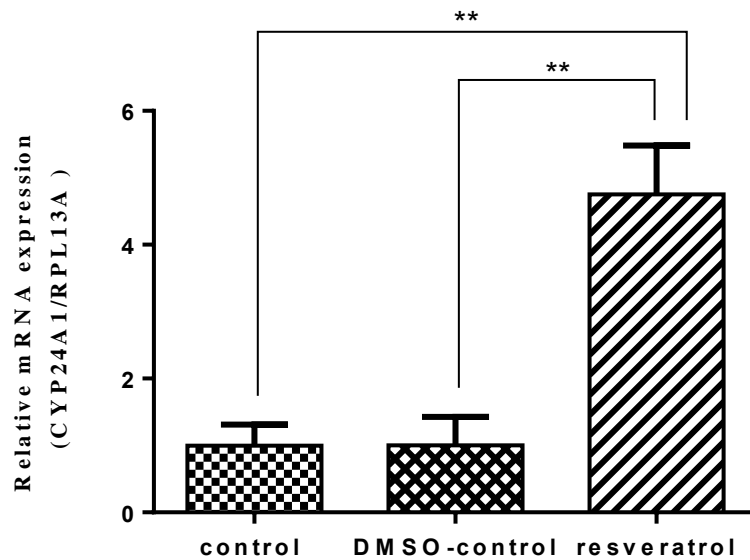
Figure 3.48 Comparison of CYP24A1 mRNA expressions between control, DMSO control and resveratrol treated HEK-293 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. ** P≤0.01.

3.3.3 Effects of Quercetin on mRNA Expression of Vitamin D Metabolizing CYPs in HUH-7 Cells

3.3.3.1 Effect of Quercetin on mRNA Expression of CYP2R1 in HUH7 Cells

Figure 3.49 shows the mean ± SD of the relative CYP2R1 mRNA expressions of control, vehicle DMSO- control and quercetin treated HUH-7 cells. Quercetin treatment increased the relative mRNA expression of CYP2R1 in HUH-7 significantly with respect to DMSO control (* P≤0.05).

93
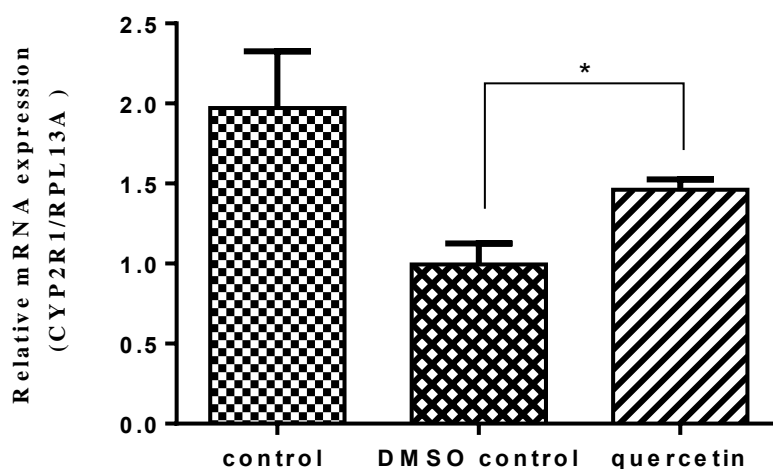
Figure 30.49 Comparison of CYP2R1 mRNA expressions between control, DMSO control and quercetin treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05.

3.3.3.2 Effect of Quercetin on mRNA Expression of CYP27A1 in HUH7 Cells

Figure 3.50 shows the mean ± SD of the relative CYP27A1 mRNA expressions of control, vehicle DMSO- control and quercetin treated HUH-7 cells. Quercetin treatment was not led to significant change in the relative mRNA expression of CYP27A1 in HUH-7 with respect to DMSO control.
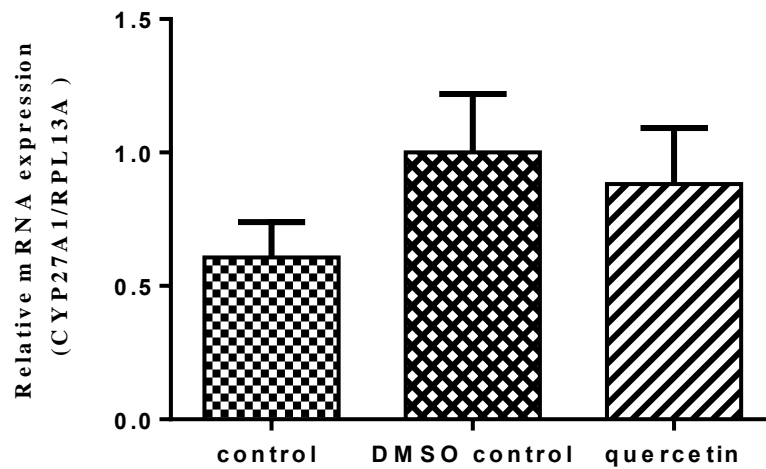
Figure 3.50 Comparison of CYP27A1 mRNA expressions between control, DMSO control and quercetin treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments.

3.3.3.3 Effect of Quercetin on mRNA Expression of CYP27B1 in HUH7 Cells

Figure 3.51 shows the mean ± SD of the relative CYP27B1 mRNA expressions of control, vehicle DMSO-control and quercetin treated HUH-7 cells. Quercetin treatment increased the relative mRNA expression of CYP27B1 in HUH-7 significantly with respect to DMSO control (**$P \leq 0.01$).
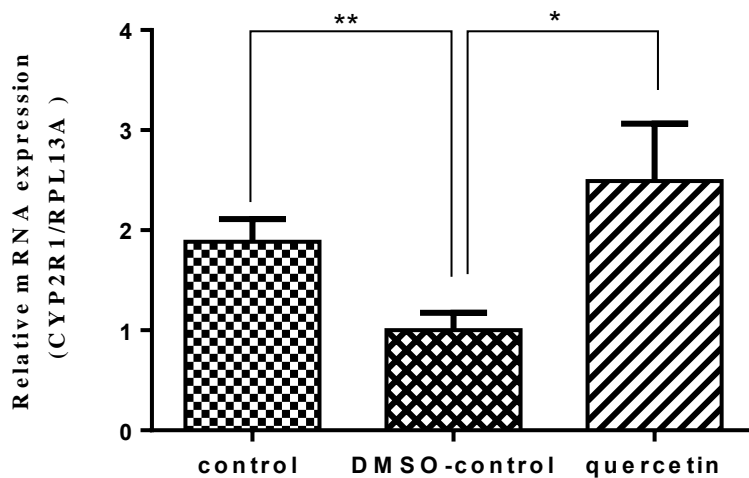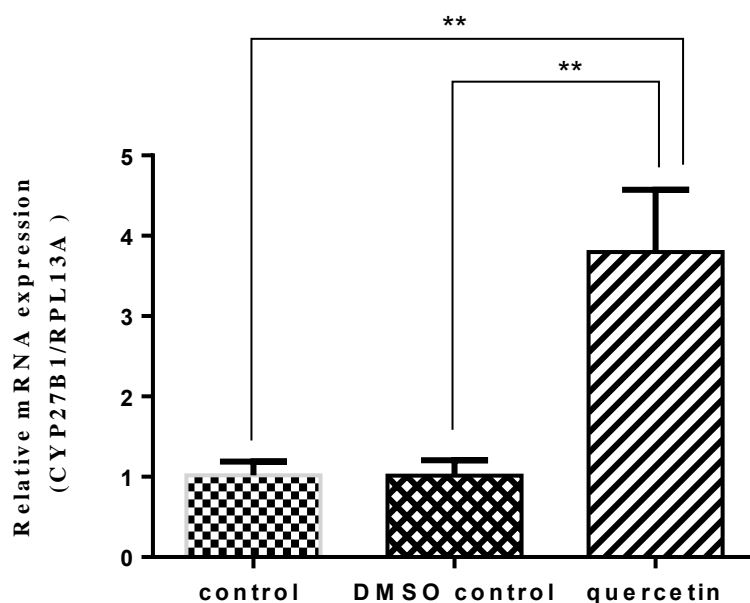
Figure 3.51 Comparison of CYP27B1 mRNA expressions between control, DMSO control and quercetin treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. **P≤0.01.

3.3.3.4 Effect of Quercetin on mRNA Expression of CYP24A1 in HUH7 Cells

Figure 3.52 shows the mean ± SD of the relative CYP24A1 mRNA expressions of control, vehicle DMSO-control and quercetin treated HUH-7 cells. Quercetin treatment increased the relative mRNA expression of CYP24A1 in HUH-7 significantly with respect to DMSO control(* P≤0.05).
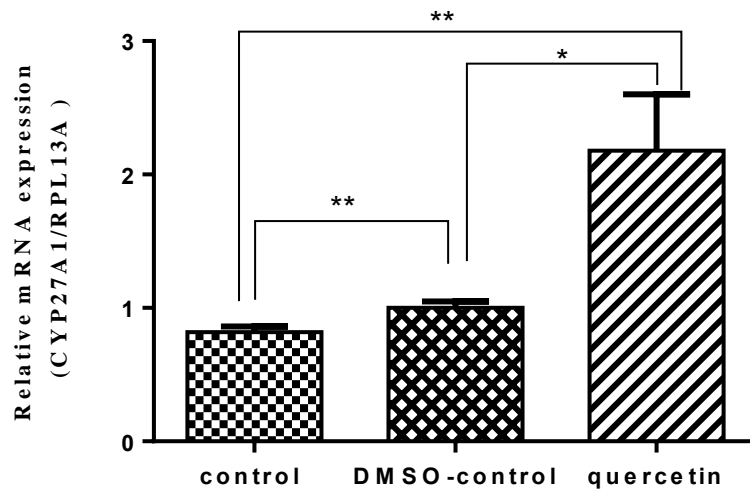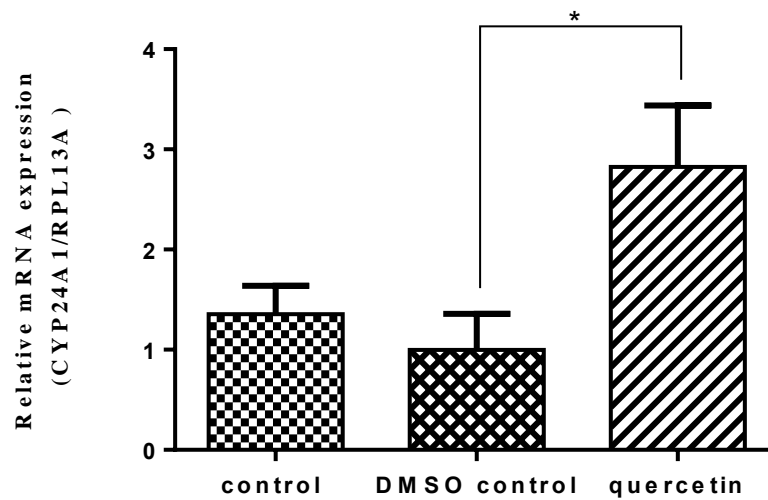
Figure 3.52 Comparison of CYP24A1 mRNA expressions between control, DMSO control and quercetin treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments.
* P≤0.05.

3.3.4 Effects of Resveratrol on mRNA Expression of Vitamin D Metabolizing CYPs in HUH-7 Cells

3.3.4.1 Effect of Resveratrol on mRNA Expression of CYP2R1 in HUH7 Cells

Figure 3.53 shows the mean ± SD of the relative CYP2R1 mRNA expressions of control, vehicle DMSO-control and resveratrol treated HUH-7 cells. Resveratrol treatment increased the relative mRNA expression of CYP2R1 in HUH-7 significantly with respect to DMSO control (**P≤0.01).
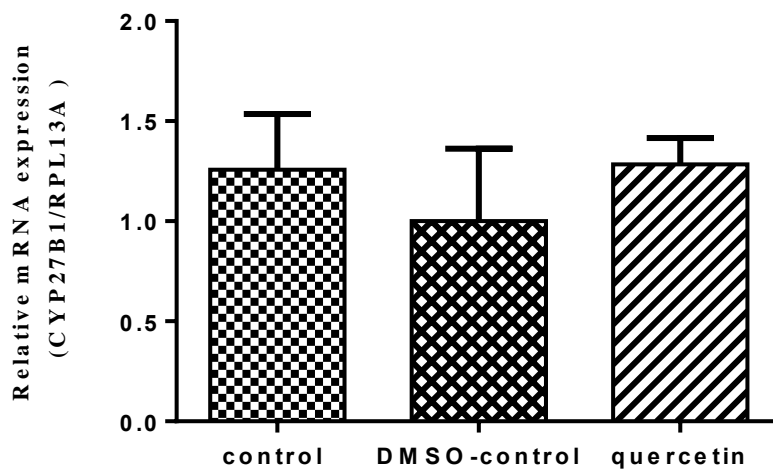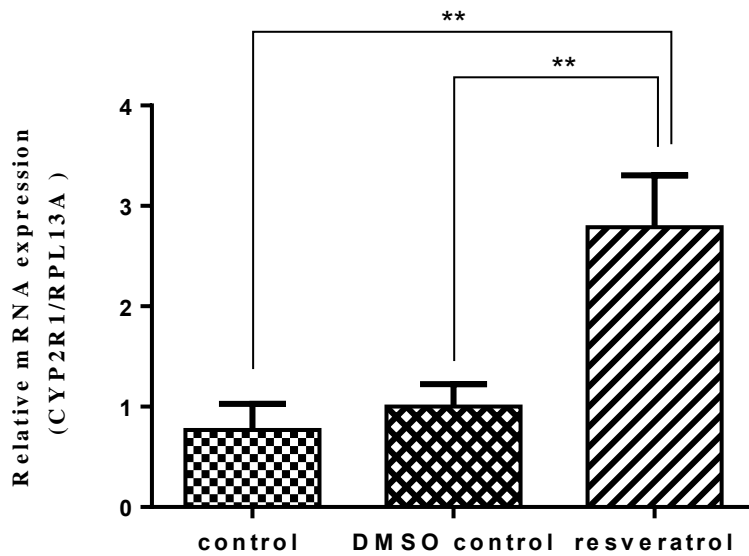
Figure 3.53 Comparison of CYP2R1 mRNA expressions between control, DMSO control and resveratrol treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. **P≤0.01.

3.3.4.2 Effect of Resveratrol on mRNA Expression of CYP27A1 in HUH-7 Cells

Figure 3.54 shows the mean ± SD of the relative CYP27A1 mRNA expressions of control, vehicle DMSO- control and resveratrol treated HUH-7 cells. Resveratrol treatment decreased the relative mRNA expression of CYP27A1 in HUH-7 significantly with respect to DMSO control (* P≤0.05).
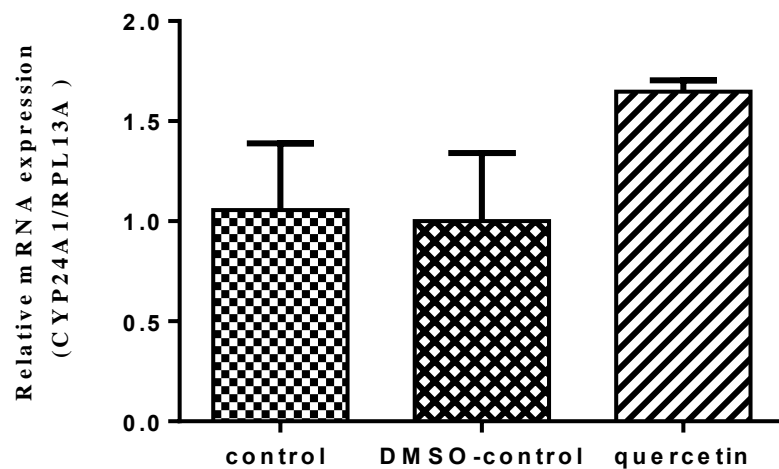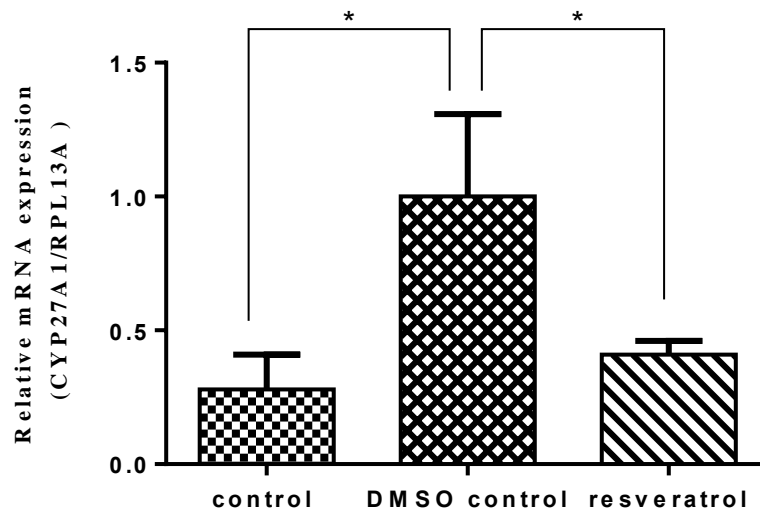
Figure 3.54 Comparison of CYP27A1 mRNA expressions between control, DMSO control and resveratrol treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05.

3.3.4.3 Effect of Resveratrol on mRNA Expression of CYP27B1 in HUH7 Cells

Figure 3.55 shows the mean ± SD of the relative CYP27B1 mRNA expressions of control, vehicle DMSO-control and resveratrol treated HUH-7 cells. Resveratrol treatment decreased the relative mRNA expression of CYP27B1 in HUH-7 significantly with respect to DMSO control (**P≤0.01).
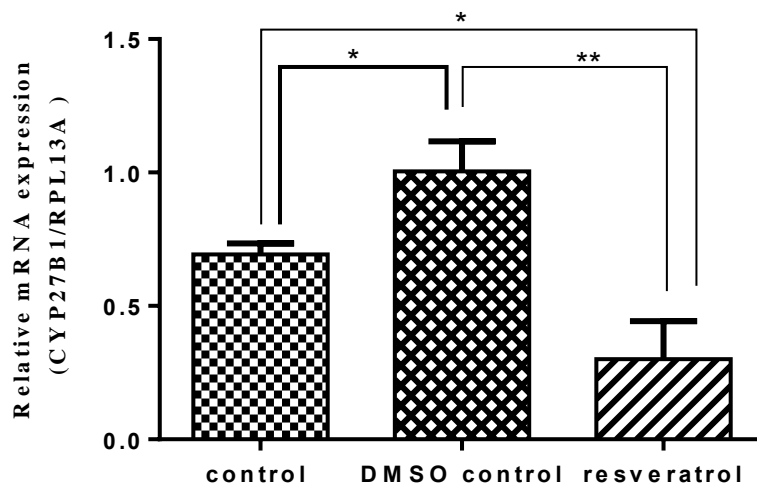
Figure 3.55 Comparison of CYP27B1 mRNA expressions between control, DMSO control and resveratrol treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05; ** P≤0.01.

3.3.4.4 Effect of Resveratrol on mRNA Expression of CYP24A1 in HUH7 Cells

Figure 3.56 shows the mean ± SD of the relative CYP24A1 mRNA expressions of control, vehicle DMSO- control and resveratrol treated HUH-7 cells. Resveratrol treatment increased the relative mRNA expression of CYP24A1 in HUH-7 significantly with respect to DMSO control (**P≤0.01).
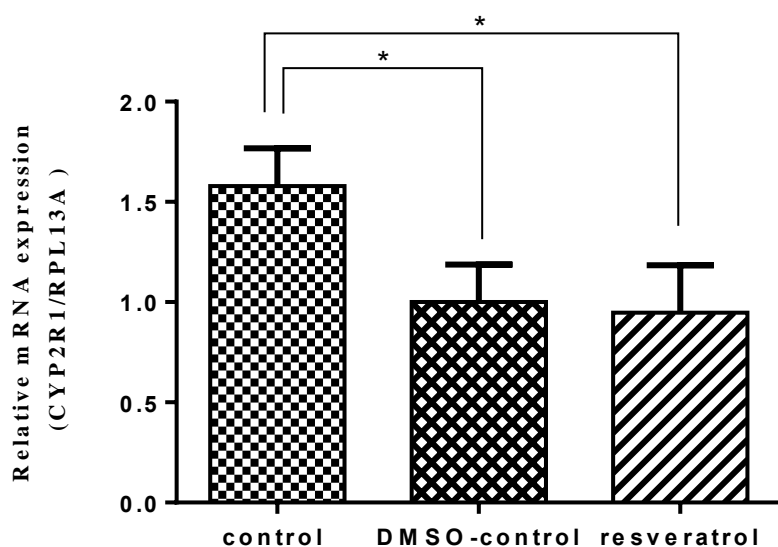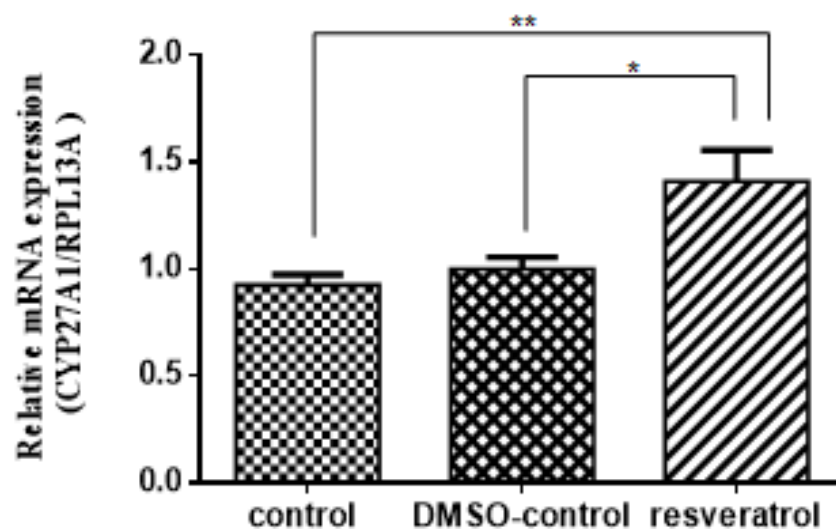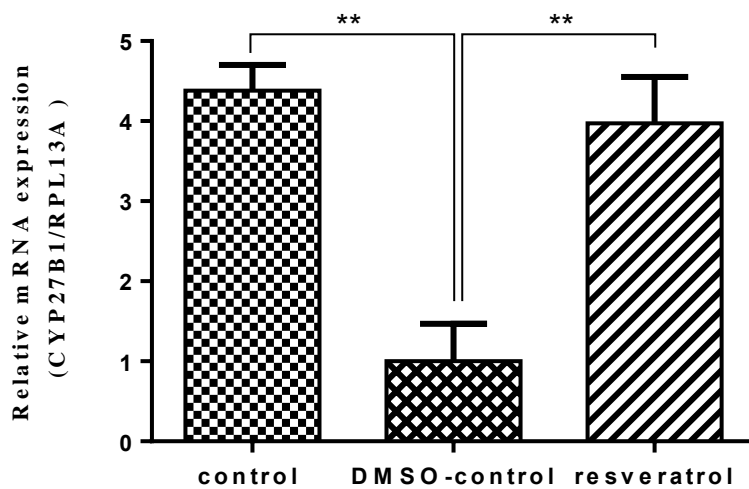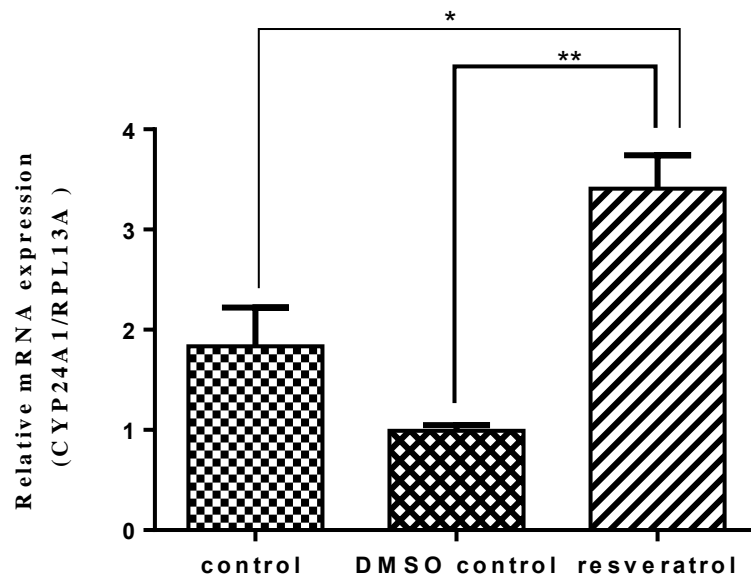
Figure 3.56 Comparison of CYP24A1 mRNA expressions between control, DMSO control and resveratrol treated HUH-7 cells. Error bars indicate ± SD. Graph represents data of three independent experiments. * P≤0.05; ** P≤0.01.

CHAPTER 4

DISCUSSION

Vitamin D is essential compound for life having role not only in the regulation of calcium metabolism but also in the regulation of cell proliferation, cell differentiation, immune responses, cardiovascular homeostasis and nervous system. However, vitamin D itself is devoid of biological activity. These wide range of actions occur with the enzymatic conversion of vitamin D to $1\alpha,25$-dihydroxyvitamin D $[1,25(OH)_2D]$. This metabolism is regulated by cytochrome P450 enzymes (CYPs). CYP2R1, CYP27A1, CYP27B1 and CYP24A1 are important enzymes having role in vitamin D metabolism. CYP2R1 and CYP27A1 are responsible for 25-hydroxylation of vitamin D. While CYP27B1 provides the synthesis of hormonally active form of vitamin D, $1,25(OH)_2D$, CYP24A1 involves in catabolism of vitamin D to prevent excessive synthesis of the hormone.

CYPs are known to be modulated by plant phenolic compounds, which are found mainly in our traditional diet. Among these phenolic compounds, quercetin and resveratrol are widely studied ones due to their abundant consumption in a diet, important roles in human health as activators or inhibitors for biochemical reaction, phytoestrogenic features, antioxidant and anticancer activities. Up to know, there are many studies about the impact of quercetin or resveratrol on some of the CYP450 enzymes. However, these studies do not contain vitamin D metabolizing CYPs.

Recently, there are few studies about interaction between VDR and these phenolic compounds. It has been reported that both quercetin and resveratrol results in VDR activation (Inoue, 2010; Lee *et al*., 2016; Dampf Stone *et al*., 2015). In addition to VDR activation, resveratrol and quercetin have been reported as having beneficial effects on cardiovascular system, cholesterol metabolism and glucose homeostasis overlapping with the effects of high amount of vitamin D concentration. Taken together, these bring forward an idea that quercetin and resveratrol can have an impact on the regulation of vitamin D and its target genes. In that reason, the effects of quercetin and resveratrol on protein and mRNA expressions of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 in HEK-293 and HUH-7 cell lines were investigated.

In this study, since vitamin D metabolizing enzymes are mainly expressed in kidney and liver, HEK-293 and HUH-7 cells were used. Moreover, trans-resveratrol was used since it is accepted as more biologically active than cis-configuration (Anisimova *et al.*, 2011; Stivala *et al.*, 2001; Mukherjee *et al.*, 2010). In order to determine treatment concentrations of quercetin and resveratrol in HEK-293 and HUH-7 cells, Alamar Blue assay was carried out. After 48 hours treatment, half maximal inhibitory concentrations (IC50) of quercetin were found as 60.72 µM and 185.31 µM for HEK-293 and HUH-7 cells, while IC50 values of resveratrol were determined as 34.36 µM and 131.93 µM for HEK-293 and HUH-7 cells, respectively. These results show that the same polyphenol has different IC50 values for two different cell lines. HEK-293 and HUH-7 cell lines belong to different tissues. Observing different IC50 values is expected result due to cell and tissue specific responses. Since, each cell line was originated from different person having unique biological characteristics, different IC50 values were reported for different cell lines treated with the same drug or compound in literature (Abdel-Latif *et al.,* 2015).

Both quercetin and resveratrol are limited solubility in water so dimethyl sulfoxide (DMSO) was used as solvent. DMSO concentrations used in these experiments were less than 1 % (v/v). While resveratrol was solved in 0.5 % DMSO, quercetin was solved in 0.35 % DMSO. It is known that DMSO has cytotoxic effects for cells at high concentration. Its toxic range changes according to the cell line. According to alamar blue assay, these concentrations used both in HEK-293 and HUH-7 cells did not cause significant viability differences. Although DMSO was not cytotoxic in this concentration in our experiments, it was observed that this DMSO concentration affected gene expressions. There are various studies about effects of DMSO on gene expression (Sumida *et al.,* 2011; Czysz *et al.,* 2015; Klinken *et al.,* 1988). For example, it was reported that DMSO has effects on some drug-metabolizing enzyme genes (Nishimura *et al.,* 2003; Wilkening and Bader, 2004; Choi *et al.,* 2009). Therefore, it was important to use vehicle DMSO control in experiments. In this experiment, we had opportunity to compare the effect of DMSO by using both normal control and DMSO control, and also the effects of quercetin and resveratrol were observed directly relative to vehicle DMSO- control.

The overall results of the mRNA and protein expression analysis of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 from DMSO control and quercetin treated HEK-293 cells are shown in Figure 4.1. According to these results, quercetin treatment caused a significant increase in mRNA expression of CYP2R1 and CYP27A1 in HEK-293 cells. However, this phenolic compound resulted in strong downregulation in CYP2R1 and CYP27A1 protein expressions in HEK-293 cells. This result indicated that post-transcriptional or posttranslational mechanisms have an important role in regulation of these two vitamin D 25-hydroxylases. Micro RNAs can play a role in post-transcriptional regulation (Abaza and Gebauer 2008, Fabian *et*

al. 2010). Moreover, phosphorylation, acetylation, glycosylation, sumoylation and ubiqutination are important regulations as post-translational modifications (Preiss and Hentze, 1998). Sumoylation is a post-translational modification process containing small ubiquitin-related modifier (SUMO) (Wilkinson and Henley, 2012). It was reported that quercetin treatment increased sumoylation levels in some cells (Lee *et al.,* 2016). One of the reasons of the decrease in CYP27A1 and CYP2R1 protein expressions with quercetin in HEK-293 cells can be sumoylation. Sumoylation of CYP27A1 and CYP2R1 can cause ubiquitination and degradation through ubiquitin-proteasome proteolytic pathway. Whether regulation of CYP27A1 and CYP2R1 with this modification or any other mechanisms, down-regulation of CYP27A1 and CYP2R1 protein expressions affects vitamin D metabolism negatively. Because, their down-regulation can decrease amount of 25(OH)D, which affects the amount of active vitamin D, $1,25(OH)_2D$. In addition, CYP24A1 protein expression was increased in response to quercetin treatment for the same cell line. It is known that CYP24A1 inactivates the hormonally active form of vitamin D. This means that quercetin can affect vitamin D metabolism negatively in HEK-293 cells.
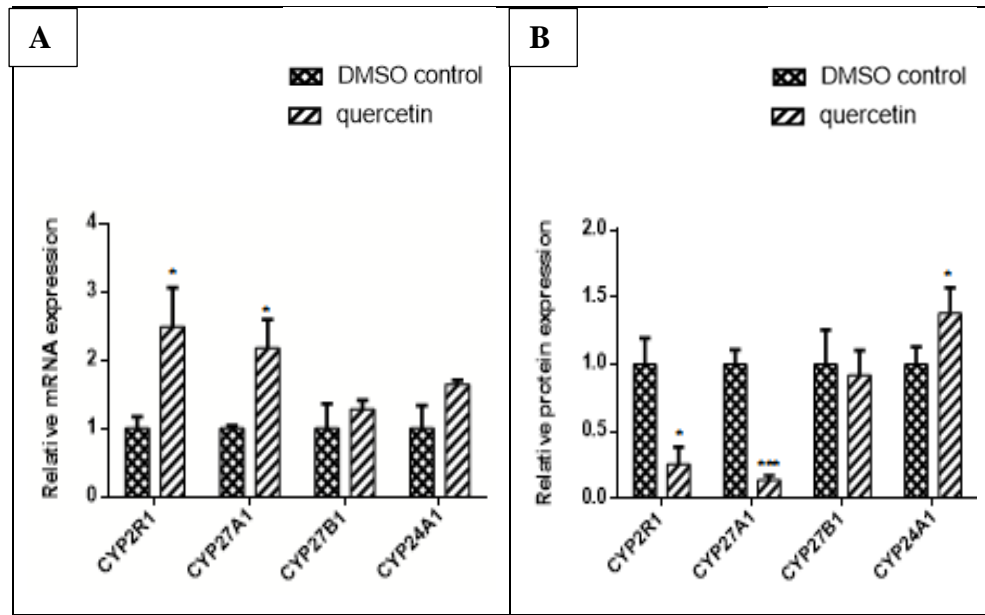
Figure 4.1 Effect of quercetin on CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA (A) and protein (B) expressions in HEK-293 cells. A: Fold change of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA expression. B: Fold change of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 protein expression. $*P \leq 0.05$; $***P \leq 0.001$.

Figure 4.2 shows the effect of resveratrol on mRNA and protein expressions of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 in HEK-293 cells. As seen in Figure 4.2, resveratrol treatment caused an increase in the mRNA expression of CYP27A1, CYP27B1 and CYP24A1 in HEK-293 cells. However, protein expressions of CYP27A1 and CYP27B1 in HEK-293 cells were decreased by resveratrol treatment. Similar to quercetin, resveratrol also caused an increase in CYP24A1 protein expression in HEK-293 cells. These results showed that there is no significant correlation between mRNA and protein expression. There are many studies about no significant correlation between transcription and translation levels. Abundance of protein cannot be described only with transcriptional regulation. The reason of this variance is the post-transcriptional, translational and post-translational regulations. For example, micro RNAs (miRNAs) are known as posttranscriptional regulators. They are 21–22-nucleotide long non-coding RNAs mediating post-transcriptional gene silencing. Protein synthesis inhibition with miRNAs is generally occurred either by translational repression due to alteration of the interaction of mRNA with translational machinery complexes or by inhibiting ribosome elongation with the induction of ribosome drop-off (Abaza and Gebauer 2008, Fabian *et al.* 2010). In addition, a decrease in half-life of proteins with an enhanced degradation rate can affect correlation (Greenbaum *et al.,* 2003). Also, some metabolites can cause conformational changes in the mRNA structure, which affects the translation efficiency (Maier *et al.,* 2009). The other reason can be RNA binding proteins, which can cause down-regulation of mRNA translation. Furthermore, protein stability can be affected by post-translational modifications such as phosphorylation, acetylatylation, glycosylation and ubiqutination (Preiss and Hentze, 1998). All of these can be the reason of that increase CYP27A1 and CYP27B1

mRNA expression does not translate into an increase in their protein expression and low correlation between CYP24A1 mRNA and protein expressions. Although increased CYP24A1 mRNA expression result was consistant with increased protein expression, transcriptional increase was more drastic and it did not translate sharply in protein expression of CYP24A1. Furthermore, overall protein expression results imply that resveratrol can affect vitamin D metabolism negatively in HEK-293 cells by causing decrease in CYP27A1 and CYP27B1 protein expressions and increase in CYP24A1 protein expression.
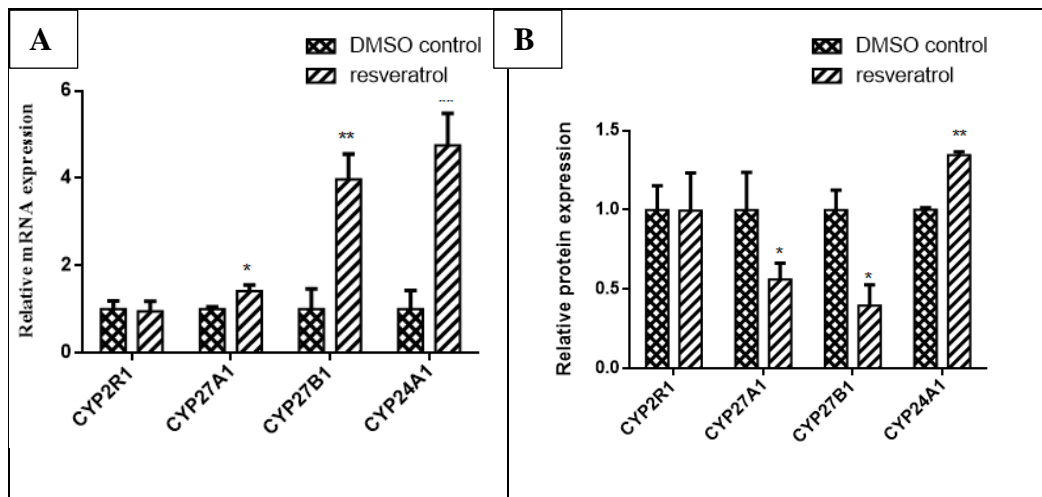
Figure 4.2 Effect of resveratrol on CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA (A) and protein (B) expressions in HEK-293 cells. A: Fold change of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA expressions. B: Fold change of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 protein expressions. $*P \leq 0.05$; $**P \leq 0.01$.

Figure 4.3 shows the effect of quercetin on mRNA expressions of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 and protein expressions of CYP27B1 and CYP24A1 in HUH-7 cells. In HUH-7 cells, quercetin treatment caused an upregulation in mRNA expression of CYP2R1, CYP27B1 and CYP24A1. In contrast to the results of mRNA expression, quercetin did not significantly affect the protein expressions of CYP27B1 and CYP24A1 in HUH-7 cells. Moreover, in HUH-7 cell line, protein expressions of CYP27A1 and CYP2R1 were not observed by western blotting technique. Although these are mainly expressed in liver and HUH-7 cell line is known as human hepatocellular carcinoma, CYP27A1 and CYP2R1 protein expressions were not detected as it was expected. However, cell lines are originated from different individuals having genetically different features. In addition, differences in the protein expressions can be seen more abundantly in cancerous cell lines. The reasons of not detecting CYP27A1 and CYP2R1 protein expressions can be some changes in the nucleotides resulting in protein instability or incomplete peptide formation because of single nucleotide change in coding region. From another point of view, HUH-7 cell line was originated from Japanese male, who is 57 years old. There are many researches about vitamin D insufficiency in Japanese population (Nakamura, 2006). A Japan can be more prone to have mutations in vitamin D-25 hydroxylases. It was reported that Japan possesses higher prevalence of cerebrotendinous xanthomatosis (CTX), caused by mutations of CYP27A1 gene (Kim *et al.*, 1994). Investigations of CYP27A1 mutations that are common in Japanese population such as R474Q and R474W reveal that such variations may alter the proper processing and functioning of CYP27A1 peptide (Kim *et al.*, 1994; Gallus *et al.,* 2006). Therefore, the reason of no observable protein expressions may be explained by one of these mutations.
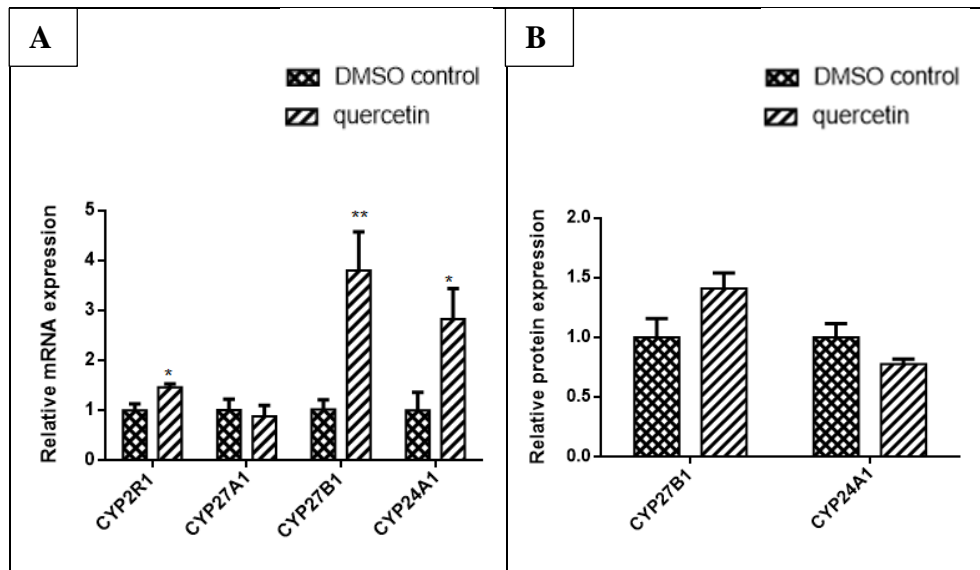
Figure 4.3 Effect of quercetin on CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA (A) and CYP27B1 and CYP24A1 protein (B) expressions in HUH-7 cells. A: Fold change of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA expressions. B: Fold change of CYP27B1 and CYP24A1 protein expressions. *P≤0.05; **P≤0.01.

As seen in Figure 4.4, while resveratrol caused an increase in mRNA expression of CYP2R1 and CYP24A1, mRNA expressions of CYP27A1 and CYP27B1 was down-regulated in HUH-7 cells. One of the reasons of transcriptional down-regulations of CYP27A1 and CYP27B1 can be the activation of sirtuin 1 (SIRT1) by resveratrol. Several studies reveal that resveratrol activates SIRT1, in the class III histone deacetylases (HDAC) (Howitz *et al.,* 2003;Vetterli and Maechler, 2011). SIRT1 plays an important role in histone deacetylation and methylation in promoter having CpG island. CYP27A1 and CYP27B1 have CpG islands in their promoter regions (Fetahu *et al.,* 2014). Thus, decrease in transcriptional levels of CYP27A1 and CYP27B1 may be explained by that activation of SIRT1 with resveratrol represses the binding of transcriptional factors to DNA by causing "closed" state of chromotin. In addition, resveratrol caused significant decrease in protein expression of CYP24A1. CYP24A1 is important enzyme to control the $1,25(OH)_2D$ levels in tissues. It was reported that elevated CYP24A1 expressions were observed in several malignancies (Höbaus *et al.,* 2016; Townsend *et al.* 2005; Parise *et al.* 2006). This high CYP24A1 expression is considered as negative effect due to inhibition of anti-tumorigenic effects of vitamin D by stimulating catabolism of $1,25(OH)_2D$. For this reason, finding specific CYP24A1 inhibitors has become one of the favorite topics in recent years. In our study, resveratrol caused a decrease in protein expression of CYP24A1 in HUH-7 cells even though mRNA overexpression of CYP24A1 was observed with the treatment of resveratrol in the same cell line. The difference in mRNA and protein expressions can be caused by the posttranscriptional regulation of CYP24A1 in HUH7 cells. MicroRNAs are accepted as important regulators in post transcription. miR-125b recognition element was identified in 3'UTR of human CYP24A1 (Komagata *et al.,* 2009) and post-transcriptional regulation of CYP24A1 by

miR125-b was reported in some studies (Komagata *et al.,* 2009; Craig *et al.,* 2014; Kaminski *et al.,* 2012). In addition, endogenous expression of miR125-b was observed in HUH-7 cells unlike HEK-293 cells (Li *et al.,* 2015). Moreover, alteration in miRNA expression profiles with resveratrol such as miR-20b, miR-21, miR-27b, miR-133 was also reported (Mukhopadhyay *et al.,* 2012; Liu *et al.,* 2013). Therefore, one of the reasons of down-regulation of CYP24A1 protein expression in HUH-7 cells after resveratrol treatment can be upregulation of miR125-b in HUH-7 cells with the effect of resveratrol.
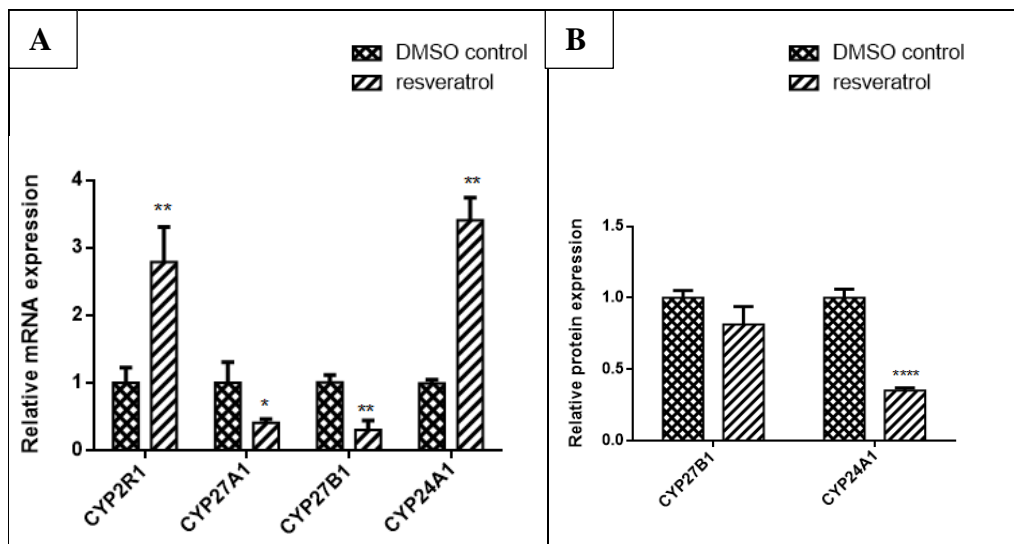
Figure 4.4 Effect of resveratrol on CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA (A) and CYP27B1 and CYP24A1 protein (B) expressions in HUH-7 cells. A: Fold change of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 mRNA expressions. B: Fold change of CYP27B1 and CYP24A1 protein expressions. *P≤0.05; **P≤0.01; ****P≤0.0001.

The comparison of two cell lines revealed that the effects of polyphenols on protein and mRNA expressions of vitamin D metabolizing CYPs were modulated differently depending on cell line. Apart from the reason that cell lines are originated from genetically different individuals, another reason can be the different concentrations of polyphenols used in cell lines. These treatment concentrations were determined according to their half maximal inhibitory concentrations. Third reason can be that HUH-7 cell line is hepatic cell. It is known that quercetin and resveratrol are rapidly metabolized in liver to their conjugate forms, which can affect their bioavailability and different experimental results can be obtained between hepatic and other cell lines.

In conclusion, the results of this study showed that mRNA and protein expressions of CYP2R1, CYP27A1, CYP27B1 and CYP24A1, are modulated by quercetin and resveratrol in HEK-293 and HUH-7 cell lines. This modulation is different depending on the type of phenolic compound and cell line. All these results show that there is no significant correlation between mRNA transcript and protein levels. Enzyme activity studies can be done as a future study to determine the actual effectiveness of these phenolic compounds on activities of Vitamin D metabolizing enzymes. In addition, HUH-7 cell line is not recommended for a use as model in future researches for CYP27A1 and CYP2R1 studies because of the lack of protein expression.

CHAPTER 5

CONCLUSION

In this study, the effects of quercetin and resveratrol on the protein and mRNA expressions of vitamin D metabolizing CYPs- CYP27R1, CYP27A1, CYP27B1 and CYP24A1 in human embryonic kidney cell line (HEK-293) and human hepatocellular carcinoma cell line (HUH-7) were analyzed for the first time.

While IC50 values of quercetin were determined as 60.72 and 185.31 µM for HEK-293 and HUH-7 cells, IC50 values of resveratrol were determined as 34.36 and 131.93 µM for HEK-293 and HUH-7 cells, respectively. Cells were treated with quercetin or resveratrol using IC50 concentrations in order to observe the effects of these phenolic compounds on protein and mRNA expressions compared to vehicle DMSO-control groups in HEK-293 and HUH-7 cells.

The results showed that quercetin and resveratrol have modulatory effects on mRNA and protein expressions of vitamin D metabolizing CYPs, CYP2R1, CYP27A1, CYP27B1 and CYP24A1 in HEK-293 and HUH-7 cell lines. Using specific cell line and/or the type of phenolic compound varied the experimental results in terms of their expressions. For both quercetin and resveratrol, generally more drastic changes were observed in HEK-293 cells than HUH-7 cells. Furthermore, CYP27A1 and CYP2R1

protein expressions were not observed in HUH-7 cells. Therefore, HUH-7 cell line is not recommended for use as a model cell line in studies including CYP27A1 and CYP2R1. In addition, there was no significant correlation between transcription and translation level, which implies post-transcriptional, translational and post translational regulations. In the light of these findings, noncoding RNAs involved in these regulations can be examined in detail and enzyme activity studies can be done to determine the actual effectiveness of these phenolic compounds on activities of vitamin D metabolizing enzymes as a future study.

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128

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131

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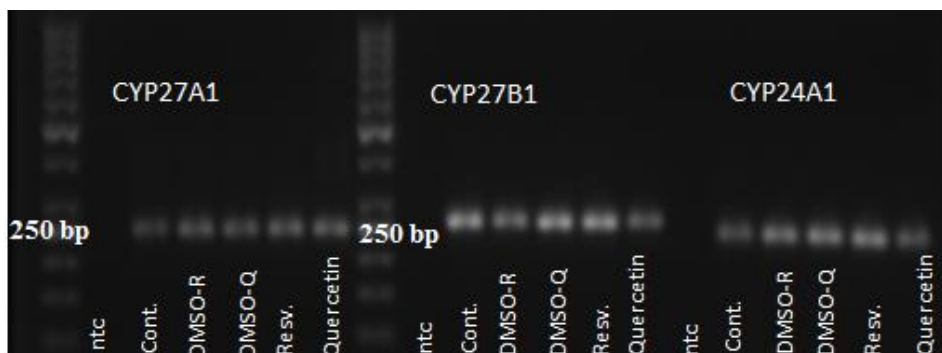## A.1 Visualization of qRT-PCR products

Figure A.1 qRT-PCR products of CYP27A1 (247 bp), CYP27B1 (265 bp), CYP24A1 (245 bp) cDNA of control, DMSO control, resveratrol and quercetin treated HEK-293 cells. NTC is the no template control.
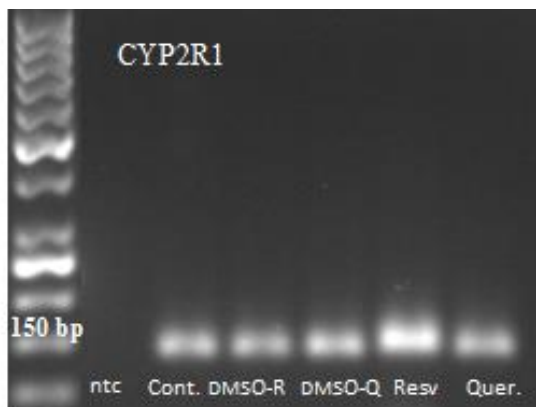

Figure A.2 qRT-PCR products of CYP2R1(133 bp) cDNA of control, DMSO control, resveratrol and quercetin treated HEK-293 cells. NTC is the no template control.
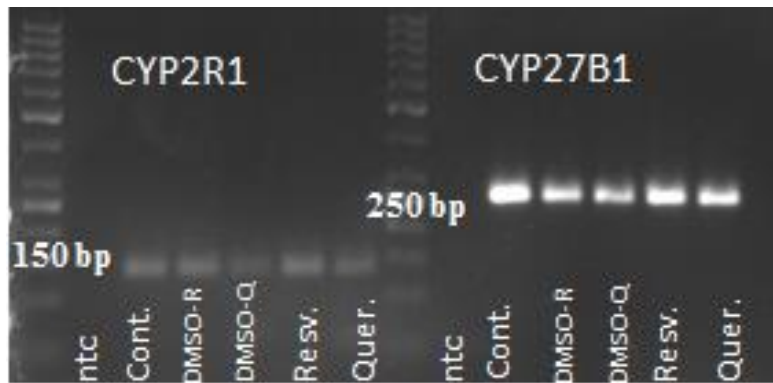
Figure A.3 qRT-PCR products of CYP2R1 (133 bp), CYP27B1 (265 bp), cDNA of control, DMSO control, resveratrol and quercetin treated HUH-7 cells. NTC is the no template control.
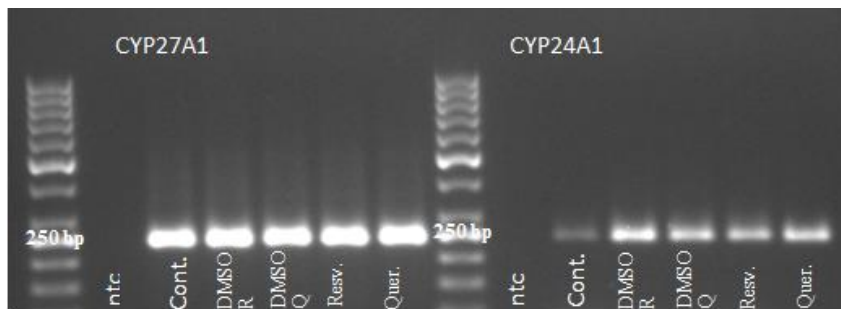

Figure A.4 qRT-PCR products of CYP27A1 (247 bp), CYP24A1 (245 bp), cDNA of control, DMSO control, resveratrol and quercetin treated HUH-7 cells. NTC is the no template control.