ANTIOXIDANT PROPERTIES OF *Salvia absconditiflora* EXTRACTS AND THEIR EFFECTS ON PHASE I AND PHASE II GENE EXPRESSIONS IN HEPG2 CELL LINE

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ANTIOXIDANT PROPERTIES OF *Salvia absconditiflora* EXTRACTS AND THEIR EFFECTS ON PHASE I AND PHASE II GENE EXPRESSIONS IN HEPG2 CELL LINE

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ABSTRACT

ANTIOXIDANT PROPERTIES OF *Salvia absconditiflora* EXTRACTS AND THEIR EFFECTS ON PHASE I AND PHASE II GENE EXPRESSIONS IN HEPG2 CELL LINE

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S.absconditiflora is one of the endemic Salvia species grown in Turkey, which is consumed as a herbal tea. Because of the presence of high amounts of vesicles on their leaves, it is very rich in bioactive compounds.

S.absconditiflora water and methanol extracts were studied for their antioxidant capacity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ABTS free radical scavenging assay. Total phenolic and total flavonoid contents were quantified by spectrophotometric methods, HPLC and LC-MS/MS analyses and the results revealed the presence of several compounds and their quantities were calculated by using 10 different standards.

June water extracts showed the highest % Radical Scavenging Activity against DPPH radical. Total flavonoid content was found as one third of the total phenolic content. April methanol extracts had the highest TPC (180.77 mgGAE/ g of dried extract) and June methanol extracts had highest TFC (21.8 mg QE/ g of dried extract).

Cytotoxic effects of *S.absconditiflora* extracts and two standards, as main phenolic components, were examined in human hepatocyte carcinoma cell line, HepG2, via XTT colorimetric and Trypan Dye Exclusion cell viability

assays. IC_{50} values in HepG2 cell line, after 48 and 72 hour incubations were determined.

Effects of *S.absconditiflora* water and methanol extracts on the gene expressions of several Phase I and II enzymes were further investigated. Among CYP450 enzymes, only five accounts for major drug metabolism, namely CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2 and water extracts induce the gene expression of all these CYP enzymes. Only CYP1A2, CYP2D6 and CYP2E1 gene expressions were induced with methanol extract. However, CYP2C9, CYP2C19 and CYP3A4 gene expressions were found to be inhibited with methanol extracts. CYP3A4 is the most important enzyme as its involved in almost all CYP450 mediated reactions. This inhibitory potential of *S.absconditiflora* methanol extract toward CYP3A4 could have potential herb-drug interaction in liver and might increase the bioavailability of co-adminestered drug and lead to toxicity. Inhibitory effect of methanol extract but not the water extract suggested that flavonoids, which exist abundantly in the methanol extract of *S.absconditiflora* may be responsible for this inhibition.

Gene expression of all GST Mu enzyme (M1-M5) was inhibited by 3-18 fold with methanol extracts whereas water extracts inhibited M1 and M2 by 1.5 fold and induce M3 and M4 by again 1.5 fold. Both water and methanol extracts decreased the gene expression of GSTP1. According to our panel results we conclude that, the methanol extract of *S.absconditiflora* shows the most potent effects on CYP enzymes. On the other hand, the aqueous, water extract shows negligible effects on CYP activities. Since most individuals consume the leaves of *S.absconditiflora* either raw or boiled with water, the findings of this study suggest that the water extract may be safe considering the herb-drug interactions.

K**eywords:** *S.absconditiflora,* HepG2 cell line, Phase I and Phase II gene expression

ÖZ

Salvia absconditiflora EKSTRELERİNİN ANTİOKSİDAN İÇERİKLERİ VE HEPG2 HÜCRELERİNDE FAZ I VE FAZ II ENZİMLERİNİN GEN EKSPRESYONLARI ÜZERİNE ETKİLERİ

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S.absconditiflora Türkiye de yetişen ve bitki çayı olarak da tüketilen endemik *Salvia* türlerinden bir tanesidir. Yaprakları üzerindeki yüksek miktardaki damarlardan dolayı *S.absconditiflora* çok aktif bileşiklere sahiptir.

*S.absconditiflora'*nın su ve metanol ekstrelerinin antioksidan kapasitelerine DPPH ve ABTS metodu ile bakıldı. Toplam fenolik ve flavanoid içerikleri spektroskopik teknik ile belirlendi. HPLC ve LC-MS/MS analizleri ile *S.absconditiflora'*nın içerisindeki bazı fenolik bileşiklerin varlığı 10 farklı standart kullanılarak belirlendi.

Haziran su ekstresi en yüksek radikal yakalama kapasitesi gösterdi. Toplam flavanoid içeriği toplam fenolik içeriğinin 1/3'ü olarak belirlendi. Nisan metanol ekstresinin 108.77 mg GAE/g ekstre ile en yüksek toplam fenolik içeriğe sahip olduğu bulundu.

S.absconditiflora 'nın ve 2 ana aktif bileşeninin sitotoksik etkileri HepG2 hücre hattı üzerinde XTT kolorimetrik ve tripan mavisi boyası ile incelendi. IC_{50} değeri 48 ve 72 saat uygulamaları ile tripan mavisi sayımı ile belirlendi.

CYP enzimleri arasında, ilaç metabolizmasında rol alan en önemli beş enzim, CYP3A4, CYP2D6, CYP2C9, CYP2C19, ve CYP1A2 olarak bilinir ve bu enzimlerin gen ifadelerine bakıldığında *Salvia* su ekstresinin bu enzimlerin gen ifadelerinin hepsini arttırdığı görüldü. Sadece CYP1A2, CYP2D6 ve CYP2E1 gen ifadelerinin metanol ekstresi ile uyarıldığı, bununla birlikte, CYP2C9, CYP2C19 ve CYP3A4 gen ifadelerinin, metanol ekstresi uygulandığında inhibe edildiği bulundu. CYP3A4 enzimi tüm CYP enzim reaksiyonları içinde en önemli enzimdir. *S.absconditiflora* metanol ekstresinin CYP3A4 üzerindeki bu inhibe edici özelliği, karaciğerde, olası bitki-ilaç etkileşimine neden olabilir ve birlikte kullanılan ilacın biyoyarılanımını arttırarak toksik etkiye sebep olabilir. *S.absconditiflora* metanol ekstresinin inhibe edici etkisinin, içerisinde bol miktarda bulunan flavonoidler tarafından sağlanabileceği düşünülmektedir.

GST Mu enzimlerinin hepsinin (M1-M5) gen ifadelerinin metanol ekstresi ile 3 ile 18 kat düştüğü görüldü. GSTM1 ve GSTM2 gen ifadeleri su ekstresi ile 1.5 kat düşerken, GSTM3 ve GSTM4 gen ifadelerinin su ekstresi ile 1.5 kat arttığı görüldü. Su ve metanol ekstrelerinin her ikiside de GST P1 gen ifadesini düştüğü gözlemlendi.

Bu çalışmada kullanılan panel sonuçlarına göre, CYP enzimleri üzerine en güçlü etkiye *S.absconditiflora* metanol ekstresinin sahip olduğu bulundu. Diğer yandan, su ekstrelerinin CYP aktiviteleri üzerinde daha az etkisi olduğu görüldü.

Çoğu kişi, *S.absconditiflora* yapraklarını çiğ ya da haşlanmış olarak tüketmektedir. Bu çalışmanın bulgularına bakıldığında su ekstrelerinin bitki-ilaç etkileşimleri dikkate alındığında güvenli olabileceğini düşünülmektedir.

Anahtar kelimeler: *S.absconditiflora*, HepG2 hücre hattı, Faz I ve Faz II gen ifadeleri

To My son, Arda Kaan

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ATCC : American type culture collection

cDNA : complementary DNA

CYP : Cytochrome P450 Monooxygenase

DMSO : Dimethyl sulfoxide

DPPH : 2,2 diphenyl 1-picrylhyrazy

EDTA : Ethylenediaminetetraaceticacid

LC-MS/MS : Liquid chromotography mass spectrometry

PCR : Polymerase chain reaction

q-RT PCR : Quantitative real time polymerase chain reaction

RSA : Radical scavenging activity

TFC : Total flavanoid content

TPC : Total phenolic content

XTT : 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium - 5-Carboxanilide

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is also named as malignant tumors and neoplasms, a large group of diseases that can affect any part of the body. Cancer is the rapid creation of abnormal cells of the body and spread to other organs, the latter process is referred to as metastasizing. These changes are the result of the interaction between a person's genetic factors and physical, chemical and biological carcinogens. Another fundamental factor is ageing for the development of cancer. Cancer is a leading cause of death worldwide in 2012 (World Cancer Report 2014). The most common cancers are lung, liver, stomach, colorectal and breast cancers, liver cancer is the second one which causes of death (745000 deaths).

1.2 Liver Cancer

Liver cancer originates in the liver and in 2012 it occurred in 782000 people and resulted in 746000 deaths, mortality rate is 95% (World Cancer Report 2014). The most frequent liver cancer is hepatocellular carcinoma (HCC). HCC is formed by liver cells, hepatocytes. 75 % of liver cancer is HCC. Hepatoblastoma is other type of cancer formed by liver cells, which is specifically formed by immature liver cells. In 2013, 735000 people died, 40% of them was due to hepatitis B, 47 % of them was due to hepatitis C and 13 % of them was to alcoholic cirhosis (GBD 2013 Mortality and Causes of Death).

Infections of hepatitis B and/or C can assist the development of hepatocellular carcinoma by the body's own immune system to attack the liver cells.

1.2.1 Nutrition and Cancer

Many epidemiological literature introduces a protective role for foods, vegetables and fruits in cancer incidence. Inhibition of chemically induced tumors could be feasible by phytochemicals in fruits and vegetables (Wattenberg, 1966). Cancer cases can be prevented by changes in dietary habits (Sarkar et al., 2004).

The ‘phyto’ is a Greek word meaning ‘plant’. Thera are more than 5000 phytochemicals in foods has been identified yet (Liu, 2004). Phytochemicals can be divided in to 5 classes, polyphenols are most studied ones and considered more powerful antioxidants than carotenoids, alkaloids, nitrogen- containing compounds and sulfur-containing compounds (Mandlekar et al., 2006).

Polyphenols; substances have an aromatic ring consist of one (phenol) or more (polyphenol) have been indicated as ‘blocking agents’ that they are capable of preventing carcinogenesis (Henderson et al., 2000). They are moderately water soluble compounds, with molecular weight of 500-4000 Da, containing more than 12 phenolic hydroxyl groups, having 5-7 aromatic rings per 1000 Da (Haslam, 1988). Plants generally produce polyphenols in defense against ultraviolet radiation or aggression by pathogens (Beckman, 2000).

More than 8000 polyphenolic compounds have been identified in various plant species. Researchers have investigated that these molecules are very good antioxidants and neutralize the subversive reactivity of reactive oxygen/nitrogen species.

Epidemiological studies have exposed that polyphenols obtain a significant protection against development of several diseases such as cardiovascular diseases (CVDs), cancer, diabetes, infections, aging, asthma etc. (Pandey and Rizvi, 2009). Since herbal plants are being ingested regularly in human diet, dietary polyphenols play many significant roles in human life. They are monitored to be effective in human health, such as in the treatment and prevention of cancer, cardiovascular diseases, and other pathologies (Yang et al. 2001, Visioli, Borsani and Galli 2000).

The main classes of polyphenols comprise phenolic acids, flavonoids, stilbenes and lignans which were shown in Figure 1.1

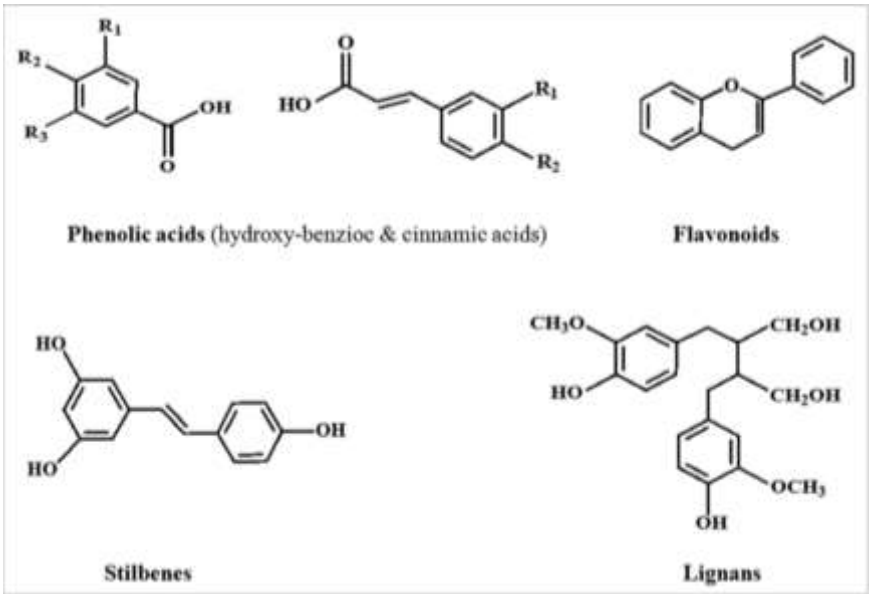

Figure 1.1 Chemical structures of polyphenols

Flavonoids are the most widespread and crucial group of polyphenol compounds. There are more than 5000 compounds currently known. They are divided into 13 classes as, chalcones, dihydrchalcones, aurones, flavones, flavonols, dihydroflavonol, flavonones, flavanol, flavandiol, anthocyanidin, isoflavonoids, biflavonoids and proanthocyanidins or condensed tannins (Harborne, 1986).

Phenolic acids are a member of nonenzymatic antioxidants, and they can scavenge free radicals and also chelate metal ions, decreasing the pro-oxidant activity. (Fridovich, 1974). Caffeic acid and gallic acid are the most known phenolic acids. Flavonoids such as quercetin and catechins are most wide polyphenols in human diet. There are more than 4000 types of flavanoids are described.

In literature, many polyphenols, such as quercetin, catechins, ellagic acid, red wine polyphenols, resveratrol, conserve and induce a reduction of the tumor or their growth on human cancer cell line (Yang et al, 2001, Arts and Hollman,2005).

1.3 Drug Metabolizing Enzymes

Drug metabolism is the biochemical modification of xenobiotics by specialized enzymatic systems. Drug metabolism include biotransformation and detoxification processes. Drug metabolism divided 3 parts; first phase I enzyme reaction such as cytochrome P450 oxidases present reactive or polar groups into xenobiotic. After these modification, compounds are conjugated to polar compounds in phase II reactions such as glutathione S-transferases (Mizuno et al., 2003)

The hepatic cytochrome P450s (CYPs) play critical roles in the metabolism of many drugs and xenobiotics (Danielson, 2002).

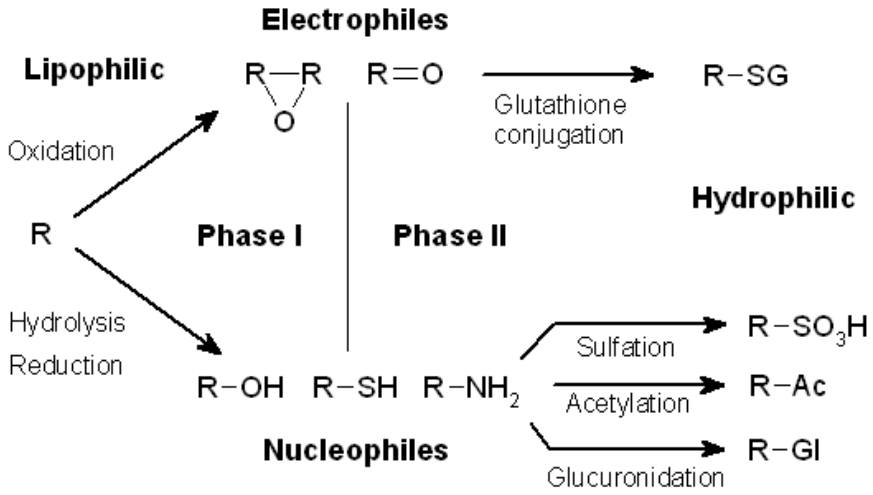

Figure 1.2 Drug detoxification reaction in the body

1.3.1 Cytochrome P450 Enzymes

Cytochrome P450 (CYP) enzymes; heme containing enzymes found in all organisms involved in the metabolism of most of the xenobiotics. The term P450 refers to a pigment that absorbs light at 450nm when reduced form is exposed to carbon monoxide (Omura & Sato 1964).

CYPs are membrane bound enzymes mainly located on the endoplasmic reticulum of the cells in a wide range of tissues. The active site of cytochrome P450 contains a heme-iron structure in the center. Figure 1.3 shows the CYPs mechanism (Berka et al., 2011)

Oxidative reactions typically require a Cytochrome P450 enzymes, NADPH and oxygen. Typical reactions were shown below;

$$O_2 + NADPH + H^+ + RH \rightarrow NADP^+ + H_2O + ROH$$

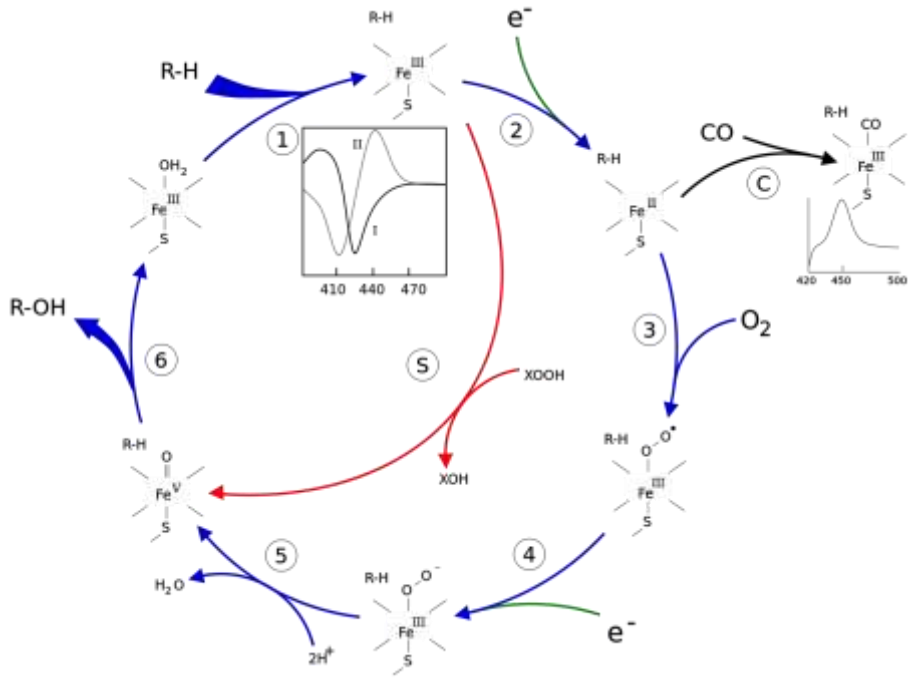

Figure 1.3 CYPs action mechanisms (Guengerich 2001).

1: Substrate binds to the heme group; 2: Substrate binding excited electron transfer from NAD(P)H via cytochrome P450 reductase; 3: Molecular oxygen binds to the resulting ferrous heme center; 4: A second electron is transferred, reducing the Fe-O_2 adduct to give a short-lived peroxo state; 5: The peroxo group formed in step 4 is rapidly protonated twice, releasing one molecule of water and forming the highly reactive species; 6: After the product has been released from the active site, the enzyme returns to its original state, with a water molecule returning to occupy the distal coordination position of the iron nucleus.

CYP genes are members of multigene family, expressed in mainly liver in which most of the drugs and endogenous compounds are metabolized by CYPs. Eighteen families with 43 subfamilies of CYP are present in humans and each of them show different functions. First 3 family are mainly play role in xenobiotic metabolism (Table 1.1) (Wang et al. 2009; Walsh et al. 2013).

Table 1.1 Human CYP enzyme family member associated with drug metabolism

Human CYP Families	Names	Function
CYP 1 (3 genes)	1A1, 1A2, 1B1	Xenobiotic and steroid metabolism
CYP 2 (16 genes)	2A6, 2A7, 2A13, 2B6, 2C8,	
	2C9, 2C18, 2C19, 2D6, 2E1,	
	2F1, 2J2, 2R1, 2S1, 2U1, 2W1	
CYP 3 (4 genes)	3A4, 3A5, 3A7, 3A43	

All members of CYP 1 family play role in xenobiotic metabolism. CYP1A1 is also known as AHH (aryl hydrocarbon hydroxylase) and it is very important enzymes involved in the metabolism of xenobiotics including drugs and procarcinogens (Wang et al. 2009; Walsh et al. 2013). Polycyclic aromatic hydrocarbons (PAH), such as benzo (a) pyrene (BP) is converted to carcinogen by the activation process (Figure 1.4). This intermediates highly reactive and covalently bind to DNA to cause carcinogenesis. CYP1A1 gene is positioned on chromosome 15 and length of mRNA is 2608bp and while molecular weight of the CYP1A1 protein is 58kDa and composed of 512 amino acids. The crystal structure of CYP1A1 protein is given in Figure 1.5 (Omiecinski et al. 1990; Meyer et al. 2002).

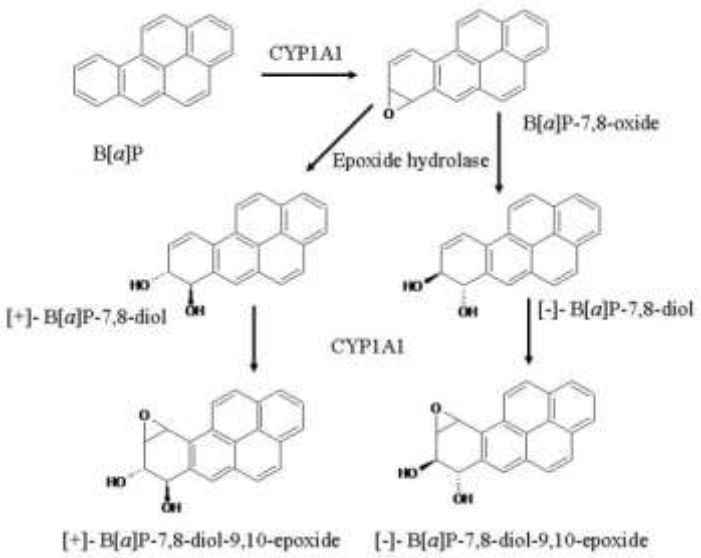

Figure 1.4 Mutagenic metabolite formation by the CYP 1A1 gene

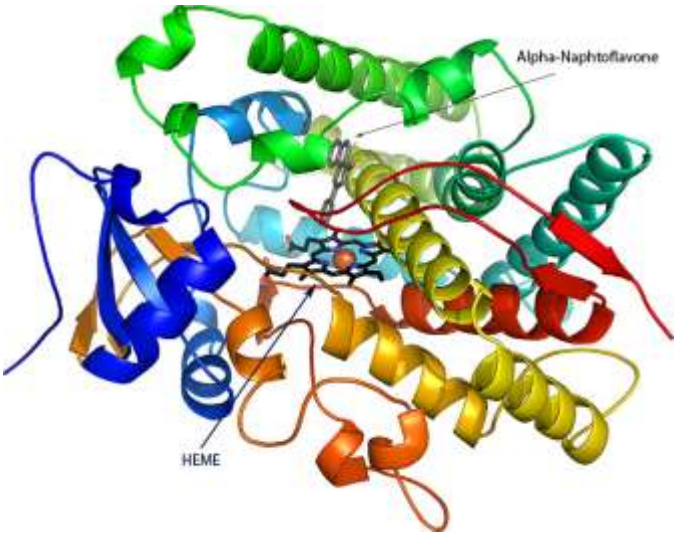

Figure 1.5 Human CYP450 1A1 with alpha-naphthoflavone (Walsh et al. 2013).

CYP1A2 localizes to the endoplasmic reticulum and it metabolizes some PAHs to carcinogenic intermediates. CYP1A2 genes are inducable genes and induced via PAHs and some vegetables such as cabbages, cauliflower and broccoli (Nelson et al., 2004). Acetaminophen (APAP) is the substrates of CYP1A2 gene. APAP cause hepatic necrosis under overdose conditions It is converted to reactive intermediate via CYP1A2 and then detoxified by

conjugation with glutathione (GSH). When decrease the GSH level intermediates reacts more extensively with hepatic proteins leading to hepatocellular damage (Jaiswal et al., 1987). The crystal structure of CYP1A2 protein is given in Figure 1.6. Some vegetables are known to increase levels of CYP1A2 such as cabbages, cauliflower and broccoli (Sanday, 2011).

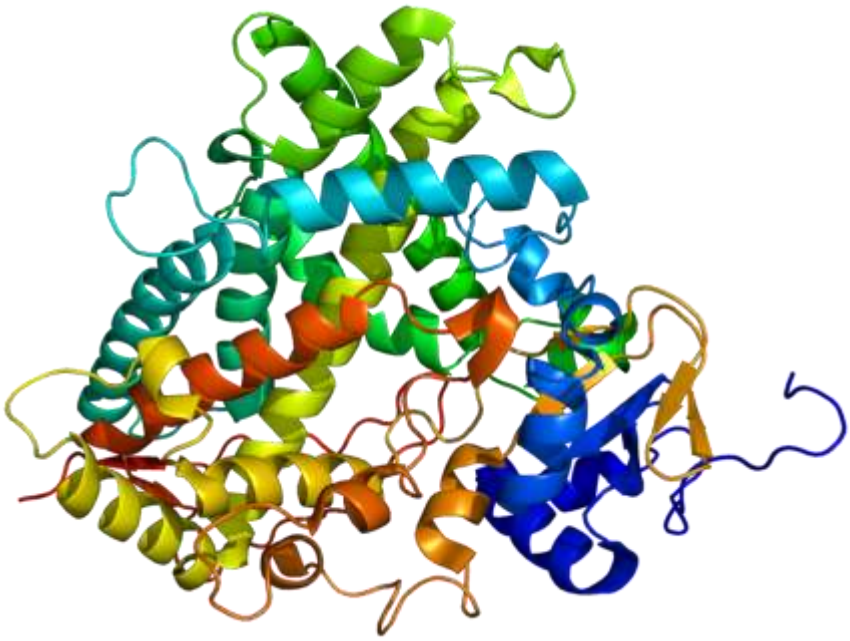

Figure 1.6 Human CYP450 1A2 (PDB)

Other forms of cytochrome P450 isozymes such as CYP3A4 and 2E1 activate the naturally occurring carcinogens respectively into highly mutagenic and carcinogenic agents (Hashimoto et al., 1993). The carcinogenic potency of PAHs, and other carcinogens and the extent of binding of their ultimate metabolites to DNA and proteins are correlated with the induction of cytochrome P450 isozymes. CYP3A4 is involved in the metabolism of approximately half the drugs that are used commercially today, including acetaminophen, codeine, diazepam, and erythromycin.

CYP3A4 is an important enzyme in the body, mainly found in the liver and in the intestine. CYP3A4 have important roles on deactivation of many

drugs are deactivated and also activation of drugs or procarcinogens to carcinogen, such as grapefruit juice and some drugs, interfere with the action of CYP3A4 (He et al., 1998; Bailey et al., 1998; Garg et al., 1998).

In humans, the CYP3A4 protein is encoded by the CYP3A4 gene found in 7th chromosome. Figure 1.7 shows the CYP3A4 protein.

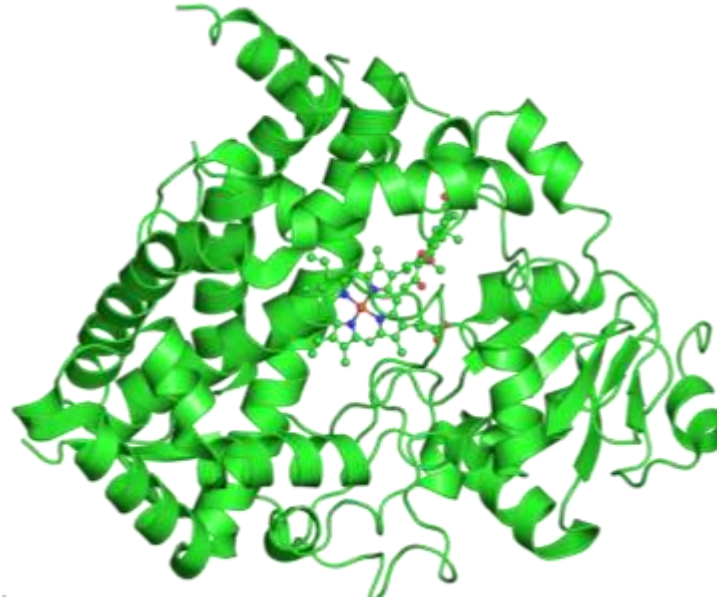

Figure 1.7 Human CYP3A4 polypeptide

CYP 2E1 ethanol inducible Cytochrome P450 form and its crystal structure is given in the Figure 1.8. CYP2E1 is mainly found in the E.R. and mitochondria and it is expressed in a range of extrahepatic tissues such as kidney, lung and brain (Botto et al. 1994)**.** CYP2E1 catalyses the conversion of drugs such as acetaminophen, pre-toxins and procarcinogens to active form (Gonzalez 2005).

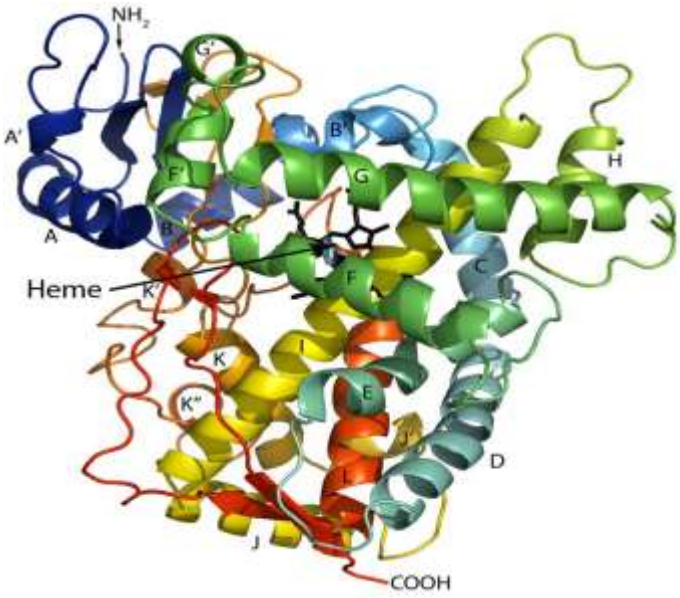

Figure 1.8 Human CYP2E1 (Porubsky et al. 2008).

According to Arinç et al. 2005; Arinç et al. 2007 , diabetes causes significant increase in both protein expression and catalytic activity of CYP2E1. Some chemicals such as resveratrol, plant phenolic compound, downregulates CYP2E1 and has potential to prevent carcinogenesis caused by catalytic activity of CYP2E1 (Carroccio et al. 1994; Celik & Arinç 2010). The expression of this enzyme is controlled by transcriptional, posttranscriptional and posttranslational actions.

1.3.2 Phase II Drug Metabolizing Enzymes

Phase II drug-metabolizing enzymes such as glutathione S-transferase, inactivate chemical carcinogens into less toxic or inactive metabolites. Phase II reactions make xenobiotics more hydrophilic and these metabolites readily eliminated from the body (Jacoby and Ziegler 1990). Conjugation reactions include glucuronidation, sulfation, methylation and also amino acid conjugations (Jacova et al., 2010). The balance of detoxification and activation reactions subject to the chemical structure of the agents, genetic background, sex, diet, age, endocrine status, and the presence of other chemicals.

Glutathione S-transferases (GSTs), catalyze the conjugation of the reduced glutathione (GSH) to xenobiotic substrates for the aim of detoxification. The GST family have three superfamilies: the cytosolic, mitochondrial, and microsomal (Sheenan et al., 2001). Cytosolic GSTs are divided into 13 classes according to their structure: alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega. The prior role of GSTs is to detoxify xenobiotics by catalyzing the nucleophilic attack by GSH on electrophilic carbon, sulfur, or nitrogen atoms of said nonpolar xenobiotic substrates (Oakley, 2011). Figure 1.9 shows the GST enzyme structure.

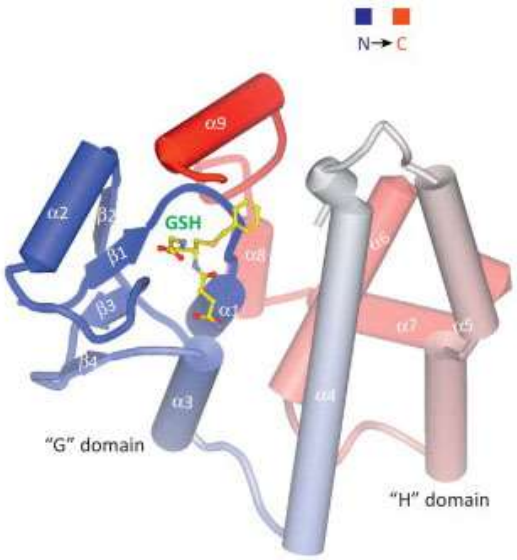

Figure 1.9 The structure of GST enzyme (Wu & Dong 2012).

GSTs have lots of function on the elimination of xenobiotics including active form of drugs and biosynthesis of important inflammatory mediator, prostaglandin, makes this enzyme family target for the drugs (Matsuoka 2000). GST S1-1 catalyzing isomerization reaction was given an example of biosynthesis reaction. It catalyses the isomerization of prostaglandin H2 to prostaglandin D2., a mediator of allergy and inflammation responses; nocodazole, inhibitor of GSTS1-1, is used as anti-allergic and anti-inflammatory drug (Weber et al. 2010). Some of the drugs are substrates of GST enzymes such cisplatin and carmustine. They are substrates of GSTP1

and GSTM1, respectively and excreted by conjugation with glutathione (Ban et al. 1996; Smith et al. 1989).

GSTs bind both the substrate at the enzyme's hydrophobic H-site and GSH at the adjacent, hydrophilic G-site, which together form the active site of the enzyme; and subsequently to activate the thiol group of GSH, enabling the nucleophilic attack upon the substrate. Most mammalian isoenzymes have affinity for the substrate 1-chloro-2, 4-dinitrobenzene (CDNB), an universal substrate, and spectrophotometric assays utilising this substrate are commonly used to report GST activity. Figure 1.10 shows the GSH and GST enzyme activity.

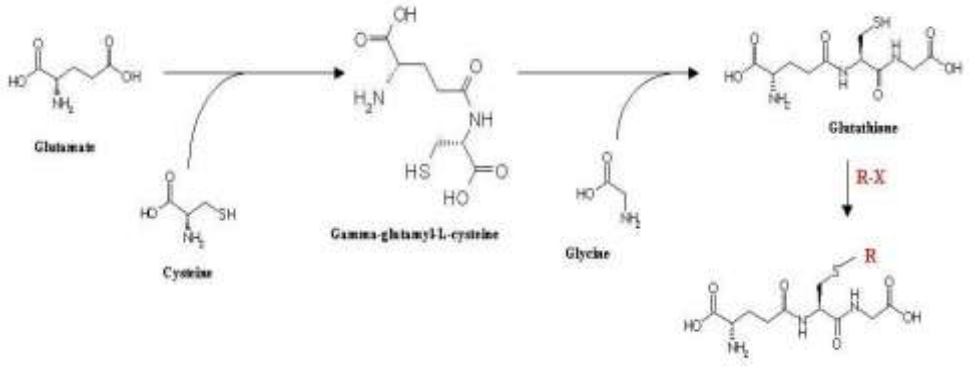

Figure 1.10 Glutathione conjugation reactions

1.3.3 Effect of Antioxidant Enzymes

Catalase is a nearly most common enzyme found in most of all living. It catalyzes the decomposition of hydrogen peroxide; reactive oxygen species, to water and oxygen (Chelikani et al., 2004). It is a very important enzyme in protecting the cell from oxidative damage. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert approximately 5 million molecules of hydrogen peroxide to water and oxygen each second (Goodsell,2004). Catalase is located in a cellular organelle peroxisome. The reaction catalyzed by catalase was showing below,

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase enzyme contains heme groups that allow the enzyme to react with the hydrogen peroxide (Boon et al., 2007). The reaction contain two steps;

$$H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O{=}Fe(IV)\text{-}E(.+)$$
$$H_2O_2 + O{=}Fe(IV)\text{-}E(.+) \rightarrow H_2O + Fe(III)\text{-}E + O_2$$

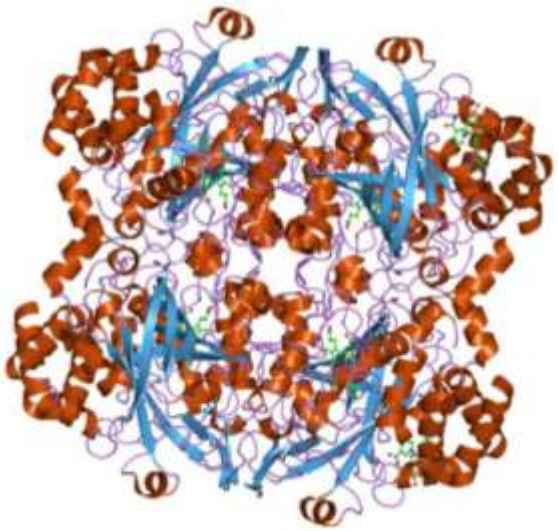

Figure 1.11 Crystalline structure of Catalase

Glutathione peroxidase (GPx) protect the organism from oxidative damage with the activity to reduce lipid hydroperoxides to corresponding alcohols and to reduce hydrogen peroxide to water (Epp et al.,1983).

Glutathione peroxidase also protect the organism from oxidative damage. Mammalian Glutathione peroxidases (GPx1, GPx2, GPx3, and GPx4) have selenium in it and they are members of selanoproteins (Katar et al., 2014). The reaction was catalyzed by glutathione peroxidase was shown below.

$$2GSH + H_2O_2 \rightarrow GS\text{–}SG + 2H_2O$$

GSH: reduced monomeric glutathione,
GS–SG: glutathione disulfide.

Glutathione reductase then reduces the oxidized glutathione to complete the cycle:

$$GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+.$$

GPx is also found in glutathione redox cycle (Figure 1.12).

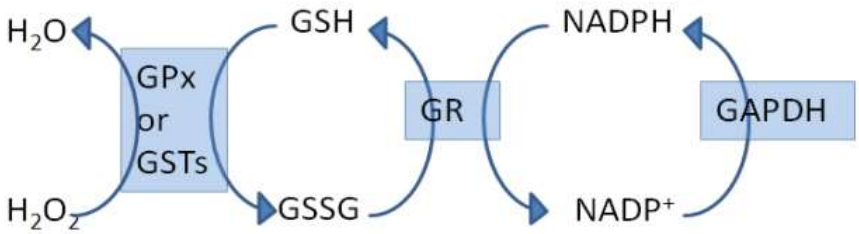

Figure 1.12 Glutathione redox cycle

GPx: Glutathione Peroxidase
GST: Glutathione S-transferase
GR: Glutatihone Reductase

1.3.4 Effects of Phytochemicals on Drug Metabolism

The relationship between drug metabolism and phytochemicals has great importance to evaluate herb-drug interactions. Phytochemicals can adjust these enzymes by transcriptional regulation or interacting with enzyme activity (Galli, 2007).

Diet and herbal remedies can regulate drug metabolizing enzyme systems, such as cytochromes P450, leading to clinically relevant drug-phytochemical interactions. Phytochemicals have the potential to both escalate and suppress cytochrome P450 activity. In the intestine, high concentrations of phytochemicals may be achieved, and alteration in cytochrome P450 activity will impress. These phytocemical and drug metabolizing enzyme interactions may have beneficial effects, such as cancer prevention (i.e., isothiocyanates in cruciferous vegetables). Grapefruit juice (GFJ) inhibited the both intestinal and hepatic CYP3A4 enzyme which catalyze most of the drugs in the market such as statins (Veronesa et al., 2003)

A sulfur containing isothiocyanate, sulforaphane (SFN) is shown as an chemopreventive phytochemical due to its role in GST induction. It is found in cruciferous vegetables such as in broccoli. Gao et al. (2010) examined that, AML12 cells treated with sulforaphane for 12 hours showed 4.3 fold increases in their GST enzyme activity. Overexpression of GST enzymes can also results in resistance toward anticancer agents and carcinogens.

1.4 Salvia Genus

The genus name "Salvia" is derived from the Latin "salvare", meaning "to heal or save. In Turkey, Salvia L. is named as "adaçayı" and is frequently consumed as tea (Baytop, 1999).

Salvia genus (Lamiaceae) includes about 900 species throughout the world and has 95 species in Turkey. Fourty five of the Salvia species are endemic to Turkey. Optimum growth condition for Salvia is well drained soil and full sun (Kamatau, 2008). The flowering months are May and June for almost all Salvia sp.

Many of Salvia sp. are used in folk medicines throughout the world, have an antioxidant, antidiabetic, antimicrobial, antitumor, antiplasmodial and anti-inflammatory properties (Ulubelen, 2003; Kamatou et al., 2008; Şenol et al., 2010). For example, *S.hydranza* ex Bentham is used as infusion for cold, diabetes, stomach disorders and antipyretic treatments. *S.nemorosa* L. is used for cold, catarrh treatment and as hemostatic. *S.multicaulis* Vahl.is used as anti-inflammatory. *S.verticillate* is used as laxative and also for cold and nausea treatment. In another study in Edremit Gulf of Balıkesir, *S.tomentosa* leaves are prepared by infusion and drunk 2 times a day for cold, flu and tonsillitis treatments (Polat and Satil, 2012). In addition, *S. officinalis* is used by local people in Maden (Elazığ) for treatment of Alzheimer, cough, flu and tonsillitis (Çakılcıoğlu et al., 2010). An Ottoman herbalist-physician lived during Sultan Mehmed the fourth used *S. triloba* for memory enhancement.

Lots of Salvia sp. are also used medicinal such as in food, cosmetics, perfumery and the pharmaceutical industry (Chalchat et al., 1998; Baylac & Racine, 2003).

A well-known antioxidant herbal plant is *Salvia officinalis* (sage), used as a popular folk medicine for the treatment of various ailments, such as antispasmodic and antiseptic (Bruneton, 1995).

According to in vitro studies, Salvia extracts have antimicrobial, anticancer, antioxidant and anti-inflammatory effects (Kamatou et al. 2008, 2010). Various Salvia sp. are shown to be beneficial for treatment of even more complicated diseases like coronary heart disease, cerebrovascular disease, hepatitis, hepatocirhosis, chronic renal failure, dysmenorrhea and neurasthenic insomnia (Lian, 1998).

1.4.1 Salvia in Turkey

Turkey is one of the richest centers in the world for plant diversity. Because, it has diverse climate conditions and it is also in a territory between Irano-Turanian, Mediterranean and Euro-Siberian. Endemism ratio is too high that almost 30% of plants are endemic (Kahraman et al., 2012).

Salvia genus is represented by totally 94 species in Turkey (Bagherpour et al., 2010). 51 of these species are endemic and endemism ratio (52.5%) is high. *S.fruticosa, S.absconditiflora, S.multicaulis, S.sclarea* and *S.tomentosa* species have economic values. Turkey's annual Salvia export is approximately 1200 tons and most of this export is constitiued by *S.fruticosa* (İpek and Gürbüz, 2010).

Salvia absconditiflora

Taxonomic Hierarchy:

Kingdom: Plantae

Subkingdom : Tracheobionta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass : Asteridae

Order : Lamiales

Family: Lamiaceae

Genus : Salvia

Species : *Salvia absconditiflora*

Common names: kara ot, kara sabla, kara salva, kara sapla

Salvia absconditiflora is an endemic perennial plant that grows on hillside and uncultivated following lands. The taxon is distributed mainly in middle Anatolian area of Turkey, in Afyonkarahisar, Ankara, Çorum, Erzincan, Kayseri, Konya, Niğde, Ordu, Sivas region. (Figure 1.13 Turkish Plants Data Service).

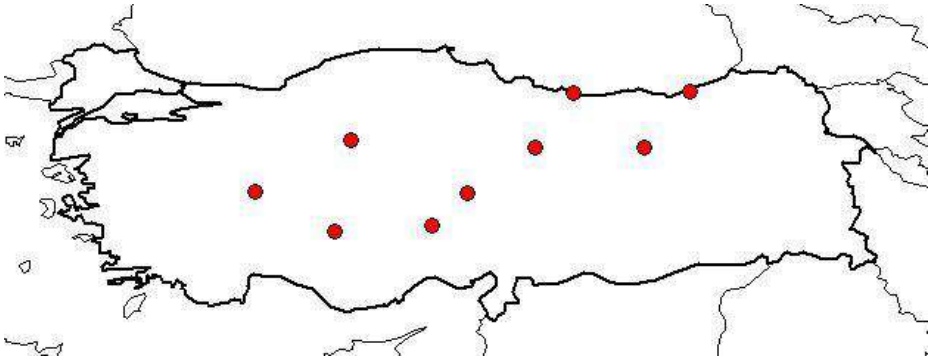

Figure 1.13 Distribution of the *S.absconditiflora* over Turkey

(http://turkherb .ibu.edu.tr/index.php?sayfa=1&tax_id=8076)

1.4.2 Antioxidant Compounds in Plants

Salvia is an important genus for polyphenol richness with more than 160 phenolics identified. The phenolic acid derivative variety and amount can differ between various Salvia species and also between different plant parts and extracts.

Polar phenolic acids are the main water-soluble components of Salvia species. Most of the phenolic acids are caffeic acid and its derivatives and, except rosmarinic acid and lithospermic acid, they are unique to Salvia species. Caffeic acid is found in dimer form mainly as rosmarinic acid that responsible for high antioxidant activity of Salvia.

Phenolic acids unique to Salvia species are salvianolic acids. Other phenolics identified in Salvia species are; benzoic acids such as 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid or protocatechuic acid, 3-4methoxy-4-hydroxybenzoic acid or vanillic acid, 2,4-dimethoxybenzoic acid, an ether linked dimer of hexyl 4-hydroxybenzoate, and coumarins; 6,7-dihydroxycoumarin (esculetin), 7-methoxycoumarin (herniarin) (Lu and Foo 2002).

Flavonoids are also commonly found in Salvia species, mainly present as flavones, flavonols and their glycosides, anthocyanins ans proanthocyanidins. The 6-hydroxyflavones, such as apigenin, luteolin, cirsimaritin, salvigenin, nepetin, cirsiliol, eupatorin, are the most significant flavonoids for Salvia genus since they characterize the species of Salvia.

1.5 Aim of the study

Many Salvia species are used in folk medicines throughout the world, because of their antioxidant, antidiabetic, antimicrobial, antitumor, and anti-inflammatory properties. One of the well-known antioxidant herbal plant is *Salvia officinalis* (sage), which is used as a popular folk medicine for the treatment of various diseases in Turkey. *Salvia absconditiflora* is an endemic species in Turkey and widely found in METU Campus.

In this study, we studied the beneficial effects of *S.absconditiflora* and carry out its biochemical characterization.

Most of the *Salvia* sp. are consumed as herbal tea and usually together with the prescribed drugs. All the xenobiotics including the drugs are metabolized in liver by Phase I and II enzymes. Most of these enzymes are polymorphic so the metabolism of these compounds are unique to each individual.

So, in this study we further investigate the metabolism of *S. absconditiflora* water and methanol extracts by HepG2 (liver) cells, emphasizing on the Phase I and II gene expressions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials

S. absconditiflora plants grow up in forested land near Biological Sciences Department in METU Campus. Plant leaves were collected from an in April, May and June 2013 and identified by Assoc. Prof. Dr. Ferhat Celep (Gazi University). Voucher specimen has been preserved in Plant Systematic Laboratory of Middle East Technical University Biological Science Department. Herbarium number is FCelep 1773.

Figure 2.1 *S.absconditiflora* (Biological Sciences Department in METU Campus)

2.1.2 HepG2 Cell Line

HepG2 cell line is a hepatocellular carcinoma cell line was purchased from ATCC (American Type Culture Collection).

2.1.3 Chemicals and Materials

All laboratory equipment are cell culture grade and sterilized. In this study we used T-25/T-75 Cell culture flasks and 6 well/ 96-well cell culture plates, 100x20 mm and 65x15 mm culture dish (Sarstedt, Germany or Grenier-Bio, Germany), Cryovials (Grenier-Bio, Germany), Micro pipettes; 1000, 200, 100, 10, 2.5 µL(Eppendorf, Germany), Multichannel Pipette 200 µL (Rainin, Mettler-Toledo, USA), and Pipetboy (Isolab, Germany). Sterile Filtered Pipette Tips; 10, 100, 200 and 1000 µL (Grenier-Bio, Germany, Axygen, USA or Rainin, USA), Serological pipettes; 25, 10, 5, 2 mL (Sarstedt or Grenier-Bio, Germany), Polypropylene centrifuge tubes; 50 mL, 15 mL, 2 mL, 1 mL, 0.5 mL (Sarstedt or Isolab, Germany) and BrightLine Hemacytometer (Marienfeld, Germany). 0.45 µm Enjector filter (Millipore, Germany).

All chemicals are cell culture grade or sterilized. In this study we used EMEM medium with L-Glutamine, Na-pyruvate and PBS (Lonza, Belgium). Fetal Bovine Serum (FBS) Cell culture tested (Sigma Aldrich, USA), Trypsin-EDTA 10X (Biochrom, Germany or Lonza, Switzerland). Glacial acetic acid, Bovine Serum Albumin, Methanol, 2,2-diphenyl-2-picryl-hydrazyl, L-Glutathione reduced, 1-chloro-2,4-dinitrobenzene, Ethacrynic acid, Ethanol absolute, Quercetin, (±)-α-Tocopherol, (-)-Catechin, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) iammonium salt, (-)-Epicatechin and (-)-Epigallocatechin gallate (Sigma-Aldrich, USA). Gallic acid monohydrate (Acros Organics, USA). Dimethyl Sulfoxide cell culture tested (AppliChem, Germany). Folin Ciocalteu Reagent, Acetonitril gradient grade, Potassium dihyrogen phosphate, Sodium hydroxide, Acetonitrile, Potassium peroxodisulfate, Trifluoroacetic

acid and Formic acid (Merck, Germany). RIPA Buffer (Cell Signalling Technology, USA). Gentamycin Sulfate and Trypan blue solution 5% (Biological Industries, Israel). Nuclease-Free Water (Hyclone, USA). Copper (II) sulfate-5-hydrate and Sodium carbonate (Riedel-de Haen-Honeywell, USA). Ethylenediaminetetraacetic acid (EDTA), Aluminum chloride and Potassium sodium tartrate (Fluka, Switzerland).

2.1.4 Instruments

The instruments used in this study are Laminar flow cabinet (NUVE, Turkey) CO_2 incubator (Heraus, Germany and NUVE, Turkey), Olympus CKX41 Inverted phase contrast microscope and BH-2 Research Microscope (Olympus, Japan), Eppendorf Centrifuge 5810 R (Eppendorf, Germany), WiseMix Vortex (Wisd Laboratory Instruments-Verkon, Czech Republic), pH meter (Mettler Toledo, USA), Spectrophotometer (Schimatsu, Japan or, Multiskan™ GO- Thermo Scientific, USA), Thermal Cycler (Biorad MyCycler, USA and Eppendorf, Germany), Real-time PCR Thermal Cycler (Corbett-QIAGEN, Netherlands), -80 °C freezer (Sanyo -86C ULT Freezer, Japan), -20°C freezer (Ugur, Turkey) +4 refrigerator (Arcelik, Turkey), microplate reader (Multiskan™ GO- Thermo Scientific, USA). AB104-S Analytical Balance (Mettler Toledo, USA), WiseBath Water Bath (Wisd Laboratory Instruments-Verkon, Czech Republic), Shaking Water Bath ST 402 (Nüve, Turkey), Autoclave OT 032 (Nüve, Turkey), Ultrasonic Cleaner (Alex Machine, Malaysia), Microwave (Beko, Turkey), Laminar Flow&Biosafety Cabins Chemocell LRCX-UV (Teknomar, Turkey), Heracell 150 CO_2 incubator (Heraeus-Thermo Scientific, USA), Alpha 1-4 LD Freeze Dryer (Christ, Germany), NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

In this study we used the commercially available kit- Cell Proliferation kit XTT Based (Biological Industries, Israel), Thermo Gene Jet RNA Purification Kit and cDNA Synthesis Kit (Thermo Scientific, USA), FastStart Universal Syber Green ROX (ROCHE, Switzerland).

2.1.5 Primers

CYP1A1, CYP1A2, CYP3A4, CYP2E1, CATALASE, GPX4, GSTM1, GSTP1, and GAPDH, gene primers were designed with NCBI BLAST and purchased from Iontek, İstanbul, Turkey (Table2.1).

Table 2.1 Primer sequences, annealing temperatures and product sizes of the genes.

Primer	Sequences (5´– 3 ´)	Product Size	TM
GAPDH forward	GAGCGAGATCCCTCCAAAAT	197	57.3
GAPDH reverse	GGCTGTTGTCATACTTCTATGG		60.6
CYP1A1 forward	TACCTCAGCAGCCACCTCCAAG	121	64.0
CYP1A1 reverse	GGCCCTGATTACCCAGAATACC		62.1
CYP1A2 forward	ATGCTCAGCCTCGTGAAGAAC	96	59.8
CYP1A2 reverse	GTTAGGCAGGTAGCGAAGGAT		59.8
CYP3A4 forward	CCTTACATATACACACCCTTTGGAAG	100	61.6
CYP3A4 reverse	GGTTGAAGAAGTCCTCCTAAGCT		60.6
CATALASE forward	GATAGCCTTCGACCCAAGCA	120	59.4
CATALASE reverse	ATGGCGGTGAGTGTCAGGAT		59.4
GPX4 forward	GAGGCAAGACCGAAGTAAACTAC	100	60.6
GPX4 reverse	CCGAACTGGTTACACGGGAA		59.4
GSTM1 forward	GAACTCCCTGAAAAGCTAAAGC	148	58.4
GSTM1 reverse	GTTGGGCTCAAATATACGGTGG		60.3
GSTP1 forward	CCTACACCGTGGTCTATTTCCC	136	62.1
GSTP1 revers	CAGGAGGCTTTGAGTGAGC		58.8
CYP2E1 forward	AGCGCTGCTGGACTACAAGG	184	61.4
CYP2E1 reverse	CCTCTGGATCCGGCTCTCAT		61.4

2.2 Methods

2.2.1 Plant Extraction- Sample Preparation

S. absconditiflora leaves were harvested on the same day (Monday) and hour (11.30 am) with 1 week intervals for 3 months.

The *Salvia* leaves had been cleaned with tap water for several times and then with distilled water. They were dried under the blotting paper with the indirect sunlight. With mortar and pestle, the dried samples become powdered. The water and methanol extracts of dried Salvia leaves were prepared with Soxhlet device. Five gram of each leaves were extracted. Two days after, extracts were obtained. Firstly methanol was removed by rotary evaporator and then resuspended with double distilled water. Extracts had been freezed 1 day in - 80 ºC and then dried by Freeze Dryer for 3 days. Extraction yield was determined by the equation showing below.

Percent extraction (w/w) = (Mass of dried extract / mass of total leaves) X 100

2.2.2 Determination of Antioxidant Capacity

2.2.2.1 Free Radical Scavenging Capacity- DPPH Assay

Free radical scavenging activity of *S.absconditiflora* extracts were determined by DPPH methods according to Blois' method (1958) with some modification. 0.05 mg/mL DPPH solution was prepared with ethanol and sonicated 30 min before use. 10 µL of each extracts (serial diluted 0.1-1 mg/mL) were mixed with 140 µL DPPH solution in 96 well plate with triplicate and plate was shaken vigorously. After 20 minute absorbance changes was determined at 517 nm by using MultiSkan^{TM} Go Elisa Plate Reader. Quercetin was used as a reference. % RSA was calculated using the following equation:

$$\% \text{ RSA (Radical scavenging activity)} = [(A_0 - A_1) / A_0] \times 100$$

A_0 : Absorbance of DPPH and dH2O mixture
A_1 : Absorbance of sample with DPPH

% RSA versus final concentrations of the extracts (mg/mL) were plotted and IC_{50} (50% effective concentration) value was calculated.

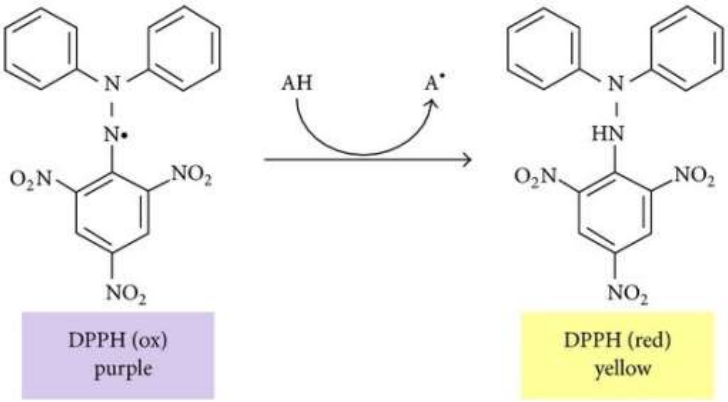

Figure 2.2 Principle of DPPH radical scavenging activity assay (Teixeira et al., 2013

2.2.2.2 Free Radical Scavenging Capacity - ABTS Assay

ABTS, free radical scavenging capacity was determined according to Re et al., (1999) with some modifications. Firstly, ABTS solution (7mM) was reacted with potassium persulfate solution ($K_2S_20_8$) to produce ABTS radical cation ($ABTS^{\bullet+}$). Then the mixture was kept in dark in room temperature for 12–16 h before using. Seven mM ABTS was mixed with 2.45mM KPS 1:1 ratio. Finally, $ABTS^{\bullet+}$ solution was diluted with 1:120 or 1:60 of methanol to an approximate absorbance unit of 0.70 (±0.02) at 734 nm.

Trolox was used as standard in this reaction. 2.5 µL of each extracts or standard solution were added to the diluted 250 µL of $ABTS^{\bullet}$ solution in 96 well plate with triplicate and mixed. After 6 min, absorbance was monitored at 734 nm using Multiskan™ GO microplate Reader (Thermo Scientific, USA). Trolox equivalent antioxidant capacity (TEAC) values were used to show free radical scavenging capacity. Percent inhibition was calculated using the formula showing below;

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100$$

A_0 : Absorbance of ABTS a nd dH2O (2.5 µl) mixture
A_1 : Absorbance of sample with ABTS

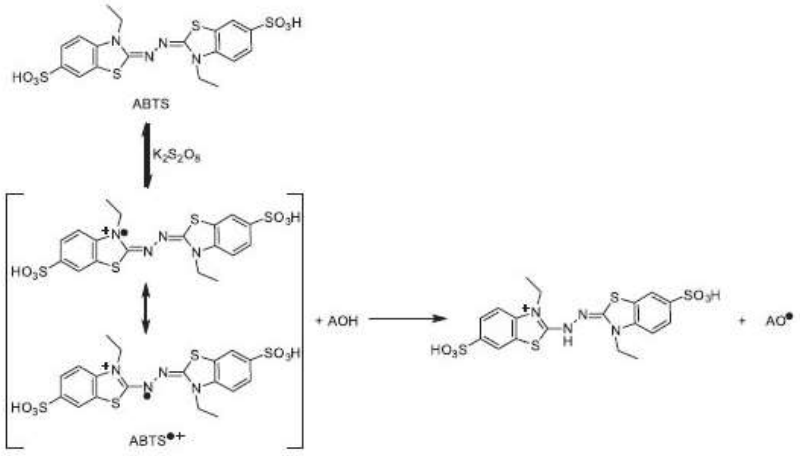

Figure 2.3 Oxidation of ABTS by potassium persulfate to generate radical cation $ABTS^{\bullet+}$ and its reaction with an antiradical compound.

2.2.3 Determination of Total Phenolic Contents (TPC) of Salvia Extracts

TPC of the *S. absconditiflora* extracts were determined by Folin–Ciocalteu method of Singleton et al. (1999) with some modifications. Twenty µL of the extracts were mixed with 100 µL of 1:4 diluted % Folin-Ciocalteu's phenol reagent and mixed by pipetting. And then 80 µL of 10% sodium carbonate solution was added in the 96 well plate with triplicate and shaken vigorously. After 30 minutes of incubation at room temperature, read the absorbances at 750 nm by using plate reader against blank that contain 20 µL ethanol instead of sample.

Gallic acid (GA) was used as a standard. GA versus absorbance plot was drawn and the equation was used to calculate milligrams of total phenolics (TP) per gram of extract, as the gallic acid equivalents (GAE).

2.2.4 Determination of Total Flavonoid Contents (TFC) of Salvia Extracts

TFC contents in the extracts were determined by aluminum chloride colorimetric assay (Zhishen et al., 1999) with some modifications. Quercetin and Catechin (+) were used as standards for the determination of TFC. During the experiment, they were dissolved in ethanol (99.5%) and varying concentrations of standards were prepared with serial dilutions (75 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 750 µg/mL).

Freeze-dried *S.absconditiflora* extracts were dissolved in dH_2O. 0.5 mg/mL, 1 mg/mL and 2 mg/mL were prepared by serial dilutions. Twenty µL of each extract or standard solutions were added into 96 well plate containing 80 µL of distilled water and then 6 µL of 5% sodium nitrite ($NaNO_2$) was added to the wells. Five minutes later, 6 µL of 10 % aluminium chloride ($AlCl_3$) solution was added. After waiting 6 minutes, 40 µL of 1 M sodium hydroxide (NaOH) was added and the total volume was completed up to 200 µL with distilled water. The absorbances were measured against blank at 510 nm using Multiskan™ GO microplate reader (Thermo Scientific, USA). Results were calculated by using the standard curve and were recorded as milligrams of total flavonoids in gram of extracts, as the quercetin equivalent (QE) and catechin equivalents (CE). Analyses were run in three replicates and expressed as average values with standard deviations.

2.2.5 Determination of Metal Chelating Activity

The iron-chelating abilities of the *S.absconditiflora* extracts were determined by the method of Dinis et al (1994) with some changes. EDTA (Ethylenediaminetetraacetic acid) was used as a standard chelating agent. Forty µL of different concentrations of extracts (2, 4, 6, 8, 10 mg/mL) and standards (0.1–5 mM) were added to 96 well microplate wells and mixed with 8 µL ferrozine (5 mM), 4 µL iron (II) chloride (2 mM) and 148 µL

absolute methanol. After 10 min incubation at room temperature, absorbance values were read at 562 nm, and IC_{50} values were calculated.

2.2.6 Determination of antibacterial activities

The antibacterial activities of *S. absconditiflora* extracts were determined by using disc diffusion susceptibility method (Bauer et al. 1966).

In this method we used *Escherichia coli* T. Escherich, *Bacillus licheniformis*, *Staphylococcus aureus* Rosenbach, *Bacillus subtilis* (Ehrenberg) Cohn, and *Agrobacterium tumefaciens* Smith and Townsend. Tetracycline, gentamicin and penicillin were used as the standard antibiotic discs. Müller Hilton Agar was used as a growth media. Microorganisms were grown at 35°C and 28°C for 18 h and then cell density were adjusted according to 0.5 McFarland standards.

Adjusted microorganism suspension (100 µL) was inoculated onto Müller Hilton Agar by spread plate technique. *S. absconditiflora* extracts were applied to the discs 20 µl each side of disc (total 40 µL) and standard antibiotic discs were placed on inoculated petri dishes. Negative controls were prepared with their solvent. Inhibition zones (IZ) around the discs after overnight incubation were measured to determine antibacterial activities of the extracts. The study was performed in triplicates for each sample.

2.2.7 RP- HPLC Analysis of phenolic compounds

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) is the most popular technique used to separate, identify and quantify each component in a mixture. To identify and quantify of different phenolic compounds in *S. absconditiflora* water and methanol extracts, serially diluted standard solutions were used to obtain the calibration curves. Recovery was determined for the overall assay by adding known amounts of standards on the original concentration of the analyzed samples. Three HPLC replicate injections were performed for standards and extracts. All *S.*

absconditiflora extract solutions were filtered through a 0.45 μm membrane filter (Millipore, Milford, MA). Twenty μL of each filtrated sample was injected into HPLC for analysis.

2.2.7.1 RP-HPLC Conditions

The analysis of phenolic compounds was carried out by Reverse Phase (RP)-HPLC. Gradient elution for rosmarinic acid and caffeic acid content of *S. absconditiflora* was carried out at a flow rate of 1.0 mL/min at 30°C. Two different mobile phases were used. Mobile phase A was 0.1% (v/v) formic acid solution in water, while mobile phase B was acetonitrile. A ratio of 88% A and 12% B was applied in the first 30 min. After 30 min, a ratio of 80% A and 20% B was used for the next 15 min. Finally, 70% A and 30% B were used after 45 min for an additional 15 min. The HPLC equipment consisted of a Shimadzu LC-20AD system including DGU-20A5 prominence degasser, SIL-20AHT prominence auto sampler, SPD-M20A prominence UV-Vis photodiode array detector, CTO-20A prominence column oven. The column was an Agilent Zorbax SB-C18 (250 mmx 3 mm, 5 μm). Data was processed by using LC Solution Programme.

Solutions of pure standards such as rosmarinic acid, gallic acid, caffeic acid, quercetin and t-resveratrol were chromatographed as external standards. All standards were dissolved in HPLC grade methanol before injection. Their ranges of concentration used were 0.7–15.0 mg/L. Phenolic compounds of extracts were defined by comparing their retention times with those of pure standards. The results were clarified as % of each compound from the total phenolic compounds.

2.2.8 Identification of phenolic compounds with LC-MS/MS

Mass spectrometry (MS) technique is based on the measurement of mass to charge ratio by ionizing chemical compounds hence production of charged molecules (Ignat et al., 2011).

LC-MS/MS analysis was performed by Ceren Biler and Ürün Duru in Central Laboratory of METU. LC-MS/MS equipments and conditions are shown in Table 2.2.

Ten different standards (10 ppm) were used and *S. absconditiflora* freeze-dried water and methanol extracts were weighed and dissolved in MS grade methanol and became 1 g/mL at final concentration. Then 3 (different months) water extracts were combined together and their names were total water and all methanol extracts were combined and the name was total methanol extract.

Table 2.2 LC-MS/MS equipments and conditions**.**

Mass spectrometry	
Device	AGILENT 6460 LCMSMS
Ionization source	ESI+ Agılent Jet Stream
Pump	AGILENT BinPump-SL (G1312B9)
Automatic Sampler	AGILENT h-ALS-SL +(G1367D)
Database	AGILENT G3793AA MassHunter Optimizer software
Nitrogen Generator	Nitrogen generator UHPLCMS 30
Analysis Mode	MRM
Gas Temperature	300^0C
Gas Flow	10 Ml/ min
Sheath gas temperature	350^0C
Capillary	4000 V (+,-)
Nozzle Voltage	500 V (+,-)
Liquid Chromatography	
Column	Zorbax SB-C18(2,1x50 mm x 1,8µ)
Mobile phase	Solvent A: 0,05% Formic acid+ 5mMAmonium format (MilliQ water) Solvent B: Methanol (MS grade, MERCK)
Flow rate	0,3 ml/min
Analysis time	13 min
Mobile phase flow mode	Graduated
Injection volume	5µL
Range of standard curve	0.01-0.025-0.05-0.1-0.5-1-5-10 ppm

2.2.9 Cell Culture

2.2.9.1 Cell Culture Conditions

HepG2; adherent, epithelial like, hepatocellular carcinoma cell lines were used. These cells were obtained from American Cell Culture Collection (ATCC; USA). They were grown in EMEM (formulation was shown in Figure 2.4) supplemented with 1% L- Glutamine, 10% fetal bovine serum, 0.2 % Gentamycin Sulfate and 1% Na-pyruvate. The medium were changed every 2-3 days. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Table 2.3 Formulation of EMEM Medium

MEM Eagle with Earle's BSS w/o L-Glutamine		12-125	LONZA			
			Concentration		Molarity	
Description	CAS #	Chemical Formula	g/L	mg/L	mM	uM
Calcium Chloride Anhydrous	10043-52-4	CaCl2	0,2	200	1,802	1,80E+03
Dextrose	50-99-7	C6H12O6	1	1,00E+03	5,551	5,55E+03
Magnesium Sulfate Anhydrous	7487-88-9	MgSO4	9,77E-02	97,67	0,811	811,415
Potassium Chloride	7447-40-7	KCl	0,4	400	5,366	5,37E+03
Sodium Bicarbonate	144-55-8	NaHCO3	2,2	2,20E+03	26,187	2,62E+04
Sodium Chloride	7647-14-5	NaCl	6,8	6,80E+03	116,359	1,16E+05
L-Arginine Monohydrochloride	1119-34-2	C6H14N4O2.HCl	0,127	1,27E+02	0,603	6,03E+02
L-Histidine Monohydrochloride Monohydrate	5934-29-2	C6H9N3O2.HCl.H2O	4,20E-02	4,20E+01	0,2	2,00E+02
L-Isoleucine	73-32-5	HO2CCH(NH2)CH(CH3)CH2CH3	5,20E-02	5,20E+01	0,396	3,96E+02
L-Leucine	61-90-5	HO2CCH(NH2)CH2CH(CH3)2	5,20E-02	5,20E+01	0,396	3,96E+02
L-Lysine Monohydrochloride	657-27-2	C6H14N2O2.HCl	7,25E-02	7,25E+01	0,397	3,97E+02
L-Methionine	63-68-3	HO2CCH(NH2)CH2CH2SCH3	1,50E-02	1,50E+01	0,101	1,01E+02
L-Phenylalanine	63-91-2	HO2CCH(NH2)CH2C6H5	3,20E-02	3,20E+01	0,194	1,94E+02
L-Threonine	72-19-5	HO2CCH(NH2)CH(OH)CH3	4,80E-02	4,80E+01	0,403	4,03E+02
L-Tryptophan	73-22-3	C11H12N2O2	1,00E-02	1,00E+01	4,90E-02	4,90E+01
L-Valine	72-18-4	HO2CCH(NH2)CH(CH3)2	4,60E-02	4,60E+01	0,393	3,93E+02
D-Calcium Pantothenate (Vitamin B5)	137-08-6	C18H32CaN2O10	1,00E-03	1,00E+00	2,10E-03	2,10E+00
Choline Chloride	67-48-1	HOCH2CH2N(CH3)3Cl	1,00E-03	1,00E+00	7,16E-03	7,16E+00
Folic Acid	59-30-3	C19H19N7O6	1,00E-03	1,00E+00	2,27E-03	2,27E+00
I-Inositol	87-89-8	C6H12O6	2,00E-03	2,00E+00	1,11E-02	1,11E+01
Niacinamide (Nicotinamide)	98-92-0	C6H6N2O	1,00E-03	1,00E+00	8,19E-03	8,19E+00
Pyridoxine Monohydrochloride	58-56-0	C8H11NO3	1,00E-03	1,00E+00	4,86E-03	4,86E+00
Riboflavin (Vitamin B2)	83-88-5	C17H20N4O6	1,00E-04	1,00E-01	2,66E-04	2,66E-01
Thiamine Monohydrochloride (Vitamin B1)	67-03-8	C12H18N4OSCl2	1,00E-03	1,00E+00	2,97E-03	2,97E+00
Phenol Red	34487-61-1	C19H14O5S	1,00E-02	1,00E+01	2,66E-02	2,66E+01
L-Tyrosine Disodium Salt, Dihydrate	122666-78-9	C9H9NO3Na2.2H2O	5,19E-02	5,19E+01	1,99E-01	1,99E+02
L-Cystine Dihydrochloride	30925-07-6	C6H12N2O4S2.2HCl	3,13E-02	3,13E+01	9,99E-02	9,99E+01
Sodium Phosphate Monobasic, Anhydrous	7558-80-7	NaH2PO4	1,22E-01	1,22E+02	1,02E+00	1,02E+03

2.2.9.2 Cell Thawing

Before thawing the cells, complete growth medium was warmed to 37°C in water bath. Nine mL of pre-warmed growth medium was taken and transferred into 15 mL sterile falcon tube. After that, cryovials were immediately transferred into a +37 °C water bath for 1-2 min and small ice crystals should remain when removed from the water bath. Outside of the cryovial were cleaned with %70 ethonol absorbant paper. Semi-thawed cell suspension was tranferred into a 15 mL sterile Falcon tube containing the growth medium. Then cell suspension was slowly mixed with gentle shaking. The tube was centrifuged at 800*xg* for 5 min and then the supernatant was discarded. The cell pellets were resuspended in growth media and the cells were seeded on T-25 flask. Cells were incubated in CO_2 incubator at 37°C. The following day, culture media was removed and the cells were washed twice with PBS, pH 7.4. After washing, normal trypsinization procedure was done and cell suspension was transferred to a T75 flask.

2.2.9.3 Cell Passaging/ Trypsinization

The cells were passaged when the confluency reached to minimum 80%. First of all, old medium was removed and then cells were washed twice with 5mL PBS solution. After washing 1 mL Trypsin/EDTA solution was added into a T25 Flask or 2 mL Trypsin/EDTA solution were added into a T75 Flask and then the flasks were incubated at 37°C with 5% CO_2 humidified incubator. In order to detach the cells, 5-8 min. were enough for HepG2 cells. Three volumes of complete medium was added into the culture flasks to get rid of the activity of trypsin. The cells were splited in a 3 new flask for every trypsinization procedure.

2.2.9.4 Cell Freezing

After normal trypsinization procedure, cell suspension was centrifuged at 1000*xg* for 5 min. The supernatant was removed and the cell pellet was resuspended in freezing medium. HepG2 cell freezing medium contains 90% (v/v) FBS and 10% (v/v) dimethylsulfoxide (DMSO). Cells were dissolved in freezing medium and then immediately put into in cryovials, then they were placed into Mr Frosty which has isopropanol in it. It was transferred to -80°C in Mr Frosty. One day after, cryovials were transferred into liquid nitrogen for long term storage.

2.2.9.5 Viable Cell Counting- Growth curve

Determining cell number and viability with a hemacytometer and trypan blue staining

HepG2 cells were seeded in 6-well plates at a density of $25x10^4$ cells/mL and 2 mL in each well. And then 6 well plates were placed into the humidified CO_2 incubator at 37°C. After 24 h, the medium of two duplicate wells were removed and washed with 1 mL of PBS. Cells were detached with 0.5 mL of trypsin and in order to inactivate trypsin, 1 mL of fresh complete medium was added to the wells. Cell suspensions were transferred into 2 seperate eppendorf tubes. After cell detachment, 1 volume of cell solution was added to 1 or 9 volume of Trypan Blue Solution (0.25 M) which was used to analyze cell viability. Cell counting was carried out using a hemocytometer (Figure 2.5) under light microscopy. Viable cells don't contain blue color whereas dead cells appear blue.

The hemocytometer contains nine squares, each of which is 1 mm^2 (Figure 2.4; A). The actual counting area contains 25 large squares (Figure 2.4; B, C, D, E, F) and each of them has 16 smaller squares (Figure 2.4; G). The cell counting take place in this 25 large squares. The area of 25 large

squares and each corner square is 1 mm x 1 mm = 1 mm^2: the depth of each square is 0.1 mm. According to that total cell concentration can be multiplied by 10000. The cells were counted for 5 to 6 different times and then average of these numbers was used to calculate the number of cells per mL using the formula:

Total Cells/mL= (Total Cell Counted x Dilution Factor x 10^4) x #of Squares

The cells were counted during 15 days and the growth curves for the HepG2 cell line were plotted with the number of cells against time. The ratio of cell suspension and trypan blue changed depending on the number of the cells.

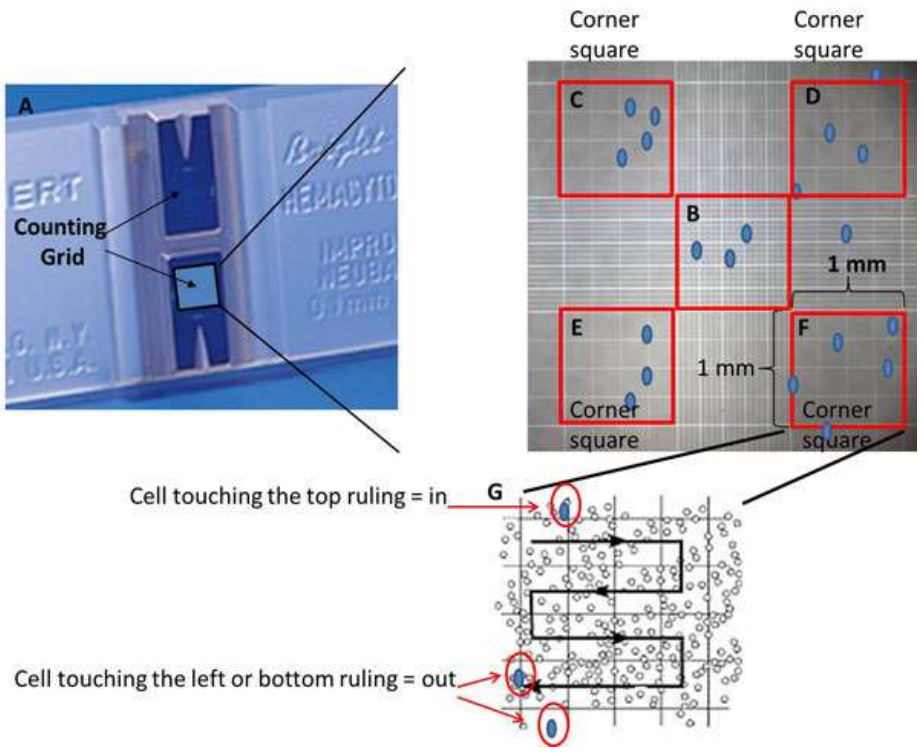

Figure 2.4 Hemocytometer chamber

2.2.10 Cytotoxicity

2.2.10.1 Preparation of Crude Extract of *S.absconditiflora,* Rosmarinic Acid and Caffeic Acid Solutions For Treatments

S. absconditiflora crude water and methanol extracts and 2 main active components; caffeic acid (CA) and rosmarinic acid (RA) were used as cytotoxic agents on the proliferation of HepG2 cells. Two different serial dilutions were done according to type of the extracts. Twenty mg crude water extracts were dissolved in 2 mL 0.2 % DMSO containing EMEM medium with phenol red (10 mg/mL) and 12 mg crude methanol extracts were dissolved in 2 mL of the same medium. Working extract solutions between 1 mg/mL to 10 mg/mL for water extracts and 0.5 to 6 mg/mL for methanol extracts were prepared by diluting the stock solutions with sterile water. DMSO concentration was kept constant because of its own cell toxicity. Final crude extract concentrations were varied from 0.5 mg/mL to 5mg/mL for water extracts and 0.25 mg/mL to 3 mg/mL for methanol extracts in each well during the treatments.

RA and CA solutions at different concentrations (from 100 μg/mL to 1000 μg/mL) were prepared by diluting the stock working solutions in 0.2 % DMSO containing EMEM medium containing phenol red. Final concentrations of RA and CA were varied from 50 to 500 μg/mL in each well during the treatments. Finally, mixtures of RA and CA working solutions of varying concentrations (from 100 μg/mL to 1000 μg/mL) were prepared by diluting the stock working solution in 0.2 % DMSO containing EMEM medium with phenol red (RA: CA; 1:1 ratio). Final concentrations of the mixture were also varied from 50 μg/mL to 500 μg/mL in each well during treatments.

2.2.10.2 XTT Cell Proliferation Assay

XTT- tetrazolium dye (2, 3 -Bis- (2-Methoxy- 4-Nitro- 5-Sulfophenyl)- 2*H*-Tetrazolium- 5-Carboxanilide) cell proliferation kit can be effectively used in cell proliferation, cytotoxicity, and apoptosis assays. Living cells convert the XTT to a water-soluble, orange colored formazan product by the mitochondrial dehydrogenase activity (Figure 2.5). The sensitivity of the XTT assay is showed to be similar to or better than the other assay. In order to obtain usable absorbance values, phenazine methosulfate (PMS) is used.

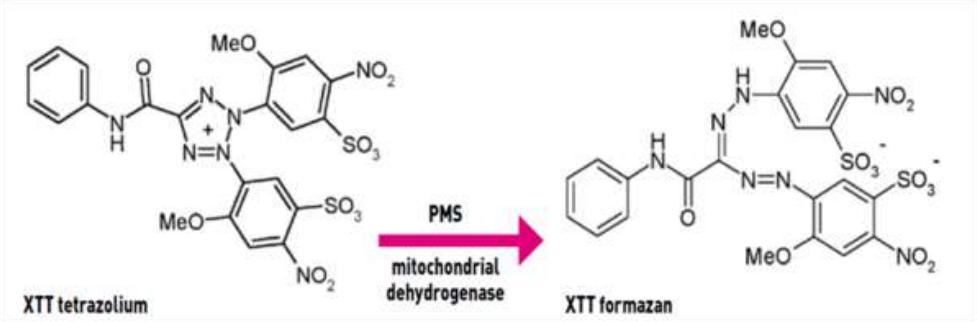

Figure 2.5 Enzymatic conversion reaction of XTT into colored formazan

The cells were cultured in a 96 well plate (100.000 cells/mL) as 100µL per well. After incubation in CO_2 for 24-h, the medium in the wells were removed and wells were washed twice with 50µL PBS. Fresh complete medium (50µL) was added in all wells. Afterwards, 50µL growth medium was added again in to second row and 50µL 0.2% DMSO were added in to third row as a control. Then 50 µL serial diluted extracts (Figure 2.6) which were in increasing order from left to right, were added in to the wells except for first three columns. The diagram of the 96-well plates for XTT assay was shown in Figure 2.6 for water extracts; XTT assay was shown in Figure 2.7 for methanol extracts and it was shown in Figure 2.8 for 2 standards and their combination. After 48 and 72-h incubation, XTT was applied to the cells. After 10 h, the absorbance values were measured at 415 nm with

microplate reader to calculate the percent viability of the cells. In all three figures B2 to F2, complete medium control, 100 µL; C2 to F2, 0.1% DMSO medium control, prepared as 50 µL 0.2% DMSO medium+50 µL complete medium, B4 to B11 and F4 to F11 *S.absconditiflora* extract concentrations. B2 to 11 and C2 to C11 columns are without the cells.

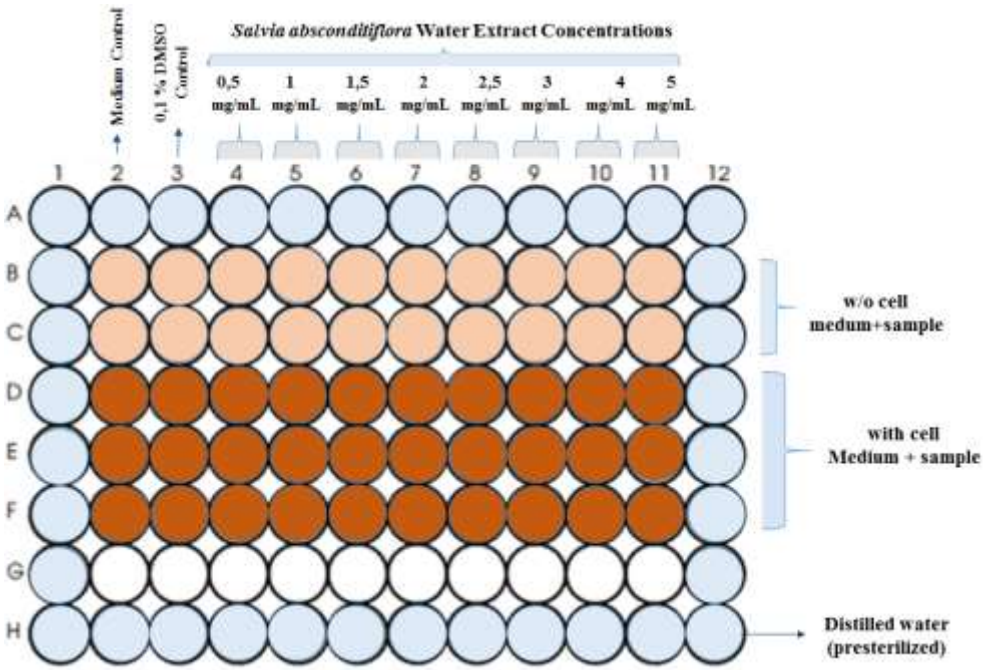

Figure 2.6 96-well plate representation of the XTT assay for water extracts.

B4 to *F4,* 0.5 mg/mL extract, prepared as 50 µL 1 mg/mL *S.absconditiflora* water extract + 50 µL complete medium; *B5* to *F5,* 1 mg/mL extract, prepared as 50 µL 2 mg/mL *S.absconditiflora* water extract + 50 µL complete medium; *B6* to *F6,* 1.5 mg/mL extract, prepared as 50 µL 3 mg/mL *S.absconditiflora* water extract + 50 µL complete medium; *B7* to *F7,* 2 mg/mL, prepared as 50 µl 4 mg/ml *S.absconditiflora* water extract + 50 µL complete medium; *B8* to *F8,* 2,5 mg/mL extract, prepared as 50 µL 5 mg/mL *S.absconditiflora* water extract + 50 µL complete medium; *B9* to *F9*, 3 mg/mL extract, prepared as 50 µL 6 mg/mL *S.absconditiflora* water extract + 50 µL complete medium; *B10* to *F10,* 4 mg/mL extract, prepared as 50 µL 8 mg/mL *S.absconditiflora* water extract + 50 µL complete

medium; *B11* to *F11*, 5 mg/mL extract, prepared as 50 µL 10 mg/mL *S.absconditiflora* water extract + 50 µL complete medium.

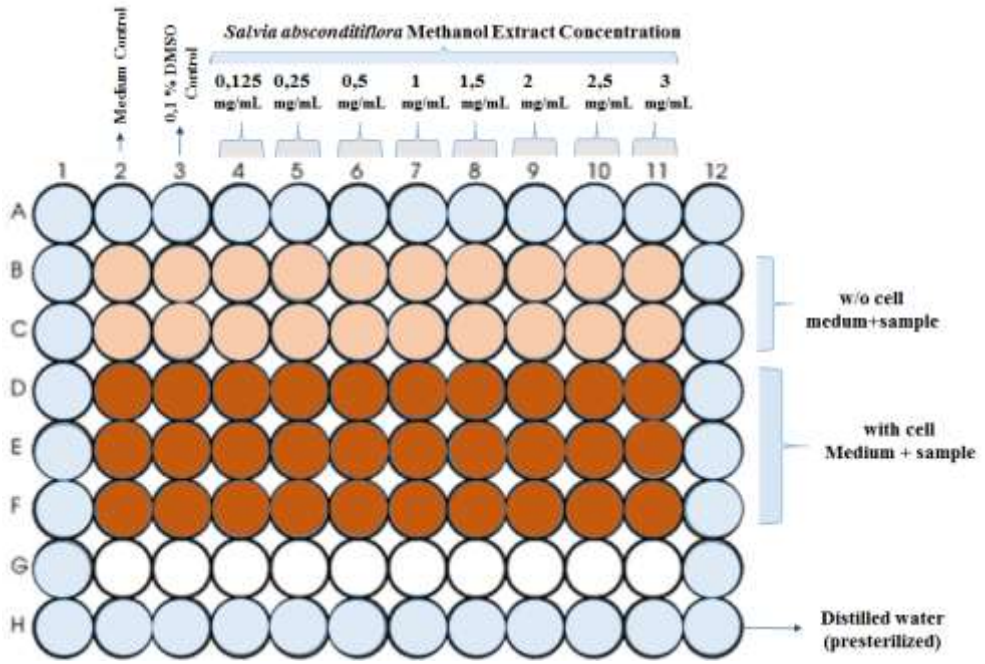

Figure 2.7 96 well plate representation of XTT assay for methanol extracts

B4 to *F4*, 0.125 mg/mL extract, prepared as 50 µL 0.25 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B5* to *F5*, 0.25 mg/mL extract, prepared as 50 µL 0.5 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B6* to *F6*, 0.5 mg/mL extract, prepared as 50 µL 1 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B7* to *F7*, 1 mg/mL, prepared as 50 µL 2 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B8* to *F8*, 1.5 mg/mL extract, prepared as 50 µL 3 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B9* to *F9*, 2 mg/mL extract, prepared as 50 µL 4 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B10* to *F10*, 2.5 mg/mL extract, prepared as 50 µL 5 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium; *B11* to *F11*, 3 mg/mL extract, prepared as 50 µL 6 mg/mL *S.absconditiflora* methanol extract + 50 µL complete medium.

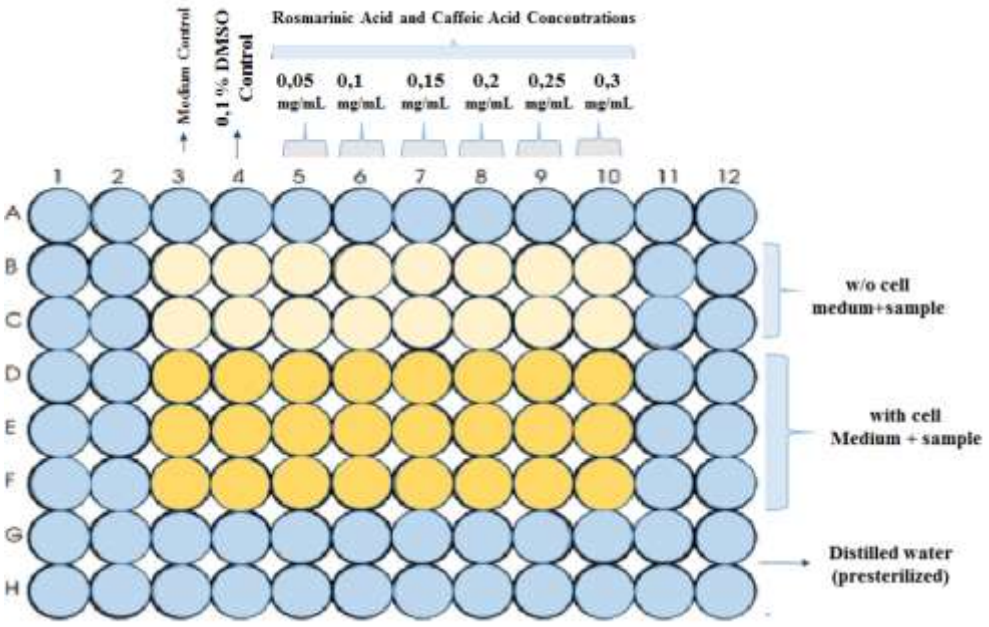

Figure 2.8 96 well plate representation of XTT assay for standards

B5 to *F5,* 50 µg/mL standard solution (RA,CA or RA+CA), prepared as 50 µL 100 µg/mL standard solution + 50 µL complete medium; *B6* to *F6,* 100 µg/mL standard solution, prepared as 50 µL 200 µg/mL standard solution + 50 µL complete medium; *B7* to *F7,* 150 µg/mL standard solution, prepared as 50 µL 300 µg/mL standard solution + 50 µL complete medium; *B8* to *F8,* 200 µg/mL standard solution, prepared as 50 µL 400 µg/mL standard solution + 50 µL complete medium; *B9* to *F9*, 250 µg/mL standard solution, prepared as 50 µL 500 µg/mL standard solution + 50 µL complete medium; *B10* to *F10*, 300 µg/mL standard solution, prepared as 50 µL 600 µg/mL standard solution + 50 µL complete medium.

2.2.11 Total RNA Isolation

2.2.11.1 Counting of cell for RNA isolation- Calculation of IC50 values

Cells were seeded into a 6 well plate with duplicate at a density of $25x10^4$ cells/mL and 2 mL in each well. After one day, worn medium was changed

and different concentrations of extract were applied to the cells. Two hour incubations were used in order to calculate IC_{50} values.

After 48 and 72-h, cells were trypsinized and counted with trypan blue. After counting the cell, IC_{50} values for 48 and 72 h incubations were obtained.

2.2.11.2 RNA Isolation

Cells were seeded into a 65x15 mm culture flask as duplicate at a density of $25x10^4$ cells/mL and 6 mL were loaded to each plate. After 1 day, worn medium was discarded and samples were added in it. Cells were cultured for 48 and 72-h and then RNA isolation is carried out according to Thermo Scientific GeneJET RNA Purification Kit as described according to the manifacturer. The worn medium was removed and cells were rinsed with PBS to remove residual medium and then PBS was discarded. The cells from the culture plate were detached by normal trypsinization. Cells were transferred into a microcentrifuge tube and centrifuged at 250*xg*. for 5 min. Supernatant was discarded and the cells were resuspend with PBS. Second centrifugation was done in same conditions and PBS was discarded. Cells were resuspended in 600 µL of lysis buffer; add 20 µL of β-mercaptoethanol to each 1 mL volume of lysis buffer. Microcentrifuge tube was vortexed for 10 s to mixed thoroughly and 360 µL of 99.5 % ethanol were added and mixed with the sample by pipetting.

Seven hundred µL of lysate was transferred to the RNA Purification Column inserted in a collection tube then tube was centrifuged for 1 min at 12000 *xg*. Flow-through was removed and placed the column back into the collection tube and repeated this step until all of the lysate has been transferred into the column and centrifuged.

The collection tube was removed with solution and placed the column into a new 2 mL collection tube then add 700 µL of Wash Buffer 1 to column and

centrifuged at 12500 *x g* for 1 min. The flow through was discarded and placed the column into the same collection tube.

600 µL of Wash Buffer 2 were added in to the column and centrifuged for 1 min at 13,000 × g., discarded the flow through and placed the purification column into the new collection tubes. 250 µL of Wash Buffer 2 was added again to the column and centrifuge at 13.500 *x g* for 2 min.

The collection tube with flow-through solution was discarded and the column was transferred to a sterile 1.5 mL RNase-free microcentrifuge tube and 50-100 µL of nuclease free waterwas added to the center of the column membrane, then centrifuge at 14.000 *x g* for 2 min to elute RNA.

2.2.11.3 Gel Electrophoresis of Total RNA

RNA gel electrophoresis was done in order to show the purity and accuracy. In the presence of an electric current, RNA migrate toward the anode during gel electrophoresis due to their negatively charge.

RNA samples were heated at 70^{o}C and run on 1.2% agarose / 0.5XTBE gel.

Twenty µL of sample was loaded in to wells with RNA loading dye. Seventyfive mA and 80 volts electric field are applied for 60 minutes. Gel photograph is obtained with UV-imaging system.

2.2.11.4 Quantification of RNA

The isolated RNA concentrations and purity were determined by using NanoDrop™ 2000 spectrophotometer (Thermo Scientific). Nuclease free water was used as blank and RNA samples were measured at 260 and 280nm. RNA concentration was calculated from equation showing below:

$$\textit{Concentration of RNA sample (ng/µL)} = 40 \times A_{260} \times \textit{Dilution factor}$$

As proteins give absorbance at 280 nm, the protein contamination in RNA samples was measured by calculating the A260/A280 ratio. The ratio of A260/A280 must be between 1.8 and 2.2. Below 1.8 refers the protein contamination while above 2.2 referring the DNA contamination.

2.2.12 Polymerase Chain Reaction

2.2.12.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

In order to synthesize complementary DNA (cDNA) from total RNA, reverse transcriptase polymerase chain reaction was carried out using Thermo RevertAid RT Kit (Thermo Scientific, USA). 1000 ng total RNA was used as a starting material. We calculate the RNA concentration and then 1000 ng equals to which µL were taken. In PCR tubes, 1000 ng total RNA was mixed with 1 µL Oligo $(dT)_{18}$ primer in total volume of 12 µL. And then, 4 µL 5X Reaction Buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2 µL 10 mM dNTP Mix and 1 µL RevertAid RT (200 U/µL) were added. The samples were then incubated for 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min. After incubation, samples were stored at -20°C.

2.2.12.2 Quantitative Polymerase Chain Reaction (qRT-PCR)

The mRNA expression levels of 4 phase I genes (CYP1A1, CYP1A2, CYP2E1 and CYP3A4), 2 phase II genes (GSTM1 and GSTP1) and 2 antioxidant enzymes genes (GPx4 and Catalase) in the HepG2 cell lines with different *Salvia* extract were determined using qRT-PCR (Corbett-QIAGEN, Netherlands) Each reaction mixture containing 5 µL FastStart Universal SYBR Green Master- ROX (Roche Diagnostic, Germany), 0.2 µL each primer (10 uM) and 1.6 µL cDNA in a total volume of 10 µL completed to nuclease free water as indicated in Table 2.3. Reaction was performed in conditions given in Table 2.4.

Table 2.4 qRT - PCR components

Components	Reaction Volume (µl)
SYBR Green PCR master mix	5 µL
Forward Primer (10µM)	0.2 µL
Reverse Primer (10 µM)	0.2 µL
cDNA	1.6 µL
RNAse free water	3 µL
Total volume	10 µL

cDNAs were diluted 50X for GSTM1, GSTP1, GPx4 and Catalase and 15X for CYP1A1, CYP1A2, CYP2E1 and CYP3A4. Standard cDNA were diluted 1/5, 1/10, 1/20, 1/40 and 1/80.

Table 2. 5 Quantitative Real Time PCR Conditions

	Cycles	**Segment**		**Temperature (°C)**	**Time**
Preincubation		1		95	10 min
Amplification	40	Denaturing		95	20 sec.
		Annealing	GAPDH	58	30 sec
			CYP1A1	60	
			CYP1A2	60	
			CYP2E1	61.5	
			CYP3A4	65	
			GSTM1	60	
			GSTP1	60.5	
			GPx4	60	
			Catalase	59.5	
		Elongation		72	20 sec

2.2.12.3 Real Time Panel For qRT-PCR

Real time panel microplate containing 88 targeted and 8 housekeeping gene primer sets (20µL per well, 10uM concentration). There are 75 RNA panel are found commercially. We selected 2 of them; Drug detoxification I and II. Targetted genes were shown in Table 2.5 and Table 2.6

Table 2.6 Human detoxification panel I list (BioRad, Germany)

HDTX-I

A1	AADAC	C1	CYP11A1	E1	CYP2J2	G1	FMO3
A2	ADH1A	C2	CYP11B1	E2	CYP2R1	G2	FMO4
A3	ADH1B	C3	CYP11B2	E3	CYP2S1	G3	FMO5
A4	ADH1C	C4	CYP17A1	E4	CYP2W1	G4	GZMA
A5	ADH4	C5	CYP19A1	E5	CYP3A4	G5	GZMB
A6	ADH5	C6	CYP1A1	E6	CYP3A43	G6	HSD17B1
A7	ADH6	C7	CYP1A2	E7	CYP3A5	G7	HSD17B10
A8	ADH7	C8	CYP1B1	E8	CYP3A7	G8	HSD17B2
A9	ALDH1A1	C9	CYP21A2	E9	CYP4A11	G9	HSD17B3
A10	ALDH1A2	C10	CYP24A1	E10	CYP4A22	G10	MAOA
A11	ALDH1A3	C11	CYP26A1	E11	CYP4B1	G11	MAOB
A12	ALDH1B1	C12	CYP26B1	E12	CYP4F11	G12	PTGS1
B1	ALDH2	D1	CYP26C1	F1	CYP4F12	H1	PTGS2
B2	ALDH3A1	D2	CYP27A1	F2	CYP4F2	H2	UCHL1
B3	ALDH3A2	D3	CYP27B1	F3	CYP4F3	H3	UCHL3
B4	ALDH3B1	D4	CYP2A13	F4	CYP4F8	H4	XDH
B5	ALDH3B2	D5	CYP2B6	F5	CYP7A1	H5	ACTB
B6	ALDH4A1	D6	CYP2C18	F6	CYP7B1	H6	B2M
B7	ALDH5A1	D7	CYP2C19	F7	CYP8B1	H7	GAPDH
B8	ALDH6A1	D8	CYP2C8	F8	DHRS2	H8	GUSB
B9	ALDH7A1	D9	CYP2C9	F9	DPYD	H9	HPRT1
B10	ALDH8A1	D10	CYP2D6	F10	ESD	H10	PGK1
B11	ALDH9A1	D11	CYP2E1	F11	FMO1	H11	PPIA
B12	CEL	D12	CYP2F1	F12	FMO2	H12	RPL13A

Table 2.7 Human Human detoxification panel II list (BioRad, Germany)

HDTX-II

A1	AANAT	C1	GSTA1	E1	NNMT	G1	UGT1A4
A2	ACSL1	C2	GSTA2	E2	NQO1	G2	UGT1A6
A3	ACSL3	C3	GSTA3	E3	NQO2	G3	UGT1A7
A4	ACSL4	C4	GSTA4	E4	PNMT	G4	UGT2A1
A5	ACSL5	C5	GSTA5	E5	PTGES	G5	UGT2A3
A6	ACSL6	C6	GSTK1	E6	SAT1	G6	UGT2B10
A7	ACSM1	C7	GSTM1	E7	SAT2	G7	UGT2B11
A8	ACSM2B	C8	GSTM2	E8	SULT1A1	G8	UGT2B15
A9	ACSM3	C9	GSTM3	E9	SULT1A2	G9	UGT2B17
A10	AGXT	C10	GSTM4	E10	SULT1A3	G10	UGT2B28
A11	AS3MT	C11	GSTM5	E11	SULT1A4	G11	UGT2B4
A12	ASMT	C12	GSTO1	E12	SULT1B1	G12	UGT2B7
B1	BAAT	D1	GSTO2	F1	SULT1C2	H1	PTGS2
B2	CCBL1	D2	GSTP1	F2	SULT1C3	H2	UCHL1
B3	CES1	D3	GSTT1	F3	SULT1C4	H3	UCHL3
B4	CES2	D4	GSTT2	F4	SULT1E1	H4	XDH
B5	CES3	D5	HNMT	F5	SULT2A1	H5	ACTB
B6	CES5A	D6	INMT	F6	SULT2B1	H6	B2M
B7	COMT	D7	MGST1	F7	SULT4A1	H7	GAPDH
B8	EPHX1	D8	MGST2	F8	SULT6B1	H8	GUSB
B9	EPHX2	D9	MGST3	F9	TPMT	H9	HPRT1
B10	GAMT	D10	NAT1	F10	TST	H10	PGK1
B11	GLYAT	D11	NAT2	F11	UGT1A1	H11	PPIA
B12	GNMT	D12	NAT5	F12	UGT1A3	H12	RPL13A

2.2.13 Protein Extraction

8-10 mL of cell suspensions were seeded in 100x20 mm tissue culture dishes at a density of $25x10^4$ cells/ mL. After one day, growth medium was removed and the cells were washed two times by using PBS buffer. Extracts were added according to their IC_{50} values. After 48 or 72-h incubations, worn growth medium was discarded and then cells were washed 2 times with ice cold PBS.

1X RIPA buffer was used to isolate the proteins. First of all, 1X RIPA buffer was prepared by diluting 10X RIPA buffer (Cell Signaling Technology) with distilled water and 1mM (PMSF) was added just prior to use to prevent protease activity.

600 µL of the diluted RIPA buffer was added into the petri dishes and lysis of the cells on ice were took place. After 5 min, cells were scraped. The lysate was collected in a sterile eppendorf tube and sonicated for 5 min. After sonication, cell suspensions were centrifuged at 14.000*xg* at +4^{o}C for 10 min. Finally supernatant was taken and stored at -80 °C for further use.

2.2.13.1 Determination of Total Protein Concentration

Protein concentrations of cell lysates were determined by the Micro Lowry method using bovine serum albumin(BSA) as a standard (Lowry et al., 1951). This method depends on the reduction of Cu^{2+} ions with peptide bonds with Folin–Ciocalteu reagent under alkaline conditions and the color produced absorbs light at a 660 nm. Absorbance values at this wavelength is proportional to the protein concentration.

Reagents:

Reagent I : 2% w/v CuSO4.5H2O
Reagent II : 2% w/v Na-K Tartarate
Reagent A : 2% w/v Na_2CO_3 in 0.1 N NaOH

Lowry ACR Reagent (alkaline cupper reagent): Reagent I, Reagent II and Reagent A were mixed with a ratio of 1:1:100 (v/v/v) respectively

Folin-Phenol Reagent: 2N stock reagent were diluted to 1N with dH_2O and kept in dark.

Bovine Serum Albumin (BSA) Protein Standards:

0.02, 0.05, 0.1, 0.15, 0.2 mg/mL

Protein Sample:

Samples were diluted 40 and 80 times.

In this method, 40 µL of samples and standards were added to the 96 well plate as triplicate. (40 µL of distilled water for blank). 200 µL Lowry ACR was then added. After 10 min. incubation at room temperature and dark environment, 20 µL Folin's Reagent was added and the wells are mixed immediately. At the end of 45 min. incubation at room temperature and dark environment, the absorbances of samples were measured at 660 nm with Multiskan™ GO microplate reader (Thermo Scientific, USA).. Protein concentration was calculated by the following formula;

Protein Concentration (mg/mL) = [(OD660nm / Slope of Standards) x Dilutions]

2.2.14 Determination of Total GST Enzyme Activity

GST enzyme activity was determined spectrophotometrically at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate and reduced glutathione (GSH) as a cofactor. (Habig et al., 1974). Enzyme activity was measured directly by measuring the conjugation of CDNB with GSH. The enzymatic reaction of CDNB with GSH was shown in Figure 2.10.

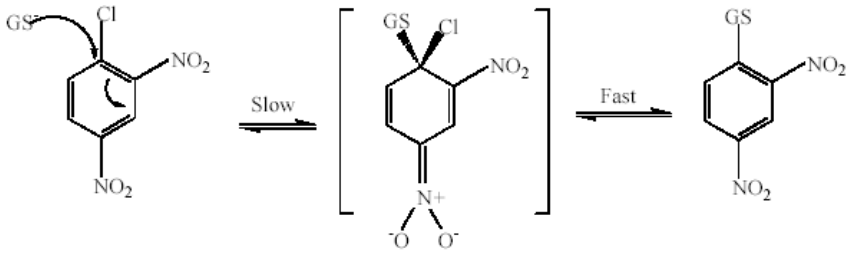

Figure 2.9 Reaction of CDNB with GSH
(Armstrong, 1991).

HepG2 cells cytosolic fraction (3.5 mg protein/mL) which was used as enzyme source, was diluted in 10 mM phosphate buffer, pH 7.4, at a ratio of 1:15. In a single well of a 96 well plate, 162.5 Ml dH2O; 50 µL 500 mM Phosphate Buffer pH 7.4; 10 µL 25 mM GSH; 15 µL enzyme source (20-50 µg protein) were added. Reaction started when 12.5 µL 20 mM CDNB was added. The constituents of the reaction mixture were shown in Table 2.4. Reactions were measured for each well at every 10 seconds for 3 minutes. For reaction blank, 15 µL water was used instead of enzyme source. The slopes of reaction blanks were subtracted from slopes of enzyme reaction. Specific activity were calculated according to the equation showing below (εCDNB = 6.29 mM-1/cm^{-1}).

Specific Activity= [((Average Slope (dA/dt)/ ε CDNB) x (250/15) x (1/ mg protein) x DF]

Table 2.7 The constituents of the reaction mixture for total GSTs Assay

Reagents	**Stock solution**	**Volume to be taken**	**Final concentration**
KPi buffer (pH: 7,4)	500 mM	50 µL	100 mM
GSH	25 mM	10 µL	1 mM
CDNB	20 mM	12,5 µL	1 mM
Sample cytosol	200-500 µg/mL	15 µL	12-30 µg/mL
dH_2O	-	162.5 µL	-

2.2.15 Determination of Glutathione Peroxidase (GPx) Activity

The GPx activity was determined by the reduction of peroxide with reduced glutathione (GSH) catalyzed by GPx and again recycling of oxidized glutathione by glutathione reductase in exess using NADPH, H^+ as a cofactor (Paglia and Valentine, 1967). Micro GPx activity was optimized in our laboratory.

Components:

- 0.1 M Tris-Cl buffer pH 8.0
- 3 mM GSH
- 0.3 mM NADPH
- 0.5 unit/ mL GR
- Cell lysate
- 35 µM H_2O_2
- 2 mM NaN_3

The reaction was initiated by the addition of H_2O_2 and the decrease in absorbance was monitored spectrophotomerically at 340 nm for 3 min. Nonenzymatic reaction was also determined with water instead of cell lysate.

Specific activity of GPx was calculated according to the following equation;

$$\textit{GPx Activity} = [(dOD_{340}/min)/0.00373\mu M^{-1}] \times (0.25/0.02) \times DF)/ \textit{mg protein}$$

2.2.16 Catalase Enzyme Activity

In order to determine Catalase activities, Aebi's method (1984) was used with some modifications. The decomposition of hydrogen peroxide (H_2O_2) was followed spectrophotometrically by the absorbance decrease at 240nm (εH_2O_2=0.00394 L/mmol.mm).

Components:

- 0.05 M KPi buffer pH 7.0
- 30 mM H_2O_2
- 2.5 x diluted dell lysate

Cell lysate were diluted 10 times with 1% (v/v) Triton X-100 in Phosphate buffer and further 2.5 fold dilutions were carried out with 50mM KPi buffer (pH 7.0). 100 µL of enzyme solution was mixed with 50 µL of 30 mM H_2O_2 and the rate of decomposition was followed by spectrophotometrically at 240nm for 3 min. with MultiSkan^{TM} GO plate reader.

Specific activity of Catalase was describe as the amount of substrate (nmol) consumed in one min by 1 mg total protein according to the following equation;

$$\textit{Catalase Activity} = [(dOD_{240}/min)/ 19.54\ (mM^{-1}\ cm^{-1})\ ] \times (0.15/0.1) \times DF)/\ mg\ protein$$

2.2.17 Statistical analysis

Statistical analyses were performed by using GraphPad Prism version 6 statistical software. All results were expressed as means with their sandard deviation (SD). One-way ANOVA and student t-test were utilized using Graphpad prism 6 statistical Package software and $p<0.05$ were chosen as the level for significance.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Extraction of S.absconditiflora

Extraction is an important process in order to get food constituents like, polyphenols, flavonoids and etc. In extraction process, liquid-solid ratio, particle size, time of extraction and liquid type might affect the efficiency of the process.

In this study, we used solid-liquid extraction technique and according to this method, *S. absconditiflora* air-dried leaves were extracted with distilled water and methanol by using Soxhlet extraction device for two days.

At first, the weight of freeze dried extracts was recorded, and the percent yield of extraction was calculated as % (w/w). Table 3.1 shows the yield of extraction process. The percent yield of extraction of leaves of *S. absconditiflora* with distilled water was found as 24.13 % (w/w) for April, 19.87% for May and 19.47 % for June samples. The percent yield of extraction of leaves of *S. absconditiflora* with methanol was found as 18.68% (w/w) for April, 18.9% for May and 18.8% for June samples.

Many extraction studies were found in literature which were performed with alcohol. In alcoholic extraction maximum phenolic compounds were taken because of they are more soluble in alcohol.

In such a study which were done our laboratory, the yield of water extract of *S.absconditiflora* was reported as 17.39 % (w/w) (Hisarlı, 2013). Bozan and coworkers (2002) showed that a yield of extraction was found 18.3% for the

methanolic extract of *S. absconditiflora*. In another study of *Salvia species* (*S. fruticosa***)** collected from Greece region was extracted with methanol extraction yield was found as 21.5 ± 2.11 (Pizzale, 2002).

Table 3.1 Yield of Extraction Process

Extracts		% Yield
Water	April	24.13
	May	19.87
	June	19.47
Methanol	April	18.68
	May	18.9
	June	18.8

3.2 Determination of antioxidant capacity

Antioxidant properties of many herbal extract are pertained to their phenolic contents. Phenolic constituents have a strong impact on the removal of free radicals from the human body. Therefore, it is important to determine the antioxidant capacity of the herbal extracts for increasing their medicinal values. There are many different methods used to evaluate the antioxidant capacity.

In this study, DPPH free radical scavenging capacity and ABTS radical scavenging capacity assays were tested for their sensitivity, simplicity and reproducibility.

3.2.1 Free radical scavenging capacity- DPPH assays

In this study, DPPH radical scavenging capacity of the *S.absconditiflora* extracts were determined according to Blois et al, (1958) as previously

described in Chapter II. Micro DPPH method was optimized with some modifications.

Accordingly, DPPH was prepared at a concentration of 1.5 x 10^{-4} M and further sonicated. The percent radical scavenging activity (%RSA) of extracts were calculated and used for determination of IC50 value, which represents the amount of the extract that cause loss of 50 % of DPPH radical activity.

The graph for percent radical scavenging activity (%RSA) versus extract concentrations as mg/mL for months was plotted and given in Figure 3.1 and Figure 3.2. DPPH incubation time was previously optimized in our laboratory (Hisarlı, 2013).

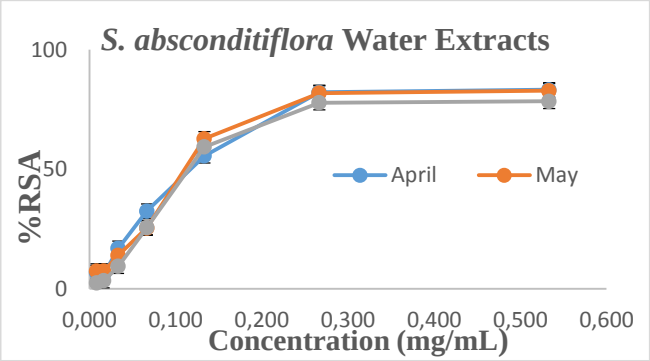

Figure 3.1 DPPH radical scavenging activity of *S. absconditiflora* water extracts.

Each point is the mean of triplicate measurements from two different experiments (n=3)

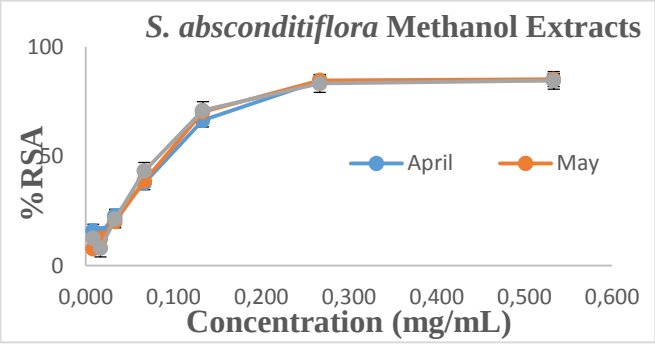

Figure 3.2 DPPH radical scavenging activity of *S. absconditiflora* methanol extracts.

Each point is the mean of triplicate measurements from two different experiments (n=3)

Table 3.2 % Radical scavenging activity (%RSA ± SD*) of *S. absconditiflora* extracts.

Extracts	**Average EC_{50} for DPPH % RSA**
April Water	0.199 ± 0.005
May Water	0.190 ± 0.002
June Water	0.216 ± 0.005
April Methanol	0.176 ± 0.002
May Methanol	0.179 ± 0.003
June Methanol	0.180 ± 0.009
α-tocopherol	17.1 ± 0.014

In the literature, the free radical scavenging capacities of the aqueous extracts of *R. officinalis* and *S. officinalis* were obtanined as 0.236 and 0.265 (IC50 [mg/ml]), respectively (Dorman *et al*.,2003). In another research, EC_{50} value of *S. fruticosa* was determined as 50.43 μg/ mL for dichloromethane, 72.07 μg/ mL for ethyacetate and 32.13 μg/ mL for methanol extracts. (F. Sezer Senol, 2010). According to the Kamatou et al, 2010, the antioxidant activity of some of *Salvia* species extracted with methanol: chloroform were specified to be 11.9 to 69.3 μg/ mL with EC_{50} values by DPPH.

According to our results, highest DPPH radical scavenging activity was observed in June water extract with a value of 0.216 ± 0.005 mg/mL, and

the lowest radical scavenging activity was obtained for May methanol extract as 0.179± 0.003 mg/mL, as given in Table 3-2.

3.2.2 Free radical scavenging capacity- ABTS assays

In this study, micro ABTS method was used with some modifications in order to evaluate ABTS radical scavenging activity of *S. absconditiflora* freeze-dried extracts. 7mM ABTS was prepared with water. In order to obtain ABTS radical cation (ABTS•1) ABTS stock solution was reacted with 2.45 mM potassium persulfate (final concentration) and incubated in the dark at room temperature for 12–16 h before. After the addition of 247.5 µL of diluted ABTS• solution to 2.5 µL of sample, 96 well plate was mixed and the absorbance was read at 734 nm. The percent radical scavenging activity (%RSA) of extracts were calculated and used for determination of IC50 value, which represented the amount of the extract that cause loss of 50 % of ABTS radical activity.

The graph for percent radical scavenging activity (%RSA) versus Trolox concentrations as mg/mL was plotted and given in Figure 3.3

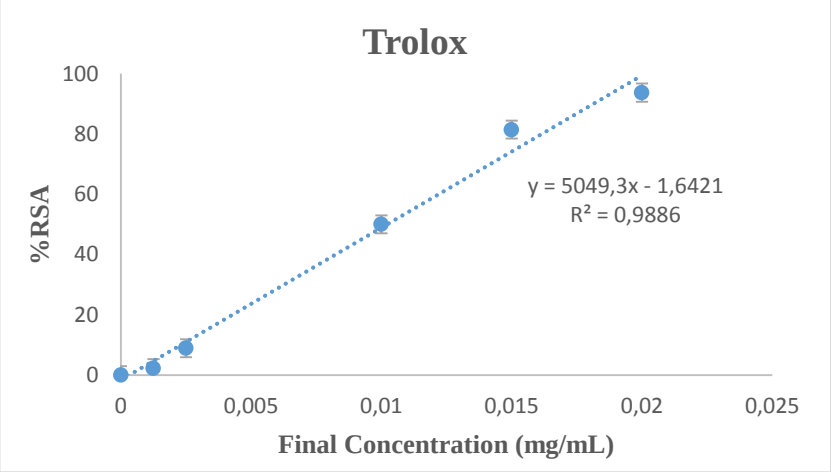

Figure 3.3 Percent radical scavenging activity (% RSA) of trolox. Each point is the mean of triplicate measurements from three different experiments (n=3).

S. absconditiflora radical scavenging activity was also calculated according to ABTS assay**.** The extracts of *S.absconditiflora* were calculated from the slope of percent radical scavenging activity that was plotted against the different concentrations of trolox standards. Results were given in Table 3.3.

Table 3.3 Free radical scavenging capacity (EC_{50}) of all extracts

Free radical scavenging capacity		
Months	***% RSA ± SD***	***mg TEAC/g ext ± SD***
AW	2.715 ± 0.57	89.78 ±11.22
MW	13.58 ± 0.23	305.17 ± 0.23
JW	39.93 ± 2.19	827.93 ± 43.63
AM	27.65 ± 1.22	584.38 ± 24.11
MM	33.24 ± 0.72	695.08 ± 14.23
JM	40.77 ± 0.80	844.55 ± 15.83

TEAC : Trolox equivalents antioxidant capacity

According to our results in June methanol extracts had the highest antioxidant capacity in both extract types.

In the literature, *S. officinalis* methanolic extracts has 1.783 ± 0.011 mmol TE/ g extract (Ünver,2009).

3.2.3 Total Phenolic Content of Salvia Extracts

Total phenolic content of *S.absconditiflora* extracts were determined according to Singleton and Rossi (1965) with some modifications. Gallic acid was used as a standard and gallic acid standard curve was used to calculate the total phenolic content in Salvia extracts. Gallic acid concentrations were used between 10 to 100 µg/mL as initial concentration, and 1 to 10 µg/mL in each well. Figure 3.5 shows the gallic acid standard curve. We used the equation of this plot to calculate the total phenolic contents of Salvia extracts.

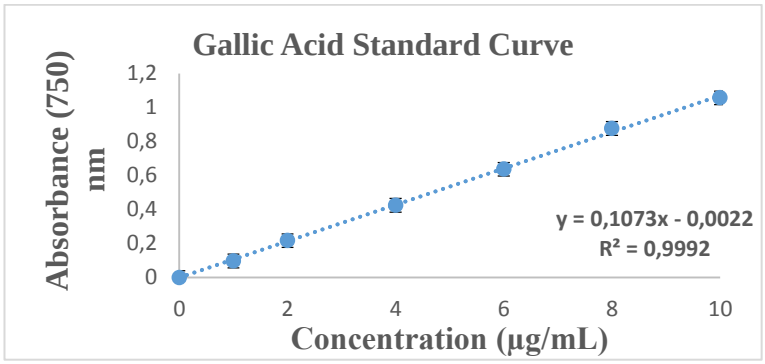

Figure 3.4 Gallic acid sandard curve.

Each point is the mean of triplicate measurements from three different experiments (n=3)

Total phenolic contents of extracts were calculated in 2 different concentrations, as 0.025 and 0.05 mg /mL for each month extract. The overall results are given in Figure 3.5.

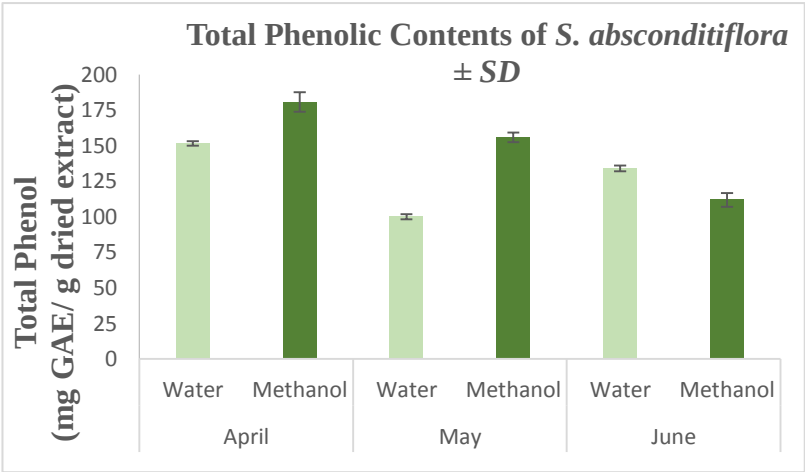

Figure 3.5 Total phenolic content of *S. absconditiflora* extracts.

Each point is the mean of triplicate measurements from three different experiments (n=3)

As seen from the Figures 3.5, the highest amount of total phenolics were observed in April-methanol extract as 180.77 ± 6.87 mg GAE/ g dried

extract and the lowest total phenol was observed in May- water extract as 99.96 ± 1.78 mg GAE/ g dried extract (Table 3.4).

Table 3.4 Total phenolic contents (*mg GAE/ g of dried extract* ± SD) of different *S. absconditiflora extracts*

	Months		
	April	**May**	**June**
Water Extracts	151.53 ± 1.53	99.96 ± 1.78	134 ± 2.02
Methanol Extracts	180.77 ± 6.87	155.81 ± 3.33	111.86 ± 4.82

These differences in overall total phenol contents can be a response to environmental conditions, like water amount and exposure to the sun light.

In the literature, total phenolic contents of methanol extracts of *S officinalis* and *S fruticosa* were 46 to 107 µg GAE /mg dry extract respectively (Lorena Pizzale, 2002). In another study, it was shown that total phenolic content for some *Salvia* sp. were extracted with methanol:chloroform were determined as 45 to 211 µg GAE / mg dry extract (Kamatou, 2010).

In different study, the antioxidant activities were evaluated according to the DPPH method. *S. officinalis* indicated the highest antioxidant activity (91%), while the lowest (11.3%) was seen in parsley (*Petroselinum crispum*) (Rababah, et al., 2011).

A another study showed that, total phenolic contents of methanol extract of *S. fruticosa* was ranged between 63.7 and 144 µg GAE/ mg extract (Papageorgiou et al.,2008).

3.2.4 Total Flavanoid Contents of Salvia Extracts

Total flavonoid content of *S. absconditiflora* extracts were determined by using aluminium chloride colorimetric method as described in Materials and Methods. Quercetin and Catechin (+) were used as standards for the determination of total flavonoid contents. The standard curves are given in Figure 3.7 and 3.8.

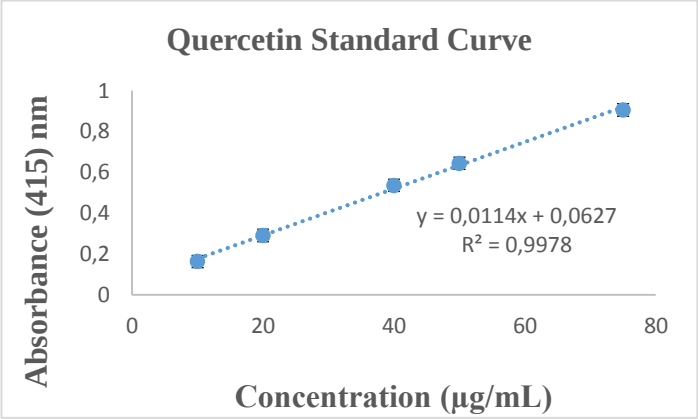

Figure 3.6 Quercetin standard curve.

Each point is the mean of triplicate measurements from three different experiments (n=3)

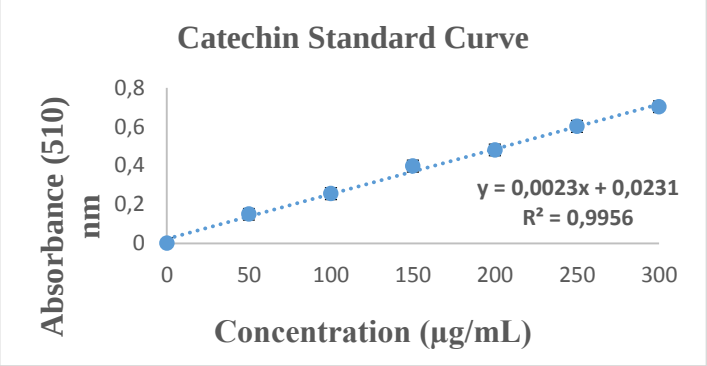

Figure 3.7 Catechin standard curve.

Each point is the mean of triplicate measurements from three different experiments (n=3)

Total flavanoid content of extracts were also calculated for 3 different concentrations of 0.025, 0.05 and 1 mg /mL for each month extract. The results are given in Figure 3.8 and Table 3.5.

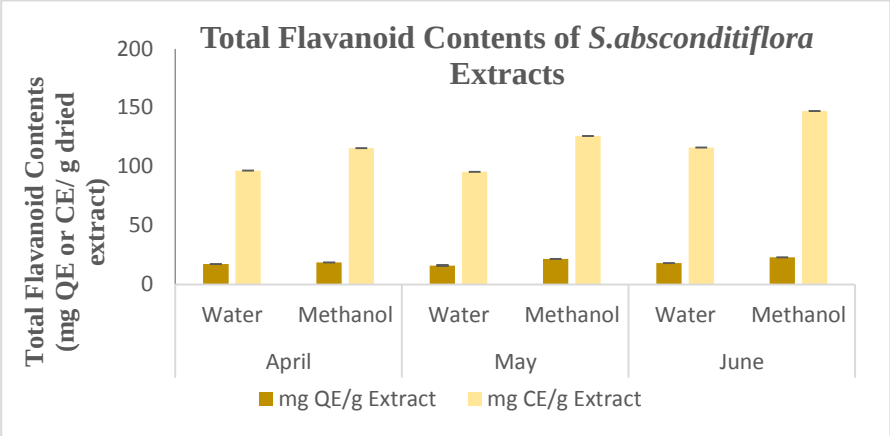

Figure 3.8 Total flavanoid contents of *S. absconditiflora* extracts.

Table 3.5 Total favanoid fontents of *S. absconditiflora* Extracts

		Months		
		April	**May**	**June**
Water Extracts	**mg QE/ g of dried extract ± SD**	17.49±0.002	16.14 ± 0.002	18.12± 0.004
	mg CE/ g of dried extract ± SD	96.8 ± 0.002	95.65 ± 0.002	116.23±0.005
Methanolic Extracts	**mg QE/ g of dried extract ± SD**	18.71±0.005	21.81 ± 0.003	22.95± 0.003
	mg CE/ g of dried extract ± SD	115.65±0.006	126.09±0.001	147.39 ±1.77

According to our results, methanol extracts of *S. absconditiflora* have more flavanoids compared to water extracts. *S. absconditiflora* extracts have lower concentration of quarcetin than catechin.

In the literature, Mediterranean Salvia species were extracted with methanol and their total flavanoid contents were destermined as 36.27 to 40.83 µg CE / mg dry extract (Dincer et al., 2013). According to the Miliauskas et al.,

2004 total flavonoid content for some *Salvia* species were measured as 1.4 to 5.7 µg Rosmarinic acid equivalents (RE)/mg dry extract when compared to the these results, water extracted *S.fruticosa* displayed much higher flavonoid content.

Substances isolated from *S. miltiorrhiza* have been documented to exhibit antioxidant capabilities, and recent studies also indicated that *S.miltiorrhiza* (SM) has anti-tumor potential. (Liu et al., 2000).

3.2.5 Determination of Metal Chelating Activity

In this study, we used 6 different concentrations of extract, ranging from 1 mg/mL to 10 mg/mL to calculate metal chelating activity.

As seen from Figure 3.9 and 3.10 we found that, both methanol and water June extracts have higher metal chelating activity at concentrations of 10 mg/ mL EDTA was used as a positive control. The lower the $IC_{50,}$ the higher the Fe^{+2} chelating capacity. IC50 values of metal chelating activity were calculated from the figures and given at Table 3.6.

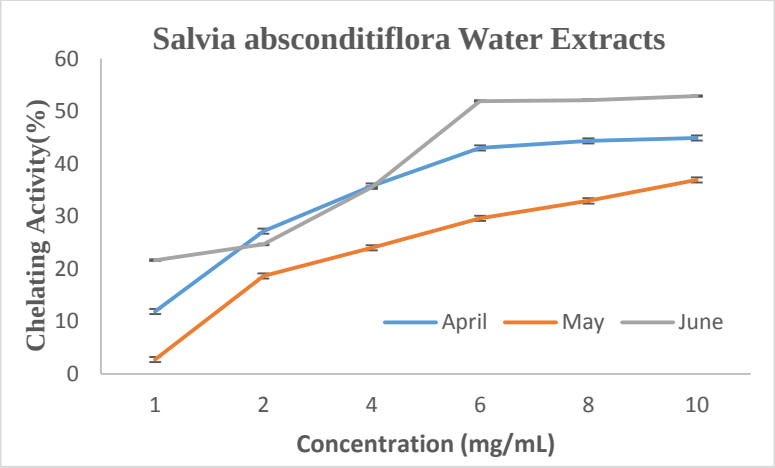

Figure 3.9 Percent metal chelating activities of *S. absconditiflora* water extracts.

Each point is the mean of triplicate measurements from three different experiments (n=3).

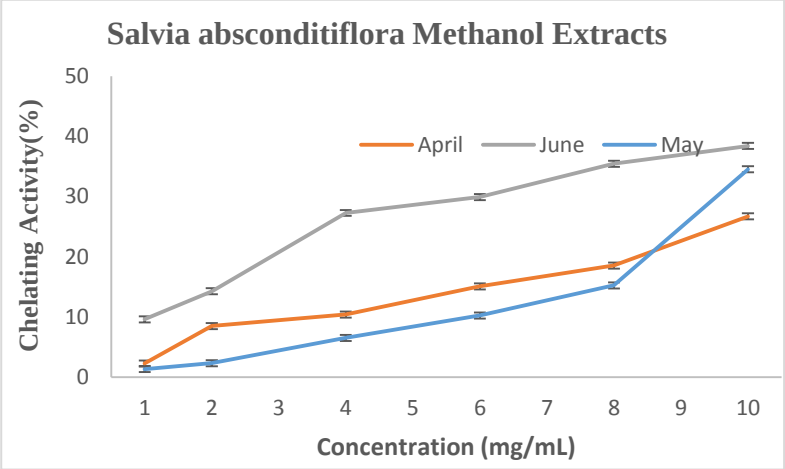

Figure 3.10 Percent metal chelating activities of *S. absconditiflora* methnol extracts.

Each point is the mean of triplicate measurements from three different experiments (n=3).

Table 3.6 Metal chelating activity of *S.absonditiflora* all extracts.

Sample	**Metal chelating activity IC50 mg/ mL ± SD**		
	April	**May**	**June**
Water Extracts	1.477 ± 0.015	1.987 ± 0.044	1.319 ± 0.043
Methanol Extracts	3.296 ± 0.052	2.405 ± 0.039	2.034 ± 0.047
EDTA	0.107 ± 0.002		

The results showed that, water extracts of *S. absconditiflora* have more chelating activity than methanol extracts.

When compared with the potent control group EDTA, our results showed that water extracts have approximately ten times lower metal chelating activity and methanol extracts have approximately twenty times lower metal chelating activity.

3.2.6 Determination of Antibacterial Activities

Antibacterial activity of *S.absconditiflora* extracts were analyzed by disc diffusion method with 5 different bacteria; two of them gram negative (*E. coli* and *A. tumefaciens*) and three of them gram positive bacteria (*B. subtilis, B. licheniformis, S. aureus*). Penicillin, gentamicin and tetracycline were used as the standard antibiotic discs. Microorganisms were grown in Müller Hilton broth agar at 35°C and 28°C for 18 h and then cell density were adjusted according to 0.5 McFarland standards. After that, 100 µl of adjusted microorganism suspension was inoculated onto Müller Hilton Agar by spread plate technique.

Inhibition zones (IZ) around the discs after overnight incubation were measured to determine antibacterial activities of the extracts. The study was performed in triplicates for each sample. One example of antibacterial activity of the extracts was shown in Figures 3.11 and the rest were given in Appendix A.

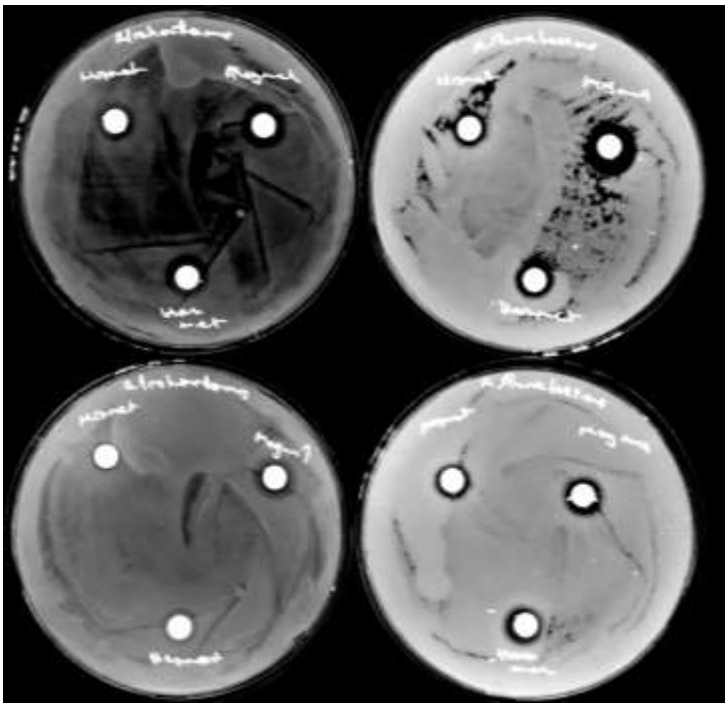

Figure 3.11 *Salvia* methanol extracts on *B.licheniformis and A.tumefaciens*

The largest IZ was measured as 13 mm with May methanol extracts of *A.tumefaciens*. Table 3.7 summarizes the antibacterial effects of Salvia extracts.

Table 3.7 Inhibitory zones of all extracts

	Water			Methanol		
	April	May	June	April	May	June
E.coli	NO	NO	NO	NO	NO	NO
S.aureus	9/8 mm	10 mm	11/10mm	8/7 mm	11/10mm	11/10mm
B.subtilis	8 mm	9 mm	9 mm	9/8 mm	9/9 mm	9/8 mm
B.licheniformis	7 mm	8 mm	8 mm	7 mm	7 mm	7/6 mm
A.tumefaciens	7 mm	7/9 mm	9 mm	9 mm	13/9 mm	9 mm

Salvia methanol extracts showed antibacterial activity on *B.subtilis* and *S.aureus*. But there is not any differences between the inhibitory zones for both strains.

Salvia methanol extracts showed antibacterial activity on *B.licheniformis* and *A.tumefaciens*. But again, there were no differences between the inhibitory zones

Both water and methanol extracts have no effect on *E.coli*. No inhibitory zone was obtained.

Salvia water extract showed an antibacterial activity on *B.licheniformis* and *A.tumefaciens*. No differences between the inhibitory zones of *B.licheniformis* and *A.tumefaciens* were seen. The rest of the figure were shown in Appendix A.

Salvia water extracts showed antibacterial activity on *B.subtilis* and *S.aureus*. But there were no differences between the inhibitory zones of *B.subtilis* and *S.aureus.*

3.3 Analysis of The Phenolic Compounds

3.3.1 RP- HPLC Analysis of Phenolic Compounds

High-performance liquid chromatography is the most popular technique which was used to separate, identify and quantify each component found in the extracts (Middlesworth and Cannell, 1998). In order to identify phenolic compounds found in S.*absconditiflora* extracts, different standard solutions were used to obtain the calibration curves for phenolic compounds.

RP-HPLC analysis of the standards was given in Table 3.8. First of all, each standards was injected individually for determination of the retention time and then mixture of the standards was performed at 280 nm The retention time of the standards were ranged from 3.76 min (gallic acid) to 42.6 min (rosmarinic acid).

Table 3.8 Retention times max wavelength (λmax) of phenolic standards

Phenolic standarts	**Retention time**	**λmax (nm)**
Gallic acid	3.76	271
(+ Catechin)	5.12	280
(-) Epicatechin	5.80	280
Epigallocatechin gallate	6.14	280
Syringic acid	6.93	274
p-Coumaric acid	9.60	310
t-Resveratrol	16.1	306
Quercetin	19.55	370
Caffeic acid	6.2	322
Rosmarinic acid	42.6	330

Qualitative analysis of the phenolic compounds in the *Salvia* extracts were based on the comparison of their retention times with the reference phenolic standards. Figure 3.12 showed the well resolved RP-HPLC chromatograms of the *S.absconditiflora* extract for detection of caffeic acid and rosmarinic acid at 322 and 330 nm, respectively. Gallic acid, syringic acid, t-resveratrol and quercetin in the extract were also monitored at 271, 274, 306 and 370 nm, respectively (Figure 3.13)

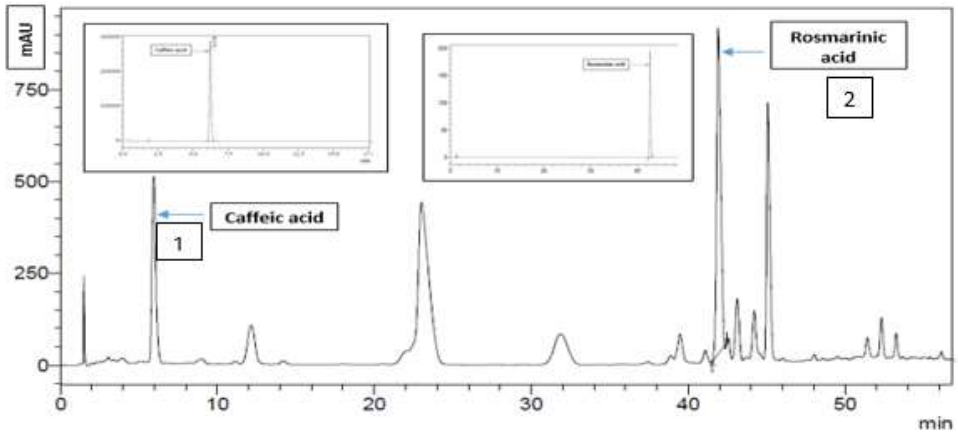

Figure 3.12 RP-HPLC chromatogram of the *S.absconditiflora* extract monitored at 330 nm. Peaks are indicated as follows; 1) caffeic acid 2) rosmarinic acid

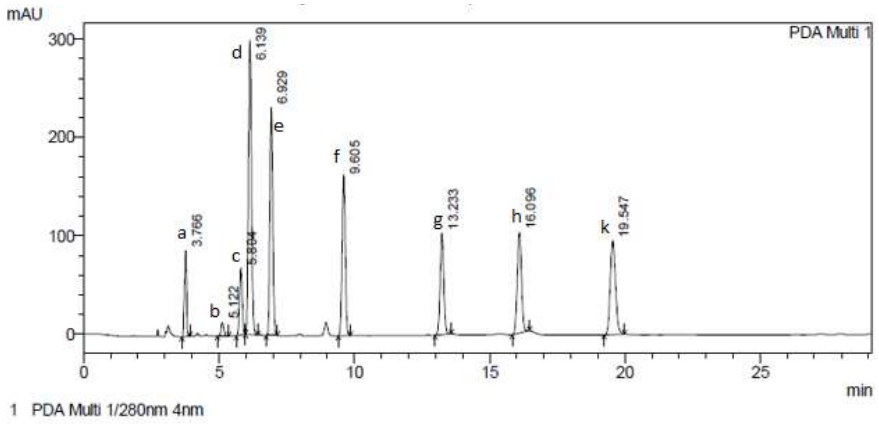

Figure 3.13 HPLC chromatogram of phenolic standarts at 280 nm with a retention times; (a; gallic acid, b; (+) catechin, c; (-)epicatechin, d; epigallocatechin gallate, e; syringic acid, f; p-

coumaric acid, g; rosmarinic acid, h; t-resveratrol, k; quercetin).

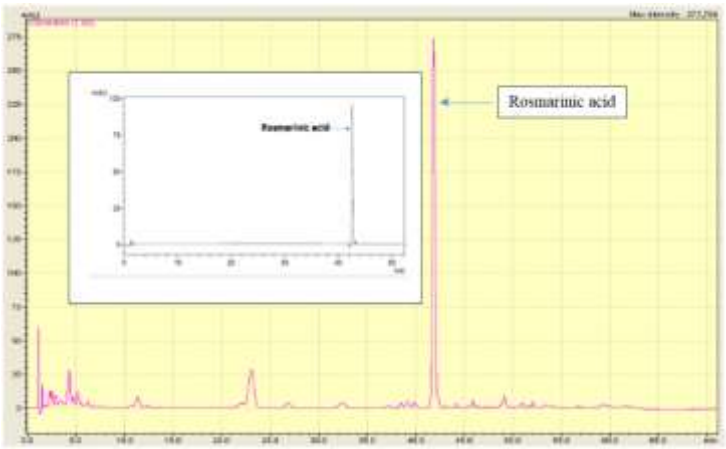

Figure 3.14 HPLC chromatogram of the Salvia extract and standard rosmarinic acid at 330 nm. with a retention time of 42.6 min.

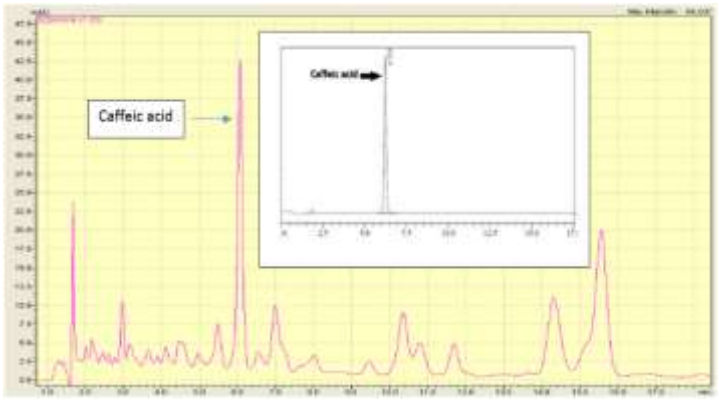

Figure 3.15 HPLC chromatogram of the Salvia extract and standard caffeic acid at 330 nm with a retention time of 6.1 min.

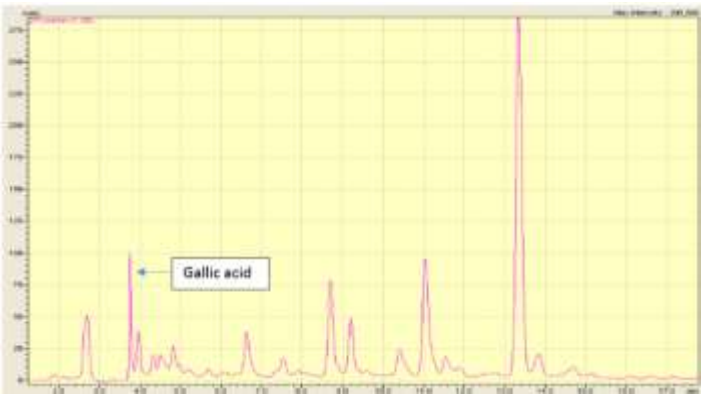

Figure 3.16 HPLC chromatogram of the Salvia extract at 271 nm. Detection of gallic acid with a retention time of 3.7 min.

Figure 3.17 HPLC chromatogram of the Salvia extract at 306 nm. Detection of t-resveratrol with a retention time of 16.1 min

Figure 3.18 HPLC chromatogram of the Salvia extract at 370 nm. Detection of quercetin with a retention time of 19.6 min.

According to our results, Salvia extracts includes lots of phenolic compound. The most abundant phenolics were found in *S. absconditiflora* was rosmarinic acid and caffeic acid.

3.3.2 Quantitation of the Phenolic Constituents in Salvia absconditiflora by RP-HPLC

The amounts of the phenolic compounds in *S.absconditiflora* were identified by HPLC methods. Different concentrations of the phenolic standards were prepared individually and the standard curves were drawn by plotting the peak area of the standard against the concentration (µg/mL).

All standards and samples were measured in duplicates and injected as 20 ppm. The amounts of the RA and CA in extracts individually were shown in Table 3.9.

Table 3.9 RA and CA content of Salvia extracts individually

Extract Name	mg C.A /g extract ± SD	mg R.A /g extract ± SD
April water	2.938 ± 0.252	23.69 ± 1.63
May water	2.388 ± 0.015	20.40 ± 0.26
Junewater	1.718 ± 0.004	19.64 ± 0.05
April methanol	4.423 ± 0.028	92.23 ± 0.03
May methanol	4.222 ± 0.122	114.75 ± 0.7
June Methanol	4.568 ± 0.011	109.39 ± 0.71

And total water and methanol extract content were shown in Table 3.10

Table 3.10 Phenolic contents of salvia water and methanolic extracts

Extract Name	mg G.A/g extract	mg Quercetin/ mg extract	mg t-resveratrol/g extract
water	1,24 ± 0,004	0,19 ± 0,050	0,06 ± 0,006
methanol	1,36 ± 0,012	0,31 ± 0,005	0,13 ± 0,003

According to our results methanolic extracts have higher concentration of phenolics rather than water extracts.

Quercetin, gallic acid and trans-resveratrol were determined as minor phenolic compounds in the *S. absconditiflora*. *Salvia* species have low amount of them. In this study, it was demonstrated that the presence of trans-resveratrol was low in *S. absconditiflora* by RP-HPLC.

Salvia species have high amount of phenolic compounds and they were widely investigated because of their medical usage. Salvianolic acids is a Phenolic acids was unique to *Salvia* sp. Rosmarinic acid and caffeic acid were highly abundant phenolic acids in *Salvia* sp. According to high amount of phenolic, *Salvia* sp. have antiinflammatory, antibacterial and antiviral activities (Petersen, 2003).

According to Dinçer et al. (2013) *S. tomentosa* methanol extract has 10.24 mg RA /g dry extract. In another study, caffeic acid content of methanolic extracts of *S. tomentosa, S. fruticosa*, and *S. officinalis* were reported as 42.77, 97.3 and 15.14 mg/g dry extract, respectively (Askun 2009)

Caffeic acid amount in the *S. absconditiflora* was determined as 15.43 mg/g dry extract (Yılmaz S, 2013).

According to Lu and Foo (2002) caffeic acid content of the *S.fruticosa* extract was determined as 15.16 mg/g dry extract. These results indicate that caffeic acid amount of water extracted *S. fruticosa* is approximately the same with the results of *S. officinalis and S. absconditiflora and lower than those of S. fruticosa* and *S. tomentosa*. The some differences in rosmarinic acid and caffeic acid contents between the results were due to the differences of the solvents used for extraction and being different subtype. The presence of caffeic acid and it's derivatives contribute to *Salvia species* a high antioxidant activity.

3.3.3 LC-MS/MS Analysis of Phenolic Compounds

This method has the combination of physical separation ability of liquid chromatography and mass differentiation capacity of mass spectrometry. It is highly sensitive and suitable for the detection of plant phenolic compounds in extracts.

In order to identify the phenolic compounds, 10 different standards were used. The LC-MS/MS result of *S.absconditiflora* water and methanol extracts for 10 selected compounds; rosmarinic acid, apigenin, p-qumaric acid, caffeic acid, gallic acid, luteolin, t-resveratrol, syringic acid, chlorogenic acid and rutin trihydrate, were plotted as abundance versus mass to charge ratio. LC-MS/MS graphs are shown in Figure 3.19-21.The standards were selected according to the literature (Roby et al., 2013).

The amount of phenolic compound identified by LC-MS/MS analysis were shown in Table 3.11. According to our results, methanol extracts of *S.absconditiflora* have higher content of phenolics than water extracts.and also rare amount of phenolics were identified such as apigenin, syringic acid and chlorogenik acid.

Lamiaceae species are known to contain high amount of phenolic acid and flavanoids. *Salvia* genus is the member of *Lamiaceae* family. *Salvia* sp. also have various phenolic acids which are the main compounds making the plant valuable for medicinal purposes. The presence of phenolic acids shows the plants' high antioxidant activity (Lu and Foo 2002). Caffeic acid is one of the most abundant phenolic acid observed in *Salvia* sp.

Table 3.11 The amount of phenolics in *Salvia* extracts with LC-MS/MS.

	ppm	
Phenolics	**Water Extract**	**Methanol Extract**
Apigenin	≤0.031	≤0.031
p-Coumaric acid	0.3108±0.0013	0.0560±0.0006
Caffeic acid	2.3561±0.0038	0.2978±0.0003
Gallik asit	≤0.125	≤0.125
Luteolin	0.7618±0.0073	0.5328±0.0057
t-Resveratrol	≤0.125	≤0.125
Syringic acid	0.2754±0.0001	≤0.039
Chlorogenik acid	≤0.25	≤0.25
Rutin trihydrate	2.0009±0.0304	3.5903±0.0809
Rosmarinic acid	44.034±0.0048	52.325±0.0688

According to our results, caffeic acid was found as 2.356 ppm in water extracts of S.*absconditiflora* indicating the antioxidant activity of the extracts. Though our methanol extracts have low CA content, methanol extracts of different *Salvia* sp. were found to have high caffeic acid like, 100-8000 ppm (Askun 2009, Coisin 2012).

Rosmarinic acid is found in species used commonly as culinary herbs. It has potential anxiolytic, antiviral, antibacterial, antioxidant and anti-inflammatory properties (Rossi et al., 2012). RA is present in both water and methanol extracts of *S.absconditiflora* indicating the presence of these properties.

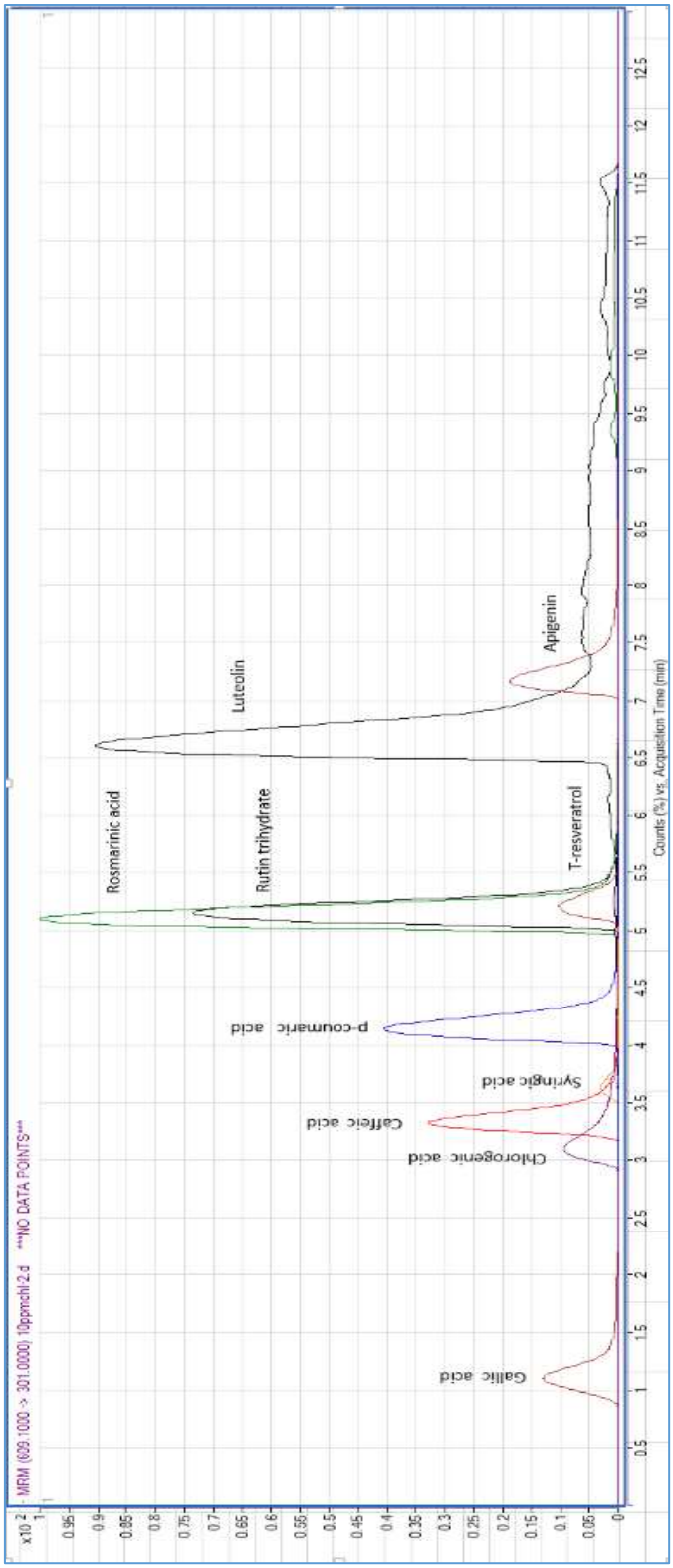

Figure 3.19 LC-MS/MS profile of 10 standards

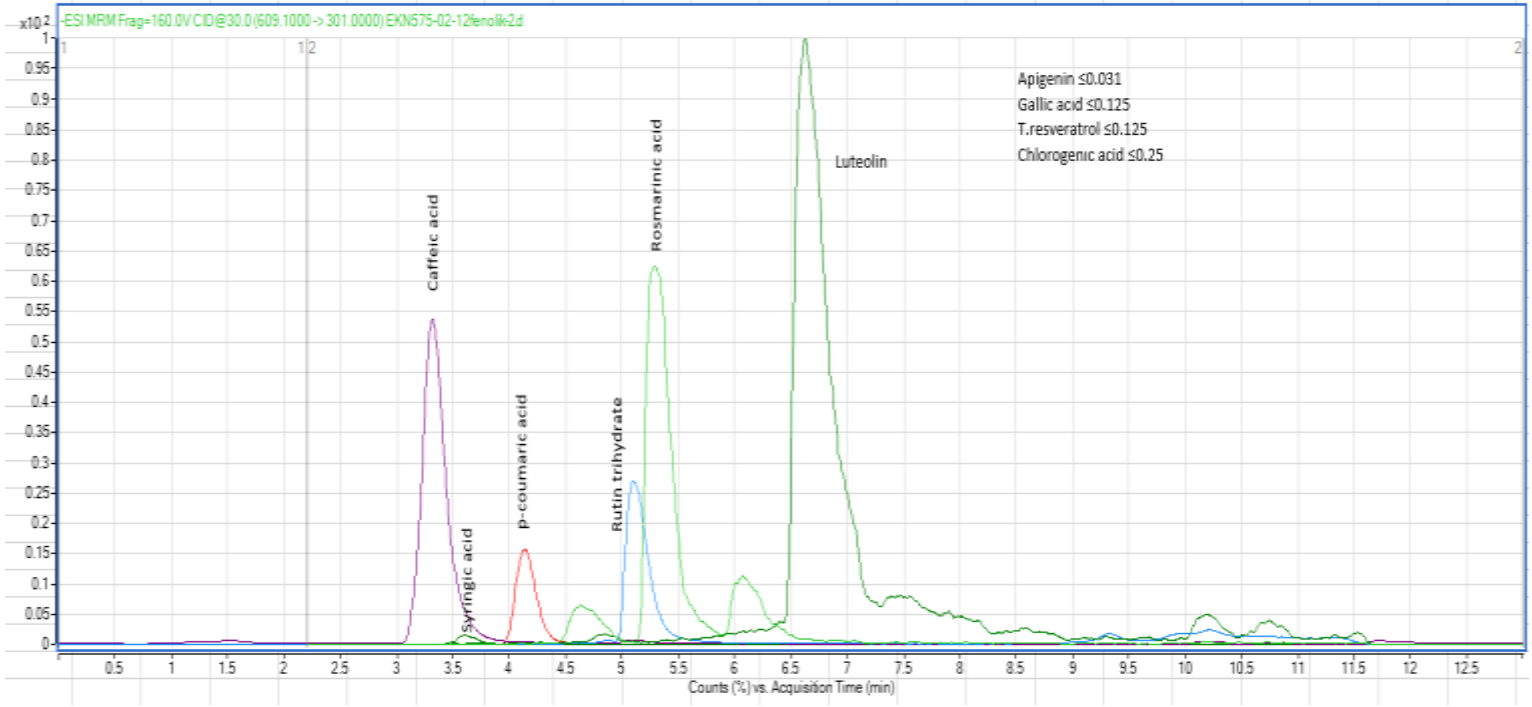

Figure 3.20 LC-MS/MS profile of *S.absconditiflora* water extract (all)

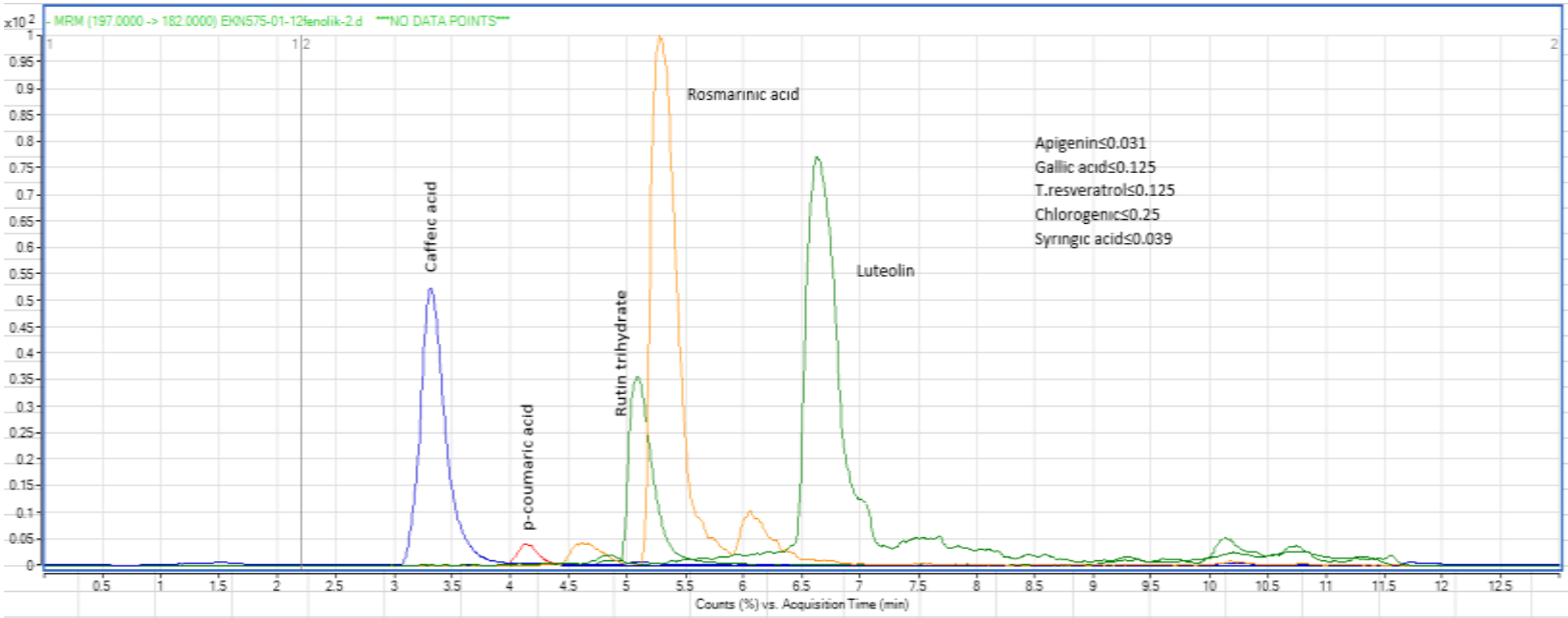

Figure 3.21 LC-MS/MS profile of *S.absconditiflora* methanol extract (all)

Rutin has anti-inflammatory, antioxidant as well as anticancer properties and it is found in many of the medicinal plants. It is composed of flavonol quercetin with the disaccharide rutinose (Sofic et al., 2010). In our results, rutin trihydrate was found in both extracts, methanol extract having the higher amount.

Salvia genus also has the flavonoid luteolin and it was shown to have antimicrobial, antioxidant, anti-inflammatory and anticarcinogenic properties (Loper-Lazaro, 2009). In our study, the level of luteolin was found as 0.762 ppm for water extracts and 0.532 ppm for methanol extracts by LC-MS/MS analysis. In the literature different *Salvia* sp. have luteolinin as its constituent. For example *S. officinalis* has 83.3 ppm, *S.tomentosa* 51.3 ppm and *S. bicolor* has 423 ppm. According to our results, luteolin was found in both extracts but at low amounts.

Salvia genus also has p-coumaric acid and it was found that, coumaric acid prevents the production of carcinogenic nitrosamines (Kikugawa, 1983). In the literature p-coumaric acid levels of methanolic extracts of *Salvia officinalis* were detected as 11.25 mg/100 g dried plant by HPLC analysis. In the same article, methanol extracts of *Salvia bicolor* was calculated as 70.27 mg/g (Taghreed, 2012). P-coumaric acid contents of *S.absconditiflora* extracts were calculated and the results showed that water extract has higher amount of p-coumaric acid but still low compared to literature.

The results showed that, methanolic extracts have higher concentration of CA than the water extracts. In all extracts, April methanol extract has the highest CA concentration. Moreover, methanol extracts have higher concentration of RA than the water extracts. In all extracts, May methanol extract has the highest RA concentration.

As a conclusion, our study showed that water and methanol extracts of *S. absconditiflora* contain high phenolic phytochemicals and have high antioxidant properties. So, it is highly proposed that cytotoxic effects of the

crude extracts of *S. absconditiflora* and its two main constituents; rosmarinic and caffeic acid, on elected cancer cell line (HepG2-Hepatocellular carcinoma) should also be indicated.

3.4 Cell Culture

3.4.1 Viable Cell Counting

Cancer cells should be supplied with nutrients and physiological conditions so that they can maintain their viability and cell division.

HepG2 cells were plated as $50X10^4$cells/well. Cells were trypsinized and counted by trypan blue day by day for 15 days. Cell number reaches the maximum value in day 12 as a density of $2.7x10^6$. After 10^{th} day, death cell number increased. Growth curve of HepG2 cell was shown in Figure 3.22.

For 15 days, cells were counted and after 10 th day death cell number increased and live cell number decreased.

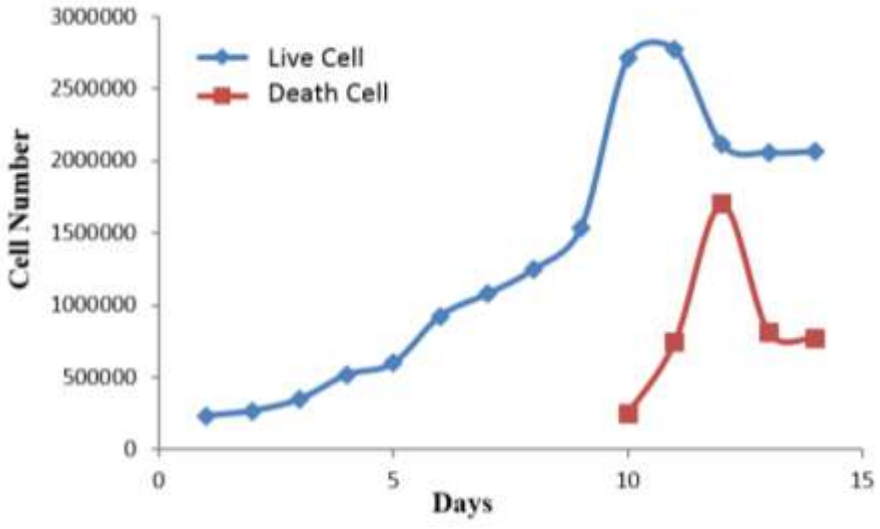

Figure 3.22 HepG2 Growth Curve

3.4.2 XTT Cell Proliferation Assay

Effects of *S.absconditiflora* water and methanol extracts on HepG2 cells were detected after 48 or 72 h incubations with extracts and standards using XTT colorimetric assay.

In the experiment, *S.absconditiflora* extracts were dissolved in medium with 0.2 % DMSO. DMSO works as a vehicle that it increases the permeability of cells and facilitates the entrance of phenolic compounds in cells and solubility of materials. Final DMSO concentration in all wells became 0.1%.

Triplicate measurements were done in our experiment and to calculate the percent viability, we used the average of them and serial diluted concentrations of samples.

XTT assay is based on the metabolically active mitochondrial dehydrogenase activity and color changes. In order to eliminate sample color we used sample blank (without cell). Percent (%) viability versus concentrations graph was plotted as in Figure 3.23 – 3.26. and IC_{50} values were determined by using GraphPad Prism Version 6.

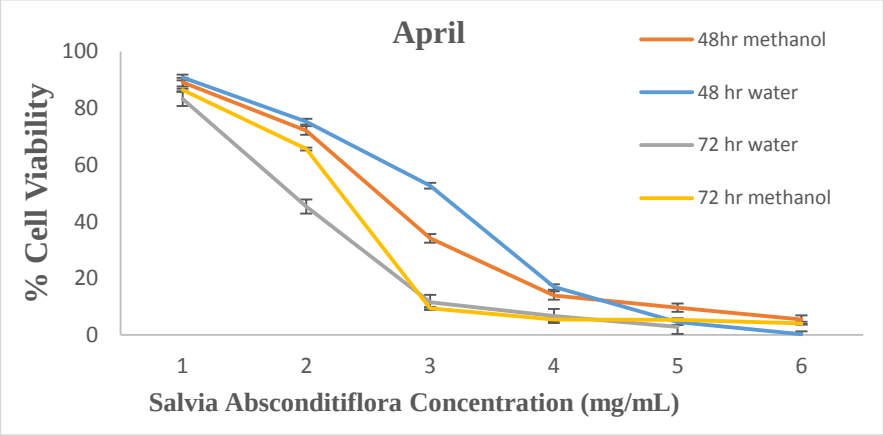

Figure 3.23 % cell viabilities of HepG2 cells in response to dose and time dependent treatment of *S. absconditiflora* April extracts 48 and 72 h incubations.

Each point is the mean of triplicate measurements from three different experiments (n=3)

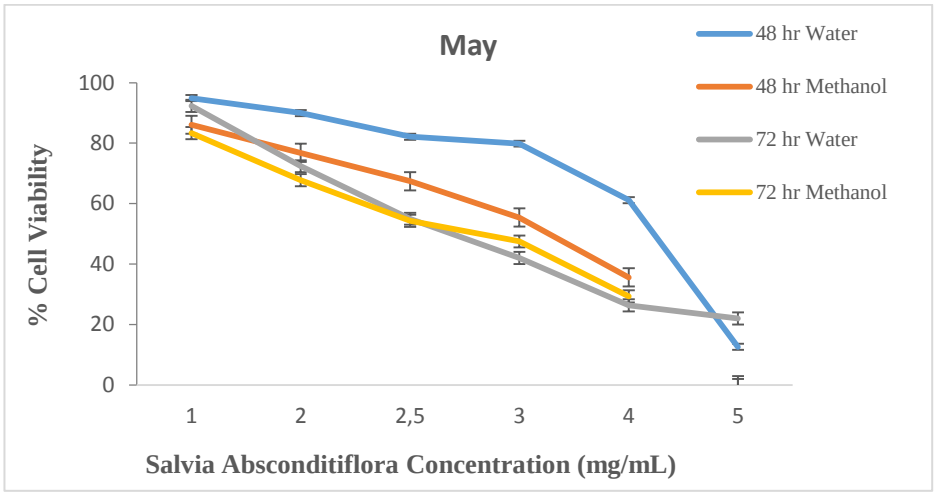

Figure 3.24 % cell viabilities of HepG2 cells in response to dose and time dependent treatment of *S. absconditiflora* May extracts 48 and 72 h incubations.

Each point is the mean of triplicate measurements from three different experiments (n=3)

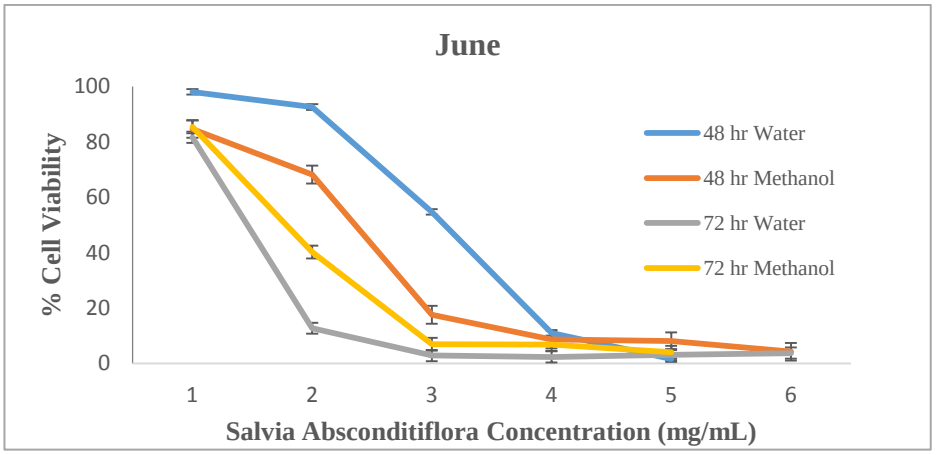

Figure 3.25 % cell viabilities of HepG2 cells in response to dose and time dependent treatment of *S. absconditiflora* June extracts 48 and 72 hr incubation.

Each point is the mean of triplicate measurements from three different experiments (n=3)

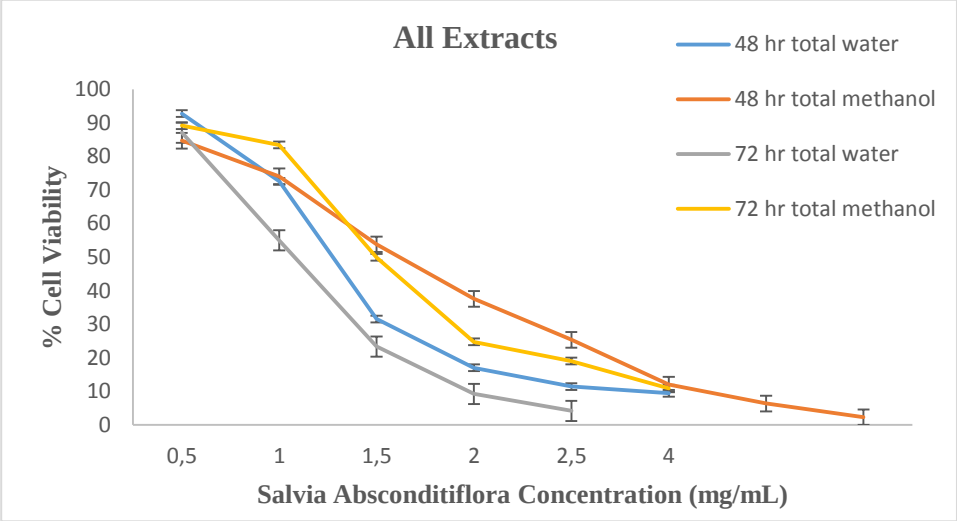

Figure 3.26 % cell viabilities of HepG2 cells in response to dose and time dependent treatment of *S. absconditiflora* All extracts 48 and 72 hr incubation.

Each point is the mean of triplicate measurements from three different experiments (n=3)

Table 3.12 IC_{50} values of *S. absconditiflora* extracts

			IC50 mg/mL ± SD
48 h Incubation	**Water**	April	2.85 ± 0.02
		May	3.78 ± 0.15
		June	2.05 ± 0.002
	Methanol	April	1.56 ± 0.004
		May	2.57 ± 0.09
		June	1.31 ± 0.003
72 h Incubation	**Water**	April	2.81 ± 0.012
		May	3.00 ± 0.11
		June	1.79 ± 0.005
	Methanol	April	1.31 ± 0.002
		May	2,19 ± 0.04
		June	0.74 ± 0.001
All Extracts	**48 h Incubation**	Water	2.93 ± 0.102
		Methanol	1.12 ± 0.05
	72 h Incubation	Water	1.68 ± 0.079
		Methanol	1.01 ± 0.1

These results indicated that IC_{50} values of 48 h incubation were higher than that of 72 h incubation. Water extracts have higher IC50 values than methanol extracts. IC_{50} values were calculated in *S.absconditiflora* treated HepG2 cells. These results indicated that IC_{50} value decreased the incubation time changes from 48 h to 72 h and from water extracts to methanol extracts (Table 3.13).

Rosmarinic acid and Caffeic acid were the most abundant component in Salvia extracts. In order to show their cytotoxic effect on HepG2 cell rather than *Salvia* , IC50 values were calculated.IC50 values were calculated after the treatment of 48 and 72 hr incubation of rosmarinic acid and caffeic acid. % cell viability were calculated (Table 3.13) These results indicated that IC50 value decreased the incubation time changes from 48h to 72h and from water extracts to methanol extracts.

Table 3.13 IC_{50} values of standards (RA and CA) on HepG2 cells

	Standards	**IC50 (µg/mL) ± SD**
48 h Incubation	Caffeic Acid	168.02 ± 0.02
	Rosmarinic Acid	145.63 ± 0.09
	R.A+C.A	135.29 ± 0.08
72 h Incubation	Caffeic Acid	125.71 ± 0.1
	Rosmarinic Acid	188.21 ± 0.11
	R.A+C.A	133.13 ± 0.08

Rosmarinic acid and caffeic acid were the most abundant components of *S.absconditiflora*. When we used these standards in order to show their effects rather than the all components of Salvia.

According to our results, IC_{50} values of Salvia extracts aproximately 15% of IC_{50} values of standards.

IC_{50} values of rosmarinic acid and caffeic acid for 48 and 72 hours incubation were calculate close to each other and also lower than those of total extracts. These results were statistically significant among the extract and standard references for 48 hours but not for 72 hours incubation.

When standard rosmarinic acid and caffeic acid were performed together in a ratio of 1:1 for the cytotoxic capacity, IC_{50} value was determined lower than those of rosmarinic acid and caffeic acid standards separately for 48 hours incubation. But the mixture of R.A + C.A was found higher than CA standard for 72 hours incubation. According to our results, the mixture of R.A + C.A has more cytotoxic effect on HepG2 cell than individual standards for 48 h incubation. The highest cytotoxic activity was shown in CA administration of 72 h incubation.

In this study, the highest cytotoxic effect for 48 h treatment was found by standard caffeic acid with rosmarinic acid and an IC_{50} value of 135.29 μg/mL. For 72 hr treatment, cytotoxic effects were found in the order of caffeic acid> CA+RA > rosmarinic acid > crude extract. In both 48 and 72 h treatment, crude extracts exposed lower cytotoxic effect.

3.4.3 Trypan Blue Dye Exclusion Assays

The effects of different *S.absconditiflora* extracts concentrations ranging from 0.25 to 3 mg/mL for water extracts and from 0.25 to 2 mg/mL for methanol extracts on the cell viability of HepG2 cells were analyzed at 48 and 72 hr. HepG2 cells that were grown in 0,1% DMSO containing medium was used as a control. Percent viability was calculated by using the cell numbers. The cell number of 0.1% DMSO was used as a control and counts as 100% (Figure 3.27-30).

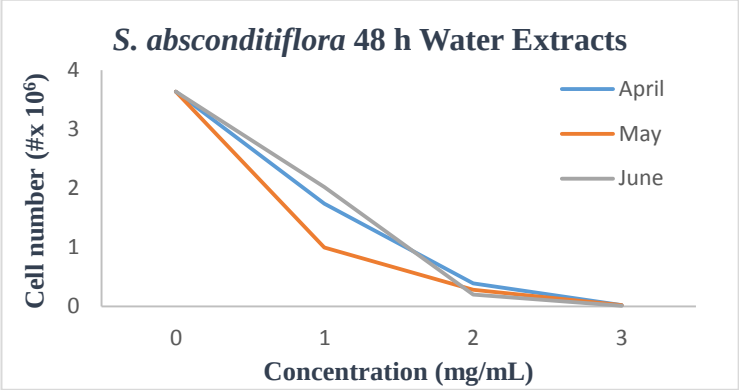

Figure 3.27 Effects on 48 h incubation of water extracts on cell number

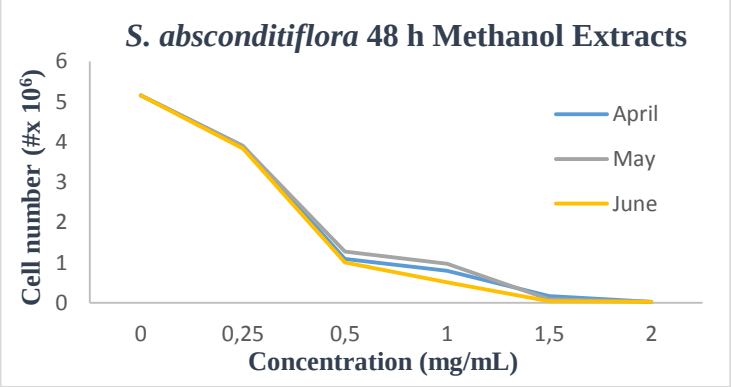

Figure 3.28 Effects on 48 h incubation of methanol extracts on cell number

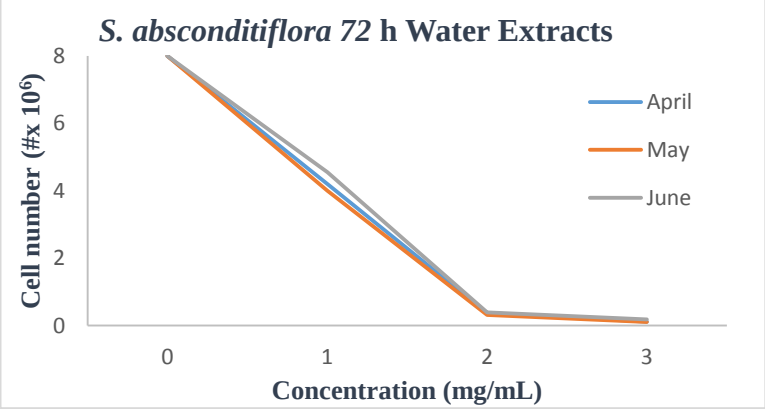

Figure 3.29 Effects on 72 h incubation of water extracts on cell number

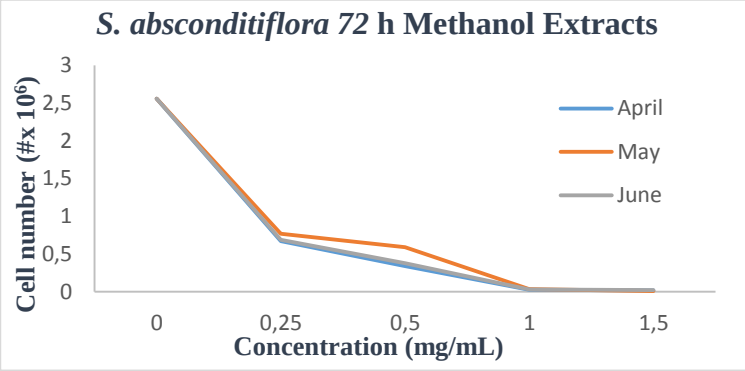

Figure 3.30 Effects on 72 h incubation of methanol extracts on cell number

S.absconditiflora water extract varying concentrations at which 50% of cells are alive (IC_{50}) were calculated for HepG2 cells as 1.884 ± 0.025 for April; 1.793 ±0.08 for May; 1.892 ±0.02 for June at 48 hr incubation and 1.226 ± (Table 3.14).

S.absconditiflora methanol extract varying concentrations at which 50% of cells are alive (IC_{50}) were calculated for HepG2 cells as 0.651 ± 0.02 for April; 0.659 ± 0.03 for May; 0.63 ± 0.06 for June at 48 hr incubation and 0.407 ± 0.02 for April; 0.421 ± 0.06 for May; 0.41 ± 0.04 for June at 72 h incubation concentrations of *S.absconditiflora* extract.

Table 3.14 IC50 values of *Salvia* extracts for RNA isolation

			IC_{50} mg/mL ± SD
48 h Incubation	**Water**	April	1.88 ± 0.03
		May	1.93 ± 0.08
		June	1.89 ± 0.02
	Methanol	April	0.65 ± 0.02
		May	0.66 ± 0.03
		June	0.63 ± 0.06
72 h Incubation	**Water**	April	1.23 ± 0.03
		May	1.20 ± 0.04
		June	1.26 ± 0.02
	Methanol	April	0.41 ± 0.02
		May	0.42 ± 0.06
		June	0.41 ± 0.04
All Extracts	**48 h Incubation**	Water	0.79 ± 0.00
		Methanol	0.74 ± 0.04
	72 h Incubation	Water	0.65 ± 0.06
		Methanol	0.59 ± 0.01

The lowest IC_{50} values of the *S. absconditiflora* extracts were determined as 0.41 ± 0.04 for June methanol.

In one of the study, the methanol extracts of six *Salvia* sp.: against HT-29 and WiDr by MTT assay were shown. Growth inhibitory activities (IC_{50} μg/ml) of crude methanolic extracts of *S. menthaefolia, S. sclarea, S. dominica, S. spinosa* for 72 h of treatment against HT-29 cells were reported as 95.3 mg/mL, 196.1 mg/mL, 119.5 mg/mL and 129.4 mg/mL, respectively (Fiore et al., 2015)

According to Loizzo et al. (2014) the antiproliferative activities of methanol extracts of nine *Salvia* sp. (collected from Iran) against Caco-2 cancer cells by sulforhodamine B (SRB) assay. The highest cytotoxic effect was investigated by *S. sclarea* with an IC_{50} value of 101.8 ± 6.4 μg/mL. Compared to the IC_{50} values, our extracts of *S. absconditiflora* exhibited approximately lower anti proliferative activity with water and methanol extracts of *Salvia species* in the litrerature.

The anti proliferative effects of two main standards of *Salvia*, caffeic acid and its derivatives RA on MCF-7 breast cancer cell line were found 5 to 50 μg/mL with an IC_{50} value of 10.7 μg/mL (Viveros Valdez et al., 2010).

The cytotoxix effects of caffeic acid against MDA-MB-231 cell lines was observed significantly at 50 μM concentration for 72 hr treatment by MTT assay (Gomes et al., 2003).

In our study, we demonstrated that the extracts of *S. absconditiflora* with its main bioactive components of caffeic acid and rosmarinic acid described by RP-HPLC indicated a good anticancer activity by inhibiting the proliferation of HepG2 cells with their low IC_{50} values. *S. absconditiflora* extracts can be a good applicant for the targeted therapy with medical purposes.

3.4.4 Morphological Analysis of HepG2 Cells by Light Microscopy

In order to observe the cytotoxic effects of *S. absconditiflora* extract on HepG2 cells, morphological changes and viability of cells were monitored under the inverted light microscope (Figure 3.31 and 3.32).

After treatment of HepG2 cells with *S.absconditiflora* extracts at IC_{50} concentrations were determined and growth inhibition in cells were observed compared with 0.1 % DMSO treated control cells.

In both time incubations, the confluency significantly decreased.

3.5 Total RNA Isolation

In our study; IC50 values of XTT and trypan blue dye exclusion assay are different from each other. This variation may be results from the cell plating area and cell number. Because in XTT, only $10x10^3$ cells were plated in 96 well but in TB dye exclusion/ RNA isolation $50x10^4$ cell were plated in 6 well plate.

After the quantification of IC50 values for RNA isolation, cells were seeded into a 65x15 mm flask as duplicate at a density of $25x10^4$cells/mL and 6 mL in each plate. After 1 day, old medium was discarded and samples were added in it. Cells were cultured for 48 and 72 h and then RNA isolation was carried out.

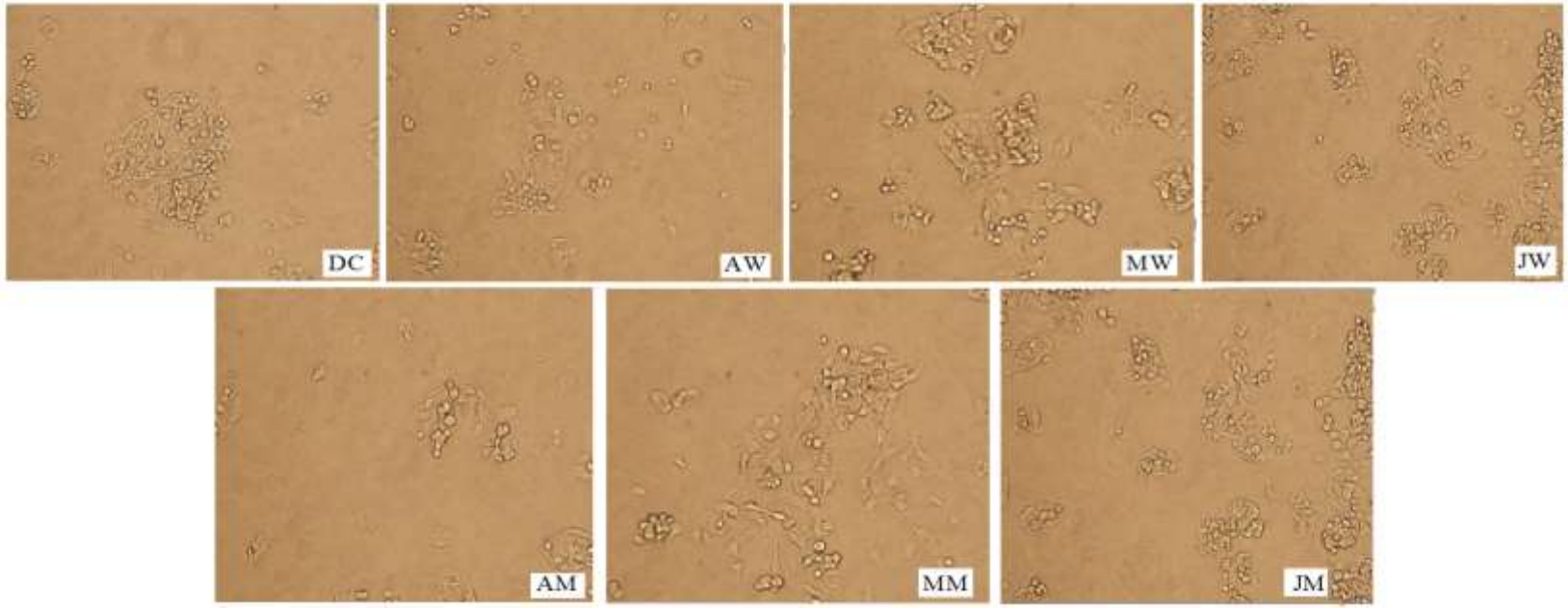

Figure 3.31 Cell Photographs of 48 h incubation of cell with cytotoxic concentration (IC_{50})

DC:DMSO Control; AW: April Water; MW: May Water; AM: April Methanol; MM: May Methanol; JM: June Methanol

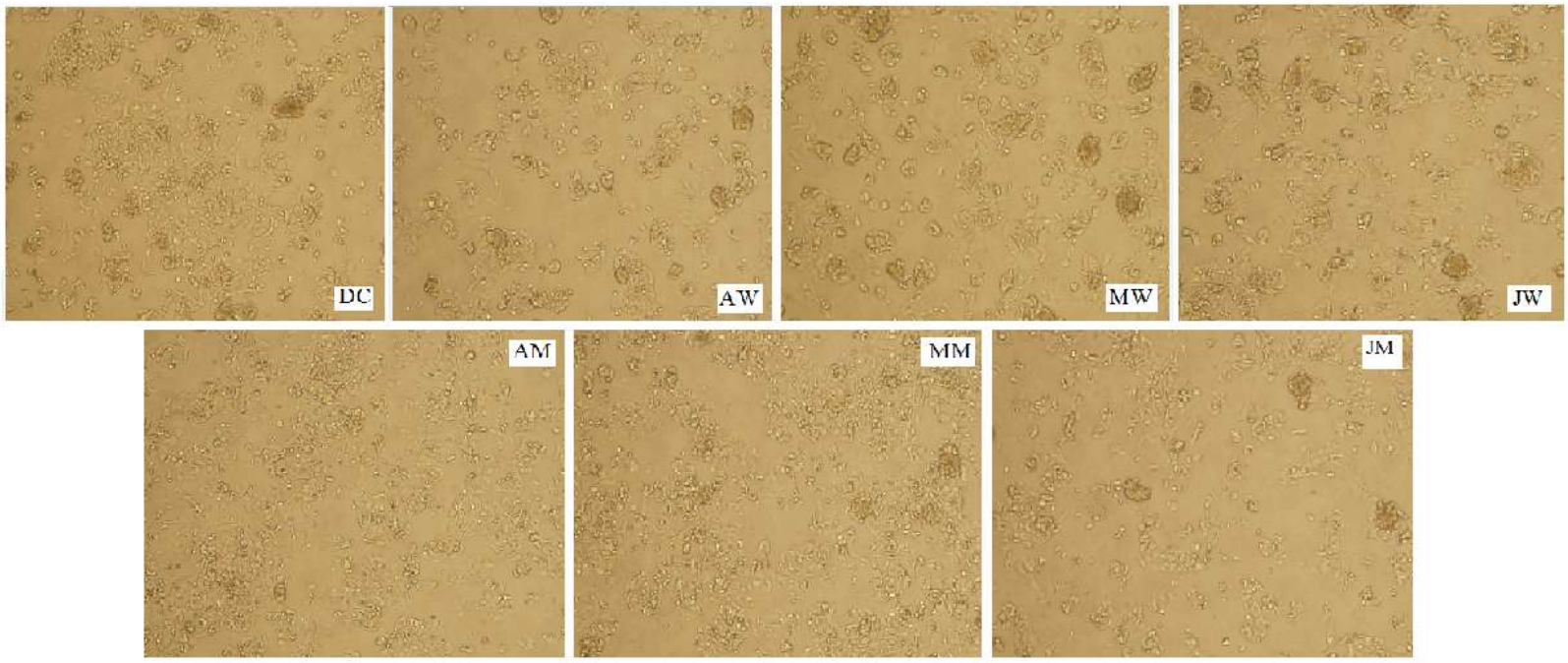

Figure 3.32 Cell Photographs of 72 h incubation of cell with cytotoxic concentration (IC_{50})

DC:DMSO Control; AW: April Water; MW: May Water; AM: April Methanol; MM: May Methanol; JM: June Methanol

3.5.1 Qualification of RNA by Agarose Gel Electrophoresis

In order to check the quality of total RNA, agarose gel electrophoresis was performed. 3 subunits of RNA (28s, 18s and 5s) without DNA contamination should be observed. We were able to observe 28s large ribosomal subunit and 18s ribosomal small unit RNA without 5s RNA. If RNA contamination was occur, we could not see any of the bands. Picture of otal RNA which were isolated from HepG2 cells according to their cytotoxic concentration of S. absconditiflora (Figure 3-33).

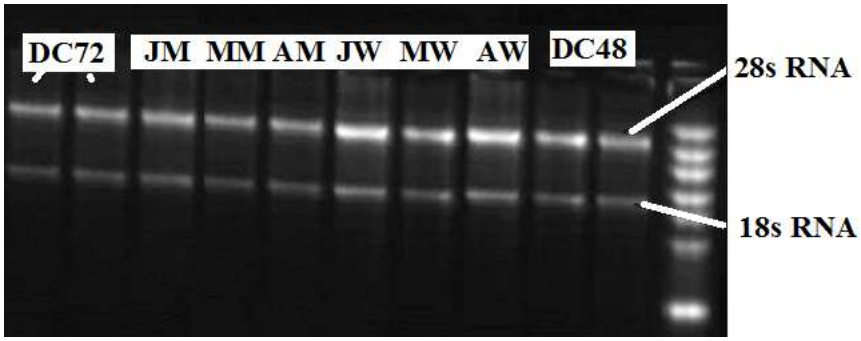

Figure 3.33 Gel photograph for Total RNA

3.5.2 Determination of RNA Purity and Concentration

In order to determine RNA purity, we used absorbance ratio. Proteins give absorbance at 280 nm, the protein contamination in RNA samples was measured by calculating the A260/A280 ratio. The ratio of A260/A280 must be between 1.8 and 2.2. Above the 2.2 refers the DNA contamination while below 1.8 referring the protein contamination. Table 3.16 showed the isolated RNA concentration and their purities.

Table 3.15 RNA concentration and purity

		Incubation (h)	RNA concentration (ng/µL)	260/280
	Control	48	589.5	2.03
		72	847.5	2.05
Water	April	48	554.6	2.02
		72	923.4	2.07
	May	48	649.6	2.03
		72	640.7	2.03
	June	48	816.6	2.05
		72	510.9	2.03
Methanol	April	48	645.4	2.04
		72	897.0	2.02
	May	48	814.1	2.07
		72	616.4	2.03
	June	48	562.1	2.04
		72	392.8	2.08

3.6 Gene Expression Analysis

Quantitative real time PCR (qRT-PCR) technique was used to determine the mRNA expression of 8 different gene in the HepG2 cell and GAPDH was used as an internal standard to calculate relative mRNA expressions. Relative mRNA expression of targeted gene were calculated by constructing a standard curve from 1:5, 1:10, 1:20, 1:40 and 1:80 dilutions of cDNA of control (Figure 3.34).

The Ct value or the threshold cycle shows that a significant increase in fluorescence was first detected. The higher the Ct value, the smaller the copy number, thus it takes more reactions to reach the detection level.

Comparative CT method ($[1+\text{Efficiency}]^{-\Delta\Delta Ct}$) was used for calculation of mRNA expression of the interested genes. When we used this technique, fold change is easily calculated. Comparison of gene expression of two different samples was calculated as shown below;

ΔΔCt = [(Ct gene of interest – Ct internal control) treated – (Ct gene of interest – Ct internal control) control]

Comparative Fold change = [1+efficiency]$^{-\Delta\Delta Ct}$

3.6.1 CYP1A1 mRNA expression in HepG2 cell line in response to *Salvia* extract treatments

The amplification plot demonstrates the changes in fluorescence of SYBR green dye I versus cycle number of CYP1A1 gene. After the cycle 22 flourescence reached to the 100 (Figure 3.35). Melt curve analysis was performed to check amplification of a specific product (Figure 3.35).

Quantitative real time PCR (qRT-PCR) technique was used to determine the mRNA expression of CYP1A1 in the HepG2 cell and GAPDH was used as internal standard to calculate relative mRNA expression of CYP1A1.

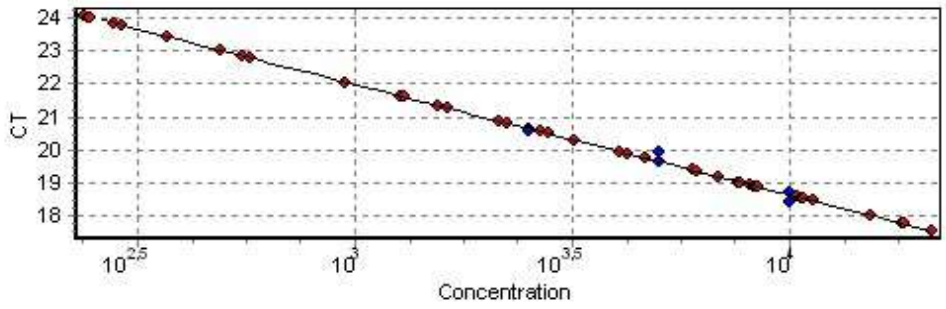

Figure 3.34 CYP1A1 standard curve generated from serial dilutions of control cDNA.

Efficiency=0.998

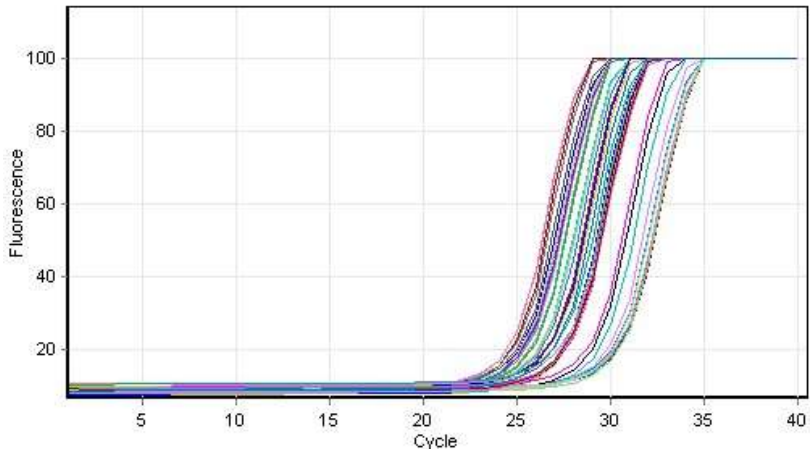

Figure 3.35 CYP1A1 amplification curve; the accumulation of fluorescence emission at each reaction cycle.

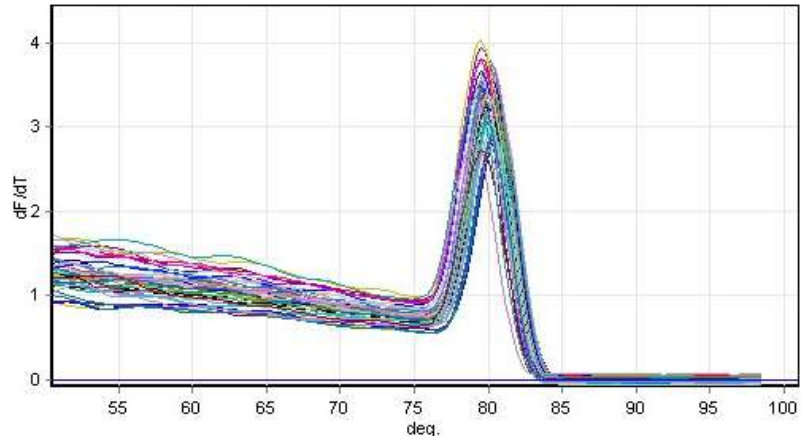

Figure 3.36 CYP1A1 melting curve; the fluorescence emission change with temperature.

Single peak means single PCR product.

Figure 3.37shows the mean ± SD of the relative CYP1A1 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP1A1 gene expression were shown in Table 3.16.

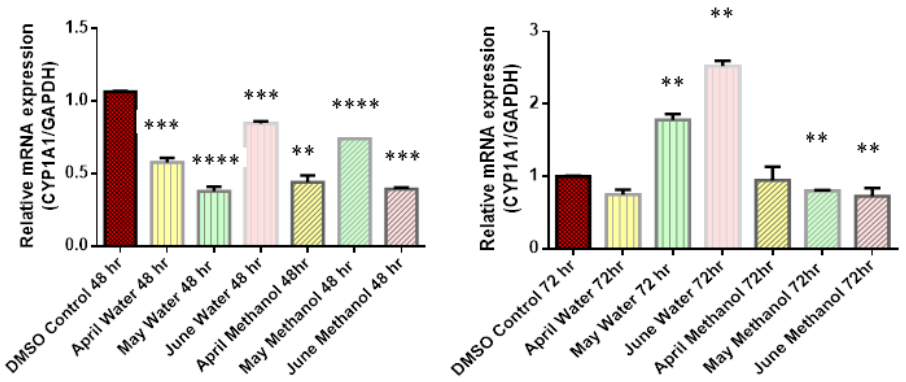

Figure 3.37 Comparison of CYP1A1 mRNA expression of HepG2 cells.

The quantifications are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

CYP 1A1 catalyzes the conversion of many pro-carcinogens to active carcinogenic compounds and hydroxylation of many endogenous substrates including the arachidonic acid and the hormones such as melatonin (Schwarz et al. 2004; Ma et al. 2005). In our results, relative mRNA expression of CYP1A1 was highly significant according to control. After 48 hr incubation of all extracts, mRNA expressions were changed (Table 3.16). May water and methanolic extracts have the most statistically significant data (**** P≤0.0001). 1.78 fold increases in the expression is observed after 72 h incubation.

In one of the study, undifferentiated HepaRG cells, the expression of most P450s was low compared with the human hepatocytes (0.2-fold) except for CYP1A1 and CYP7A1, which were comparable with human hepatocytes (Kanebratt and Andersson, 2008)

Table 3. 16 Fold changes of CYP1A1 gene expression

Fold Change of CYP1A1 gene Expression		
	48 h Incubation	**72 h Incubation**
AW	1.72 ↓	1.33 ↓
MW	2.63 ↓	1.78 ↑
JW	1.17 ↓	2.36 ↑
AM	1.92 ↓	1.05 ↓
MM	1.08 ↓	1.25 ↓
JM	2.08 ↓	1.37 ↓

3.6.2 CYP1A2 mRNA expression in HepG2 cell line in response to *Salvia* extract treatments

CYP1A2 standard curve, amplification curve and melting curve were shown in Appendix A.

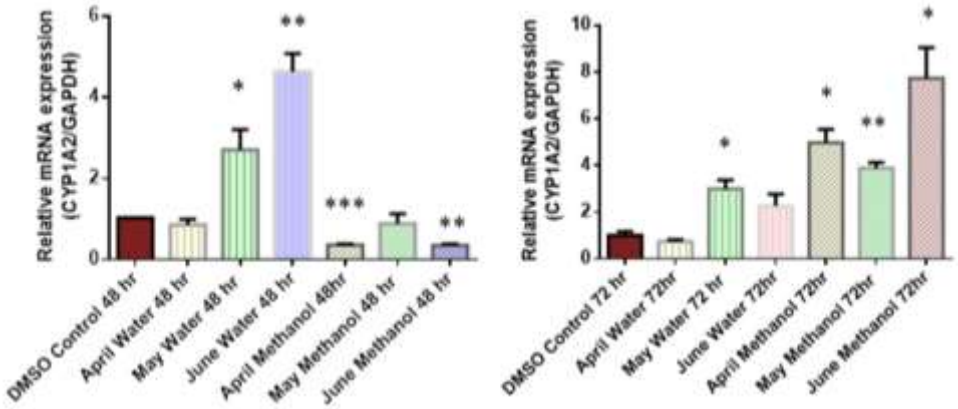

Figure 3.38 Comparison of CYP1A1 mRNA expression of HepG2 cells.

The valuess are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

Figure 3.38 shows the mean ± SD of the relative CYP1A2 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP1A2 gene expression in response to *Salvia* extract treatment were shown in Table 3.17.

CYP 1A2 catalyzes many detoxification and synthesis of compounds like cholesterol and it is localized in the endoplasmic reticulum. This enzyme is induced by many compounds such as polycyclic aromatic hydrocarbons (PAHs). In our results, after 48 h incubation only April water extract did not have any significance (Table 3.17). April methanol extracts had the most statistically significant data (*** $P \leq 0.001$).

Table 3.17 Fold changes of CYP1A2 gene expression in response to *Salvia* extracts

	48 h Incubation	**72 h Incubation**
AW	1.09 ↓	1.61 ↓
MW	1.75 ↑	2.84↑
JW	2.17 ↑	1.94 ↑
AM	1.69 ↓	4.85 ↑
MM	1.06 ↓	3.27 ↑
JM	1.72 ↓	6.07 ↑

Some of the extracts were decrease the expression of CYP1A2 but others increase the gene expression.

In the literature, CYP1A1 in primary hepatocyte were 10 times lower than HepG2 cells (Westerink, 2007).

3.6.3 CYP2E1 mRNA expression in HepG2 cell line in response to *Salvia* extract treatments

CYP2E1 standard curve, amplification curve and melting curve were shown in Appendix A.

Figure 3.39 shows the mean ± SD of the relative CYP2E1 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP2E1 gene expression were shown in Table 3.18.

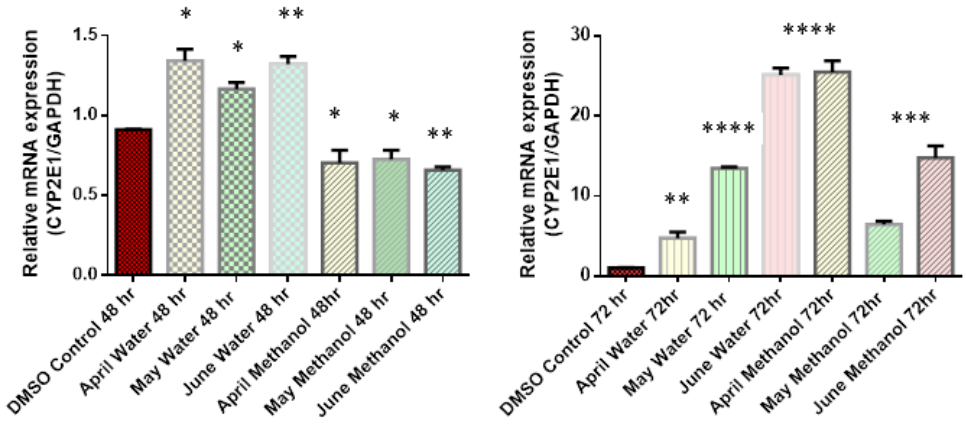

Figure 3.39 Comparison of CYP2E1 mRNA expression of HepG2 cells.

The values are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

Most of the drugs undergo detoxification by CYP2E1, either directly or by facilitated removal from the body. Also, many substances are bioactivated by CYP2E1 to form their active compounds. All Salvia extracts have significant effects on the gene expression of CYP 2E1 (Table 3.18). Except April water extract which have p value ≤0.01 but others have highly statistically significant; p values ≤0.0001 according to the control.

After 72 h of incubation in all extracts CYP 2E1 gene expression is increased, maximum value is obtained with April methanol extract with 29 fold increase.

Table 3.18 Fold changes of CYP2E1 gene expressionin response to *Salvia* extract treatment

	48 h Incubation	**72 h Incubation**
AW	1.254 ↑	4.74 ↑
MW	1.1 ↑	13.46↑
JW	1.32 ↑	25.15 ↑
AM	1,43 ↓	29.189 ↑
MM	1,38 ↓	8.7 ↑
JM	1,52 ↓	14.49 ↑

3.6.4 CYP 3A4 mRNA expression in HepG2 cell line in response to *Salvia* extract treatment

CYP3A4 metbolizes approximately 50% of the drugs that are commercially used (Viganati et al., 2005). CYP3A4 standard curve, amplification curve and melting curve were shown in Appendix A.

Figure 3.40 shows the mean ± SD of the relative CYP3A4 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP3A4 gene expression were shown in Table 3.19.

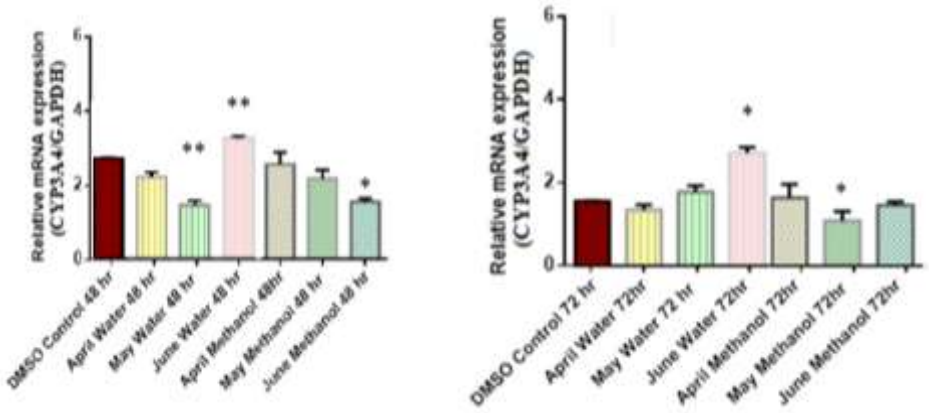

Figure 3.40 Comparison of CYP 3A4 mRNA expression of HepG2 cells.

The values are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

CYP 3A4 enzyme plays important roles in xenobiotic detoxification and activation of prodrugs which were mainly found in the liver.

According to our results, only April water 72 h incubation has not statistically significant data. June water and methanol extracts have the most statistically significant data (P≤0.001).

Table 3.19 Fold changes of CYP3A4 gene expression in response to *Salvia* extract treatment

	48 h Incubation	**72 h Incubation**
AW	2 ↓	1.1↓
MW	1.3↓	0.7↑
JW	1.43↑	1.4↑
AM	5 ↑	0.3↑
MM	2 ↓	1.1↓
JM	1.5 ↓	1.3↓

3.6.5 GSTP1 mRNA expression in HepG2 cell line in response to *Salvia* extract treatment

GSTP1 standard curve, amplification curve and melting curve were shown in Appendix A.

Figure 3.41 shows the mean ± SD of the relative GSTP1 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and its fold change was accepted as 1. Fold changes of GSTP1 gene expression were shown in Table 3.20

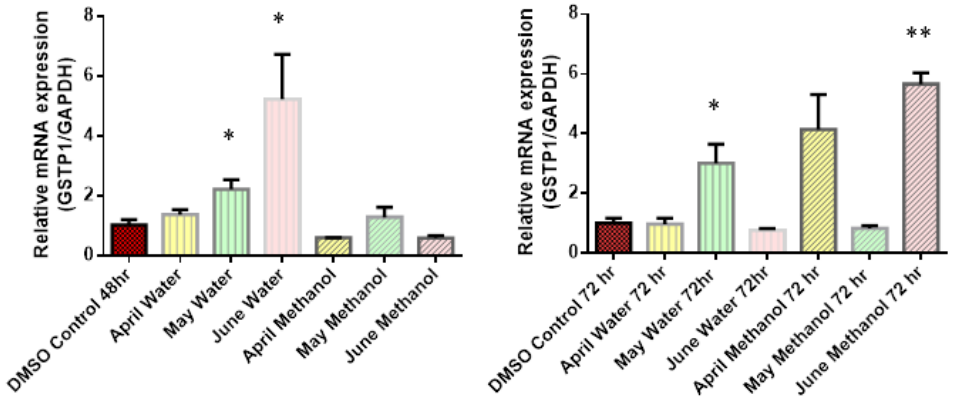

Figure 3.41 Comparison of GSTP1 mRNA expression of HepG2 cells.

The values are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

Table 3.20 Fold changes of GSTP1 gene expression in response to *Salvia* extract treatment

	48 h Incubation	**72 h Incubation**
AW	1.39 ↑	0.98 ↑
MW	2.23 ↑	3.02 ↑
JW	5.24 ↑	0.61 ↓
AM	1.64 ↓	4.14 ↑
MM	2.29 ↑	0.75 ↓
JM	1.67 ↓	7.82 ↑

Cytololic Glutathione-S-Transferase family have 13 isoenzymes including alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega. They show extensive substrate specificity. These enzymes combine reduced glutathione to xenobiotics and detoxify them. GST pi gene is polymorphic but GSTP1 is the most abundant and functional one. According to our result only 2 of the 48 h incubation (MW and JW) and 2 of the 72 h incubated GSTP1 mRNA expressions were statistically significant (Table 3.20). Gene expression drastically increased in June methanol extract after 72 h incubation.

3.6.6 GSTM1 mRNA expression in HepG2 cell line in response to *Salvia* extract treatment

GSTM1 standard curve, amplification curve and melting curve were shown in Appendix A.

Figure 3.42 shows the mean ± SD of the relative GSTM1 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and its fold change was accepted as 1. Fold changes of GSTM1 gene expression were shown in Table 3.21

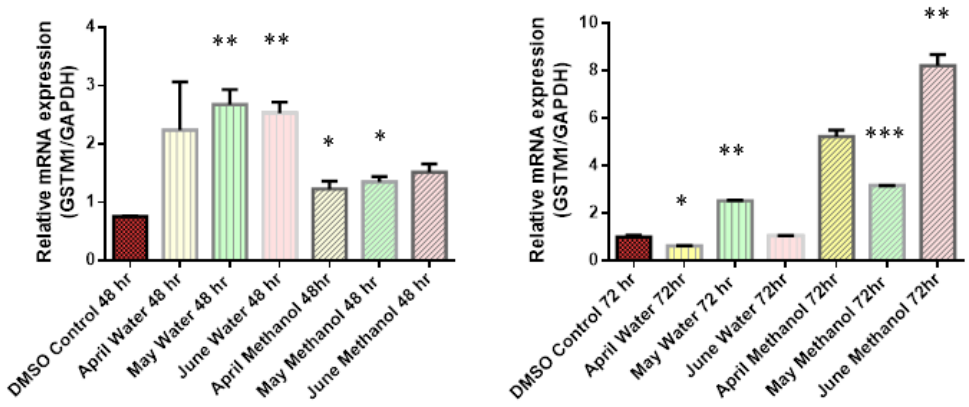

Figure 3.42 Comparison of GSTM1 mRNA expression of HepG2 cells.

The quantifications are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

All extracts induce GSTM1 expression after 48 h of incubation indicating an oxidative stress condition, though only one of the extracts; April water; decreased its expression when incubated for further 72 h but most of them also induce the GSTM1 expression.

Table 3. 21 Fold changes of GSTM1 gene expression in response to *Salvia* extract treatment

	48 h Incubation	**72 h Incubation**
AW	3.30 ↑	0.62 ↓
MW	2.61 ↑	2.52 ↑
JW	2.53 ↑	1.05 ↑
AM	2.61 ↑	5.22 ↑
MM	1.35 ↑	3.16 ↑
JM	1.51 ↑	8.21 ↑

The GST mu enzymes dexotifies electrophilic compounds, such as carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione. GSTM1 gene is polymorphic in human population, and these genetic polymorphisms in this

enzymes are important risk factors in diseases such as cancer due to increased susceptibility to environmental toxins.

3.6.7 GPx4 mRNA expression in HepG2 cell line

GPx4 standard curve, amplification curve and melting curve were shown in Appendix A.

Figure 3.43 shows the mean ± SD of the relative GPx4 mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and its fold change was accepted as 1. Fold changes of GPx4 gene expression were shown in Table 3.23.

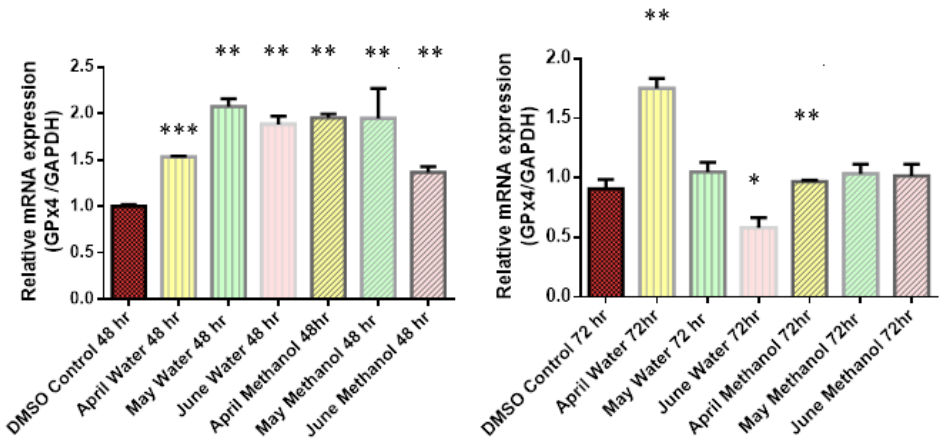

Figure 3.43 Comparison of GPx4 mRNA expression of HepG2 cells.

The values are expressed as mean ± SD of the relative expression.
* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

Glutathione Peroxidase 4 (GPx4), is an antioxidant enzyme. In mammalian glutathione peroxidase family divided 8 isoenzymes (GPx1-8). GPx4 catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, and lipid peroxides at the expenditure of reduced glutathione and functions in the protection of cells against oxidative stress. All extracts induce GPx4 expression after 48 h of incubation indicating an oxidative stress condition,

though only one of them of the extracts; June water, decreased its expression when incubated for further 72 h.

Table 3.22 Fold changes of GPx4 gene expression in response to *Salvia* extract treatment

	48 h Incubation	**72 h Incubation**
AW	1.53 ↑	1.51 ↑
MW	2.25 ↑	1.14 ↑
JW	1.77 ↑	0.58 ↓
AM	1.84 ↑	0.97 ↓
MM	1.95 ↑	1.04 ↑
JM	1.37 ↑	1.02 ↑

3.6.8 Catalase mRNA expression in HepG2 cell line in response to *Salvia* extract treatment

Catalase standard curve, amplification curve and melting curve were shown in Appendix A.

Figure 3.44 shows the mean ± SD of the relative Catalase mRNA expressions of HepG2 cells. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed. Gene expressions in 0.1% DMSO treated cDNA was used as controls and its fold change was accepted as 1. Fold changes of Catalase gene expression were shown in Table 3.23.

Catalase is a common enzyme found in most of the living organisms which are exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al., 2004).

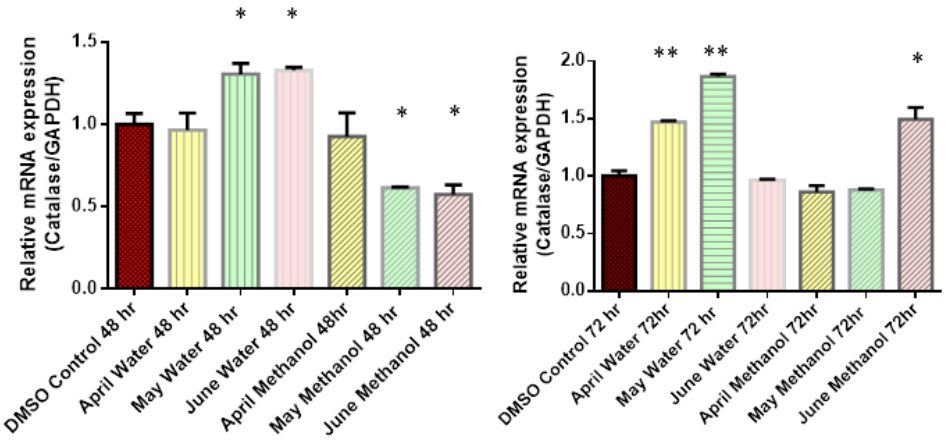

Figure 3.44 Comparison of Catalase mRNA expression of HepG2 cells. The values are expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

Table 3.23 Fold changes of Catalase gene expression in response to *Salvia* extract treatment

	48 h Incubation	**72 h Incubation**
AW	0.97 ↓	1.47 ↑
MW	1.31 ↑	0.87 ↑
JW	1.33 ↑	0.84 ↓
AM	0.93 ↓	0.86 ↓
MM	0.62 ↓	0.87 ↓
JM	0.66 ↓	1.34 ↑

Unlike GPx4 expressions, catalase showed mostly decreased expressions with especially methanol extracts. Methanol extracts increased GPx expression especially at 48 h incubation. Oxidative stress produced an imbalance between the produced reactive oxygen species and the biological system's ability to detoxify these ROS or to repair the resulting damage. The oxidative defense mechanism is made up of several components including these antioxidant enzymes.

In 48 h incubation, water extracts induce the gene expression of Catalase but methanol extracts decrease the gene expression. In 72 h incubation, nearly all extracts induce the gene expression except April and May methanol.

3.7 Panel Assay

Recently panel assay is a more preferred technique. It is easy and we can perform up to 100 PCR arrays with one panel in a reaction volume of 20µL. Microplate containing 88 targeted genes with 8 housekeeping gene primer sets (20 µL per well, 10 µM concentration).

Figure 3.45 shows the dilutions of panel to final primer concentration is 0.1 µM.

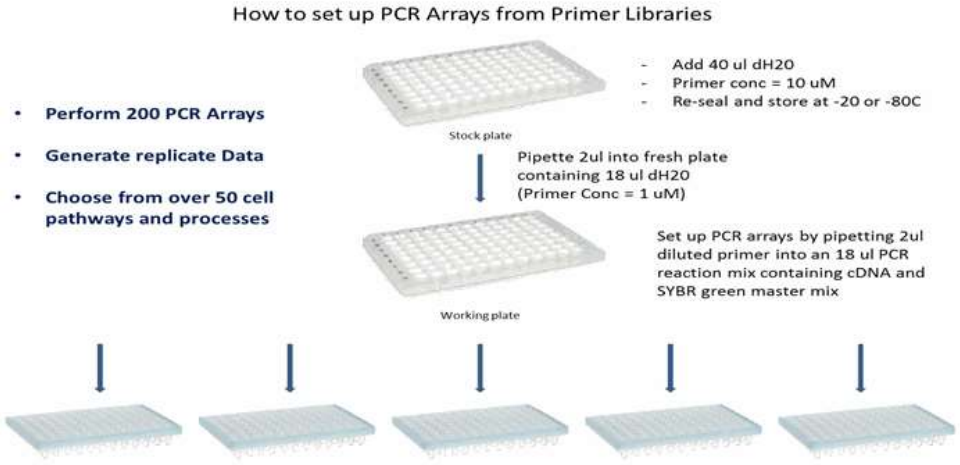

Figure 3.45 Serial dilutions of panel primers

After dilutions, reaction mixture was prepared to a total volume of 10 or 20 µL. Reaction mixture contains 3 µL cDNA (15 X diluted), 2 µL primer (0.1µM) and 5 µL SYBER Green Master mix. Table 3.24 shows the fold change values of Phase I genes and Table 3.25 shows the fold change values of the Phase II genes.

Some of the primers were not active in panel so no gene expression results were obtained. Still in others, with treatment, some of the gene expressions were not observed, so no results were given in Table 3.24.

Table 3.24 The fold change values of Phase I genes. ACTB was used as internal standard.

		HDTX-I TM48	HDTX-I TW48			HDTX-I TM48	HDTX-I TW48
WELL	SHORT NAME	Fold change	Fold change	WELL	SHORT NAME	Fold change	Fold change
A1	AADAC	1,35	2,02	E1	CYP2J2	1,20	-1,89
A2	ADH1A	1,10	1,61	E2	CYP2R1		
A3	ADH1B	2,16	5,88	E3	CYP2S1	3,19	2,72
A4	ADH1C	-2,58	-1,40	E4	CYP2W1	-44,54	3,23
A5	ADH4	-2,32	88,76	E5	CYP3A4	-3,35	3,10
A6	ADH5	1,47	4,18	E6	CYP3A43	2,66	5,57
A7	ADH6	-0,68	1,21	E7	CYP3A5	1,12	1,79
A8	ADH7	-3,06	1,12	E8	CYP3A7	-7,48	1,55
A9	ALDH1A1	3,72	>100	E9	CYP4A11	1,66	-1,75
A10	ALDH1A2	-11,68	-35,36	E10	CYP4A22	-6,71	-16,60
A11	ALDH1A3	-2,89	-2,13	E11	CYP4B1	-3,55	-6,25
A12	ALDH1B1	-13,30	-3,17	E12	CYP4F11	7,50	1,35
B1	ALDH2	1,11	8,23	F1	CYP4F12	2,15	4,18
B2	ALDH3A1	1,37	>100	F2	CYP4F2	4,28	2,10
B3	ALDH3A2	3,85	8,11	F3	CYP4F3	6,31	2,60
B4	ALDH3B1		>100	F4	CYP4F8	-66,61	-12,50
B5	ALDH3B2			F5	CYP7A1	-9,78	-3,85
B6	ALDH4A1	3,46	1,41	F6	CYP7B1	-1,40	-6,66
B7	ALDH5A1	-5,64	1,02	F7	CYP8B1	-13,21	-1,15
B8	ALDH6A1	-4,96	1,78	F8	DHRS2	-1,38	-1,12
B9	ALDH7A1	-3,84	23,88	F9	DPYD	-3,52	-1,23
B10	ALDH8A1	-40,23	6,72	F10	ESD	2,44	-1,56
B11	ALDH9A1	1,12	3,42	F11	FMO1	-7,62	-11,11
B12	CEL	-1,08	>100	F12	FMO2	-2,04	-12,50
C1	CYP11A1	2,30	4,79	G1	FMO3	-7,81	-7,69
C2	CYP11B1	-100,00	-1,35	G2	FMO4		
C3	CYP11B2	0,76	-1,59	G3	FMO5		
C4	CYP17A1	16,63	16,37	G4	GZMA	-4,44	1,88
C5	CYP19A1	1,36	22,35	G5	GZMB	-5,99	-1,85
C6	CYP1A1	10,19	>100	G6	HSD17B1	1,37	-1,11
C7	CYP1A2	1,13	8,89	G7	HSD17B10	-1,28	-1,54
C8	CYP1B1	6,28	20,24	G8	HSD17B2	-1,29	1,69
C9	CYP21A2	4,53	-3,42	G9	HSD17B3	-55,28	3,61
C10	CYP24A1	2,44	2,46	G10	MAOA	-2,36	-1,41
C11	CYP26A1			G11	MAOB	-1,06	-1,75
C12	CYP26B1	-2,86	-1,06	G12	PTGS1	-2,71	
D1	CYP26C1	3,04	-1,28	H1	PTGS2	1,22	1,90
D2	CYP27A1	1,98	3,09	H2	UCHL1	-8,47	-1,25
D3	CYP27B1	-2,66	1,64	H3	UCHL3	1,09	1,03
D4	CYP2A13	319,92	-1,16	H4	XDH	-4,03	-2,00
D5	CYP2B6	-4,42	0,30	H5	ACTB		
D6	CYP2C18	-12,28	94,15	H6	B2M	2,72	5,63
D7	CYP2C19	-12,69		H7	GAPDH	-1,86	-1,09
D8	CYP2C8	-2,15	1,61	H8	GUSB	-1,61	0,88
D9	CYP2C9	-15,13	2,27	H9	HPRT1	-1,62	-2,17
D10	CYP2D6	4,76	1,84	H10	PGK1	-4,69	1,05
D11	CYP2E1	4,02	>100	H11	PPIA	-1,51	1,19
D12	CYP2F1	-10,28	-6,66	H12	RPL13A	-0,58	3,49

Most of the drugs are metabolized first with CYP450 enzymes. The most important enzymes are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

In Phase I panel assay, water extracts induce the gene expression of all above mentioned CYP enzymes. Methanol extracts change the gene expressions. CYP1A2, CYP2D6 and CYP2E1 gene expression were induced with total methanol extract. CYP2C9, CYP2C19 and CYP3A4 gene expression were decrease with total methanol extracts.

According to our results, CYP11B1 gene expression was decreased by around 100 fold when the cells were treated with metanol extract. In adrenal glands, it plays critical roles in cortisol and corticosteroid synthesis (Martinez-Aguayo and Fardella, 2009). While cortisol maintains blood sugar level, and have critical roles on immune system, corticosteroids play role in aldosterone synthesis which control the blood pressure (Moraitis et al., 2013).

Similarly, CYP2A13 gene expression was increased 100 fold with methanol extract treatment. This enzyme which is found in liver and also in extrahepatic tissues, plays an important role in tobacco toxicity and toxicity releated tumor in respiratory tract (Su et al., 2000).

On the other hand, CYP2W1 gene expression was found to be decreased by 44 fold with metanol extract treatment. CYP2W1 overexpression has been reported in a variety of human cancers. The enzyme is induced selectively in colon cancers and for this reason the enzyme is critical drug target for cancer diseases (Gomet et al., 2007). Recently it was found that, CYP2W1 could play an important role in human hepatocellular carcinoma and might serve as a valuable prognostic marker and potential target for gene therapy in the treatment of HHC (Zhang et al., 2014).

CYP4F8 gene expression was decreased 66 fold again with metanol extract. CYP4F8 is a member of arachidonic acid pathway and identified as a

therapeutic target, together with PLA2G7, HPGD, EPHX2 genes in prostate cancer (Stark K,et al.,2003).

According to our results, the methanol extract of *S.absconditiflora* shows the most potent effects on CYP enzymes, whereas the aqueous, water extract shows less effect on gene expressions.

Our panel results showed that, CYP17A1 gene expression was increased aproximately 17 fold with water extract treatment. This gene plays an important role in cholesterol synthesis and play role in prostate cancer progression and development (Rai et al., 2014). The drug abiraterone which is used to treat castration-resistant prostate cancer blocks the biosynthesis of androgens by inhibiting the CYP17A1 enzyme (Gomez et al., 2014).

CYP19A1 gene expression was increased aproximately 23 fold with water extract treatment. This enzyme named also aromatase, is an enzyme responsible for a key step in the biosynthesis of estrogens in gonads. Aromatase inhibitors, which stop the production of estrogen in postmenopausal women, are also used in the treatment of estrogen receptor positive breast cancers (Mononen et al., 2006).

CYP27A1 gene expression was increased aproximately 3 fold with *S.absconditiflora* water extract. This enzyme plays role in cholesterol degradation. Cholesterol is converted to bile acid to be removed from the body (Cali and Russell, 1991).

CYP7A1 gene expression was decreased aproximately 4 fold with water extract treatment. This enzyme plays an important role in cholesterol synthesis and degradation. If mutation occur in this gene, it can be observed hyperlipidemia (Pullinger et al., 2002). Due to its important regulatory role in cholesterol catabolism, decreased CYP7A1 levels may lead to hypercholesterolemia.

Table 3. 25 The fold change values of Phase II genes. RPL13A was used as internal standard

		HDTX-II TM48	HDTX-II TW48			HDTX-II TM48	HDTX-II TW48
WELL	SHORT NAME	Fold change	Fold change	WELL	SHORT NAME	Fold change	Fold change
A1	AANAT	-89,3	-6,3	E1	NNMT	-7,19	-1,35
A2	ACSL1	-1,26	1,57	E2	NQO1	-2,4	3,78
A3	ACSL3	-8,99	1,46	E3	NQO2	-2,3	2,92
A4	ACSL4	-18,28	>100	E4	PNMT	-18,8	-9,9
A5	ACSL5	1,90	-1,51	E5	PTGES	-12,3	3,65
A6	ACSL6	-100	-27	E6	SAT1	-1,72	1,18
A7	ACSM1	-8,26	1,22	E7	SAT2	-5,8	-2,49
A8	ACSM2B	-56,22	1,29	E8	SULT1A1	-7,4	3,93
A9	ACSM3	-6,16	2,11	E9	SULT1A2	-33,6	1,22
A10	AGXT	-100		E10	SULT1A3	-8,85	2,23
A11	AS3MT	-9	1,62	E11	SULT1A4	-38	-4,09
A12	ASMT	-2,76	-8,2	E12	SULT1B1	-18	-1,1
B1	BAAT	-2,52	7,18	F1	SULT1C2	-6,5	-1,3
B2	CCBL1	-2,55	-10	F2	SULT1C3	-6,8	-2,7
B3	CES1	-4,22	4,39	F3	SULT1C4	-20,8	-2,4
B4	CES2	-54,85	-1,73	F4	SULT1E1	-55,7	-7,6
B5	CES3	-3,76	-1,15	F5	SULT2A1	-100	-3
B6	CES5A	-21,65	-7,17	F6	SULT2B1	-1,95	-5,6
B7	COMT	-26,24	9,92	F7	SULT4A1		
B8	EPHX1	-6,95	4,08	F8	SULT6B1	-3,36	-3,5
B9	EPHX2	-34,5	-1,57	F9	TPMT	-4,17	1,46
B10	GAMT	-100	-3,9	F10	TST	-6,6	1,22
B11	GLYAT	-14,5	-16,8	F11	UGT1A1		1,98
B12	GNMT	-19,76	-2,6	F12	UGT1A3	-29	1,21
C1	GSTA1	-28,8	-8,65	G1	UGT1A4		-4
C2	GSTA2	-66,95	-1,55	G2	UGT1A6	-11	1,57
C3	GSTA3	-53,76	-2,8	G3	UGT1A7	-42	3,60
C4	GSTA4	-36,33	1,41	G4	UGT2A1	-2,6	-1,9
C5	GSTA5	-7,72	-6,6	G5	UGT2A3	-2,55	-1,1
C6	GSTK1	-7,33	1,41	G6	UGT2B10	-14	-2,2
C7	GSTM1	-17,96	-1,4	G7	UGT2B11	-12,6	46,44
C8	GSTM2	-8,6	-1,5	G8	UGT2B15	-4,9	-1,9
C9	GSTM3	-12,26	1,33	G9	UGT2B17	-100	-22
C10	GSTM4	-2,58	2,42	G10	UGT2B28	-3,44	-4,5
C11	GSTM5		-4,9	G11	UGT2B4	-5,85	1,82
C12	GSTO1	-1,74	4,07	G12	UGT2B7	-13,6	-1,2
D1	GSTO2	-2,55	-1,26	H1	UGT3A1	-34	-11,6
D2	GSTP1	-1,29		H2	UGT3A2	-50,4	2,55
D3	GSTT1	-24,9	-1,4	H3	UGT8	-3	-1,45
D4	GSTT2	-6,55	4,88	H4	XDH	-15	-17
D5	HNMT	-31,96	3,65	H5	ACTB		
D6	INMT	-6,6	-3,18	H6	B2M	-3,1	18,31
D7	MGST1	-1,45	3,38	H7	GAPDH		
D8	MGST2	-3,57	1,32	H8	GUSB		1,98
D9	MGST3	-5,47	1,58	H9	HPRT1	-1,03	-1,1
D10	NAT1	-5,61	2,06	H10	PGK1	-41	10,64
D11	NAT2	-25,1	7,60	H11	PPIA	-59	-4,12
D12	NAT5	-16,8	-2,4	H12	RPL13A	1,00	1,00

Xenobiotics were not fully detoxified by the CYP450 enzymes, they enter the Phase II enzyme reaction to be excreated from the body. The most important Phase II enzymes are GSTs. In Phase II panel, 16 subfamilies of GST enzyme were found. GST Mu enzyme (M1-M5) gene expressions were decreased with the administration of total methanol extracts. Water extracts change the gene expression of all GST Mu gene expression. Both total water and methanol extracts decrease the gene expression of GSTP1 enzyme.

3.8 Determination of Protein Amount

The protein amount of treated HepG2 cell were calculated according to the Lowry method. Bovine serum albumin (BSA) was used as a standard. Serial diuted BSA were used to drawn standard curve (Figure3.48).

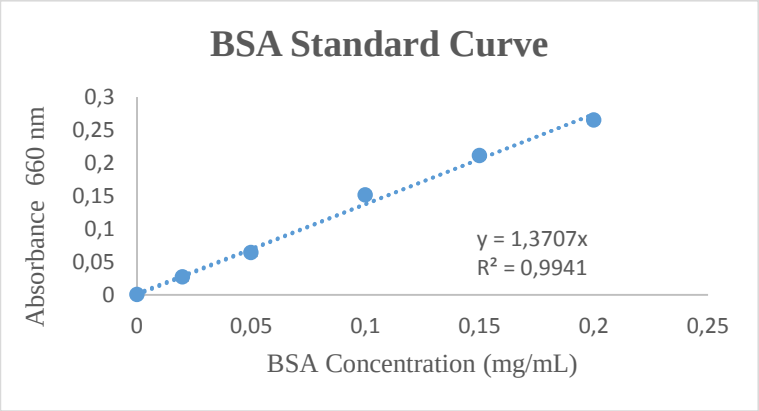

Figure 3.46 The standard curve of BSA for protein amount determination

Table 3.26 shows the protein amounts of Salvia treated HepG2 cells for 48 and 72 h incubation.

According to our results DMSO controls have the higher concentration of protein rather than treated ones. In 48 h incubation, the lowest protein amount was found in total methanol extract, 3.88 ± 0.37 mg/mL.

Protein amount of 72 h incubation was higher than the 48 h incubation. The lowest protein amount was found in total methanol extract, 5.78 ± 0.54 mg/mL in 72 h incubation.

Table 3.26 Protein amount of treated HepG2 cell lysate

Samples	Protein Concentration (mg/mL)
DC48	6.14 ± 0.45
TW48	4.89 ± 0.52
TM48	3.88 ± 0.37
DC72	6.30 ± 0.17
TW72	5.94 ± 0.10
TM72	5.78 ± 0.54

3.9 Enzyme Activities

3.9.1 GST Enzyme Activity

GSTs are the major Phase II detoxifying enzymes. Therefore, we studied the effect of *S.absconditiflora* extracts on GST enzyme activities of HepG2 cells.

GST enzyme activity in HepG2 cell controls were determined as 138.24 ± 0.01 nmoles/min/mg protein (mean ± SE) for 48 h incubation and 101.08 ± 0.01 nmoles/min/mg protein (mean ± SE) for 72 h incubation (Table 3.27).

Table 3.27 Total GST Activity (nmol/min/mg protein)

Samples	GST Specific Activity nmoles/min/mg protein ± SD
DC48	138.24 ± 0.01
TW 48	212.65 ± 001
TM48	212.70 ± 0.01
DC72	101.08 ± 0.01
TW72	107.24 ± 0.02
TM72	155.33 ± 0.01

Figure 3.47 showed GST enzyme activity of hour incubation. GST activity of DMSO controls were accepted as 100% activity, changes in other samples' activities were calculated with respect to 0.1% DMSO control.

According to Sakallı E., 2012, the major isozymes contributing to CDNB conjugation in cell lines were most probably GST Mu, Pi and Omega which are the supressors of apoptosis. Increase of the GST activities results in the decrease of apoptosis.

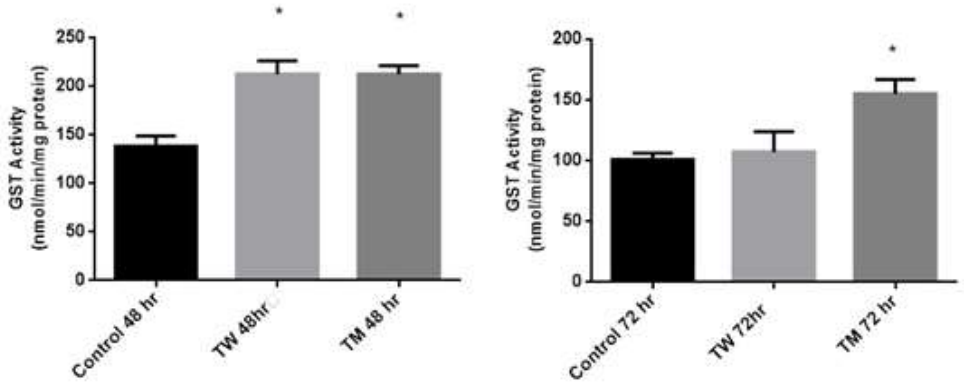

Figure 3.47 Effects of TW and TM extracts on GST Enzyme activities of 48 h and 72 h incubation. * $p \leq 0.05$

According to the control, TW and TM extracts induce the GST activities significantly (Figure 3.47) both hour incubation.

The highest specific activity was found in TM 48 h incubation as 212.70 ± 0.01 nmoles/min/mg protein (mean ± SD). Total water and total methanol have approximately same result on GST enzyme activity at 48 h incubation.

3.9.2 Catalase Activity

Catalase are the major antioxidant enzymes found nearly all organism. Therefore, we studied the effect of *S.absconditiflora* extracts on Catalase enzyme activities of HepG2 cells.

Catalase enzyme activity in HepG2 cell controls were determined as 15.66 ± 0.89 nmoles/min/mg protein (mean ± SE) for 48 h incubation and 11.45 ± 0.14 nmoles/min/mg protein (mean ± SE) for 72 h incubation (Table 3.28).

Table 3.28 Total Catalase Specific Activity (nmol/min/mg protein)

Sample	Catalase Activity nmol/min/mgprotein ± SD
DC48	15.66 ± 0.89
TW 48	16.6 ± 0.22
TM48	28.24 ± 0.47
DC72	11.45 ± 0.14
TW72	14.90 ± 0.61
TM72	9.83 ± 0.16

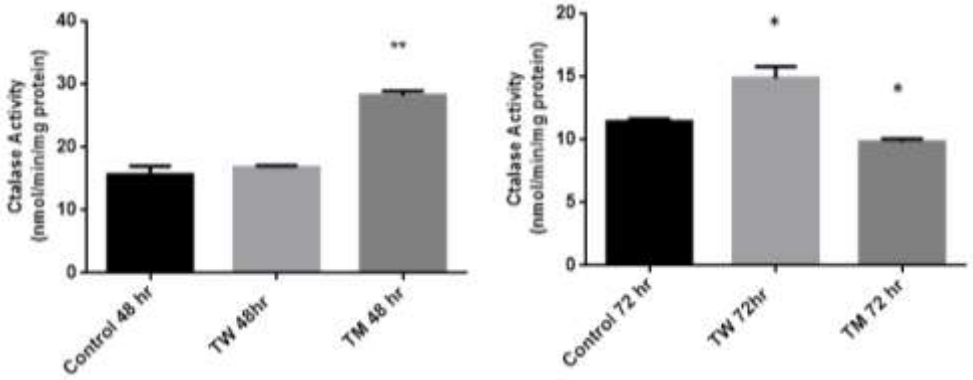

Figure 3.48 Effects of TW and TM extracts on GST Enzyme activities of 48 h and 72 h incubation.

* $p \leq 0.05$

Figure 3.48 showed Catalase enzyme activity of hour incubation. GST activity of DMSO controls were accepted as 100% activity, changes in other samples' activities were calculated with respect to 0.1% DMSO control.

Compared to control, water extract induce catalase activities in both at 48 and 72 hour incubations (Figure 3.48). However while with 48 h methanol

treatment, catalase activity increased, the activity decreased significantly at 72 h.

The highest specific activity was found with methanol, 48 h incubation as 28.24 ± 0.47 nmoles/min/mg protein (mean ± SD).

3.9.3 GPx Activity

GPx is an antioxidant enzyme. It catalyzes the conversion of H_2O_2 to two molecules of water. In this conversion GSH is converted to glutathione disulfide (GS-SG).

GPx enzyme activity in HepG2 cell in controls were determined as 6.275 ± 0.39 nmoles/min/mg protein (mean ± SD) for 48 h incubation and as 6.649 ± 0.38nmoles/min/mg protein (mean ± SD) for 72 h incubation. The control GPx activities were approximately the same. (Table 3.28)

Table 3.29 GPx Specific Activity (nmol/min/mg protein)

Samples	GPx Specific Activity nmoles/min/mg protein ± SD
DC48	6.28 ± 0.39
TW 48	22.27 ± 0.49
TM48	19.00 ± 0.12
DC72	6.65 ± 0.38
TW72	11.28 ± 1.60
TM72	4.35 ± 0.41

According to the control, in 48 h incubation TW and TM extracts induce the GPx activity significantly (Figure 3.48).

The highest specific activity was found in TW 48 h incubation as 22.273 ± 0.49 nmoles/min/mg protein (mean ± SD).

In 72 hr incubation, TW extract induce the GPx activity but TM extract decrease the GPx activity significantly.

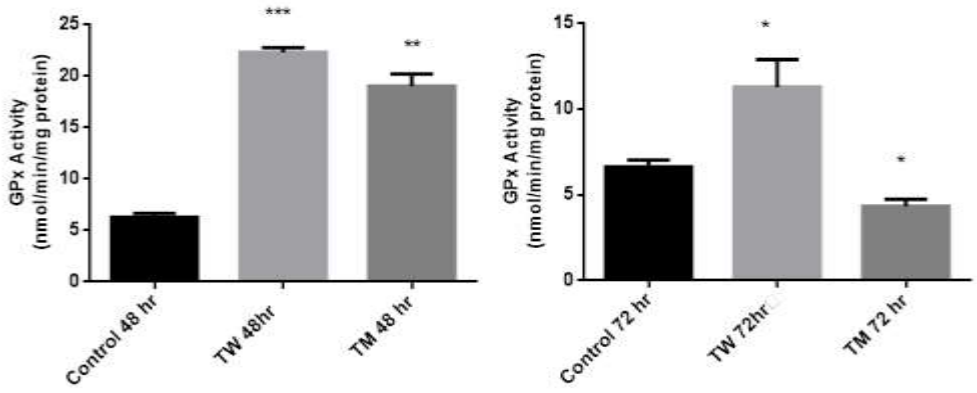

Figure 3.49 Effects of TW and TM extracts on GPx activity of 48 h and 72 h incubation.

* $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$

Reactive oxygen species are highly produced in tumor cells (Szatrowski, 1991). Mitochondrial defects and a decreased expression of antioxidant enzymes can cause this situation (Oberley, 1997; Hu et al., 2005).

Thus, many reports indicated decreased catalase levels in a wide variety of tumors and cancer cell lines compared to normal cells. (Sato et al., 1992; Kwei et al., 2003)

According to literature, overexpression of catalase in MCF-7 cells caused a 7- fold increase in catalase activity but provokes a 40% decrease in the expression of both glutathione peroxidase and peroxiredoxin II (Glorieux et al., 2011).

Our results shows a good correlation with those of the literature. GPx activity increased 3 folds compared to the catalase activity in HepG2 cells.

All GST, catalase and GPx are the members of antioxidant system. At 48 h incubation, both water and methanol extracts increase the activities of GST

and GPx. Only Catalase activity at 48 h water extract treatment did not show any change in activity compared to controls. According to these results, antioxidant system is activated at 48 h with S.absconditiflora especially methanol extracts.

CHAPTER 4

CONCLUSION

Consumption of polyphenol rich plants was suggested for their antioxidant, antimicrobial, antichelating and antitumor ability. Plants containing phenols and flavonoids have impact on the expression of several genes as induction or inhibition. Since most of the drugs including chemotherapeutic agents are metabolized by Cytochrome P450s (CYPs) which are known as phase I detoxification enzymes and by GSTs which are part of Phase II enzymes, uncontrolled intake of these plants may threaten human health by affecting the drug metabolism. So, it is important to investigate the potential effects of such plants on the gene expressions of drug metabolizing enzymes before consumption.

In this work, water and methanol extracts of *S.absconditiflora* leaves were analyzed first, for their antioxidant, antimicrobial, antichelating activities and growth inhibitory effects on human hepatocyte carcinoma cell line, HepG2. A species of sage, *S.absconditiflora*, grows endemically in several regions in Turkey and its leaves are commonly consumed as herbal tea.

Both water and methanol extracts of *S.absconditiflora* leaves were characterized in terms of flavonoid and polyphenol contents by HPLC and LC MS/MS methods. The results showed that, methanol extracts had more phenolics and the most abundant phenolics are Rosmarinic acid and Caffeic acid.

Effects of *S.absconditiflora* water and methanol extracts on the gene expressions of several Phase I and II enzymes were also studied. Among

CYP450 enzymes, CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2 are the major drug metabolizing enzymes. Especially CYP3A4 is the most important enzyme as its involved in almost all CYP450 mediated reactions. Our results showed an inhibitory potential of *S.absconditiflora* methanol extract toward CYP3A4 which could have potential herb-drug interaction in liver and might increase the bioavailability of co-administered drug and lead to toxicity.

According to our results, we further conclude that, the methanol extract of *S.absconditiflora* shows the most potent effects on CYP enzymes, whereas the aqueous, water extract shows negligible effects on gene expressions. Since most individuals consume the leaves of *S.absconditiflora* either raw or boiled with water, the findings of this study suggest that the water extract may be safe considering the herb-drug interactions.

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APPENDIX A

Antimicrobial activities of *S. absconditiflora and* gene expression of drug metabolizing enzymes

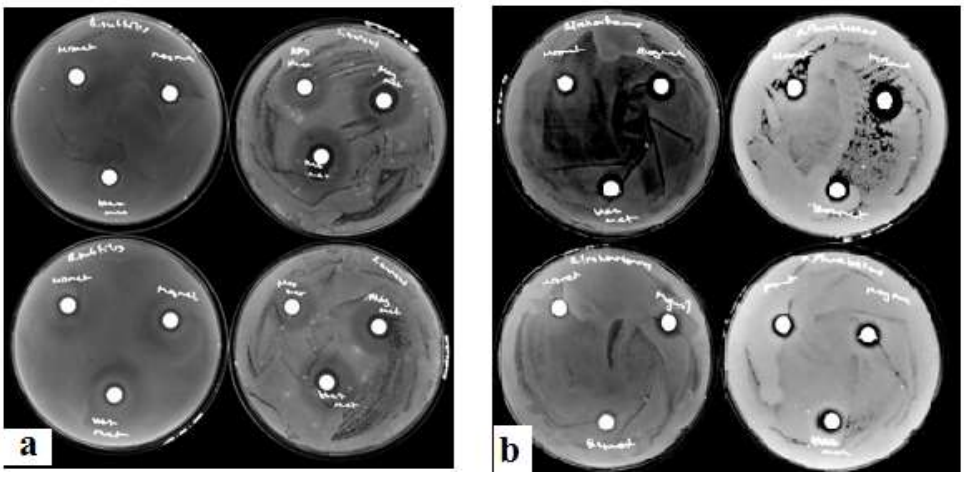

Figure A.1 Effects of *Salvia* Methanol extracts on *B.subtilis* and *S.aureus (a) ;* on *B.licheniformis* and *A.tumefaciens(b)*

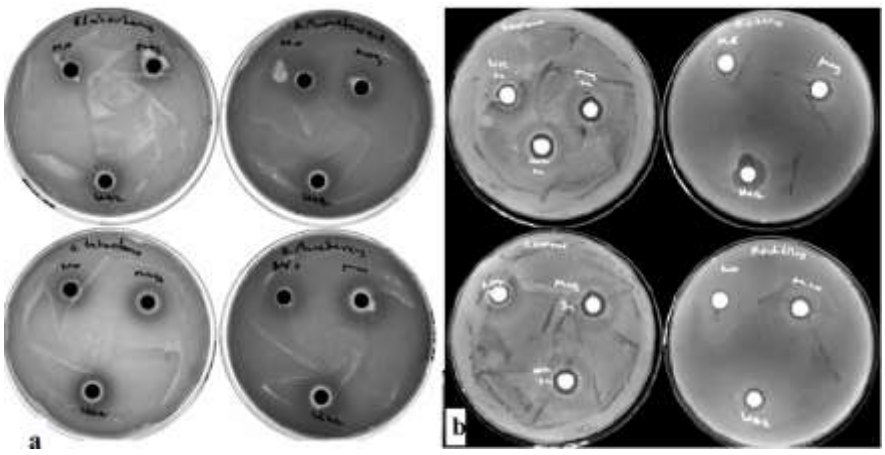

Figure A.2 Effects of *Salvia* water extracts on *B.licheniformis and A.tumefaciens (a);* on *B.subtilis* and *S.aureus (b)*

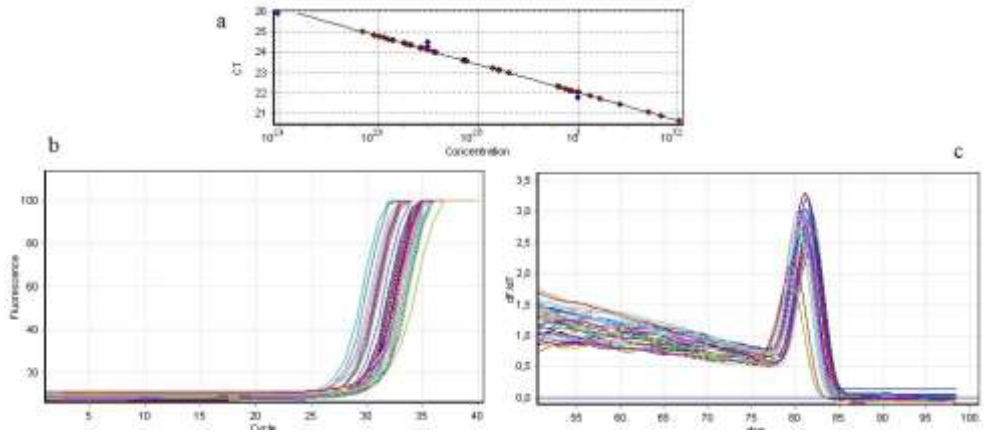

Figure A.3 CYP2E1 Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product.

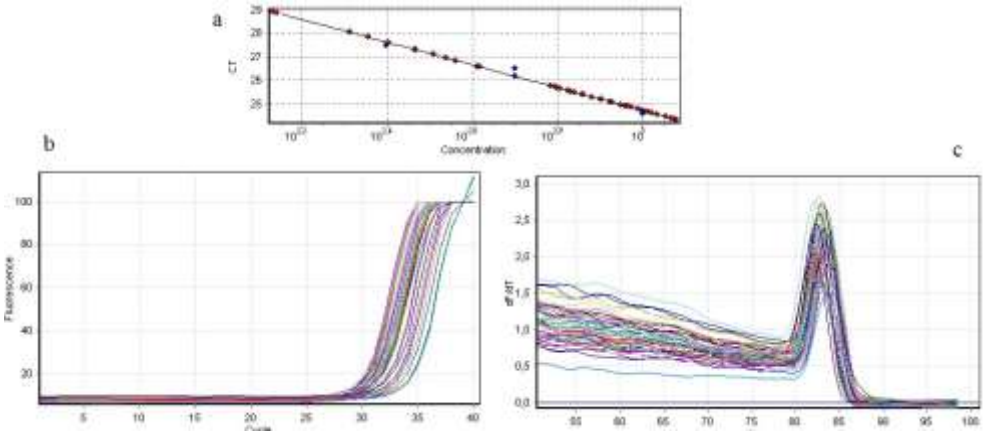

Figure A.4 CYP1A2 Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product.

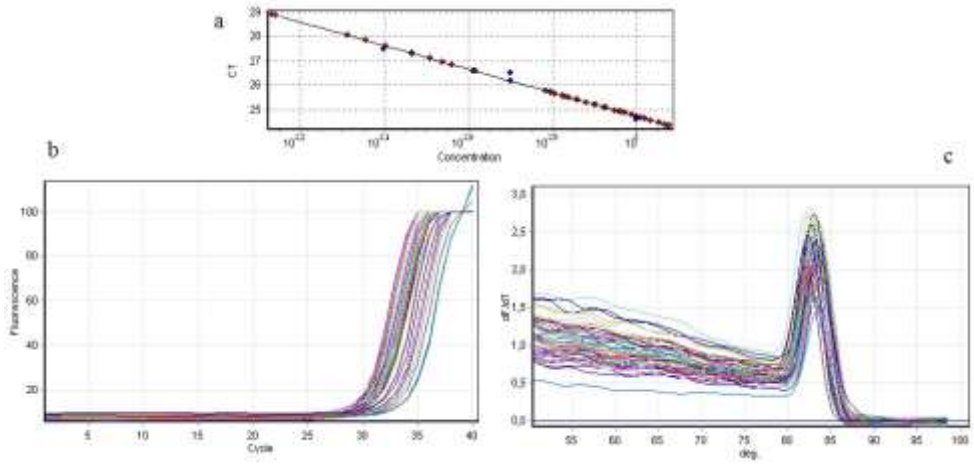

Figure A.5 CYP3A4 Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product.

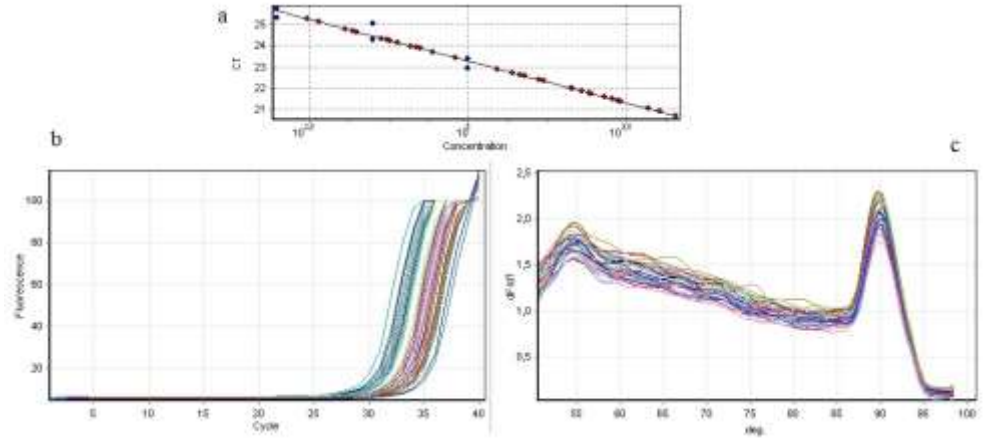

Figure A.6 GSTP1 Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product

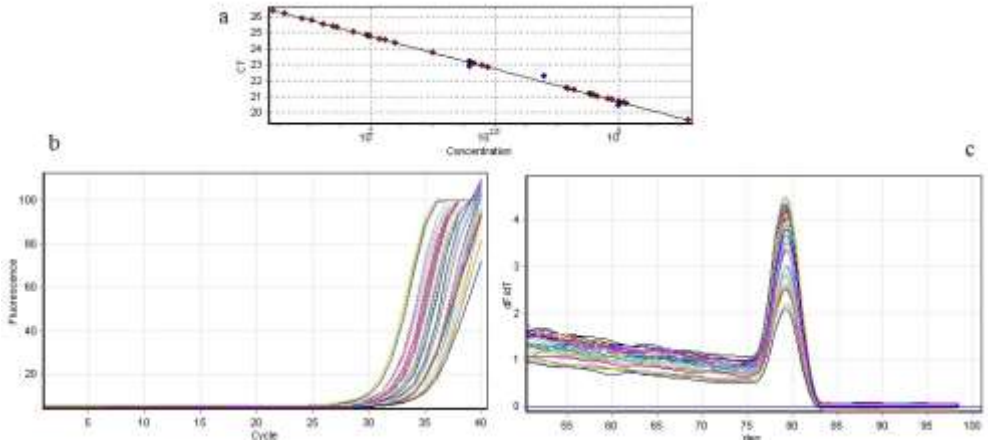

Figure A.7 GSTM1 Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product

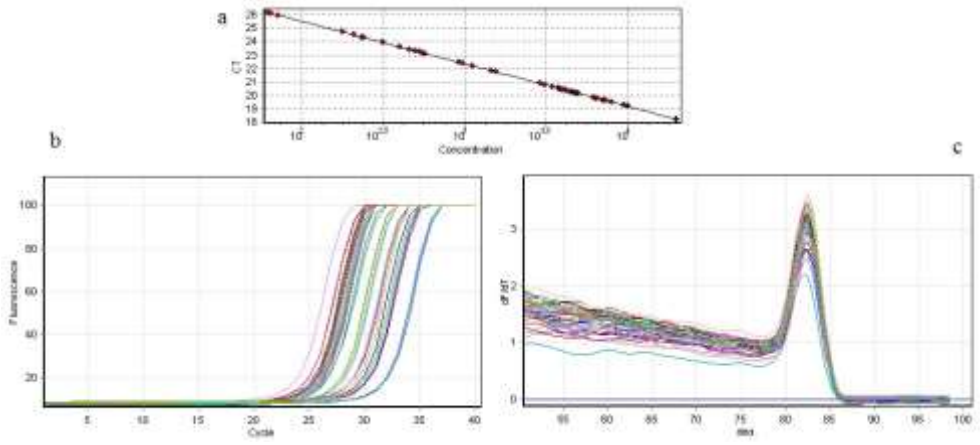

Figure A.8 GPx4 Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product

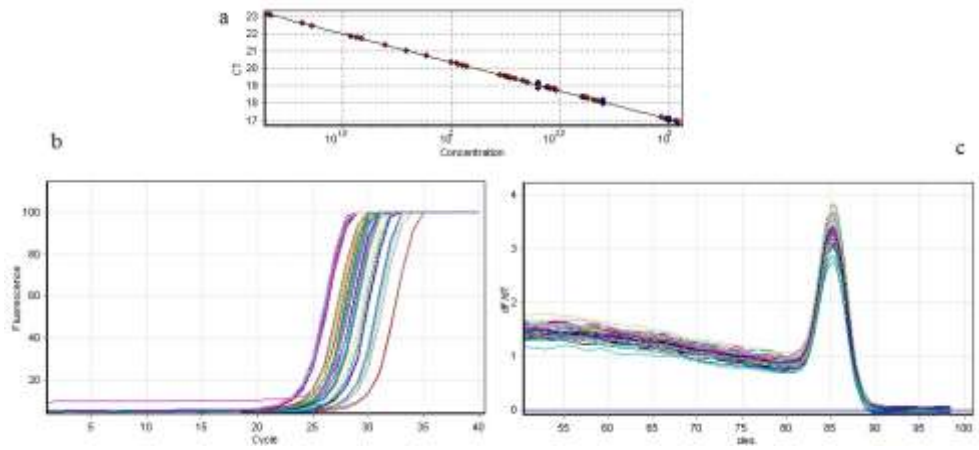

Figure A.9 Catalase Standard curve generated from serial dilutions of control cDNA(a);Amplification curve the accumulation of fluorescence emission at each reaction cycle (b); Melting curve the fluorescence emission change versus temperature (c). Single peak means single PCR product

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PUBLICATIONS

Full paper published in a peer reviewed journal covered by SCI, SSCI or AHCI core list

- Sadi, G, Emsen B, Kaya A, Kocabaş A, Çınar S, İrtem Kartal D (2015) Cytotoxicity of some edible mushrooms extracts over liver hepatocellular carcinoma cells in conjunction with their antioxidant and antibacterial properties. Pharmacognosy Magazine 05/2015; 11(42):6-18. DOI:10.4103 /0973-1296.157665
- Sadi, G, İrtem Kartal D, Guray T (2013). Regulation of Glutathione S-Transferase Mu with type 1 diabetes and its regulation with antioxidants Turkish Journal of Biochemistry 04/2013; 38(1):106-114. DOI:10.5505 /tjb.2013.96720

Abstract of a paper presented at and published in the proceedings of a refereed conference regularly held by an international organization

- Irtem Kartal D, Sadi G, Guray NT. Effect of the bioactive components of *Salvia absconditiflora* on gene expressions of HepG2 cell. 40th FEBS Congress (4-9 July 2015, Berlin, Germany). FEBS Journal 282 (2015) 56–408, 265
- Altay A, Irtem Kartal D, Sağdıçoğlu Celep G, Guray NT, Bozoğlu FT Antioxidant activities of *Salvia fruticosa* and its effects on HT-29 cell line. 40th FEBS Congress (4-9 July 2015, Berlin, Germany). FEBS Journal 282 (Suppl. 1) (2015) 56–408, 269
- Irtem Kartal D, Altay A, Yurteri G, Guray NT. Antioxidant activities of Propolis and Its Bioactive Components, and Their Effects on CYP1A1 Gene Expression in HT29 Adenocarcinoma Cell Line. 13th European ISSX Meeting (22-25 June 2015, Glasgow, Scotland) ISSX Abstract Book P,51
- Irtem Kartal D, Sadi G, Guray NT. The effects of *Salvia absconditiflora* water extract on HepG2 liver cell line. The FEBS EMBO 2014 Conference **(**30 August to 4 September 2014, Paris, France) FEBS Journal 281 (Suppl. 1) (2014) 65–783, 606
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Abstract of a paper presented at and published in the proceedings of a refereed, regularly held on national conference

- Kartal DI, Sadi G, Güray T (2008) Diabetic Rat Liver GST Alpha and Mu Gene Expressions and Activities. 20th National Biochemistry Congress (29Oct-1 Nov 2008, Nevşehir, Turkey) Turkish Journal of Biochemistry 33(S1): 142-143

NATIONAL SCIENTIFIC GRANTS

1. TUBİTAK MS Scholarship
2. TUBİTAK PhD Scholarship