

THE EFFECT OF *SALVIA ABSCONDITIFLORA* EXTRACT ON THE GENE EXPRESSIONS  
OF GSTO1 AND GSTZ1 IN MCF-7 AND MDA-MB-231 CELLS

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THE EFFECT OF *SALVIA ABSCONDITIFLORA* EXTRACT ON THE GENE EXPRESSIONS  
OF GSTO1 AND GSTZ1 IN MCF-7 AND MDA-MB-231 CELLS

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## ABSTRACT

### THE EFFECT OF *SALVIA ABSCONDITIFLORA* EXTRACT ON THE GENE EXPRESSIONS OF GSTO1 AND GSTZ1 IN MCF-7 AND MDA-MB-231 CELLS

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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, *S.absconditiflora* is very rich in active compounds.

*S.absconditiflora* water extract was investigated for its antioxidant capacity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Total phenolic and total flavonoid contents were quantified by spectrophotometric methods.

LC-MS/MS analyses revealed the presence and quantities of caffeic acid, luteolin rutin and coumaric acid.

Cytotoxic effects of water extract of *S.absconditiflora* on breast cancer cell lines (MCF-7 and MDA-MB-231) were examined via XTT colorimetric assay and Trypan Dye Exclusion cell viability assay. IC<sub>50</sub> values for each cell line at 24 and 48 hours were determined. The results indicated that water extract of leaves of *S.absconditiflora* could inhibit cell proliferation in MCF-7 and MDA-231 cells in dose dependent but not in time dependent manner.

Effects of *S.absconditiflora* water extract on the expression of glutathione-S-transferases (GSTs) in MCF-7 and MDA-MB-231 cells were investigated with qRT-PCR technique. IC<sub>50</sub> values calculated in XTT experiment for 24h incubation was used as cytotoxic extract concentration. It was found that treatment of MCF-7 cells with 1,558 mg/ml of extract enhanced an increase in expression as 2 and 2,8 fold in GSTO1 and GSTZ1 genes, respectively. Treatment of MDA-MB-231 cells with 1,131 mg/ml of extract resulted in 1,57 fold increase for GSTO1 and 1,56 fold increase for GSTZ1.

Keywords: *Salvia absconditiflora*, Polyphenols, Antioxidant activity, Glutathione S-Transferases, GSTO1, GSTZ1

## ÖZ

### MCF-7 ve MDA-MB-231 HÜCRELERİNDE *SALVIA ABSCONDITIFLORA* EKSTRAKTININ GSTO1 VE GSTZ1 GEN EKSPRESYONLARI ÜZERİNE ETKİSİ

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*S. absconditiflora* Türkiye’de yetişen ve bitki çayı olarak tüketilen endemik bir türdür. Yapraklarında bulunan daha fazla kesecik nedeniyle, *S. absconditiflora* ekstreleri aktif bileşikler bakımından zengindir.

*S. absconditiflora*’nın sulu ekstresinin antioksidan kapasitesi, 2,2-Difenil-1-pikrihidrazil (DPPH) serbest radikal süpürme tekniğiyle araştırıldı. Total fenolik ve total flavonoid miktarları spektrofotometrik tekniklerle belirlendi.

Sıvı Kromatografisi-Kütle-Kütle Spektrometresi kullanılarak var olan kafeik asitin, luteolin, rutin ve kumarik asitin miktarları tayin edildi.

*S. absconditiflora*’nın sulu ekstresinin meme kanserli hücre hatlarındaki (MCF-7 ve MDA-MB-231) sitotoksik etkisi, kolorimetrik XTT yöntemi ve Tripan mavisi boyama ile yapılan canlılık testi tekniğiyle incelendi. Her bir hücre hattında 24 ve 48 saat için IC<sub>50</sub> konsantrasyonu belirlendi. *S. absconditiflora*’nın sulu ekstresinin MCF-7 ve MDA-MB-231 hücrelerinin proliferasyonlarını, zamandan bağımsız şekilde ve doza bağlı olarak engellediği görüldü.

*S. absconditiflora*’nın sulu ekstresinin glutatyon-s-transferaz enzimlerinin ekspresyonları üzerindeki etkisi MCF-7 ve MDA-MB-231 hücrelerinde qRT-PCR tekniğiyle araştırıldı. Hücreler 24 saat boyunca ekstreyle inkübe edildi ve elde edilen XTT sonuçlarına göre hesaplanan IC<sub>50</sub> konsantrasyonları ile bu hücrelere uygulama yapıldı. Yapılan deneyler sonrasında, 1,558 mg/ml ekstre uygulanan MCF-7 hücrelerinde GSTO1 gen ekspresyonunun 2 kat, GSTZ1 gen ekspresyonunun ise 2,8 kat arttığı görüldü. 1,131mg/ml ekstre uygulanan MDA-MB-231 hücrelerinde GSTO1 geninin ekspresyonunda 1,57 kat, GSTZ1 geninin ekspresyonunda ise 1,56 kat artış bulundu.

Anahtar Kelimeler: *Salvia absconditiflora*, Polifenol, Antioksidant aktivite, Glutatyon-S-Transferaz, GSTO1, GSTZ1

*To my family,  
To Prof. Dr. Mesude İşcan,*

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## LIST OF ABBREVIATIONS

ARE: Antioxidant Response Element  
HER-2: Human Epidermal Growth Factor Receptor 2  
ER: Estrogen Receptor  
Keap1: Kelch-like ECH-associated protein 1  
Nrf2: Nuclear factor-erythroid 2-related factor 2  
CDK: Cyclin Dependent Kinases  
Apaf-1: Apoptotic Protease Activating Factor  
PI3K: Phosphoinositide 3-kinase  
PKC: Protein Kinase C  
PBS: Phosphate Buffered Saline  
DMSO: Dimethyl sulfoxide  
DEPC: Diethylpyrocarbonate  
GST: Glutathione S-transferases  
IC<sub>50</sub>: Inhibitory Concentration 50  
MCF-7: Michigan Cancer Foundation - 7  
MDA-MB-231: Monroe Dunaway Anderson - Metastatic Breast  
XTT: 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner salt  
dNTP: Deoxy Nucleotide Triphosphate  
qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction



## CHAPTER 1

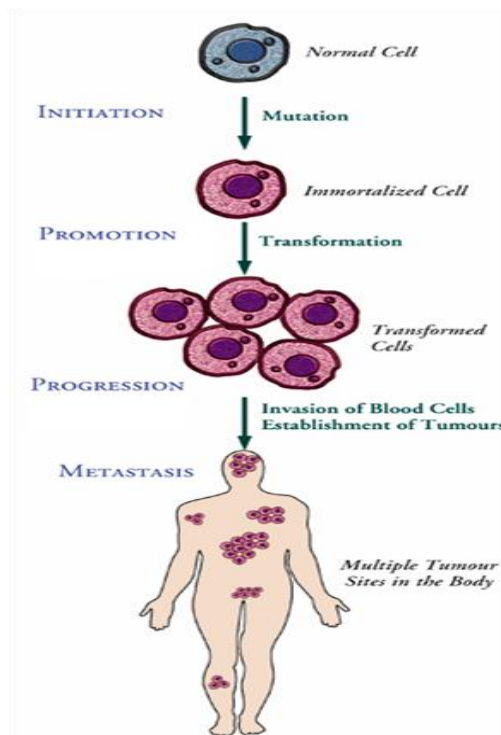
### INTRODUCTION

#### 1.1 Cancer

Cancer is the world's second biggest fatal disease after cardiovascular disease. According to World Health Organization (WHO) cancer factsheet (2012), about 12, 7 million new cancer cases occurred in 2008 and this is going to increase 22 million new cases each year by 2030. Lung, breast, colorectal and stomach cancers constitute the 40% of all cancer cases, worldwide.

Cancer is the end stage of a multi sequence process, called carcinogenesis. It results from transformation in healthy cells to pre-cancerous form and finally into tumor. Carcinogenesis is divided into 3 stages which are termed as initiation, promotion and progression (Figure 1-1). Initiation step starts when DNA of a cell or population of cells is harmed by external (e.g. chemicals, radiation, tobacco, infections) and internal factors (e.g. hormones). A non-lethal mutation occurs in DNA that cause changes in cell. If the damage is not repaired, it can lead to genetic mutation.

During promotion step, these mutated cells start proliferating in clonal expansion to form multicellular premalignant tumor cell population. This process concludes with formation of an identifiable focal lesion. This step is a reversible since continuous stimulus for tumor promotion is needed.



**Figure 1–1** Illustration of the carcinogenesis as initiation, promotion and progression (Brudnak, 2000)

During progression step, preneoplastic cells develop in to tumors. This step is irreversible and characterized by genetic instability and chromosome integrity disruption. This newly formed tumor cells can form their own blood circulation system called angiogenesis. Therefore they are capable of metastasize (Figure 1-1) (Sporn and Suh, 2000; Valko *et al.*, 2006).

## 1.2 Breast Cancer

Breast cancer is the most common type of cancer among women in the world (WHO, 2012). Each year, about 1, 38 million new breast cancer cases occur and result with 458 000 deaths in the world (IARC Globocan, 2008). In Turkey, 24% of total women cancer cases constitute the breast cancer. According to Turkish Health Ministry resources, the estimated number of breast cancer patients was over 50,000 in 2011 and the numbers are expected to increase in the future (<http://www.tbccm.org/2011/05/current-state-of-breast-cancer-and-infrastructure-in-turkey/>).

Breast consists of glands (lobules) that produce milk, ducts that transfer milk from lobules to the nipple, and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels). Breast cancers begin in the cells that line the ducts (ductal carcinoma), chiefly. Other common one is lobular carcinoma that begins in the cells that line the lobules and a small portion begin in other tissues (<http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-what-is-breast-cancer>).

Breast cancer has a heterogeneous profile that can be divided in to 5 categories according to their estrogen receptor, progesterone receptor, human epidermal growth receptor-1 and cytokeratins 5/6 expressions profile, which are luminal A, Luminal B, Basal-like, HER-2(+)/ER(-), normal breast like (Hsiao *et al.*, 2010) .

Genetic factors constitute the less than 10% of the breast cancer cases. 90% of the breast cancer cases are related to environmental factors, dietary intakes, body weight and physical activity (World Health Organization (WHO) cancer factsheet, 2012).

It has been found that oxidative damage has a big implication on the development, progression and metastatic state of the breast cancer. In a study, DNA base damage which occurred due to <sup>•</sup>OH attack detected as elevated 8-oxo-dH adducts levels was found as an important factor in breast cancer occurrence (Malins and Haimanot, 1991; Waris and Ahsan, 2006).

## 1.3 Free Radicals and Cancer

Free radicals are molecules that contain one or more unpaired electrons in their orbitals. The presence of unpaired electron ensures an extensive reactivity for a free radical.

When free radicals are formed from oxygen, they are called reactive oxygen species (ROS) which are the most significant ones for the biological systems. ROS are generated during irradiation by UV light, X-rays and gamma rays. They can be present as pollutant in atmosphere. They are the products of metal catalyzed reactions. They are also produced by mitochondria catalyzed electron transport chain and by other mechanisms. ROS play beneficial roles such as infectious disease defense, mitogenic response induction and cellular signaling system regulation. However, when their balance in the body is disrupted, they can damage cell structures including lipids, proteins and nucleic acids.



Superoxide anion ( $O_2^{\bullet-}$ ) is the main ROS. It is capable of reacting with other molecules by enzyme or metal catalyzed reactions to get secondary reactive oxygen species.

Redox active metals (Fe, Cu) play important role for various free radical species generation. When superoxide amount exceed the limits, it act as an oxidant. Superoxide radical participates in Haber-Weiss reaction ( $O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + \bullet OH + OH^-$ ) and combines a Fenton reaction which leads to reduction of  $Fe^{3+}$  by superoxide anion and producing  $Fe^{2+}$  and oxygen ( $Fe(III) + O_2^{\bullet-} \rightarrow Fe(II) + O_2$ ).

Overall reaction



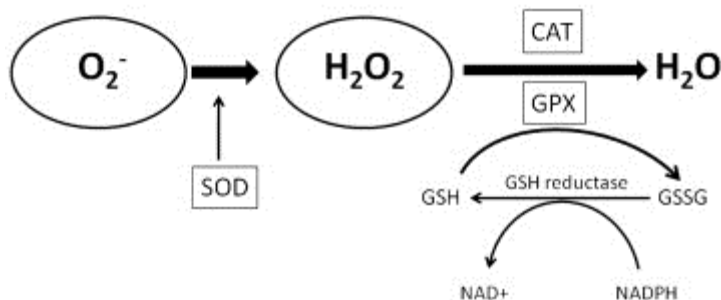
Hydroxyl radical ( $\bullet OH$ ) is a highly reactive species that generated by diverse mechanisms. It can be produced by ionizing radiation ( $H_2O \rightarrow H^+ + OH^{\bullet}$ ) and photolytic decomposition of alkyl hydroperoxides.

Peroxy radicals ( $ROO^{\bullet}$ ) are other oxygen derived radicals that involved in DNA cleavage and protein backbone modification. Together with superoxide anion, they amplify the DNA damage induction (Wang *et al.*, 2011; Klaunig *et al.*, 2010; Valko *et al.*, 2006).

Oxidative stress has roles in all three steps of carcinogenesis. Causing gene mutations and structural conversions in DNA, ROS plays roles in initiation step. Blockage of cell-to-cell communication, modification of secondary messenger system and induction of abnormal gene expression happens in promotion step contributed by ROS causes increase in cell proliferation or decrease in apoptosis of abnormal cells. Finally, further changes in altered cell population are also triggered by ROS in progression step (Reuter *et al.*, 2010).

In addition to these, chronic inflammation related to ROS has been shown as an inducer of cancer. Neutrophils and macrophages release high quantities of superoxide anion, hydroxyl radical and hydrogen peroxide during inflammation. Inflammatory cells can activate procarcinogens such as aromatic amines or aflatoxins via ROS-dependent mechanisms. Also, they can induce DNA base damages. One of matrix metalloproteinases, gelatinases which has an important role in tumor invasion and metastasis was found to be upregulated by ROS related to chronic inflammation ( Reuter *et al.*, 2010).

There are several mechanisms found in living cells to balance this oxidative stress categorized as enzymatic and non-enzymatic antioxidants. One of the most effective enzymatic antioxidant defense systems is superoxide dismutases. It has three forms, namely, cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extracellular SOD (EC-SOD). It catalyzes the dismutation of  $O_2^{\bullet-}$  to  $O_2$  and less reactive hydrogen peroxide ( $H_2O_2$ ). Another enzyme, catalase, is located in peroxisome that converts hydrogen peroxide to water and molecular oxygen. Glutathione peroxidase is found in two forms as selenium independent (GST) and selenium dependent (GPx). It converts hydrogen peroxide to water and oxygen with the help of glutathione which is the most important molecule for antioxidative defense mechanism. These three enzymes are all together providing the primary antioxidant defense mechanism (Figure 1-2).



**Figure 1–2** Mechanisms of enzymatic antioxidants which are Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPX)

The non-enzymatic defense system includes vitamin C, E, A and phytochemicals like polyphenols ( Vaisi-Raygani *et al.*, 2007 ; Wang *et al.*,2011; Valko *et al.*, 2006 ).

## 1.4 Phytochemicals

According to studies, more than two thirds of the cancer cases can be prevented by changes in dietary intakes (Sarkar *et al.*, 2004). Wattenberg (1966) demonstrated that inhibition of chemically induced tumors could be possible by phytochemicals in fruits and vegetables.

The ‘phyto’ of the word phytochemicals is a Greek word meaning ‘plant’. Thus, phytochemicals means plant chemicals that there has been identified more than 5000 phytochemicals in foods (Liu, 2004).

Phytochemicals can be divided in to 5 classes, carotenoids, alkaloids, nitrogen- containing compounds, sulfur-containing compounds and polyphenols (Mandlekar *et al.*, 2006). Within these compounds, polyphenols are most studied ones and considered more powerful antioxidants than others (Dai and Mumper, 2010). In addition, they have been indicated as ‘blocking agents’ that they are capable of preventing carcinogenesis (Henderson *et al.*, 2000).



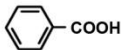
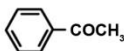
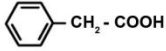
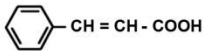
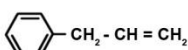
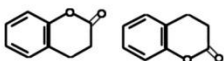


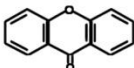
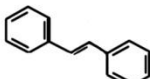
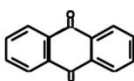
### 1.4.1 Polyphenols

Polyphenols are products of secondary metabolism of plants and represent one of the most numerous and widely distributed groups of substances in plants. Plants produce polyphenols for several purposes such as plant pigmentation, reproduction and protection against bacterial pathogens or UV light (Oksana *et al.*, 2012).

8000 known phenolic structures are distributed in the plant kingdom (Harborne, 1986) and are originated from two main pathways, namely, the shikimate pathway and the acetate pathway (Harborne, 1989).

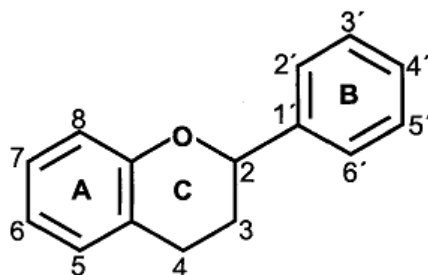
The ‘phenolic’ or ‘polyphenol’ is defined chemically as a substance which has an aromatic ring consist of one (phenol) or more (polyphenol) hydroxyl substituents, includes functional derivatives (glycosides, esters, methyl ethers etc.). According to their basic skeleton, polyphenols can be divided into 10 different classes (Harborne, 1989) (Table 1-1).

**Table 1-1** Major classes of polyphenolic compounds (Bravo, 1998)

Class	Basic Skeleton	Basic Structure
Simple Phenols	$C_6$	
Benzoquinones	$C_6$	
Phenolic acids	$C_6-C_1$	
Acetophenones	$C_6-C_2$	
Phenylacetic acids	$C_6-C_2$	
Hydroxycinnamic acids	$C_6-C_3$	
Phenylpropenes	$C_6-C_3$	
Coumarins, isocoumarins	$C_6-C_3$	
Chromones	$C_6-C_3$	
Naftoquinones	$C_6-C_4$	
Xanthones	$C_6-C_1-C_6$	
Stilbenes	$C_6-C_2-C_6$	
Anthraquinones	$C_6-C_2-C_6$	
Flavonoids	$C_6-C_3-C_6$	
Lignans, neolignans	$(C_6-C_3)_2$	
Lignins	$(C_6-C_3)_n$	

Flavonoids are the most commonly found polyphenols with 4000 species. It consists of flavonols (e.g. quercetin), anthocyanin (e.g. cyanidin), flavones (e.g. apigenin), isoflavones (e.g. daidzein), flavanones (e.g. naringenin) and flavonols (e.g. catechin) with a configuration of  $C_6-C_3-C_6$ . (Figure 1-3). Flavonoids are found in plants as aglycones and glycoside derivatives. Most common form of these compounds is the flavonols and their glycosides.

Flavonoids have important roles in lessening disease incidences such as cancer and cardiovascular disease (Bravo, 1998; Ignat *et al.*, 2011).



**Figure 1–3** Basic structure and enumeration of flavonoids (Bravo, 1998)

Other common form, phenolic acids can be found in free and bound forms through ester, ether or acetal bonds. They can be divided into 2 subgroups. Hydroxybenzoic acids such as gallic, vanilic and syringic acids have  $C_6-C_1$  structure and found in free form. Hydroxycinnamic acids are aromatic compounds such as caffeic, ferrulic p-coumaric acid that they have  $C_6-C_3$  structure. They have been found in bound form that they are linked covalently to cell wall polysaccharides or lignin (Bravo, 1998; Ignat *et al.*, 2011).

Tannins are high molecular weight polyphenols. They have two forms as hydrolysable (gallic acid derivatives) and condensed (proanthocyanidins). They have metal chelating, antioxidant and protein precipitating properties.

Stilbenes are polyphenols that mainly found in glycosylated forms. Resveratrol is the main example of stilbenes. Lignans have importance in cancer chemotherapy and have various pharmacological effects (Ignat *et al.*, 2011).

Polyphenols are found fundamentally in leaves and outer parts of the plants because of lightning. Their levels vary greatly between plants to plants, even in the same species due to growth condition differences in plants (Bravo, 1998).

Dietary sources and forms, absorption and micro flora processing rates in GI tract, liver metabolism, blood kinetic and overall cellular uptake of variety of polyphenols changes according to type of polyphenols (Galli, 2007).

Aglycones, free simple phenolics, flavonoids and phenolic acids are extractable (solubilized) phenolics, which can directly be absorbed through the small intestinal mucosa (Bravo, 1998). More specifically, most absorbable polyphenols in humans are isoflavones and gallic acid ensuing catechins, flavanones and quercetin glucosides (Galli, 2007).

Beverages are one of the most common sources of polyphenols such as wines, tea and fruit juices. It has been found that drinking green tea contains catechin that lowers the chances of developing heart disease and cancer. Wine is a very important source of polyphenolic antioxidants. Coffee contains chlorogenic acid in high amount that give coffee its antioxidant property (Ignat *et al.*, 2011).

#### 1.4.1.1 Scavenging of Reactive Metabolites with Polyphenols

The antioxidant capacity of plasma increases when polyphenol rich foods and beverages are consumed (Scalbert *et al.*, 2005). According to a study performed by Leighton *et al.* (1999),

daily intake of 240mg wine, rich in resveratrol, strongly reduced the level of oxidized DNA base 8-hydroxydeoxyguanosine (8-OHdG) in blood leukocyte.

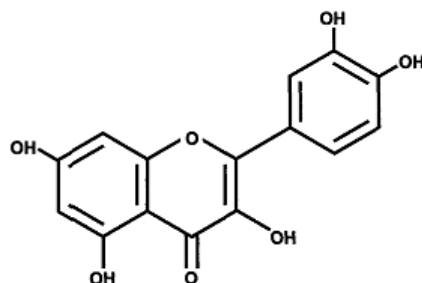
Phenolic compounds have ability to donate a hydrogen atom due to their phenolic hydroxyl groups to a free radical (R). They can also delocalize an unpaired electron by their conjugated aromatic system.



As illustrated above, when phenolic compounds (POH) donate hydrogen atoms to free radicals (R), phenoxy radical intermediates (PO $\cdot$ ) are formed. These intermediates are relatively stable molecules and so they suppress the free radical chain reaction. When they react with other free radicals, they terminate the propagation (Scalbert *et al.*, 2005; Dai *et al.*, 2010).

Also, some phenolic compounds with catechol and gallol groups are able to conjugate with transition metals to prevent generation of free radicals. They can form complexes with Cu<sup>2+</sup> and Fe<sup>2+</sup> or relatively weaker interactions with Cu<sup>+</sup>. In addition, they can enhance autooxidation of Fe<sup>2+</sup> (Dai and Mumper, 2010).

Antioxidant potential of polyphenols depends on their chemical structure. Flavonoids are very powerful radical scavengers because of their structural elements includes an *o*-diphenolic group, a 2-3 double bond conjugated with 4-oxo function and hydroxyl groups in positions 3 and 5. Quercetin as a flavonol contains all these groups so it is a very potent antioxidant (Bravo, 1998).



**Figure 1–4** Structure of quercetin ( Bravo, 1998)

In addition to these, it is indicated that instead of individual compound, combination of polyphenols provides greater antioxidant response (Dai *et al.*, 2010).

#### 1.4.1.2 Antiproliferative and Apoptotic Effects of Polyphenols

Phenolic compounds are able to interact with cell signaling pathways by which way they have roles in cell growth and apoptosis regulation.

Cell cycle control relies on interactions between cyclins and cyclin-dependent kinases (CDKs). CDK inhibitors (CDKIs) are the authorities for this interactions' control. When Cyclin A binds to CDK2, cell growth is progressed through S phase of the cell cycle (Brown *et al.*,

2012). In one of the study, it has been found that red wine polyphenols decrease the cyclin A gene expression by inhibiting its transcription factor expression (Lijima *et al.*, 2000). Also increased expression of CDK inhibitors and decreased expression of cyclin A and B genes results in stop in G0/G1 and G2/S phases of cell cycle which leads to decline in cell growth (Brown *et al.*, 2012). Hou *et al.* (2004) stated that EGCG (e (-)-epigallocatechin-3-gallate) found in tea (*Camellia senensis*) has been shown to inhibit CDKs, directly. And in several cell lines such as breast, prostate and head cancers, it induced cell cycle arrest in G0/G1 phase.

Apoptosis is a programmed cell death that are carried out through 2 pathways; extrinsic and intrinsic (mitochondrial) pathways. Extrinsic pathway is activated when a specific ligand bind to cell surface death receptor (DR) which are tumor necrosis factor (TNF) receptor superfamily. After subsequent changes, procaspase 8 binds to death inducing signaling complex.

Intrinsic pathway is activated by agents such as ionizing radiation and chemicals which increase the oxidative stress of the cells. Consequently, mitochondrial membrane potential is disrupted and cytochrome c, Apaf-1 (apoptotic protease activating factor 1), endonuclease G and AIF (apoptosis inducing factor) are released from mitochondria. A supra molecular complex called apoptosome is formed by Cytochrome c, Apaf-1, dATP and procaspase 9.

From these both pathways procaspase 8 and 9 are cleaved to their active form. Then, they activate procaspase 3. Both of these activated caspases play role in cytoskeleton cleavage and DNA degradation.

Membrane potential of mitochondria is regulated through Bcl2 families include proapoptotic (e.g Bax ,Bcl-Xs) and anti-apoptotic (e .g Bcl-2, Bcl-XL) members (Giovannini and Masella ,2012 ; Brown *et al.*, 2012 ).

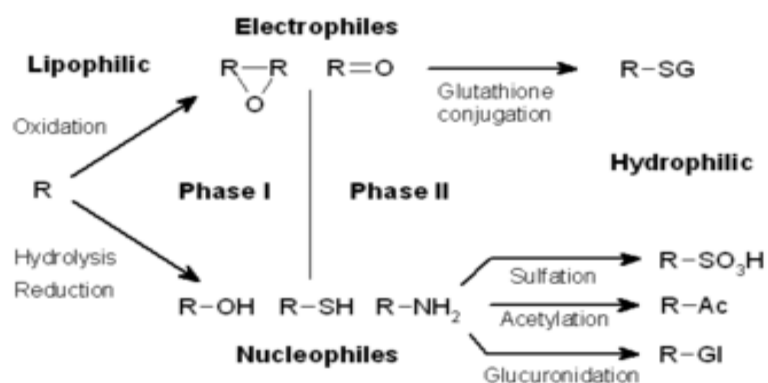
Brown *et al.* (2012) has studied on the antiproliferative effects of berry components in colon cancer models and they have found that ellagic acid decreased the expression of Bcl-XL. It also enhances the cytochrome c levels in parallel with caspase 9 and 3 activation.

In another study Karna *et al.* (2011) investigated the Sweet potato greens extracts (SPGE) as source of anthocyanins and phenolic acids by using PC3 prostate cancer cell line. They found that SPGE decreases Bcl-2 and increase BAX expression. It also enhances cytochrome c release which was detected by cleavage of caspase substrate, poly (adenosine diphosphate-ribose) polymerase.

## 1.5 Drug Metabolizing Enzymes

Drug metabolizing enzymes accomplish biotransformation of food components, drugs and xenobiotics in order to facilitate their excretion from the body. These enzymes are located in the intracellular membranes and cytosol within the cell. They can be classified in 2 groups called phase I and phase II enzymes. Phase I enzymes carry out oxidation, reduction and hydrolytic reactions. They add functional groups in to chemicals such as -OH, -COOH, -SH, -O- or NH<sub>2</sub> so they make these chemicals slightly soluble (Gonzalez *et al.*, 2005). However, some chemicals can generate electrophilic intermediates with Phase I metabolism that can leads to mutations and genetic lesions (Mandlekar *et al.*, 2006). Phase I enzymes includes cytochrome P450s (CYP), flavin containing monooxygenases (FMO) and epoxide hydrolases (EH) (Gonzalez *et al.*, 2005).

Phase II enzymes form conjugates to Phase I products. Thus, they make them more hydrophilic and these metabolites readily eliminated from the body. Conjugation reactions include glucuronidation, sulfation, and methylation and also amino acid conjugations. Phase II enzymes are UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and various methyltransferases (Figure 1-5) (Jancova *et al.*, 2010).



**Figure 1–5** General mechanism of Phase I and Phase II enzymes in drug metabolism ([http://en.wikipedia.org/wiki/File:Xenobiotic\\_metabolism.png](http://en.wikipedia.org/wiki/File:Xenobiotic_metabolism.png))

### 1.5.1 Modulations on Drug Metabolizing Enzymes via Phytochemicals

Changes in phase I and phase II enzymes levels are important factors for carcinogens metabolism since they can activate or eliminate carcinogens hazard. Since drug metabolism was also conducted by these enzymes, their modulation by phytochemicals has great importance to evaluate herb-drug interactions. Phytochemicals can regulate these enzymes by transcriptional regulation or interacting with enzyme activity (Galli, 2007).

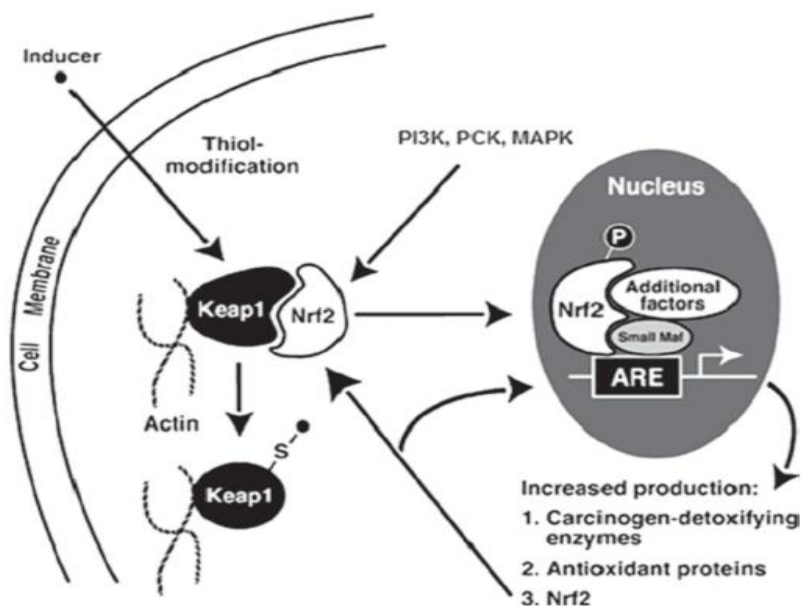
For example, Kimura *et al.* (2010) showed that CYP3A4 and CYP2C9 inhibition with 60 different polyphenols. CYP3A4 is known as a major drug metabolizer with more than 50% of clinical drugs. CYP2C9 metabolizes drugs such as warfarin and phenytoin. In their study they used human CYP3A4 and CYP2C9 microsomes expressed in Baculovirus-insect cell as a model. They concluded that most coumarins, flavonols and flavones (at concentration of 100 $\mu$ M) inhibited the CYP3A4 and CYP2C9 activity more than 80%. Especially, quercetin, phloretin, chrysin, apigenin and acacetin exhibited 100% inhibition against CYP3A4 and kaempferol, isorhamnetin, galangin, dicoumarol and naringenin showed 100% inhibition toward CYP2C9 activity.

Veronesa *et al.* (2003) examined that Grapefruit juice (GFJ) inhibited the CYP3A4 enzyme which catalyze most of the drugs in the market such as statins. They conducted two phases (phase1, n=8 male; phase 2, n=16 male) randomized, placebo controlled crossover studies. They applied erythromycin breath test (EBT) and measured oral midazolam (metabolized by CYP3A4) pharmacokinetics from blood samples. They investigated different parameters to find the way of inhibition. They concluded that grape fruit juice could inhibit both intestinal and hepatic CYP3A4 in dose-dependent manner. In another study conducted by De Castro *et al.* (2006) it was found that naringin, naringenin and furanocoumarins in GFJ are responsible for this CYP3A4 inhibition.

In an experiment conducted by Walle *et al.* (2000), UGT1A1 enzyme level was increased with flavone chrysin treatment of HepG2 and Caco-2 cell lines. Also, quercetin, naringenin, tangeretin and galangin rise up the UGT1A1 enzyme level (Moon *et al.*, 2006).

The increase in Phase II enzymes is seen as a major chemopreventive action of phytochemicals (Galli, 2007). The mechanism under this induction is related to Nrf-2 transcription factor which is a member of the basic leucine zipper transcription factor family. It is normally found in cytosol associated with cytoplasmic inhibitor Keap1. When an inducer is present, it disrupts this interaction and allows Nrf2 to translocate to nucleus. Inducer may lead to cleavage of disulfide bonds between Keap 1 and Nrf2 or may act with multiple kinase

pathways. MAPK, phosphoinositide 3-kinase (PI3K) and protein kinase c (PKC) can regulate translocation of Nrf2 to the nucleus. In nucleus, Nrf2 make complex with Maf proteins and bind to ARE (antioxidant/ electrophile response element), thereby phase II enzyme gene transcription is activated (Figure 1-6) (Moon *et al.*,2006; Bausova and Skalova,2012).



**Figure 1–6** Proposed pathway for the induction of Phase II genes by phytochemicals (Bousova and Skalova, 2012)

Phytochemicals enhance the release of Nrf2 from Keap1. Nrf2 then accumulates in the nucleus and transactivates the AREs of many Phase II genes as well as Nrf2 itself.

In one of the studies, silymarin isolated from milk thistle effectively restrain the colonic and ACF (aberrant crypt foci) induced by azoxymethane that is the intermediate of colonic carcinogen DMH (1, 2-dimethyl hydrazine) in liver and large intestine of male F344 rats. Induction of GST and QR enzyme activities by silymarin was shown as a reason of this suppression (Kohno *et al.*, 2002).

## 1.6 Glutathione S-Transferases (GSTs)

GST enzymes can be categorized in to 3 groups as cytosolic, mitochondrial and microsomal. Cytosolic and mitochondrial GSTs are soluble enzymes; however, microsomal GSTs are membrane associated proteins.

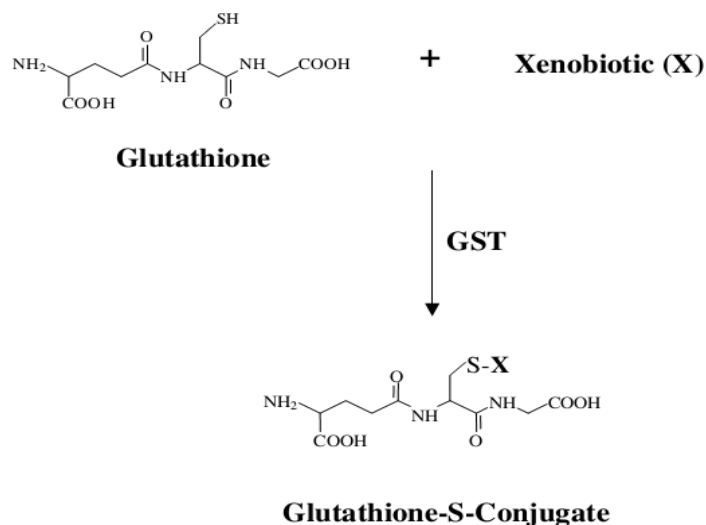
Cytosolic GSTs constitute the largest GST family and have specific activities. There are 7 classes of cytosolic GSTs according to their amino acid sequence similarities in human which are Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta classes. They are essentially located in cytoplasm.

Glutathione S Transferases have N-terminal and C-terminal domains. Also, they have glutathione (GSH) binding site (G-site) and hydrophobic site (H-site). Properties of H-site amino acid residues show differences among GSTs resulting in substrate specificity.

GSTs are the enzymes that are found in all living organisms. Basically, they are the phase II detoxifying enzymes that they metabolize the electrophilic substrates containing carbon,



nitrogen or sulfur atom by conjugation reaction with endogenous tripeptide glutathione (GSH) that results in less reactive, more water soluble products (Figure 1-7) (Bausova and Skalova,2012).



**Figure 1–7** Formation of glutathione conjugate (Townsend and Tew, 2003)

However, in some cases, glutathione conjugation can cause formation of more reactive compounds than parent can. This property is used in cancer chemotherapy as taking advantage of reactive metabolites produced with GST metabolism in tumor. For example, cytotoxic drug TER286 (or TLK 286) is metabolized with GSTs. As a result, active metabolite cyclophosphamide chemotherapeutic is formed which is an effective drug for breast cancer treatment.

GSTs also have selenium independent glutathione peroxidase activity. In addition to other enzymatic antioxidants, it can also protect cells against various reactives formed when membranes was attacked. For example, GSTs can metabolize cholesteryl hydroperoxides and fatty acid hydroperoxides to their corresponding alcohols. Therefore, they protect membranes by preventing formation of following epoxides and reactive carbonyls.

GSTs also have importance in synthesis of biologically important arachidonic acid metabolites.

GSH conjugation behaves like a flag for enhance signaling by which way it facilitate the export of the conjugates from cell (Hayes *et al.*, 2005; Hayes and Pulford, 1995).

Glutathione transferases can act in cis-trans isomerization reactions. Isomerization of maleyloacetoacetate to formylacetoacetate by GSTZ-1 enzyme in tyrosine catabolism can be represented as an example.

Soluble GSTs can change function of other intracellular proteins by binding to them. GSTO1 is able to contact with ryanodine receptors of calcium channel and thus it can prevent cells to go apoptosis by calcium channel mobilization from intracellular stores. (Sherrat and Hayes, 2002; Awasthi, 2007)

Induction in GST enzymes is seen as a chemopreventive strategy. To illustrate, a sulfur containing isothiocyanate, sulforaphane (SFN), found in cruciferous vegetables such as in broccoli, is shown as an chemopreventive phytochemical due to its role in GST induction. In one of the studies, AML12 cells treated with 10µM sulforaphane for 12 hours showed 4,3 fold increases in their GST enzyme activity. They also showed that SFN enhanced the

conjugation potential of aflatoxin B1-8, 9-epoxide (AFBO), which is a hepatocarcinogenic intermediate of Aflatoxin B, with GSH 35 fold (Gao *et al.*, 2010).

On the other hand, overexpression of GST enzymes can also results in resistance toward anticancer agents and carcinogens. Piaggi *et al.* (2010) found that GSTO1 gene overexpression leads to resistance toward cisplatin drug. They conducted studies by using HeLa cells in which GSTO1-1 enzyme was transfected. At 20 $\mu$ M cisplatin concentration, they observed 80% survival in cells with overexpressed GSTO1-1 enzyme, on the other hand, 20% survival in controls. They concluded that GSTO1-1 overexpression led to decrease in apoptosis by activating survival mechanisms and inhibiting apoptosis related JNK activation pathway.

## 1.7 Salvia Genus

Salvia is the largest genus of the Lamiaceae family. This genus has notable diversity in their secondary compounds, growth forms, floral morphology and pollination. Salvia is distributed in 3 regions of the world. It comprises around 1000 species as at least 500 species in Central and South America, 200 species in westerns Asia and 100 species in eastern Asia (Kahraman *et al.*, 2012).

As an important medicinal plant Salvia has been used since ancient times. Origin of the name Salvia stems from the Latin word *salvare* meaning heal. 'Why should man die when sage grows in his garden?' is an ancient saying that shows the importance of Salvia for health (Mohammed, 2011).

### 1.7.1 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 (Karaman *et al.*, 2012).

Salvia genus is represented by totally 94 species in Turkey (Bagherpour *et al.*, 2011). 51 of these species are endemic and endemism ratio (52.5%) is high. *S.fruticosa*, *S.abscuditiflora*, *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 constituted by *S.fruticosa* (Ipek and Gürbüz, 2010).

### 1.7.2 Ethnobotanical Use of Salvia

In a survey completed in east Anatolia in Turkey, Altındag and Öztürk (2011) found that people drink various Salvia species for different health purposes. For example, *S.hydranxa* 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.*, 2011). An Ottoman herbalist-physician lived during Sultan Mehmed the fourth used *S. triloba* for memory enhancement.

In addition to these, there are laboratory studies that show antimicrobial, anti-inflammatory, antioxidant, chemopreventive, hypoglycemic and many other properties of Salvia genus. For

example, Salvianolic acid B isolated from *S.miltiorrhiza* Bge has been found to decrease the DMBA (7,12-dimethylbenz (a) anthracene) induced squamous cell carcinoma (Wei *et al.*, 2012). Çadırcı *et al.* (2012) reported the presence of anti-inflammatory activities of *S. fruticosa*, *S.verticillata*, and *S.trichoclada* by using a carrageenan-induced inflammatory paw edema model that n-butanol extract of Turkish sage *S.fruticosa* showed the highest activity. *S.officinalis* methanolic extract that intraperitoneally administrated in diabetic rats significantly decreased the level of serum glucose in 3 hours that shows its hypoglycemic effect (Eidi *et al.*, 2006). Replacement of water with *S.officinalis* tea for 14 days in mice and rats diet resulted in significant increase in liver GST activity (24% and 10%, respectively). Also in these rats' hepatocyte primary culture, they examined increased GSH content and GST activity that shows the high antioxidant potential of *S.officinalis* (Lima *et al.*, 2005). *S.officinalis* also has an antimicrobial and astringent activity, which is used in dental care preparations (Baricevic and Bartol, 2005).

*S.miltiorrhiza* root ( Danshen) has a popularity as a 'supergrade' medicine in China that it is used in the treatment of myocardial infarction and angina pectoris. The Danshen product 'Fufang Danshen Diwan' is the first Chinese herbal medicine that has been sold in 16 countries of the world and approved by FDA for clinical tests in USA (Figure 1-8).



**Figure 1–8** Commercial tablets of Danshen (*S.miltiorrhiza* root) (Wu *et al.*, 2012)

Although it is used in cardiovascular disease treatment, it also has antioxidative, anti-inflammatory and anticancer properties related to their abitenoids, diterpene quinone pigments especially Tanshinone IIA ( Wu *et al.*, 2012).

### 1.7.3 Polyphenolics of Salvia

Salvia is an important genus for polyphenol richness with more than 160 phenolics identified. Polar phenolic acids are the main water-soluble components of Salvia. Most of the phenolic acids are caffeic acid 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.

Flavones, flavonols and their glycosides are highly distributed flavonoids in Salvia species. Especially, 6-hydroxylated flavones have taxonomic importance for Salvia genus. Apigenin and luteolin and their 6-hydroxylated derivatives are main flavones. Kaempferol and quercetin methyl ethers are mostly found flavonols (Lu *et al.*, 2002).

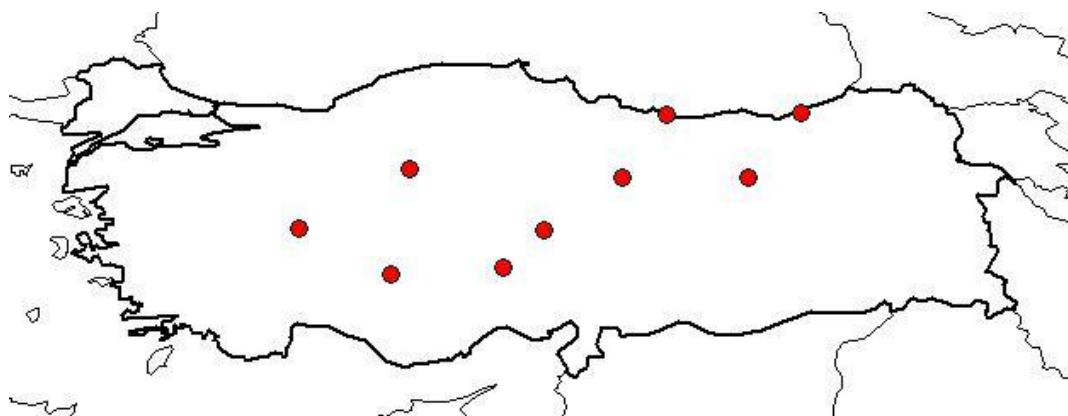
## 1.8 *Salvia Absconditiflora*

### Taxonomic Hierarchy

Kingdom: Plantae  
Subkingdom: Tracheobionta  
Division: Magnoliophyta  
Class: Magnoliopsida  
Subclass: Asteridae  
Order: Lamiales  
Family: Lamiaceae  
Genus: *Salvia*  
Species: *Salvia absconditiflora*

([http://turkherb.ibu.edu.tr/index.php?sayfa=1&tax\\_id=8076](http://turkherb.ibu.edu.tr/index.php?sayfa=1&tax_id=8076) )

Kara ot (Van), Kara şabla, Kara şalva, Kara şapla are the vernacular names of the taxon. *S.absconditiflora* is a perennial plant that grows in rocky limestone slopes, dry steppe, fallow fields and roadsides. *S.absconditiflora* is an endemic species in Turkey that grows in Afyonkarahisar, Ankara, Çorum, Erzincan, Kayseri, Konya, Niğde, Ordu and Sivas that illustrated in Figure 1-9.



**Figure 1–9** Distribution of *Salvia absconditiflora* in Turkey

There are limited numbers of studies about *S.absconditiflora* and its effects. Akin *et al.* (2010) investigated the essential oil composition of *S.absconditiflora*. They found major oil constituents of *S.absconditiflora* as camphor (19,1%), 1,8-cineole(16,4%), borneol ( 11,9%), viridiflorol ( 11,5%) and bornyl acetate( 2,4%) . They indicated that 1,8-cineole responsible for anti-microbial potential of *S.absconditiflora*.

Şenol *et al* (2010) found that *S.absconditiflora* extracts show high anticholinesterase activity (over 70%). In another study conducted by the same group, they observed significant wound healing activity in animals with ethanol extract of *S.absconditiflora* in the circular excision wound model. That response of the extract was told as similar to the reference drug Madecassol® (Süntar *et al.*2011).

Although *S.absconditiflora* leaves are consumed as herbal tea (Kahraman *et al.*, 2012), there is not enough published data about its anti-carcinogenic properties.

## 1.9 Breast Cancer Cell Lines

Cancer cell lines are the mirrors of primary tumors in copy number and gene expression abnormalities. One of the concerns about the cell lines is the stability preservation in genomic and expression patterns over multiple passages. However, it has been proved that cell lines do not accumulate new recurrent aberrations over time (Neve *et al.*, 2006). They are also good sources for studying the effect of xenobiotics without damaging animals.

### 1.9.1 MCF-7

MCF-7 (Michigan Cancer Foundation – 7) is an invasive ductal carcinoma that was taken from 69 years old Caucasian woman with pleural effusion. It shows many characteristics of differentiated mammary epithelium such as ability to process estradiol via cytoplasmic estrogen receptors. MCF-7 consist of luminal cells that appear more differentiated and form tight cell-cell junctions (Neve *et al.*, 2006; <http://www.atcc.org/attachments/17392.pdf> )

### 1.9.2 MDA-MB-231

MDA-MB-231 (Monroe Dunaway Anderson - Metastatic Breast) is an adenocarcinoma taken from 51 years old Caucasian woman with pleural effusion from metastatic site. It is estrogen and progesterone receptor negative cell line. MDA-MB-231 consist of Basal B cells which are less differentiated than luminal ones and they show a more mesenchymal like appearance and high invasiveness (Neve *et al.*, 2006; <http://www.atcc.org/attachments/17400.pdf>).

## 1.10 Scope of the Study

Medicinal herbs have important roles in various diseases such as cancer. Many studies are being conducted to examine herbs and their chemical constituents in order to investigate the principles of their actions. These improvements enlighten the use of those natural compounds in drug discovery.

*Salvia* species are being investigated highly due to presence of different chemical constituents which showed antioxidant, anti-proliferative , anti-inflammatory and many other properties. There are some novel chemicals have been explored in these species and that enhance formulation of new drug compounds to treat many diseases.

*Salvia absconditiflora* is an endemic species in Turkey, which is consumed as a herbal tea. There are some researches directed with *Salvia absconditiflora* about its effect on wound healing and Alzheimer disease. However, there is no study available with *Salvia absconditiflora* water extracts about its role in drug metabolism and chemoprevention.

This thesis study was designed to investigate antioxidant capacity and phenolic composition of *Salvia absconditiflora* water extract as well as its effects on breast cancer cell lines and GST genes expressions.

The water extract of the *Salvia absconditiflora* was investigated for its radical scavenging property via DPPH antioxidant activity assay. Its polyphenolic constituents were evaluated with liquid chromatography mass spectrometry (LC-MS/MS) technique, as well as their total content was quantified with total phenol and total flavonoid spectrophotometric assays.

Trypan blue dye exclusion and XTT assays were applied to examine the cytotoxic property of *Salvia absconditiflora* water extract for different extract concentrations at 24 and 48 hours.

In addition to these, *Salvia absconditiflora* water extract was examined for its effect on expressions of Glutathione S-Transferases (GSTO1 and GSTZ1) via qRT-PCR in order to evaluate its role on drug metabolism.

This study is the first in literature considering *Salvia absconditiflora* as a herbal tea to evaluate in terms of antioxidant capacity and polyphenol content, as well as, cytotoxic role in MCF-7 and MDA-MB-231 breast adenocarcinoma cells lines and influence on Phase 2 drug metabolizing genes expressions. This preliminary study can supply information for studying drug metabolism, discovery and drug-herb interactions.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Materials

##### 2.1.1 Cell Lines

MCF-7 and MDA-MB-231 (human breast adenocarcinoma cell lines) were bought from ATCC (American Type Culture Collection).

##### 2.1.2 Plant Material

*Salvia absconditiflora* leaves were collected from a forested land near Biological Sciences department in METU campus in November 2010, April, June and July 2011 according to leadership of Assoc. Prof. Ferhat Celep. 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*

##### 2.1.3 Chemicals and Other Materials

2, 2-diphenyl-1-picrylhydrazyl (DPPH), catechin hydrate (+), Folin-Ciocalteu's phenol reagent, Ethidium Bromide, agarose was bought from Sigma (Sigma Chemical Company, Saint Louis, Missouri, USA).

Dimethyl sulphoxide (DMSO) was purchased from AppliChem GmbH (Darmstadt, Germany). Roswell Park Memorial Institute medium (RPMI 1640 with L-Glutamine and 25mM HEPES), Phosphate buffer saline ( PBS) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for cell culture and 10X trypsin were purchased from Lonza Ltd.(Verviers, Belgium). Fetal Bovine Serum (FBS) (Heat-inactivated)

was purchased from Biochrom AG (Berlin, Germany). XTT cell proliferation kit for 1000 tests, gentamycin and 0.5% (w/v) trypan blue were purchased from Biological Industries (Haemek, Israel).

PerfectPure™ RNA isolation kit and Real MasterMix SYBR ROX was purchased from 5 Prime GmbH (Hamburg, Germany).

RevertAid First strand cDNA synthesis kit was purchased from Thermo Fisher Scientific (USA).

25-cm<sup>2</sup> (T25) and 75-cm<sup>2</sup> (T75) tissue culture flasks, 6-well and 96 well microplates (flat and round bottom), and cryovials (sterile, non-pyrogenic, DNase and RNase free) were obtained from Greiner Bio-One, Germany.

#### 2.1.4 Primers

GSTO1, GSTZ1 and beta-actin gene primers were purchased from Iontek, İstanbul, Turkey. GSTO1 and GSTZ1 primer pairs were taken from the literature (Scharmach *et al.*, 2009).

**Table 2-1** Primers used in this study

Primer Set	Sequence( 5'-3')	Amplicon size (bp)
β-actin reverse	TTGAAGGTCTAAACATGAT	201
β-actin forward	CAGAGCAAGAGAGGCATCCT	
GSTO1-1 reverse	CAGCTTCTTCCCTGGGTATG	82
GSTO1-1 forward	GGAAAACAGTCAGGGTCAGC	
GSTZ1 reverse	ATGGTAGGGTAGGGGTGAG	114
GSTZ1 forward	CACAGCGGGCATATACTGTG	

## 2.2 Methods

### 2.2.1 *Salvia Absconditiflora* water extract preparation

After collection from the field, *S. absconditiflora* leaves were washed with tap water and put on a filter paper. Then, they were allowed to dry out in the dark.

Dried leaves were grinded harshly into small pieces by hand. Then, they were weighed and mixed with 50°C dH<sub>2</sub>O at a ratio of 1:10 (w/v) in dark bottles. Then the bottles were kept in ultrasonicator for 30 min (Bandelin Sonorex Model RK 100H, Berlin, Germany) and in 50°C water bath (Nüve BS302 ) for 90min. After that, extracts were filtered through double-layered cheesecloth and the volume was recorded. After freezing at -80°C, the extracts were lyophilized with freeze dryer for 3 days. The extracted powder was weighed in order to calculate yield of extraction (w/w) and stored at -20°C in dark bottle.



## 2.2.2 Detection of antioxidant capacity of *Salvia absconditiflora* water extracts

### 2.2.2.1 DPPH assay

Free radical scavenging activity of *S. absconditiflora* water extract was determined by DPPH (2,2-Diphenyl-1-picrylhydrazyl) technique based on Blois' method (1958).

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is a simple, sensitive and rapid technique which is highly used for detection of radical scavenging capacity of foods such as fruits, vegetables, juices and wines. It is a reproducible technique and needs only UV-spectrophotometer to do measurements. Due to its free electron delocalization, DPPH is a stable nitrogen free radical. This delocalization gives it a deep violet color in solution which can be measured with UV-spectrophotometer in range of 515-520 nm wavelengths (Chen *et al.*, 2013; Molyneux, 2004; Villano *et al.*, 2007).

When DPPH solution is mixed with an H<sup>+</sup> donor, the molecule turns in to non-radical reduced form. The color of solution alters into a pale yellow color due to its picryl group still present (Figure 2-2). The basis of the method depends on the measurement of this decolorization.

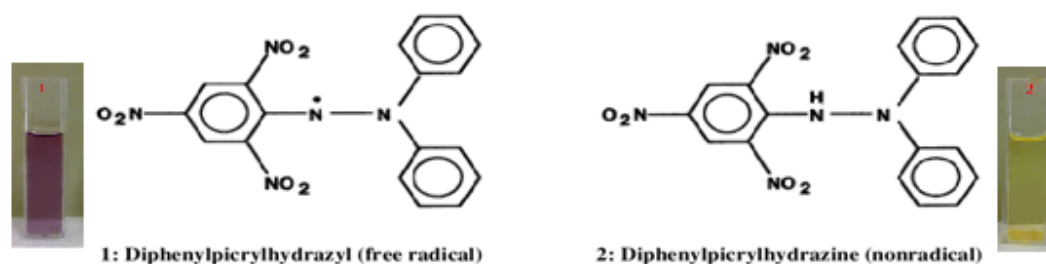


Figure 2–2 DPPH in radical and non-radical forms ( Modified from Molyneux, 2004)



As illustrated above, DPPH<sup>•</sup> is a free radical that reacts with AH which is an antioxidant in foods. With this reaction, R<sup>•</sup> radical species is formed. It reacts with another DPPH<sup>•</sup> radical and DPPH-R is formed as final product (Brand-Williams *et al.*, 1995).

The reaction in this process is biphasic. That starts with a fast decline in absorption in the first few minutes, continues with a slower reaction and results with equilibrium (Villano *et al.*, 2007).

For the experiment, DPPH<sup>•</sup> was dissolved in 99.5% ethanol in dark bottle, as concentration becomes 0,05mg/ml. It was mixed thoroughly and waited in ultrasonicator for at least 30 min.

*S. absconditiflora* freeze-dried extracts were dissolved in dH<sub>2</sub>O. Then the serial dilutions at different concentrations were prepared. These are 0,1 mg/ml, 0,25 mg/ml, 0,5mg/ml, 0,75 mg/ml, 1 mg/ml, 1,25 mg/ml, 1,5 mg/ml, 2 mg/ml, 2,5 mg/ml and 3 mg/ml.

Absorbance of DPPH was measured against ethanol at 517 nm in order to check whether it is ready or not for the experiment. After that, 100µl of each concentration was mixed with 1400µl DPPH solution in tubes in duplicates, vortexed and they were incubated at room

temperature in dark for 30 min , which was optimized before. Tubes were poured into cuvette and measured at 517 nm with spectrophotometer (Shimadzu UV-160A, Japan) against 99,5 % ethanol as reference cuvette. Sample blank (100µl of each sample mixed with 1400µl dH<sub>2</sub>O) absorbance was measured in order to eliminate the absorbance effects of *S.absconditiflora* itself. Quercetin was also measured in different concentrations as a positive control.

The result of the assay is interpreted using the EC<sub>50</sub> concentration parameter, which is the concentration of the *S.absconditiflora* extract that causes 50% loss of DPPH free radical activity. It is calculated from % RSA (Radical Scavenging Activity Percentage) versus concentration curve. There is an inverse relationship between EC<sub>50</sub> value and antioxidant capacity.

$$\%RSA = \frac{Abs_{BLANK} - (Abs_{SAMPLE} - Abs_{SAMPLE\ BLANK})}{Abs_{BLANK}} \times 100 \quad (2-3)$$

Where;

Abs = Absorbance at 517nm

Abs<sub>BLANK</sub> = Absorbance of DPPH (1400 µl) and dH<sub>2</sub>O (100 µl) mixture

Abs<sub>SAMPLE</sub> = Average absorbance of sample with DPPH

Abs<sub>SAMPLE BLANK</sub> = Average absorbance of sample with dH<sub>2</sub>O

% RSA was plotted against the concentration of the sample and the EC<sub>50</sub> values were determined using Graphpad Prism version 5 (Graphpad Software, San Diego, California, USA).

### 2.2.2.2 Total phenol assay

Total phenolic components of the freeze-dried *S.absconditiflora* water extracts were analyzed by Folin–Ciocalteu method as described by Singleton (1999) with slight modifications using gallic acid as a standard.

In the experiment, gallic acid standard solutions were prepared at different concentrations with dH<sub>2</sub>O as serial dilutions, ranging from 25 to 150 µg/ml. *S.absconditiflora* freeze-dried extracts were diluted in dH<sub>2</sub>O with serial dilutions as 0,5 mg/ml, 0,75 mg/ml and 1mg/ml. Folin-Ciocalteu reagent (2N) was diluted in 1:9 ratios with dH<sub>2</sub>O. 100 µl lyophilized extract or gallic acid standard was mixed with 1ml Folin Ciocalteu reagent and 800 µl aqueous Na<sub>2</sub>CO<sub>3</sub> (1M) in tubes. They were vortexed and incubated for 15 min at room temperature in dark. After that, the mixtures were measured at 765 nm spectrophotometrically in duplicates. Sample blanks (100 µl sample / standard mixed with 1800µl dH<sub>2</sub>O) were also measured to get rid of any background due to extract.

Total phenol values were calculated using the gallic acid standard curve' slope and expressed in terms of mg gallic acid equivalents (GAE) / g of freeze-dried extract.

$$\text{mg GAE / g dry extract mass} = \frac{(Abs_{SAMPLE} - Abs_{SAMPLE\ BLANK}) - Abs_{BLANK}}{\text{SLOPE}} \times \text{DF} \quad (2-4)$$

Where;

Abs = Absorbance at 765nm

Slope = Obtained from standard curve (Absorbance versus concentrations of Gallic Acid)

Abs<sub>SAMPLE</sub> = Average absorbance of sample with reactants

Abs<sub>SAMPLE BLANK</sub> = Average absorbance of sample wells without reactants (only dH<sub>2</sub>O)

Abs<sub>BLANK</sub> = Average absorbance of reactants and dH<sub>2</sub>O mixture

DF= Dilution Factor

### 2.2.2.3 Total flavonoid assay

The total flavonoid content in the extracts was detected by modified aluminum chloride colorimetric assay (Zhishen *et al.*, 1999).

Quercetin used a standard for determination of total flavonoid content in each months extract (November, April, June and July). During experiment, it was dissolved in ethanol (99, 5%). Then varying concentrations of quercetin was prepared with serial dilutions.

Catechin (+) as a standard in total flavonoid calculation of mixed extract was dissolved in ethanol (99,5 %). Then, different concentrations of catechin(50 µg/ml, 125 µg/ml, 200 µg/ml, 250 µg/ml and 300 µg/ml) was prepared as serial dilutions.

*S.abscoditiflora* freeze-dried water extract was dissolved in dH<sub>2</sub>O. 0,5mg/ml, 1mg/ml and 2mg/ml were prepared by serial dilutions.

0,2ml sample / catechin (quercetin) standard was mixed with 0,75ml 5% (w/v) sodium nitrite (NaNO<sub>2</sub>). The mixtures were incubated for 5 min at room temperature. Then, 0,15 ml 10% (w/v) aluminum chloride was added and after 6 min incubation 0,5 ml 1M NaOH was added. Finally, total volume was completed to 3 ml with dH<sub>2</sub>O in tubes and mixed thoroughly. The absorbance was measured at 465 nm with spectrophotometer (Shimadzu UV-160A, Japan). The samples were measured in duplicates. Sample Blank was not measured. Because, extract did not give absorbance at 465 nm without reactants.

Total flavonoid content of the extracts were determined by the help of catechin + (or quercetin) standard curve slope and it was expressed as mg catechin (or quercetin) equivalents / g of freeze-dried extract.

$$\text{mg CE(QE) / g dry extract mass} = \frac{\text{Abs}_{\text{SAMPLE}} - \text{Abs}_{\text{BLANK}}}{\text{Slope}} \times \text{DF} \quad (2-5)$$

Where,

Abs = Absorbance at 465nm

Abs<sub>SAMPLE</sub> = Average of absorbance value of extract with reactants

Abs<sub>BLANK</sub> = Average absorbance value of reactants and dH<sub>2</sub>O mixture

Slope= Obtained by standard curve (Absorbance versus concentration of catechin (+) or quercetin graphic )

DF= Dilution Factor

### 2.2.3 Identification of phenolic compounds with LC-MS/MS

MS (mass spectrometry) is a very important technique for structural characterization of polyphenols. The technique is based on 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 Dr.Tamay Şeker in Central Laboratory of METU. Standards were used was 10 ppm. *Salvia absconditiflora* freeze-dried water extract was weighed and dissolved in 50% methanol solvent and became 0,362 g/ml at final.

**Table 2-2** LC-MS/MS equipments and conditions for analysis of *S.absconditiflora* phenolics

<b>Mass spectrometry</b>	
Device	AGILENT 6460 LCMSMS
Ionization Source	ESI+Agilent 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 °C
Gas Flow	10 mL/min
Sheath gas temperature	350 °C
Capillary	4000 V (+, -)
Nozzle Voltage	500 V (+, -)
<b>Liquid Chromatography</b>	
Column	Zorbax SB-C18 (2,1 x 50 mm x 1,8 µ)
Mobile phase	Solvent A: 0,05 % Formic acid+ 5 mM Amonium format ( MilliQ water) Solvent B: Metanol (MS grade ,MERCK)
Flow rate	0,3 ml/min
Analysis time	13 min
Mobile phase flow mode	Graduated
Injection volume	5 µL
Range of standard curve	0.01 – 0.025 – 0.05 – 0.1 – 0.5 – 1 – 5 – 10 ppm

## 2.2.4 Cell Culture

### 2.2.4.1 Cell lines and growth conditions

MCF-7 (Michigan Cancer Foundation – 7) and MDA-MB-231 (Monroe Dunaway Anderson - Metastatic Breast) monolayer cell lines were grown in RPMI 1640 with phenol red including 10% heat-inactivated fetal bovine serum (FBS) and 0,2% (50 mg/ml) gentamycin in the flasks. They were incubated in Hepa filtered Heraeus Hera Cell 150 incubator at 37°C with 95% humidified air and 5% CO<sub>2</sub>. Cell culture experiments were carried on HERA safe Class II Biological safety laminar flow.

### 2.2.4.2 Thawing

Frozen cells in cryovials taken from nitrogen tank in cold room were defrosted at room temperature. Then, they (1 ml) were transferred into T25 tissue culture flask. The volume was completed to 5 ml with RPMI 1640 complete medium and T25 flask was incubated in CO<sub>2</sub> incubator at 37°C for 24 hours.

### 2.2.4.3 Viable cell counting

Trypan Blue is a dye that only penetrates across the membranes of dead/non-viable cells. Viable cells are seen as small, round and retractile while non-viable cells are observed as swollen and dark blue under light microscope when trypan blue is applied. Before usage, trypan blue stock solution (0.5 % (w/v)) is diluted in 1:1 ratio with PBS (Dulbecco's Phosphate Buffered Saline).

In the experiment 20µl trypan solution and 20µl cell suspension were mixed thoroughly in order to obtain homogenous mixture. A coverslip was put on to a Neubauer hemacytometer (Bright-line, Hausser Scientific, Horseam, PA, USA). 10 µl of the mixture was filled in each of the two counting chambers of hemacytometer by placing the micropipette at the midside of the coverslip and the suspension was allowed to fill the space by capillary action.

Each chamber of hemacytometer consists of 9 squares each of which is 1mm<sup>2</sup>. Coverslip sits 0,1mm over the chambers. Therefore, the volume of each square becomes 0,1mm<sup>3</sup> or 10<sup>-4</sup> ml. In order to calculate number of cells per ml in suspension, number of cells counted in middle square is multiplied by 10<sup>4</sup> since the volume of 1 square is 10<sup>-4</sup> ml.

Cell number / ml = the average cell number of the two chambers x DF x 10<sup>4</sup>      **(2-5)**

DF= Dilution factor that was done with Trypan Blue

### 2.2.4.4 Cell passaging

When the cells in flask reached 80% confluency, the medium was discarded. The flask was washed with 2 ml PBS (Dulbecco's Phosphate Buffered Saline) for two times in order to get rid of all the waste materials and serum which includes trypsin inhibitors. Then 2 ml trypsin was added in and the flask was incubated in CO<sub>2</sub> incubator for 2-5 min. When detachment was observed under inverted light microscopy, flask was taken from incubator and growth medium was put in immediately to stop trypsin activity since trypsin exposure can cause damage in cells after some time. In order to separate cells from each other and eliminate the clumps, pipetting was done in suspension and finally cells were divided into 2 flasks.

### 2.2.4.5 Freezing

Freezing of the cells was accomplished with freezing medium prepared by mixing 10 % dimethylsulfoxide (DMSO) and 90% fetal bovine serum (FBS). DMSO is a cryoprotectant that lowers the freezing point.

After trypsinization of cells, detached cells were mixed with growth medium and counted as described in 2.2.4.3. Then they were centrifuged at 100g for 5min. The supernatant, which includes medium, dead cells and waste products, was completely discarded. Pellet includes cells was re-suspended with freezing medium according to number of cells (1ml Freezing medium / 10 million cells). After pipetting thoroughly, cell suspension was put in cryovials as 1 ml for each. The cryovials were stored at – 80°C in a isopropyl alcohol containing box to provide gradual freezing and the next day they were placed in liquid nitrogen tank (-190°C) for long term storage.

## 2.2.5 Cytotoxic effects of *S. absconditiflora* on MCF-7 and MDA-MB-231 cell lines

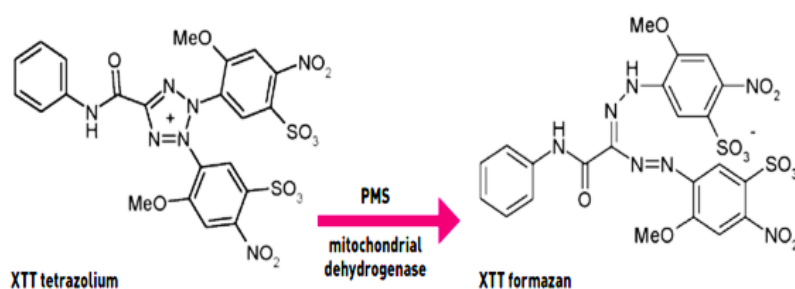
### 2.2.5.1 Cell treatment with *S. absconditiflora* water extract

For cytotoxicity experiments, MCF-7 and MDA-MB-231 cell lines were seeded at a density of  $1 \times 10^5$  cells/ml into 96 or 24 well plates and incubated at 37°C for 24 hours in CO<sub>2</sub> incubator. Next day, stock DMSO solution was diluted with RPMI 1640 growth medium to obtain 0,2% DMSO. Then, plant extract was dissolved in 0.2% DMSO to a definite concentration. Serial dilutions were prepared with this stock working solution in varying concentrations ranging from 0,5 to 10 mg/ml. After that, cells were treated with these concentrations and plate was incubated in CO<sub>2</sub> incubator for 24 and 48 hours.

### 2.2.5.2 XTT

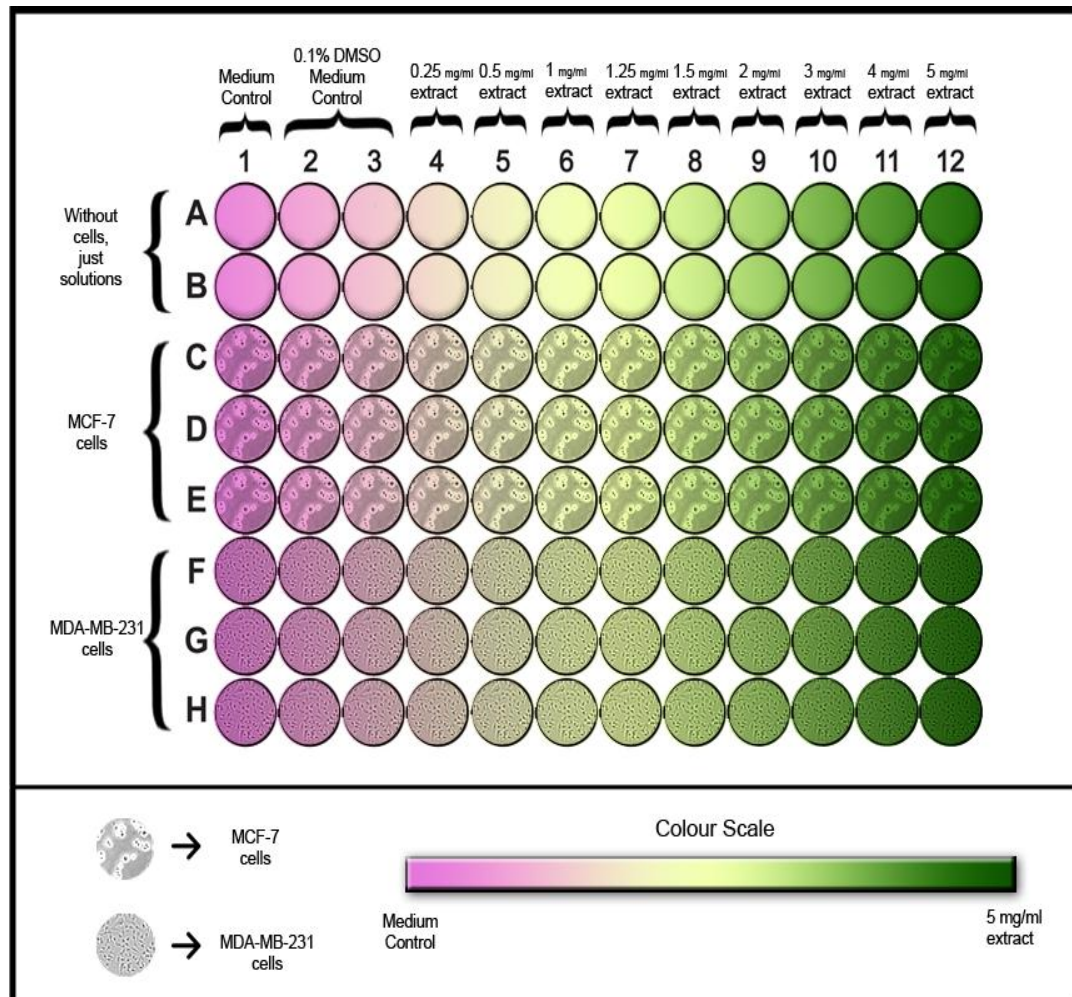
The cytotoxic effects of *S. absconditiflora* in MCF-7 and MDA-MB-231 cell lines were studied with Cell Proliferation XTT Kit of Biological Industries in conformity with manufacturer's instructions.

The basis of this technique is the reduction of tetrazolium salt XTT by metabolically active cells to orange colored formazan by succinate dehydrogenase enzymes of mitochondria respiratory chain (Figure 2-3). The viable cells with intact mitochondrial and cellular membrane have active dehydrogenases. Therefore, the greater the number of active cells, the greater the activity of mitochondrial enzymes of the cells and the higher the concentration of the dye formed. The intensity of this newly formed dye can be measured and quantified at 415nm with ELISA reader.



**Figure 2–3** Demonstration of reduction in tetrazolium salt into formazan in viable cells (<http://www.applichem.com/en/literature/applications/no-12-cell-proliferation-assay-xtt/>)

The cells were cultivated in a 96 well plate (100.000 cells/ml) as 100µl per well. No cells were seeded in to the first two row of plate. After incubation in CO<sub>2</sub> incubator for 24 hours, the medium in the wells were discharged and wells were washed with 50ul PBS. Fresh medium (50µl) was added in all wells. Then 50µl extracts in different concentrations in ascending order from left to right column were added in to wells except first two columns. 50µl growth medium is added in to first row and 50µl 0.2% DMSO added in to second row as a control. The visual model of the 96 well plates for XTT assay was demonstrated in Figure 2-4.



**Figure 2-4** 96 well plate representation of XTT assay. A1 to H1, complete medium control, 100 µl; A2 to H2 and A3 to H3, 0,1% DMSO medium control, prepared as 50 µl 0,2% DMSO medium + 50 µl complete medium, A4 to H4 0,25 mg/ml *S.absconditiflora* extract, prepared as 50 µl 0,5 mg/ml *S.absconditiflora* extract + 50 µl complete medium, A5 to H5 0,5 mg/ml *S.absconditiflora* extract, prepared as 50 µl 1 mg/ml *S.absconditiflora* extract + 50 µl complete medium; A6 to H6 1 mg/ml *S.absconditiflora* extract, prepared as 50 µl 2 mg/ml *S.absconditiflora* extract + 50 µl complete medium; A7 to H7, 1,25 mg/ml *S.absconditiflora* extract, prepared as 50 µl 2,5 mg/ml *S.absconditiflora* extract + 50 µl complete medium; A8 to H8, 1,5 mg/ml *S.absconditiflora* extract, prepared as 50 µl 3 mg/ml *S.absconditiflora* extract + 50 µl complete medium; A9 to H9, 2mg/ml *S.absconditiflora* extract, prepared as 50 µl 4 mg/ml *S.absconditiflora* extract + 50 µl complete medium; A10 to H10, 3 mg/ml *S.absconditiflora* extract, prepared as 50 µl 6 mg/ml *S.absconditiflora* extract + 50 µl complete medium; A11 to H11 4 mg/ml *S.absconditiflora* extract, prepared as 50 µl 8 mg/ml *S.absconditiflora* extract + 50 µl complete medium, A12 to H12 5 mg/ml *S.absconditiflora* extract, prepared as 50 µl 10 mg/ml *S.absconditiflora* extract + 50 µl complete medium. A1 to A12 and B1 to B12 columns are without cells

After addition of the extracts and medium, plate was incubated for 24 or 48 hours in CO<sub>2</sub> incubator at 37°C.

For XTT to be ready for the experiment, 0,1ml activation solution containing PMS (N-methyl dibenzopyrazine methyl sulfate) was added in to 5ml XTT reagent. Then, 50 µl of this prepared solution was added into each well.

Following the incubation with XTT reagent for 20 hours in CO<sub>2</sub> incubator at 37°C, the dye intensity was measured at 415 nm with Bio-tek ELISA reader linked to a PC supplied with KC Junior program.

The first two rows without cells were used as blank in order to remove interferences due to extract itself and the serum at this wavelength.

The results were expressed in terms of percentage cellular viability with respect to concentrations and calculated as ;

$$\% \text{Cell Viability} = \frac{\text{Avg. OD}_{415} \text{ of treated well}_{w/\text{cell}} - \text{Avg OD}_{415} \text{ of treated well}_{w/\text{out cell}}}{\text{Avg. OD}_{415} \text{ of DMSO control well}_{w/\text{cell}} - \text{Avg OD}_{415} \text{ of DMSO control}_{w/\text{out cell}}} \times 100 \quad (2-6)$$

3 independent experiments were performed and each one was triplicate wells. According to dose-response curve drawn according to percent viability, IC<sub>50</sub> was calculated with Graphpad Prism Version5.

### 2.2.5.3 Trypan Blue Exclusion

MCF-7 and MDA-MB-231 cell lines were seeded into 24 well plates at a density of 100000 cells/ml as 1ml in each well. After incubation for 24 hours, medium was discharged, wells were washed with PBS and 500µl fresh medium was added in each well. Then, extract was diluted as described in 2.2.5.2 to be 0,5mg/ml, 1mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, 6 mg/ml, and 8 mg/ml in stock. Then 500µl of these solutions were added into wells and the plates were left for 24 or 48 hours incubation. After the incubation period, medium was discharged and the wells were washed with PBS for two times. 100µl trypsin was added in each well and incubated for 2-5 minutes in CO<sub>2</sub> incubator. When detachment was observed 400µl growth medium was added in each well and collected into eppendorf tubes. 50µl of the suspension was mixed with 50 µl trypan blue dye and after homogenous mixing, 10 µl of mixture was filled in the chambers of hemacytometer. Cells treated with 0,1% DMSO in well was used as negative control and its result was evaluated as 100% viability and other concentrations' values were calculated accordingly. % Viability versus concentration graphic (dose-response curve) was drawn and the IC<sub>50</sub> value was calculated with Graphpad Prism V5.

$$\% \text{ Cell Viability} = \frac{\text{Avg. of number of cells counted in varying extract concentration}}{\text{Avg. of number of cells counted in 0,1\% DMSO treated cells}} \times 100 \quad (2-7)$$

## 2.2.6 Gene expression analysis of quantitative RT-PCR

### 2.2.6.1 Isolation of Total RNA

5 PRIME Perfect Pure™ Purification System was used for total RNA isolations of MCF-7 and MDA-MB-231 cell lines. Isolation procedure was applied as manufacturer's specifications.



MCF-7 and MDA-MB-231 cells (500.000cells/well) were seeded in 6 well plates by applying 2ml each. Next day, medium (1ml) was refreshed and cells were treated with the IC<sub>50</sub> concentration, 0,2% DMSO and medium, and incubated for 24 or 48 hours. At the end of incubation period, medium was removed and wells were washed with PBS. Then 400µl lysis solution was added in each well and the plate incubated at room temperature by rocking gently for 5 minutes. After vigorous pipetting, 400µl lysed cells were added in to purification column. Centrifugation was done at 15000g for 1 min and purification column was transferred in to a new collection tube. 400µl Wash 1 solution was added in to purification column. Centrifugation was done at 15000g for 1 min. Purification column was transferred to a new collection tube. 50µl DNase Solution prepared before (2,6 ml DNase Buffer was added in to the lyophilized DNase I, mixed and stored at -20°C) was added into the purification column and incubated at room temperature for 15 min. After that, 200µl DNase Wash solution was added to column and centrifuged at 15000g for 1 min. Then, 200µl DNase Wash solution again was added to the purification column and centrifuged at 15000g for 2 min. Purification column was transferred to a new collection tube. 200µl Wash 2 solution was added to the same purification column. Centrifugation was done at 15000g for 1 min. 200µl Wash 2 solution was added again in the same purification column and centrifugation was done at 15000g for 2 min. Purification column was transferred into a new collection tube. 50µl Elution solution was added to the purification column and centrifugation was done at 15000g for 1 min. This time purification column was discarded. 50µl solution in collection tube was divided as 10 µl and 40µl into new collection tubes. 10µl solution was used for quantitation and visualization. 40µl isolated RNA was kept at -80C until it was used for cDNA synthesis.

### **2.2.6.2 Nanodrop analysis**

Nanodrop 2000 was used for RNA purity and concentration measurements. There are two parameters to evaluate the purity of isolated RNA which are ratio of absorbance at 260nm to 280 nm and 260nm to 230nm.

### **2.2.6.3 Qualification of RNA**

In order to show isolated RNA purity and integrity, agarose gel electrophoresis was performed.

Before starting experiment, DEPC (diethyl dicarbonate) treated water was prepared. For its preparation, 1ml DEPC and 1000ml dH<sub>2</sub>O were mixed. After overnight incubation, it was autoclaved.

10X TBE buffer (Tris / Borate / EDTA) was prepared by mixing 0,89M Tris, 0,89M Boric Acid and 20mM EDTA in 500ml DEPC water (pH=8,3).

Before electrophoresis, gel apparatus was cleaned with detergent, distilled water and ethanol, respectively.

2g agarose powder was weighed and dissolved in 100 ml 1X TBE buffer. Then, it was melted in microwave oven. After that, 1µl EtBr (0,5mg/ml) was added in it and it was poured in to gel apparatus. Gel comb was placed and agar was let to dry. When it became solid, 10µl RNA, 2µl loading dye mixture was applied into wells of gel. Also low range RNA ladder was applied in a well. Samples were run at 90V for 30 min. RNA bands were visualized under UV transilluminator ( Vilber Lourmat Infinity Capt ,France) and captured.

#### 2.2.6.4 Complementary DNA (cDNA) Preparation

After RNA isolation, complementary DNA (cDNA) was prepared prior to quantitative real time PCR experiment. RevertAid™ M—MuLV Reverse Transcriptase was used for synthesis of first strand cDNA that it is able to synthesis of cDNA up to 13kb.

2µg RNA, 1 µl oligo (dT) 18 primer was mixed in eppendorf and completed final volume 12µl with ultrapure DEPC treated water. Oligo (dT) 18 primer was chosen because it is selectively synthesizing cDNA only from poly (A) tailed mRNA. After that, the mixture was incubated at 65°C for 5 min in BioRad Thermal cycler (USA). After incubation was finished, eppendorf tube was chilled on ice. Then, 4µl 5X reaction buffer, 1µl RiboLock RNase Inhibitor (20 u/µl), 2µl 10mM dNTP mix and 1µl RevertAid M-MuLV Reverse Transcriptase (200 u/µl) was added in eppendorf tube, respectively. After that, mixture was incubated for 5 min at 25°C followed by 60min incubation at 45°C and terminated by heating at 70°C for 5 min in thermal cycler. cDNA synthesis reaction components were illustrated in Table 2-3. Reverse transcription reaction product was stored in -20°C for short storage and at -80°C for longer storages.

**Table 2-3** cDNA synthesis reaction components

Components	Amount (µl)
Template RNA	Varies to obtain 2µg RNA
Oligo (dT) 18 primer	1
5X Reaction Buffer	4
RiboLock RNase Inhibitor (20u/µl)	1
10mM dNTP Mix	2
RevertAid-M-MuLV Reverse Transcriptase (200u/µl)	1
Nuclease free water	varies
<b>Total volume</b>	<b>20</b>

#### 2.2.6.5 Primers

All primers were obtained in lyophilized form from Iontek, Turkey. Before used, primers dissolved in DEPC treated ultrapure water to obtain final concentration 100µM for each primer. Then, primers were diluted with DEPC treated ultrapure water to a concentration of 200 nm. Then primer were aliquoted and stored at -20 ° C.

#### 2.2.6.6 Quantitative RT-PCR

In order to explore the expression levels of GSTO1 and GSTZ1 genes, Real MasterMix SYBR ROX kit of 5Prime was used in quantitative real time PCR. 9µl SYBR Green-ROX, 2µl forward, 2µl reverse primer and 5 µl DEPC treated ultrapure water was mixed. Then 2µl cDNA was added into mixture (Table 2-4). qRT-PCR was performed using the instrument of Corbett Research that reaction conditions was shown in Table 2-5.

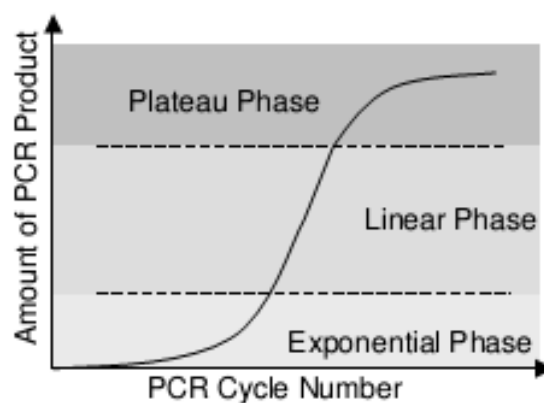
**Table 2-4** qRT - PCR components

Components	Reaction Volume (µl)
2,5X Real Master Mix SYBR ROX / 20X SYBR Solution	9
Forward Primer	2
Reverse Primer	2
cDNA	2
RNAse free water	5
<b>Total Volume</b>	<b>20µl</b>

**Table 2-5** Quantitative Real Time PCR Conditions

Analysis Mode	Cycles	Segment	Temperature °C	Time
Preincubation	1		95	5min
Amplification	40	Denaturing	94	30sec
		Annealing	53	30sec
		Extension	72	30sec
Cooling	1		30	30sec

PCR includes three phases shown in Figure 2-4. which are exponential phase in which PCR product double during each cycle, linear phase that linear increase occurs in PCR product as PCR reagents become limited and the plateau phase with no change in number of PCR product due to depleted reagents ( Yuan *et al.*, 2006).

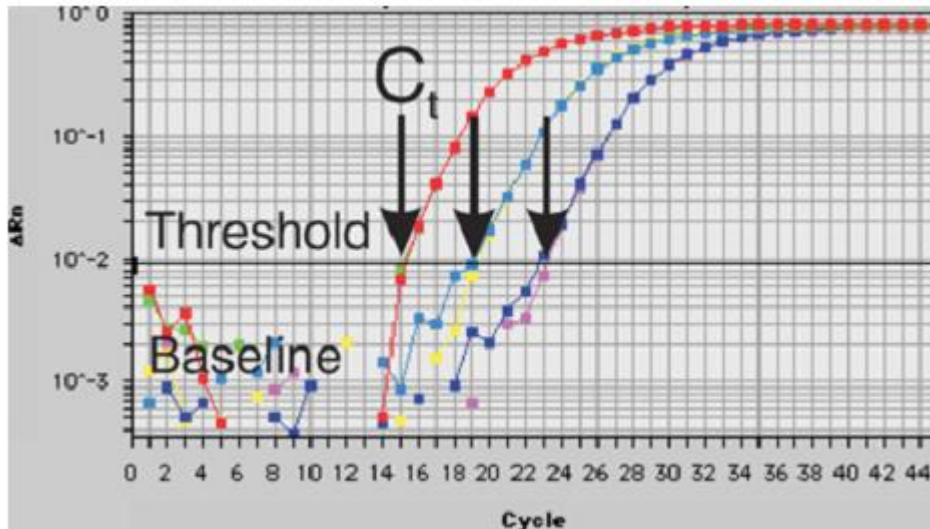


**Figure 2-5** Theoretical plot of PCR product amount versus cycle number. Three phases of qRT- PCR was pointed which were exponential phase, linear phase and plateau phase (Yuan *et al.*, 2006)

For fluorescent signal formation, SYBR Green is used most widely. When the dye in solution is found as unbound, it gives just a little fluorescence. As the number of double stranded

DNA increases, increasing amount of dye binds to these newly formed ds DNA. This is result in increase in the fluorescence signal monitored in real time

During exponential phase of the PCR cycle, fluorescent signal of the reporter dye crosses a threshold (baseline level) in the certain cycle. It is called threshold cycle ( $C_T$ ) (Figure 2-5).  $C_t$  value enables the real time quantitation of qRT-PCR. As the copy number of the target gene increases,  $C_t$  value becomes lower since fluorescent signal comes sooner as illustrated in Figure 2-5 (Bustin and Mueller, 2005).



**Figure 2-6** qRT-PCR amplification curve, plotting fluorescence signal versus cycle number (Bustin and Mueller, 2005)

**Table 2-6** cDNA Dilutions for qRT-PCR

Standard	Copy number
No dilution	$10^4$
1/10	$10^3$
1/100	$10^2$
1/1000	$10^1$

Before the experiment, serial dilutions of cDNA were done as shown in Table 2-6. The target gene and a reference gene for internal control were included in each run as triplicate tubes. As an internal control,  $\beta$ -actin housekeeping gene was used.

In order to analyze data, comparative  $C_T$  method ( $2^{-\Delta\Delta C_t}$ ) was applied for calculations. The technique is advantageous due to its simplicity and presentation of data as 'fold change'.

Comparison of gene expression of two different samples was calculated as shown in Equation 2-8.

$$\Delta\Delta Ct = [ ( Ct \text{ gene of interest} - Ct \text{ internal control} )_{\text{SAMPLE A}} - ( Ct \text{ gene of interest} - Ct \text{ internal control} )_{\text{SAMPLE B}} ]$$

(2-8)

$$\text{Comparative Fold change} = 2^{-\Delta\Delta Ct}$$

Sample A was extract treated cDNA and sample B was 0,1% DMSO treated control cDNA. In this way, the data was explicated as the expression of gene of interest relative to internal control  $\beta$ -actin in extract treated sample compared to control.

### 2.3 Statistical Analysis

All experiments were performed in triplicate unless otherwise noted. Results were expressed as mean  $\pm$  standard deviation.

The statistical significance of differences between groups were analyzed using One way ANOVA followed by Tukey's Multiple Comparison Test using the GraphPad Prism version 5 (GraphPad Software, California, USA). A statistically significant difference was considered to be at  $p < 0.01$ .

IC<sub>50</sub> values were calculated in Prism using log inhibitor versus normalized response curve with variable slopes that reported with  $p < 0,05$  significant.

## CHAPTER 3

### RESULTS AND DISCUSSION

*Salvia* species are widely distributed in Turkey with 94 species. Endemism ratio is very high in Turkey as 52%. *Salvia absconditiflora* is one of these endemic species that are distributed in Central Anatolia. *Salvia absconditiflora* leaves contains high amount of vesicles so it contains various active compounds in itself. Due to consumption of its leaves as a herbal tea, it is important to analyze its effects on human metabolism.

In this study, *Salvia absconditiflora* leaves were collected at different months as November, April, June and July. Water extraction procedure was carried out with each month collected leaves by freeze-drying small aliquots. Then, antioxidant capacity, total phenol and total flavonoid contents were determined. Finally, all the leaves of each month were gathered together and they were freeze dried after water extraction. Freeze dried extract was characterized with its antioxidant capacity, total phenol and total flavonoid content followed by its cytotoxic activity determination in MCF-7 and MDA-MB-231 cells.

#### 3.1 Extraction

Extraction is an important process for the isolation and the characterization of polyphenolic compounds. In all extraction methods, solvent extraction is one of the most commonly used techniques.

Most of the laboratories choose classic extraction techniques due to their simplicity and cheapness. There are two techniques for solvent extraction depends on biomass types, which are liquid-liquid extraction and solid-liquid extraction. Solid-liquid extraction technique is widely used for recovery of many important food constituents such as phytochemicals from plants.

Since chemical structure and interactions of polyphenols in foods are not well known, it is important to find optimum conditions to get desired compounds in the extract. Temperature, pH, liquid-solid ratio, particle size and time of extraction influence the efficiency of extraction. Polyphenols can easily be oxidized so prolong incubation can cause damages. High temperature and alkaline environment can result in degradation due to hydrolysis, internal redox reactions and polymerization. Also high temperatures can cause solvent loss (Ignat *et al.*, 2011; Dent *et al.*, 2012).

In one of the study, effects of different solvents, temperature and time on composition and total phenol content of *Salvia officinalis* has been investigated. According to their study, for extraction with water as a solvent, highest total phenol content was reached at 60°C rather than 90°C. Also 90 min incubation gave higher total phenol content instead of 30 or 60 min incubation at 60°C (Dent *et al.*, 2012).

In our experiment, *Salvia absconditiflora* air-dried leaves were extracted with distilled water at 50°C for 90 minutes in water bath in place of higher temperatures in order to prevent the damaging effects of the heat on some polyphenols. Then the extract was freeze dried as explained in 'Methods & Materials' part. The weight of freeze dried extract was recorded, and the percent yield of extraction was calculated as % (w/w).

The percent yield of extraction with distilled water after mixing each months' ( November, April, June and July) leaves of *S.abscconditiflora* was found as 17, 39 %( w/w). Unfortunately, there is no available data for comparison of yield in literature due to lack of experiments done with *S.abscconditiflora* water extract. However, there are studies conducted with other solvent types for *S.abscconditiflora*. In one of the study, it was found that methanolic extract of *S.abscconditiflora* had 18, 3% yield (Bozan *et al.*, 2002). In another study, ethanolic extract of *S.abscconditiflora* gave 21, 33% yield (Süntar *et al.*, 2011).

### 3.2 Determination of Antioxidant Capacity of *Salvia abscconditiflora*

In order to calculate antioxidant activity of compounds, DPPH is an easy and rapid assay. In various laboratories different protocols are applied which differ in initial DPPH concentration (22,5 µM-250 µM), incubation time (5- 60 min) and reaction solvent (ethanol, methanol).

The basis of the assay depends on the number of reducing DPPH molecule according to structural confirmation of flavonoids and phenolic acids. Position of phenolic hydroxyl groups, presence of double bonds or their conjugation to hydroxyl groups and ketonic groups affects the activity of the reaction.

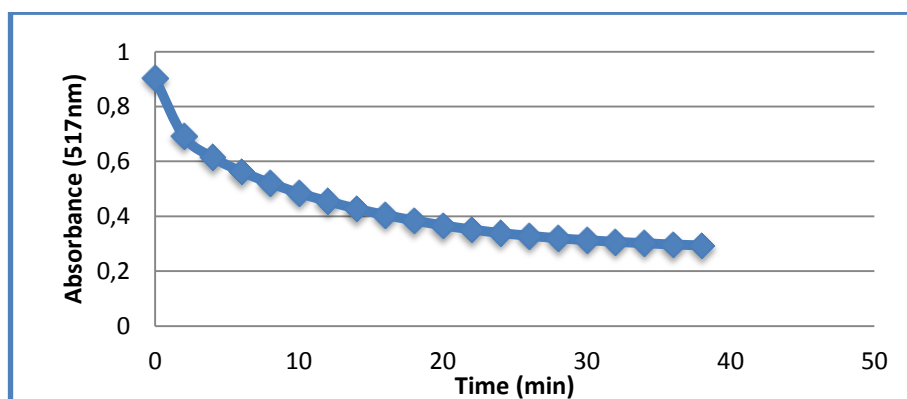
Due to these differences, it is suitable to find the optimum incubation time for extract in which reaction should be followed until reduction of DPPH has reached to plateau. When we concern solvent types, it was found that both ethanol and methanol are good solvents for assay without interfering (Sharma and Bhat, 2009; Brand-Williams *et al.*, 1995; Mishra *et al.*, 2012).

In our project, method applied by Blois (1958) was modified to evaluate DPPH radical scavenging activity of *Salvia abscconditiflora* freeze-dried water extracts. The Radical Scavenging Activity (RSA) of extract was calculated and used for determination of EC<sub>50</sub> value, which indicated the amount of the extract cause loss of 50 % of DPPH radical activity.

Antioxidant capacity of freeze-dried water extract of *S.abscconditiflora* leaves was investigated by measuring their DPPH radical scavenging activity.

Since *S.abscconditiflora* leaves were collected in 4 different months (November, April, June and July), DPPH radical scavenging activity was measured both for each months 'extracts and for mixture.

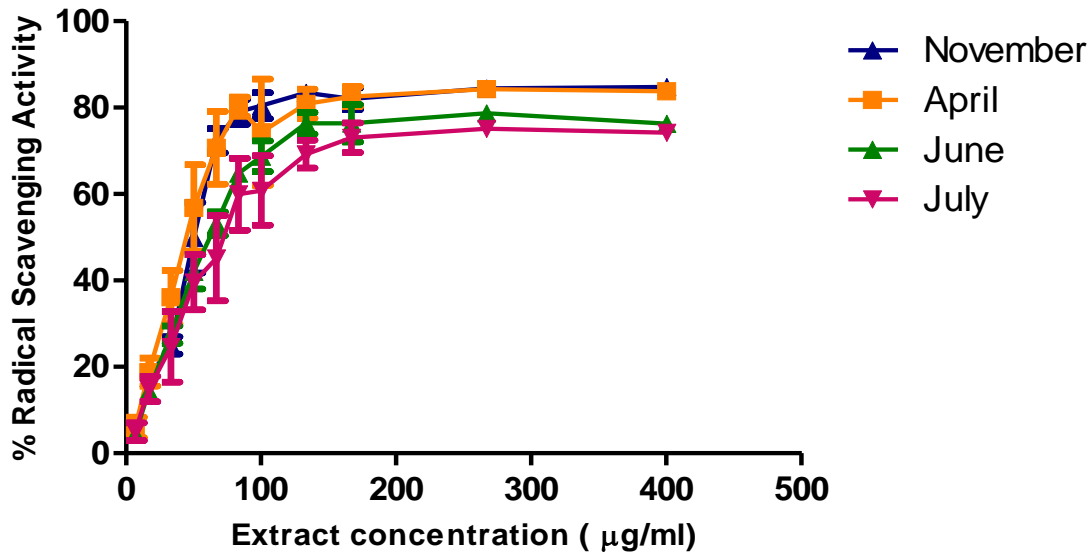
They were monitored at 517nm after 30 min incubation, which was optimized previously as shown in Figure 3-1.



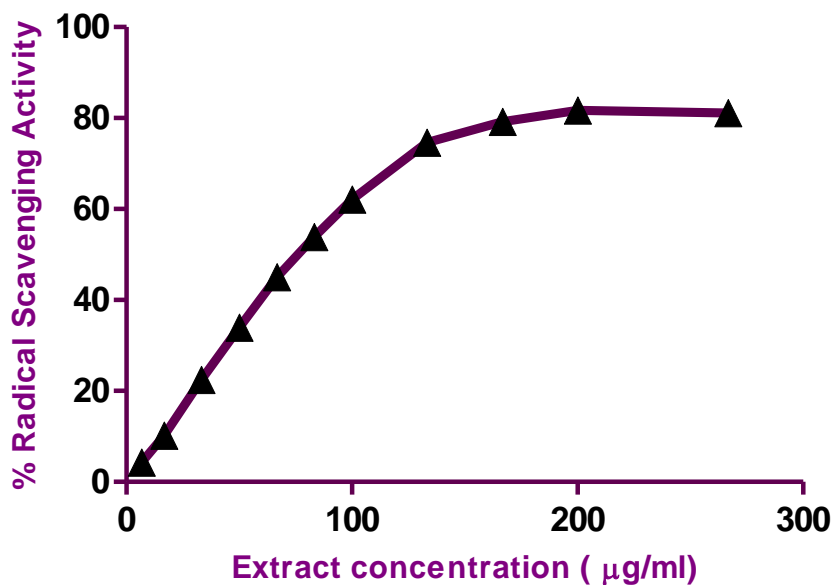
**Figure 3–1** Time optimization of DPPH. Decrease in the absorbance was recorded once per 1 min for 40min at room temperature. The final concentration of the *S.abscconditiflora* extract was 266,7 µg / ml. There was a rapid decrease of absorbance at 0–20min was followed by a slow reduction of the DPPH at 20- 40min.

Percent DPPH radical scavenging activity of extracts were carried out for different 11 final concentrations between the values 6,667-400  $\mu\text{g}$  of plant crude extract per ml for each month and 6,7-266,7  $\mu\text{g}$  / ml for mixture measurement.

The graphic for percent DPPH radical scavenging activity (%RSA) versus extract concentrations in  $\mu\text{g}$  / ml for months was plotted as shown in Figure 3-2 and for mixture as shown in Figure 3-3.



**Figure 3–2** Percent DPPH scavenging activities (% RSA) of *Salvia absconditiflora* leaves water extracts according to collected months. Each point is the mean of duplicate measurements from four different sets of experiments (n=4) (\* p<0,01 compared to and analyzed by one way ANOVA).



**Figure 3–3** Percent DPPH scavenging activities (% RSA) of *Salvia absconditiflora* leaves water extracts after mixing



Also quercetin was used as a positive control for the DPPH assay. Its final 6 concentrations were ranging from 1,7 - 66,7 µg / ml (Figure 3-4).

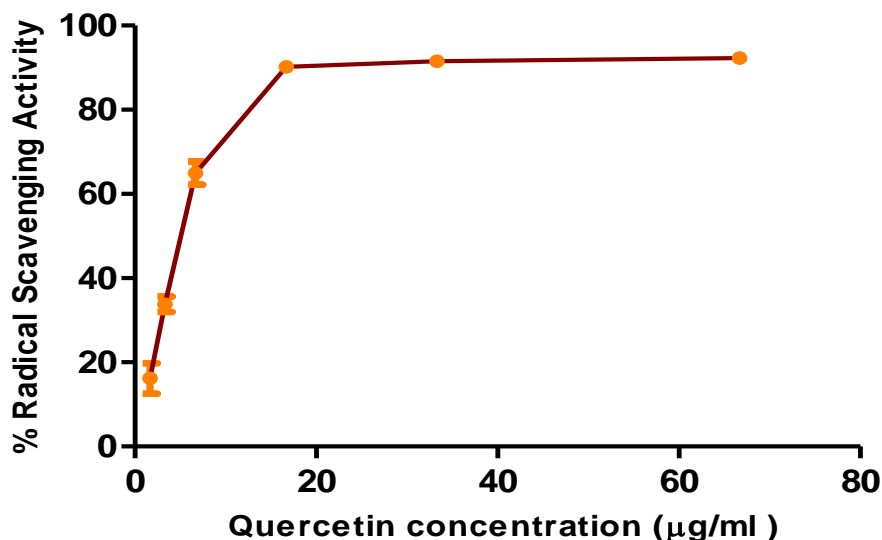


Figure 3–4 Percent DPPH scavenging activity (% RSA) of quercetin

The EC<sub>50</sub> values were determined using GraphPad Prism version 5. The results of the DPPH experiments were given in Table 3-1 which demonstrates %RSA and EC<sub>50</sub> values of *S. absconditiflora* for each month (November, April, June, and July) and for mixture.

Table 3-1 Antioxidant activity (EC<sub>50</sub> µg/ml ± SD\*) and % Radical Scavenging Activity (%RSA ± SD\*) in *S. absconditiflora* water extract

SAMPLES	Antioxidant Activity (EC <sub>50</sub> µg/ml ± SD*)	Maximum % Radical Scavenging Activity (%RSA ± SD*)
November	47,78 ± 1,054	84,493 ± 1,059
April	43,93 ± 1,058	83,076 ± 2,166
June	61,83 ± 1,036	78,708 ± 0,909
July	74,06 ± 1,052	75,163 ± 0,477
Mixed	73,78 ± 1,95	81,729 ± 0,079
Quercetin	4,784 ± 0,421	92,264 ± 0,772

\*SD was derived from four independent experiments

In Table 3-1, maximum radical scavenging activity of extracts and quercetin at the concentration range used in the experiments were also listed.

In all samples, increase in antioxidant capacities (%RSA) was observed depending on increase in concentration up to reach a plateau corresponding to a certain concentration.

When we investigate the EC<sub>50</sub> values of each month, highest DPPH radical scavenging activity was observed in April with 43,93 ± 1,058, on the other hand, the lowest radical scavenging activity was observed in July with 74,06 ± 1,052 µg/ml as shown in Table 3-1.

This difference between months can be due to stress conditions that *S. absconditiflora* come up with. In one of the study, it was found that low temperature conditions lead to increase in phenolic composition of wheat leaves ( Olenichenko *et al.*,2006). In another study radical scavenging activity of methanolic extract of *S.officinalis* aerial parts exposed to drought stress increased significantly by 17.77 and 61.48% under moderate water deficiency and severe water deficiency, respectively ( Bettaieb *et al.*,2011).

Phenolic compounds are known as antioxidant molecules. This can be the reason of the increase observed in antioxidant potential of *S.absconditiflora*.

Also when we look at the % RSA values, although there were no significantly difference between months statistically ( $p>0.01$ ), lower %RSA values were observed in June and July extracts in comparison to November and April extracts.

According to Kamatou *et al.* (2008),  $EC_{50}$  values more than 100 $\mu$ g/ml was demonstrated as poor antioxidants. Also they stated that *Salvia* species that they examined in South Africa showed DPPH radical scavenging activity ranging from 1,61 to 74,50  $\mu$ g/ml. In our experiment, *S.absconditiflora* water extract DPPH radical scavenging activity was 73,78  $\pm$ 1,95  $\mu$ g /ml after mixing which was in the range of this literature.

Süntar *et al.* (2011) evaluated the DPPH radical scavenging capacity of *S.cyanescens* and *S.absconditiflora* ethanol extracts. Both of them showed high scavenging activity with more than 80%, but *S.absconditiflora* was more active than *S.cyanescens* in lower concentrations. Also in FRAP test, other antioxidant capacity test based on ferric reducing ability of antioxidant, *S.absconditiflora* showed twice higher activity than *S.cyanescens*.

Unfortunately, there is no published data available with DPPH activity of *S.absconditiflora* water extract for comparison. In one of the study, %RSA value of *S.absconditiflora* petroleum ether extract was 74,6 % and chloroform extract was 71,1% ( Orhan *et al.*,2007). In our experiment, *Salvia absconditiflora* water extract % RSA value is 81,729 % after mixing which is higher than this literature. This may be explained with polarity differences. Petroleum ether and chloroform are non-polar solvents, and water is a polar solvent. *Salvia absconditiflora* leaves may contain more polar compounds than nonpolar compounds that lead to increase in antioxidant capacity of extract.

$EC_{50}$  value of quercetin was found as 4,784  $\pm$  0,421  $\mu$ g/ml. In the doctoral thesis conducted in our laboratory by Uyar (2011),  $EC_{50}$  value was found as 4.49 $\pm$  2.54  $\mu$ g/ml which is agree with our value.

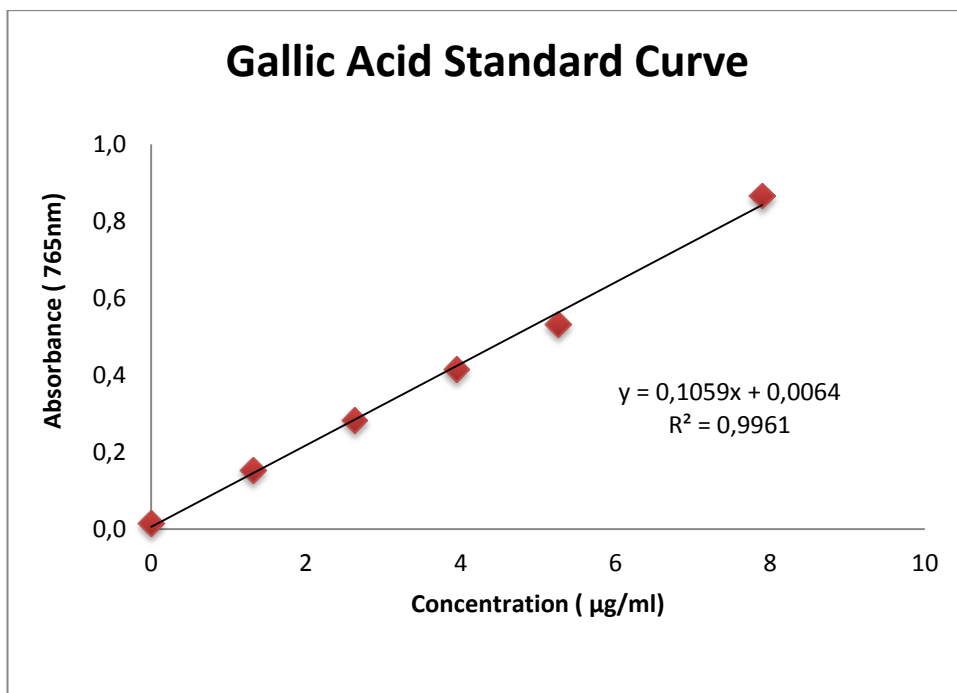
### **3.3 Determination of Total Phenolic Content of *Salvia absconditiflora***

Total phenolic contents of *S.absconditiflora* freeze-dried water extracts were determined by Folin-Ciocalteu reagent assay based on the method of Singleton and Rossi (1965). The results were shown as mg phenolic equivalents of gallic acid (GAE) in g of extract.

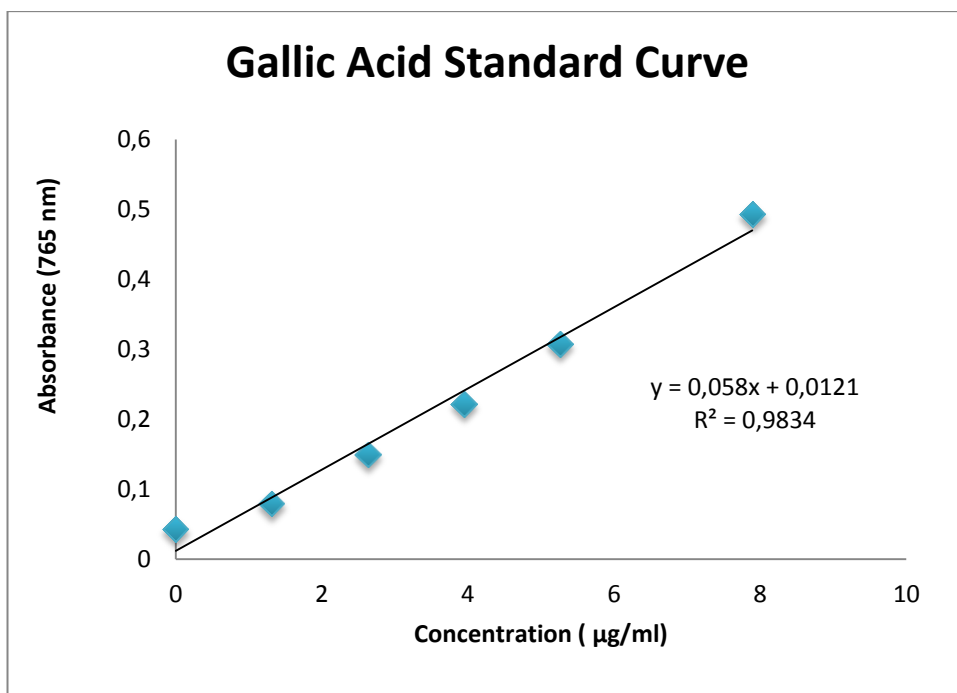
Total phenolic compounds of each months' extracts and mixture of each extracts were determined as mg equivalents of gallic acid per g of freeze-dried extracts of *S.absconditiflora*.

Absorbance values of gallic acid in different concentrations ranging from 1,315 to 7,895  $\mu$ g/ml were measured at 765 nm. From these recorded data, the final concentration vs. absorbance graph was plotted as a standard curve as in Figure 3-5 and Figure 3-6.

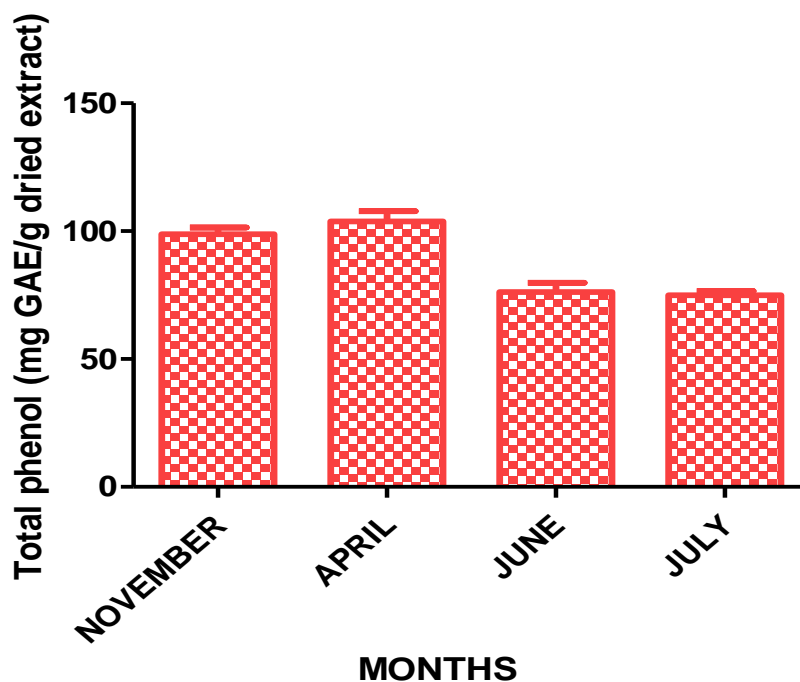
Total phenol content of extracts were calculated in 3 different concentrations in final 0,039,0,053 and 0,079 mg /ml for each month extract and 0,026, 0,039 and 0,079 mg /ml for mixture extract.



**Figure 3–5** Gallic Acid standard curve for total phenol content measurements of each months' extract



**Figure 3–6** Gallic Acid standard curve for total phenol content measurements of mixed extract



**Figure 3-7** Total phenol content of *Salvia absconditiflora* leaves water extracts according to collected months expressed as mg GAE per dried extract. (\* p<0.01 compared to and analyzed by one-way ANOVA with Tukey's Multiple Comparison Test).

When the total phenol content of each month was compared ( November, April, June, July), there was a statistically( $p>0,01$ ) difference among November and June, November and July, April and June and April and July. The highest amount of total phenol was observed in extract of April as  $103,865 \pm 3,916$  mg GAE/ g dried extract and the lowest total phenol amount was observed in July extract as  $75,118 \pm 1,793$  mg GAE/ g dried extract ( Figure 3-7). These differences between total phenol contents can be in response to biotic/abiotic stresses. In a study, total phenol content was 4,07 times higher in moderately water deficit group than control group in *Salvia officinalis* methanol extract that drought stress resulted in induction of synthesis and accumulation of polyphenols ( Bettaieb *et al.*,2011).

**Table 3-2** Total phenol contents of *S.absconditiflora* water extracts in different months( November, April, June,July) and mixture as expressed in gallic acid equivalents (GAE).

SAMPLES	Total Phenol (mg GAE/g dried extract ± SD*)
November	98,931 ± 2,605
April	103,865 ± 3,916
June	76,743 ± 3,955
July	75,118 ± 1,793
Mixed	137,408 ± 6,898

TP GAE : Total phenolic contents mg equivalents of gallic acid / g of plant extract  
 \*SD : derived from three independent experiments

After mixing, total phenol content of *S. absconditiflora* leaves freeze dried water extract became  $137,408 \pm 6,898$  mg GAE/ g dried extract which became the highest total phenol content when compared with each month. This could be the reason of collection of different types of phenols in the mixture extract that some of phenols may not be found in high amount in specific month. In the literature, total phenol content of indigenous *Salvia* species of Southern Africa was ranging in between 45 to 211 mg/ g of GAE dry sample (Kamatou *et al.*, 2008). Regarding these results, our *S. absconditiflora* water extract was in this range.

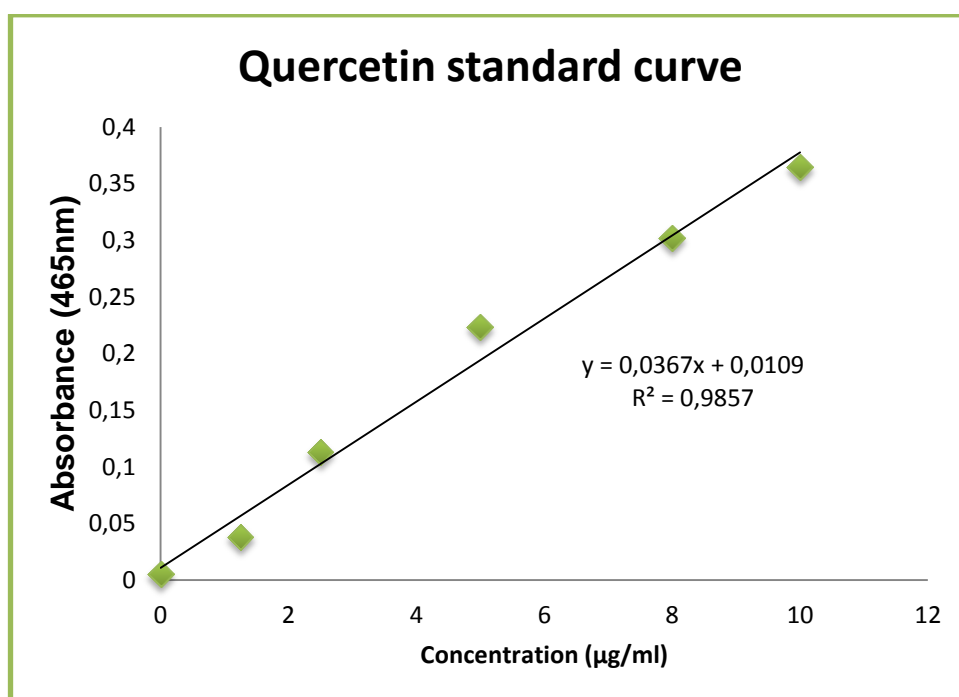
*S. absconditiflora* water extract was also showed higher total phenol content than *Salvia halophila* water extract which was  $58,46 \pm 0,44$  mg GAE/ g dried extract of aerial material (Koşar *et al.*, 2011).

Dent *et al.* (2012) found that total phenol content of *Salvia officinalis* leaves' water extract which was prepared in 60°C with 90 min incubation was  $61,68 \pm 1,20$  mg gallic acid / g dry extract. *Salvia absconditiflora* leaves water extract prepared with 90 min incubation at 50°C showed higher total phenol content than *Salvia officinalis*.

### 3.4 Determination of Total Flavonoid Content of *Salvia absconditiflora*

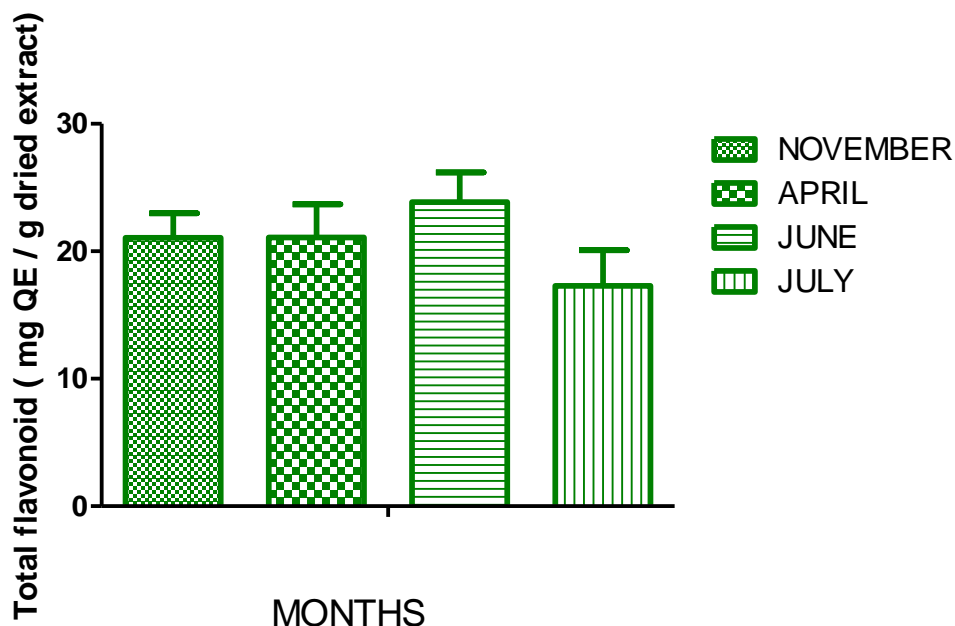
Total flavonoid content of *Salvia absconditiflora* water extract was determined by using aluminium colorimetric assay as described in Materials and Methods part.

Before mixing each months' extract, quercetin was used as a standard (Figure 3-8).



**Figure 3–6** Quercetin standard curve for total flavonoid content determination of each month ( November, April, June and July)

Total flavonoid content calculation was applied for extract in months was done with 4 different concentrations of *S. absconditiflora* water extract which were 0,05, 0,1, 0,15 and 0,2 mg/ml in final.



**Figure 3-7** Total flavonoid content of *Salvia absconditiflora* leaves water extracts according to collected months expressed as mg quercetin per dried extract. (\* p<0, 01 compared to and analyzed by one way ANOVA)

According to one way ANOVA test (p<0, 01), there was not any difference between total flavonoids content of each month of *Salvia absconditiflora* water extracts expressed as quercetin equivalent (Figure 3-9). Among months, June showed the highest and July showed the lowest total amount of flavonoid as 23,853 ± 2,336 and 17,297 ± 2,792 mg QE equivalent/ g dried extract, respectively (Table 3-3).

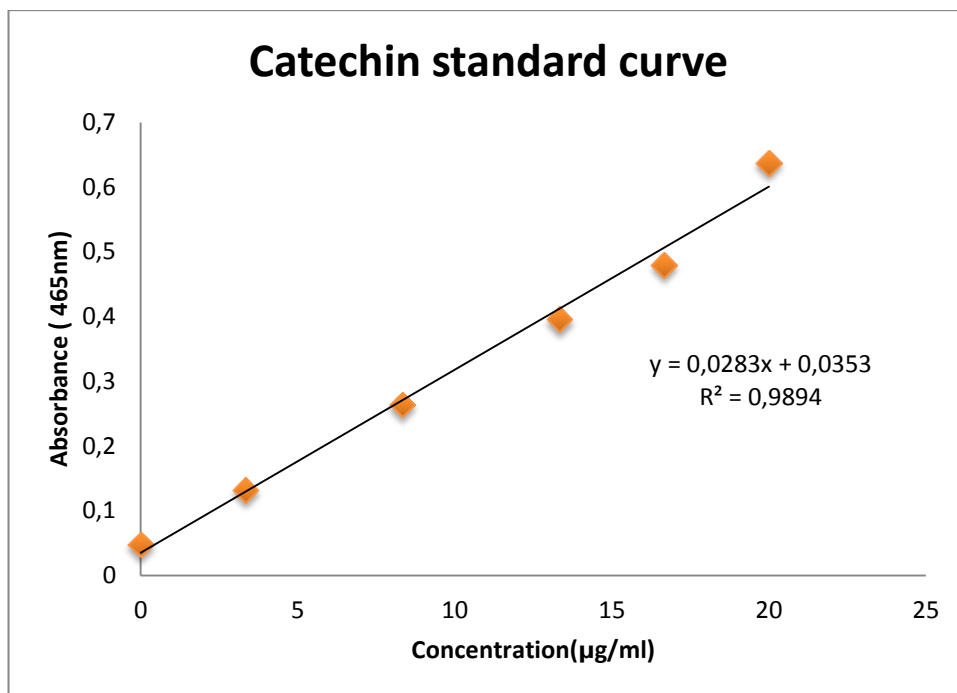
In one of the study, *Salvia miltiorrhiza* water extract had total flavonoid content 133,93±0,98 mg quercetin per gram extract which is higher than total flavonoid content of each months' water extract of *S.absconditiflora* freeze-dried leaves (Ravipoti *et al.*,2012).

**Table 3-3** Total flavonoid content of *S.absconditiflora* water extracts in different months (November, April, June,July) as expressed in quercetin equivalents

SAMPLES	Total Flavonoid (mg quercetin/g dried extract ± SD*)
November	21,066 ± 1,915
April	21,081 ± 2,607
June	23,853 ± 2,336
July	17,297 ± 2,792
Mixed	-

\*SD derived from three independent experiments

The results were too low with quercetin as a equivalent for total flavonoid assay because quercetin is a powerful flavonol. In order to understand the total flavonoid content markedly, (+) catechin was started to use as a standard after mixing (Figure 3-10) and total flavonoid content was expressed as mg of catechin equivalents (CE) per gram of *Salvia absconditiflora* freeze-dried water extract.



**Figure 3–8** Catechin standard curve for total flavonoid content determination of mixed extract

Total flavonoid content of the mixed extract was calculated in 2 different concentrations as 0,067 and 0,133mg/ml of extract.

The flavonoid content of *S.abscconditiflora* leaves' water extract was 66,923±1,432 mg CAE per gram dried *S.abscconditiflora* extract (Table 3-4).

**Table 3-4** General view of antioxidant activity, total phenol and total flavonoid content of *S.abscconditiflora* water extract , GAE: Gallic Acid Equivalents

SAMPLES	Antioxidant Activity (EC <sub>50</sub> µg/ml± SD)	Maximum % Radical Scavenging Activity (%RSA± SD)	Total Phenol (mg GAE/g dried extract± SD)	Total Flavonoid (mg quercetin/g dried extract± SD)
November	47,78 ± 1,054	84,493 ±1,059	98,931 ± 2,605	21,066 ± 1,915
April	43,93 ± 1,058	83,076 ±2,166	103,865 ± 3,916	21,081 ± 2,607
June	61,83 ± 1,036	78,708 ±0,909	76,743 ± 3,955	23,853 ± 2,336
July	74,06 ± 1,052	75,163 ±0,477	75,118 ±1,793	17,297 ± 2,792
Mixed	73,78 ± 1,95	81,729 ±0,079	137,408 ± 6,898	<b>66,923 ± 1,432*</b>
Quercetin	4,784 ± 0,421	92,264 ±0,772	-	-

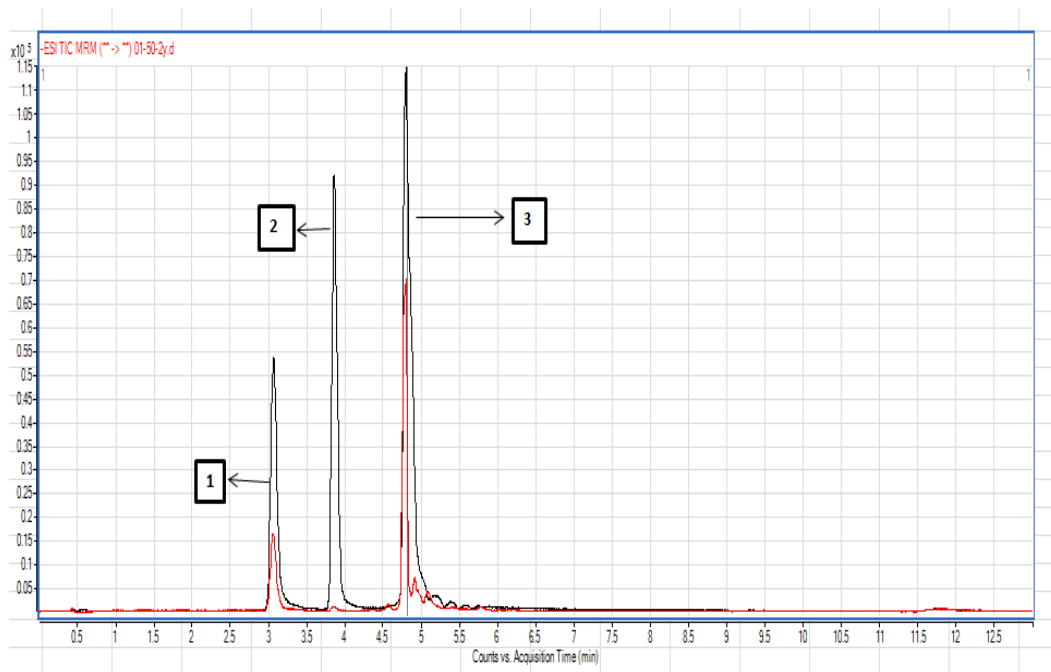
\*mg catechin per gram dried *S.abscconditiflora* extract

As a result of all these experiments correlation between antioxidant capacity, total phenol and total flavonoid content was observed among each months' extract. As the phenol content increased, the antioxidant capacity of the extract was also increased. When mixed extract was examined, it was shown that flavonoids constituted a significant amount in phenol content which was correlated with LC-MS/MS result.

### 3.5 LC-MS/MS Analysis

The technique combines both physical separation ability of HPLC and mass differentiation capacity of mass spectrometry. It is highly sensitive and suitable for detection of phenolic compounds in extracts.

For the experiment, internal standards were used in the experiment in order to obtain reliable results which had identical chemical properties with the compounds that would be analyzed. The LC-MS/MS result of *S.abscconditiflora* extract for 4 selected compounds were plotted as abundance versus mass to charge ratio shown in Figure 3-11.



**Figure 3–9** LC-MS/MS profile of *S.abscconditiflora* water extract. Black lines show 10ppm standard and red lines show the sample. Peak assignment : 1; Caffeic acid , 2; Coumaric Acid, 3; Luteolin and Rutin

Caffeic acid, coumaric acid, luteolin and rutin was chosen to investigate according to literature( Lu and Foo, 2002).

**Table 3-5** LC-MS/MS results of *S.abscconditiflora* extract as caffeic acid, coumaric acid, luteolin and rutin content

Caffeic Acid (ppm)	Coumaric Acid (ppm)	Luteolin (ppm)	Rutin (ppm)
121,03 ± 7,39	2,2359 ± 0,0122	236,73 ± 0,95	195,06 ± 4,55

Caffeic acid and its derivatives constitute the major water soluble phenolic acids of genus *Salvia*. Caffeic acid is found in an important position in the Lamiaceae family that metabolites of *Salvia* consist of caffeic acid. Caffeic acid also has antioxidant and anti-inflammatory properties. Caffeic acid amount was 121,03 ppm in *S.abscconditiflora* extract. In one of the literature, caffeic acid content was calculated according to HPLC experiment as 118,5 ppm



for *Salvia aethiopsis* and 118,8ppm for *Salvia officinalis* which was similar to our results. However there were also other *Salvia* species that showed higher or lesser amount of caffeic acid amount when compared with *S.abscoditiflora* ( Coisin *et al.*, 2012).

Coumaric acid is another phenolic acid found in *Salvia*. However its amount is less than caffeic acid and its derivatives (Lu and Foo, 2002). According to LC-MS-MS data, it was found as 2,2359 ppm. When compared with caffeic acid amount, this was too low as expected.

Luteolin, 3',4',5,7-tetrahydroxyflavone, is one of the most abundant flavonoid within *Salvia* species. Luteolin shows anti-inflammatory, anti-allergy and anti-cancer properties ( Lin, 2008; Lu and Foo, 2002). In the LC-MS/MS experiment, luteolin content of *S.abscoditiflora* water extract was found as 236,73 ppm. Coisin *et al.*( 2012) found that *Salvia protensis* and *Salvia ringens* contained similar luteolin contents as 114,6 and 132,4 ppm, respectively. On the other hand, *S.fruticosa* luteolin content was found as 335,6 ppm in another study analyzed with HPLC, which was higher than our result (Askun *et al.*, 2009).

Rutin is a flavonoid glycoside that compose of flavonol quercetin and the disaccharide rutinose. It is found in many of the medicinal plants such as *Salvia* species ( Sofic *et al.*, 2010). Rutin has anti-inflammatory, antioxidant as well as anticancer properties. Khalifa *et al.*(1983) found that rutin had the ability to decrease the permeability and fragility of blood vessels and could be classified as vitamin P. *S.abscoditiflora* rutin content was 195,06 ppm. In one of the study, rutin was a prominent compound in *S.tomentosa* with 866,9 ppm, on the other hand, it was not detected in *S.fruticosa* (Askun *et al.*,2009).

### 3.6 Cytotoxicity of *Salvia abscoditiflora* in MCF-7 and MDA-MB-231 Cell Lines

#### 3.6.1 XTT Assay

Effects of *S.abscoditiflora* water extract on metabolically active MCF-7 and MDA-MB-231 cells were detected after 24 or 48 hour incubation with extract using XTT colorimetric assay

XTT is a tetrazolium salt. Succinate dehydrogenase enzymes of mitochondria of metabolically active cells reduce this tetrazolium salt into an orange colored formazan product .The absorbance of this formazan product proportional to number of active cells was measured using ELISA plate reader.

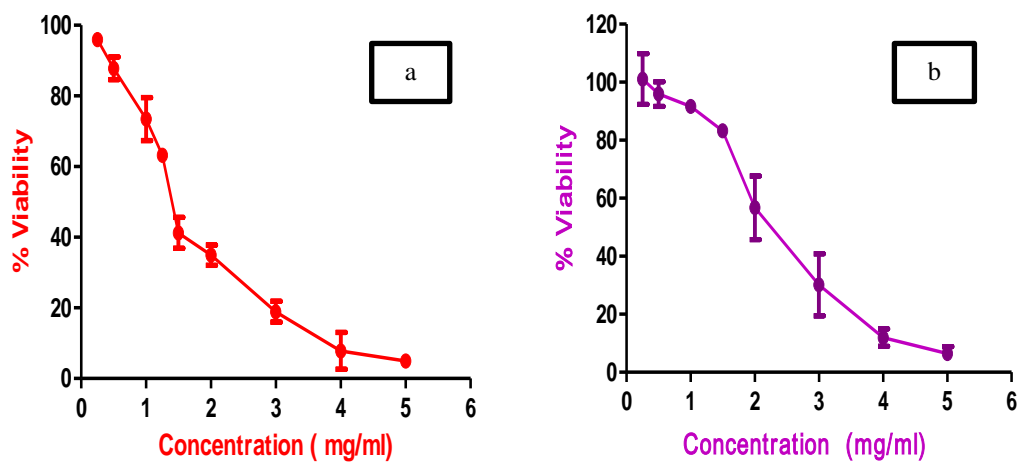
In the experiment, *S.abscoditiflora* water extracts were dissolved in 0,2% DMSO. DMSO works as a vehicle that it increases the permeability of cells and facilitates the entrance of phenolic compounds in cells. Final DMSO concentration in all wells became 0,1%.

Averages of triplicate measured absorbance values in wells treated with a defined concentration of extract was calculated for 24 and 48 hours. Absorbance values obtained in different extract concentrations were converted into percentage viability by setting 0,1% DMSO treated wells' absorbance value as 100%.

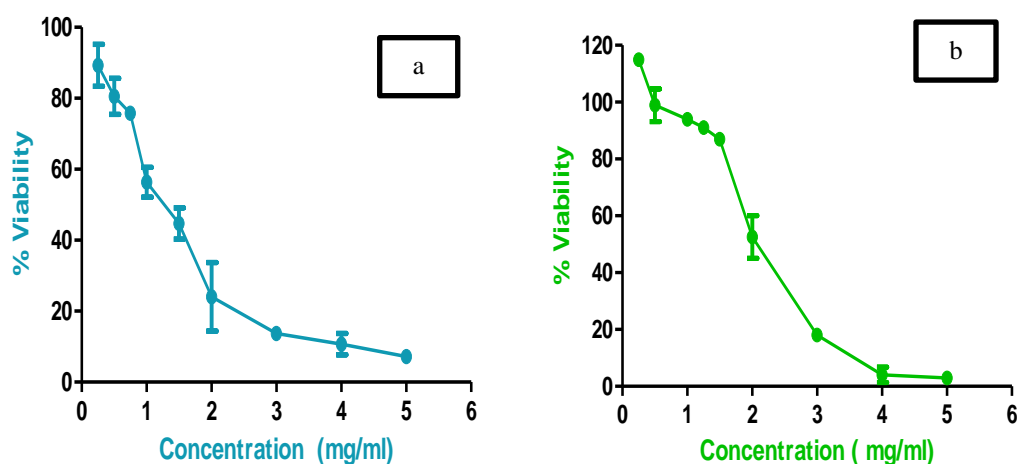
There were concerns about the usage of XTT technique in herbal extracts. It was indicated that cytotoxicity tests with tetrazolium salts give false-negative results since extracts reduce tetrazolium salts also in the absence of living cells. In addition to this, different antioxidant molecules containing free thiol groups were found to be able to reduce tetrazolium salts. In another study, serum in the growth medium could induce the formation of formazan products in a dose dependent manner ( Shoemaker,2004 ; Talorete *et al.*,2006).

In order to prevent these interferences, control wells without cells were included in the XTT measurements and absorbance of average of blank wells ( cells without extract) were subtracted from sample wells to observe direct action of the *S.abscoditiflora* water extract on MCF-7 and MDA-MB-231 cells.

Percent (%) viability versus concentrations graphic was plotted as in Figure 3-12 and Figure 3-13. and IC<sub>50</sub> values were determined by using GraphPad Prism Version 5.



**Figure 3-10** Effects of *S. absconditiflora* leaves water extract on MCF-7 cells after 24 and 48 hour treatments. a:24 hours treatment, b:48 hours treatment



**Figure 3-11** Effects of *S. absconditiflora* leaves water extract on MDA-MB-231 cells after 24 and 48 hour treatments. a: 24 hours treatment, b: 48 hours treatment

IC<sub>50</sub> values were calculated in treated MCF-7 cells as  $1,558 \pm 0,251$  and  $2,18 \pm 0,355$ mg/ml for 24 and 48 hours, respectively. These results showed that IC<sub>50</sub> value increased as incubation time changes from 24h to 48h.

IC<sub>50</sub> values were calculated as  $1,131 \pm 0,079$  and  $2,1 \pm 0,09$  mg/ml for 24 and 48h, respectively in treated MDA-MB-231 cells. These results showed that IC<sub>50</sub> values also increased in MDA-MB-231 cells (Table 3-6).

**Table 3-6** IC<sub>50</sub> values and % viability (Viability at highest extract concentration) obtained by XTT assay

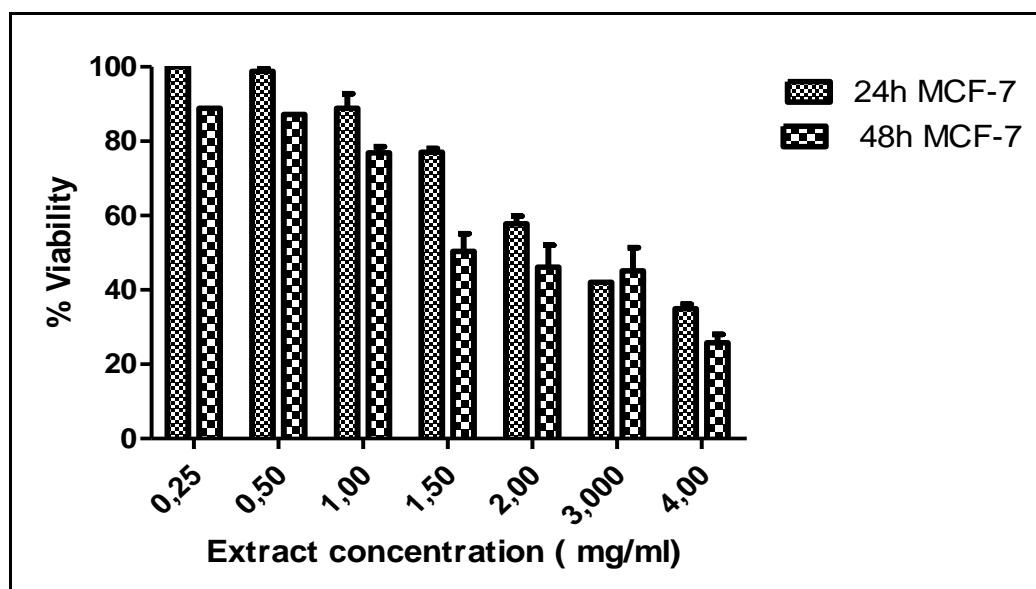
Cell Lines	Incubation Time (h)			
	24hr		48hr	
	IC50 (mg/ml)	% Viability	IC50 (mg/ml)	% Viability
<b>MCF-7</b>	$1,558 \pm 0,251$	$4,942 \pm 0,548$	$2,18 \pm 0,355$	$6,492 \pm 2,3564$
<b>MDA-MB-231</b>	$1,131 \pm 0,079$	$7,230 \pm 1,336$	$2,1 \pm 0,09$	$2,926 \pm 0,369$

### 3.6.2 Trypan Blue Dye Exclusion Assay

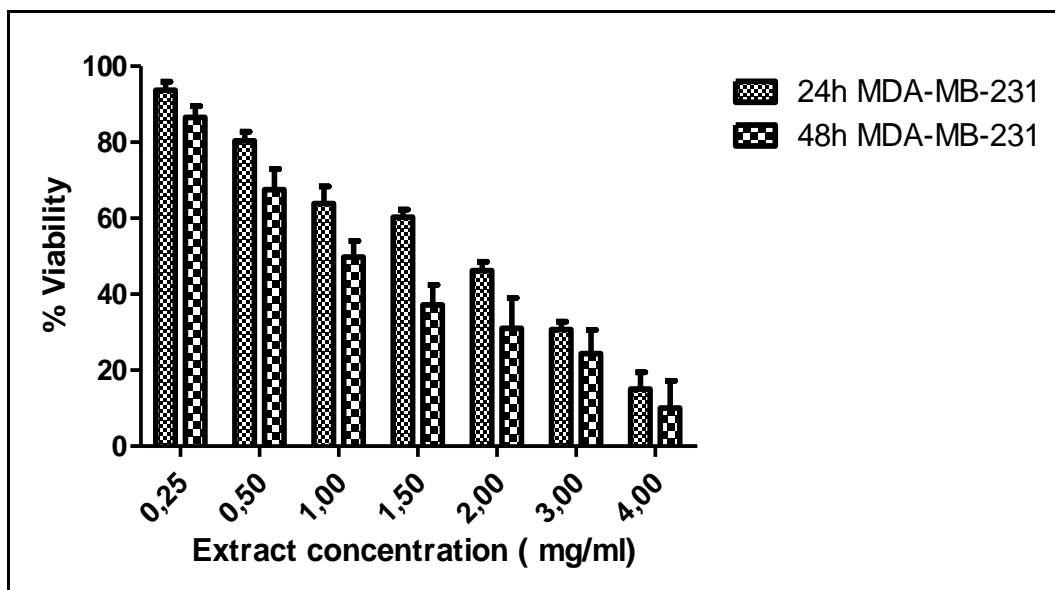
Trypan Blue Exclusion Assay is one of the principle cell culture technique for cell counting. The basis of the technique is the entrance of the trypan dye into dead cells.

The effects of different *S.absconditiflora* extract concentrations ranging from 0,25 to 4 mg/ml on the cell viability of MCF-7 and MDA-MB-231 cells were analyzed at two different time points ( 24 and 48 h).

Cells that were grown in 0,1% DMSO containing medium was used as a control. Averages of countings in different concentrations of extract were converted into percent viability by setting control(0,1% DMSO) counts as 100% ( Figure 3-14 and 3-15).



**Figure 3–12** Effects of *S.absconditiflora* leaves water extract on cell survival in MCF-7 cells by the TBE assay. MCF-7 cells were precultured in 24-well plates for overnight and then incubated with 0,25 – 4 mg/ml of extract.



**Figure 3-13** Effects of *S. absconditiflora* leaves water extract on cell survival in MDA-MB-231 cells by the TBE assay. MDA-MB-231 cells were precultured in 24-well plates for overnight and then incubated with 0,25 - 4mg/ml of extract.

*S. absconditiflora* water extract concentrations at which 50% of cells are alive ( $IC_{50}$ ) were calculated for MCF-7 cells as  $2,635 \pm 0,048$  and  $1,982 \pm 0,284$  mg/ml after 24 and 48-hour incubations, respectively.

$IC_{50}$  values were calculated as  $1,626 \pm 0,073$  and  $1,009 \pm 0,162$  mg/ml for 24 and 48 hours, respectively, in MDA-MB-231 cells treated with varying concentrations of *S. absconditiflora* extract (Table 3-7).

**Table 3-7**  $IC_{50}$  values obtained by TBE Assay.

Cell Lines	Incubation Time (h)	
	24hr	48hr
	$IC_{50}$ (mg/ml)	$IC_{50}$ (mg/ml)
<b>MCF-7</b>	$2,635 \pm 0,048$	$1,982 \pm 0,284$
<b>MDA-MB-231</b>	$1,626 \pm 0,073$	$1,009 \pm 0,162$

### 3.6.2.1 Evaluation of XTT and TBE results

When we compare  $IC_{50}$  values between XTT and TBE assays, the values were not correlated with each other. However, comparison is not necessarily an important parameter for assays' reliability. Even a perfect correlation does not mean the assay results are the same (Ulukaya, 2008).

Both TBE and XTT  $IC_{50}$  values could be analyzed together other than comparison to get a conclusion.

**Table 3-8** IC<sub>50</sub> values in MCF-7 and MDA-MB-231 cells obtained by XTT and TBE Assay after treatment of *S.abscconditiflora* extract for 24h and 48h.

Cell Lines	IC <sub>50</sub> (mg/ml) ( XTT)		IC <sub>50</sub> (mg/ml) ( TBE)	
	Incubation Time		Incubation Time	
	24h	48h	24h	48h
<b>MCF-7</b>	1,558 ± 0,251	2,18 ± 0,355	2,635 ± 0,048	1,982 ± 0,284
<b>MDA-MB-231</b>	1,131 ± 0,079	2,10 ± 0,09	1,626 ± 0,073	1,009 ± 0,162

There were differences in IC<sub>50</sub> values between XTT and TBE assay results both for 24 and 48h incubation in MDA-MB-231 cells. While increase in IC<sub>50</sub> values for XTT assay was observed, decrease in IC<sub>50</sub> values for TBE assay was observed with increase in incubation of plates with extract. Same results between XTT and TBE assay could be accomplished if the wash out step before trypsinization in TBE assay was not applied.

MDA-MB-231 cells were detached in the prolong presence of *S.abscconditiflora* extract and these non-attached cells were thrown away during experiment. As a result, they could not be counted although they were viable, so IC<sub>50</sub> value was decreased. As the time pass, detachment ratio was increased and difference in IC<sub>50</sub> values between two assays were increased.

It is conceivable that, as long as extract is found in its environment, MDA-MB-231 cells could not find themselves a place to attach and so cannot be fed and die. Therefore, the results can be explained as cancer preventive ability of *S.abscconditiflora* on MDA-MB-231 cells come from its anti-metastatic property .

It was stated that adhesion molecules are important for progression of invasiveness and metastasis. Tanshinone1 isolated from *Salvia miltiorrhiza* root was shown to reduce expression of ICAM-1 and VCAM-1 which are molecules involved in cell to cell and cell to extracellular matrix (ECM) interactions and are necessary for metastasis ( Nizamutdinova *et al.*, 2008).

As a future study, the expression levels of ICAM and VCAM can be analyzed in the presence of *S.abscconditiflora* water extract in order to show the molecular basis of this detachment.

MCF-7 cells gave different respond toward *S.abscconditiflora* extract compared with MDA-MB-231 cells. According to TBE assay, number of MCF-7 cells decreased as incubation time changed from 24 to 48 h whereas, their metabolic activity was increased as shown in XTT assay. Remaining MCF-7 cells started fighting with extract by increasing their aggressiveness through increase in their metabolic activity. Their resistance toward extract was proved while TBE experiment was performed. In detachment of MCF-7 cells with trypsin application, higher incubation time was required when compared with MDA-MB-231 cells.

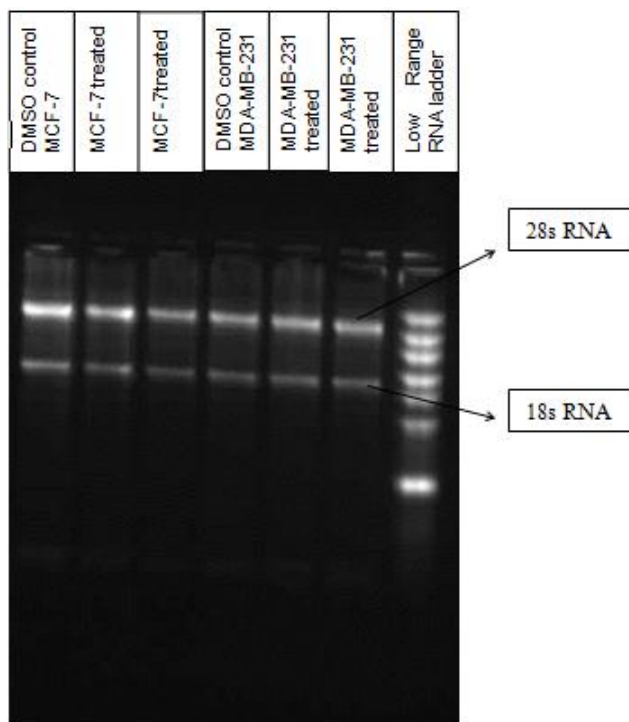
It is indicated that local extracellular matrix environment (ECM) of breast cancer can protect cancer cells against apoptosis by providing a survival signal or blocking a death signal. Resistance of MCF-7 cells can be attributed to induced integrin affinity or by clustering integrin which generates increased adhesive avidity in response to *S.abscconditiflora* extract (Brakebusch *et al.*, 2002).

### 3.7 Modulation of the Levels of GSTO1 and GSTZ1 Enzymes by *S.absoconditiflora* in MCF-7 and MDA-MB-231 Cells

#### 3.7.1 Qualification of RNA by Agarose Gel Electrophoresis

In order to check quality and intactness of isolated total RNA, agarose gel electrophoresis was performed as described in Materials and Methods part. 3 subunits of RNA (28s, 18s and 5s) without DNA contamination should be observed. Ratio of the intensity of RNA bands between 28s and 18s also should be 2:1.

We were able to observe 28s large ribosomal subunit and 18s ribosomal small unit RNA without 5s RNA. It was enough to check the intactness of RNA. If RNA was contaminated with Rnase, we could not see any of the bands (Figure 3-16).



**Figure 3–14** Total RNA isolated from MCF-7 and MDA-MB-231 cells which were treated with 0, 1% DMSO or with cytotoxic concentration ( $IC_{50}$ ).

#### 3.7.2 Determination of RNA Purity and Concentration

260/280 value should be 2 for 'pure' RNA. Lower values indicate contaminations. 260/230 value is another measurement to show nucleic acid purity. It should be in the range of 2.0-2.2. Isolation of total RNA without contamination is an important parameter for further RT-PCR experiments.

**Table 3-9** Results of nanodrop measurements of isolated total RNAs of MCF-7 and MDA-MB-231 cells

<b>MCF-7</b>	<b>[RNA] ng/µl</b>	<b>260/280</b>	<b>260/230</b>
Treated	2586,15	2,09	2,13
0,1%DMSO control	2566	2,08	2,15
<b>MDA-MB-231</b>			
Treated cells	1238,67	2,09	2,12
0,1%DMSO control	812,83	2,07	2,2

### 3.7.3 Expression Analysis of GSTO1 and GSTZ1 enzymes

Cytosolic GSTs are the largest family in Glutathione-S- Transferases family and they have 8 isoenzymes including alpha, mu, omega, pi, theta and zeta classes. These isoenzymes shows extensive substrate specificity and their expression patterns change in different tissues.

GSTs play an important role in detoxification of numbers of drugs used in cancer treatment such as cyclophosphamide, busulfan and cisplatin. GSTs also catalyze many other drugs such as antifungal drugs (Terbinafine), antiulcer drugs (Pantoprazole) or diuretic drugs (Spironolactone). GSTs act on reduction of these drugs reactivity so facilitate their elimination. Therefore, changes in GST activity can significantly affect the biological and pharmacokinetic activity of these drugs.

Moreover, overexpression of GST in tumor cells can lead to resistance toward chemotherapy drugs that result in survival of cancer cells (Bousova and Skalova, 2012).

Scharmach *et al.* (2009) stated that among GST isoenzymes, GSTO1 and GSTZ1 showed highest baseline expression levels in MCF-7 cells.

In order to receive MCF-7 and MDA-MB-231 cell lines response toward *S.abscconditiflora* water extract definitely, GSTO1 and GSTZ1 enzymes were chosen among GSTs to observe changes in expression levels.

GST omega 1 is an enzyme that has unusual properties when compared with other GSTs. It has a cysteine residue in its active site although other GSTs have tyrosine or serine residues. In addition, GSTO1 has an extra N-terminal tag of 19 amino acids. GSTO1 participates in protein glutathionylation reactions by catalyzing thioltransferase reactions. Moreover, it has antioxidant defense property with its dehydroascorbate reductase activity. It shows MMA (monomethylarsonic) and DMA (dimethylarsonic) reductase activity in arsenic metabolism. GSTO1 participates in inflammatory mediator interleukin-1 $\beta$  activation and ryanodine receptor calcium release channels alteration. Polymorphism in GSTO1 enzyme could be related to hepatocellular carcinoma, cholangiocarcinoma and breast cancer. GSTO1 enzyme overexpressed cells show resistance toward chemotherapeutic drug cisplatin, which prevents anticancer treatment (Piaggi *et al.*, 2010).

GST zeta 1, a novel gene that was discovered via human EST ( Expressed Sequence Tag) database, is expressed in many tissues of the body and has high degree of sequence conservation. It involves in the oxygenation of dichloroacetic acid (DCA) to glyoxylic acid. DCA is found in drinking water and carcinogenic. On the other hand, it is important in management of congenital lactic acidosis due to its ability in mitochondrial pyruvate dehydrogenase stimulation. GSTZ1 also catalyzes t-butyl and cumene hydroperoxides with its glutathione peroxidase activity. Biotransformations of dihaloacetic acids, fluoroacetic

acid, 2-halopropanoic acid and 2,2-dichloropropanoic acid were catalyzed by glutathione dependent GST zeta 1. GSTZ1 enzyme is identical with maleylacetoacetate isomerase (MAAI). Both of them are responsible for the glutathione dependent cis-trans isomerization of maleylacetoacetate to fumarylacetoacetate which is the second to last step in phenylalanine and tyrosine degradation pathways. Deficiency in this pathway can lead to alcaptonuria, phenylketonuria, and tyrosinemia (Board, 1997; Board *et al.*, 2001; Board and Anders, 2005; Blackburn *et al.*, 2006).

For qRT-PCR experiment, total RNA was isolated from MCF-7 and MDA-MB-231 cells as control and treated. Gene expressions in 0,1% DMSO treated cells were used as controls and the fold change was accepted as 1. IC<sub>50</sub> values calculated for 24h in XTT experiment was applied on cells as treatment.

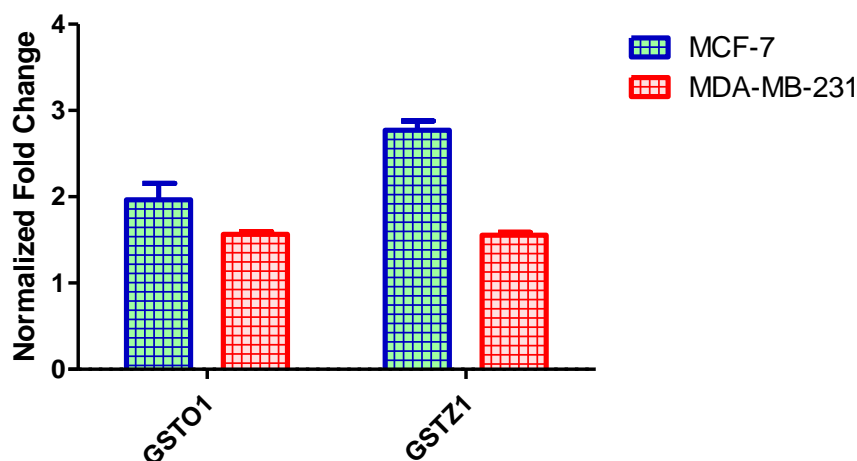
**Table 3-10** Effects of *S.abscconditiflora* on GSTO1 and GSTZ1 expression in MCF-7 and MDA-MB-231 cells

Genes / Cell lines	MCF-7	MDA-MB-231
GSTO1	↑ 2,0	↑ 1,57
GSTZ1	↑ 2,8	↑ 1,56

In MDA-MB-231 cells, GSTO1 and GSTZ1 expressions were induced as 1,57 and 1,56 fold , respectively . These were not significant changes that both of them were lower than 2 fold.

In MCF-7 cells, 2 fold increase for GSTO1 gene and 2,8 fold increase for GSTZ1 gene were observed. The response of MCF-7 cells was slightly higher than MDA-MB-231 cells toward GSTO1 and GSTZ1 genes. This could be the consequence of increased metabolic activity in MCF-7 cells to fight with *S.abscconditiflora* extract.

Transcriptional mechanisms of GST induction are very complicated to understand due to presence of lots of cross talks and repressors. As a result, much weaker induction can be observed in GST than CYP. Also, Bousova and Skalova (2012) stated that there are differences among GST isoforms toward inducers since they encounter slightly different mechanisms for induction.



**Figure 3-15** Expressions of GSTO1 and GSTZ1 genes in MCF-7 and MDA-MB-231 cells upon treatment with IC<sub>50</sub> concentrations of *S.abscconditiflora* extract for 24 hours. The values are the average of triplicate measurements from two biological replicates.



## CHAPTER 4

### CONCLUSIONS

Generally, consumption of herbs rich in polyphenols was recommended for their antioxidant ability especially during chemotherapy in order to protect cells against oxidative stress. Herbs containing phenols and flavonoids have impact on GST genes as induction or inhibition of their expression. Since most of the chemotherapeutic agents and many other types of drugs are metabolized by GSTs as Phase II detoxification enzymes, this induction or inhibition should not be bypassed that uncontrolled intake may threaten human health via affecting drug metabolism. Hence, it is important to investigate the potential effects of herbs on GST enzymes before consumption.

In this thesis work, *S.absconditiflora* leaves water extract were analyzed for the first time in order to show its influence on GST gene expressions on breast cancer cell lines. *S.absconditiflora* species grows only in Turkey and its leaves are consumed as herbal tea. Our analysis was showed that, the extract did not have an important effect on GST expressions.

*Salvia absconditiflora* water extract was also characterized in terms of antioxidant and polyphenol content, as well as the growth inhibitory effect on MCF-7 and MDA-MB-231 cells. *S.absconditiflora* water extract inhibited breast cancer cells in a dose dependent manner.

Consequently, this thesis study is a preliminary work to show the effects of drinking *S.absconditiflora* tea. As a future study, investigations can be performed in healthy breast cancer cells. In addition to this, changes in other drug metabolizing enzymes and drug transporter enzymes expressions could be analyzed to get a clear conclusion

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