ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF *SALVIA ABSCONDITIFLORA* AND EFFECTS ON CYP1A1, CYP1B1 GENE EXPRESSIONS IN BREAST CANCER CELL LINES

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ABSTRACT

ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF *SALVIA ABSCONDITIFLORA* AND EFFECTS ON CYP1A1, CYP1B1 GENE EXPRESSIONS IN BREAST CANCER CELL LINES

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Salvia genus is a widely cultivated genus and used in medicine for various purposes as having antimicrobial, antioxidant, anticarcinogen and anti-inflammatory features. In this study the aim was to investigate phenolic composition of *Salvia absconditiflora* and understand the possible effects of those constituents in cancer related drug metabolizing enzymes. Salvia absconditiflora showed 80,43 % Radical Scavenging Activity against DPPH radical. Total flavonoid content was found as one third of total phenolic content. Presence of important phenolic acids and flavonoids such as caffeic acid, luteolin, coumaric acid are validated with LC-MS/MS analysis. Cytotoxicity of *Salvia absconditiflora* treatment on MCF-7 and MDA-MB-231 breast cancer cell lines were investigated through XTT and TBE assays both dose and time dependent manner. Cell proliferation was inhibited 50 % by different IC_{50} values calculated in different assays and different time intervals. This suggests that two breast cancer cell lines response in a different way to cytotoxic treatments. Cancer related drug metabolizing enzyme gene modulations were investigated with qRT-PCR. CYP1A1 and CYP1B1 were up-regulated in MCF-7 but down-regulated in MDA-MB-231 cells in response to *Salvia absconditiflora* treatment.

Keywords: *Salvia absconditiflora*, polyphenols, cytotoxicity, antioxidant, CYP1A1, CYP1B1

ÖZ

SALVIA ABSCONDITIFLORA'NIN ANTİOKSİDAN VE SİTOTOKSİK ÖZELLİKLERİ VE MEME KANSERİ HÜCRE HATLARINDA CYP1A1 VE CYP1B1 GEN İFADELERİ ÜZERİNE ETKİLERİ

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Salvia yaygın olarak yetiştirilen ve antimikrobiyal, antioksidan, antikarsinojen ve antiinflamatuvar özellikleri sebebiyle tıpta çeşitli amaçlar için kullanılan bir türdür. Bu çalışmanın amacı *Salvia absconditiflora*'nın fenolik birleşimini araştırmak ve bu bileşenlerin kanser bağlantılı ilaç metabolize eden enzimler üzerindeki etkisini anlamaktır. *Salvia absconditiflora* DPPH radikaline karşı % 80,43 radikal sönümleme aktivitesi göstermiştir. Toplam flavonoid içeriği toplam fenolik içeriğinin üçte biri olarak bulunmuştur. Kafeik asit, luteolin, kumarik asit gibi bazı önemli fenolik asit ve flavonoidlerin varlığı LC-MS/MS analizi ile tasdik edilmiştir. XTT ve TBE metodları ile *Salvia absconditiflora*'nın MCF-7 ve MDA-MB-231 meme kanseri hücre hatları üzerindeki doza ve zamanı bağlı sitotoksik etkisi araştırılmıştır. Farklı metodlar sonucu hücre çoğalmasının % 50 engellenmesine sebep olan IC_{50} konsantrasyonu için değişken değerler elde edilmiştir. Bu verilerden iki farklı meme kanseri hücre hattının sitotoksik muameleye farklı yollarla cevap verdiği saptanmıştır. Kanserle bağlantılı ilaç metabolize eden enzimlerin gen ifadeleri kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile incelenmiştir. *Salvia absconditiflora* muamelesi sonucu MCF-7 hücrelerinde CYP1A1 ve CYP1B1 genlerinin ifadelerinin arttığı, MDA-MB-231 hücrelerinde ise azaldığı gözlenmiştir.

Anahtar Kelimeler: *Salvia absconditiflora*, polifenoller, sitotoksite, antioksidan, CYP1A1, CYP1B1

To my family,
To Prof. Dr. Mesude İşcan

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LIST OF ABBREVIATIONS

Abs: Absorbance
AhR: Aryl hydrocarbon receptor
ATCC: American type culture collection
B[a]p: Benzo[a]pyrene
cDNA: complementary DNA
CYP: Cytochromo P450 Monooxygenase
DEPC: Diethylpyrocarbonate
dH_2O: distilled water
DMSO: Dimethyl sulphoxide
DPPH: 2,2-diphenyl-1-picrylhydrazy
E_2: Estradiol
EDTA: Ethylenediaminetetraacetic acid
ER:Estrogen receptor
EROD: Ethoxyresorufin-O-deethylase
FBS: Fetal bovine serum
HAA:Heterocyclic aromatic amine/amide
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her-2: Human Epithelial Growth Factor Receptor 2
LC-MS/MS : Liquid chromatography mass spectrometry
PAH: Polycyclic aromatic hydrocarbon
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
qRT-PCR: quantitative real time polymerase chain reaction
RPMI: Roswell Park Memorial Institute Medium
RSA: Radical scavenging activity
SNP: Single nucleotide polymorphism
TBE: Tryphan Blue Exclusion
TBE buffer: Tris borate EDTA buffer
TCDD: 2,3,7,8-Tetrachlorodibenzodioxin
UP dH_2O: Ultrapure water
XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER 1

INTRODUCTION

1.1 *Salvia* genus

Salvia genus (Lamiaceae) includes about 900 species throughout the world and has 95 species in Turkey, which are divided into seven sections as *Eusphace* Benth., *Hymenosphace* Benth., *Aethiopis* Benth., *Plethiosphace* Benth., *Horminum* (Moench) Dumort *Drymosphace* Benth. and *Hemisphace* Benth. (Sener, 2011). Fourty five of the *Salvia* species are endemic to Turkey. Optimum growth condition for *Salvia* is well drained soil and full sun (Kamatau, 2008). The flowering months are May and June for almost all *Salvia* species.

The name *Salvia* is derived from the Latin word “salvere” which means “to heal” corresponding to the curing properties of the herb (Grieve, 1984).

It is shown that different *Salvia* species have many healing properties for various medicinal conditions such as diarrhoea, flu, urticaria, febrile attacks, tuberculosis, liver diseases and stomach problems (Watt and Breyer-Brandwijk, 1962; Clebsch, 2003; Van Wyk and Wink, 2004; Amabeoku et al., 2001).

According to in vitro studies, *Salvia* extracts have antimicrobial, anticancer, antioxidant and antiinflammatory effects (Kamatou et al. 2008,2010). Various *Salvia* species are shown to be beneficial for treatment of even more complicated diseases like coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea and neuroasthenic insomnia (Li, 1998).

1.1.1 *Salvia absconditiflora*

Taxon: *Salvia absconditiflora*
Taxonomic Hierarchy:
Kingdom: Plantae
Subkingdom : Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass : Asteridae
Order : Lamiales
Family: Lamiaceae
Genus : *Salvia*
Species : *Salvia absconditiflora*
Common names: kara ot, kara sabla, kara salva, kara sapla

Figure 1.1 *Salvia absconditiflora*

Salvia absconditiflora is an endemic perennial plant that grows on hillside and uncultivated following lands. The taxon is distributed mainly in middle Anatolian area of Turkey, in Afyonkarahisar, Ankara, Çorum, Erzincan, Kayseri, Konya, Niğde, Ordu, Sivas region (Figure 1.2) (Turkish Plants Data Service).

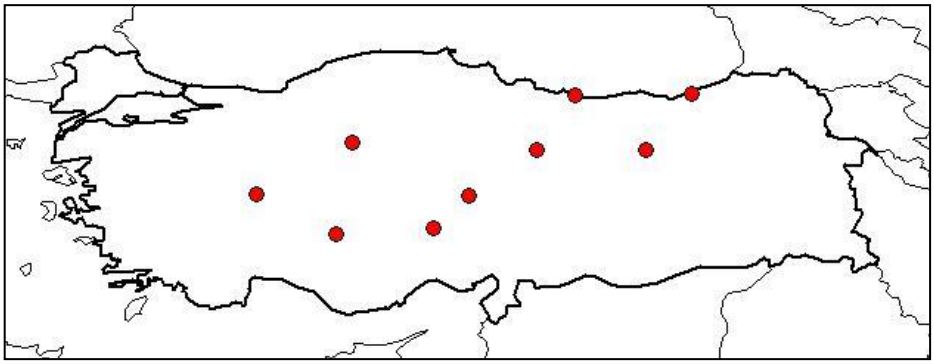


Figure 1.2 Distribution of the Taxon *Salvia absconditiflora* over Turkey
http://turkherb.ibu.edu.tr/index.php?sayfa=1&tax_id=8076

1.2 Antioxidant Compounds in Plants

1.2.1 Phenolic Compounds

Phenolics are organic compounds, consisting of a hydroxyl group and an aromatic hydrocarbon ring. Natural polyphenols exist either as simple phenol molecules, or highly polymerized forms such as tannins. Usually they are found as conjugated to a sugar residue. Also, they can be conjugated with carboxylic and organic acids, amines, and lipids or associated with other phenols (Bravo 1998).

According to Harborne, there are 8000 phenolic structures. They are produced by two different pathways which are shikimate pathway and the acetate pathway (Figure 1.3).

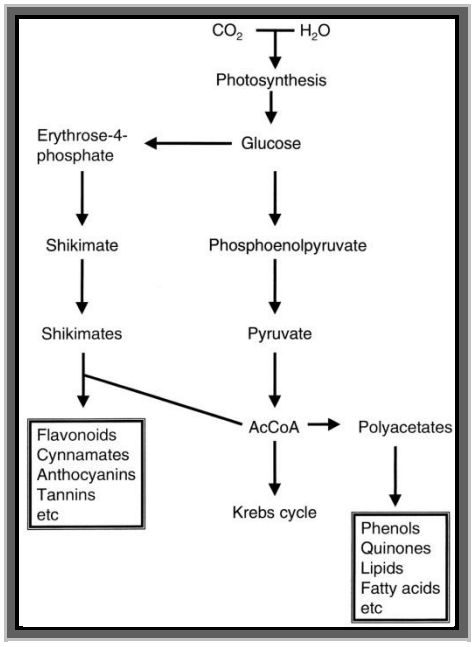
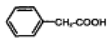
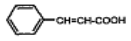
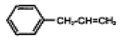
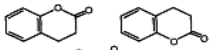
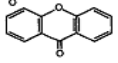
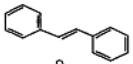
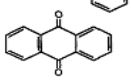


Figure 1.3 Two main pathways for production of phenolic compounds: Shikimate and Acetate Pathway.

Due to their chemical structure polyphenols can be divided into 10 classes (Table 1.1) (Harborne, 1989).

Table 1.1 Main Classes of Polyphenolic Compounds

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	$(C_6$-$C_3)_n$	

Polyphenol is defined by Haslam and colleagues as generally, moderately water soluble compunds, with molecular weight of 500-4000 Da, containing more than 12 phenolic hydroxyl groups, having 5-7 aromatic rings per 1000 Da (Haslam, 1988).

Polyphenols, the most abundant secondary metabolites in plants are characteristic chemical defenses against predators. Because of their ability to make complex structures with proteins due to their chemical structure, as they contain multiple potential binding sites provided by phenolic groups and aryl rings present on molecule, they have a harsh taste and they produce dryness and roughness in the mouth, and in addition they can also easily make complex with polysachharide structures of the cellular matrix. This feature of polyphenols provides an antiherbivore and antipathogenic activity for most of the plants (Feeny 1970, Haslam 1988).

Since herbal plants are being consumed regularly in human diet, dietary polyphenols play many important roles in human life. They are observed to be effective in human health, such as in the treatment and prevention of cancer, cardiovascular diseases, and other pathologies (Yang et al. 2001, Visioli, Borsani and Galli 2000).

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

1.2.2 Free Radicals

A free radical is a chemically reactive molecule that has an unpaired electron. They are produced in living systems as a result of normal metabolic activities such as autooxidation, enzymatic oxidation or respiration or they are generated in the organism when subjected to exogenous sources such as ionizing radiation, drugs which can undergo redox cycling, or xenobiotics that can create reactive species in situ (Freeman and Crapo 1982).

When a covalent bond between two atoms or molecules is broken, newly formed atoms remain with one unpaired electron, which makes them free radicals. Free radicals turn a surrounding molecule into a new free radical by stealing an electron. Then, this newly formed radical tries to return to its ground state by stealing electrons from cellular structures or molecules. This leads to free radical chain reactions (Halliwell, 1985).
Free radicals are named as reactive oxygen species, if they involve oxygen. Inner mitochondrial membrane is the place for electron transport chain, which uses oxygen to produce energy in the ATP form. During these reactions electron escape occurs frequently, leading to formation of highly damaging reactive oxygen species (ROS). Most common types of reactive oxygen species are superoxide anion (O_2-), hydroxyl radical (OH.), singlet oxygen ($_1O_2$) and hydrogen peroxide (H_2O_2) (Halliwell, 1985).

Free radicals play beneficial role in human body, in killing the bacteria by phagocytosis or in cell signalling and signal transduction (Saran et al. 1999, Cadenas 2004). Besides, they have harmful effect because they destroy the oxidative status of the organism which leads to several health problems.

1.2.3 Antioxidant Activity of Polyphenols

An antioxidant is any substance that protects the molecules from oxidative damage by giving up its own electron to free radicals. To improve the free radical scavenging activity and reduce the damage caused by free radicals, aerobic organisms evolved antioxidant defense systems. There are both enzymatic and nonenzymatic antioxidants. Nonenzymatic antioxidants include phenolic acids, ascorbic acid, uric acid, vitamin E, vitamin A, selenium, thiols, glutathione, carotenes and melatonin. Superoxide dismutase, catalase and glutathione peroxidase enzymes are the members of enzymatic antioxidant systems (Fridovich 1974).

Polyphenols are good antioxidants. They can scavenge free radicals and also chelate metal ions, decreasing the pro-oxidant activity.

1.2.4 Polyphenolics of *Salvia*

Salvia plants are good sources of phenolic acids. The phenolic acid derivative variety and amount can differ between different Salvia species and also between different plant parts and extracts.

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

Caffeic acid and its dimer form rosmarinic acid are the major ones in *Salvia*, which are important for their biochemistry as being building block of many plant metabolites (Gerhardt, 1983). Caffeic acid oligomers also have significance in therapeutic point of view. Phenolic compounds have many biological activities such as antioxidant, antiplatelet, antitumor and antiviral activity (Lu and Foo 2002).

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

1.2.5 Quantification of Polyphenols

Phenolic compounds can be analyzed after extraction from the plant source. Solubility of phenolic compounds depend on the solvent system used, degree of polymerization of phenolics and interferences of other compounds which may form insoluble complexes. So there is no proper procedure which is used for extraction of all phenols or a specific group of phenolic compounds.Thus phenolic extracts are always mixtures of different types of phenols which are soluble in the solvent system used. Some of the solvent systems used for extraction of phenolics are methanol, ethanol, acetone, water, ethyl acetate and propanol, dimethylformamide, and their combinations (Naczk and Shahidi 2004).

Several chromatographic and spectroscopic methods are developed for quantification of phenols. They can be separated and quantified by gas chromatography or high performance liquid chromatography techniques. Spectroscopic assays are based on the determination of various

structural groups present on phenols. Folin-Ciocalteu assay is commonly used for determination of total phenolic content which is based on reduction of phosphomolybdic-phosphotungstic acid reagent to a blue colored compound by phenolic compounds in an alkaline solution (Folin, Karsner and Denis 1912). While being a main technique for quantification because of its simplicity and low cost, it can unfortunately only estimate the total content of phenols. For quantification of proanthocyanidins, vanillin method is used. Total flavonoid content can be measured by $AlCl_3$ assay proposed by Zhishen et al. (Zhishen, 1999).

1.3 Cancer

Cancer is a term, used for a group of a various diseases in which cells have lost division control and lead to abnormal cell growth. The reasons for a cell to become cancerous are genetic alterations which are responsible for the regulation of cell division and cell death. Oncogenes and tumor suppressor genes are regulatory genes that play important role in cancer. A mutation in proto-oncogene, which is a normal gene found in many organisms including humans, taking part in signal transduction and execution of mitotic signals, may turn it into a tumor inducing agent by elevating the expression levels of oncogenes and their protein products (Todd and Wong 1999). On the other hand, tumor suppressor genes are the genes that prevent a cell to progress to cancer. They code for proteins that regulate cell cycle, repair DNA damage or promote apoptosis. Mutations in those genes increase the risk of cancer (Figure 1.4).

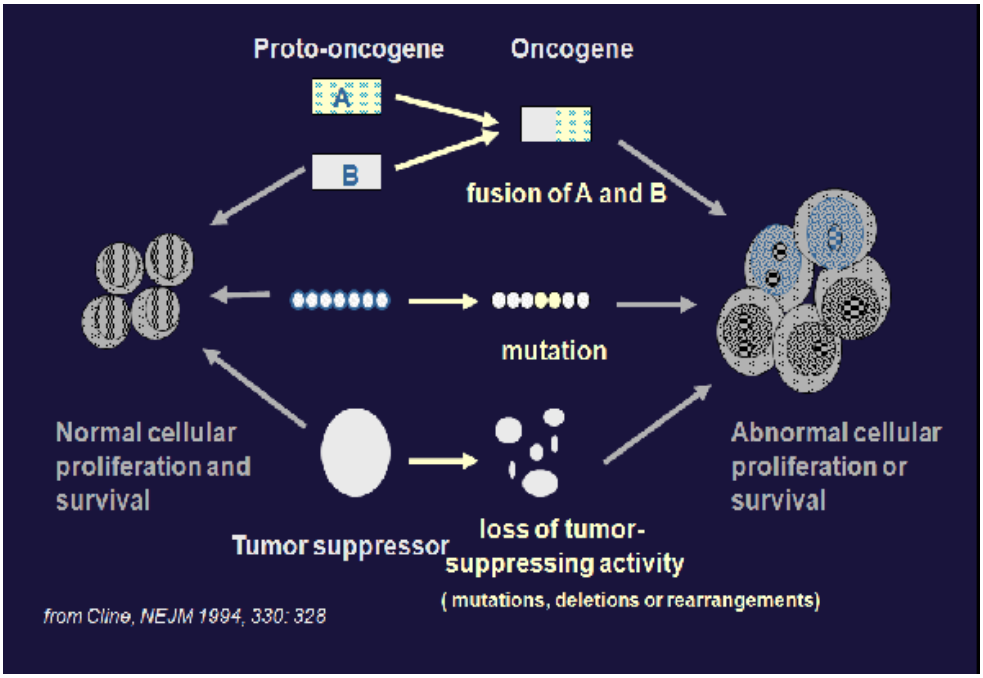


Figure 1.4 Oncogenes and Tumor Supressor Genes

Accumulation of mass of tissue constituted from uncontrolled cell divisions form tumor. Benign tumors are not cancerous, do not spread to other parts of the body and often can be removed without harm. On the contrary malignant tumors are cancerous; they invade nearby tissues, travel through blood and metastasize to other organs.

Cancer development process called carcinogenesis has 3 stages consisting of initiation, promotion and progression. Initiation involves damage to DNA, chromosome or to the epigenome that regulates the gene expression. Oxidative stress is the major driving force for initiation. Promotion is a long term stage of cellular growth of genomically unstable cells assisted by inflammation. During the progression stage, while multiplying, cells add more damage to their genome, evolving themselves into an invasive malignant tumor (Poirier 1987).

There are different types of cancer, but nearly all have similar abnormal physiology that shows malignant growth (Hanahan and Weinberg 2000). Self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evading apoptosis are the acquired capabilities of cancer (Figure 1.5).

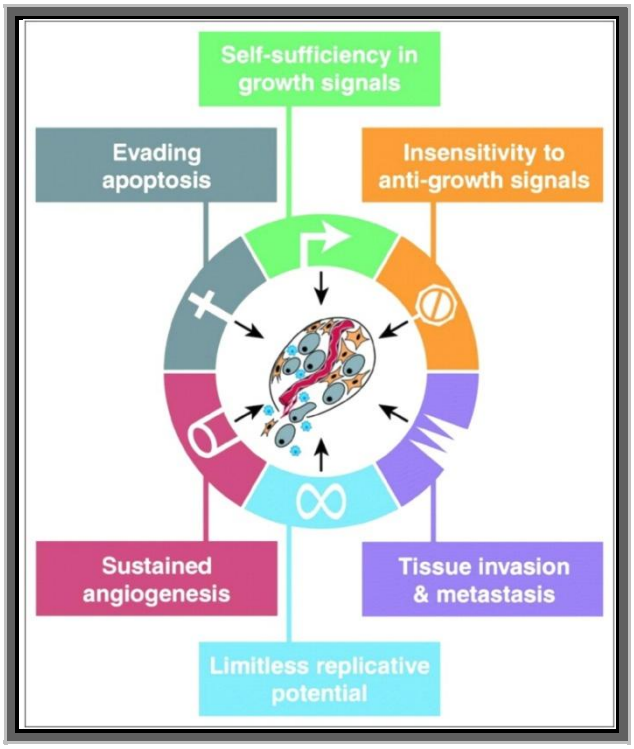


Figure 1.5 Acquired Capabilities of Cancer

Cancer may be hereditary, may pass between generations with inherited DNA damage, or can be acquired with environmental factors. Risk factors are smoking, ultraviolet radiation, ionizing radiation, diet, nutrition, alcohol, chemicals, hormone replacement therapy and life style.

1.3.1 Breast Cancer

Cancer is the second reason for death among other diseases worldwide. According to incidence rates prostate cancer and breast cancer are the two major cancers. In Turkey, in 2011 the recorded number of breast cancer patients was approximately 50.000, reflecting an increased in each year. Risk factors of breast cancer in Turkish women are age, late menopause, nulliparity and family history (http://www.tbccm.org/2011/05/current-state-of-breast-cancer-and-infrastructure-in-turkey/).

Breast cancer originates from the inner lining of milk ducts or the lobules of breast tissue. Rapid uncontrolled cell division is followed by spread from breast to lymph nodes or other organs. Invasive ductal carcinoma is malignant and abnormal proliferation of neoplastic cells in breast tissue. Ductal carcinoma in situ is the most common type in which noninvasive but potentially malignant cancer cells multiple in milk ducts. Lobular carcinoma is a form of tumor which primarily affects the lobules of gland.

17B-estradiol (E_2), the main estrogen in breast tissue plays an important role in the development of breast cancer. E_2 can cause breast cancer via 2 pathways as being a substrate for phase I drug metabolizing enzymes and as being a ligand for estrogen receptor (Figure 1.6). Oxidation metabolites of E_2 can cause DNA damage whereas E_2 itself stimulates cell proliferation and gene expression through estrogen receptor (Figure 1.7) (Parl et al. 2009).

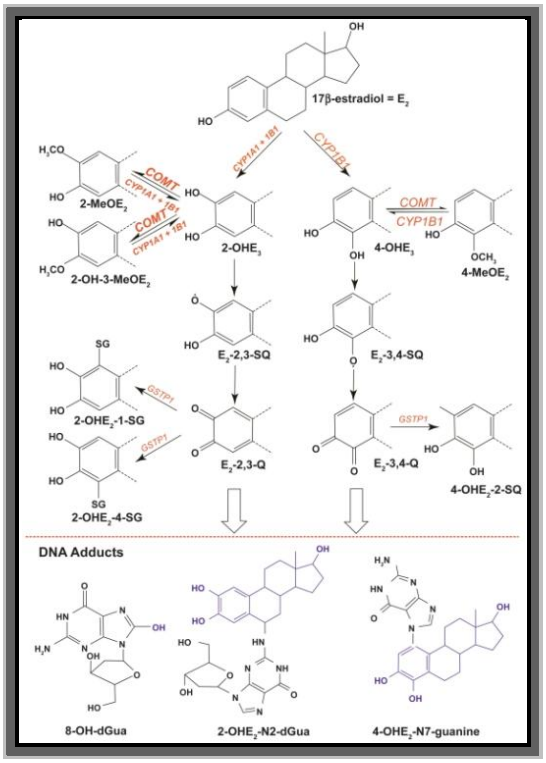


Figure 1.6 Oxidative estrogen metabolism causes DNA adduct formation.

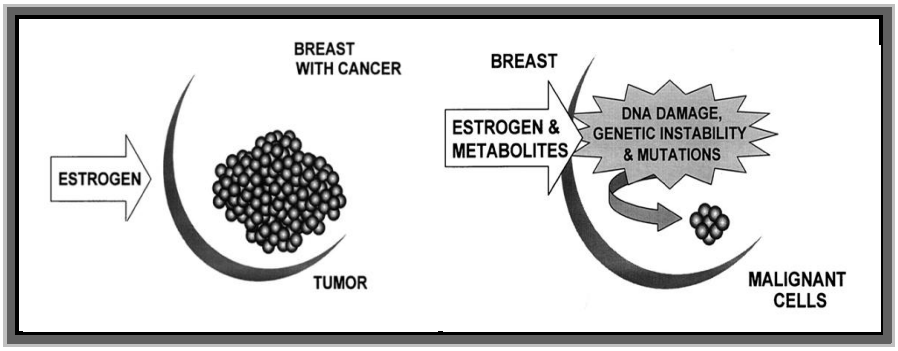


Figure 1.7 Initiation and Promotion of Breast Cancer by Estrogen

1.3.2 MCF-7 and MDA-MB-231 cell lines

MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman. The epithelial cells were isolated from adenocarcinoma. MCF-7 cell line is commonly used in breast cancer studies since it has retained some of the ideal characteristics particular to mammary epithelium. The most important feature is to have estrogen receptors. Another property of MCF-7 cell line is that cells are sensitive to cytokeratin. When they are cultured in vitro, the cell line forms domes and grow in monolayers (Figure 1.8).

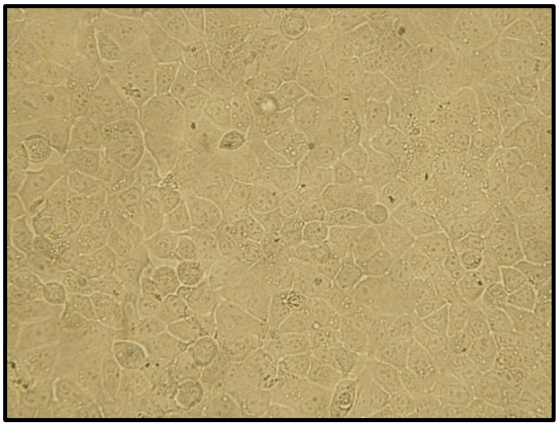


Figure 1.8 MCF-7 cell line

MDA-MB-231 breast cancer cell line was first obtained from a 51-year-old Caucasian woman patient in 1973 at M. D. Anderson Cancer Center. The epithelial cells were isolated from adenocarcinoma of breast mammary gland. They appear as spindle shaped cells. They lack estrogen receptor and Her-2 receptor (Human epidermal growth factor receptor 2), which makes them invulnerable to hormonal therapy. The cells have invasive phenotype, they are aggressive and metastatic (Figure 1.9).

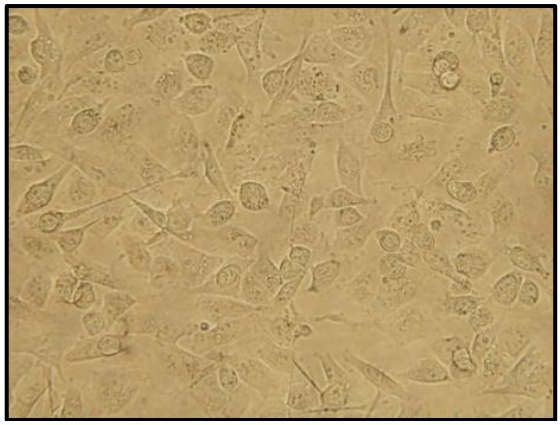


Figure 1.9 MDA-MB-231 cell line

1.3.3 Medicinal Plants and Cancer Chemoprevention

Great achievements have been made in treatment and prevention of cancer but still there are significant deficiencies and more improvement is remained. Chemotherapy is a useful treatment but has adverse side effects. Plant derived products for cancer treatment may reduce undesired side effects. Novel natural products provide innovation in drug discovery for cancer prevention and treatment.

Since 1960s, after National Cancer Institute (United States) started to screen antitumor activity of plant extracts, interest on chemoprevention of medicinal plants and their extracts increased. It is shown that dietary plants like fruits, vegetables, spices, cereals,edible roots prevent cancer by inducing cellular defense systems such as increasing detoxification and stimulating antioxidant enzymes and inhibiting inflammation (Kwon et al. 2007). Plant metabolites called phytochemicals have anticarcinogenic and antimutagenic properties. They interfere with tumor promotion and progression. Among phytochemicals, phenolic compounds are under great investigation since they have wide variety of bioactivities. For example, phenolic acids, ellagic acid and resveratrol, increased the expression of apoptotic genes, thus inhibiting cell proliferation in prostate cancer (Narayanan et al. 2002). In another study, when MDA-MB-231 cell intercardiac injected mouse was treated with curcumin, inhibition of matrix metalloproteinase which degrades basement membrane and extracellular matrix, causing cancer cell mobility making the cell invasive and metastatic was observed (Weng and Yen 2012).

1.4 Drug Metabolizing Enzymes

Drug metabolism is the biochemical process in which the living organism modifies the xenobiotics by specialized enzyme systems to detoxify or activate the substances. After taken into body, a pharmaceutical compound follows those 4 steps which determine the drug levels and kinetics of drug exposure to the tissue; absorption, distribution, metabolism and finally excretion (ADME).

Drug metabolism consists of metabolic pathways which biotransform the molecules in a way that detoxifies and deactivates the poisonous compounds or sometimes activates the inactive prodrugs.

Drug metabolism is divided into 2 phases. (Figure 1.10) Phase I enzymes are Cytochrome P450 oxidases which perform oxidation, hydrolysis or reduction reactions. They add reactive groups such as hydroxyl radical to the xenobiotics by using oxygen and NADH. These reactive intermediates are

then further metabolized by Phase II enzymes, which perform conjugation reactions such as glucuronidation, sulfation, acetylation, methylation and glutathione and amino acid conjugations, that transform the xenobiotics into water soluble compounds so that they can be excreted through urine or bile.

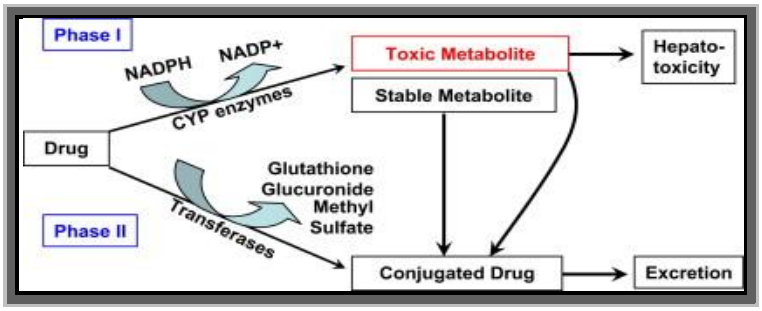


Figure 1.10 Phase I and Phase II reactions.

Drug metabolism is influenced by several factors as shown in Figure 1.11. Inhibition or induction of drug metabolizing enzymes may affect the efficacy of drug or may cause drug mediated toxicity. Nutrients can also have influence on drug disposition by inhibiting or inducing several enzymes. For example CYP3A4 is the most important member of cytochrome P450 superfamily of enzymes metabolizing a wide range of drugs and take place in synthesis of cholesterol, steroids and other lipids. It was interesting that grapefruit juice was found as an inhibitor of CYP3A4. So, plasma concentration levels of antidepressant drugs such as amitripytline, sertraline, trazodone, nefazonode and clomipramine that are substrates of CYP3A4 may increase when taken together with grapefruit juice, leading to toxicity (Bailey et al. 2004) (Okan, 2009). Similar inhibitory effects were observed in other drugs like HMG-CoA reductase inhibitors; statins (Ishigami et al. 2001) (Rowan, 2010). There are also other factors that contribute to variation of biotransformation between individuals like genetic polymorphism, disease, age and gender (Figure 1.11).

Figure 1.11 Factors influencing drug metabolism.

Genetic polymorphisms of human Cytochrome P450, N-Acetyltransferase, Glutathione S-transferase and Epoxide Hydrolase enzymes were shown to have significant effects on the pharmacological activity and the presence of side effects of drugs (Wormhoudt, Commandeur and Vermeulen 1999). Databases based on meta-analysis of the literature show the impact of alterations in gene expression of an enzyme from the knowledge of the turnover rate of the enzymes and fold induction of genes obtained from in vitro studies, thus predicting the effect of drug-drug interactions and bringing light for precautions against drug toxicity.

1.5 Cytochrome P450s

Cytochrome P450s are the most significant Phase I drug metabolizing enzymes. They metabolize a variety of xenobiotics such as therapeutic drugs or dietary constituents and some important metabolic intermediate compounds like steroids. The common Cytochrome P450 monooxygenase reaction is shown in equation Equation 1.1 (Figure 1.12). One feature of Cytochrome P450s is being hemoprotein because of containing heme cofactor. Thus their color is red. When the enzyme is complexed with CO (in reduced state) it gives maximum absorption at 450nm.

Equation 1.1: $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$

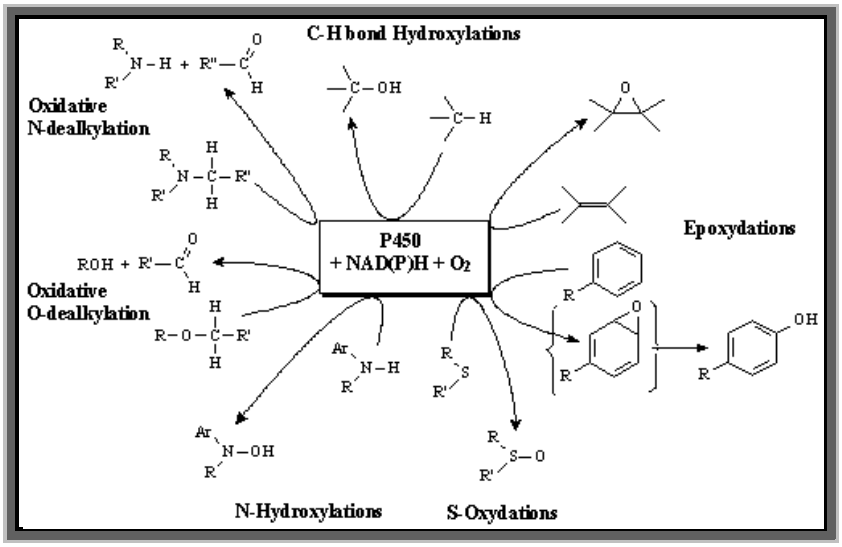


Figure 1.12 Monooxygenase reactions catalyzed by Cytochrome P450.

More than 11.500 CYP proteins are known in all domains of life (Nelson, 2010). The human CYPs can be divided into 3 major groups. CYP 5-51 families have high affinity to their substrates and conserved well during evolution. CYP 1-3 families have less affinity to their substrates and less conserved during evolution thus they are polymorphic. CYP 4 family has role in both fatty acid metabolism and xenobiotic metabolism. The CYP 1-3 families take part in 80% of all phase I metabolism of therapeutic drugs (Ingelman-Sundberg 2004).Some CYP enzymes have various substrates, whereas some catalyze only one reaction as in the case of CYP19 catalyzing aromatization reaction. CYP3A4 is the most important CYP in drug metabolism and CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6 are the other important enzymes in drug metabolism.
Cytochrome P450 1A1 is involved in oxygenation of polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines/amides (HAAs), the demethylation of aminoazodyes and the dealkylation of phenacetin and caffeine. Those reactions provide the conversion of the agents to more polar metabolites, thereby increasing their excretion. But oxygenation of those procarcinogens forms more reactive ultimate carcinogens leading to formation of DNA and protein adducts, thus causing tumor formation and toxicity.

Benzo[a]pyrene (B[a]p) induces CYP1A1 gene by activating aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, therefore activates the CYP1A1 enzyme, through transcription. For the metabolic clearance of substrates the induction of CYP1A1 can be two folds. However, high induction brings additional risk for cancer. CYP1A1 was shown to be the most important enzyme in the bioactivation of B[a]p to the ultimate carcinogenic B[a]P-7,8-diol-9,10-epoxide metabolites in human lung cancer. (Uppstad et al. 2010).

On the contrary, fluoroquinolones; widely used antimicrobials for infectious diseases, are shown to have inhibitory effect on CYP1A1 (Regmi et al. 2005). Modulation of an enzyme by one substrate will affect the metabolism of the other substrate of the same enzyme, resulting in unexpected drug-drug interactions (Ma and Lu 2007). For example, administration of enoxacin, a fluoroquinolone antibacterial agent decreased the clearance of the coadministered drug theophylline, a methylxanthine drug used in therapy for respiratory diseases (Wijnands, Vree and van Herwaarden 1986).

CYP1B1 sequence shows 40% homology with CYP1A1. Gene expression is also regulated by AhR as CYP1A1. The enzyme mediates cytotoxic effects of PAHs and HAAs (Kurzawski, 2012). It has the

highest catalytic activity toward PAHs, therefore it is the most potent inducer of mammary tumors and lung cancer (Simada, 1996).

Single nucleotide polymorphisms (SNPs) in CYP1B1 cause altered metabolism of hydroxylation of estradiol which show significant associations with various cancers by influencing susceptibility of individuals against carcinogens (Lewis et al. 2003).

CYP1B1 was shown to have important role in fetal development. CYP1B1 enzyme metabolizes oxidative synthesis of retinoic acid from retinol. Retinoic acid is the ligand for various nuclear receptor proteins, thus regulates morphogenesis. Mutations in CY1B1 cause primary congenital glaucoma (Kaur, Mandal and Chakrabarti 2011).

Both CYP1A1 and CYP1B1 take part in estrogen metabolism as E_2 hydroxylases in human (Spink et al. 1992, Spink et al. 1997). In breast tissue, the main estrogen 17B-estradiol is the substrate of CYP1A1 and CYP1B1, and ligand for Estrogen Receptor. Oxidation of E_2 causes the formation of 2-OH and 4-OH catechol estrogens, which cause DNA damage, thus breast cancer. On the other hand E_2 stimulates cell proliferation and gene expression by binding to Estrogen Receptor (Parl et al. 2009). Therefore modulation of CYP1A1 and CYP1B1 enzymes are important determinant for the fate of E_2 in breast cancer.

1.6 Scope of the Study

Medicinal herbs are important natural products being valuable candidates for drug discovery in many diseases especially in cancer.

Salvia species are one of the important group of medicinal plants because of the presence of polyphenols in their structures. Besides being used as traditional medication, *Salvi*a species also have nutritional value as drinking tea.

Salvia absconditiflora is an endemic species in Turkey. Even there exist some studies on the role of *Salvia absconditiflora* in wound healing and Alzheimer's disease, there is no study conducted on antioxidant, cytotoxic, and cancer chemopreventive effects of *Salvia absconditiflora* water extract. According to chemical profile of *Salvia absconditiflora*, phenolic acids and flavonoids are the major constituents which are known to be responsible from antioxidant properties, chemoprevention and tumor suppression.

The aim of this study was to investigate the chemical composition of *Salvia absconditiflora* water extract and to study its antioxidant and cytotoxic effects on two different breast cancer cell lines MCF-7 and MDA-MB-231.

In this study antioxidant, cytotoxic and cancer chemopreventive effects of *Salvia absconditiflora* water extract were evaluated for the first time in literature. Also investigations on effects of *Salvia absconditiflora* water extract treatment on MCF-7 and MDA-MB-231 cell lines were preliminary in this field.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Salvia absconditiflora leaves were collected from METU campus in November 2010, April, June, July 2011 with the guidance of Dr. Ferhat Celep (Herbarium no: FCelep 1773).

2.1.2 MCF-7 and MDA-MB-231 cell lines

MCF-7 and MDA-MB-231 cell lines were obtained from ATCC (American Type Culture Collection).

2.1.3 Chemicals and Other Materials

Water was distilled and purified using a Milli-Q system (Millipore, Bedford, MA, USA). Ultrapure (UP) water was obtained from New Human Power I Scholar UV water purification system.

99.5% extra pure ethanol was obtained from Dop Organik Kimya (Turkey).

Aluminium Chloride ($AlCl_3$) was from Merck.

2,2-diphenyl-1-picrylhydrazyl (DPPH), agarose powder, ethidium bromide, 2N Folin Ciaceltau reagent, catechin (+) hydrate were purchased from Sigma Aldrich.

Diethylpyrocarbonate (DEPC) 97 %, Dimethyl sulphoxide (DMSO) was obtained from AppliChem GmBH (Germany).

Roswell Park Memorial Institute Medium (RPMI-1640) with L-glutamine,with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 25mM cell culture medium, Phosphate Buffered Saline (PBS) without calcium and magnesium buffer solution and trypsin 10X for cell culture were purchased from Lonza Biowhittaker (Belgium). Fetal Bovine Serum (FBS) was supplied from Biochrom AG (Berlin).

XTT based colorimetric assay, cell proliferation kit and Tryphan Blue Solution 0.5% were obtained from Biological Industries.

Revert Aid First Strand cDNA Synthesis Kit (cDNA kit) was purchased from ThermoScientific. RNA isolation kit and quantitative real time PCR kit (SYBR ROX 2.5X real mastermix) were from 5-prime company.

2.1.4 Primers

CYP1B1, CYP1A1 and beta- actin gene primers were purchased from Iontek, Istanbul, Turkey.

Table 2.1 Primers for RT-PCR

Gene	sequence 5' to 3'		
	forward primer	reverse primer	size
beta actin	CAG AGC AAG AGA GGC ATC CT	TTG AAG GTC TAA ACA TGA T	201bp
CYP1B1	AAC GTC ATG AGT GCC GTG TGT	GGC CGG TAC GTT CTC CAA ATC	360bp
CYP1A1	TAG ACA CTG ATC TGG CTG C	GGG AAG GCC CAT CAG CAT C	146bp

2.2 Methods

2.2.1 Preparation of Salvia absconditiflora Water Extracts

Salvia absconditiflora leaves were washed with distilled water and placed on filter paper to let air dry at room temperature in dark. Dried plant leaves were grinded into small pieces roughly by hand. Water extraction was performed with 1:10 (w/v) ratio of dry plant in distilled water, at 50°C, 30 minutes in ultrasonicator further followed by 90 minutes incubation in a hot water bath (Nüve Bs 301) in a brown bottle. The infusion was filtered, the volume was recorded and then freezed at -80°C in Sanyo Ultra-Low Temperature Freezer. Lyophilization was performed for 3 days. The extracted powder was weighed and stored at -20°C in brown bottle until use.

2.2.1.1 Absorption Spectrum of Salvia absconditiflora Water Extract

The absorption spectrum of *Salvia* absconditiflora water extract was recorded against dH_2O between 200nm to 600nm at different concentrations ranging from 0,1 mg/ml to 10 mg/ml in Shimadzu UV-1800 spectrophotometer.

2.2.1.2 LC-MS/MS Analysis of Salvia Absconditiflora Water Extract

Liquid Chromatography Mass Spectrometry analysis was performed in METU MBB RD Center (Ankara, Turkey).
For Liquid chromatography Zorbax SB-C18 (2.1 x 50mm x 1.8 µ) colon was used. Mobile phase consisted from 2 solvent system as; Solvent A: 0.05 % Formic acid + 5mM ammonium formate (MilliQ dH_2O) and Solvent B: Methanol (MS grade). Flow rate was 0,3 ml/min., analysis duration was 13 minutes with gradual mobile phase flow and injection volume was 5 µL. Standard curve range was between 0,01 to 10 ppm (0,01-0,025-0,05-0,1-0,5-1-5-10 ppm).
Mass Spectrometry was performed in Agilent 6460 LC-MS/MS by using ESI+Agilent Jet Stream as ionization source, Agilent BinPump-SL (G1312B9) as pump, Agilent h-ALS-SL+ (G1367D) as automatic sampler, UHPLCMS 30 as nitrogen sampler. Analysis mode was selected as MRM. Gas temperature was 300°C, sheath gas temperature was 350°C, gas flow was 10 ml/minute, capillary and nozzle

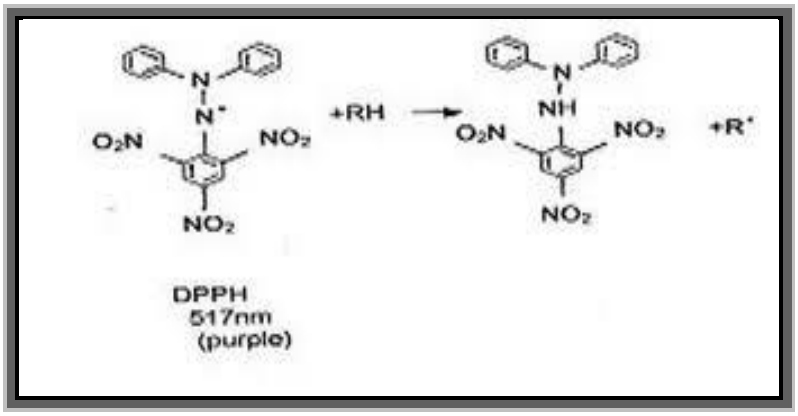
voltage were 400 V and 500 V respectively. The software used for the analysis was Agilent G3793AA MassHunter Optimizer.

2.2.2 Determination of in vitro Antioxidant Activity

The spectrophotometric measurements depicted in this part were performed with Shimadzu UV-160A UV visible recording spectrophotometer (Japan).

2.2.2.1 Free Radical Scavenging Activity by DPPH Method

Free radical scavenging activity of freeze-dried *Salvia absconditiflora* water extract was measured according to DPPH method stated by Blois (1958). DPPH free radical scavenging activity is the basic common antioxidant assay. It is a simple and accurate method. The basis of this assay is the ability of DPPH radical to capture hydrogen atom of a free radical (Figure 2.1). DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a free radical which has a purple color in solution. When scavenged by antioxidant molecules and phenolic compounds, it is reduced to diphenylpicryl hydrazine, which has yellow color in solution. This property allows visual monitoring of the reaction, and free radical scavenging activity can be calculated by measuring the absorption changes at 517nm. The results can be demonstrated in terms of Trolox Equivalents, which is commercial vitamin E and used as standard, or EC_{50}, corresponding to amount of antioxidant required to decrease the initial DPPH radical concentration by 50% (Sharma, 2009).



2.1 DPPH radical scavenging.

Freeze-dried *Salvia absconditiflora* water extracts were dissolved in dH_2O to prepare serial dilutions at different concentrations as; 0,5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 8 mg/ml. Hundred µl of each concentration was mixed with 1400 µl DPPH ($1.5x10^{-4}$ M) that is dissolved in 99,5 % ethanol. The reaction tubes were incubated for 30 minutes in dark at room temperature. The absorbance against Ethanol as reference was measured at 517 nm using Shimadzu UV visible recording spectrophotometer. Quercetin was used as standard. In order to eliminate the absorbance effect of *Salvia absconditiflora*, the absorbance of sample blank solutions consisting of 100µl of each concentration plus 1400 µl dH_2O, at 517 nm were also measured. To obtain relevant results in each experiment, absorbance of DPPH was measured against ethanol and recorded. The radical scavenging activity (%RSA) was calculated by using the equation 2.1.

Equation 2.1:

Radical Scavenging Activity (% RSA) = Abs (blank) – [Abs (sample) – Abs (sample blank)] / Abs (blank) X 100

2.2.2.2 Determination of Total Phenolic Content

Total phenolic content of freeze-dried *Salvia absconditiflora* water extract was measured according to Folin-Ciocalteu method stated by McDonald et al. (2001) with slight modifications by using gallic acid as standard. Folin-Ciocalteu method is based on the reduction of phosphutungstate-phosphomolybdate complex by phenolic compounds. The reaction product gives blue color and its absorbance can be measured at 765 nm spectrophotometrically.

Gallic acid standards were prepared by dissolving gallic acid in dH_2O. From the stock solution different concentrations were obtained by serial dilutions from 25µg/ml to 150 µg/ml.

Freeze-dried *Salvia absconditiflora* water extracts were dissolved in dH_2O to prepare serial dilutions at different concentrations as; 0,2 mg/ml, 0,5 mg/ml, 0,75 mg/ml, 1 mg/ml.
100 µl *Salvia absconditiflora* sample/ gallic acid standard was mixed with 800 µl 1M Na_2CO_3 and 1 ml 1:9 diluted 2N Folin Ciocalteu reagent (Table 2.2). The mixtures were incubated for 15 minutes at room temperature and absorbance was measured at 765 nm. The samples were analyzed as duplicates. In order to eliminate the effect of sample on absorbance, reagent blanks (100 µl *Salvia absconditiflora* sample + 1800 µL dH_2O) were measured and subtracted from absorbance values of samples. The total phenol content of the *Salvia absconditiflora* extracts was calculated according to standard curve of gallic acid as shown in equation 2.2 and expressed in terms of mg gallic acid equivalents (GAE) / g of dry extract mass.

Table 2.2 Determination of total phenolic content protocol

	Concentration	Volume added	Dilution factor
Sample	0,2 /0,5 /0,75/ 1 mg/ml	100 µl	1:19
Na_2CO_3	1M	800 µl	1:2
Folin Ciocalteu	1:9 diluted 2N	1ml	1:1,9

Equation 2.2:

mg GAE /g dry extract mass= [(Abs(sample)- Abs(sample blank)) – Abs (blank)]/ slope X DF

2.2.2.3 Determination of Total Flavonoid Content

Total flavonoid content of freeze-dried *Salvia absconditiflora* extract was measured according to the aluminum chloride colorimetric assay described by Zhishen et al. (1999) with slight modifications by using catechin as standard.

Catechin standards were prepared by dissolving catechin in dH_2O. From the stock solution different concentrations were obtained by serial dilutions from 50 µg/ml to 300 µg/ml.

Freeze-dried *Salvia absconditiflora* water extracts were dissolved in dH_2O to prepare serial dilutions at different concentrations as; 0,5 mg/ml, 1 mg/ml, 2 mg/ml. 0,2 ml *Salvia absconditiflora* extract sample/ catechin was mixed with 0,75 ml 5% $NaNO_2$ and incubated for 5 minutes. Then 0,15 ml 10 % $AlCl_3$ was added. After 6 minutes, 0,5 ml 1M NaOH was added and the total volume was completed up to 3 ml with dH_2O (Table 2.3). The absorbance was measured at 510 nm.

Table 2.3 Determination of total flavonoid content protocol

	Concentration	**Volume added**	**Incubation time**
Sample	0,5 /1 /2 mg/ml	0,2 ml	-
$NaNO_2$	5 %	0,75 ml	-
			5 minutes
$AlCl_3$	10 %	0,15 ml	
			6 minutes
NaOH	1 M	0,5 ml	-
dH_2O	-	1,6 ml	-

The total flavonoid content of the *Salvia absconditiflora* extracts was calculated according to standard curve of catechin as shown in equation 2.3 and expressed in terms of mg catechin equivalents (CE) / g of dry extract mass.

Equation 2.3:

mg CE /g dry extract mass= [Abs(sample) – Abs (blank)]/ slope X DF

2.2.3 Cell Culture

2.2.3.1 Cell Culture Conditions

MCF-7 and MDA-MB-231, human mammary gland breast cancer cell lines, were cultured in RPMI-1640 growth medium in which 10 % heat-inactivated fetal bovine serum (FBS) and 0.2 % (50 mg/ml) gentamicine were added. Incubation conditions for the cultures were 37°C with 95 % air and 5 % CO_2 in Hepa filtered Heraeus Hera Cell 150 incubator. The experiments were performed in a HERAsafe Class II Biological Safety laminar flow. The culture medium were refreshed in 2-3 days to provide cells proper conditions for growth.

2.2.3.2 Cell Thawing and Freezing

The frozen cells placed in cryovials kept in nitrogen tank were defrosted at room temperature. Cells were transferred to T25 tissue culture flask which contains 10 ml growth medium and incubated in CO_2 incubator at 37°C for 24 hours. Next day, medium was removed and the flask was washed with 2ml PBS. Then to detach the cells, 2 ml trypsin was added to the flask and incubated in 37°C for 3-4 minutes. 5 ml growth medium was added to deactivate trypsin. Immediately the cell suspension was transferred to T75 tissue culture flask and the volume was completed to 10 ml with growth medium.

After the cells reached 70 % confluency in T75 culture flask, the medium was removed and cells were washed with 2 ml PBS for 2 times. After the cells were detached with trypsin, growth medium was added to stop trypsin activity. The cells were centrifuged (at 100g, room temperature, 5 minutes) to remove leaked cell waste and medium. The cell pellet was resuspended with freezing medium in 10 % DMSO, 90 % FBS. The cells were kept as 1 ml aliquots in cryovials. The cryovials were frosted stepwise by keeping at -80°C for 1 day and placed in liquid nitrogen tank for long term storage.

2.2.4 Cytotoxicity Assays

2.2.4.1 Viability Measurement of *Salvia absconditiflora* Treated Cells using XTT Assay

The cytotoxic effect of freeze dreid *Salvia absconditiflora* water extract treatment on MCF-7 and MDA-MB-231 cell lines were investigated by using Cell Proliferation Kit (Biological Industries).

Cells were seeded in 96-well micro plates as 50 µl of $2x10^4$ cell suspension in RPMI-1640 growth medium and incubated at 37°C in 5% CO_2 incubator for 24 hours. The cells were seeded in 96 well plate as shown in Figure 2.2. Blank wells were left without cells but only complete medium containing 0.1 % DMSO was added. Next day the medium was aspirated and the attached cells were washed with PBS. Different concentrations of plant extract were prepared by serial dilutions with growth medium. 50 µl plant extracts + 50 µl of fresh medium (containing 0.2% DMSO so that final DMSO concentration is 0.1%) were added in each well.

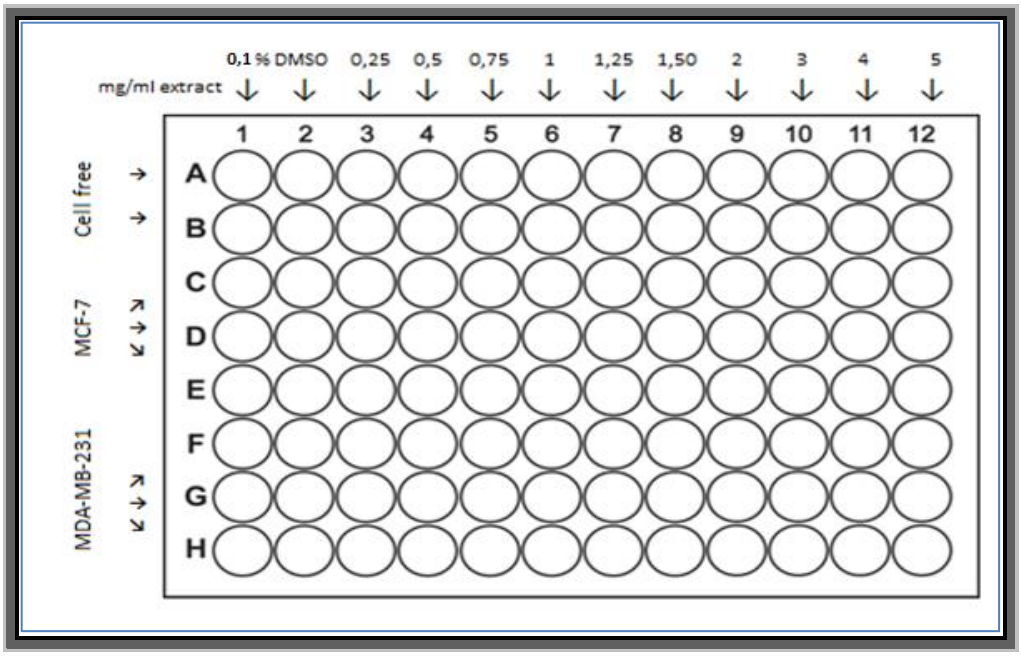


Figure 2.2 XTT assay organization in 96- well plate. First two lines are cell free, next three lines were incubated with MCF-7 cells and the following three lines were incubated with MDA-MB-231 cells. *Salvia absconditiflora* extract treatment was performed with different concentrations as indicated above each column. 0,1 % DMSO treatment was used as control.

The plate was incubated at 37°C in 5 % CO_2 incubator for 24 hours or 48 hours. Afterwards, 100 µL of phenazine metho-sulfate (activator) is added to 5ml XTT reagent, and 50 µl of this solution was applied to each well. The plate was incubated at 37°C in 5 % CO_2 incubator for 5 hours (5 to 20 hours incubation duration is valid). The mitochondrial enzymes of alive cells reduce the tetrazolium salts to formazan (Figure 2.3). This chromogenic product can be measured at 415 nm. Therefore relative viabilities of cells after treatment with different concentrations of *Salvia absconditiflora* extract can be measured by measuring the absorbance differences resulted by formazan formation. For this purpose, 96 well micro plate was measured with Bio-tek ELISA reader (Elx808-Bio-tek, Germany) and analysed with KC Junior program. The results were calculated as shown in Equation 2.5 and depicted as percent viability with respect to extract concentration. With the statistical analysis, IC_{50} (the plant extract concentration required to reduce viability 50 %) was found.

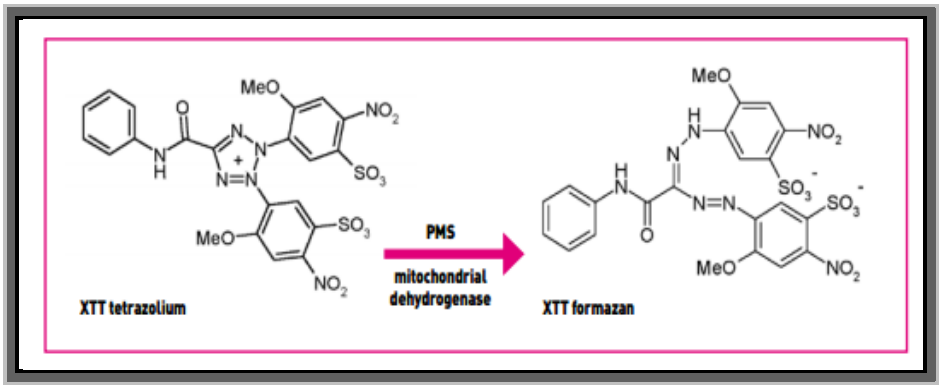


Figure 2.3 Reudction of XTT tetrazolium to XTT formazan by mitochondrial dehydrogenase in the presence of phenazine metho-sulphate.

Equation 2.5:

% cell viability = [Abs (extract treated cells) – Abs (extract in cell free medium)] / [Abs (untreated cells) – Abs (cell free medium)] x 100

2.2.4.2 Viability Measurement of *Salvia absconditiflora* Treated Cells with Tryphan Blue Exclusion Method

Both MCF-7 and MDA-MB-231 cells were seeded separately in 24 well plates as $1x10^5$ cells in 1 ml RPMI-1640 growth medium and incubated at 37°C in 5 % CO_2 incubator for 24 hours. Next day the medium was aspirated and the attached cells were washed with PBS. Different concentrations of plant extract were prepared by serial dilutions with growth medium. 500 µl plant extracts + 500 µl of fresh medium (containing 0.2 % DMSO so that final DMSO concentration is 0.1 %) were added in each well. 1000 µl growth medium containing 0.1% DMSO was added to the control wells. The plate was incubated at 37°C in 5 % CO_2 incubator for 24 hours or 48 hours. Afterwards, the medium was discarded, the cells were washed with PBS and detached with 500 µl trypsin. (To activate trypsin the plates are kept at 37°C for 2-3 minutes.) Trypsin was deactivated by adding 500 µl growth medium. Suspended cells were collected in an eppendorf tube. 50 µl of cell suspension was stained with 50 µl 0.25 % (w/v) tryphan blue solution and counted with Heamacytometer (Neubauer) under light microscope (Olympus BH-2) (Figure 2.4).

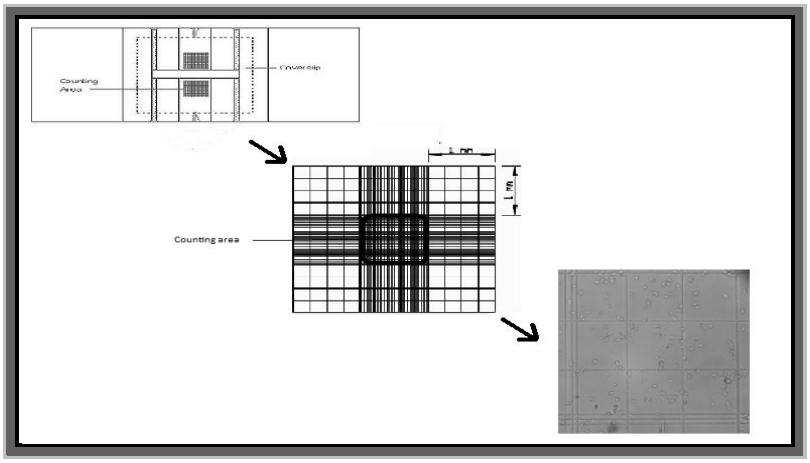


Figure 2.4 Viable cell counting with Heamocytometer

Percent cell viability was calculated from equation 2.6. With the statistical analysis IC_{50} values (the plant extract concentration required to reduce cell viability 50 %) for 24 hour treatment and 48 hour treatment were found.

Equation 2.6:

%viability= (cell count for a given concentration / cell count for DMSO control) x100

2.2.4.3 Light Microscopic Analysis

The viability of cells were examined under light microscope. The confluency of control cells and treated cells were compared.

2.3 Gene Expression Analysis by qRT-PCR

2.3.1 Isolation of Total RNA from MCF-7 and MDA-MB-231 cells

5-prime Manual PerfectPure RNA isolation kit was used for isolation of total RNA from MCF-7 and MDA-MB-231 cells as described by the company's procedure.

Cells were cultured in 6 well plates as 25 x 10^4 cells per 2 ml for each well. Second day, the cells were treated either with 0,1 % DMSO or *Salvia absconditiflora* IC_{50} concentrations. Third day, the medium was discarded and the cells were harvested by adding 400 µl Lysis Solution per well. The plate was incubated with Lysis Solution by rocking the plate gently for 5 minutes at room temperature. The solution was pipetted up and down to homogenize and lyse the cells. The lysed cells were added to a purification column and centrifuged at 15000xg for 1 minute in Eppendorf 5810R centrifuge. The purification column was then transferred to a new collection tube. The column was washed with 400 µl Wash 1 Solution by centrifugation at 15000xg for 1 minute. In order to reduce the DNA contamination and to increase RNA yield, optional DNase treatment was used. 50 µl DNase Solution was added to Purification Column and incubated at room temperature for 15 minutes. DNase was washed away by adding 200 µL DNase wash solution and centrifugation at 15000xg for 1 minute

and adding 200 µL DNase wash solution again and centrifugation at 15000xg for 2 minutes. Afterwards, 200 µL wash 2 solution was added and spinned at 15000xg for 1 minute and one more 200 µL wash 2 solution was added and further spinned at 15000xg for 2 minutes. Finally, the purification column is transferred to a new collection tube. 50 µL elution solution is added and eluted by centrifugation at 15000xg for 1 minute. purification column is discarded and collection tube containing the eluted purified RNA is kept on ice, and stored at -80°C.

Before the procedure all the glass and plastic materials were washed with 0.1 % DEPC (Diethyl Pyrocarbonate) solution, incubated overnight in order to prevent RNA degredation. Then autoclaved to convert DEPC to CO_2 and ethanol to be inactivated.

2.3.2 Electrophoresis of RNA

One percent agarose gel was prepared by adding 1 gr agarose to 100 ml 1X TBE (Tris Borate EDTA) buffer. In addition 0.5 µg/ml ethidium bromide was added to the solution to make the RNA bands visible under UV light. Ang the gel is left to dry at room temperature inside of the electrophoresis apparatus. With 1µl loading dye 10 µl of RNA was loaded to the gel and run with 90V generated by power supply. In addition RNA ladder was loaded in one well to check the sizes of RNA fragments. Afterwards, the gel was visualized under UV transilluminator (Vilber-Lourmat), and RNA integrities were observed.

2.3.3 Measurement of RNA Concentration with Nanodrop

RNA concentration was measured with Nanodrop 2000 spectrophotometer (Thermo Scientific) by applying 1 µl of RNA into the device. Elution buffer which was used in last step of RNA isolation was used as blank solution. A260/280 ratio shows the contamination of any protein, phenol or other compounds that absorb light near 280nm. This value should be approximately 2.0. A260/230 ratio also indicates nucleic acid purity. The ratio should be between 2.0- 2.2.

2.3.4 Complementary DNA (cDNA) Synthesis

cDNA was synthesized from purified RNA with Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's guideline. Approximately 2 µg of template RNA was added into a sterile, nuclease-free tube on ice. 1 µl Oligo$(dT)_{18}$ primer (15-20 pmol) was added. The volume is completed to 12 µL with ultrapure (UP) water. The tube is mixed gently and spinned shortly in Hettich Zentrifugen EBA12 and incubated at 65°C for 5 minutes in Bio-Rad MyCycler Thermal Cycler (USA). 4 µl 5X Reaction Buffer, 1 µl RiboLock TNase Inhibitor, 2 µl 10mM dNTP Mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase) were added. The tube was mixed gently and spinned shortly. The tube is placed on thermal cycler and incubated for 60 minutes at 42°C and the reaction was terminated by heating at 70°C for 5 minutes. The reverse transcription product was stored in -20°C for less than one week before use.

2.3.5 Primer Preparation

Required amount of RNase free water (which is recommended by the primer supplier company) were added to lyophilized primers. Then they were diluted at a concentration of 2000 µM and aliquoted. The aliquots were stored at -20°C.

2.3.6 Quantitative Real Time PCR

cDNA was used as template for quantitative real time polymerase chain reaction. 5 prime Real Mastermix SYBR ROX 2,5X was used for amplification. 125 µL of 20X SYBR solution was added into a tube (1 ml) of the 2,5X RealMasterMix SYBR ROX and mixed well. 9 µL of 2,5X RealMasterMix SYBR ROX (final concentration: 1X), 2 µl of forward primer, 2 µl of reverse primer (final concentration 200 nM) and 2 µl of cDNA were added and the volume was completed to 20 µl by addition of 5 µl UP water (Table 2.4). The reaction tube was mixed well and centrifuged. The qRT-PCR reaction conditions are given at Table 2.5. The qRT-PCR was performed in Qiagen RotorGene Q by using RotorGene Q Series Software program.

Table 2.4 qRT-PCR mixture preparation

Component	Volume	Final Concentration
2,5X RealMasterMix SYBR ROX	9 µl	1X
Forward primer	2 µl	200 nM
Reverse primer	2 µl	200 nM
cDNA	2 µl	
UP dH_2O	5 µl	

Table 2.5 Real Time PCR Conditions

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

2.4 Statistical Analysis

GraphPad Prism version 6 (GraphPad Software, San Diego, California, USA) was used for data analysis and graphs. All experiments were performed as triplicates and the results were expressed as mean ± standard deviation.

CHAPTER 3

RESULTS AND DISCUSSION

Salvia genus, embracing approximately 900 species, is a plant on the focus of pharmacology studies since it has been used in traditional herbal medicine for centuries. Although antioxidant and cytotoxic properties of many Salvia species are investigated frequently, the literature on *Salvia absconditiflora* is not broad enough. Besides, it is a wild plant which can grow in harsh conditions, in diverse areas, making the plant easy to handle in middle Anatolia. When the *Salvia absconditiflora* leaves were examined under light microscope, it was observed that the leaves have many vesicles indicating the presence of active compounds. *Salvia absconditiflora* leaves are commonly used as tea especially to treat cold and flu. So in this study the antioxidant and cytotoxic effects of the endemic herb, *Salvia absconditiflora* was studied in order to understand the healing effect of this species when consumed as warm tea.

3.1 Extraction Yield

The yield of the active ingredients depends on extraction conditions, solvent polarity, extraction temperature and time. In this study the effect of *Salvia absconditiflora* as a drinking tea was studied so the extraction was performed with water at 50°C for 2 hours as described in Chapter II.The extraction yield was calculated by Equation 3.1 as 17.39 % (w/w). Most of the studies in literature were carried out with alcohol in order to extract maximum amount of phenolic compounds since they are more soluble in alcohol. In such a study, *Salvia sclarea* collected from Konya region in June was extracted in 1:10 methanol at 60°C for 6 hours and the extraction yield was found as 21,58 ± 2,11 (Tulukcu, 2009).

Equation 3.1:

% yield= (weight of freeze-dried extract powder / weight of dry leaves) x 100

3.2 Absorption Spectrum of *Salvia absconditiflora* Water Extract

The absorption spectrum *Salvia absconditiflora* water extract was recorded against dH_2O between 200nm and 600nm at room temperature for different concentrations ranging from 10mg/ml to 100µl/ml . The absorption curve drawn for 2mg/ml is shown in Figure 3.1.

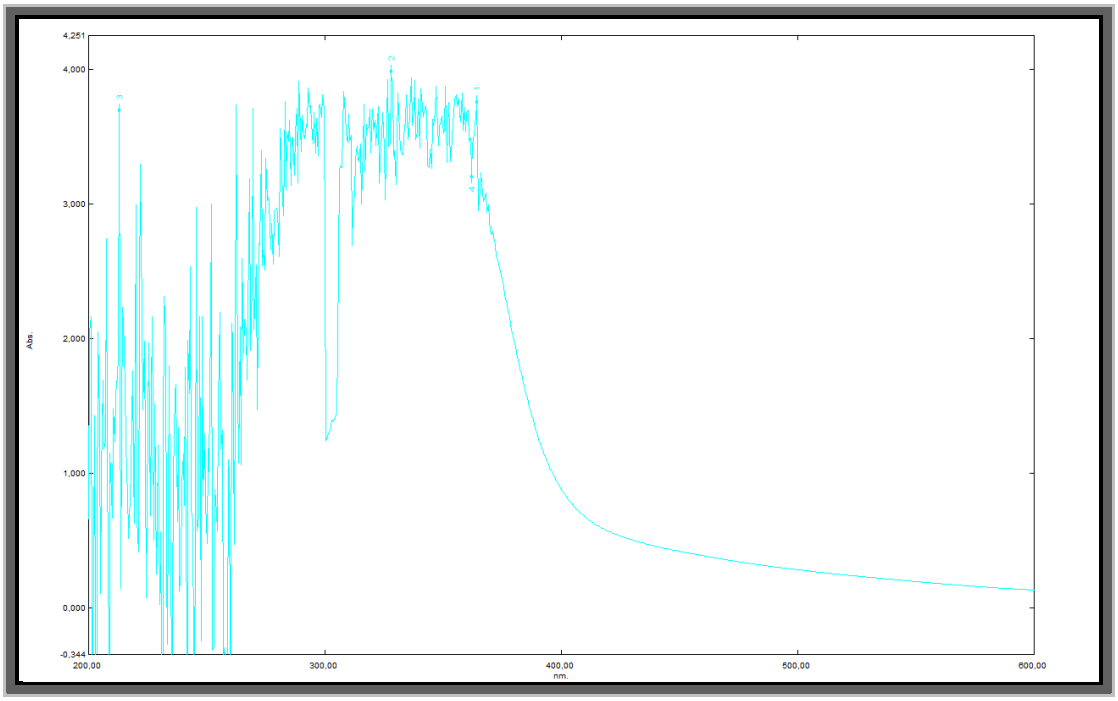


Figure 3.1 Absorption Spectrum of 2mg/ml *Salvia absconditiflora*.

Drawing the absorption spectrum curve is very important for standardization of different extractions. It can be used as technical standard to show if there is variability between different extractions. The extractions are valid if the curves of two different extractions are identical. In this study, all the plant material was extracted concurrently. Therefore absorption spectrum is not necessary for standardization. Though, the absorption spectrum was taken at different wavelength in order to observe the maximum peaks (λ_{max}).

Table 3.1 and 3.2 summarize the maximum peaks observed in different runs of variable concentrations.

Some of the phenolics which were shown to be present at high concentrations in *Salvia* species are caffeic acid, salvianolic acid, luteolin, quercetin and p-coumaric acid which have λ_{max} at 327nm, 286nm, 348/359nm, 285nm and 310nm respectively (Gould et al. 2000 and Xu et al. 2008). (http://home.cc.umanitoba.ca/~adam/lab/hplc/index.shtml). As observed from the maximum peaks, luteolin and caffeic acid are the major phenolics found in *Salvia absconditiflora*. Salvianolic acid, which is unique to *Salvia* genus is also present in *Salvia absconditiflora*.

Table 3.1 Concentrations of Caffeic acid and Luteolin in various *Salvia absconditiflora* extract

caffeic acid 327nm		luteolin 255/348nm	
concentration (mg/ml)	λ_{max} (nm)	concentration (mg/ml)	λ_{max} (nm)
3	324	0,6	259
2	328	0,2	260
0,6	329	0,1	250
0,5	328	6	350
0,4	329	1	349
0,2	328	0,6	345
0,1	329		

Table 3.2 Concentrations of Coumaric acid and Salvinolic acid in various *Salvia absconditiflora* extract

coumaric acid 310nm		salvianolic acid 286nm	
concentration (mg/ml)	λ_{max} (nm)	concentration (mg/ml)	λ_{max} (nm)
6	308	6	282
6	310	1	285
0,5	313	0,4	284
0,5	315		

There can be a slight change at λ_{max} according to pH since it can affect the stability of phenolic compounds (Friedman and Jürgens 2000).

In this method, the solution is not separated with any technique into detectable molecules. Therefore various compounds are being detected in a mixture altogether. For sensitive detection of compounds other techniques such as LC-MS/MS analysis is required.

3.3 Liquid Chromatography-Mass Spectrometry (LC-MS/MS) Analysis

LC-MS is a combined technique for general detection and identification of chemicals in a complex mixture. After LC separations, Mass Spectrometry, by measuring the mass-to-charge ratio of charged molecules, determines the composition of a sample (Pitt 2009).

In this study, caffeic acid, cumaric acid, luteolin and rutin were chosen as standard phenolic acids since they were shown to be present in Salvia species relatively higher than other phenolic acids (Lu and Foo 2002). 392 mg/ml *Salvia absconditiflora* extract was dissolved in 50% Methanol. 1:50 diluted sample was applied to LC-MS/MS device. The standard mixture was applied as 10 ppm (10 mg/L). Comparison of retention times data, which correspond to masses of the unknowns, of *Salvia*

absconditiflora extract and standard compounds revealed the relative abundance of phenolic compounds in question in the extract as shown in Figure 3.2 and Table 3.3.

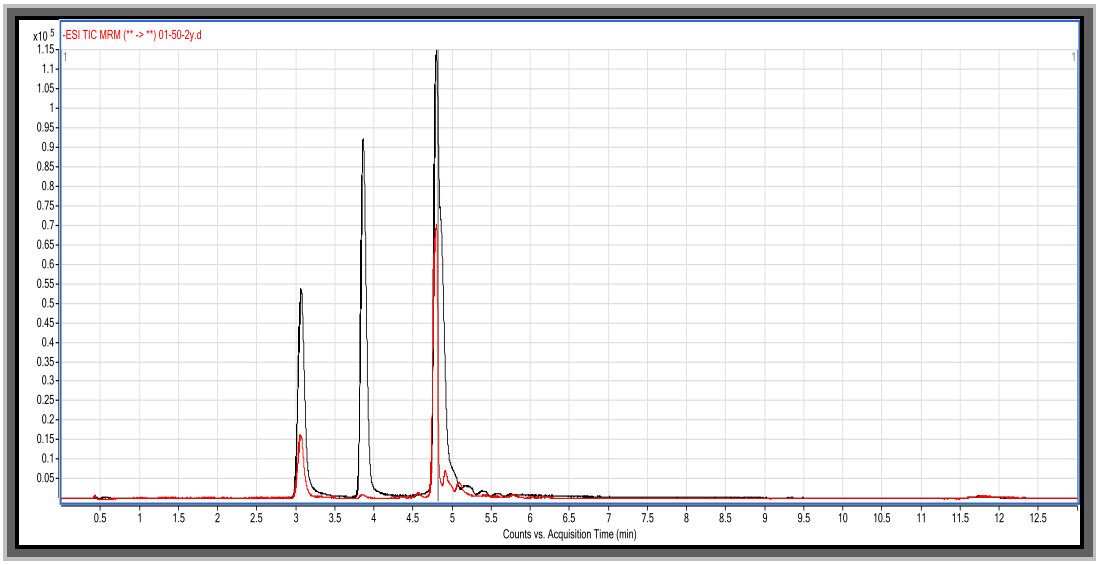


Figure 3.2 LC-MS/MS chromatography of 10ppm standard mixture (black line) and *Salvia absconditiflora* extract (7,84mg/ml, in 50% Methanol) (red line). First peak: caffeic acid, second peak: coumaric acid, third peak: luteolin+rutin.

Table 3.3 LC-MS/MS Analysis

Phenolic Compound	ppm	mg/g dry *Salvia absconditiflora* sample
Caffeic acid	121,03 ± 7,39	15,43
Coumaric acid	2,24 ± 0,01	0,29
Luteolin	236,73 ± 0,95	30,20
Rutin	195,06 ± 4,55	24,88

Phenolic acids in various *Salvia* species are widely investigated, since they are the main compounds making the plant valuable for medicinal approaches. Caffeic acid is the most abundant phenolic acid in *Salvia* genus. Methanol extracts of *Salvia fruticosa*, *Salvia tomentosa* and *Salvia officinalis* were shown to contain 7638,6, 335,5 and 118,8 ppm respectively (Askun 2009, Coisin 2012). In this study, caffeic acid was found as 121,03 ppm. The presence of caffeic acid suggests that *Salvia absconditiflora* has a high antioxidant activity (Lu and Foo 2002).

p-Coumaric acid, also known as p-hydroxy-cinnamic acid, is the most abundant coumaric acid isomer in nature. It prevents formation of carcinogenic nitrosamines (Kikugawa, 1983). In *Salvia officinalis* methanol extract p-coumaric acid levels were detected as 11,25 mg/100 g dried plant, a similar amount with caffeic acid present in the same sample according to HPLC analysis. In *Salvia bicolor* methanol extract it was found as 70,27 mg/g (Taghreed,2012). LC-MS/MS analysis indicated the presence of coumaric acid in *Salvia absconditiflora*, but with relatively small amount (2,24 ppm). This result showed that coumaric acid could be extracted with methanol.

The flavonoid luteolin is present in many herbs, including *Salvia* genus, and was shown to have antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic properties (Loper-Lazaro, 2009). According to HPLC analysis, levels of luteolin were found as 355,6 ppm in *Salvia fruticosa*, 51,3 ppm in *Salvia tomentosa*, 83,3 ppm Salvia officinalis and 423 ppm in *Salvia bicolor*. In LC-MS/MS analysis of *Salvia absconditiflora*water extract luteolin showed the highest amount compared to other 4 standard phenolic acids, with a value of 236,73 ppm.

Rutin was the second highest compound among other 4 phenolic acids investigated in LC-MS/MS of *Salvia absconditiflora* also with a value of 195 ppm. It was found as the most prominent phenolic compound in *Salvia tomentosa* methanol extract (866,9 ppm). Most of the phenolic compounds are extracted with maximum yield in alcohol.

The presence and concentration of the constituents of a plant is variable between species. Variation of chemical profile is related to harvesting period. It also depends on the extraction method.

3.4 Antioxidant Efficiency of *Salvia absconditiflora*

There is a great interest on antioxidant activities of herbs and nutrients since their constituents are shown to have effects on the removal of free radicals from human body. Therefore the tests to estimate the antioxidant efficiency are very necessary to increase the value of various herbs and nutrients.

DPPH free radical scavenging activity (RSA) assay, a method by Blois, with slight modifications is a simple, rapid and low costing method, and was used to determine the antioxidant capacity of *Salvia absconditiflora* extract.

Before the determination of the antioxidant capacity of the *Salvia absconditiflora* water extract, the assay conditions should be optimized. Assay conditions can change depending on the sample material and the equipment used (Molyneux, 2004).

3.4.1 Incubation Time Optimization of DPPH Assay

Use of DPPH radical for investigation of antioxidant capacity of compounds is an easy and rapid way but also requires optimization for different compounds. The radical scavenging activity should be measured at a steady state where all the DPPH radicals are reduced by the compound. Therefore incubation time for DPPH assay for *Salvia absconditiflora* was optimized by measuring absorbance at 517nm in 1 minute intervals for 40 minutes. Absorbance at 517nm versus time graph was plotted and the steady state was shown (Figure 3.3). 30 minutes was selected as time required for DPPH incubation for *Salvia absconditiflora*.

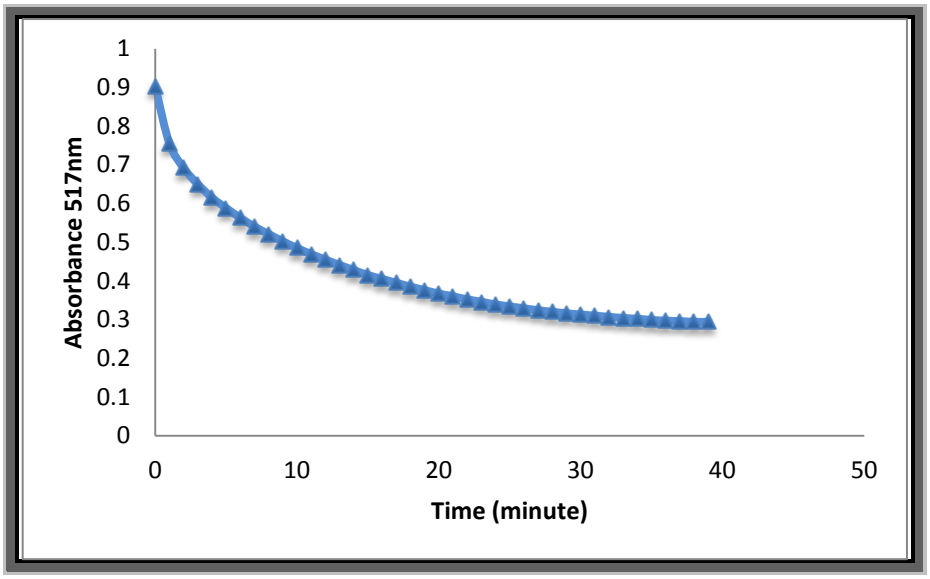


Figure 3.3 Reaction Kinetics of DPPH. Incubation time optimization for DPPH.

3.4.2 Determination of Antioxidant Capacity of *Salvia absconditiflora* by DPPH Assay

The results were interpreted as % RSA versus different extract concentrations (Figure 3.4). EC_{50}, concentration of extract that causes 50% loss of DPPH activity (color) was calculated from the graph.

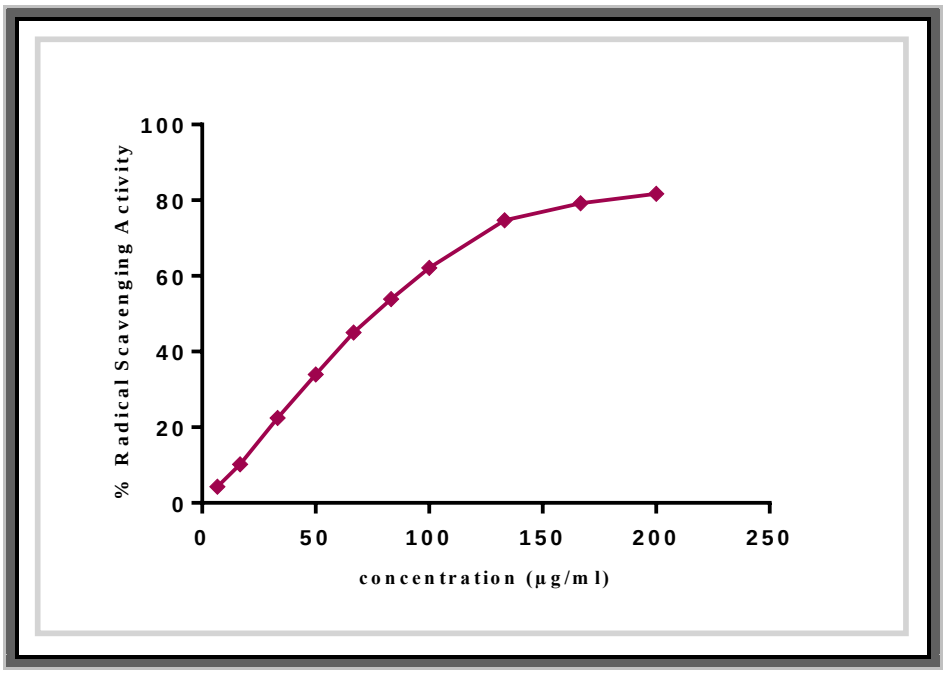


Figure 3.4 Percent DPPH scavenging activity (% RSA) of *Salvia absconditiflora* water extract. Each point is the mean of triplicate measurements from three different experiments (n=3).

Quercetin was used as standart in the assay. Percent DPPH scavenging activity of different concentrations of quercetin was plotted and shown in Figure 3.5. EC_{50} values of both quercetin and Salvia absconditiflora water extract are given at Table 3.4.

Table 3.4 Antioxidant Capacity of *Salvia absconditiflora* and Quercetin according to DPPH method

Sample	Antioxidant Activity	Maximum %RSA
	(EC_{50} µg/ml ±sd)	(%RSA ± sd)
Salvia absconditiflora extract	73,62 ± 0,47	80,43 ± 1,37
Quercetin	4,78 ± 0,35	92,29 ± 0,76

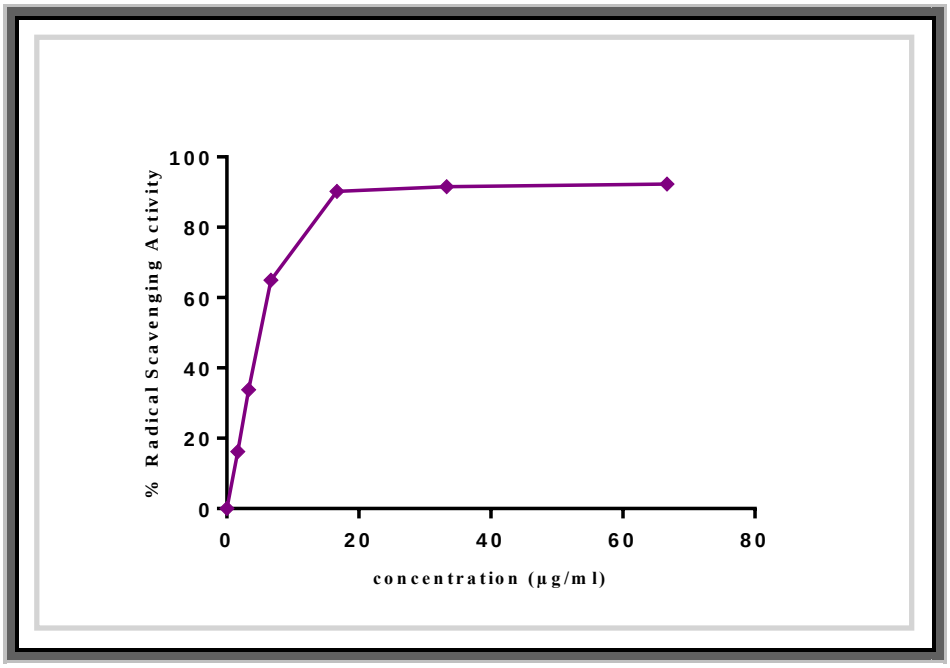


Figure 3.5 Percent DPPH scavenging activity (%RSA) of quercetin. Each point is the mean of triplicate measurements from three different experiments (n=3).

From the graph, EC_{50} value was calculated as 4,78 ± 0,35 µg/ml and maximum % RSA was calculated as 92,29 ± 0,76.

Potent radical scavengers are useful in the prevention of diseases (Zainol, 2003), thus natural antioxidants are becoming very important due to health issues. Due to this concern, previously it was stated that antioxidant activity of some solvent extracts of *Salvia* species were showing antioxidant activity with EC_{50} values between 1,61 and 74,50 µg/ml using DPPH assay (Kamatou et al. 2005). When compared to literature, by using DPPH, the radical scavenging activity of *Salvia absconditiflora* water extract is found as 73,62 ± 0,47 µg/ml indicating that *Salvia absconditiflora* is a good antioxidant candidate. This finding is also supported by comparing % maximum radical scavenging of *Salvia absconditiflora* (80,43 ± 1,37) with quercetin (92,29 ± 0,76), a powerful phenolic antioxidant compound.

3.5 Determination of Total Phenolic Content of *Salvia absconditiflora*

Total phenolic content of *Salvia absconditiflora* water extract was measured according to Folin-Ciocalteu method stated by McDonald *et al.* (2001) with slight modifications by using gallic acid as standard. This method is widely used since it is simple, sensitive and precise. The results are shown as mg gallic acid equivalents per gram dry mass of *Salvia absconditiflora* extract in Table 3.5, by using gallic acid standard curve (Figure 3.6).

Table 3.5 Total phenol content of *Salvia absconditiflora* extract expressed in gallic acid equivalents (GAE)

mg/ml	mg GAE / g dry extract ± sd
0,75	146,50 ± 18,32
1,00	148,60 ± 18,44
average	147,55 ± 17,56

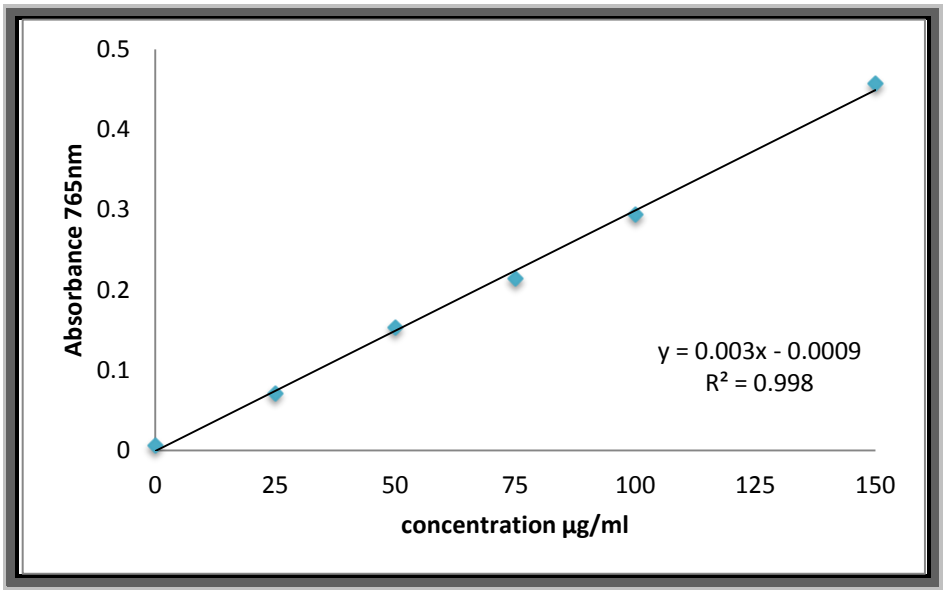


Figure 3.6 Gallic acid standard curve. Each point is the mean of triplicate measurements from three different experiments (n=3)

In the literature, total phenolic content for some *Salvia* species which were extracted with methanol:chloroform, were measured as 45 to 211 mg gallic acid equivalents per gram of dry sample. Compared to methanol extraction water extracted *Salvia absconditiflora* showed a high total phenol content 147,55 ± 17,56 mg GAE / g dry extract ± sd. High total phenolic content shows strong association with antioxidant activity (Kamatou, 2006).

3.6 Determination of Total Flavonoid Content of *Salvia absconditiflora*

Total flavonoid content of *Salvia absconditiflora* extract was measured according to the aluminum chloride colorimetric assay described by Zhishen *et al.* (1999) with slight modifications by using catechin as standard. According to the standard curve drawn with catechin, total flavonoid content of *Salvia absconditiflora* extract was determined as mg catechin equivalents per gram dry mass of extract (Figure 3.7, Table 3.6).

Table 3.6 Total flavonoid content of *Salvia absconditiflora* extract expressed in catechin equivalents (CAE)

mg/ml	mg CAE/ g extract ± sd
0,5	65,88 ± 3,31
1,0	67,42 ± 2,74
2,0	63,19 ± 4,23
average	65,32 ±3,66

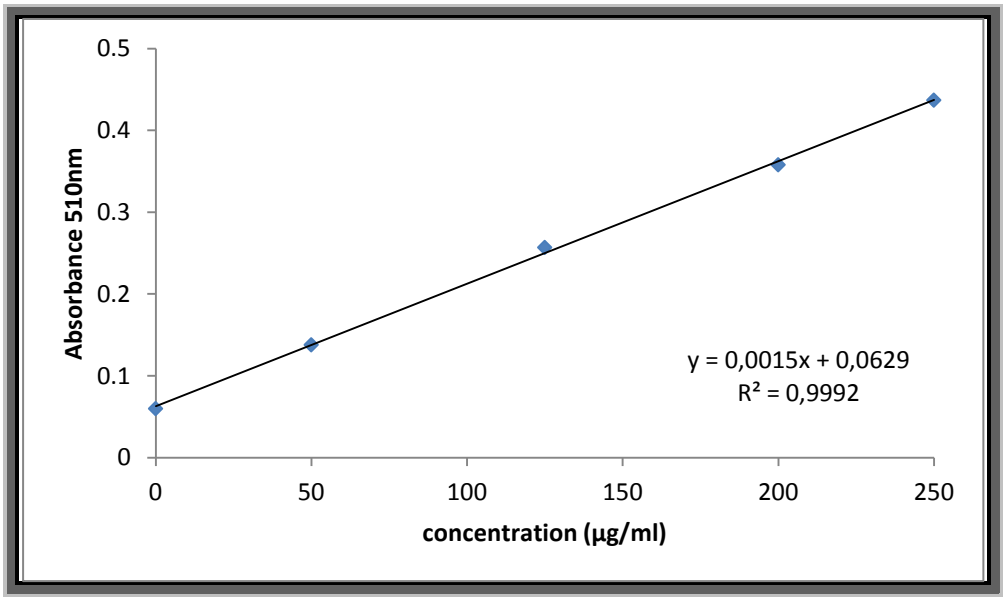


Figure 3.7 Catechin standard curve. Each point is the mean of triplicate measurements from three different experiments (n=3)

Total phenol and total flavonoid contents were measured by using different concentrations of *Salvia absconditiflora* extract (0,5 to 2 mg/ml). The identical results obtained for different concentrations showed that the assay results are independent from concentration. The reason for choosing this concentration range was, the lower or higher concentrations were out of spectrophotometrical measurement range. In addition measurements for lower concentrations scattered and deviated thus giving unpredictable data.

From the table 3.7, summary of the antioxidant capacity of *Salvia absconditiflora*, total phenol and total flavonoid content can be compared. Approximately one third of total phenol is found to be flavonoids.

Table 3.7 Summary of Antioxidant Capacity of *Salvia absconditiflora* extract.

Sample	Antioxidant Activity	Maksimum %RSA	total phenol content	total flavonoid content
	(EC_{50} µg/ml ± sd)	(%RSA ± sd)	(mg GAE/g dry extract ± sd)	(mg CAE/g extract ± sd)
Salvia absconditiflora	73,62 ± 0,47	80,43 ± 1,37	147,55 ± 17,56	65,32 ±3,66
Quercetin	4,78 ± 0,35	92,29 ± 0,76		

3.7 Cytotoxicity of *Salvia absconditiflora* in MCF-7 and MDA-MB-231 cells

3.7.1 XTT Assay

The cytotoxic effect of *Salvia absconditiflora* water extract treatment on MCF-7 and MDA-MB-231 cell lines were investigated by XTT assay. Mitochondrial enzymes such as dehydrogenases and reductases of metabolically active cells reduce the tetrazolium salt (XTT; 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) to formazan dye which has orange color and can be measured spectrophotometrically.

Cell viability for each concentration of extract treatment was depicted as % viability, assuming the control (0.1% DMSO) as 100 % viable. To eliminate the effect of plant on absorption, the cultured cells without extract treatment were used. Effect of *Salvia absconditiflora* on MCF-7 and MDA-MB-231 cell lines were investigated in both time (24/48 hours) and dose (0,25-5,0 mg/ml) dependent manner. Percent viability versus concentration graphs were plotted by Graphpad Prism for each cell line and for each incubation duration as shown in Figure 3.9. IC_{50} values, concentration of extract required to decrease cell viability by 50 %, were calculated and given at Table 3.8.

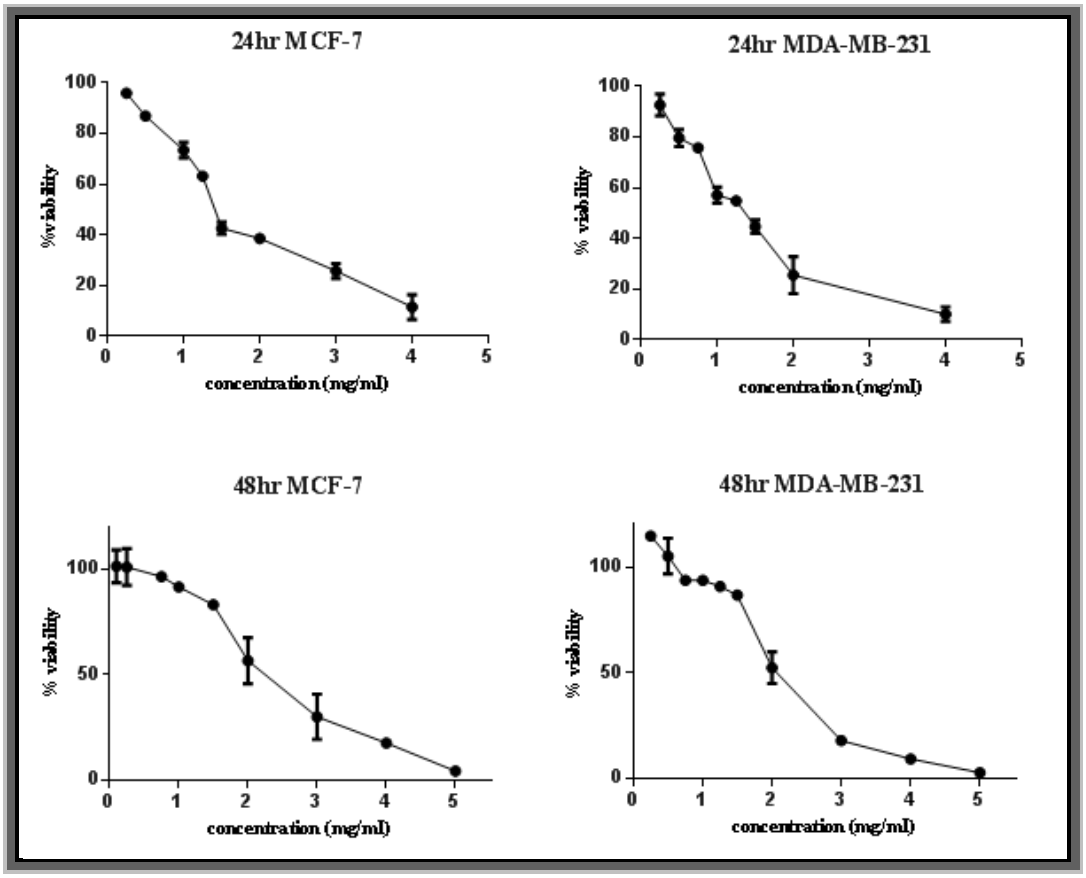


Figure 3.8 Viabilities of MCF-7 and MDA-MB-231 cells in response to dose and time dependent treatment of *Salvia absconditiflora* according to XTT assay. Each point is the mean of triplicate measurements from three different experiments (n=3)

Table 3.8 Concentrations of *Salvia absconditiflora* required to decrease the viability of cells 50 % according to XTT assay.

IC_{50} (mg/ml)		
Cell line	Incubation time	
	24hours	**48hours**
MCF-7	1,514 ± 0,101	2,285 ± 0,320
MDA-MB-231	1,195 ± 0,056	2,117 ± 0,097

3.7.2 Viable Cell Counting with Typhan Blue Exclusion Method

The principle of tryphan blue exclusion (TBE) cell viability test is based on the cell membrane permeability where, damaged or unimpaired membranes of dead cells cannot exclude the dye whereas living cells with intact membrane can exclude it. TBE cell viability test with haemocytometer

is commonly used because of its simplicity but it has limitations as low accuracy and operator dependency. (Kim et al. 2011).

Cell viability for each concentration of extract treatment was expressed as % viability, assuming the control (0.1% DMSO) as 100% viable. Effect of *Salvia absconditiflora* on MCF-7 and MDA-MB-231 cell lines were investigated in both time (24/48 hours) and dose (0,25-5,0 mg/ml) dependent manner. % Viability versus concentration graphs were plotted by Graphpad Prism for each cell line and for each incubation duration (Figure 3.9). IC_{50} values, concentration of extract required to decrease cell viability by 50%, were calculated (Table 3.9).

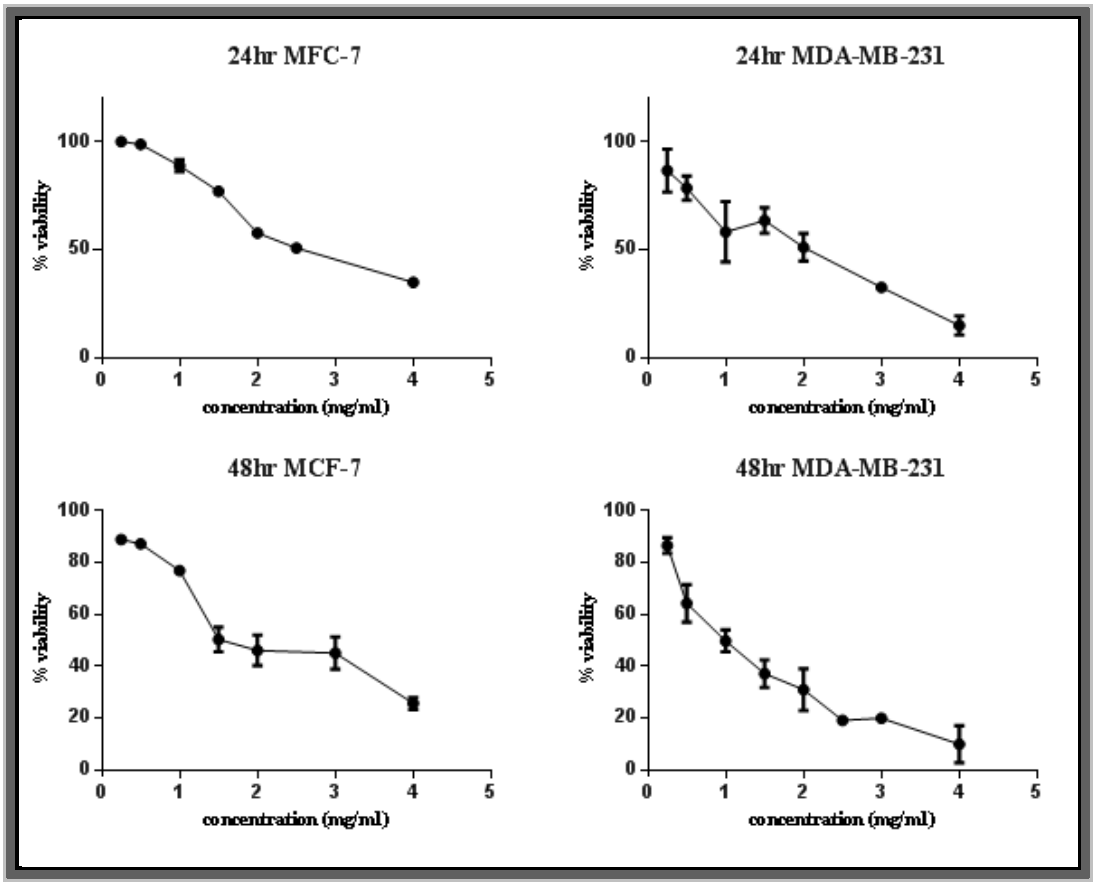


Figure 3.9 Viabilities of MCF-7 and MDA-MB-231 cells in response to dose and time dependent treatment of *Salvia absconditiflora* according to TBE assay. Each point is the mean of triplicate measurements from three different experiments (n=3)

Table 3.9 Concentrations of *Salvia absconditiflora* required to decrease the viability of cells 50 % according to TBE assay.

IC_{50} (mg/ml)		
Cell line	Incubation time	
	24hours	**48hours**
MCF-7	2,641 ± 0,043	1,961 ± 0,284
MDA-MB-231	1,662 ± 0,064	0,947 ± 0,178

3.7.3 Cell Proliferation Evaluation by XTT and TBE assay

The differences between IC_{50} concentrations found for each cell line from different assays are because of different principles of the assays. XTT assay is based on the metabolic activity of viable cells that metabolise the tetrazolium salts to formazan dyes. In this assay formazan dye production is measured spectrophotometrically. TBE assay is based on the permeability of the membrane which excludes the dye in viable cells. In this assay viable cells, which do not permit the dye and shine bright are counted with light microscope. XTT is a cell proliferation assay for investigating the cytotoxic effect of a compound based on the response of the population. On the other hand in TBE assay, individual responses to a compound are observed. Both have advantages and disadvantages but it cannot be stated that one of them gives more reliable results. To validate accuracy, several methods should be performed. When choosing an assay, experimental factors should be taken into account such as chemicals and experimental models.

Metastasis is hematogenous spread of cancer cells to distant organs and colonization to form secondary lesions (Fidler 2003). With the knowledge of MCF-7 cells are slightly metastatic and MDA-MB-231 cells are strongly metastatic, the different IC_{50} values obtained from XTT and TBE assays can be evaluated (Winnard et al. 2008). In both time period treatments (24 and 48 hours) and in both assays (XTT and tryphan) the IC_{50} values for MCF-7 is greater than MDA-MB-231 as shown in Table 3.10 and Figure 3.10. In 48hour treatments, XTT IC_{50}s are greater than TBE IC_{50} in both cell lines.

Table 3.10 Summary of Cytotoxicity of *Salvia absconditiflora* extract on MCF-7 and MDA-MB-231 cells.

IC50 (mg/ml)	24 hours		48 hours	
	XTT	TBE	XTT	TBE
MCF-7	1,514 ± 0,101	2,641 ± 0,043	2,285 ± 0,320	1,961 ± 0,284
MDA-MB-231	1,195 ± 0,056	1,662 ± 0,064	2,117 ± 0,097	0,947 ± 0,178

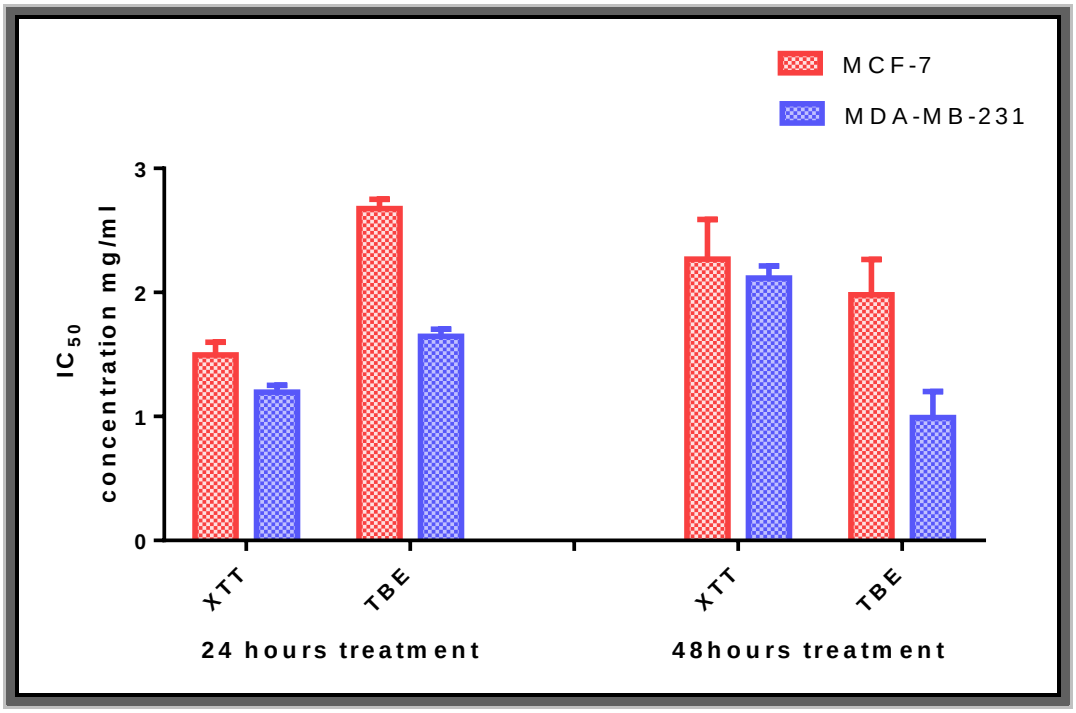


Figure 3.10 *Salvia absconditiflora* IC_{50} concentrations of MCF-7 and MDA-MB-231 cell lines according to XTT and TBE assays.

In TBE cell counting, the non attached cells are washed away during the procedure. Since MDA-MB-231 cell line is strongly metastatic, the non attached cells but still viable cells are lost before the cell count. This causes a false effect in viability thus decrease in the IC_{50} concentration. Those non attached aggressive metastatic cells incorporate to viability in XTT assay, therefore increase the IC_{50} value. This can be supported with light microscopic analysis by visualizing the nonattached but alive cells floating in culture dish. The reason for obtaining higher IC_{50} in XTT in 48 hours treatment is also similar. As the cells are subjected to the extract for a longer time period, cells become more aggressive and try to survive by increasing their metabolic activity. The survived ones contribute to viability with higher metabolic activity therefore a higher value of IC_{50} concentration is obtained.

As being an antimetastatic cell line MCF-7 cell lines tend to increase their adhesion to the surface. This is also supported by the high amount of trypsin requirement in the experiments to detach the MCF-7 cells.

The ability of the extract to interfere with cell adhesion might be beneficial. Cell adhesion was shown to modulate drug response and prevent cell death. Extracellular microenvironment affects drug response and triggers drug resistance in cancer cells (Hazlehurst, Landowski and Dalton 2003). Presence of several compounds in extracellular matrix which influence cell-cell or cell-ECM interactions plays role in acquisition of drug resistance. (White, Rayment and Muller 2006). Luteolin was shown to have inhibitory effect on HGF-mediated migration and invasion in HepG2 cells. It also inhibited cell scattering and cytoskeleton change which are important phenomena in cell adhesion (Lee et al. 2006) . Luteolin and caffeic acid significantly inhibited in vivo invasion of human PC-3 prostate cancer cells (Lansky et al. 2005).

The dose of *Salvia absconditiflora* used in this study might not have shown consequential results but by increasing the bioavailability, the extract might be a good candidate for targeted therapy rather than using it as drinking tea for medical purposes.

3.7.4 Light Microscopic Analysis

For investigation of cytotoxic effects of *Salvia absconditiflora* extract on MCF-7 and MDA-MB-231 cells morphological changes and viability of cells were analyzed under inverted light microscope.

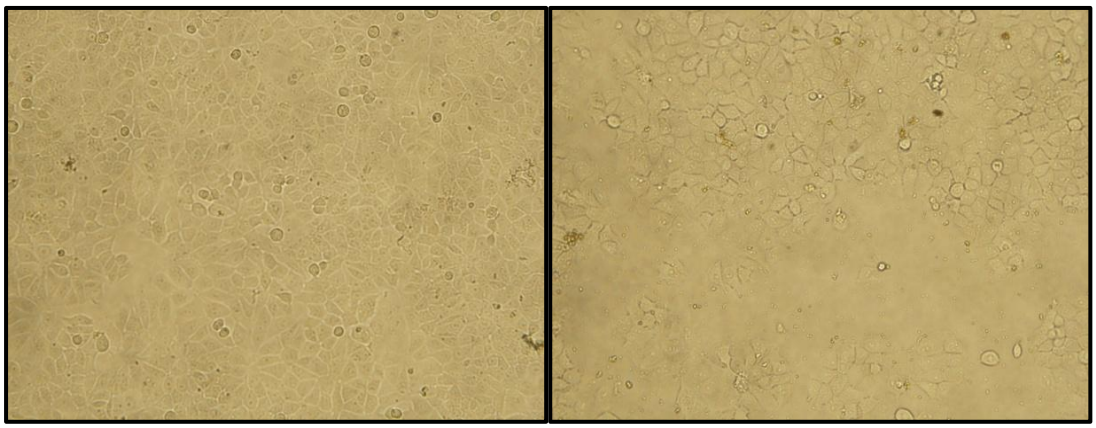


Figure 3.11 MCF-7 cells. Left: 24 hours 0,1 % DMSO treated control. Right: 24 hours IC_{50} concentration (1,514 mg/ml) *Salvia absconditiflora* treated.

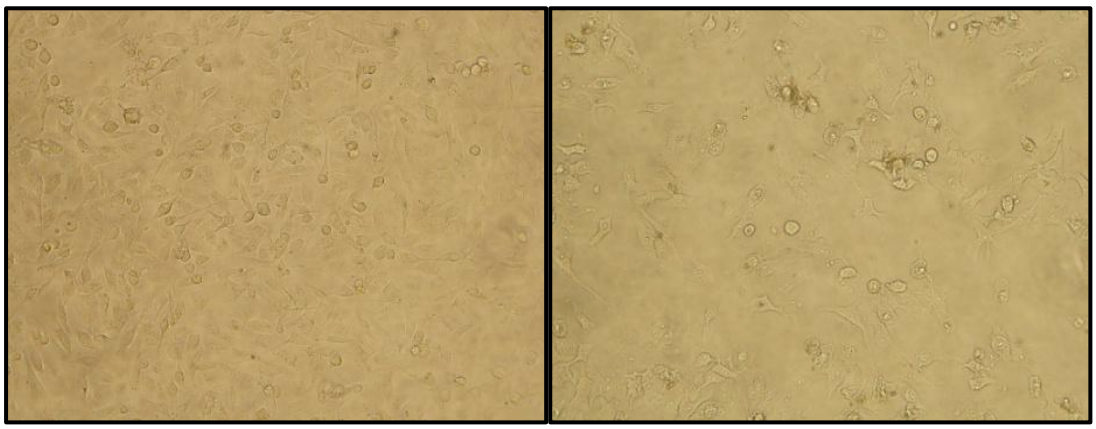


Figure 3.12 MDA-MB-231 cells. Left: 24 hours 0,1 % DMSO treated control. Right: 24 hours IC_{50} concentration (1,195 mg/ml) *Salvia absconditiflora* treated.

After incubation with *Salvia absconditiflora* extract cell growth inhibition in cells were illustrated comparing with 0,1 % DMSO control treated cells. In both cell lines, the confluency significantly decreased. Dead cells are observed in treated MDA-MB-231 cells.

3.8 Gene Expression Analysis of CYP1A1 and CYP1B1 Modulated by *Salvia absconditiflora* in MCF-7 and MDA-MB-231 cells

3.8.1 Qualification of RNA by Agarose Gel Electrophoresis

Isolated RNA was evaluated for purity by agarose gel electrophoresis (Figure 3.13). Lane 1 is DMSO control for MCF-7, Lane 2 and 3 are for treated MCF-7, Lane 4 is for DMSO control MDA-MB-231, Lane 5 and 6 are for treated MDA-MB-231 RNA. From up to bottom first bands indicate 28S RNA and second bands indicate 18S RNA. The image indicates the RNA integrity and purity. But for quantification other techniques are used.

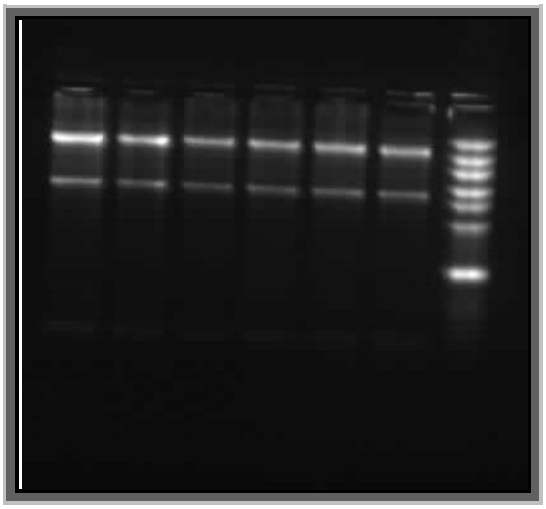


Figure 3.13 Agarose gel electrophoresis of RNA. First lane: MCF-7 DMSO control, second and third lane: MCF-7 treated, fourth lane: MDA-MB-231 DMSO control, fifth and sixth lane: MDA-MB-231 MDA-MB-231 treated. Upper bands indicate 28S RNA, lower bands indicate 18S RNA.

3.8.2 Determination of RNA Purity and Concentration by Nanodrop

RNA concentration was measured with Nanodrop technique. Purity of RNA was verified by this technique (Table 3.8). A260/280 desginates contamination of protein, phenol or other compounds that absorb light near 280nm. This value should be approximately 2.0. A260/230 also indicates nucleic acid purity. The ratio should be between 2.0- 2.2. According to data obtained from Nanodrop there was no contamination in the isolated RNA.

Table 3.11 Quantificaiton and determination of purity of RNA

		RNA concentration		
		µg/µL ± sd	**A260/280**	**A260/230**
MCF-7	**treated**	3742,05 ± 49,99	2,1	2,18
	DMSO control	2460,35 ± 3,75	2,08	2,14
MDA	**treated**	1366 ± 12,59	2,1	2,19
	DMSO control	1252 ± 20,79	2,1	2,21

3.8.3 Expression Analysis of CYP1A1 and CYP1B1 with Quantitative Real Time PCR

The effects of IC_{50} cytotoxic concentration (1,514 mg/ml for MCF-7 and 1,195mg/ml for MDA-MB-231) treatmentof *Salvia absconditiflora* water extract on gene modulation was investigated by qRT-PCR by measuring the expression levels of CYP1A1 and CYP1B1 in MCF-7 and MDA-MB-231 cells. The changes in expression levels of corresponding genes in MCF-7 and MDA-MB-231 cells are shown in Table 3.12 and Figure 3.13. Gene expressions were shown as fold changes which are calculated by using $2^{-\Delta\Delta Ct}$ method (Equation 3.1).

Equation 3.1: $2^{\Delta\Delta Ct}=2^{Ct\ (treated\ cells)\ -\ Ct\ (control\ cells)}$

As control 0,1 % DMSO treated cells were used. β-actin gene was used as internal control, which is a highly conserved gene and used as internal control in gene expression analysis (Hanukoglu, 1983).

Table 3.12 Effect of *Salvia absconditiflora* treatment on CYP1A1 and CYP1B1 gene expressions in MCF-7 and MDA-MB-231 cell lines.

Genes	**Cell line**	
	MCF-7	**MDA-MB-231**
CYP1A1	2,46 ↑	3,86 ↓
CYP1B1	1,46 ↑	3,10 ↓

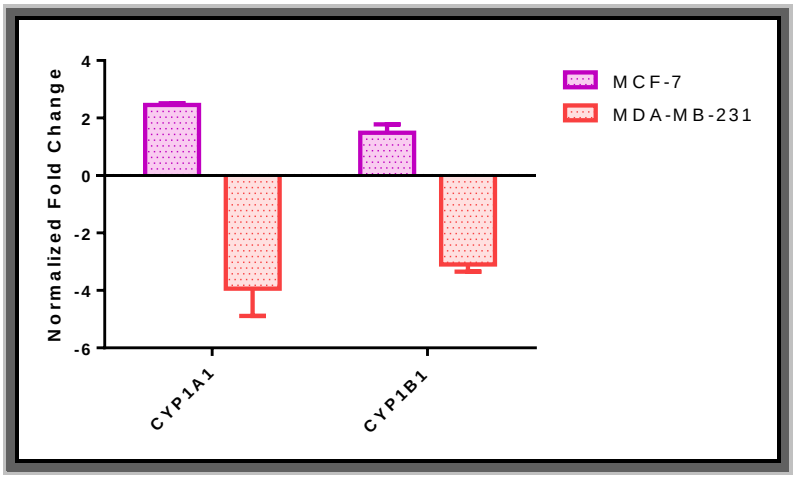


Figure 3.14 Gene expression modulations by *Salvia absconditiflora* water extract in MCF-7 and MDA-MB-231 cells

As shown in Table 3.9 and Figure 3.14 expression of CY1A1 and CYP1B1 increased in IC_{50} (1,514mg/ml) *Salvia absconditiflora* extract treated MCF-7 cells by 2,46 and 1,46 folds respectively. On the contrary the IC_{50} (1,195mg/ml) *Salvia absconditiflora* extract treatment decreased the expressions of those genes inMDA-MB-231 cells 3,86 folds for CYP1A1 and 3,10 folds for CYP1B1. *Salvia absconditiflora* extract treatment slightly upregulated the expression of CY1A1 and CYP1B1 in MCF-7 cells whereas distinctly downregulated the expressions in MDA-MB-231 cells.

Dietary or therapeutic agents interfere with gene modulation in various cancer types. Polyphenolic cocoa extract (PCE) that is rich in flavonoids causes over expression of CYP1A1 in MCF-7 cells. PCE activates AhR which binds to promoter of CYP1A1 in MCF-7 cells, leading to transcriptional activation. Estrogen Receptor and AhR interaction upon treatment with PCE causes CYP1A1 induction in ER positive MCF-7 cells (Oleaga,2012).
Genistein, a natural flavonoid found in soy, is an inhibitor of CYP1A1 mediated EROD enzyme activity (Shon, 2006). Enzyme activity increase may be correlated with upregulation of corresponding gene.
TCDD (2,3,7,8-Tetrachlorodibenzodioxin), a carcinogenic compound enhances E_2 metabolism through activating AhR, as an antagonist. TCDD treatment induced CYP1A1 expression in MCF-7 cells which have epithelial morphology, whereas did not affect CYP1A1 expression in MDA-MB-231 cells which have fibroblastic or mesenchymal morphology. However CYP1B1 was induced in both cell lines in response to TCDD treatment (Spink,1998).
In this study on *Salvia absconditiflora* slight increase of CYP1A1 expression suggesting that the plant may have estrogenic activity thereby affecting only MCF-7 cells. But the decrease in CYP1A1 expression in MDA-MB-231 cells indicates that the plant has also an anticarcinogenic effect on Estrogen Receptor negative MDA-MB-231 cells, when compared to TCDD mediated induction.

CHAPTER 4

CONCLUSION

Salvia is an important genus, investigated highly by researches and used in medicine because of its curative properties in a variety of diseases. This study was carried out with endemic *Salvia absconditiflora* water extract.

The aim was to understand the phenolic composition of *Salvia absconditiflora* and to highlight the antioxidant, cytotoxic and cancer chemopreventive effects of the constituents of *Salvia absconditiflora* when it is used as drinking tea.

Salvia absconditiflora was found as a good radical scavenger by DPPH method. Phenolic composition which is the main determining factor for antioxidant activity was examined by measuring total phenol and total flavonoid content and presence of some of the phenolic acids and flavonoids such as caffeic acid and luteolin were validated with LC-MS/MS analysis.

Cytotoxic effects of *Salvia absconditiflora* on MCF-7 and MDA-MB-231 cell lines were examined by XTT and TBE methods in dose and time dependent manner. Cell proliferation was inhibited 50 % by *Salvia absconditiflora* concentrations of 1,514/2,285 mg/ml in MCF-7 and 1,195/2,117 mg/ml in MDA-MB-231 cells in 24 hours and 48 hours treatment respectively in XTT assay, indicating an increased cellular activity in both cell lines for prolonged incubation with *Salvia absonditiflora*. However, decreased cell viability was observed in TBE assay, with 2,641/1,961 mg/ml IC_{50} in MCF-7 and 1,662/0,947 mg/ml IC_{50} in MDA-MB-231 cells for 24 hours and 48 hours treatment respectively. Comparison of the results suggests that MCF-7 and MDA-MB-231 cells increase their carcinogenic activity when subjected to *Salvia absconditiflora* for increased duration. Investigations on CYP1A1 and CY1B1 gene expression patterns resulted in up-regulation in MCF-7 but down-regulation in MDA-MB-231 when treated with *Salvia absconditiflora* IC_{50} concentrations found in XTT assay. Slight changes in gene expression do not indicate that *Salvia absconditiflora* has interference with drug metabolism but reveals an accountable effect which can be further searched for higher concentrations further. These findings show that *Salvia absconditiflora* is a good candidate for targeted therapy and drug discovery.

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