ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF *SALVIA FRUTICOSA M.* AND ITS EFFECTS ON GENE EXPRESSIONS OF SOME CYP450 AND ANTIOXIDANT ENZYMES IN HT-29 CELL LINE

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

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ABSTRACT

ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF *SALVIA FRUTICOSA M.* AND ITS EFFECTS ON GENE EXPRESSIONS OF SOME CYP450 AND ANTIOXIDANT ENZYMES IN HT-29 CELL LINE

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Salvia genus is a widely cultivated as a perennial herb and used in medicine for various purposes due to its antimicrobial, antioxidant, anticarcinogen and anti-inflammatory properties.

In this study, the phenolic composition of water extract of *Salvia fruticosa* and possible effects of those constituents in cancer related drug metabolizing enzymes are investigated. *S. fruticosa* extract displayed high Radical Scavenging Activity against DPPH and ABTS radicals and also showed high Fe^{2+} Chelating Activity . Total flavonoid content was found as three fourth of total phenolic content. Presence of important phenolic acids and flavonoids such as rosmarinic acid, caffeic acid, syringic acid, trans resveratrol and quercetin were validated with RP-HPLC analysis.

Cytotoxicity of *S. fruticosa* extract, rosmarinic acid and caffeic acid treatments on HT-29 colon cancer cell line was investigated by XTT and TBE assays by dose and time dependent manners and IC_{50} values were calculated.

Cancer related drug metabolizing enzyme gene modulations were also investigated using qRT-PCR. While some phase I and phase II antioxidant enzymes were up-regulated some were down-regulated in HT-29 cells in response to duration of *S. fruticosa* treatment were observed.

Keywords: *S. fruticosa*, HT-29 cell line, polyphenols, cytotoxicity, antioxidant enzymes, Phase I and Phase II enzyme gene expressions.

ÖZ

SALVIA FRUTICOSA M. BİTKİSİNİN ANTİOKSİDAN VE SİTOTOKSİK ÖZELLİKLERİ VE HT-29 HÜCRE HATTINDA BULUNAN BAZI CYP450 VE ANTİOKSİDAN ENZİMLERİN GEN İFADELERİ ÜZERİNE ETKİLERİ

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Salvia bitki türü yaygın olarak yetiştirilen ve antimikrobiyal, antioksidan, antikarsinojen ve antiinflamatuvar özellikleri sebebiyle tıpta çeşitli amaçlar için kullanılan bir türdür.

Bu çalışmada *Salvia fruticosa* su ekstresinin fenolik bileşimini araştırmak ve bu bileşenlerin kanser bağlantılı ilaç metabolize eden enzimler üzerindeki etkisini anlamaktır. *S. fruticosa* ekstresi DPPH ve ABTS radikallerine karşı yüksek sönümleme aktivitesi ve yüksek oranda Demir (II) iyonları şelatlama aktivitesi göstermiştir. Ekstrenin toplam flavonoid içeriği toplam fenolik içeriğinin dörtte üçü olarak bulunmuştur. Rosmarinik asit, kafeik asit, sirinjik asit, trans resveratrol ve kuversetin gibi bazı önemli fenolik asit ve flavonoidlerin varlığı RP-HPLC ile tasdik edilmiştir.

XTT ve TBE metodları ile *S. fruticosa* ekstresinin ve fenolik bileşenlerinden rozmarinik asit ve kafeik asitin HT-29 kolon kanser hücre hattı üzerindeki doza ve zamana bağlı sitotoksik etkisi araştırılmıştır. Farklı metodlar sonucu hücre çoğalmasının % 50 engellenmesine sebep olan IC_{50} konsantrasyon değerleri hesaplanmıştır.

Kanserle bağlantılı ilaç metabolize eden enzimlerin gen ifadeleri kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile incelenmiştir *S. fruticosa* muamelesi sonucu HT-29 hücrelerindeki bazı faz I, faz II enzimleri ile bazı antioksidan enzimlerin gen ifadelerinin arttığı bazılarının ise azaldığı gözlenmiştir.

Anahtar kelimeler: *S. fruticosa*, HT-29 hücre hattı, polifenoller, sitotoksisite, antioksidan enzimler, Faz I ve Faz II enzimlerinin gen ifadeleri.

To my mother

my family

and

to my dearest, Mehtap

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This study is dedicated to my family, whose help, advice, patience, support, and love are undeniable and unforgettable. Without them I couldn't have done this.

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LIST OF ABBREVIATIONS

ATCC	: American type culture collection
cDNA	: complementary DNA
CYP	: Cytochrome P450 Monooxygenase
DMSO	: Dimethyl sulfoxide
DPPH	: 2,2 diphenyl 1-picrylhyrazy
EDTA	: Ethylenediaminetetraaceticacid
PCR	: Polymerase chain reaction
q-RT PCR	: Quantitative real time polymerase chain reaction
RSA	: Radical scavenging activity
TFC	: Total flavanoid content
TPC	: Total phenolic content
XTT	: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*- Tetrazolium - 5-Carboxanilide

CHAPTER 1

INTRODUCTION

1.1 *Salvia Genus*

Salvia commonly known as sage derived from the Latin word "salvere" means to heal or save that correspond to the curing features of the herb (Grieve, 1984).

Salvia species have been used for more than 60 different ailments ranging from aches to epilepsy (Kumar et al., 2010). *Salvia* extracts have antimicrobial, anticancer, antioxidant and anti-inflammatory effects (Kamatou et al., 2008).

There are around 900 species of *Salvia* in the worldwide, 95 of which are currently represented in Turkey (Şenol et al., 2010; Celep et. al., 2009). Commonly grown and consumed sage in Turkey is *S. fruticosa* (Baytop, 1984). It is one of the important exported medicinal plants of Turkey (Baytop, 1984; Akgül, 1993). *S. fruticosa* spontaneously grows mainly in Western and Souther Anatolia. The leaves of the plant is regularly consumed as tea and to treat some disorders in Anatolian folk medicine. It has been used for its diuretic, antiperspirant, wound healing, and antiseptic properties (Baytop,1984; Bayram, 2001), antimicrobial, antihypertensive, antidiabetic and spazmolytic activities (Baytop, 1984), to treat diarrhea, eye disease (Giwell et al., 2013). It is also used as a hypoglycemic herb and against inflammations, hepatitis, and tuberculosis (Pitarokil, et al., 2003). Essential oil of *S. fruticosa* is used as an antiseptic agent on open wounds and burns and is applied onto the abdomen for dysmenorrhea, digestive troubles, heartburn, flatulence, cold, stomachache, cut, wound and burn (Gürdal, 2013). Pharmacological properties of Salvia genus was shown in Figure 1.1

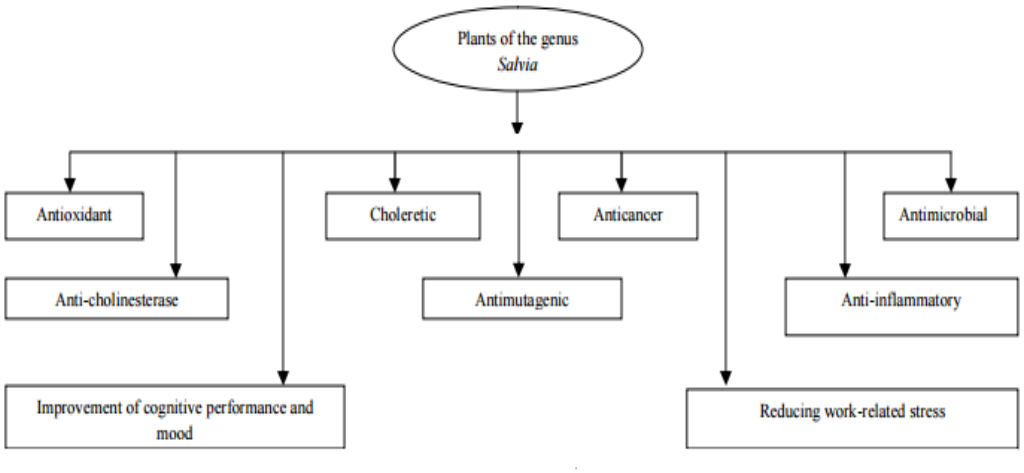


Figure 1.1 Pharmacological properties of Salvia genus

1.1.1 *Salvia fruticosa*

Taxonomic Hierarchy:

Kingdom: Plantae

Subkingdom : Tracheobionta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass : Asteridae

Order : Lamiales

Family: Lamiaceae

Genus : *Salvia*

Species : *S. fruticosa* Miller

Figure 1.2 *S. fruticosa*

S. fruticosa is an perennial herb that native to the eastern Mediterranean including Italy, the Canary Islands and North Africa. The taxon is distributed mainly in eastern Mediterranean of Turkey, in Antalya, Aydın, İzmir, Muğla and Tekirdağ (Figure 1.3, Turkish Plants Data Service).

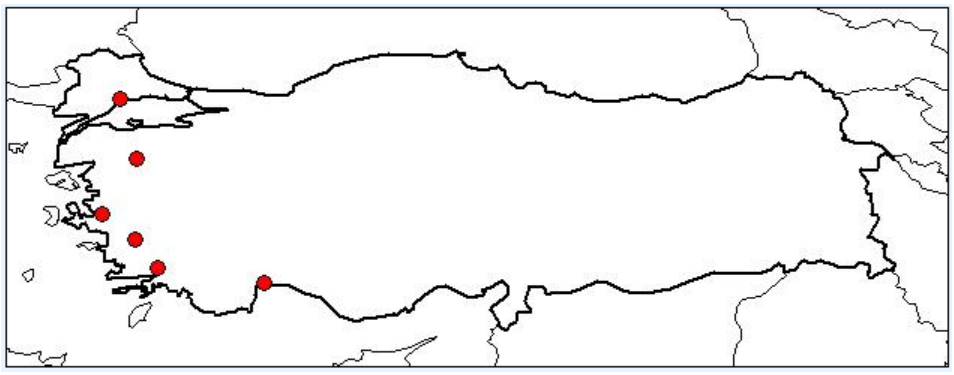


Figure 1.3 Distribution of *S. fruticosa* over Turkey

(http://www.tubives.com/index.php?sayfa=1&tax_id=8036)

1.2 Cancer

Cancer is a type of disease in which cells are divided abnormally and hurts the tissues. Cancerous cells are capable of passing to other tissues as well as faraway places in the body by metastasis. Cancer development process is divided into 3 stages named as initiation, promotion and progression. Initiation step cause damage to DNA and chromosome that regulates the gene expression of the cell. Promotion is a long term stage of cellular growth of genomically unstable cells assisted by inflammation. In the progression stage, cells cause more damage to their genome, evolving themselves into an invasive malignant tumor (Poirier, 1987). Different types of cancers share similar abnormal physiology showing malignant growth (Hanahan and Weinberg, 2000). There are many factors leading to formation of cancer. It can be hereditary that pass between generations with inherited DNA damage, or it may be acquired with environmental factors including, ultraviolet radiation, ionizing radiation, smoking, diet, nutrition, alcohol, chemicals and life style. According to World Cancer Report (2014), Cancer is a leading cause of death on worldwide in 2012 and the most common cancer types are lung, liver, stomach, colorectal and breast cancers**.**

1.2.1 Colorectal Cancer

Colorectal cancer also known as colon cancer or rectal cancer is one of the leading causes of death worldwide. It is the third most common malignant disease among men and the second among women making up about 10% of all cases (Ku et al., 2012; Winawer, 2007). According to the data reported by Turkish Ministry of Health, colorectal cancer is the third most commonly diagnosed cancer in women and men respectively for the years 2007-2008 in Turkey. 1 million colorectal cancer is diagnosed every year and 500.000 patients die because of the disease (Winawer, 2007). However, colorectal cancers are one of the most preventable types of cancers since they are highly related with nutritional habits and life style (Stracci et al., 2014). Human, animal, and cell line studies indicate that many herbal products can exert significant chemopreventive effects (Enayat et al., 2013).

1.3 Free Radicals and Cancer

Free radicals are molecules or atoms containing one or more unpaired electrons in their orbitals. The presence of unpaired electron increase the reactivity of the free radicals.

Reactive oxygen species (ROS) that are formed from oxygen molecules are the most significant radicals for the biological systems. There are many factors in the formation of ROS. They can be generated by environmental factors such as tobacco smoke, UV light, X-rays and gamma rays. They can also be produced by mitochondria catalyzed electron transport chain and by other mechanisms in other biological systems.

ROS have beneficial effects on infectious disease defense, mitogenic response induction and cellular signaling system regulation. On the other hand,if their balance in the body is disrupted, ROS can be harmful for cell structures including lipids, proteins and nucleic acids. Superoxide anion ($O2^{\bullet}$) is one of the main ROS. Superoxide anion is able to react with other molecules by enzyme or metal catalyzed reactions leading to formation of secondary reactive oxygen species.

Redox active metals (Fe, Cu) have important factors for the generation of various free radical species. When the amount of superoxide exceed the treshold, it acts as an oxidant. Superoxide radicals take part in Haber-Weiss reaction ($O2^{\bullet-}+H_2O_2 \rightarrow O_2+{}^{\bullet}OH+OH^{-}$) and combines a Fenton reaction leading to reduction of $Fe^{3}+$ by superoxide anion and producing Fe^{2+} and oxygen.

$$(Fe\ (III) + O2^{\bullet-} \rightarrow Fe\ (II) + O2).$$

Overall reaction:

$$Fe\ (II) + H2O2 \rightarrow Fe\ (III) + {}^{\bullet}OH+OH^{-}$$

Hydroxyl radicals (•OH) generated by diverse mechanisms are highly reactive species. It can also be produced by ionizing radiation ($H_2O \rightarrow H^+ + OH^\bullet$) and photolytic decomposition of alkyl hydroperoxides.

Peroxy radicals ($ROO^\bullet$) have some roles in DNA cleavage and protein backbone modification. DNA damage inductions are amplified by superoxide anion and peroxy radicals when present together (Wang et al., 2011; Klaunig et al., 2010; Valko et al., 2006).

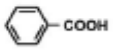
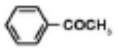
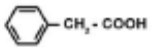
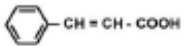
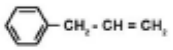
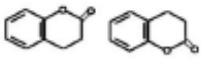
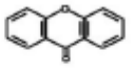
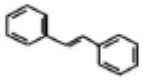
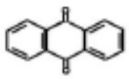
Oxidative stress takes some roles in the formation of carcinogenesis by gene mutations and structural conversions in DNA in initiation step; preventing the cell-to-cell communication, modification of secondary messenger system and induction of abnormal gene expression in promotion step; further changes in altered cell population triggered by ROS in progression step (Reuter et al., 2010).

Chronic inflammation related to ROS has been demonstrated to be inducer of cancer. During inflammation, superoxide anion, hydroxyl radical and hydrogen peroxide are released by neutrophils and macrophages. Procarcinogens such as aromatic amines and aflatoxins can be activated by inflammatory cells via ROS-dependent mechanisms. They can also induce DNA base damages. One of matrix metallaproteinases, gelatinases having important role in tumor invasion and metastasis was found to be upregulated by ROS related chronic inflammation (Reuter et al., 2010).

1.4 Polyphenols and Cancer

Phenolic compounds that exist either as simple phenol molecules, or highly polymerized forms are composed of a hydroxyl group and an aromatic hydrocarbon ring. They are generally found as conjugated with a sugar residue, carboxylic and organic acids, amines, and lipids or associated with other phenols (Bravo, 1998). Polyphenols are divided into different classes according to their basic structure (Harborne, 1989). Chemical Structures of Polyphenols was given in Table 1.1.

Table1.1 Chemical Structures of Polyphenols

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	
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	$(C_6$-$C_3)_n$	

Polyphenols are produced by plants for several functions such as plant pigmentation, reproduction and protection against bacterial pathogens or UV light (Oksana et al., 2012). Due to their ability to make complex structures with proteins, polyphenols have a harsh taste, producing dryness and roughness in the mouth. They can also make complex with polysachharide structures of the cellular matrix.

These properties of polyphenols provides an antiherbivore and antipathogenic activity for most of the plants (Feeny 1970; Haslam 1988).

Consumption of fresh fruits and vegetables has an important impact on human health, particularly by prevention of degenerative diseases such as cardiovascular diseases, neurological diseases and cancer. Therefore, they form an integral part of the human diet (Ku et al., 2012).

It was demonstrated that polyphenols are very good antioxidants neutralizing the adverse effects of reactive oxygen or nitrogen species and thus, leading to prevention of carcinogenesis (Henderson et al., 2000). Phenolic acids, a member of nonenzymatic antioxidants, can scavenge free radicals and also chelate metal ions, decreasing the pro-oxidant activity (Fridovich, 1974).

Phenolic compounds are capable of donating a hydrogen atom by their phenolic hydroxyl groups to a free radical (R). An unpaired electron of a free radical can also be delocalized by conjugated aromatic system of phenolic compounds (Figure 1.4).

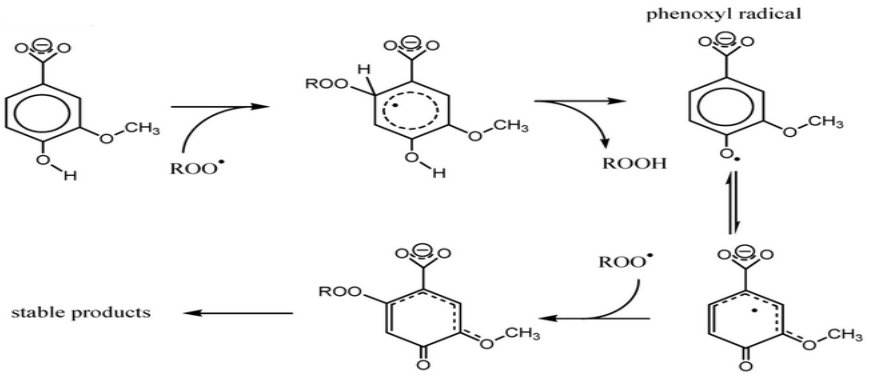


Figure 1.4 Delocalization of a free radical by phenolic compounds

As shown above, phenoxy radical intermediates (PO•) are formed in the result of donation of hydrogen atoms to free radicals (R) by phenolic compounds (POH). Because of the high stability of these intermediates, they can easily suppress the free radical chain reaction. Upon the phenoxy radical intermediates react with other

formed free radicals, they terminate the propagation (Scalbert et al., 2005; Dai et al., 2010).

Many polyphenols, such as quercetin, catechins, ellagic acid, resveratrol, play important roles for the reduction of the tumor or their growth on human cancer cell line (Yang et al, 2001; Arts and Hollman, 2005).

It was indicated that instead of individual compound, combination of polyphenols provides greater responses (Dai et al., 2010). Phenolic acids are highly abundant in *Salvia* species. Salvianolic acids A-K or yunnaneic acids A-H are unique to *Salvia* species. Benzoic acids for example 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic 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) are other identified phenolics in *Salvia* species (Lu and Foo, 2002).

The most commonly found polyphenols in plants are flavonoids mainly in the forms of flavones, flavonols and their glycosides, anthocyanins and proanthocyanidins. Flavonoids play important roles on lessening some diseases such as cancer and cardiovascular disease (Bravo, 1998; Ignat et al., 2011). Basic structure of flavonoids was shown in Figure 1.5.

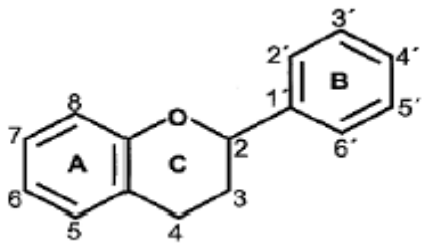


Figure 1.5 Basic structure of flavonoids

The most significant flavonoids for Salvia species are luteolin, apigenin, cirsimaritin, nepetin, cirsiliol, salvigenin eupatorin. *S. fruticosa* contains high amount of phenolic compounds such as caffeic acid, vanillic acid, ferulic acid, rutin,

apigenin, quercetin, catechin, and luteolin but, mainly rosmarinic acid (Lu and Foo, 2002; Aşkun et al., 2009).

1.5 Antiproliferative and Apoptosis Properties of Polyphenols

Phenolic compounds take some roles in cell growth and apoptosis regulation by interacting with cell signaling pathways.

Cell cycle control based on the interactions between cyclins and cyclin-dependent kinases (CDKs). CDK inhibitors (CDKIs) are the authorities for this interactions' control. When Cyclin A combines to CDK2, cell growth is progressed through S phase of the cell cycle (Brown et al., 2012). The cell cycle, mitosis and meiosis was given in Figure 1.6.

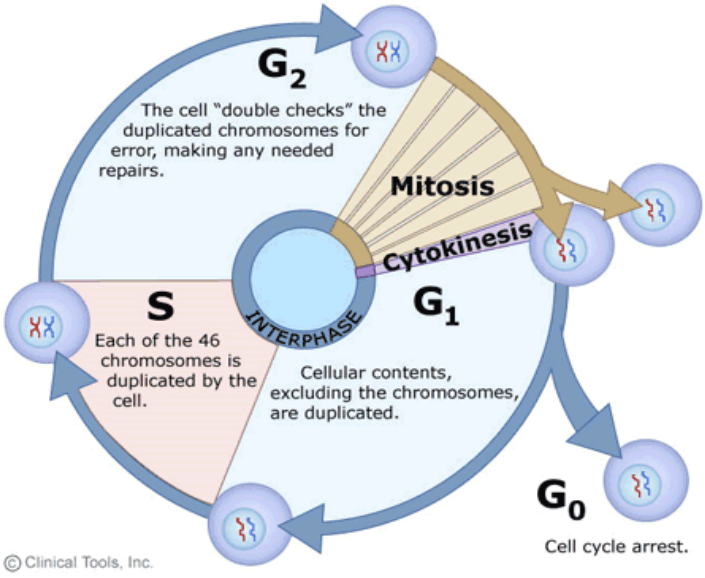


Figure 1.6 The cell cycle, mitosis and meiosis

(Virtual Genetics Education Centre)

In one of the study, it was demonstrated that polyphenols in red wine decrease the cyclin A gene expression by inhibiting its transcription factor expression (Lijima et al., 2000).

It was also shown that increased expression of CDK inhibitors and decreased expression of cyclin A and B genes resulted in stop in G0/G1 and G2/S phases of cell cycle leading to decline in cell growth (Brown et al., 2012).

(−)-epigallocatechin-3-gallate (EGCG) found in tea (*Camellia senesis*) was shown to inhibit CDKs, directly. It induced cell cycle arrest in G0/G1 phase in several cell lines such as breast, prostate and head cancers (Hou et al., 2004).

Apoptosis, a programmed cell death, are carried out through 2 pathways that are extrinsic and intrinsic (mitochondrial) pathways. Extrinsic pathway is activated upon a specific ligand binds to cell surface death receptor (DR) which is a tumor necrosis factor (TNF) receptor superfamily. After that, procaspase 8 binds to death inducing signaling complex. On the other hand, intrinsic pathway is activated by agents such as ionizing radiation and chemicals that increase the oxidative stress of the cells. Consequently, when mitochondrial membrane potential is disrupted, cytochrome c, Apaf-1 (apoptotic protease activating factor 1), endonuclease G and AIF (apoptosis inducing factor) are released from mitochondria. A supra molecular complex that is called apoptosome is formed by Cytochrome c, Apaf-1, dATP and procaspase 9 (Almagro MC and Vucic D, 2012). The intrinsic and extrinsic apoptotic pathways was shown in Figure 1.7.

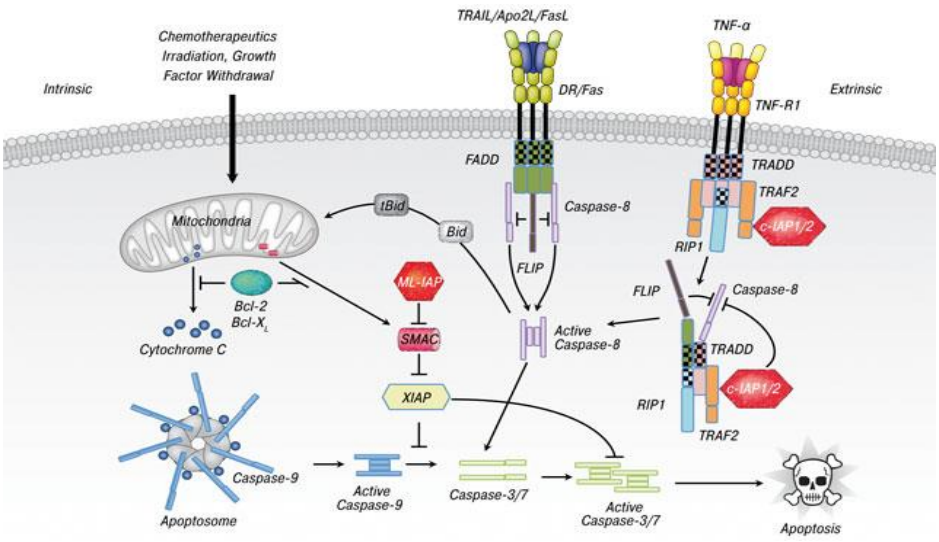


Figure 1.7 The intrinsic and extrinsic apoptotic pathways

Procaspase 8 and 9 are cleaved to their active form by these both pathways and then, they activate procaspase 3. These activated caspases have an important role in cytoskeleton cleavage and DNA degradation.

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

Antiproliferative effects of berry components on colon cancer models was studied and it was found that ellagic acid decreased the expression of Bcl-XL and increased the cytochrome c levels in parallel with caspase 9 and 3 activation (Brown et al., 2012).

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

1.6 Phase I and Phase II Drug Metabolizing Enzymes

Drug metabolism is the biochemical process that living organism modifies the xenobiotics to detoxify or activate the substances by specialized enzyme systems. Xenobiotic compounds follow 4 steps in the body; absorption, distribution, metabolism and excretion. After taken in to body, a pharmaceutical compound follows the metabolic pathways in which it is detoxified and deactivated or sometimes transformed to active poisonous compounds by drug metabolism.

Drug metabolism is divided into 2 phases; Phase I and Phase II enzymes. Phase I enzymes are Cytochrome P450 oxidases that perform oxidation, hydrolysis and reduction reactions by adding reactive groups such as hydroxyl radical to the

xenobiotics. Then these reactive intermediates are further metabolized by Phase II enzymes performing conjugation reactions such as glucuronidation, sulfation, acetylation, methylation and glutathione and amino acid conjugations. Consequently, xenobiotics are converted into water soluble compounds so that they can easily be excreted through urine or bile. Phase I and Phase II reactions was shown in Figure 1.8.

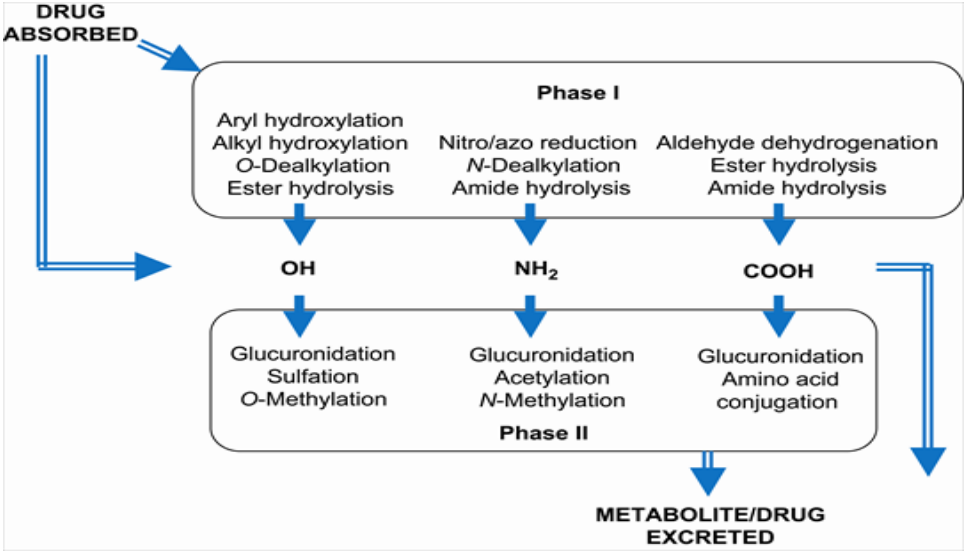


Figure 1.8 Functions of Phase I and Phase II Enzymes in Drug Metabolism

1.6.1 Phase I Drug Metabolizing Enzymes

Cytochrome P450 (CYP) enzymes are a superfamily of heme-enzymes found in all organisms involved in the metabolism of most of the xenobiotics and endogenous compounds including fatty acids and hormones. The term P450 refers to a pigment that absorbs light at 450 nm upon reduced form is exposed to carbon monoxide (Omura & Sato 1964). Figure 1.9 shows the Cytochrome P450 enzymes and their regulations.

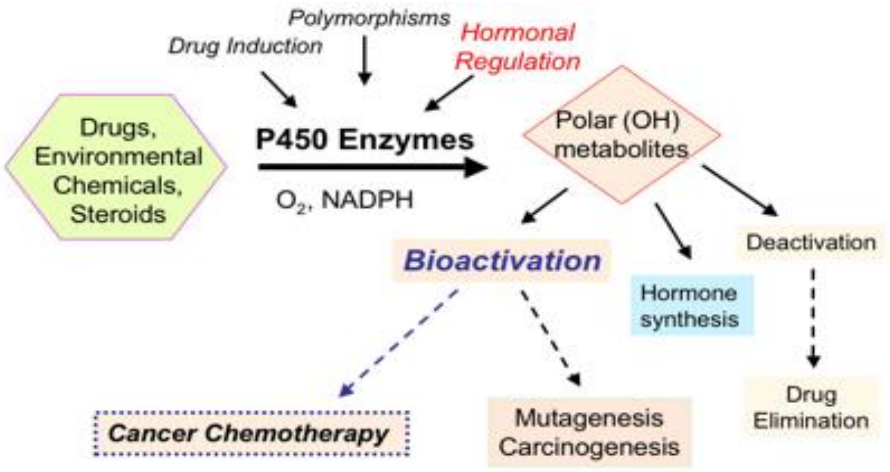


Figure 1. 9 Cytochrome P450 enzymes and their regulations

(http://people.bu.edu/djw)

CYPs are membrane bound enzymes mainly located on the endoplasmic reticulum of the cells in a wide range of tissues. Reactions of xenobiotics are generally catalyzed by CYPs using NADPH and O_2. displayed below;

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

The active site of CYPs includes a heme-iron complex in the center called as ferriprotoporphyrin 9. Common catalytic mechanism for the oxygenation of a substrate by CYPs were shown in Figure 1.10.

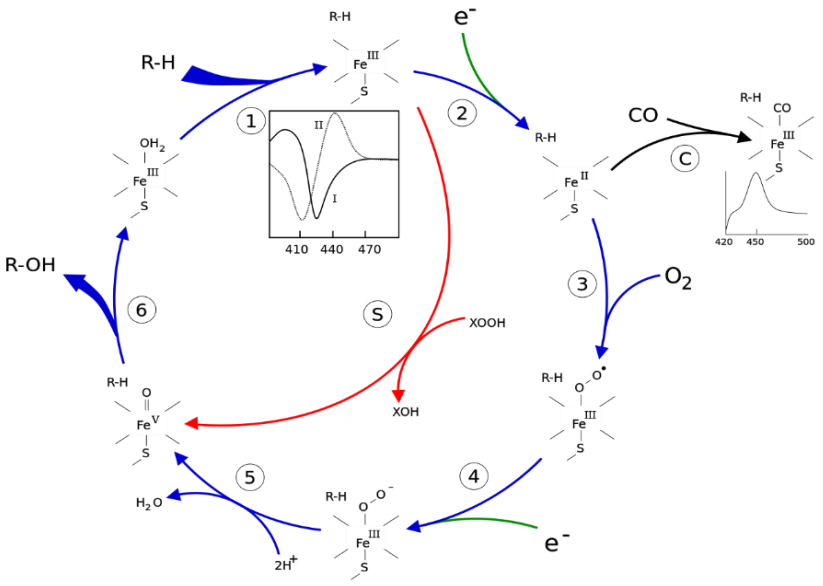


Figure 1.10 Catalytic cycle for the oxidation of substrates by CYPs (Guengerich, 2001).

The catalytic cycle for the oxidation of substrates by CYPs include the following steps; In the first step, substrate is bound to ferric (Fe^{3+}) form of cytochrome P450 enzyme. In the second step, NADPH-P450 reductase transfers an electron from NAD(P)H reducing the Fe^{3+} to Fe^{2+} (ferrous). In the third step, O_2 binds to the reduced enzyme-substrate complex that form an unstable structure leading to generation of ferric iron and superoxide anion.

In the fourth step, NADPH-P450 reductase catalyze the second reduction by reducing the molecular oxygen and forming "activated oxygen"-CYP-substrate complex. In the fifth step, H_2O is released by leading to generation of high-valent FeO^{3+} and finally, it is followed by the dissociation of the product from the enzyme (Guengerich, 2001).

Many isoforms of Cytochrom P450 enzymes are expressed in a wide range of tissues containing kidney, lung, brain, colorectal and gastrointestinal tract (Omiecinski et al., 1990; Adali et al., 1996; Meyer et al., 2002; Arinç et al., 2007).

In humans, 18 families with 43 subfamilies of CYP are present. Each isoform of CYPs displays different functions (Table 1.2). CYP1, CYP2 and CYP3 families take roles mainly in xenobiotic metabolism (Wang et al. 2009; Walsh et al., 2013).

Table 1.2 Human Cytochrome P450 gene families (Nelson, 2009)

Human CYP Families	Names	Function
CYP1 (3 genes)	1A1, 1A2, 1B1	Xenobiotic metabolism
CYP2 (16 genes)	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1	Xenobiotic and steroid metabolism
CYP3 (4 genes)	3A4, 3A5, 3A7, 3A43	Xenobiotic and steroid metabolism
CYP4 (12 genes)	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Fatty acid metabolism
CYP5 (1 gene)	5A1	Thromboxane A_2 synthesis
CYP7(2 genes)	7A1, 7B1	Bile acid biosynthesis
CYP8 (2 genes)	8A1, 8B1	Prostacyclin synthesis and bile acid biosynthesis
CYP11 (3 genes)	11A1, 11B1, 11B2	Steroid biosynthesis
CYP17 (1 gene)	17A1	Estrogen and testosterone biosynthesis
CYP19 (1 gene)	19A1	Estrogen hormone biosynthesis
CYP20 (1 gene)	20A1	Drug metabolism and cholesterol biosynthesis
CYP21 (1 gene)	21A2	Steroid biosynthesis
CYP24 (1 gene)	24A1	Vitamin D degradation
CYP26 (3 genes)	26A1, 26B1, 26C1	Retinoic acid metabolism
CYP27 (3 genes)	27A1, 27B1, 27C1	Bile acid biosynthesis and vitamin D_3 activation
CYP39 (1 gene)	39A1	Cholesterol biosynthesis
CYP46 (1 gene)	46A1	Cholesterol biosynthesis
CYP51 (1 gene)	51A1	Cholesterol biosynthesis

1.6.1.1 CYP 1A1

CYP1A1 (EC 1.14.14.1) known as aryl hydrocarbon hydroxylase is localized on the membrane of endoplasmic reticulum and mitochondria of extrahepatic tissues including lung and gastrointestinal tract and also in cytosol of kidney and brain tissues (Omiecinski et al., 1990; Meyer et al., 2002). The gene of CYP1A1 is located on chromosome 15 with 2608 bp length of mRNA while molecular weight

of the CYP1A1 protein is 58kDa that composed of 512 amino acids. The crystal structure of CYP1A1 protein is shown in Figure 1.11.

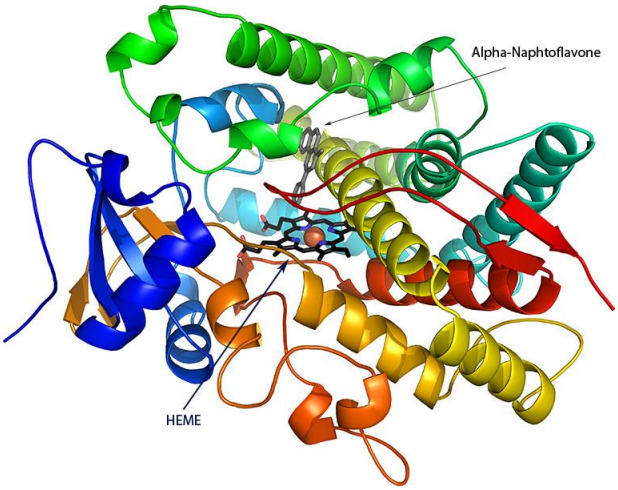


Figure 1.11 Human CYP1A1 with alpha-naphthoflavone (Walsh et al. 2013)

Many polycyclic aromatic hydrocarbons (PAHs) are metabolized to carcinogens by CYP1A1 via epoxidation reactions forming epoxide intermediates leading to more active metabolites, diol epoxides by the epoxide hydrolase (Wei et al., 1996). Conversion of inactive Benzo[*a*]pyrene (BaP) to mutagenic metabolite, BaP-7,8-diol-9,10-epoxide, forming DNA adducts mainly with deoxyguanosine is an example of catalytic activity of CYP1A1 (Androutsopoulos et al., 2009). Figure 1.12 displays the conversion of BaP to mutagenic metabolite by CYP1A1.

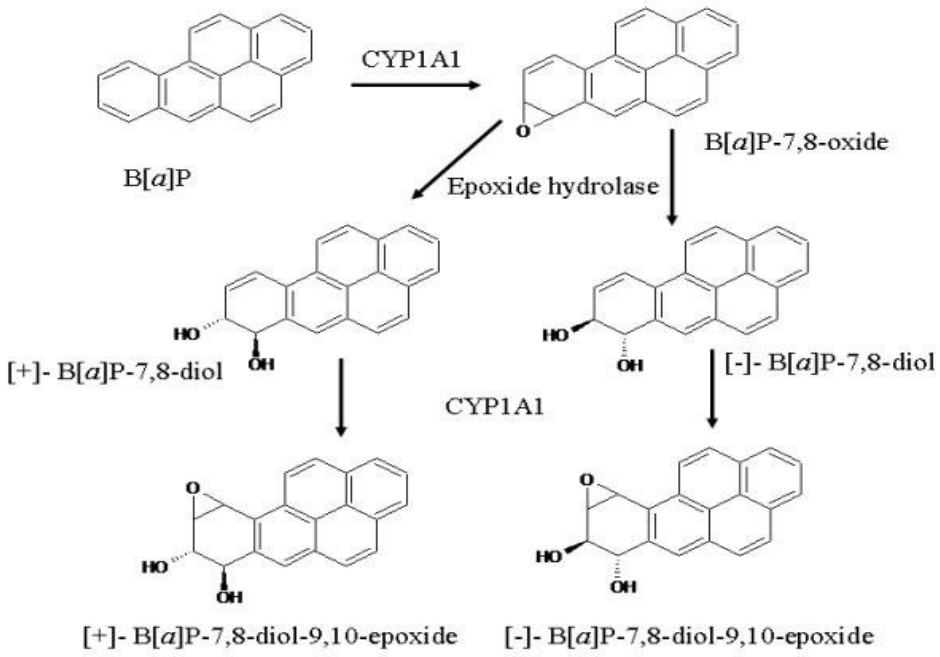


Figure 1.12 Conversion of BaP to mutagenic metabolite by CYP1A1

1.6.1.2 CYP 1A2

CYP1A2 (EC 1.14.14.1) localizes to the endoplasmic reticulum. Some polycyclic aromatic hydrocarbons (PAHs) are metabolized to carcinogenic intermediates by CYP1A2. Xenobiotic substrates for CYP1A2 enzyme include caffeine, aflatoxin B1, and acetaminophen but, mainly cigarette smoke. Expression of CYP1A2 is induced by various dietary constituents such as cabbages, cauliflower and broccoli (Nelson et al., 2004).

Acetaminophen (APAP), substrates of CYP1A2 gene, cause hepatic necrosis under overdose conditions. It is converted to reactive intermediate by CYP1A2 and then detoxified by conjugation with glutathione (GSH). Upon the decreasing the GSH level, intermediates reacts more extensively with hepatic proteins that leads to hepatocellular damage (Jaiswal et al., 1987). The crystal structure of CYP1A2 protein is given in Figure 1.13.

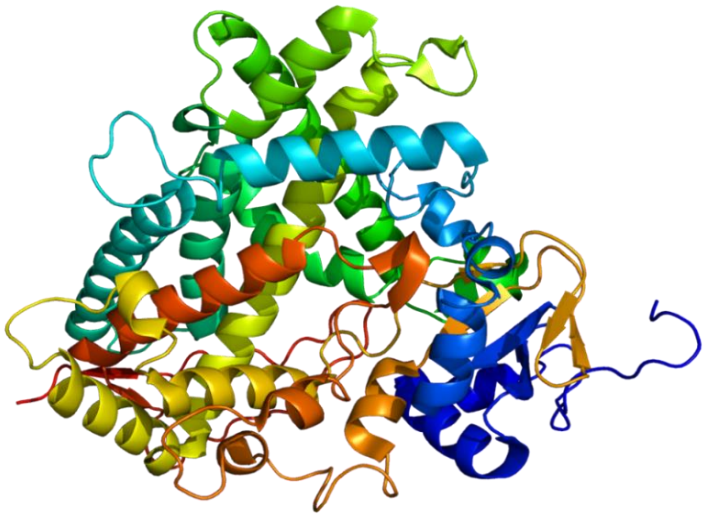


Figure 1.13 Polypeptide structure of Human CYP 1A2 (PDB)

1.6.1.3 CYP 2E1

CYP2E1 (EC 1.14.13) gene located on chromosome 10 with 62 kDa molecular weight is composed of 493 amino acids. The crystal structure of CYP2E1 was given in Figure 1.14.

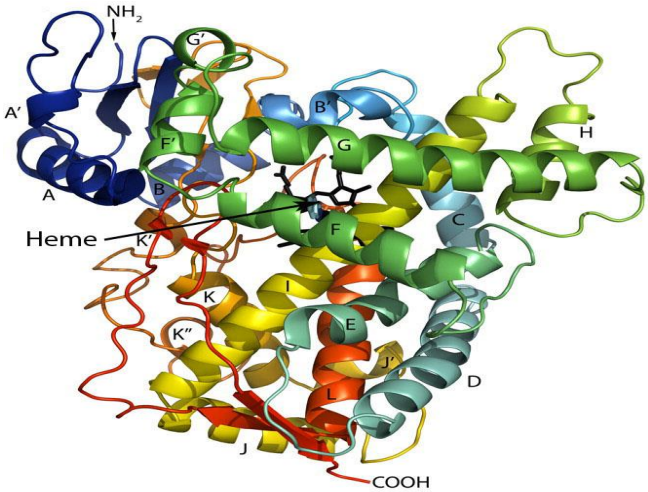


Figure 1.14 Polypeptide structure of Human CYP2E1 (Porubsky et al., 2008).

CYP2E1 is mainly expressed in the E.R. and mitochondria and also found in plasma membrane of hepatocytes (Seliskar & Rozman, 2007). In addition, CYP2E1 is expressed in a wide range of extrahepatic tissues such as kidney, lung and brain (Botto et al., 1994). The metabolism of both endogenous substrates, such as acetone, fatty acids; and exogenous substrates, such as carbon tetrachloride, nitrosamines, chloroform are carried out by CYP2E1 enzyme (Gonzalez, 2005). It is also responsible for the formation of alcohol mediated reactive oxygen species (Cederbaum, 2006; Guengerich et al., 1991).

Conversion of drugs such as acetaminophen, pre-toxins and procarcinogens to active form are catalyzed by CYP2E1 (Gonzalez, 2005). It was reported that there is a strict correlation between CYP2E1 expression and nasopharyngeal carcinoma susceptibility among cigarette smokers. Because cigarette smoke contains *N*-nitrosonornicotine, metabolized by CYP2E1, causes DNA methylation and carcinogenesis (Hildesheim & Anderson, 1997).

Several factors such as genetic factors, age, diet, drugs and chemicals affect the CYP2E1 gene expression of. It was reported that diabetes induces the protein expression and catalytic activity of CYP2E1 (Arinç et al., 2005; Arinç et al., 2007).

It was demostrated that gene expression of CYP2E1 was induced by ethanol while the expression of CYP2E1 was downregulated by resveratrol that is a plant phenolic compound having potential to prevent carcinogenesis (Carroccio et al. 1994; Celik & Arinç, 2010).

1.6.1.4 CYP 3A4

CYP3A4 (EC 1.14.13.97) is an important enzyme in the drug metabolism, mainly located in the liver and in the intestine. In humans, the CYP3A4 enzyme is encoded by the CYP3A4 gene located in the chromosome 7. Polypeptide structure of CYP3A4 was given in Figure 1.15.

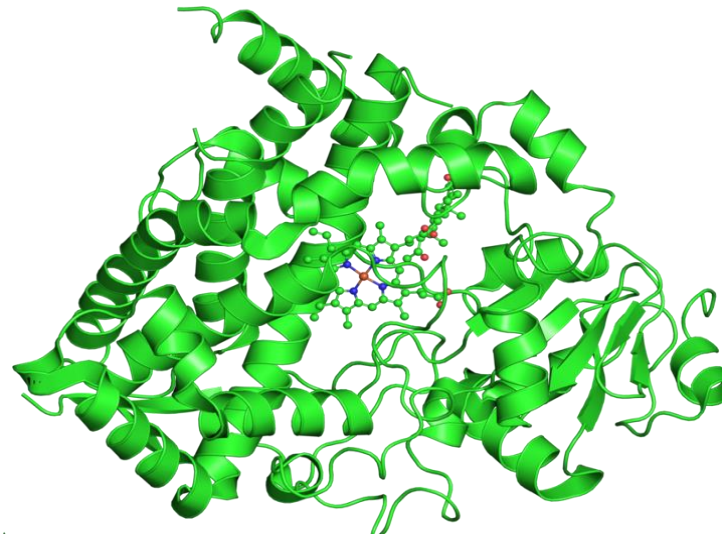


Figure 1.15 Polypeptide structure of Human CYP3A4

CYP3A4 plays important roles on deactivation of many drugs and also activation of drugs or procarcinogens to carcinogen agents. It is involved in the metabolism of nearly half the drugs used commercially today, such as acetaminophen, codeine, diazepam, and erythromycin. (Hashimoto et al., 1993).

It was demonstrated that some substances, such as grapefruit juice and some drugs, interfere with the action of CYP3A4 increasing the bioavailability of drugs (He et al., 1998; Bailey et al., 1998; Garg et al., 1998).

The expression of Cytochrom P450 enzymes are controlled by transcriptional, posttranscriptional and posttranslational actions (Umeno et al., 1988; Lieber et al., 1978; Roberts et al., 1995).

1.6.2 Phase II Drug Metabolizing Enzymes

Xenobiotics or active chemical carcinogens are converted to less toxic or inactive metabolites by Phase II drug-metabolizing enzymes such as Glutathione S-Transferase (GSTs), UDP-glucuronosyltransferases (UGTs), Sulfotransferases (SULTs) shown in Figure 1.16.

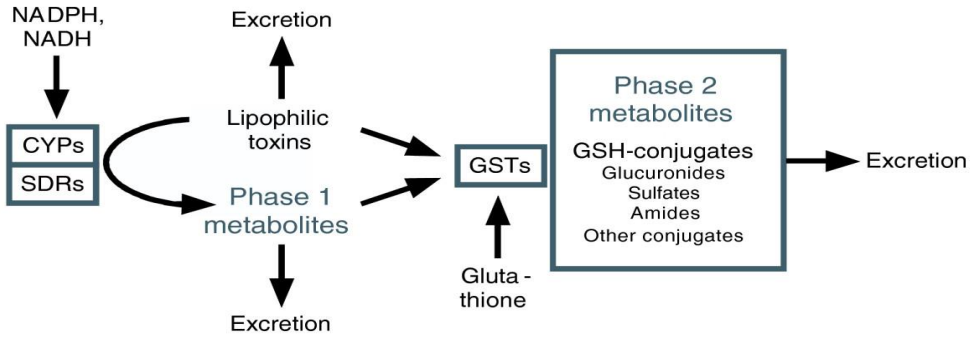


Figure 1.16 Conjugation of xenobiotics by Phase II reactions

(http://www.genomebiology.com/2007/8/7/R132/figure/F3?highres=y)

Conjugation of many xenobiotics to more hydrophilic metabolites are catalyzed by Phase II reactions for the elimination readily from the body (Jacoby and Ziegler, 1990). Conjugation reactions are classified as glucuronidation, sulfation, methylation and amino acid conjugations (Jacova et al., 2010). Genetic background, chemical structure of the agents, sex, endocrine status, age, diet, and the presence of other chemicals determine the balance of detoxification and activation reactions.

Phase I and phase II enzymes are regulated by phytochemicals via transcriptional regulation or interacting with enzyme activity (Galli, 2007).

UDP- Glucoronyl transferase 1A1 (UGT1A1) was induced by flavone chrysin treatment in HepG2 and Caco-2 cell lines. Also, UGT1A1 enzyme level rised up with quercetin, naringenin, tangeretin and galangin treatments (Moon et al., 2006).

1.6.2.1 Glutathione S-Transferases (GSTs)

GSTs (EC 2.5.1.18) are the enzymes found in all living organisms. They are the phase II detoxifying enzymes metabolizing the electrophilic substrates containing carbon, nitrogen and sulfur atom by conjugation reaction with endogenous tripeptide glutathione (GSH) resulting in less reactive and more water soluble

products (Bausova and Skalova, 2012). Figure 1.17 shows the glutathione conjugate formation.

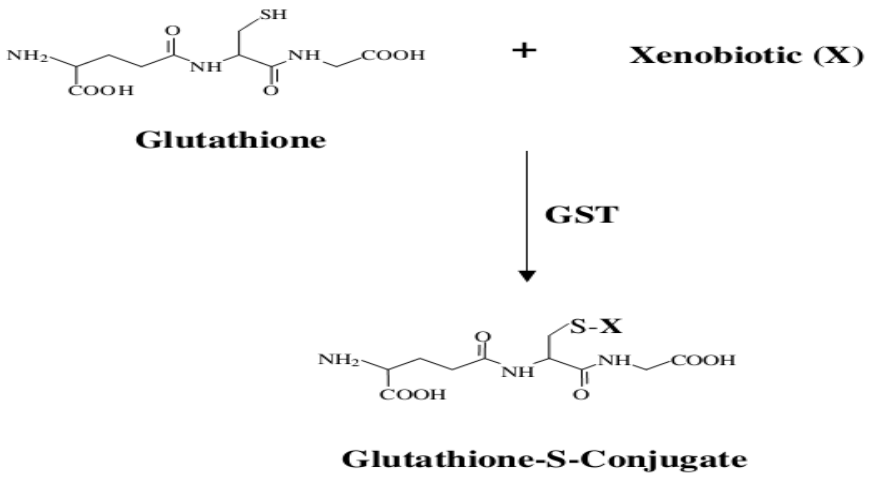


Figure 1.17 Glutathione conjugate formation (Townsend and Tew, 2003)

GST enzymes are categorized as cytosolic, mitochondrial and microsomal (Sheenan et al., 2001). while cytosolic and mitochondrial GSTs are soluble enzymes, microsomal GSTs are membrane associated proteins. The largest GST family are composed of cytosolic GSTs having specific activities. According to amino acid sequence similarities, cytosolic GSTs are classified as alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega.

Glutathione S Transferases contain N-terminal and C-terminal domains having glutathione (GSH) binding site (G-site) and hydrophobic site (H-site). Features of H-site amino acid residues display differences among GSTs resulting in substrate specificity (Figure 1.18).

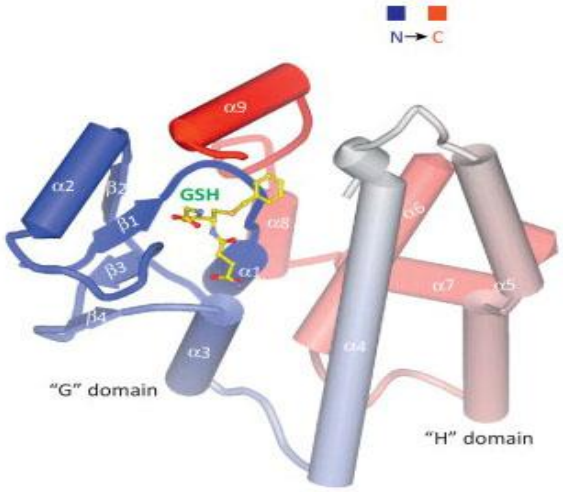


Figure 1.18 3D structure of GST enzyme (Wu & Dong, 2012)

In addition to the elimination of xenobiotics, GSTs plays important role for the biosynthesis of important inflammatory mediator, prostaglandin, making this enzyme family target for the drugs (Matsuoka, 2000).

Convertion of prostaglandin H2 to prostaglandin D2 that is a mediator of allergy and inflammation responses is catalyzed by GST S1-1. Nocodazole, inhibitor of GSTS1-1, is used for anti-allergic and anti-inflammatory drug (Weber et al., 2010).

Some of the drugs including cisplatin and carmustine are substrates of GSTP1 and GSTM1 enzymes, respectively and they are excreted by conjugation with glutathione (Ban et al., 1996; Smith et al., 1989).

On the other hand, in some cases, glutathione conjugation leads to formation of more reactive compounds. This feature of GSTs is used for cancer chemotherapy. Cytotoxic drug TER286 or TLK 286 is metabolized with GSTs resulting in the formation of active metabolite cyclophosphamide chemotherapeutic that is an effective drug for breast cancer treatment (Morgan et al., 1998).

GSTO1 is capable of contacting with ryanodine receptors of calcium channel by preventing the cells to go apoptosis by calcium channel mobilization from intracellular stores (Sherrat and Hayes, 2002; Awasthi, 2007).

Overexpression of GST enzymes results in resistance against anticancer agents and carcinogens. It was demonstrated that overexpression of GSTO1 gene caused to resistance towards cisplatin drug leading to decrease in apoptosis by activating survival mechanisms and inhibiting apoptosis related JNK activation pathway in HeLa cells (Piaggi et al., 2010). Figure 1.19 displays the survival mechanisms and inhibiting apoptosis related JNK activation pathway.

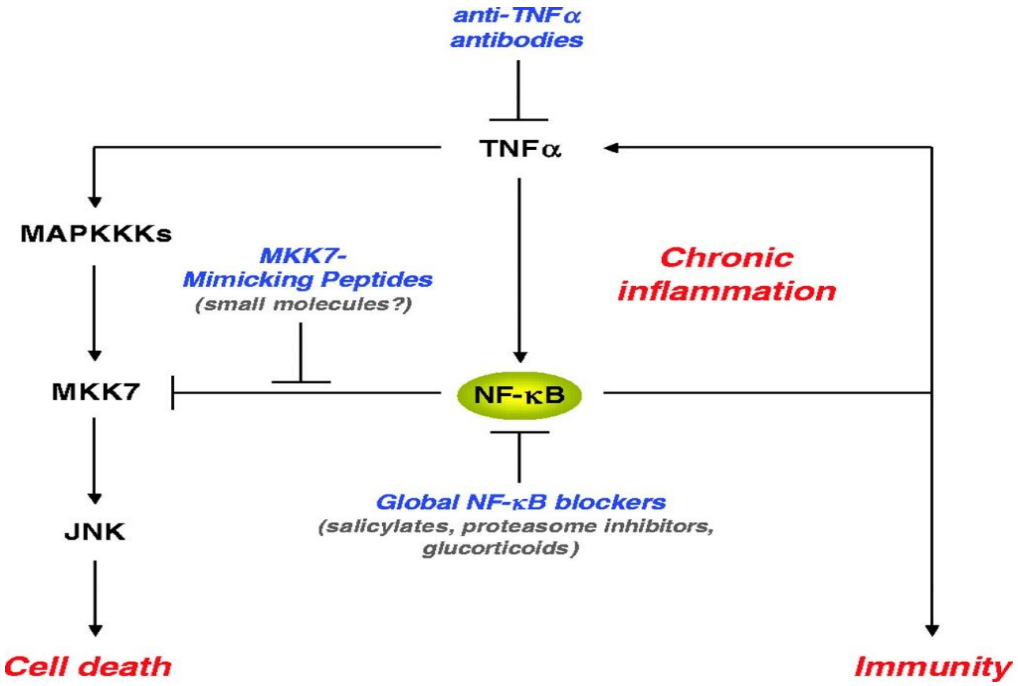


Figure 1.19 Survival mechanism and inhibiting apoptosis related JNK activation pathway (http://jcs.biologists.org/content/117/22/5197/F3.expansion.html)

1.6.3 Antioxidant Enzymes

Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. These reactons produce free radicals, in turn, starting chain reactions (Figure 1.20).

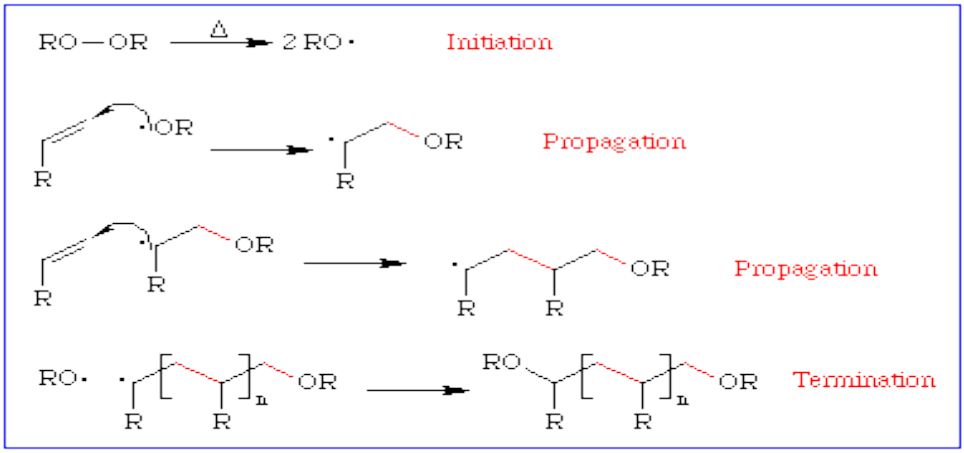


Figure 1.20 Formation and termination of free radicals (https://en.wikipedia.org/wiki/Photo-oxidation_of_polymers)

Free radicals cause damage or death to the cells. Antioxidants terminate these chain reactions by removing free radical intermadiates and inhibit other oxidation reactions (Figure 21). They do this by being oxidized themselves. Antioxidants are often reducing agents such as thiols, ascorbic acid (vitamin C), or phenols (Sies H., 1997).

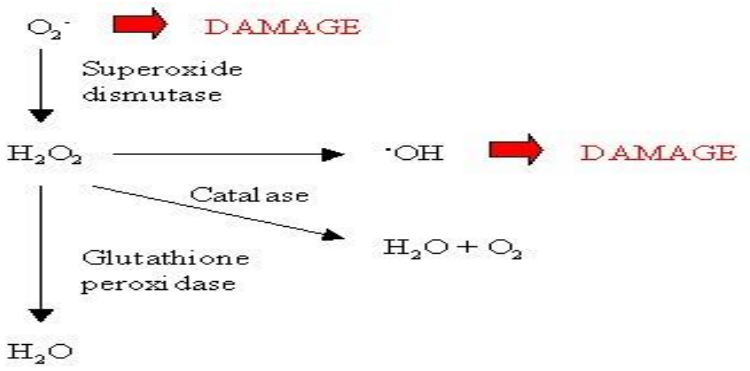


Figure 1.21 Termination of superoxide radical by antioxidant enzymes (www. drproctor.com; Free radicals and human diseases)

Plants and animals have complex systems of multiple types of antioxidants including glutathione, vitamin C, Vitamin A and vitamin E as well as enzymes such as catalase, superoxide dismutase (SOD) and Glutathione peroxidases (GPx).

Antioxidant enzymes can be inhibited, induced and activated by endogenous effectors. They have important functions for the regulation of metabolic pathways in the cells.

1.6.3.1 Catalase

Catalase (EC 1.11.1.6) is a heme containing tetrameric protein with a molecular weight of 240 kD. Figure 1.22 displays the cristal structure of Catalase

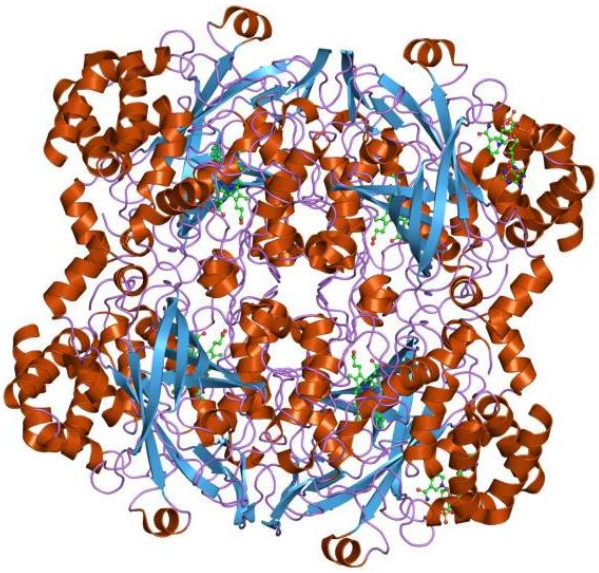


Figure 1.22 Cristal structure of Catalase

It is one of the most common enzymes found in most of all living organisms. It catalyzes the decomposition of hydrogen peroxide to water and oxygen molecule (Figure 1.23).

Figure 1.23 Decomposition of hydrogen peroxide by Catalase

(www. worthington-biochem.com)

In addition, catalase have peroxidative function converting peroxides (ROOH) into alcohol (ROH) and water. Thus, cells are protected from the adverse effects of lipid peroxidations (Chelikani et al., 2004). Catalase is mainly located in peroxisomes (Davias et al., 1979), cytosol of human neutrophils (Ballinger et al., 1994) and heart mithocondria of rats (Radi et al., 1991). Hydrogen peroxide is mainly degraded by GPx in erythrocytes that have a high amounts of non-peroxisomal catalase (Nicholls, 1972).

1.6.3.2 Glutathione Peroxidase (GPx)

Glutathione Peroxidase (EC 1.11.1.9) are homotetrameric water soluble enzymes (Figure 1.24), generally located in cytosolic and mithocondrial compartments of cells (Epp et al., 1983).

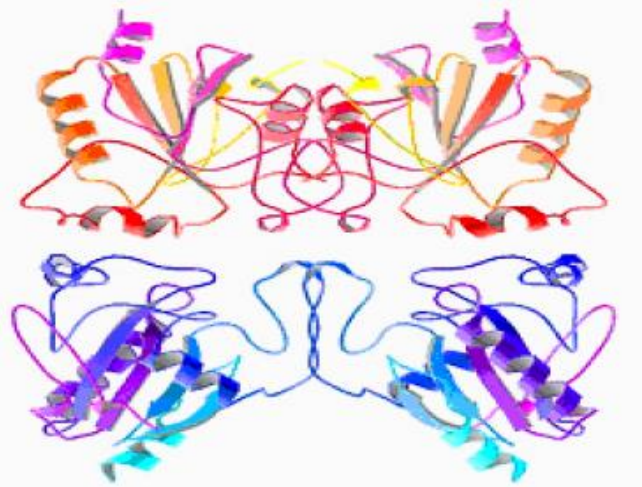


Figure 1.24 Cristal structure of GPx

It catalyzes the reduction of hydrogen peroxides to water and alcohols at the expense of GSH (Flohe, 1989 and Ursini et al., 1995). GPx protect the organism from oxidative damage. Active sites of Glutathione peroxidases (GPx1, GPx2, GPx3, and GPx4) contain selenium in it and they are members of selanoproteins (Flohe et al., 1973, Rotruck et al., 1973 and Stadtman, 1991). The reaction in Glutathione redox cycle catalyzed by GPx was shown in Figure 1.25.

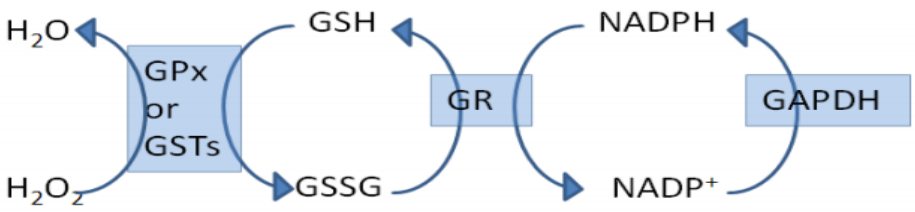


Figure 1.25 Glutathione redox cycle (**GPx:** Glutathione Peroxidase, **GST:** Glutathione S-transferase, **GR:** Glutatihone Reductase, **GSH:** reduced monomeric glutathione, **GS–SG**: glutathione disulfide)

1.8 Scope of thesis

Salvia species with the presence of polyphenols in their structures are one of the important group of medicinal plants. *Salvia species* are used in folk medicines for various purposes due to their antioxidant, anticarcinogen, antimicrobial and anti-inflammatory properties. Most *Salvia species* are consumed as herbal tea in addition to traditional medication. *S. fruticosa* that is a well known antioxidant herbal plant is native to Mediterranean Countries.

In this study, it was aimed to investigate the chemical composition of *S. fruticosa* water extract and to study its antioxidant and cytotoxic effects on HT-29 cell line.

Many herbal plants including *S. fruticosa* are consumed generally as drinking tea together with the prescribed drugs. Many xenobiotics and drugs are metabolized by Phase I and II enzymes. Many of these enzymes are polymorphic. Therefore, the metabolism of these chemicals are unique to each individual.

For this reason, in this study it was also aimed to investigate the metabolism of *S. fruticosa* water extract by HT-29 cells and its emphasizing on the Phase I and II gene expressions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

The plant material *S. fruticosa M.* was collected from Germiyan Village, Çeşme, İzmir, Turkey in 2012 and identified by Musa Dogan (Department of Biology, Middle East Technical University, Ankara, Turkey) and Assoc. Prof. Dr. Ferhat Celep (Nevşehir University, Turkey) and the voucher specimens were preserved at Laboratory of Plant Systematic, Department of Biological Sciences, Middle East Technical University, Ankara, Turkey.

2.1.2 HT-29 Cell line

HT29 is a colorectal adenocarcinoma cell line was purchased from ATCC (American Type Culture Collection).

2.1.3 Chemicals and Materials

Antioxidant assays; 2,2-diphenyl-2-picryl-hydrazyl (Sigma, USA) 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (Sigma, USA), Ethylenediamine tetraacetic acid (EDTA) (Fluka, Switzerland), Potassium peroxodisulfate (Merck, Germany), (±)-α- Tocopherol (Sigma, USA),

Total phenol and flavonoid assays; Folin Ciocalteu Reagent (Merck, Germany), Sodium hydroxide (Merck, Germany), Sodium carbonate (Riedel-de Haen-Honeywell, USA), Aluminum chloride (Fluka, Switzerland), Gallic acid

monohydrate (Acros Organics, USA), Quercetin (Sigma, USA), (-)-Catechin (Sigma, USA).

Protein determination and enzyme activity; Bovine Serum Albumin (Sigma, USA), Potassium sodium tartrate (Fluka, Switzerland), Copper(II) sulfate-5-hydrate (Riedel-de Haen-Honeywell, USA), Potassium dihyrogen phosphate (Merck, Germany), Dipotassium hyrogen phosphate (Merck, Germany), L-Glutathione reduced (Sigma-Aldrich, USA), 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich, USA), Ethacrynic acid (Sigma, USA).

Cell culture; Mc Coy's 5A medium with L-Glutamine (Lonza, Belgium), Na-pyruvate (Lonza, Belgium), PBS (Lonza, Belgium), Dimethyl Sulfoxide cell culture tested (Appli Chem, Germany), Trypan blue solution 5% (Biological Industries, Israel), RIPA Buffer (Cell Signalling Technology, USA), Nuclease-Free Water (Hyclone, USA).

HPLC analysis; Glacial acetic acid (Sigma, USA), Methanol (Sigma-Aldrich, USA), Acetonitril gradient grade (Merck, Germany), Ethanol absolute (Sigma-Aldrich, USA), Formic acid (Merck, Germany), Trifluoroacetic acid (Merck, Germany), (-)-Epicatechin (Sigma, USA), (-)-Epigallocatechin gallate (Sigma, USA), Rosmarinic acid (Sigma, USA), caffeic acid (Sigma, USA), syringic acid (Sigma, USA), p-coumaric acid (Sigma, USA), t-resveratrol (Sigma, USA),

2.1.4 Instruments

Laminar flow Class II Safety Cabinet (Heraus, Germany), CO_2 incubator (Heraus, Germany), Olympus CKX41 Inverted phase contrast microscope (Olympus, Japan), BH-2 Research Microscope (Olympus, Japan), Eppendorf Centrifuge 5810 R (Eppendorf, Germany and SİGMA 2-5 centrifuge, Germany), WiseMix Vortex (Wisd Laboratory Instruments-Verkon, Czech Republic), pH meter (Mettler Toledo, USA), Spectrophotometer (Schimatsu, Japan or, Multiskan™ GO- Thermo Scientific, USA), PCR Thermal Cycler (Biorad MyCycler, USA and Eppendorf,

Germany), Real-time PCR Thermal Cycler (Corbett-QIAGEN, Netherlands), Chemiluminescence imaging system (DNR Systems MF-ChemiBIS 3.2, Israel), -80 °C freezer (Sanyo -86C ULT Freezer, Japan), -20°C freezer (Ugur, Turkey), +4 refrigerator (Arcelik, Turkey), ELISA plate reader (Multiskan™ GO- Thermo Scientific, USA), AB104-S Analytical Balance (Mettler Toledo, USA), WiseBath Water Bath (Wisd Laboratory Instruments-Verkon, Czech Republic), Shaking Water Bath ST 402 (Nuve,Turkey), Autoclave OT 032 (Nuve, Turkey), Ultrasonic Cleaner (Alex Machine, Malaysia), MyCycler Thermal Cycler (BIO-RAD, USA), Microwave (Beko, Turkey), Laminar Flow&Biosafety Cabins Chemocell LRCX-UV (Teknomar, Turkey), Heracell 150 CO_2 incubator (Heraeus-Thermo Scientific, USA), Alpha 1-4 LD Freeze Dryer (Christ, Germany), NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), Cryo.s (Greiner bio-one, Germany), Pasteur pipettes (Isolab, Germany), Multi pipette 20-200 µl (Rainin-Mettler Toledo, USA), Multi pipette 5-50 µl (Thermo Scientific, USA).

Commercially available kits used in this study are Cell Proliferation kit XTT Based 1000 Rxn (Biological Industries, Israel), Thermo Gene Jet RNA Purification Kit 250 prep. and cDNA Synthesis Kit (Thermo Scientific, USA), FastStart Universal Syber Green ROX (ROCHE, Switzerland).

2.1.5 Primers

CYP1A1, CYP1A2, CYP3A4, CYP2E1, Catalase, GPX4, GSTM1, GSTP1, and GAPDH, gene primers were designed with NCBI BLAST and purchased from Iontek, İstanbul, Turkey (Table 2.3).

2.2 Methods

2.2.1 Preparation of *S. fruticosa* water extract

The aerial parts of the plant was dried at room temperature, protected from direct sun light. Briefly, 20 g of the dried plant was mixed with tap water (400 mL) and left to boiling for 20 min. After filtration of the solvent, the water extract was concentrated under vacuum by evaporating to dryness and lyophilization was performed for 48 hours. The extracted powder was weighed and stored at -20°C in brown bottle until use. The residue was dissolved in water for the experimental analysis. Extraction yield was determined using the following equation 2.1.

Equation 2.1:

Percentage extraction (w/w) = [Mass of SF (in extracted solution) / mass of total SF] X 100

2.2.2 Determination of Total Phenolic Content

Total phenolic content of the plant extract was determined by using the method of Singleton and Rossi (1965) with some modifications. 20 µL of the extract was mixed with 1N, 100 µL of 20 % Folin-Ciocalteu's phenol reagent and 80 µL of 10 % sodium carbonate solution in the 96 well plate and shaken vigorously. After 30 minutes of incubation at room temperature, absorbance values were recorded at 750 nm against blank that contain 20 µL ethanol without any sample using Elisa reader. Results were evaluated using gallic acid standart curve and recorded as milligrams of total phenolics (TP) per gram of extract, as the gallic acid equivalents (GAE) shown in equation 2.2. Analyses were run in three replicates and the results were expressed as average with the standart deviations.

Equation 2.2:

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

2.2.3 Determination of Total Flavonoid Content

Total flavonoid content was determined by using the method of Zhishen et al. (1999) with some modifications. 20 µL of extract or standart solution of catechin was added into 96 well plate containing 80 µL of distilled water. 6 µL of 5% sodium nitrite was added to the wells. After 5 minutes, 6 µL of 10 % aluminium chloride solution was added. At 6^{th} minute, 40 µL of 1 M sodium hydroxide was added and the total volume was completed up to 200 µL with distilled water. The solution was mixed well and the absorbance was measured against prepared blank at 510 nm using Elisa reader. Results were calculated by using the catechin standart curve and were recorded as milligrams of total flavonoids in gram of extract, as the catechin equivalents (CE) shown in equation 2.3. Analyses were run in three replicates and expressed as average values with standart deviations.

Equation 2.3:

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

2.2.4 Determination of Antioxidant Activity

Antioxidant activity of the extract was evaluated by three different methods, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), (ABTS$^{\bullet}$) radicals scavenging method and Metal (Fe^{2+}) chelating activity test.

2.2.4.1 Free Radical Scavenging Capacity by DPPH Method

The stable DPPH$^{\bullet}$ scavenging activity was determined according to Blois et al, (1958) with some modifications. 140 µL of 0.05 mg/mL DPPH solution in ethanol and 10 µL of extract were mixed in 96 well plate and reaction mixture was shaken vigorously. Decrease of absorbance in the mixture was determined after 20 minute at 517 nm due to depletion of DPPH radical (Figure 2.1) by using Elisa reader. α-tocopherol was employed as the reference. Radical scavenging activity was calculated using the following equation 2.4.

Equation 2.4:

RSA (Radical scavenging acitivity) % = [(A_0 –A_1) / A_0] X 100

Where A_0 is the absorbance of the control reaction (containing all reagents except the test sample) and A_1 is the absorbance of the extracts/reference.

According to the results, RSA% vs final concentrations of the extracts (mg/mL) was plotted and IC_{50} (50% effective concentration) value was calculated. Analyses were run in three replicates.

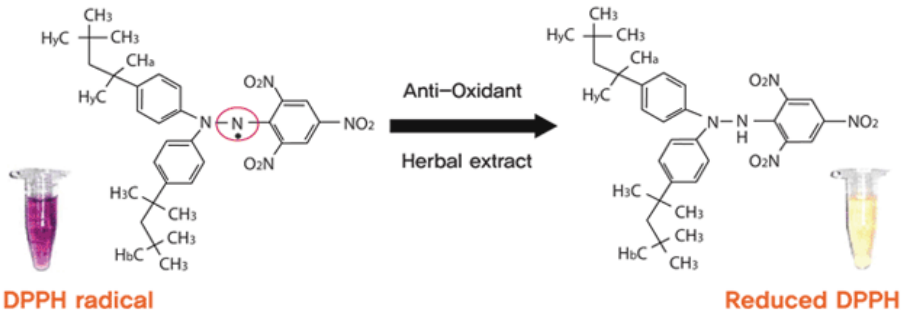


Figure 2.1 Chemical reaction of DPPH radical by antioxidants

2.2.4.2 Free Radical Scavenging Capacity by ABTS Method

2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet}$) method was performed according to Re et. al (1999) with some modifications. In this procedure; first, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution with potassium persulfate solution ($K_2S_20_8$) shown in figure 2.2. The mixture then allowed to stand in the dark at room temperature for 12–16 h before using. (7mM ABTS mixed with 2.45mM KPS 1:1 ratio. 76.8mg ABTS was dissolved in 20ml water and 13,25 mg KPS was dissolved in 20 ml water). Finally, $ABTS^{\bullet+}$ solution was diluted with 120 mL of methanol to an approximate absorbance unit of 0.70 (±0.02) at 734 nm

2.5 µL of extract or standart solution was added to the diluted 250 µL of ABTS• solution in 96 well plate and mixed. Absorbance was monitored at 734 nm using Elisa reader. After the initial mixing of the reactants, time was recorded in every minute from 1st to 6th. Trolox was used as standart to calculate the trolox equivalent antioxidant capacity (TEAC) values. Percent inhibition was calculated using the following equation 2.5.

Equation 2.5:

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

Where A_0 is the absorbance of the control reaction (containing all reagents except the test sample) and A_1 is the absorbance of the extracts. Analyses were run in three replicates and expressed as average values with standart deviation.

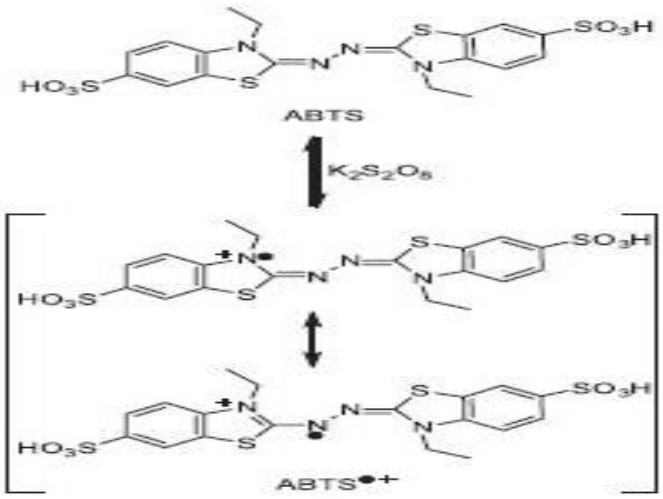


Figure 2.2 Formation of ABTS radical cation (Pannala, 2001)

2.2.4.3 Fe2+ Chelating Activity

The chelating activity (*ChA*) of the substances was investigated according to methods of Dinis et al. (1994) with some modifications. Different concentrations of the investigated extracts and EDTA between 0.5 to 4 mg/mL and 0,093 to 3.72 mg/mL were prepared, respectively. 50 µL of the working solutions of the extracts/

EDTA were placed to 96 well plate containing 185 µL dH_2O, and 5 µL of 2 mM FeCl2 solution was added. After 5 min, the reaction was initiated by adding 10 µL of 5 mM ferrozine solution. Absorbance at 562 nm was recorded after 10 min of incubation at room temperature. A reaction mixture containing methanol (50 µL) instead of substance solution was used as a control. EDTA was used as the chelating standard. ChA % was calculated using the following equation 2.6.

Equation 2.6:

$$ChA\ \% = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 is the absorbance of the control reaction (containing all reagents except the test sample) and A_1 is the absorbance of the extracts/reference. Chelating activity was expressed as IC_{50}, the concentration that chelates 50% of Fe2+ ions. Analyses were run in three replicates and expressed as average values with standart deviation.

2.2.5 RP- HPLC Analysis of *S. fruticosa* water extract

High-performance liquid chromatography is the most popuplar technique used to separate the components in a mixture, to identify each component, and to quantify each component. For identification and quantification of different phenolic compounds in the water extract of *S. fruticosa*, a series concentrations of different standart solutions were used to obtain the calibration curves of phenolic compounds. Recovery was determined for the overall assay by adding known amounts of standart on the original concentration of the analyzed samples. Three HPLC replicate injections were performed for standards and plant extracts. All samples were filtered through a 0.45µm membrane filter (Millipore, Milford, MA). An aliquot of 20 µL of the filtrate was injected into HPLC for analysis.

2.2.5.1 RP- HPLC Conditions

Two different elution system was performed for the analysis of phenolic compounds in *S. fruticosa*. Gradient elution for rosmarinic acid and caffeic acid content of *S. fruticosa* was carried out at a flow rate of 1.0 mL/min at 30°C. Mobile phase A was 0.1% (v/v) formic acid solution in water, while mobile phase B was acetonitrile. A ratio of 88% A and 12% B was applied in the first 30 min. After 30 min, a ratio of 80% A and 20% B was used for the next 15 min. Finally, 70% A and 30% B were used after 45 min for an additional 15 min (Liu et al., 2013). The column was a Agilent Zorbax SB-C18 (250 mmx 3 mm, 5 μm).

The other applied gradient elution conditions to determine the phenolic compounds in the extract was as follows: 0 min. 20% B; 0–10 min. from 20% to 30% B; 10–40 min. from 30% to 40% B; 40–60 min. from 40% to 60% B; 60–80 min. from 60% to 80% B; and finally, 90 min. from 80% to 20% B. The mobile phase was composed of (A) 0.1% (v/v) formic acid in water and (B) acetonitrile (Soraia I. et al., 2010). The column was operated at 30 °C and flow rate was 1 mL/min. The column was a Inertsil ODS-3 (250 mm x 4,6 mm, 5 μm).

The HPLC equipment consisted of a Shimadzu LC-20AD system including DGU-20A5 prominence degasser, SIL-20AHT prominence autosampler, SPD-M20A prominence UV-Vis photodiode array detector, CTO-20A prominence column oven. Data was processed on a Intel pentium IV PC computer by using LC Solution Programme.

2.2.6 Cell Culture

2.2.6.1 Cell Culture Conditions

HT29 cell line that is the the most widely used a adenocarcinoma cell line were grown in medium McCoys's 5A containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) solution. Cultures were incubated at 37°C with 5% carbon dioxide (CO_2) and 95% humidity in incubator. Studies of the cell culture

were performed in Metisafe Class II Safety Cabinet**.**The growth mediums of culture were renewed with fresh medium in 2-3 days in order to constant the appropriate growth conditions.

2.2.6.2 Cell Thawing

Cryotubes that were taken from the liquid nitrogen were defrosted at 37°C water bath and transferred to T75 cell culture flask that contain growth medium. Cultured cells were incubated in CO_2 incubator at 37°C. After 24 hours, medium was renewed with fresh one to remove dimethylsulfoxide (DMSO) and then placed in CO_2 incubator.

2.2.6.3 Cell Passaging

When cells were 80% confluent, the medium in the T75 flask was removed and cells were washed with 10 mL of phosphate buffered saline (PBS). After PBS were pipetted from the flask without causing any distribution to the attached cells, 2 ml of prewarmed trypsin/EDTA solution were added to the flask and placed to the CO_2 incubator at 37°C for 5-10 min. 8 mL of fresh growth medium was added to the flask for inactivation of trypsin. 2 mL of the cell suspansion mixture was transferred into new T75 flasks and 7 to 8 mL of fresh growth medium was added and the splitted cell cultures were placed at 37°C, CO_2 incubator. This procedure was repeated in every 3-4 times in a week.

2.2.6.4 Cell Freezing

When cells were 80% confluent, the medium in the T75 flask was removed and cells were washed with 10 mL of phosphate buffered saline (PBS). After PBS were pipetted from the flask without causing any distribution to the attached cells, 2 mL of prewarmed trypsin/EDTA solution were added to the flask and placed to the CO_2 incubator at 37°C for 5-10 min. Upon all the cells were detached, 5 to 6 mL of fresh growth medium was added into the flask for inactivation of the trypsin. The cell suspansion in the T75 were transferred into a 15 mL falcon tube and centrifuged at 1000 x g for 5 minutes at 37°C. After centrifugation, supernatant was removed

from the falcon tube and pellet was re-suspended in 3 mL freezing medium that contain 10 % DMSO in a complete growth medium supplemented with serum. Finally, the cell suspension were transferred to cryotubes and immediately placed in the -80°C Freezer. After a week, cryotubes were transferred to liquid nitrogen tank for longer term storage at about -196 °C.

2.2.6.5 Cell counting and Growth curve

HT-29 cells from adenocarcinoma cell lines were seeded in 6-well plates at a density of $5x10^5$cells /well and placed into the CO_2 incubator at 37°C. After 24 hour, the medium of different two wells were removed and and washed with 1 mL of PBS. After cells were detached with 0,5 mL of trypsine, 2 mL of fresh complete medium was added to the wells. Detached cell suspansions were transfered to seperate eppendorfs and stained with 0,25 % (w/v) trypan blue. Cell counting was carried out using hemocytometer under light microscopy. The cells were counted during 30 days and groth curve of the HT-29 cell line was plotted with the number of cells against time. The ratio of cell suspension and tryphan blue changed depending on the number of the cells. 10 µl of mixed cell suspansion was placed on 2 chambers of the hemocytometer (Figure 2.3). After cells were counted , the average of two chambers were taken and the cell number per ml was calculated as follows;

Cell number / ml = The average cell number of the two chambers x DF x 10^4

DF= Dilution factor that was done with Trypan Blue

10^4= Factor calculated from the dimensions of hemocytometer

The dimentions of each chamber on the hemocytometer were 1cm lengh, 1 cm width and 0.1 cm height, having volume of 0.1 cm^3 or 10-4 mm^3 (10-4 mL).

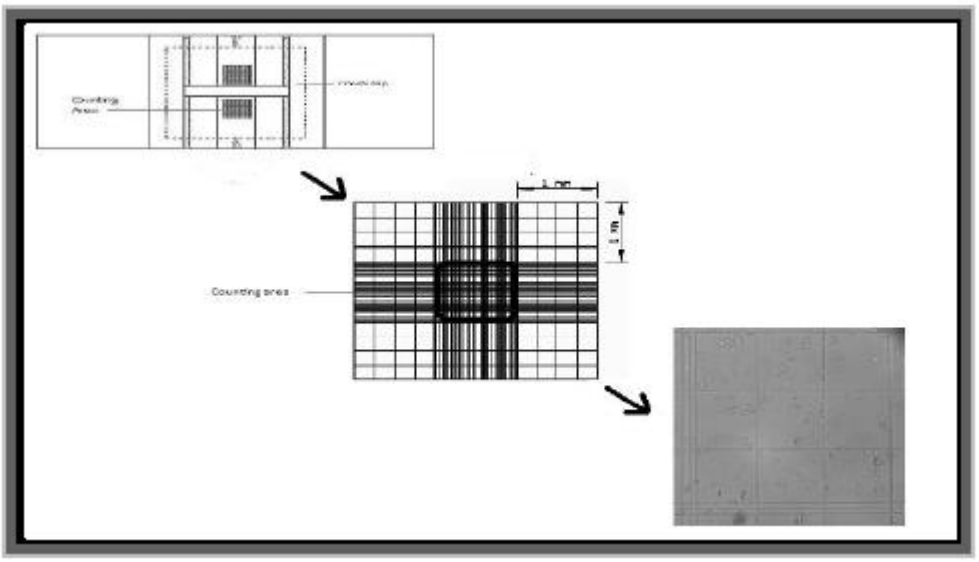


Figure 2.3 Viable cell counting with Hemocytometer

2.2.7 Cytotoxicity Assays

2.2.7.1 Preparation of *S. fruticosa extract,* Rosmarinic acid and Caffeic acid working solutions

Water extract of *S. fruticosa* and two main active components, rosmarinic acid (RA) and caffeic acid (CA) were used as cytotoxic agents on the proliferation of HT-29 cell line. First, 20 mg crude extracts of *S. fruticosa* was dissolved in 4 mL 0.2 % DMSO containing McCoys's 5A medium with phenol red (5 mg/mL). Working crude extract solutions between 100 µg/mL to 3200 µg/mL were prepared by diluting the stock working solution. DMSO concentration was kept constant. Final crude extract concentrations were varied from 50 µg/mL to 1600 µg/mL in each well during treatments. Second, working solutions of RA and CA solutions at different concentrations (from 100 µg/mL to 1000 µg/mL) were prepared by diluting the stock working solutions of RA and CA in 0,2 % DMSO containing McCoys's 5A medium with phenol red. Final concentrations of RA and CA were varied from 50 µg/mL to 500 µg/mL in each well during treatments. Third, mixture of RA and CA working solutions of varying concentrations (from 100 µg/mL to 1000 µg/mL) were prepared by diluting the stock working solution in 0,2 % DMSO

containing McCoys's 5A medium with phenol red (RA: CA; 1:1 ratio). Final concentrations of the mixture were varied from 50 µg/mL to 500 µg/mL in each well during treatments.

2.2.7.2 Viability Measurement of *S. fruticosa,* Rosmarinic acid and Caffeic acid with XTT Assay

The cytotoxic effects of water extract of *S. futicosa*, rosmarinic acid and caffeic acid in HT-29 cells were investigated by using Cell Proliferation XTT Kit Biological Industries according to manufacturer's instructions.

The tetrazolium salt XTT was reduced to orange colored formazan compounds by the activity of mitochondrial enzymes in metabolic active cells (Figure 2.4). The formed product formazan was water soluble therefore, it can easily be quantitated using with ELISA reader at 415 nm.

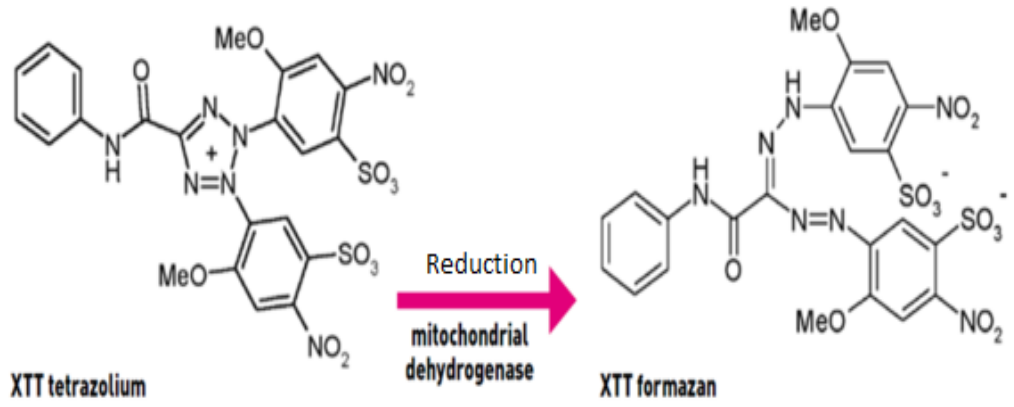


Figure 2.4 Reduction of XTT tetrazolium to XTT formazan by mithocondrial dehydrogenase in the presence of phenazine metho-sulphate

100 µl of HT-29 cells (100.000 cells/mL) were seeded into 96 well plates and left for 24 hours incubation to attach and grow. After that time, medium of the wells

were discarded, 50 µL fresh complete medium was added and cells were treated with 50 µL of working solutions of *S. fruticosa*, rosmarinic acid and caffeic acid, seperately. After treatments, cells were left for 48 and 72 hours incubation. One of the control wells contain only growth medium (100 µL), the other control wells contain 0.1 % DMSO in growth medium. The 96 well plate representation of XTT assays (Figure 2.5.1; 2.5.2; 2.5.3).

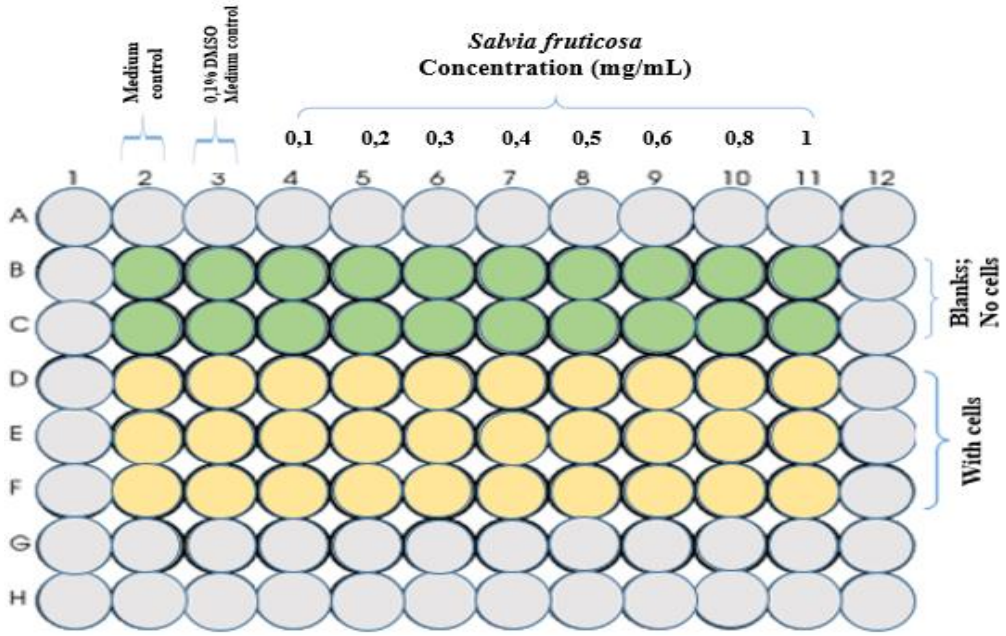


Figure 2.5 96 well plate representation of XTT assay of *S. fruticosa*

B2 and C2, no cells, complete medium control, 100 µl; B3 and C3, no cells, 0.1% DMSO medium control, prepared as 50 µL 0.2% DMSO medium + 50 µL complete medium, B4 to F4, 0.1 mg/mL extract, prepared as 50 µl 0.2 mg/mL extract + 50 µL complete medium, B5 to F5, 0.2 mg/mL extract, prepared as 50 µl 0.4 mg/mL extract + 50 µL complete medium; B6 to F6, 0.3 mg/mL extract, prepared as 50 µL 0.6 mg/mL extract + 50 µL complete medium; B7 to F7, 0.4 mg/mL extract, prepared as 50 µl 0.8 mg/mL extract + 50 µL complete medium; B8 to F8, 0.5 mg/mL extract, prepared as 50 µL 1 mg/mL extract + 50 µL complete medium. B9 to F9, 0.6 mg/mL extract, prepared as 50 µL 1.2 mg/mL extract + 50 µL complete

medium. B10 to F10, 0.8 mg/mL extract, prepared as 50 µL 1.6 mg/ml extract + 50 µL complete medium. B11 to F11, 1 mg/mL extract, prepared as 50 µl 2 mg/mL extract + 50 µL complete medium.

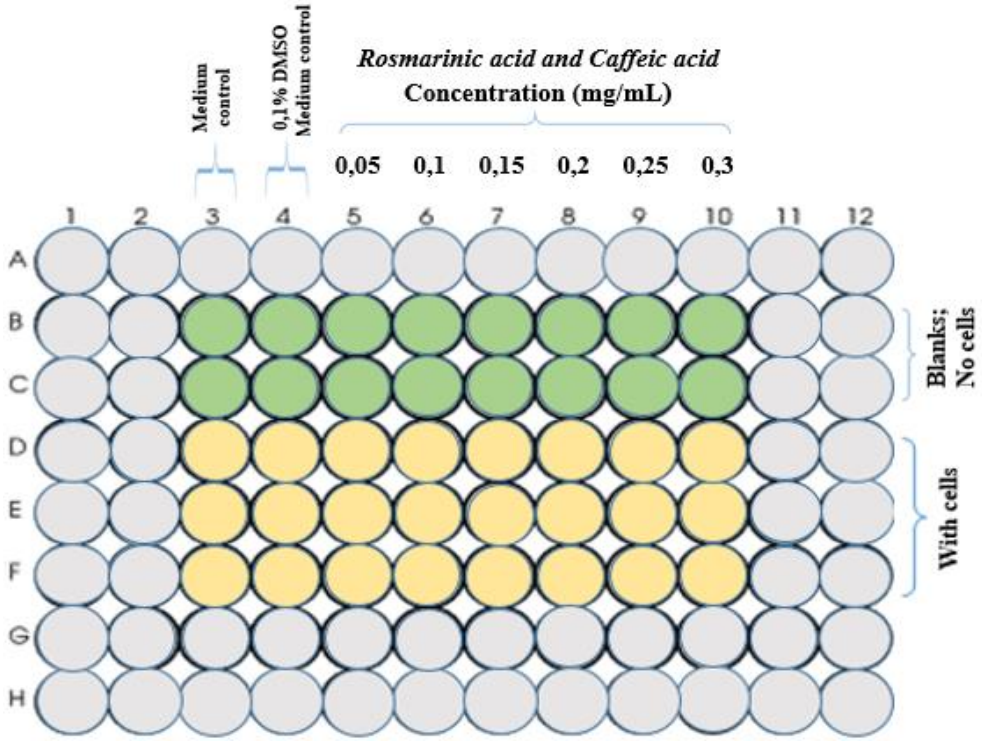


Figure 2.6 96 well plate representation of XTT assay of rosmarinic acid (RA), caffeic acid (CA)

B3 and C3, no cells, complete medium control, 100 µL; B4 and C4, no cells, 0.1% DMSO medium control, prepared as 50 µL 0.2% DMSO medium + 50 µL complete medium, B5 to F5, 0.05 mg/mL *R.A* and C.A, prepared as 50 µL 0.1 mg/mL *R.A* and C.A + 50 µL complete medium, B6 to F6, 0.1 mg/mL *R.A* and C.A, prepared as 50 µL 0.2 mg/mL *R.A* and C.A + 50 µL complete medium; B7 to F7, 0.15 mg/mL *R.A* and C.A, prepared as 50 µL 0.3 mg/mL *R.A* and C.A + 50 µL complete medium; B8 to F8, 0.2 mg/mL *R.A* and C.A, prepared as 50 µL 0.4 mg/ml *R.A* and C.A + 50 µL complete medium; B9 to F9, 0.25 mg/mL *R.A* and C.A, prepared as 50 µl 0.5 mg/mL *R.A* + 50 µL complete medium. ; B10 to F10, 0.3 mg/mL *R.A* and C.A, prepared as 50 µL 0.6 mg/mL *R.A* and C.A + 50 µL complete medium.

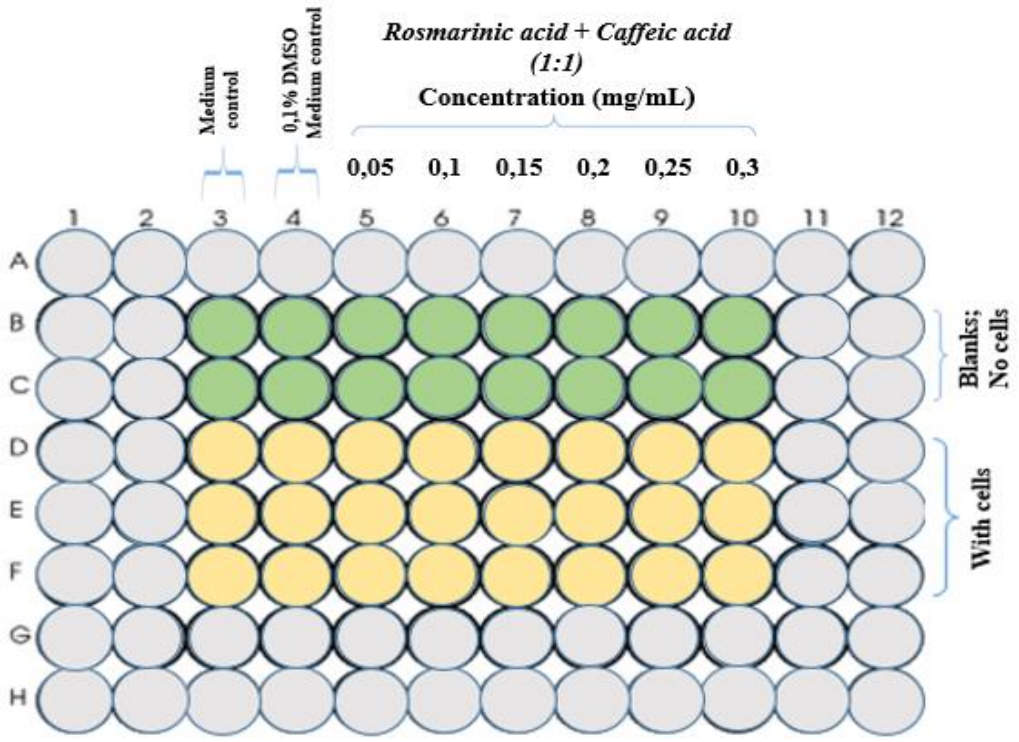


Figure 2.7 96 well plate representation of XTT assay of rosmarinic acid (RA)+ caffeic acid (CA)

B3 and C3, no cells, complete medium control, 100 µL; B4 and C4, no cells, 0.1% DMSO medium control, prepared as 50 µL 0.2% DMSO medium + 50 µL complete medium, B5 to F5, 0.05 mg/mL R.A+*C.A*, prepared as 50 µL 0.1 mg/mL R.A+*C.A* + 50 µL complete medium, B6 to F6, 0.1 mg/mL R.A+*C.A*, prepared as 50 µL 0.2 mg/mL R.A+*C.A* + 50 µL complete medium; B7 to F7, 0.15 mg/mL R.A+*C.A*, prepared as 50 µL 0.3 mg/mL R.A+*C.A* + 50 µL complete medium; B8 to F8, 0.2 mg/mL R.A+*C.A*, prepared as 50 µL 0.4 mg/mL R.A+*C.A* + 50 µL complete medium; B9 to F9, 0.25 mg/mL R.A+*C.A*, prepared as 50 µL 0.5 mg/mL R.A+*C.A* + 50 µL complete medium. B10 to F10, 0.3 mg/mL R.A+*C.A*, prepared as 50 µL 0.6 mg/mL R.A+*C.A* + 50 µL complete medium.

After the incubation, 50 µL of reaction mixture prepared by adding 100 µL activation reagent to 5 ml XTT reagent was added to each well. After plates were incubated with XTT reagent mixture for 8 hours in CO2 incubator at 37 ^{0}C, the

absorbance was read at 415 nm with ELISA reader. Blanks at different concentrations with no cells were subtracted than that of sample solutions with cells. % cell viability was calculated using the following equation 2.7.

Equation 2.7:

$$\% \text{ cell viability} = [\text{Abs (extract treated cells)} - \text{Abs (extract in cell free medium)}] / [\text{Abs (untreated cells)} - \text{Abs (cell free medium)}] \times 100$$

According to the results, % cell viability vs final concentration of the samples were plotted and IC_{50} (Sample concentrations required to reduce viability 50 %) value was calculated.

2.2.7.3 Viability Measurement of *S. fruticosa* treated Cells with Tryphan Blue Exclusion Method

2 mL of HT-29 cells were seeded in 6 well plates as 250.000 cells/mL for each well and incubated for 24 hours in CO_2 incubator at 37ºC. The following day, medium was refreshed and the cells were treated with working solutions of *S. futicosa* at different concentrations. 1mL plant extracts + 1mL of fresh medium (containing 0.2 % DMSO so that final DMSO concentration is 0.1 %) and the plates was incubated at 37°C in 5 % CO2 incubator for 48 and 72 hours. When the incubation periods were completed, the medium was discarded and cells were washed with PBS and detached with 500 µL trypsin. 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 and 10 µL of mixed cell suspansion was placed on 2 chambers of the hemocytometer under light microscope. Percent cell viability was calculated from the following equation 2.8.

Equation 2.8:

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

According to the results, % Cell viability vs final concentrations of the extracts (mg/mL) was plotted and IC_{50} value of the plant extract for 48 and 72 hours treatments were determined.

2.2.8 Gene Expression Analysis

2.2.8.1 Isolation of Total RNA from HT-29 Cells

Isolation of total RNA from HT-29 cells were performed according to Thermo Scientific GeneJet RNA Prufication Kit. 2 mL of HT-29 cells were seeded in 6 well plates as 250.000 cells/mL for each well and incubated for 24 hours in CO_2 incubator at 37°C. The following day, medium was refreshed and the cells were treated with *S. futicosa* at a concentration of IC_{50} (mg/mL) values and incubated for 48 and 72 hours in CO_2 incubator at 37°C. When the incubation time periods completed, the medium was discarded washed with PBS buffer. After PBS was removed and discarded, the cells in the culture plate were detached by trypsinization. The cells were transfered into microcentrifuge tubes and pelleted by centrifugation for 5 min at 250 × g. Supernatants were discarded. The cells were resuspend and 600 µL of lysis buffer supplemented with β mercaptoethanol was added and vortexed for 10 s to mix thoroughly. 360 µL of ethanol (96-100%) was added and mixed the samples by pipetting. 700 µL of lysate was transferred to purification columns inserted in collection tubes and centrifuged for 1 min at ≥12000 × g. Flow-through was discarded and the purification columns were placed back into the collection tubes. 700 µL of Wash Buffer 1was added to RNA purification columns and centrifuged for 1 min at ≥12500 × g. The flowthrough was discarded and the purification columns were placed back into the collection tubes. 600 µL of Wash Buffer 2 was added to RNA purification columns and centrifuged for 1 min at ≥13000 × g. The flowthrough was discarded and the purification columns were placed back into the collection tubes. 250 µL of Wash Buffer 2 was

added to RNA purification columns and centrifuged for 1 min at ≥13500 × g. 100 µL of nuclease-free water was added to RNA purification columns and centrifuged for 1 min at ≥14000 × g. to elute RNA. Purified RNA was stored at -80°C until use.

2.2.8.2 Determination of RNA Concentration

Concentration of the isolated RNA was quantified by measuring the absorbance at 260 nm. Purity was assessed by the 260/280 nm ratio. The ratio of OD260/OD280 must be between 1.8 and 2.2. Below 1.8 refers the DNA contamination while above 2.2 referring the protein contamination. The optical density of 1.0 corresponded to the 40 µg/mL for RNA. The concentration and purity of the RNA were measured at NanoDrop™ 2000 (Thermo Scientific). Concentration of RNA is calculated using the equation 2.9.

Equation 2.9:

A260 X 40 X Dilution factor = µg/ml RNA concentration

2.2.8.3 cDNA Synthesis

Components:

5X Reaction Buffer : 250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DDT
M-MuLV ReverseTranscriptase: (200u*/µL)
Ribolock: RNase inhibitor (20u*/µL)
dNTP Mix: Deoxyribonucleotide triphosphate (10mM)
Oligo $(dT)_{18}$ Primer

cDNA synthesis was performed according to RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). Two step protocol was performed. In the first step, 2 µg of total RNA isolated from HT-29 cell line was mixed with 1 µL of oligo dT primer in an eppendorf tube on ice and the volume of the mixture

was completed to 12 µL with nuclease-free distilled water. In the second step, the foollowing components were added in the indicated order (Table 2.1).

Table 2.1 cDNA Synthesis Reaction Mixture

5X Reaction Buffer	4 µL
RiboLockTM RNase Inhibitor	1 µL
10 mM dNTP Mix	2 µL
RevertAidTM M-MulV Reverse Transcriptase	1 µL
Total	20 µL

Following mixing and centrifuging briefly, the tube was incubated for 60 minutes at 42 ^{o}C and the reaction was terminated by heating at 70 ^{o}C for 10 minutes. The syntesized cDNA was kept and stored at -80 ^{o}C for further use.

2.2.8.4 Quantitative Reverse Transcription PCR (qRT-PCR)

The mRNA expressions of CYP1A1, CYP1A2, CYP2E1, CYP3A4, GSTM1, GSTP1, GPx and Catalase genes in HT-29 cell line were analyzed by quantitative Real Time PCR (qRT-PCR) using Corbett Rotor Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137).

10 µL of final reaction mixture containing 1.6 µL cDNA (50-100 ng), 0.2 µL of 10 mM forward primer, 0.2 µL of 10 mM reverse primer, 5 µL of 1 X Maxima® SYBR Green qPCR Master Mix (Fermentas, Glen, Burnie, MD) and 3 µL of RNase free distilled water (Table 2.2).

Table 2.2 qRT-PCR mixture preparation

Component	Volume	Final Concentration
1X RealMasterMix SYBR ROX	5 µL	0.5X
Forward primer	0.2 µL	0.2 mM
Reverse primer	0.2 µL	0.2 mM
cDNA	1.6 µL	
Nuclease free water	3 µL	

The qRT-PCR program consisted of the following cycling profile; initial melting at 95 °C for 10 minutes, amplification and quantification program repeated 40 times containing melting at 95 °C for 20 seconds, annealing at 58-62°C (depending on the gene) for 30 seconds and extension at 72 °C for 20 seconds with a single fluorescent measurement was added. In order to confirm the PCR product, Melting curve analysis of the amplification product was carried out at the end of each amplification reaction.

Quantities of specific mRNAs in the sample were measured with corresponding gene and relative standard curve method. Each Standard curve was derived from dilution series (1:5, 1:15, 1:30, 1:40, 1:80) of selected standard cDNA for each gene. Light cycler quantification software was used to draw the standard curve. No template control (NTC) was used to detect any contamination. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used as an internal standard.

In order to evaluate the results, ΔΔCt method was used.

$$\Delta Ct = Ct\ (target) - Ct\ (normalizer)$$

ΔCt is the difference between the CT values of the target and the normalizer that is GAPDH . $\Delta\Delta C = \Delta Ct\ (sample) - \Delta Ct\ (reference)$

$$\text{Comparative expression level} = 2^{-\Delta\Delta Ct}$$

Subtraction of ΔCt value of control from ΔCt value of treatments gave the ΔΔCt value and $2^{-\Delta\Delta Ct}$ gave comparative expression level. So if the ΔCt values of the samples are greater than control there is a down regulation of gene expression with respect to control, and if the ΔCt values of the samples are smaller than DMSO control there is an up regulation of gene expression with respect to control. PCR reaction conditions and primer sequences for 9 genes were shown in Table 2.3.

Table 2.3 Primer sequences, annealing temperatures and product sizes of 9 genes.

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

2.2.9 Protein Extraction

Protein extraction was performed according to Cell Signaling Technology procedure. HT-29 cells ($25x10^4$ cell/ mL) were seeded to 100 x 15mm tissue culture petri dish for protein extraction. When the cells reached to 80% confluency, growth

medium of petri dish was removed and the cells were washed with cold (4 °C) PBS buffer to remove residual medium. 400 µL of 1X RIPA buffer that is prepared from commercially available 10X RIPA buffer and 1mM phenylmethanesulfonyl fluoride (PMSF) was added into the dish for lysis of the cells. The cells were scraped after dish was incubated on ice for 5 minutes. The lysate was sonicated for 5 minutes and centrifuged at 14000 x g in a cold microfuge for 10 minutes. Supernatant was taken and stored at -80 °C for further use.

2.2.10 Determination of Protein Concentration

Reagents:

Reagent I: 2% $CuSO_4.5H_2O$

Reagent II: 2% Na-K Tartrat

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Alkaline Copper Reagent: Mix reagents I, II and A in ratio 1: 1: 100 respectively.

Folin-Phenol Reagent: Dilute 2N Folin-Phenol reagent with distilled water at 1:1 ratio.

Protein concentration determination was performed according to Lowry method. Bovine serum albumine (BSA) protein was used as standart to drawn standart curve. Protein concentration of the samples were calculated using the slope of the standart curve. Cytosolic fractions prepared from HT-29 cells were diluted as 1/40, 1/80 in distilled water. 30 µL of them were placed into 96 well plates as triplicates. 150 µL of Lowry alkaline copper reagent (ACR) was prepared mixing the reagents I, II and A in ratio 1: 1: 100, respectively. Following 10 minutes incubation at room temperature, 15 µl Folin Phenol Reagent was added and mixed for 10 seconds. After 45 minutes incubation at room temperature, the intensity of the color was measured by ELISA reader at 660 nm. The intensity of the color is directly proportional to the protein concentration.

2.2.11 Screening of Total Glutathione S-Transferase (GST) Activities

GST activity was determined spectrophotometrically by monitoring the thioether formation between 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate and GSH as cofactor at 340 nm (Habig et. al., 1974). The enzymatic reaction of CDNB with GSH was shown in (Figure 2.6).

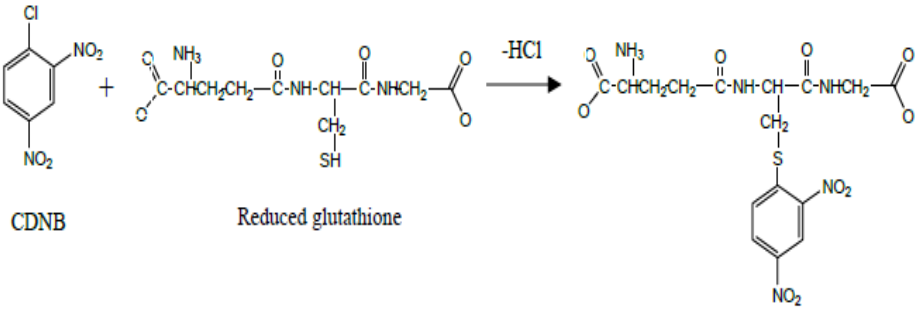


Figure 2.8 Reaction of 1-chloro-2, 4-dinitrobenzene (CDNB) with GSH (Armstrong, 1991).

HT 29 cells cytosolic fraction (5 mg protein/mL) was diluted in 10 mM phosphate buffer, pH 7.4, at a ratio of 1:15 and used as GST enzyme source in reaction medium. In a single well of a 96 well plate, 162.5 µL dH2O; 50 µL 500 mM Phosphate Buffer pH 7.4; 10 µL 25 mM GSH; 15 µL enzyme source (20-50 µg protein) were added. Reaction was started after addition of 12.5 µL CDNB. Reactions were measured for each well at every 10 seconds for 5 minutes. For reaction blank, 15 µL water was used instead of enzyme source. The slopes of reaction blanks were subtracted from slopes of enzyme reaction. GST activity was expressed as unit/mg where one unit of enzyme was defined as one nmol product formed in one minute Specific activity calculations were done according to the following equation 2.10. (ε CDNB is 6,29 mM-1/cm-1). The constituents of the reaction mixture was shown in Table 2.4.

Equation 2.10;

$$\textbf{Spesific Activity}: \frac{\text{Average Slopes (dA/dt)} \times 250 \times 1 \times DF}{\sum \text{CDNB} \times 15 \times \text{mg protein}}$$

Table 2.4 The constituents of the reaction mixture for total GST Assay

Reagents	Stock solution	Volume to be taken	Final concentration
KPi buffer (pH: 7.4)	500 mM	50 µL	100 mM
GSH	25 mM	10 µL	1 mM
CDNB	20 mM	12,5 µL	1 mM
Sample cytosol	200-500 µg/mL	15 µL	12-30 µg/mL
dH_2O	-	162.5 µL	-

2.2.12 Screening of Glutathione Peroxidase (GPx) Activities

GPx activity was performed according to (Paglia and Valentine, 1967) with some modifications. The principle of GPx activity was based on the screening of NADPH oxidation spectrophtometrically at 340 nm together with glutathione peroxidase that utilize oxidized glutathione and NADPH as a substrate. Since oxidized glutathione is produced by GPx in the reaction mixture, depletion degree of NADPH is direct indicative of GPx activity. The enzymatic reaction was shown in Figure 2.7.

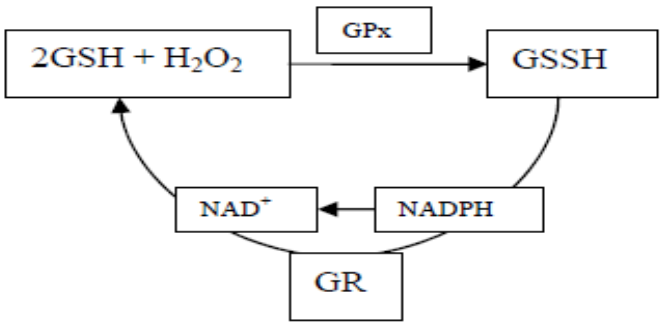


Figure 2.9 Cycle of GPx Assay

HT 29 cells cytosolic fraction (5 mg protein/mL) was used as GPx enzyme source in reaction medium. In a single well of a 96 well plate, 105 µL Tris-HCl buffer; 25 µL GSH; 25 µL glutathione reductase; 20 µL cytosolic fraction were added and incubated for 3 minutes at room temperature. Reaction was started after addition of 25 µL H_2O_2. Reactions were measured for each well at every 10 seconds for 4 minutes. For reaction blank, 20 µL buffer was used instead of enzyme source. GPx activity was expressed as unit/mg where one unit of enzyme was defined as one nmol NADPH consumed in one minute. Specific activity calculations were done according to the following equation 2.11. (ε 340 is 0.00373 $\mu M^{-1}/cm^{-1}$). The constituents of the reaction mixture was shown in Table 2.5.

Equation 2.11;

GPx Activity: ([(ΔOD/min340)/ ε 340] x (250/20) x DF)/ mg protein

Table 2.5 The constituents of the reaction mixture for GPx Assay

Reagents	Stock solution	Volume to be taken	Final concentration
Tris-HCl buffer; pH 8.0	0.1 M	105 µL	0.042 M
Reduced Glutathione (GSH)	30 mM	25 µL	3 mM
NADPH	3 mM	25 µL	0.3 mM
Glutathione Reductase	1.25 unit	25 µL	0.125 unit
Hydogen peroxide (H_2O_2)	350 µM	25 µL	35 µM
Sodium Azide (NaN_3)	200 mM	25 µL	20 mM
Cell lysate	5 mg/mL	20 µL	0.5 mg/mL

2.2.13 Screening of Catalase Activities

Catalase activity was performed according to Aebi method (1974) with some modifications. The principle of the catalase activity is based on the monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm by the catalase enzyme. The enzymatic reaction was shown in Figure 2.8.

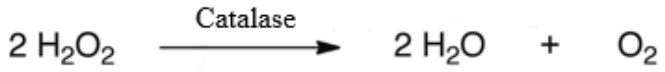


Figure 2.10 Decompositions of Hydrogen peroxide by Catalase

Reagents

50 mM KPi buffer, pH 7.0

30 mM H_2O_2

Triton X-100 (1% v/v)

Before starting the reaction, cytosolic fractions that contain catalase were diluted with 1% (v/v) Triton X-100 in ratio of 1:9 to release the catalase in peroxisomes to the assay medium. Then, further 2.5 fold dilutions were done with 50 mM KPi buffer. In a single well of a 96 well plate, 100 µL of cytosolic cell fraction used as catalase enzyme source was added to each well and reaction was started by the addition of 50 µL of hydrogen peroxide. For reaction blank, 100 µL buffer was used instead of enzyme source. Catalase activity was expressed as unit/mg where one unit of enzyme was defined as one nmol hydrogen peroxide substrate consumed in one minute. Specific activity calculations were done according to the following equation 2.12. (ε 240 is 19.54 mM^{-1}/cm^{-1}).

Equation 2.12:

Catalase Activity: ([(ΔOD/min / ε 240] x (150/100) x DF)/ mg protein

2.2.14 Statistical Analysis

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

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Extraction of *S. fruticosa*

There are many factors effecting the yield of active componenets of plants depending on the extraction conditions such as solvent polarity, extraction temperature and time. In this study, the effects of *S. fruticosa* was aimed as a drinking tea. Therefore, the extraction of the plant was performed with hot water for 20 minutes as described in Chapter II. The extraction yield was calculated as 16.5 ± 1.5 % (w/w) by equation 2.1.

In literature, many extraction studies were performed with alcohol for the extracton of maxium phenolic compounds because they are more soluble in alcohol. In such a study, *S. fruticosa* collected from Greece (Crete) region was extracted with methanol extraction yield was found as 21.5 ± 2.11 (Lorena Pizzale, 2002). In another study, the yield of water extract of *Salvia species* was reported as 17.39 % (w/w) (Hisarlı, 2013).

3.2 Determination of Total Phenolic Content of *S. fruticosa*

Total phenolic content of water extract of *S. fruticosa* was determimed by the method of Singleton and Rossi (1965) with some modifications by using the equation 2.2 previously defined in Chapter II with slight modifications. This method is widely used due to it's simple, sensitive and precise. Gallic acid was used as a standart in this assay. The phenolic content of the extract was determined in terms of gallic acid equivalent (GAE) by using the gallic acid standard curve (Figure 3.1). The results were given as μg gallic acid equivalents per mg dry mass extract in Table 3.1.

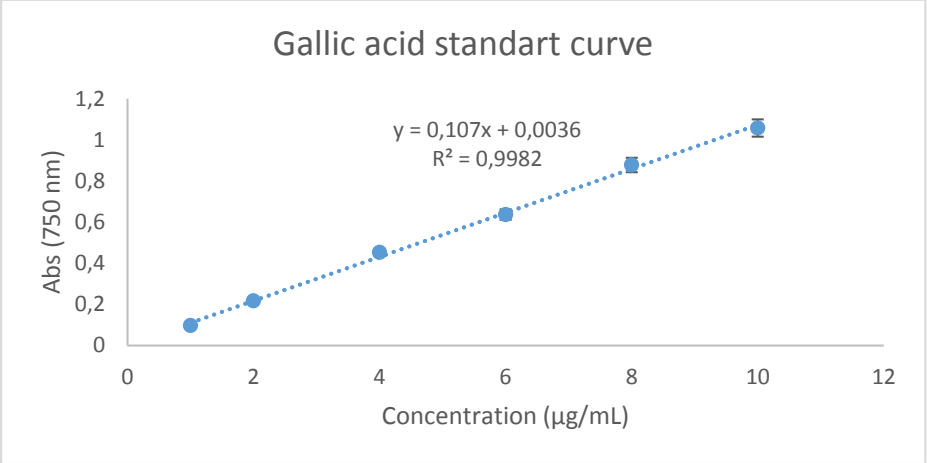


Figure 3.1 Gallic acid standart curve. (Each point is the mean of triplicate measurements from three different experiments, n=3).

Table 3.1 Total phenol content of *S. fruticosa* extract in gallic acid equivalents

Concentration (mg/mL)	**Total Phenol Content** µg GAE / mg dry extract + SD
0.25	178.84 ± 2.15
0.5	171.66 ± 2.61
Average	175.2 ± 5.07

In the literature, total phenolic content for some *Salvia* species from South Africa that were extracted with methanol:chloroform were determined as 45 to 211 µg GAE / mg dry extract (Kamatou, 2010).

Another research group reported the phenolic content of some salvia species, *S officinalis and S fruticosa*, that were extracted with methanol were 46 to 107 µg GAE / mg dry extract and 61 to 113 µg GAE / mg dry extract, respectively (Lorena Pizzale, 2002).

In addition, total phenolic content of some salvia species collected from Mediterranean and extracted with methanol were stateted to be 5.3 to 87.7 µg GAE / mg dry extract (Rababah et al., 2010).

Total phenolic content of methanol extract of *S. fruticosa* was ranged between 63.7 and 144 g GAE µg/ mg extract (Papageorgiou et al., 2008).

When compared with methanol extraction, water extracted *S. fruticosa* displayed a high total phenol content with 175.2 ± 5.07 µg GAE /mg dry extract ± sd.

3.3 Determination of Total Flavonoid Content of *S. fruticosa*

Total flavonoid content of *S. fruticosa* was measured according to the aluminum chloride colorimetric assay previously described by Zhishen et al. (1999) with slight modifications.In this assay, catechin was used as standard. According to the catechin standard curve (Figure 3.2), total flavonoid content of *S. futicosa* extract was determined as µg catechin equivalents per mg dry mass of extract shown in (Table 3.2).

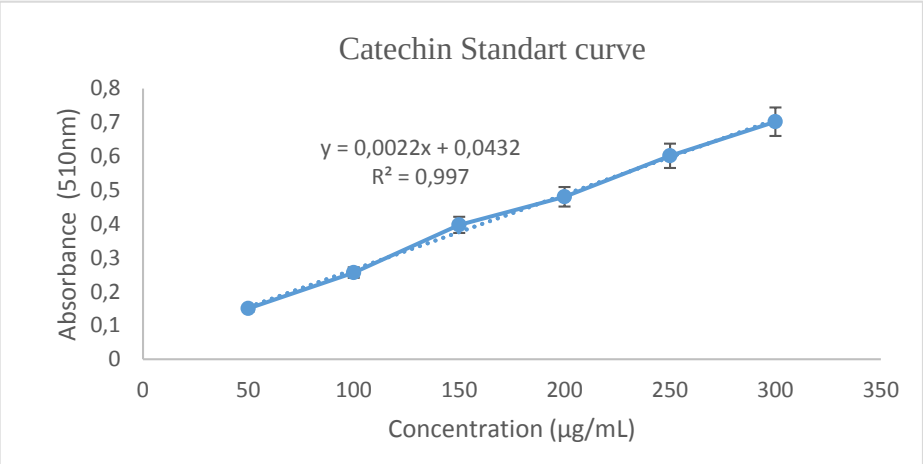


Figure 3.2 Catechin standart curve. (Each point is the mean of triplicate measurements from three different experiments, n=3)

Table 3.2 Total flavonoid content of *S. fruticosa* extract in catechin equivalents

Concentration (mg/mL)	**Total Flavonoid Content** (µg CE / mg dry extract + SD)
0.25	105.4 ± 3.12
0.5	108.9 ± 2.61
Average	107.14 ± 2.47

In the literature, there are not many studies related with application of total flavonoid content assays. In one research, it was reported total flavonoid content for some *Salvia* species from Mediterranean countries that were extracted with methanol: water were determined as 36.27 to 40.83 µg CE / mg dry extract (Dinçer et al., 2013).

In other study, total flavonoid content for some *Salvia* species were measured as 1.4 to 5.7 µg RE / mg dry extract (Miliauskas et al., 2004).

when compared to the these results, water extracted *S. fruticosa* displayed much higher flavonoid content. High total phenolic and flavonoid content is correleted with strong antioxidant activity (Kamatou, 2006).

3.4 Antioxidant Efficiency of *S. fruticosa*

Antioxidant properties of many plant extract is attributed to their phenolic contituents because these groups have a strong impact on the removal of free radicals from human body. Therefore, it is important to determine the antioxidant capacity of the plant extracts for increasing the their values. There are some different methods to evaluate the antioxidant capacity. In this study, DPPH radical scavenging capacity, ABTS radical scavenging capacity and metal chelating activity methods were selected for their sensitivity, simplicity and reproducibility.

3.4.1 Determination of Antioxidant Capacity of *S. fruticosa* by DPPH Method

DPPH radical scavenging capacity of the extract was determined according to Blois et al, (1958) previously defined in Chapter II by using the equation 2.4. The results were expressed as RSA % versus different extract concentrations (Figure 3.3). IC_{50} values were calculated from the plot of % RSA versus different extract concentrations (Table 3.3). The lower the IC_{50}, the higher the antioxidant capacity. In this study, α tocopherol was used as a positive control.

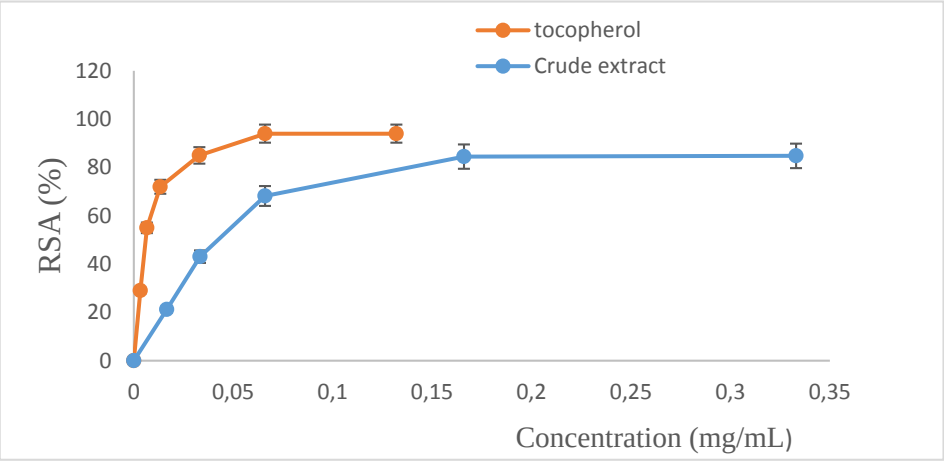


Figure 3.3 Percent DPPH scavenging activity (%RSA) of *S. fruticosa* extract. (Each point is the mean of triplicate measurements from three different experiments, n=3)

Table 3. 3 Antioxidant Capacity of *S. fruticosa* and α-tocopherol by DPPH method

	Antioxidant Activity	**Maximum RSA (%)**
Sample	IC50 µg/ mL ± SD	RSA % ± SD
S. fruticosa extract	34.9 ± 0.009	84,8 ± 0.85
α-tocopherol	17.1 ± 0.014	94 ± 1.02

In the literature, it was reported that antioxidant activity of some of *Salvia* species extracted with methanol: chloroform were stated to be 11.9 to 69.3 μg/ mL with EC_{50} values by DPPH assay (Kamatou et al. 2010).

In another research, EC_{50} value of dichloromethane, ethyacetate and methanol extracts of *S. fruticosa* was detemined as 50.43 μg/ mL, 72.07 μg/ mL and 32.13 μg/ mL, respectively (Senol, S F. et al., 2010).

The free radical scavenging activities of the aqueous extracts of *R. officinalis* and *S. officinalis* obtanined from Finland were reported as 0.236 and 0.265 (IC50 [mg/ml]), respectively (Dorman et al., 2003).

In this study, antioxidant activity of the *S. fruticosa* extract with an IC_{50} (34.9 μg / mL) value determined by DPPH method shows a strong antioxidant capacity compared to the literature studies. On the other hand, it was found that α-tocopherol a powerful antioxidant compound with an IC_{50} (17.1 μg/ mL) value shows two times more antioxidant capacity than *S. fruticosa* extract.

3.4.2 Determination of Antioxidant Capacity of *S. fruticosa* by ABTS Method

ABTS assay is another method to evaluate the antitoxidant capacity of biological samples. One advanteges of this method is due to its high precision and easy applicability for the determination of antitoxidant capacity of plant extracts.

In this study, antitoxidant capacity of *S. fruticosa* was performed according to Re et. al (1999) previously described in Chapter II by using the equation 2.5. Trolox was used as a standart and percent radical scavenging activity of trolox was plotted against the different concentrations of trolox (Figure 3.4). TEAC value of the water extract of *S. fruticosa* was calculated from the slope of percent radical scavenging activity that was plotted against the different concentrations of trolox. Results were given in Table 3.4.

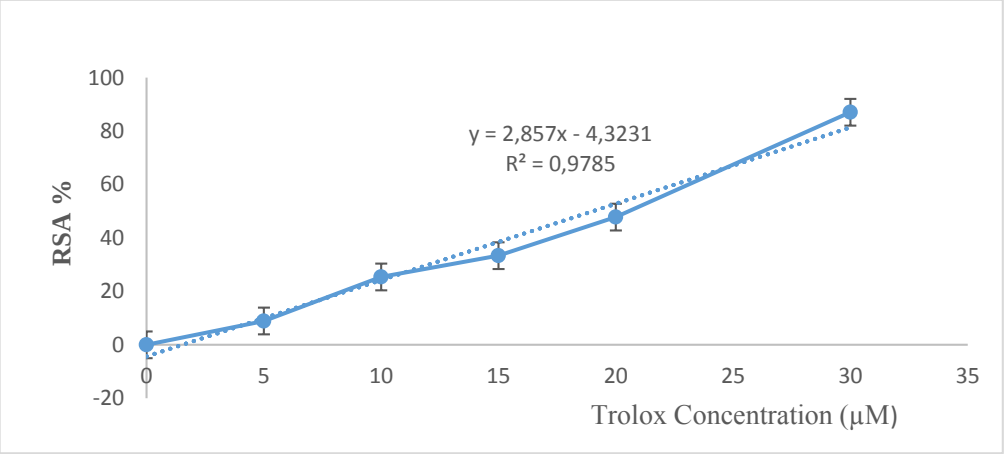


Figure 3.4 Percent radical scavenging activity (% RSA) of trolox**.** (Each point is the mean of triplicate measurements from three different experiments, n=3).

Table 3. 4 Trolox equivalent antioxidant capacity (TEAC) of *S. fruticosa extract*

Concentration (µg/mL)	TEAC value (mmol TE/ g extract ± SD)
2,5	1.23 ± 0.0043
5	1.31 ± 0.0052
10	1.15 ± 0.0046
Average	1.23 ± 0.1900

In this assay, trolox equivalent antioxidant capacity (TEAC) value was determined as 1.23 ± 0.19 (mmol TE/ g extract). In the literature, the TEAC value of methanol ectracted *Salvia officinalis* was reported as 1.783 ± 0.011 mmol TE/ g extract (Ünver et al., 2009). These results were highly compatible with each other and the results of DPPH method.

3.4.3 Determination of Fe^{+2} Chelating Activity of *S. fruticosa*

Fe^{+2} Chelating capacity of the extract was screened according to Dinis et al (1994) previously defined in Chapter II by using the equation 2.6. The results were expressed as RSA % versus different extract concentrations (Figure 3.5). EDTA

was used as a positive control. Results with an IC_{50} (mg/mL) values of extract and EDTA were given in Table 3.5. The lower the $IC_{50,}$ the higher the Fe^{+2} chelating capacity.

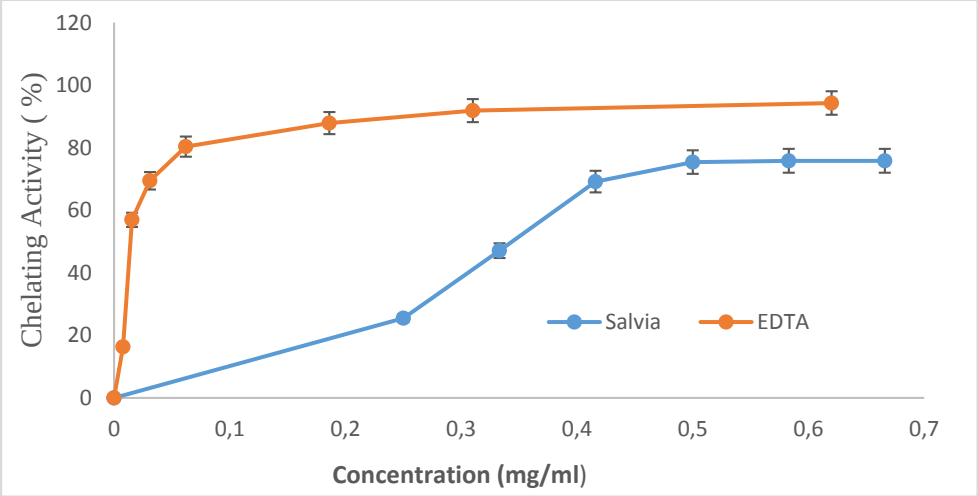


Figure 3. 5 Fe^{+2} Chelating Activity of S. futicosa with max. RSA (%) (Each point is the mean of triplicate measurements from three different experiments, n=3).

Table 3.5 Fe^{+2} Chelating Activity of *S. fruticosa* with maximum RSA (%)

	Fe^{+2} Chelating Activity	**Maximum RSA (%)**
Sample	IC50 mg/ mL ± SD	RSA % ± SD
S. fruticosa extract	0.43 ± 0.0035	75.8 ± 0.92
EDTA	0.107 ± 0.002	94.3 ± 1.08

In this study, IC50 value for Fe^{+2} Chelating capacity of the extract was determined as 0.43± 0.0035 mg/ mL. In the literature, IC50 value for methanol extract of *S. fruticosa* was found as 0.21 ± 0.09 mg/ mL (Ebrahimzadeh, 2008). Compared to the literature, the extract showed two times lower metal chelating activity. When compared with the potent control group EDTA, it showed four times lower metal chelating activity.

This finding supports that *S. fruticosa* with it's small IC_{50} value for DPPH and Ferrous ion Chelating actvitiy methods and also its high TEAC value with ABTS method, it is a good antioxidant candidate.

3.5. RP- HPLC Analysis

3.5.1 Optimization of Chromatographic Conditions

Reverse-Phase High Performance Chormatography (RP-HPLC) is widely used technique for the identification and quantitation of natural products (Cannell, 1998). In this study, it was aimed to identify the bioactive components in *S. fruticosa* by RP-HPLC.

Different elution systems were carried out to optimize the choromatographic conditions. Two different gradient system were performed to get the best resolution of the phenolic compounds previously mentioned in section 2.2.5.1.

Figure 3.6 and 3.7 display the different chromatograms of HPLC analysis for 10 references standart at 280 nm, 322 nm and 330 nm by RP-HPLC photodiode array dedector.The literature reported that optimal detection of phenolic compounds was at 280 nm and 320 nm (Escarpa, 1998 and Liu et al, 2013).

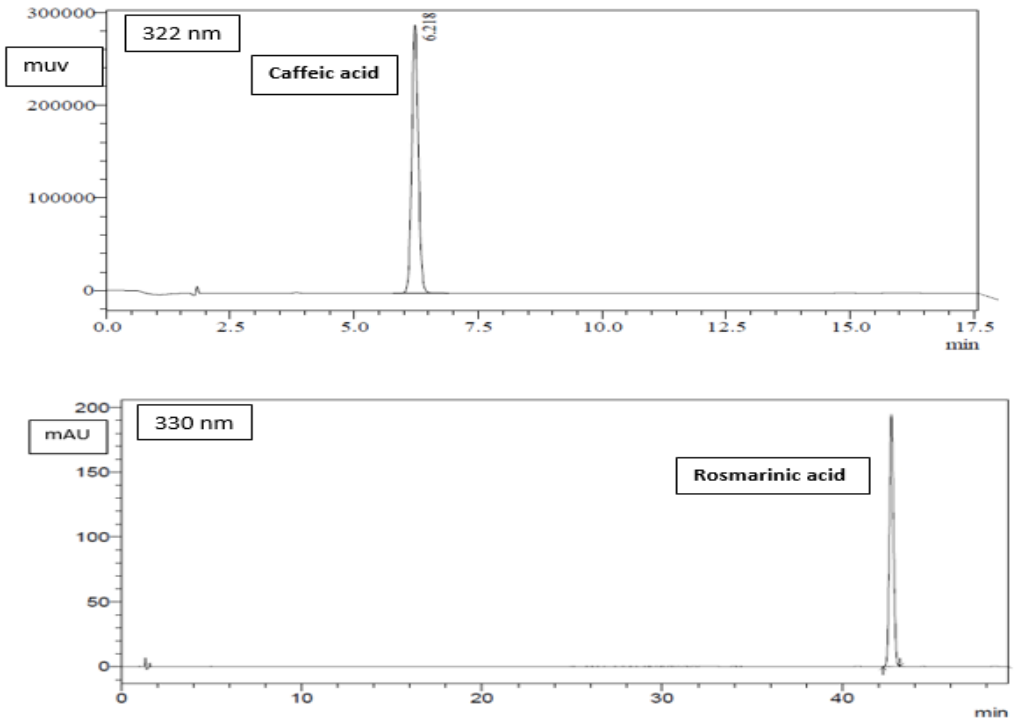


Figure 3. 6 RP-HPLC chromatogram of caffeic acid and rosmarinic acid standarts at 322 and 330 nm with their retention times

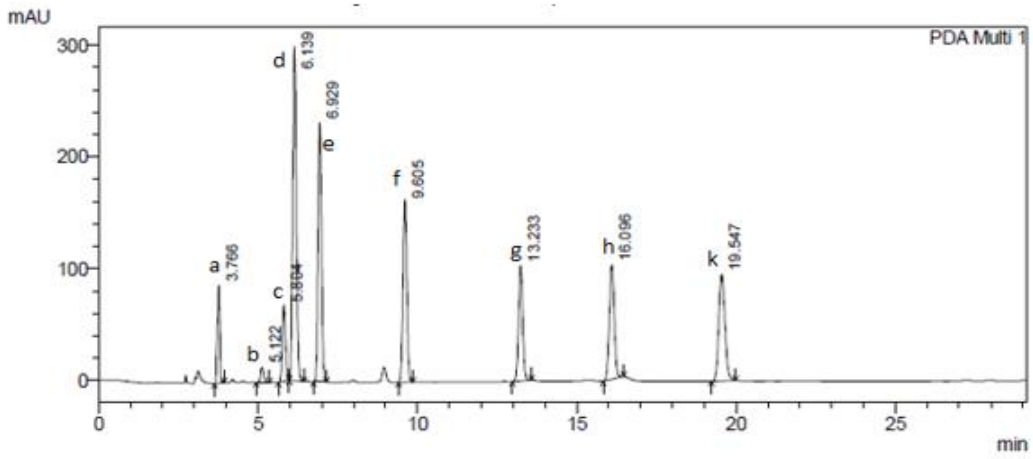


Figure 3. 7 HPLC chromatogram of phenolic standarts mixture at 280 nm with retention times; (a; gallic acid, b; (+) catechin, c; (-) epicatechin, d; epigallocatechin gallate, e; syringic acid, f; p-coumaric acid, g; rosmarinic acid, h; t-resveratrol, k; quercetin).

RP-HPLC analysis of the individual standarts is given in Table 3.6. First of all, each standarts was injected individually for determination of the retention time and chromatographic characteristics and then mixture of the standarts was performed at 280 nm shown in Figure 3.7.

Rosmarinic acid and caffeic acid were not carried out in a mixture in Figure 3.6. The retention time of the standarts were ranged from 3.76 min (gallic acid) to 42.6 min (rosmarinic acid).

Table 3. 6 Retention times max wavelength (λmax) of phenolic standarts

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

3.5.2 Identification of the Phenolic Constituents in *S. fruticosa* by RP-HPLC

Qualitative analysis of phenolic compounds in the plant extract were based on the comparasion of their retention times with the reference phenolic standarts. Figure 3.8 displays the well resolved RP-HPLC chromatograms of the S. futicosa extract for detection of caffeic acid and rosmarinic acid at 322 and 330 nm, respectively. Gallic acid, syringic acid, t-resveratrol and quercetin in the extract were monitored at 271, 274, 306 and 370 nm, respectively (Figure 3.9). Analytical conditions were previously described in Chapter 2.

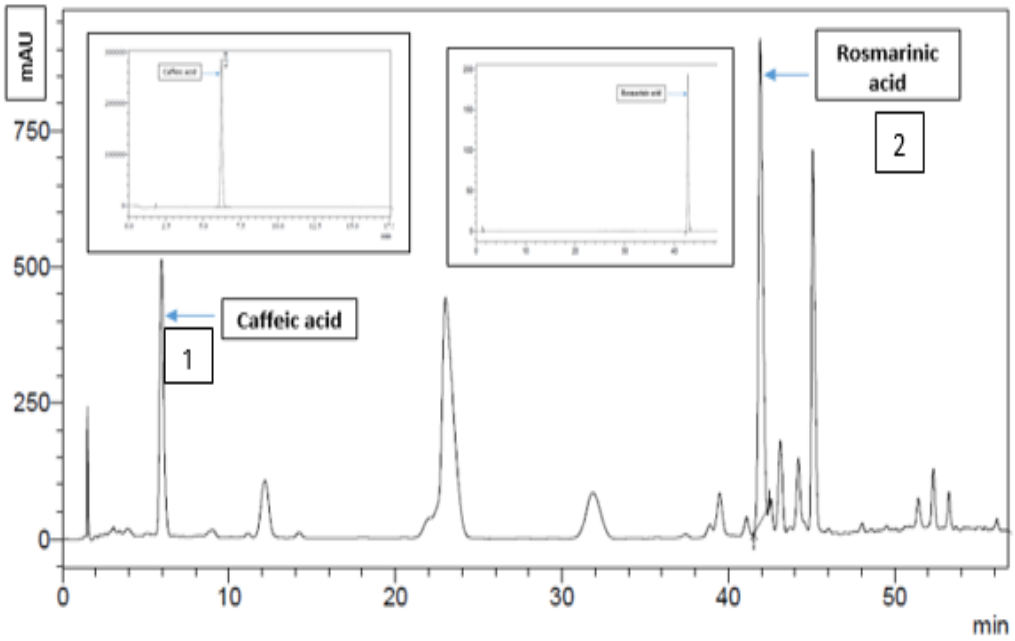


Figure 3.8 RP-HPLC chromatogram of the *S. fruticosa* extract monitored at 330 nm. Peaks are indicated as follows; 1) caffeic acid 2) rosmarinic acid, respectively.

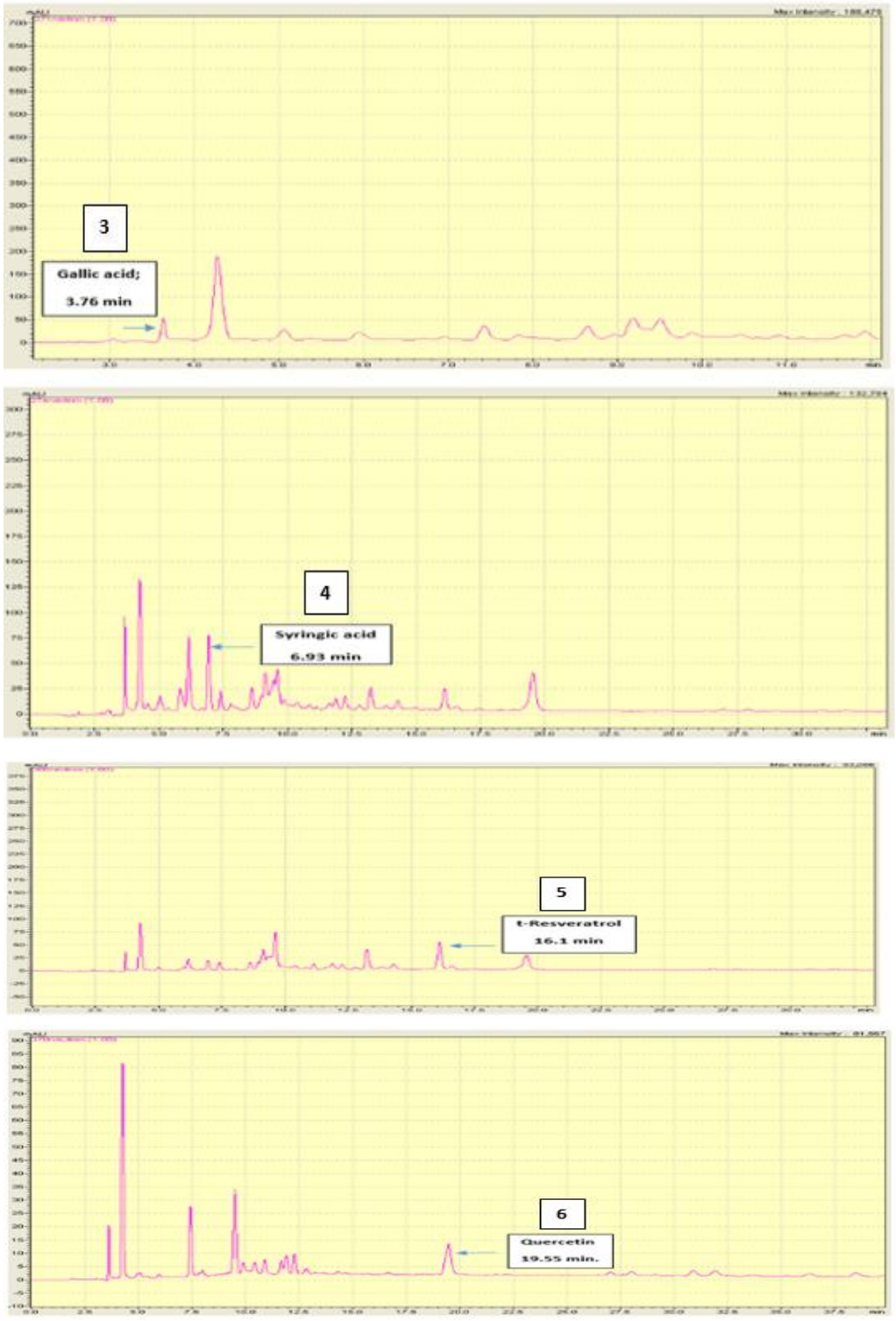


Figure 3.9 RP-HPLC chromatogram of the *S. fruticosa* extract monitored at 271, 274, 306 and 370 nm. Peaks are indicated as follows; 3) gallic acid 4) syringic acid, 5) trans resveratrol and 6) quercetin, respectively.

3.5.3 Quantitation of the Phenolic Constituents in *S. fruticosa* by RP-HPLC

For quantitation of the amounts of the phenolic compounds identified in the *S. fruticosa* extract, various concentrations of the phenolic standards were prepared individually. Standart curves were prepared by plotting the peak area of the standard against the concentration (μg/mL). All the standart samples were measured in duplicates and injected as 20 ppm. The amounts of the identified phenolic compounds in the extract were shown in Table 3.7.

Table 3.7 RP-HPLC Analysis of *S. fruticosa* extract

Phenolic compounds	**mg/g dry extract ± SD**
Rosmarinic acid	59.3 ± 3.82
Caffeic acid	15.16 ± 0.4
Gallic acid	0.56 ± 0.024
Syringic acid	0.74 ± 0.0104
Trans resveratrol	0.17 ± 0.06
Quercetin	0.73 ± 0.04

Phenolic compounds in various *Salvia* species have been widely investigated because these compounds make the plants valuable for medicinal approaches. Rosmarinic acid and caffeic acid are highly abundant phenolic acids in *Salvia species.* Rosmarinic acid that is an ester of caffeic acid is commonly found in *Salvia species.* It has a number of biological properties such as antiinflammatory, antibacterial and antiviral (Maike Petersen, 2003).

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In the literature, it was reported that methanol extracts of *S. officinalis, S. glutinosa, S. aethiopis* and *S. sclarea* contain rormarinic acid as follows 19.5, 47.3, 41.1, 13.3 mg/g dry extract, respectively (Donata Bandoniene, 2005). An other research group determined the rosmarinic acid content of methanol extract of *S. tomentosa* as 10.24 mg/g dry extract (Dinçer et. al, 2013).

In this study, rosmarinic acid and caffeic acid were determined as major phenolic compounds respectively. Rosmarinic acid content was deterined as 59.3 ± 3.3 mg/g dry extract. Our water extracted plant showed more rosmarinic acid than that of the results of them.

In the literature, Caffeic acid content of methanol extracts of *S. fruticosa*, *S. tomentosa* and *S. officinalis* were reported as 97.3, 42.77, 15.14 mg/g dry extract, respectively (Askun, 2009; Coisin 2012). Caffeic acid amount in the *S. absconditiflora* was determined as 15.43 mg/g dry extract (Yılmaz, S., 2013).

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

Quercetin, gallic acid, syringic acid and trans resveratrol were determined as minor phenolic compounds in the *S. fruticosa.* Quercetin and syringic acid amount were approxiamtely the same and determined as 0.73 and 0.74 mg/g dry extract and followed by gallic acid and trans resveratrol as 0.56 and 0.17 mg/g dry extract, respectively. In the literature, quercetin amount in *S. tomentosa* was calculated as 0.59 mg/g dry extract (Dinçer et. al, 2013).

In another research reported that quercetin makes up 2.52 percent of *S. officinalis L*. (Sarhan et. al, 2013). In this study, quercetin content of *S. fruticosa* was calculated as 0.95 %.

Quercetin that is considered as a potent antioxdiant because of its high capability of scavenging free radicals, it has a high effect on the inhibition of lipid

peroxidation (Hollman, 1997; Sakanashi, 2008). In addtion, it was shown to reduce inflammation (Boots, 2011) and to display antiviral, antibacterial, anticarcinogenic and anti-proliferative effects (Walle, 2004; Braganhol, 2006; Marchand, 2002).

In the literature however, there were not found any worth mentioning study related with trans-resveratrol and gallic acid contents in *Salvia species*. This is because of thier lower abundance in these plants. In this study, it was demonstrated the presence of trans-resveratrol and gallic acid are highly low in *S. futicosa* by RP-HPLC.

As a conclusion, this study showed that water extract of *S. fruticosa* contains high phenolic phytochemicals and have high antioxidant properties. Because of their high antioxidant capacities, some plant extracts are considered to have high cytotoxic potentials on a wide range of cancer cell lines (Boivin, 2009; Ju, 2004). So, it is highly recommended that cytotoxic effects of the crude extract of *S. fruticosa* and its two main constituents; rosmarinic acid and caffeic acid, on selected cancer cell line (HT-29 adenocarcinoma) should also be demonstrated.

3.6 Cell Culture Assay

3.6.1 Viability of HT-29 Cells

When the physical and chemical conditions are changed, most of the cells are affected from this situation because of their sensibility to environments. Cancer cells should be supplied with nutrients and physiological conditions so that they can sustain their viability and cell division.

The presence of the cells were regularly monitored under the inverted microscope during the days. In order for plotting the growth curve of cell viability, a 24 hr or 48 hr period time were needed. After this time period of incubation, cells were counted and this was recerded as zero time. Further counting was repeated for every this time period during 20 days.

For the evaluation of the cell viability using dye exclusion technique with trypan blue, a hemocytometer was used. A 20 µL of the cell suspansion were examined under the light microscope.

In this study, HT-29 cells were seeded at a density of 100000 cells/mL in a 6 well plate and incubated at 37 ^{0}C in 5% CO_2 incubator. Two wells were counted at each time point and the remaining wells were allowed to growing. The numbers of cells were plotted against the growth time (Figure 3.10).

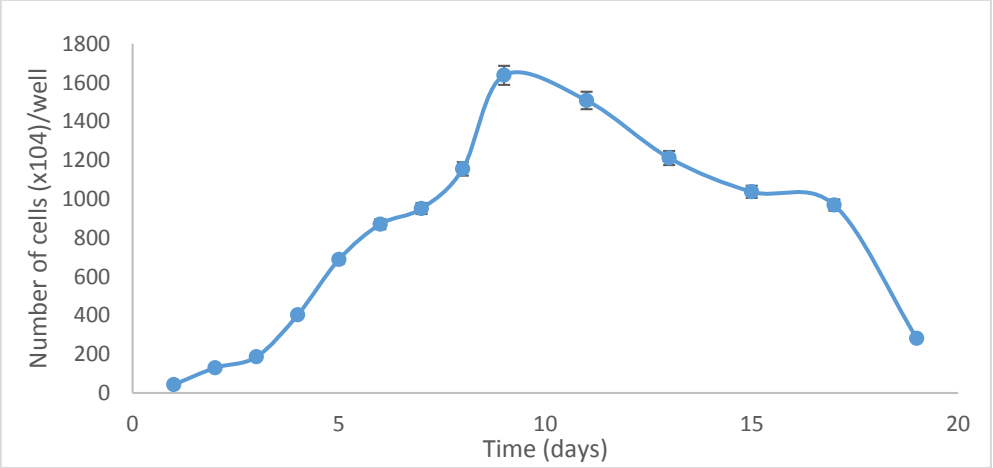


Figure 3.10 The growth curve of HT-29 cells during 20 days by trypan blue counting

3.6.2 Cytotoxicity of *S. fruticosa* extract, rosmarinic acid and caffeic acid in HT-29 cells

3.6.2.1 XTT Cell Cytotoxicity Assay

The cytotoxic effect of *S. fruticosa* extract and its main components, rosmarinic acid and caffeic acid, treatments on HT-29 human colon adenocarcinoma cells were investigated by XTT assay.

XTT is a tetrazolium salt. Succinate dehydrogenase enzymes of mitochondria of metabolically active cells reduce this tetrazolium salt into an orange colured formazan product .The absorbance of this formazan product proportional to number of active cells was measured using ELISA plate reader at 415 nm.

Cell viability for each concentration of the samples treatment was depicted as % viability, assuming the control as 100 % viable. To eliminate the effect of samples on absorption, the cultured cells without samples treatment were used. Effect of *S. fruticosa* extract, rosmarinic acid and caffeic acid on HT-29 cells were investigated in both time (48/72 hours) and dose dependent manner. Percent viability versus concentration graphs were plotted for each sample and each incubation time shown in Figure 3.11 and 3.14.

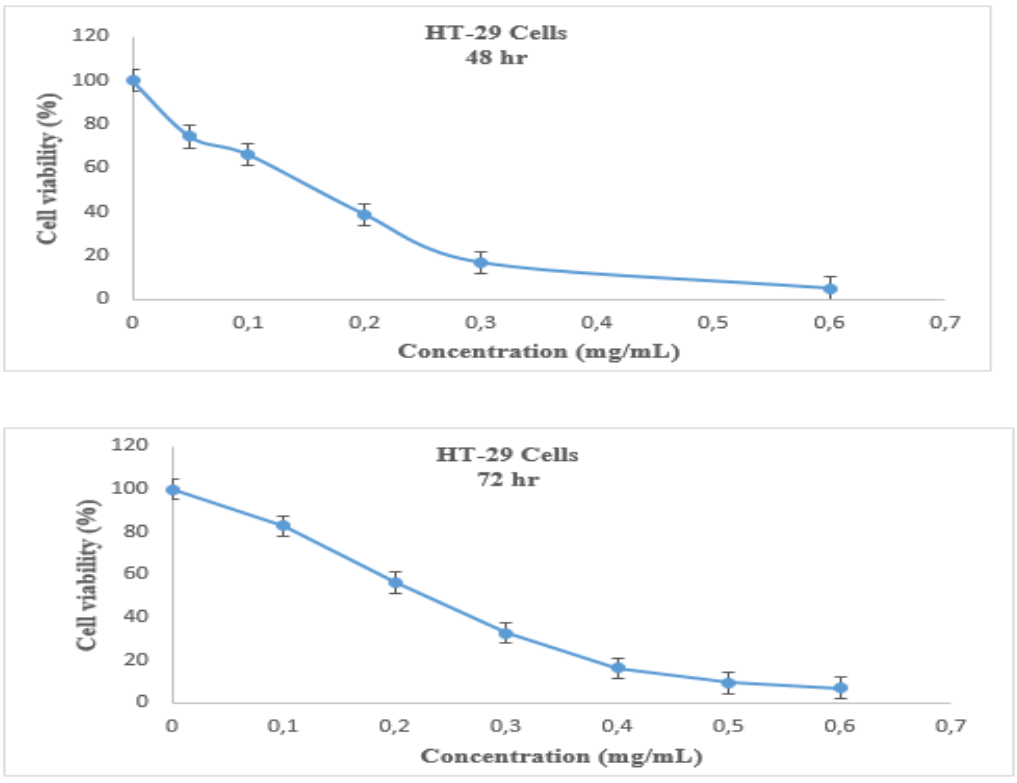
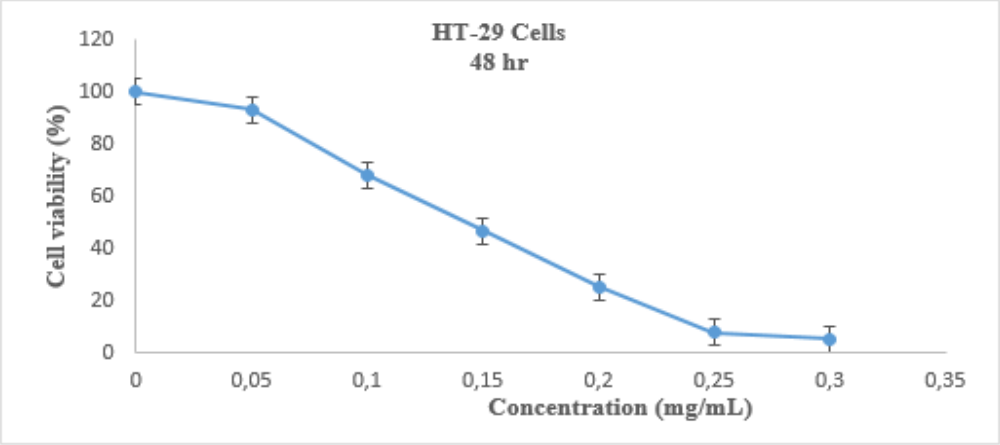


Figure 3.11 Viabilities of HT-29 cells in response to dose and time dependent treatment of *S. fruticosa* according to XTT assay. Each point is the mean of triplicate measurements from three different experiments (n=3)



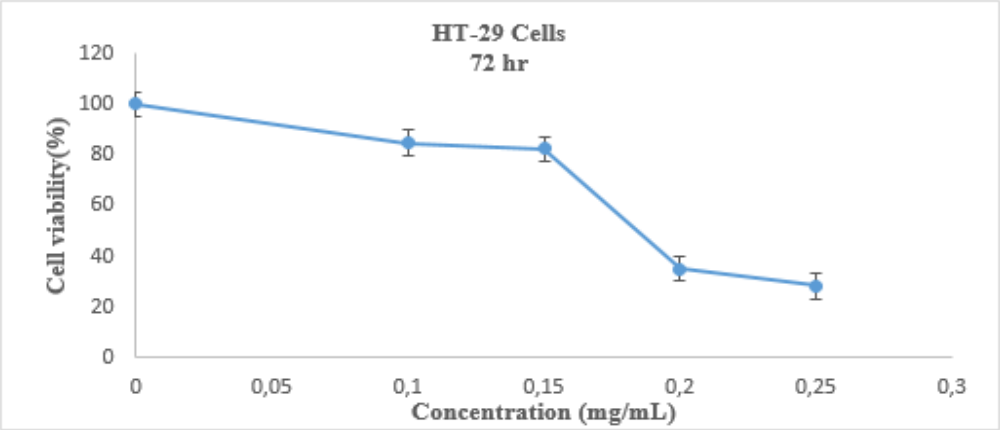
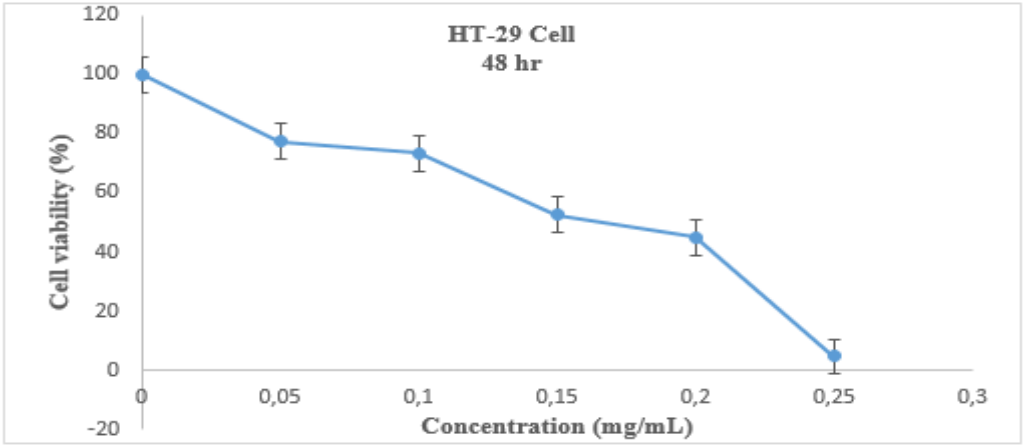


Figure 3.12 Viabilities of HT-29 cells in response to dose and time dependent treatment of rosmarinic acid according to XTT assay. Each point is the mean of triplicate measurements from three different experiments (n=3)



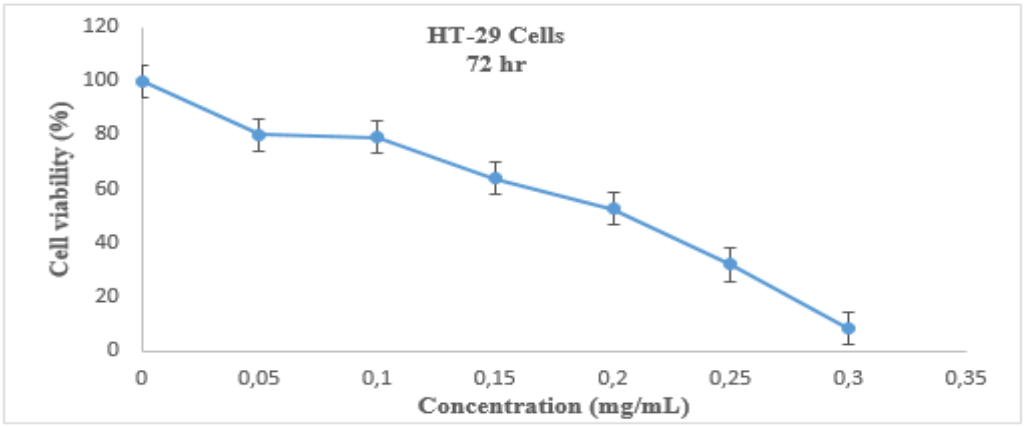
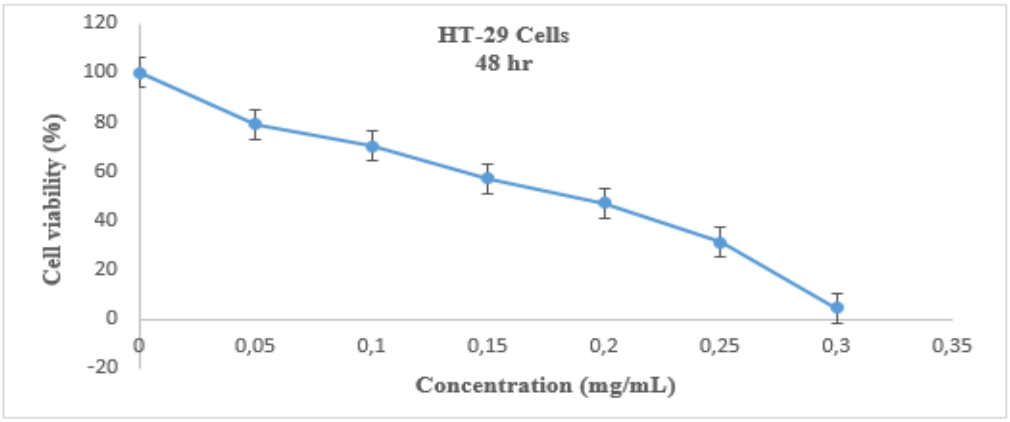


Figure 3.13 Viabilities of HT-29 cells in response to dose and time dependent treatment of caffeic acid according to XTT assay. Each point is the mean of triplicate measurements from three different experiments (n=3)



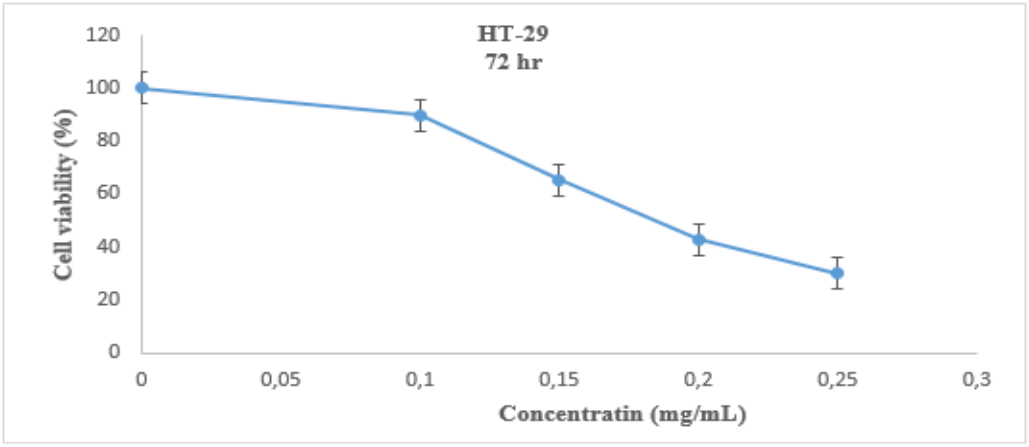


Figure 3.14 Viabilities of HT-29 cells in response to dose and time dependent treatment of rosmarinic acid + caffeic acid according to XTT assay. Each point is the mean of triplicate measurements from three different experiments (n=3)

Cell viabilities were measured at 48hr and 72 hr after treatment with crude extract, rosmarinic acid and caffeic acid standarts on the HT-29 cancer cells. All the agents displayed a significant cytotoxic activity against HT-29 cancer cells in a dose dependent manner.

More than 90 % cytotoxicty on the HT-29 cancer cells were observed at the concentration of 0.6 mg/mL for the *S. fruticosa* extract for 48 and 72 hr incubation (Figure 3.11).

Cytotoxic effect of standart rosmarinic acid against HT-29 cancer cells showed more than 90 % cell death at the concentration of 0.3 mg/mL for 48 and 72 hr incubation. This concentration was determined as 0.25 mg/mL and 0.3 mg/mL for caffeic acid at 48 and 72 hr incubation, respectively (Figure 3.12 and 3.13).

It was also studied the cytotoxic effect of the mixture of rosmarinic acid and caffeic acid in a ratio of 1:1. More than 90 % cell death was observed at the concentration of 0.3 mg/mL for 48 and 72 hr incubation with the mixture of (R.A) and (C.A) of 1:1 (Figure 3.14).

IC_{50} values of the crude extract and other reference standards against HT-29 human colon adenocarcinoma cells, concentration of the sample required to decrease cell viability by 50 %, were shown in Table 3.8 and Figure 3.15; 3.16. Lower the IC_{50} value shows the higher antiproliferative activity.

Table 3.8 Comparison of IC_{50} values of the crude extract and standarts against HT-29 colon cancer cell line for cytotoxic capacity by XTT method. Experimental values were given as means as ± standart deviation

HT-29 Cell line IC_{50} (mg/mL) ± S.D		
Agents	Incubation	
	48 hr	72 hr
Crude extract	0.185 ± 0.0025	0.229 ± 0.0148
Rosmarinic acid (R.A)	0.148 ± 0.0036	0.184 ± 0.0033
Caffeic acid (C.A)	0.149 ± 0.0021	0.181 ± 0.0063
R.A + C.A	0.169 ± 0.005	0.184 ± 0.0076

IC_{50} values for the *S. fruticosa* extract concentrations at which 50% of cells are viable were calculated as 0.185 ± 0.0025 and 0.229 ± 0.0148 mg/mL for 48 and 72 hours incubation, respectively. IC_{50} value for 48 hours incubation was found lower than that of 72 hours incubation.

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

When standard rosmarinic acid and caffeic acid were applied together in a ratio of 1:1 for the cytotoxic capacity, IC_{50} value was determined higher than those of rosmarinic acid and caffeic acid standards seperately and lower than that of crude extract for 48 hours incubation. On the other hand, IC_{50} value of the mixture of R.A + C.A was found similar to the IC_{50} values of R.A and C.A seperately and lower than that of crude extract for 72 hours incubation. In this study, the highest cytotoxic effect was exhibited by rosmarinic acid with an small IC_{50} value. These results were statistically significant.

In this study, the highest cytotoxic effect for 48 hr treatment was exhibited by standard rosmarinic acid with an IC_{50} value of 0.148 mg/mL followed by caffeic acid and crude extract with an IC_{50} value of 0.149 mg/mL and 0.185 mg/mL, respectively. For 72 hr treatment, cytotoxic activities were found in the order of caffeic acid> rosmarinic acid >crude extract. In both time 48/72 hr treatment, crude extract exhibited lower cytotoxic effect.

Infact, it is expected that IC_{50} value of an agent should be getting low, as the cells are subjected to the agent for a longer time period. The opposite situation can sometimes occur because cells become more aggressive and try to survive by increasing their metabolic activity during a longer time period. This can cause to be observed a higher value of IC_{50} concentration. In this study therefore, IC_{50} values of the agents for 72 hours incubation were higher than those of 48 hours incubation (Table 3.8).

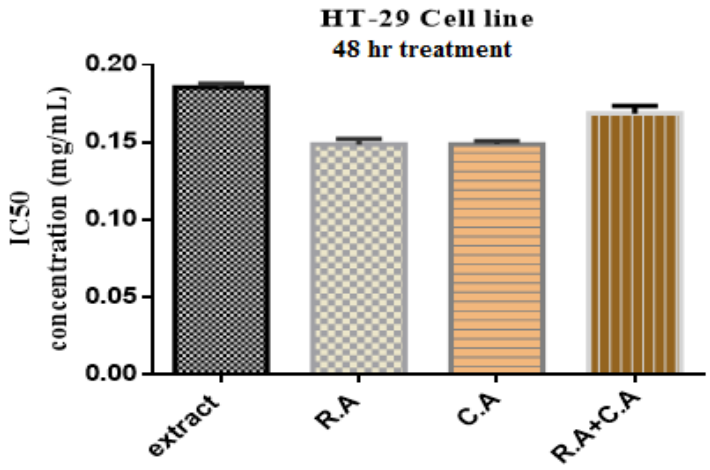


Figure 3.15 Comparision of IC_{50} values of *S. fruticosa* and standard referenences for 48 hours incubation against HT-29 cell line according to XTT assay (one way anova; $p < 0.05$).

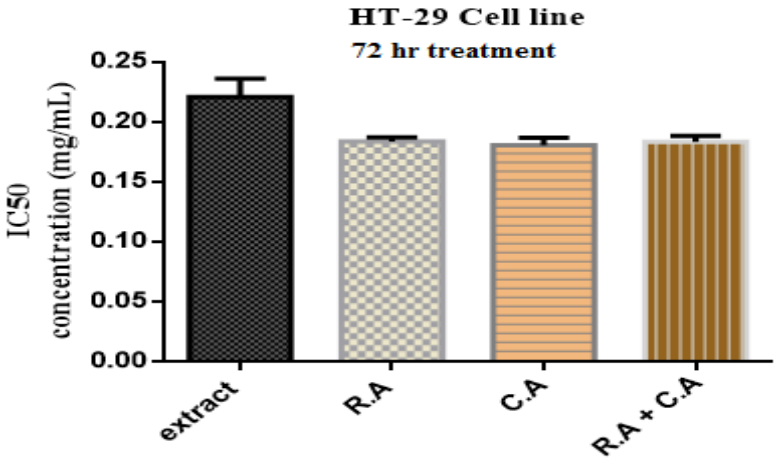


Figure 3.16 Comparision of IC_{50} values of *S. fruticosa* and standard referenences for 72 hours incubation against HT-29 cell line according to XTT assay (one way anova; $p < 0.05$).

3.6.2.2 Viable Cell Counting with Tryphan Blue Exclusion Method

In addition to XTT method, tryphan blu exclusion method (TBE) was performed for cell viability to inhibit the absorbance interference of the extract.

The main principle of TBE assay is based on the cell membrane permeability. Damaged or unimpaired membranes of dead cells cannot exclude the dye but living cells with intact membrane can exclude it. TBE is a commonly used method due to its simplicity whereas it has some limitations such as low accuracy and operator dependency (Kim et al., 2011). This method is also performed for IC_{50} value determination before protein and RNA isolation from the cells are carried out.

Cell viability for each concentration of the extract treatment was depicted as % viability, assuming the control as 100 % viable. To eliminate the effect of the extract on absorption, the cultured cells without samples treatment were used. Effect of *S. fruticosa* on HT-29 cell line was investigated in both time (48/72 hours) and dose (0,05-03 mg/ml) dependent manner. % Viability versus concentration graphs were plotted for each incubation duration (Figure 3.17). IC_{50} values, concentration of extract required to decrease cell viability by 50%, were determined shown in Table 3.9.

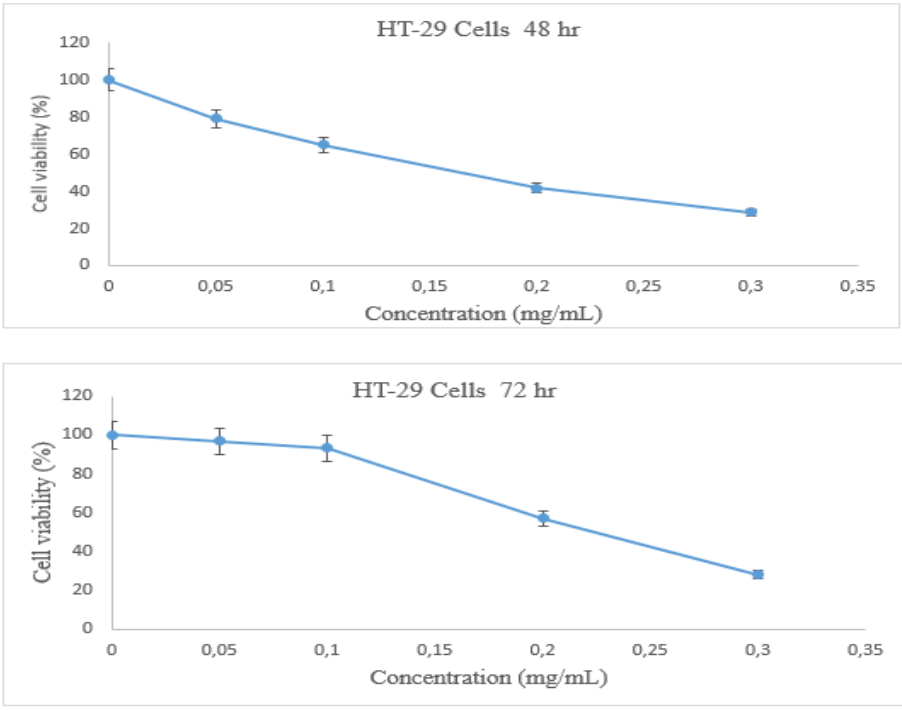


Figure 3.17 Viabilities of HT-29 cells in response to dose and time dependent treatment of *S. fruticosa* according to TBE assay. Each point is the mean of triplicate measurements from three different experiments (n=3)

Table 3. 9 Concentrations of *S. fruticosa* required to decrease the viability of cells 50 % according to TBE assay

HT-29 Cell line IC_{50} (mg/mL) ± S.D		
Agent	Incubation	
	48 hr	72 hr
S. fruticosa extract	0.174 ± 0.0063	0.228 ± 0.0032

IC_{50} values of the *S. fruticosa* extract were determined as 0.174 ± 0.0063 and 0.174 ± 0.0063 mg/mL for 48 hr and 72 hr incubation, respectively. XTT IC_{50} values for 48 hr and 72 hr treatments were determined very close to TBE IC_{50} values in HT-29 cell line (Figure 3.18).

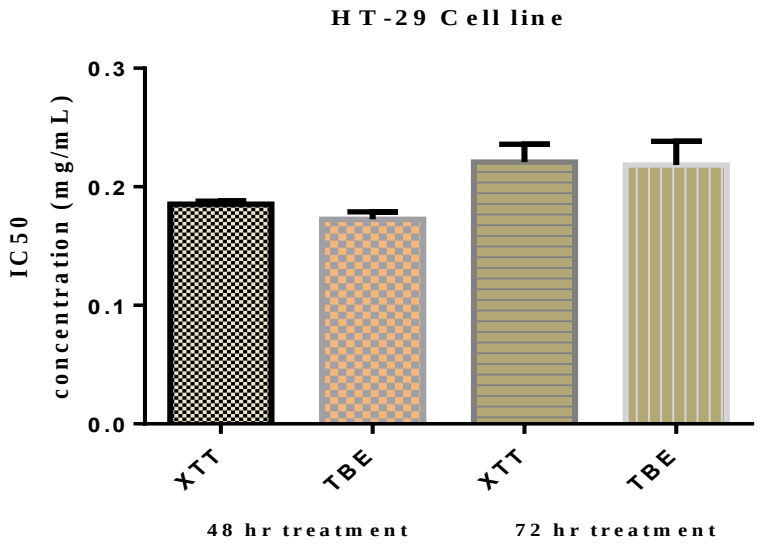


Figure 3.18 *S. fruticosa* IC_{50} concentrations of HT-29 cell line according to XTT and TBE assays. (one way anova, $p < 0.05$ selected for statistically significant)

Giovina Fiore et al. (2012) published antiproliferative activity of the methanol crude extracts of six *Salvia* species: *S. dominica*, *S. lanigera*, *S. menthaefolia*, *S. palaestina*, *S. sclarea* and *S. spinosa* against human colorectal adenocarcinoma cells (HT-29 and WiDr) by MTT assay. Growth inhibitory activities (IC_{50} μg/mL) of crude methanolic extracts of *S. menthaefolia*, *S. sclarea*, *S. dominica*, *S. spinosa* for 72 hr of treatment against HT-29 cells were reported as 95.3, 196.1, 119.5 and 129.4, respectively.

In the same study, inhibitory activities (IC_{50} μg/mL) of crude methanolic extracts of *S. menthaefolia*, *S. sclarea*, *S. dominica*, *S. spinosa* for 72 hr of treatment against WiDr cells were reported as 89.6, 194.4, 175.5 and 152.5, respectively.

In that study, the highest antiproliferative activity was exhibited by *S. menthaefolia* and the lowest antiproliferative activity was exhibited by *S. sclarea*. It was observed that the same plant extract exhibited different antiproliferative activity against different cell lines.

In our study, water extract of *S. fruticos*a displayed an antiproliferative activity for 72 hr of treatment against HT-29 cells with an IC_{50} value of 229 and 228 μg/mL by XTT and TBE method. When compared the IC_{50} values of the *Salvia species*, *S. fruticosa* displays a little low antiproliferative activity than those of the literature. This is due to the being used different subtypes of the plant, different antiproliferation methods and different extraction methods.

Wilson et al. (2012) reported the antiproliferative effects of water extracts of *S. fruticosa* and *S. officinalis* and of their main phenolic compound rosmarinic acid against two human colon carcinoma cell lines, HCT15 and CO115 by BrdU incorporation assay. SF and SO extracts showed their antiproliferative activities in the range of 12.5-50 μg/mL against HCT15 cells by significantly decreasing the levels of BrdU incorporation from 26.2% to 4.7%. On the other hand it was not observed significant antiproliferation effecst by *S. fruticosa* and *S. officinalis* in the range of 12.5-50 μg/mL against CO115 cells. Compared to two colon carcinoma cells, HCT15 cells were observed more sensitive to the sage extracts.

In the same study, rosmarinic acid individually did not display any antiprolifetaion activity in both colon cancer cell lines for 48 hr treatment. In the light of these results, it could be conclude that other active phenolic compounds in *S. fruticosa* and *S. officinalis* are responsible for the antiproliferative effects.

Monica Rosa Loizzo et al. (2014) investigated the antiproliferative activities of methanol extracts of nine *Salvia species* (collected from Iran) namely *S. sclarea, S. atropatana, S. sahendica, S. hydrangea, S. xanthocheila, S. macrosiphon, S. glutinosa, S. chloroleuca and S. ceratophylla* against Caco-2 cancer cells by sulforhodamine B (SRB) assay. The highest cytotoxic effect was exhibited by *S. sclarea* with an IC_{50} value of 101.8 ± 6.4 μg/mL. This is followed by *S. hydrangea, S. chloroleuca, S. atropatana and S. glutinosa* with an IC_{50} values of 121.2 , 123.4, 124.4, 142.8 μg/mL, respectively. Compared to the IC_{50} values, Our water extract of *S. fruticosa* displayed approximately similar antiproliferative activity with methanol extracts of *Salvia species* in the litrerature.

In another research, methanol, hexane and methylene chloride extracts of *Salvia leriifolia* were evaluated for their antiproliferative activities against colorectal adenocarcinoma (Caco-2) cells by sulforhodamine B (SRB) assay (Tundis et al., 2011). The highest cytotoxic effect was observed by methylene chloride extract with an IC_{50} value of 28.1 ± 1.6 μg/mL and followed by hexane with an IC_{50} value of 52.1 ± 1.3 μg/mL. Methanol extract showed the lowest antiproliferation activity with an higher than IC_{50} value of 100 μg/mL. These results show that methylene chloride fraction of the extract include more active phenolic compounds than other fractions displaying more cytotoxic activity against Caco-2 cancer cell line.

In the literature, there is no many research about the cytotoxic effects of caffeic acid and its derivatives against adenocarcinoma cell lines. In one research, It was reported that caffeic acid showed its antiproliferative effect in the range of 5 to 50 μg/mL with an IC_{50} value of 10.7 μg/mL against MCF-7 breast cancer cell line for 48 hr treatment by WST-1 assay (Valdez et al., 2010).

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

In todays, antiproliferation tests have been frequently studied because of it's relation with anticancer activity. One of the main advantages of cytotoxicity assays is that possible cellular proliferation mechanisms can be observed at once. Therefore, it can be focusud on the new plant extracts for discovery of new anti-cancer agents. 5-Fluorourasil was discovered as an chemotherapy drug for colon cancer treatment by this approach.

It was reported many fruit extracts have the capability of inhibiting the proliferation of different types of cancer cells (Camarda, 2007; Wang, 2008; Neto, 2008). All phytochemicals for example phenolic acids, flavonoids, carotenoids and anthocyanins are responsible for anti-proliferative activities to some extent.

Cristina P. R. Xavier et al. (2009) evaluated the antiproliferative and proapoptotic effects of water extracts of *S. fruticosa* in human colon carcinoma cell lines, HCT15 and CO115 through the MAPK/ERK and PI3K/Akt signalling pathways. The results showed that apoptosis was induced in both cell lines, but cell proliferation was inhibited by the sage extract only in HCT15 cells. ERK phosphorylation was inhibited by sage extract in HCT15 cells. Whereas it was not observed any effects on Akt phosphorylation in CO115 cells. Therefore, cytotoxic activity of sage extract seems to be attributed, at least in part, to the inhibition of MAPK/ERK pathway.

Liu et al. (2004) proposed that potent antiproliferative activities of fruits and vegetables are due to the synergistic actions between the complex mixtures of phytochemicals. For instance, pomegranate juice exhibited more cytotoxic activity in different human colon cancer cells compared to the its bioactive compounds individually.

Present study demonstrates that water extract of *S. fruticosa* with its main bioactive components rosmarinic acid and caffeic acid identified by RP-HPLC displayed a good anticancer activity by inhibiting the proliferation of HT-29 colon cancer cells with their low IC_{50} values. *S. fruticosa* extract can be a good candidate for targeted therapy for medical purposes in addition to using it as a drinking tea.

3.6.3 Morphological Analysis of HT-29 Cells by Light Microscopy

In order to observe the cytotoxic effects of *S. fruticosa* extract on HT-29 cells, morphological changes and viability of cells were analyzed under inverted light microscope (Figure 3.19 and 3.20).

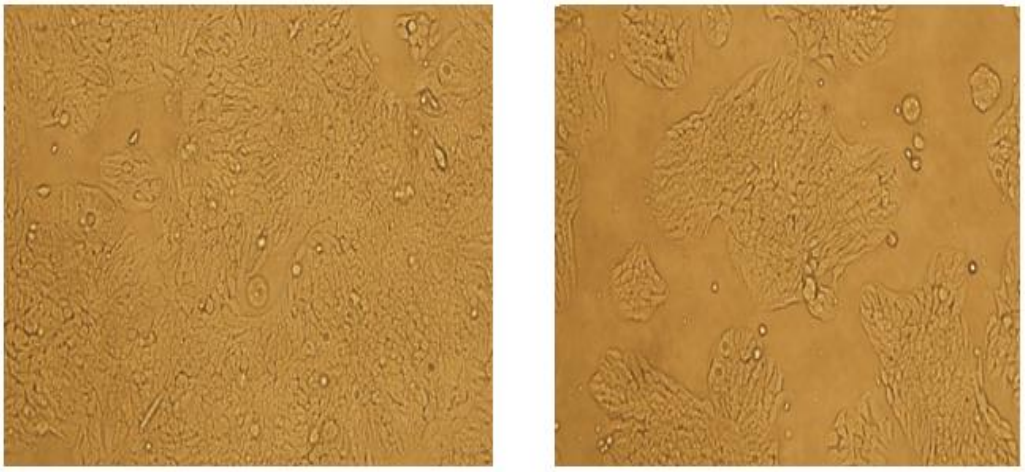


Figure 3. 19 HT-29 cells. Left: 48 hours 0,1 % DMSO treated control. Right: 48 hours IC_{50} concentration (0.174 mg/mL) *S. fruticosa* treated.

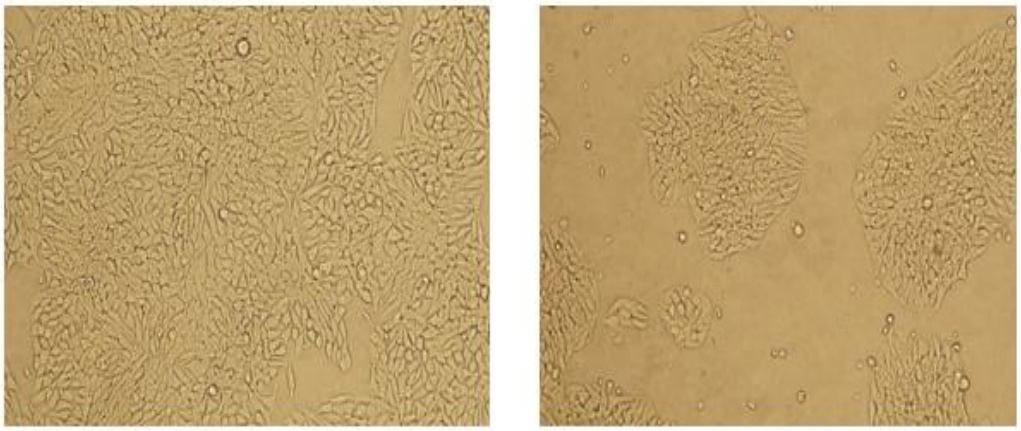


Figure 3. 20 HT-29 cells. Left: 72 hours 0,1 % DMSO treated control. Right: 72 hours IC_{50} concentration (0.228 mg/mL) *S. fruticosa* treated.

After treatment of HT-29 cells with water extract of *S. fruticosa* at IC_{50} concentrations, growth inhibition in cells were observed compared with 0,1 % DMSO control treated cells. In both time incubations (48 /72 hr), the confluency significantly decreased. Some of the cells were observed being round and detached.

3.7 mRNA Expression Analysis of Phase I and Phase II enzymes Modulated by *S. fruticosa* in HT-29 Cells

3.7.1 Agarose Gel Electrophoresis for Qualification of RNA

Isolated RNA form HT-29 cells was evaluated for quality by agarose gel electrophoresis. RNA integrity and purity were dependent for the observation of 28S RNA and 18S RNA. These subuints were easily observed at the end of the gel electrophoresis. In order for quantification of the RNA, other techniques were used.

3.7.2 Determination of RNA Purity and Concentration

Quantity of the RNA was determined reading the absorbance at 260 nm. Quality of the RNA molecule was checked observing the A_{260}/A_{280} and A_{260}/A_{230} ratios. A_{260}/A_{280} ratio should be 1.8-2.2 range. Above 2.2 shows the protein contaminatin and below 1.8 refers the DNA contamination. It was also observed the A_{260}/A_{230} ratio to checked the carbohydrate and phenolic contaminations. Isolated RNA concentration and purity was shown in Table 3.10.

Table 3. 10 Concentration and Purity of RNA molecules

	Incubation hour	**RNA concentration (ng/µl)**	**260/280**
Control	48	1535	2.06
	72	1440.5	2.08
S. fruticosa extract	48	875	2.08
	72	1180.7	2.12

3.7.3 CYP1A1 mRNA expression in HT-29 Cell line

mRNA expression of CYP1A1 in the HT-29 cells was performed by Quantitative real time PCR (qRT-PCR) technique. In order to calculate relative mRNA expression of CYP1A1, GAPDH was used as internal standard.

Relative mRNA expression of CYP1A1 was determined from the standard curve of control cDNA with a dilutions of 1:5, 1:10, 1:20, 1:40 and 1:80 (Figure 3.21). Changes in fluorescence of SYBR green dye I versus cycle number of CYP1A1 gene exhibit the amplification plot (Figure 3.22). Amplification of specific product was checked by melt curve analysis (Figure 3.23).

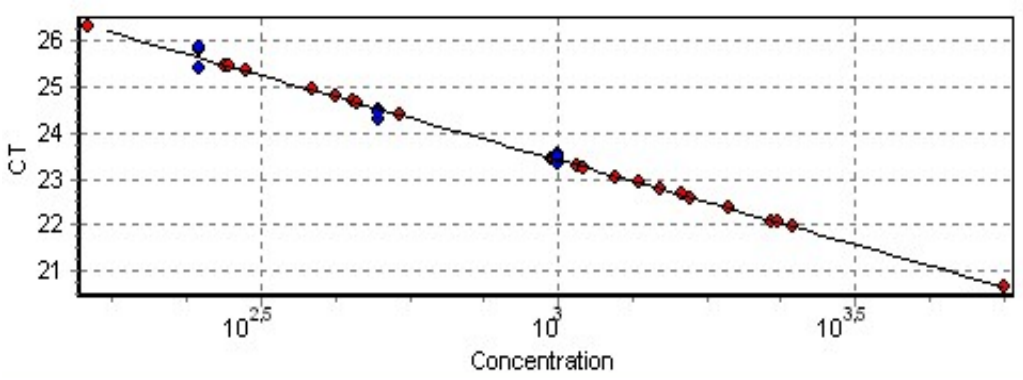


Figure 3.21 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of CYP1A1 mRNAs in HT-29 cell line. Efficiency: 0.973

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

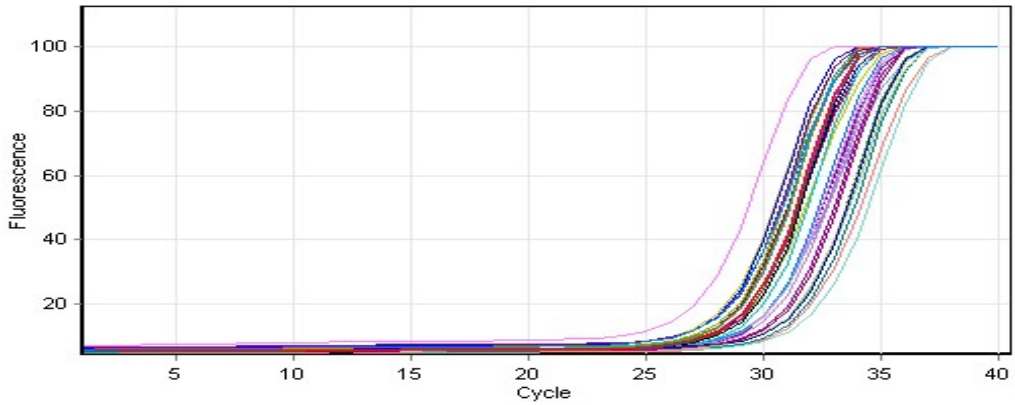


Figure 3.22 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

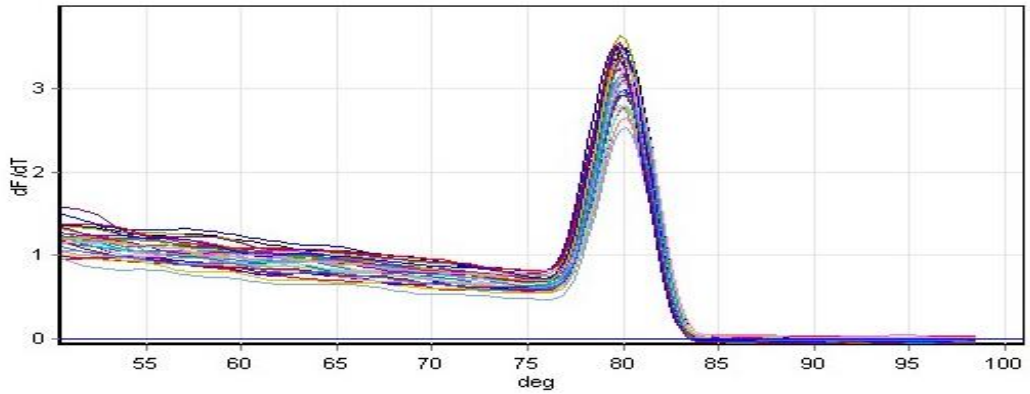


Figure 3.23 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

Relative CYP1A1 mRNA expression in HT-29 cell line was determined according to Livak method (Livak & Schmittgen 2001). Formulation for Livak ($2^{-\Delta\Delta ct}$) method is shown below;

$\Delta\Delta Ct$ = [(Ct gene of interest – Ct internal control) treated – (Ct gene of interest – Ct internal control) control]

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

Figure 3.24 displays the mean relative CYP1A1 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP1A1 gene expression in HT-29 cells were shown in Table 3.11.

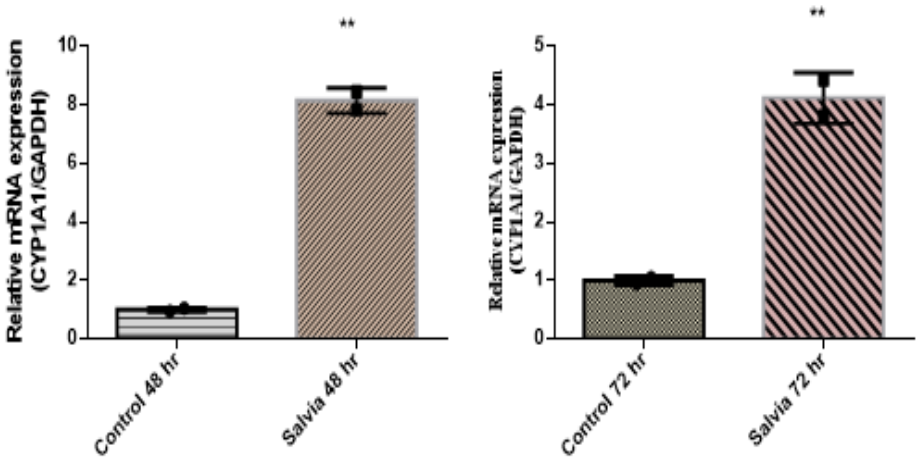


Figure 3.24 Comparison of CYP 1A1 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3. 11 Fold changes of CYP1A1 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
CYP1A1	8.14 ↑	4.12↑

As shown in Table 3.12 and Figure 3.23, expression of CY1A1 treated with *S. fruticosa* extract at the concentrations of IC_{50} values for 48 hr and 72 hr incubation in HT-29 cells increased by 8.14 and 4.12 folds respectively. 48 hr incubation caused approximately two times more upregulation of CY1A1 compared to the 72 hr incubation.

3.7.4 CYP1A2 mRNA expression in HT-29 Cell line

Figure 3.25 displays the mean relative CYP1A2 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP1A2 gene expression in HT-29 cells were shown in Table 3.12. Standard cuve, amplification curve and melting curve of CYP1A2 were displayed in Appendix A.

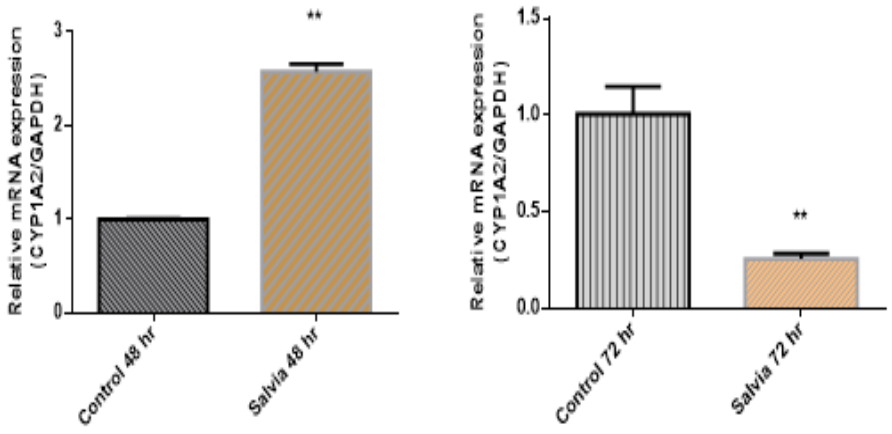


Figure 3.25 Comparison of CYP 1A2 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3. 12 Fold changes of CYP1A2 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
CYP1A2	2.57 ↑	3.89 ↓

As shown in Table 3.13 and Figure 3.23, gene expression of CYP 1A2 treated with *S. fruticosa* extract for 48 hr incubation increased by 2.57 folds in HT-29 cells. On the other hand, 72 hr incubation decreased the expression of CYP1A2 gene by 3.89 folds.

3.7.5 CYP2E1 mRNA expression in HT-29 Cell line

Figure 3.26 displays the mean relative CYP 2E1 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP2E1 gene expression in HT-29 cells were shown in Table 3.13. Standard cuve, amplification curve and melting curve of CYP2E1 were displayed in Appendix A.

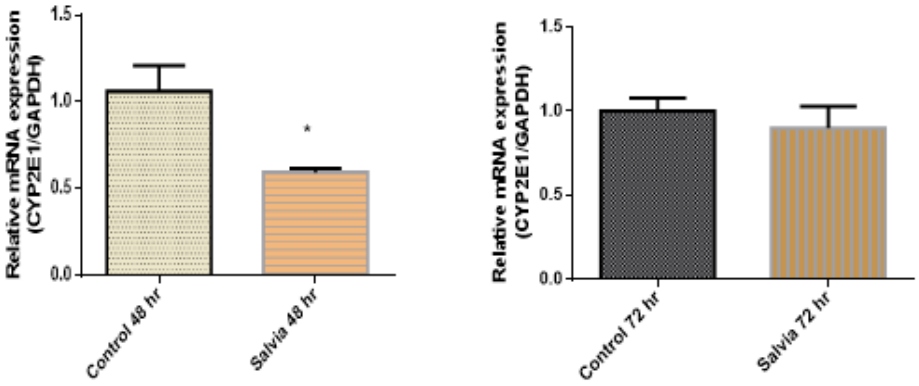


Figure 3.26 Comparison of CYP 2E1 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3.13 Fold changes of CYP2E1 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
CYP2E1	1.72 ↓	NS

As shown in Table 3.14 and Figure 3.26, gene expression of CYP 2E1 treated with *S. fruticosa* extract for 48 hr incubation decreased by 1.72 folds in HT-29 cells. On the other hand, 72 hr incubation did not cause any significant changes on the expression of CYP 2E1 in HT-29 cells.

3.7.6 CYP3A4 mRNA expression in HT-29 Cell line

Figure 3.27 displays the mean relative CYP 3A4 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of CYP 3A4 gene expression in HT-29 cells were shown in (Table 3.14). Standard cuve, amplification curve and melting curve of CYP 3A4 were displayed in Appendix A.

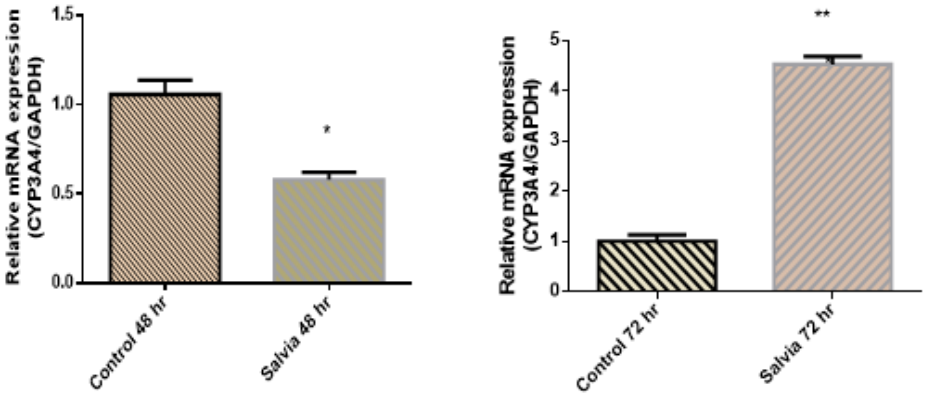


Figure 3.27 Comparison of CYP 3A4 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05, ** P≤0.01; *** P≤0.001; NS, not significant

Table 3. 14 Fold changes of CYP 3A4 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
CYP3A4	1.75 ↓	4.02 ↑

As shown in Table 3.15 and Figure 3.26, gene expression of CYP 3A4 treated with *S. fruticosa* extract for 48 hr incubation decreased by 1.75 folds in HT-29 cells. On the other hand, 72 hr incubation increased the expression of CYP 3A4 gene by 4.02 folds in HT-29 cells.

3.7.7 GSTM1 mRNA expression in HT-29 Cell line

Figure 3.28 displays the mean relative GSTM1 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of GSTM1 gene expression in HT-29 cells were shown in Table 3.15. Standard cuve, amplification curve and melting curve of GSTM1 were displayed in Appendix A.

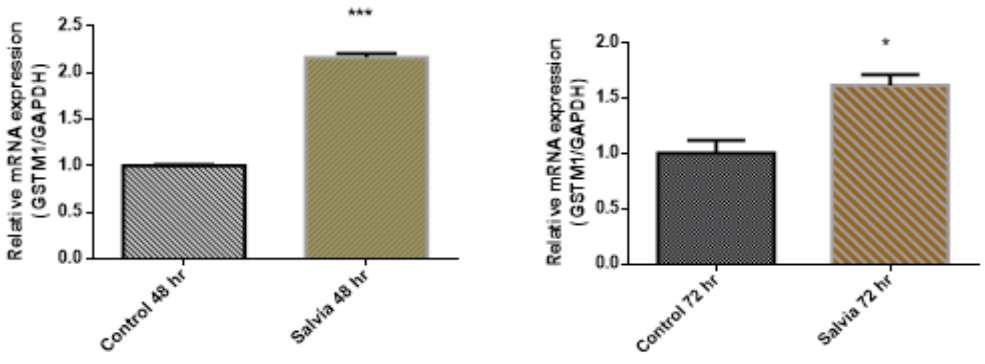


Figure 3.28 Comparison of GSTM1mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3.15 Fold changes of GSTM1 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
GSTM1	2.17 ↑	1.62 ↑

As shown in Table 3.16 and Figure 3.27, expression of GSTM1 treated with *S. fruticosa* extract at the concentrations of IC_{50} values for 48 hr and 72 hr incubation in HT-29 cells increased by 2.17 and 1.62 folds respectively. 48 hr incubation caused more upregulation of GSTM1 compared to 72 hr incubation.

3.7.8 GSTP1 mRNA expression in HT-29 Cell line

Figure 3.29 displays the mean relative GSTP1 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of GSTP1 gene expression in HT-29 cells were shown in Table 3.16. Standard cuve, amplification curve and melting curve of GSTP1 were displayed in Appendix A.

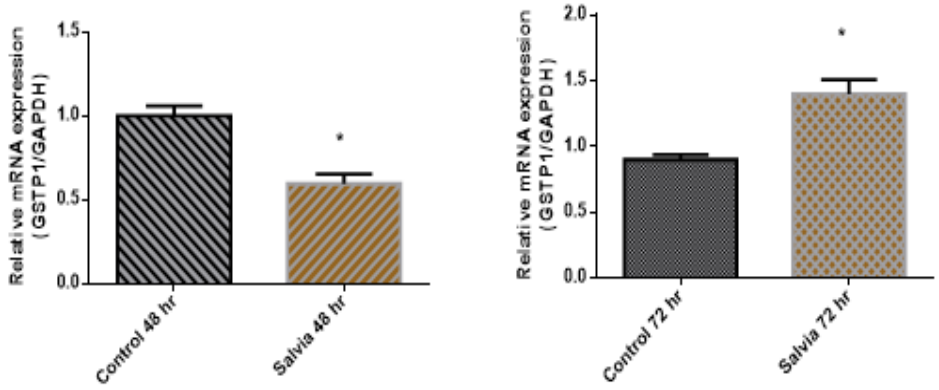


Figure 3.29 Comparison of GSTP1 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3.16 Fold changes of GSTP1 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
GSTP1	1.68 ↓	1.3 ↑

As shown in Table 3.17 and Figure 3.28, gene expression of GSTP1 treated with *S. fruticosa* extract for 48 hr incubation decreased by 1.68 folds in HT-29 cells. On the other hand, 72 hr incubation increased the expression of GSTP1 gene by 1.3 folds in HT-29 cells.

3.7.9 GPx4 mRNA expression in HT-29 Cell line

Figure 3.30 displays the mean relative GPx4 mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of GPx4 gene expression in HT-29 cells were shown in Table 3.17. Standard cuve, amplification curve and melting curve of GPx4 were displayed in Appendix A.

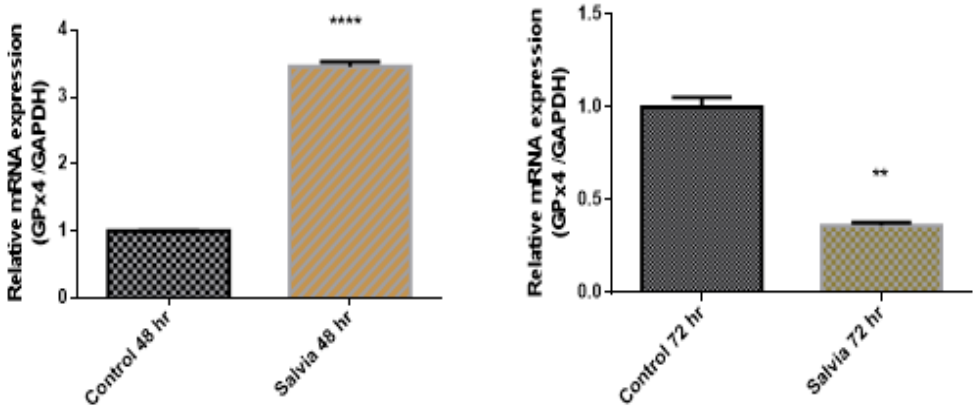


Figure 3.30 Comparison of GPx4 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3. 17 Fold changes of GPx4 gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
GPx4	3.5 ↑	2.17 ↓

As shown in Table 3.18 and Figure 3.29, gene expression of GPx4 treated with *S. fruticosa* extract for 48 hr incubation increased by 3.5 folds in HT-29 cells. On the other hand, 72 hr incubation decreased the expression of GSTP1 gene by 2.17 folds in HT-29 cells.

3.7.10 Catalase mRNA expression in HT-29 Cell line

Figure 3.31 displays the mean relative Catalase mRNA expressions in HT-29 cells with ± SD. The data was statistically analyzed by student's t test method. Gene expressions in 0,1% DMSO treated cDNA was used as controls and the fold change was accepted as 1. Fold changes of Catalase gene expression in HT-29 cells were shown in Table 3.18. Standard cuve, amplification curve and melting curve of Catalase were displayed in Appendix A.

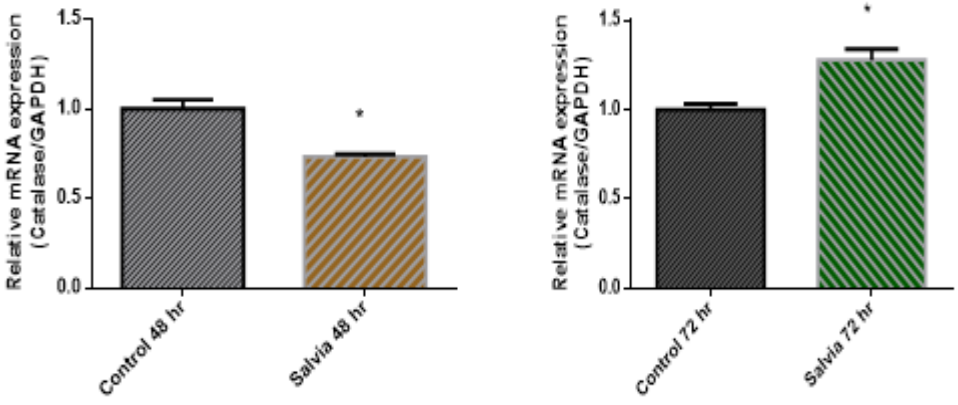


Figure 3.31 Comparison of GPx4 mRNA expression in HT-29 cells. The quantifications were expressed as mean ± SD of the relative expression. * P≤0.05; ** P≤0.01; *** P≤0.001; NS, not significant

Table 3. 18 Fold changes of Catalase gene expressions in HT-29 cell line

HT-29 Cell line		
Genes	**48 hr incubation**	**72 hr incubation**
Catalase	1.37 ↓	1.28 ↑

As shown in Table 3.19 and Figure 3.30, gene expression of Catalase treated with *S. fruticosa* extract for 48 hr incubation decreased by 31.37 folds in HT-29 cells. On the other hand, 72 hr incubation increased the expression of GSTP1 gene by 1.28 folds in HT-29 cells.

3.8 Screening of *S. fruticosa* extract on Phase II Enzyme Activities

3.8.1 Determination of Protein Concentration

Protein concentrations of *S. fruticosa* treated HT-29 cells were calculated by lowry method previously mentioned in Chapter II. Bovine serum albumin (BSA) was used as a standard. BSA standard curve was shown in Figure 3.32.

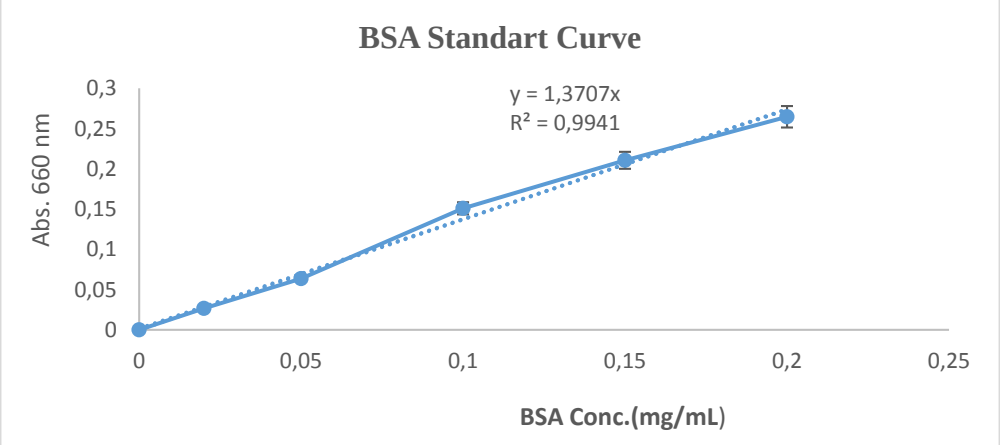


Figure 3.32 Bovine serum albumin standart curve constructed by Lowry method.

Protein concentrations of *S. fruticosa* treated HT-29 cells for 48 hr and 72 hr incubation were displayed in Table 3.19.

Table 3. 19 Protein Concentrations of *S. fruticosa* treated HT-29 cells

Groups	Protein Concentration (mg/mL) ± S.D
Control 48 hr	5.82 ± 0.69
S. fruticosa treated 48 hr	5.63 ± 0.97
Control 72 hr	5.79 ± 0.62
S. fruticosa treated 72 hr	6.99 ± 0.5

The highest amount of protein was obtained from 72 hr treated *S. fruticosa* cell lysate with the concentration of 6.99 ± 0.5 mg/mL. The protein concentrations of the other groups were in the order of 5.82 ± 0.69, 5.63 ± 0.97, 5.79 ± 0.62 mg/mL, respectively.

3.8.2 Screening of *S. fruticosa* on Glutathione S-Transferase Activity

Because GSTs are the major Phase II detoxifying enzymes in addition to their important roles in the cells such as apoptosis, the effect of *S. fruticosa* extract on GST enzyme activities in HT-29 cells were aimed to be studied. Screening of the *S. fruticosa* extract on GST enzyme activity was performed according to Habig et al., (1974) mentioned in Chapter II. Comparision of 48 and 72 hr treatments of *S. fruticosa* extract on GST enzyme activity on HT-29 cells were given in Table 3.20 and Figure 3.33.

Table 3 20 Effects of *S. fruticosa* extract on GST Activity in HT-29 Cells

Groups	GST Activity nmoles/min/mg protein ± SD
Control 48 hr	145.8 ± 3.46
S. fruticosa treated 48 hr	180.8 ± 3.8
Control 72 hr	215.16 ± 6.35
S. fruticosa treated 72 hr	147.13 ± 7.92

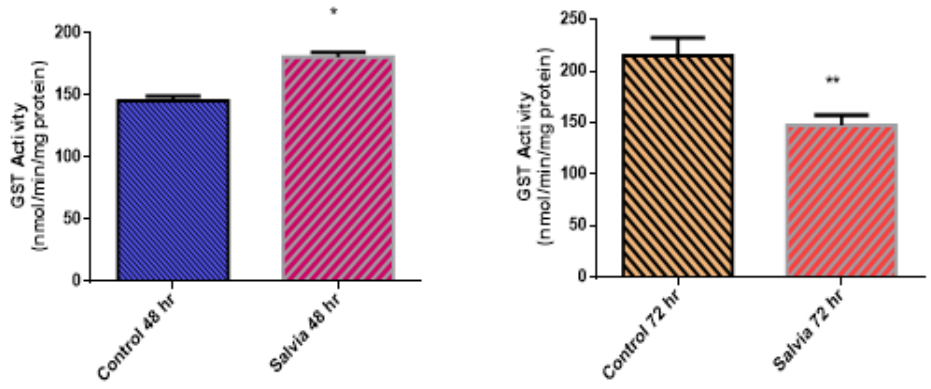


Figure 3.33 Comparision of the 48 and 72 hr treatments of *S. fruticosa* extract on GST enzyme activity on HT-29 cells (Statistically significant, $p < 0.05$).

GST enzyme activities of HT-29 cell control groups for 48 hr and 72 hr incubation were determined as 145.8 ± 3.46 and 215.16 ± 6.35 nmoles/min/mg protein, respectively and *S. fruticosa* trated groups for 48 hr and 72 hr incubation were determined as 180.8 ± 3.8 and 147.13 ± 7.92 nmoles/min/mg protein, respectively. After *S. fruticosa* treatment at the concentration of IC_{50} value for 48 hr incubation, in HT-29 cells, GST activity showed an statistically significant increase about 20% with respect to the control group. On the other hand, when incubation duration was increased up to 72 hr, GST activity displayed an statistically significant decrease about 32 % with respect to the control group.

3.8.3 Screening of *S. fruticosa* on Glutathione Peroxidase Activity

Glutathione peroxidase is one of the major antioxidant enzymes protecting the organism from oxidative damage. Among the biochemical functions of GPx are to reduce lipidhydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Flohe, 1989 and Ursini et al., 1995). Therefore, it was aimed to be studied the effects of the *S. fruticosa* extract on GPx activity in this study. Determinations of the effect of the *S. fruticosa* extract on GPx activity was carried out according to Paglia and Valentine (1967) mentioned in Chapter II. Comparision of 48 and 72 hr treatments of *S. fruticosa* extract on GPx enzyme activity on HT-29 cells were given in Table 3.21 and Figure 3.34.

Table 3. 21 Effects of *S. fruticosa* extract on GPx Activity in HT-29 Cells

Groups	**GPx Activity nmoles/min/mg protein ± SD**
Control 48 hr	23.9 ± 1.22
S. fruticosa treated 48 hr	38.2 ± 0.8
Control 72 hr	25.62 ± 1.1
S. fruticosa treated 72 hr	17.1 ± 0.33

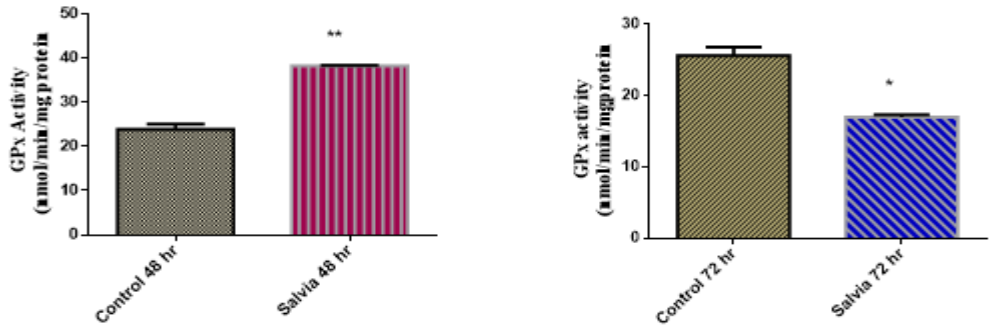


Figure 3.34 Comparision of the 48 and 72 hr treatments of *S. fruticosa* extract on GPx enzyme activity on HT-29 cells (Statistically significant, $p < 0.05$).

GPx enzyme activities of HT-29 cell control groups for 48 hr and 72 hr incubation were determined as 23.9 ± 1.22 and 25.62 ± 1.1 nmoles/min/mg protein, respectively and *S. fruticosa* trated HT-29 cells for 48 hr and 72 hr incubation were determined as 38.2 ± 0.8 and 17.1 ± 0.33 nmoles/min/mg protein, respectively. After *S. fruticosa* treatment at the concentration of IC_{50} value for 48 hr incubation, in HT-29 cells, GPx activity of the cells showed an statistically significant increase about 60 % with respect to the control group. While incubation time was increased up to 72 hr at the concentration of IC_{50}, GPx activity showed an statistically significant decrease about 33 % with respect to the control group.

3.8.4 Screening of *S. fruticosa* on Catalase Activity

Catalase is one of the major antioxidant defense components that primarily catalyze the decompostion of hydrogen peroxide (H_2O_2) to water and oxygen molecules. It shares this function with Glutathione peroxidase (GPx). At high concentration of H_2O_2, peroxides are preffered as substrate for Catalase. While, in the presence of low hydrogen peroxide, they are metabolised by GPx (Yu, 1994). Screening of Catalase activity was performed according to Aebi method (1974) previously mentioned in Chapter II. Comparision of 48 and 72 hr treatments of *S. fruticosa* extract on Catalase activity on HT-29 cells were shown in Table 3.22 and Figure 3.35.

Table 3. 22 Effects of *S. fruticosa* extract on Catalase Activity in HT-29 Cells

Groups	**Catalase Activity nmoles/min/mg protein ± SD**
Control 48 hr	11.01 ± 0.62
S. fruticosa treated 48 hr	6.83 ± 0.32
Control 72 hr	12.51 ± 0.73
S. fruticosa treated 72 hr	16.4 ± 0.38

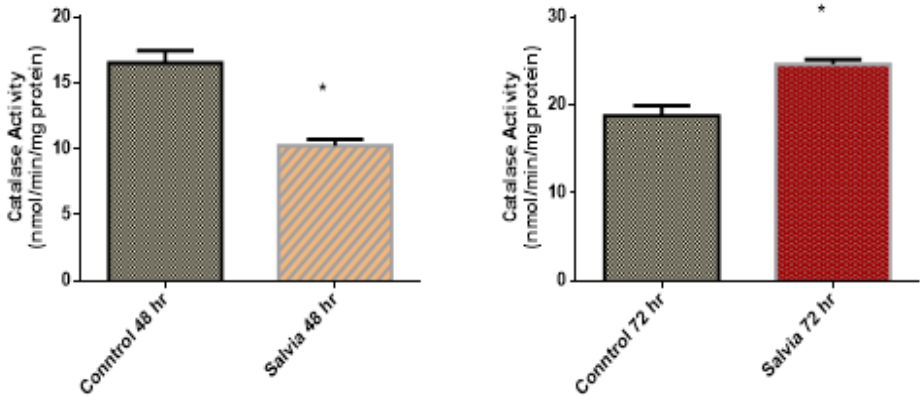


Figure 3.35 Comparision of the 48 and 72 hr treatments of *S. fruticosa* extract on Catalase activity on HT-29 cells (Statistically significant, $p < 0.05$).

Catalase activities of HT-29 cell control groups for 48 hr and 72 hr incubation were determined as 11.01 ± 0.62 and 12.51 ± 0.73 nmoles/min/mg protein, respectively and *S. fruticosa* trated HT-29 cells for 48 hr and 72 hr incubation were determined as 6.83 ± 0.32 and 16.4 ± 0.38 nmoles/min/mg protein, respectively. After *S. fruticosa* treatment at the concentration of IC_{50} value for 48 hr incubation, in HT-29 cells, Catalase activity of the cells showed an statistically significant decrease about 38 % with respect to the control group. While incubation time was increased up to 72 hr at the concentration of IC_{50}, Catalase activity showed an statistically significant increase about 31 % with respect to the control group.

CHAPTER 4

CONCLUSION

Plants that contain polyphenols play critical roles on the gene expression of many enzymes. Because many chemotherapeutic and other types of drugs are metabolized by phase I and Phase II detoxification enzymes, uncontrolled use of these plant can be hazard for human health by affecting drug metabolism. Therefore, it is obligatory to investigate the potential effects of these plants on the gene expression of enzymes before their consumption.

Salvia is an important genus highly investigated by researchers. It is used for alternative medicinal therapy and consumed as herbal tea due to its high polyphenol contents.

In this study, water extract *of S. fruticosa* leaves was analyzed for determine the main phenolic contents by RP-HPLC, total phenolic and flavonoid contents, antioxidant and antichelating activities and growth inhibitory effect on HT-29 cell line. It was also investigated the potential effects of the *Salvia* extract on Phase I and II enzyme gene expressions.

S. fruticosa was found as a good radical scavenger by DPPH, ABTS and Fe^{2+} chelating methods. Phenolic composition that is the main determining factor of antioxidant capacity was determined by measuring total phenol and flavonoid content by Folin Ciocalteu and aluminium chloride colorimetric methods, respectively. Presence of some of the phenolic compounds such as rosmarinic acid, caffeic acid, syringic acid, trans resveratrol and quercetin were validated with RP-HPLC analysis.

Cytotoxic effects of *S. fruticosa* extract and its main bioactive components, rosmarinic acid and caffeic acid, on HT-29 cell line were examined by XTT and TBE methods with dose and time dependent manners.

Cell proliferation of HT-29 cells was inhibited by *S. fruticosa* extract, rosmarinic acid and caffeic acid with an IC_{50} values of 0.185 mg/mL, 0.148 mg/mL and 0.149 mg/mL for 48 hr incubations, respectively and 0.229 mg/mL, 0.184 mg/mL and 0.181 mg/mL for 72 hr incubations respectively in XTT assay, indicating that HT-29 cells increase their carcinogenic activity while subjected to *S. fruticosa* for increased duration.

Investigations on phase I gene expression resulted in up-regulation of CYP1A1 and CYP1A2 but down-regulation of CYP2E1 and CYP3A4 for 48 hr incubation, and also up-regulation of CYP1A1 and CYP3A4 but down-regulation of CYP1A2 for 72 hr incubation when treated with *S. fruticosa* extract with an IC_{50} concentrations determined in XTT assay. On the other hand, Investigations on phase II enzymes gene expression resulted in up-regulation of GSTM1 but down-regulation of GSTP1 for 48 hr incubation while up-regulation of GSTM1 and GSTP1 for 72 hr incubation.

As for antioxidant enzymes, it was found that treatment of *S. fruticosa* extract for 48 hr resulted in up-regulation of GPx4 and down-regulation of catalase but down-regulation of GPx4 and up-regulation of catalase for 72 hr incubation.

Slight changes in gene expressions of phase I and phase II enzymes do not indicate that *S. fruticosa* has interference with drug metabolism but it reveals an accountable effect that should be further searched for higher concentrations. These findings demonstrate that *S. fruticosa* is a good candidate for targeted therapy and drug discovery.

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APPENDIX A

Gene Expression Of Drug Metabolizing Enzymes

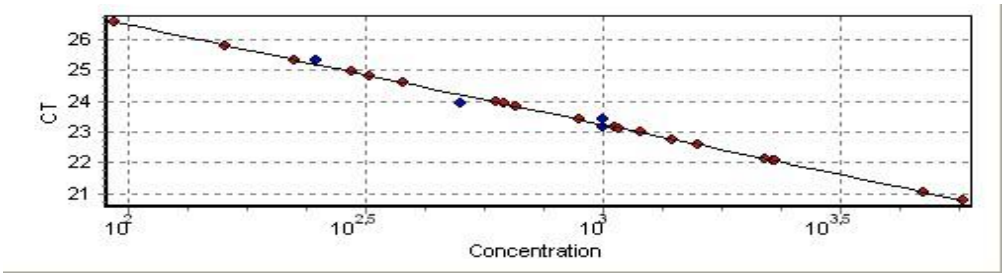

Figure A.1 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of CYP1A2 mRNAs in HT-29 cell line. Efficiency: 1.03

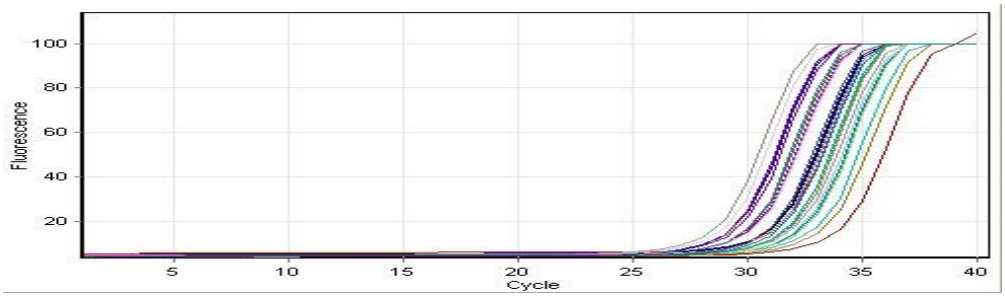

Figure A.2 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

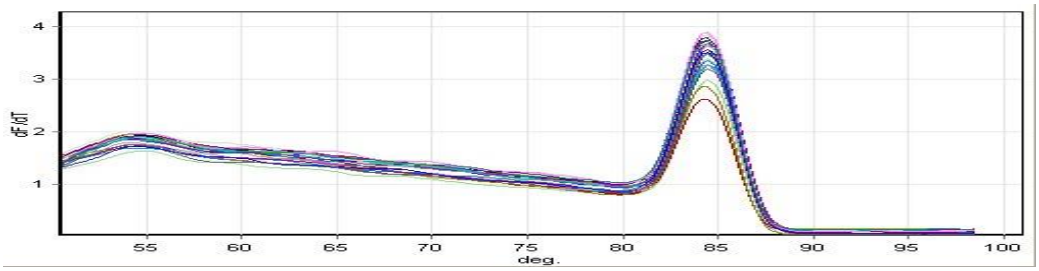

Figure A.3 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

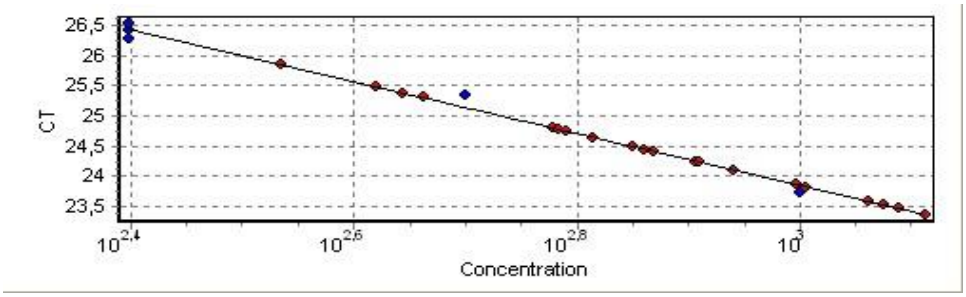

Figure A.4 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of CYP2E1 mRNAs in HT-29 cell line. Efficiency: 0.93

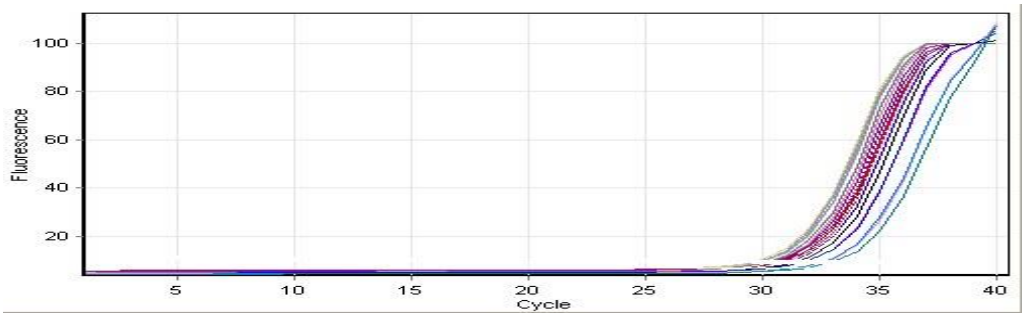

Figure A.5 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

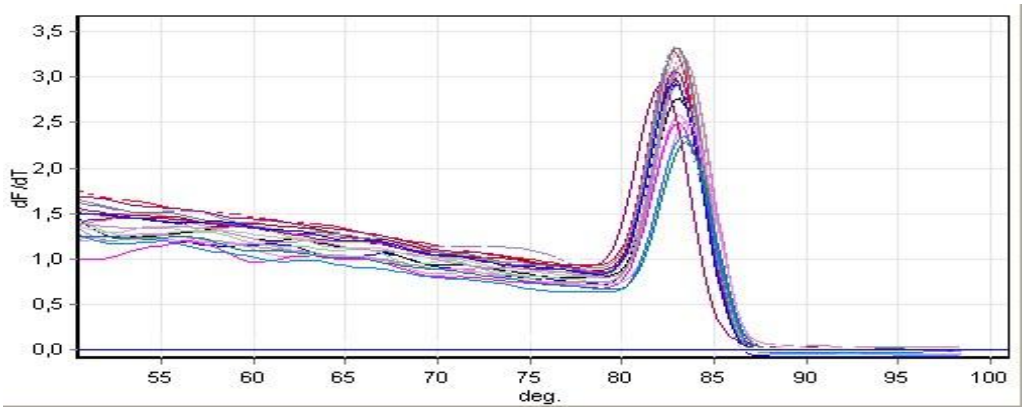

Figure A.6 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

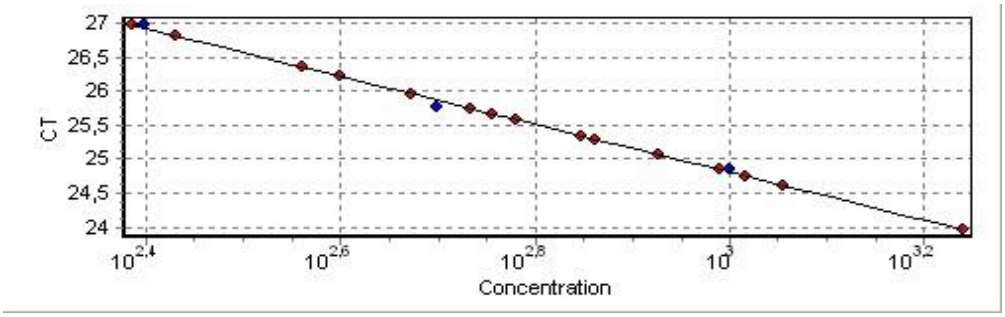

Figure A.7 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of CYP3A4 mRNAs in HT-29 cell line. Efficiency: 0.98

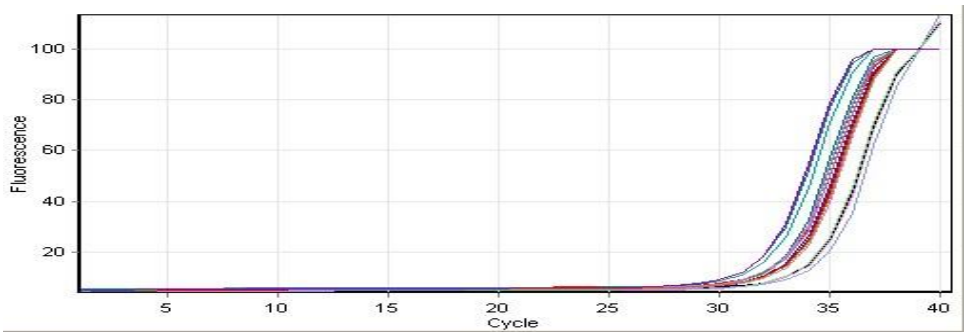

Figure A.8 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

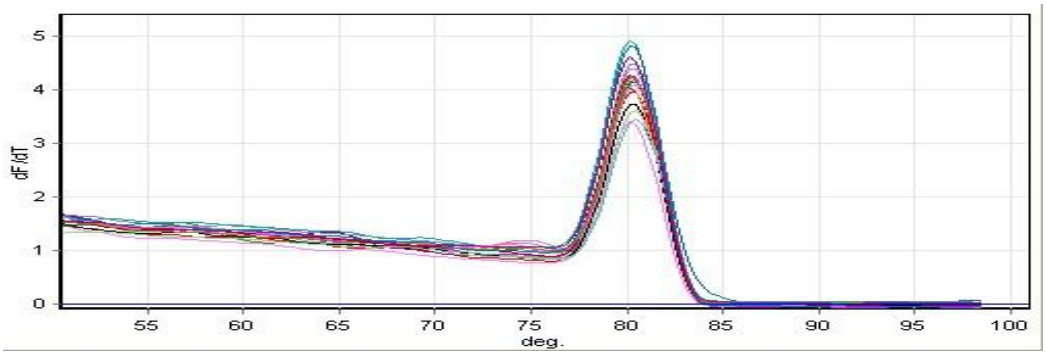

Figure A.9 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

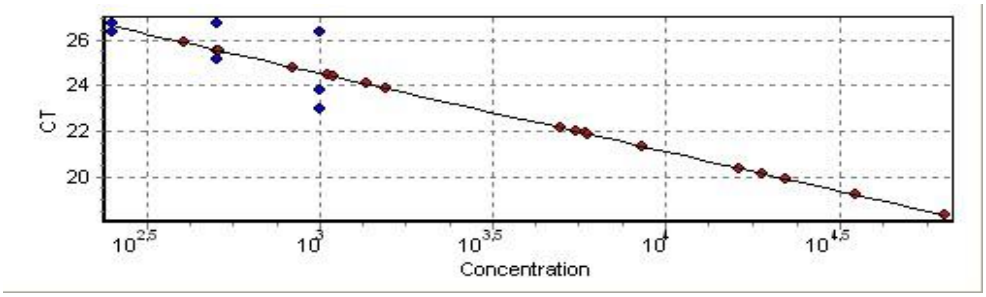

Figure A.10 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of GSTM1 mRNAs in HT-29 cell line. Efficiency: 0.96

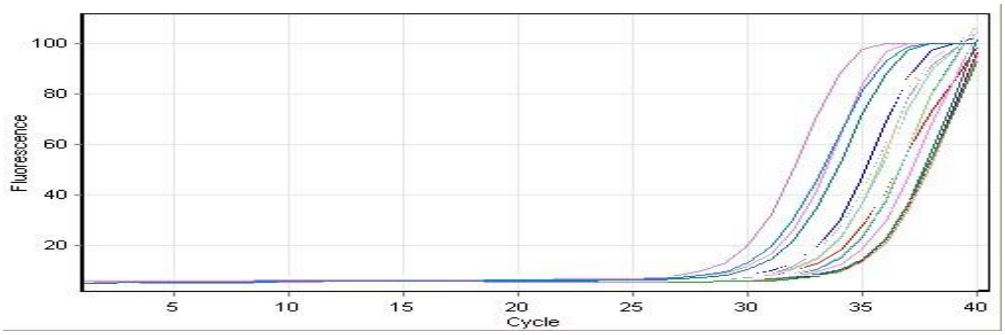

Figure A.11 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

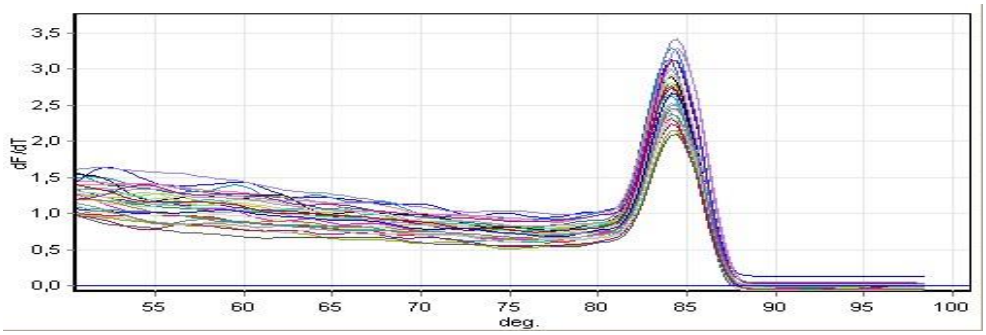

Figure A.12 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

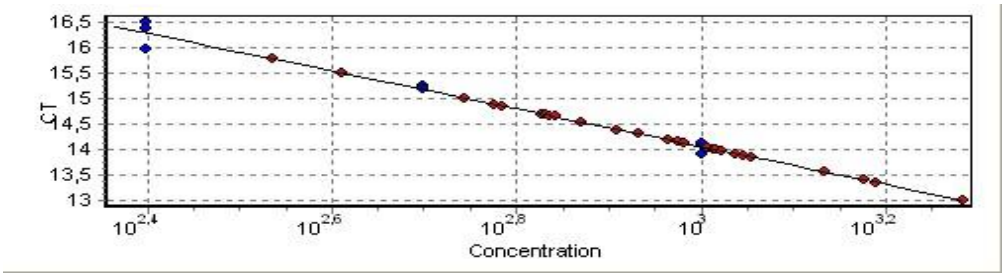

Figure A.13 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of GSTP1 mRNAs in HT-29 cell line. Efficiency: 0.87

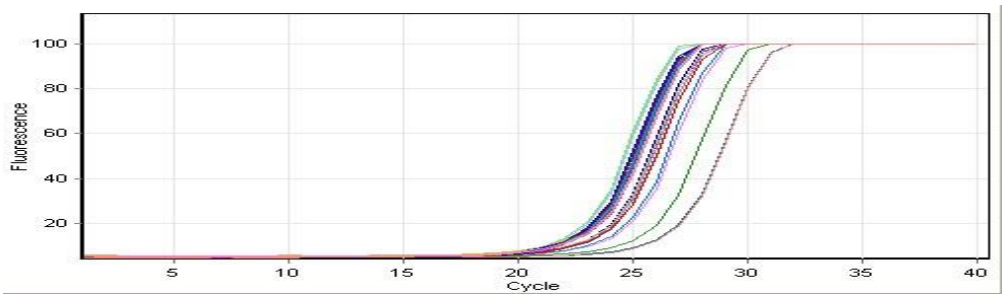

Figure A.14 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

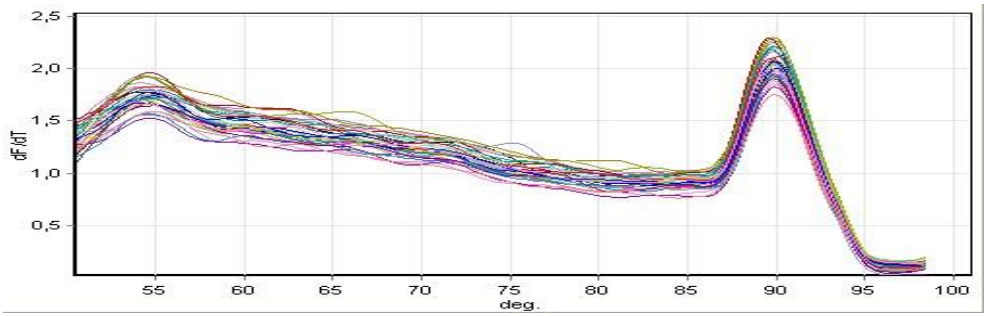

Figure A.15 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

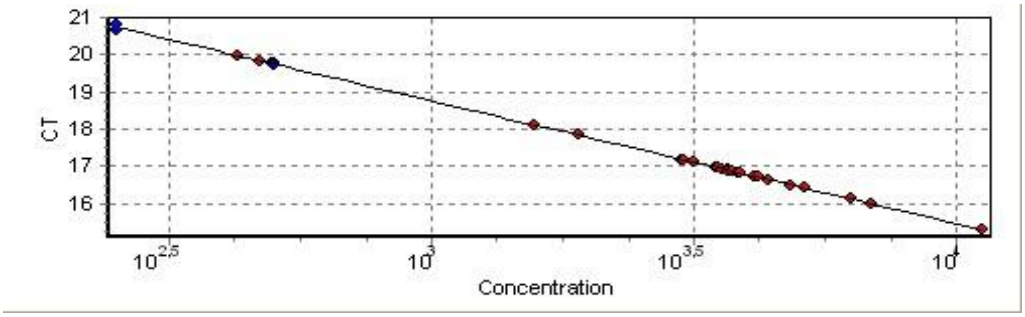

Figure A.16 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of GPx4 mRNAs in HT-29 cell line. Efficiency: 1.01

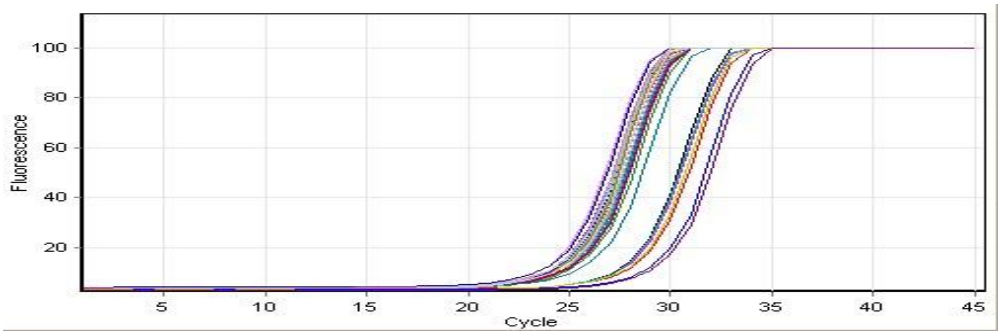

Figure A.17 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

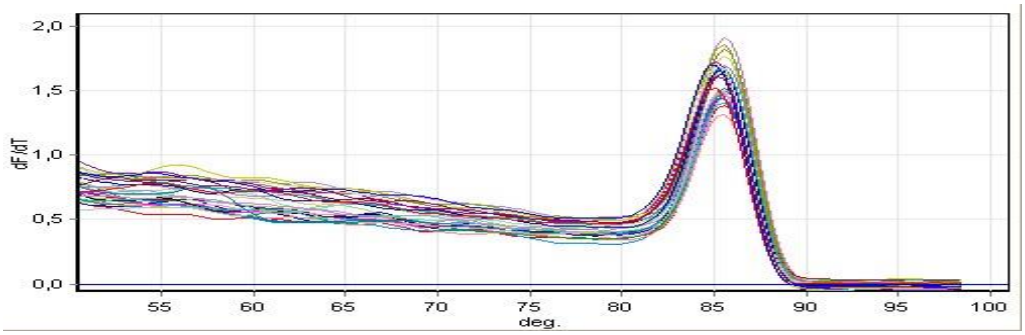

Figure A.18 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

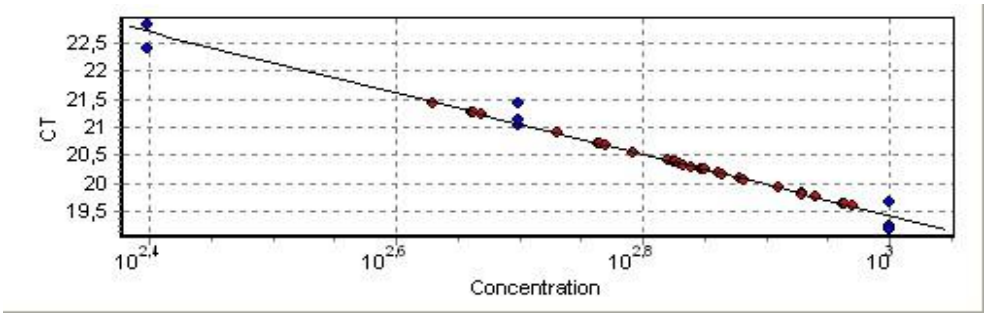

Figure A.19 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of Catalase mRNAs in HT-29 cell line. Efficiency: 0.98

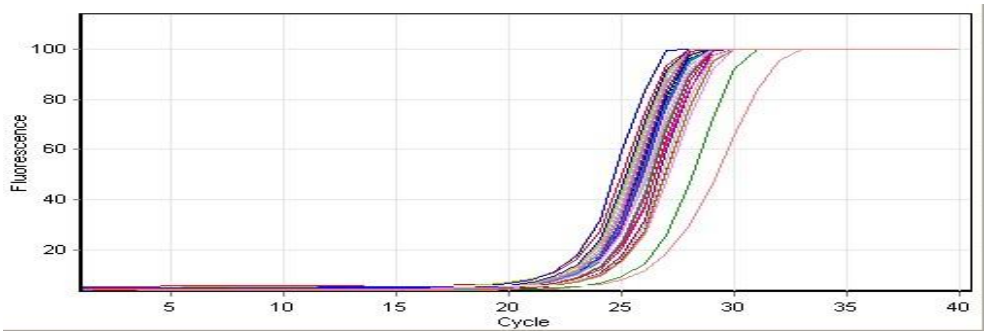

Figure A.20 Amplification curve; the accumulation of fluorescence emission at each reaction cycle.

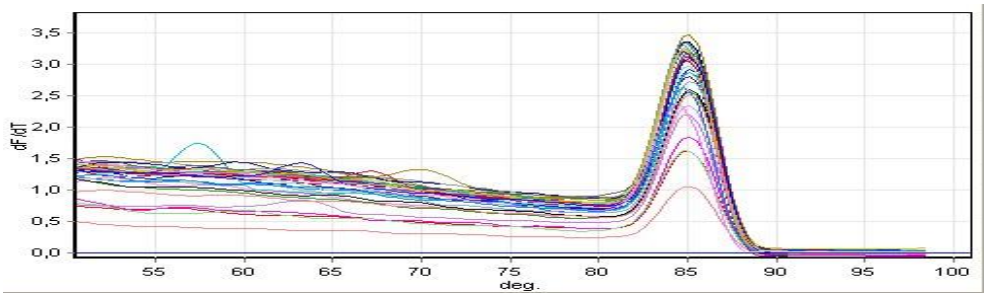

Figure A.21 Melting curve; the fluorescence emission change versus temperature. Single peak means single PCR product.

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B.S (1999-2003): Atatürk University, Faculty of Art and Sciences, Department of Chemistry

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International Society for the Study of Xenobiotics (ISSX)

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Basic Cell Culture Certificate, Hacettepe University, 10-17 of May, 2014.

Certificate of Laboratory Animal Use, Hacettepe University, 10-17 of May, 2014.

Chemical Laboratory Safety Training, METU, 2010

PUBLICATIONS

Publications in International Journals

- Antioxidant Potential, Cytosolic GST Inhibition Activity and Cytotoxic Effects of *Salvia fruticosa* on Colon Cancer Cells, Nutrition and Cancer (Submitted, 2014).

International Conference Proceedings

- Altay A, Irtem Kartal D, Sağdıçoğlu Celep G, Guray NT, Bozoğlu FT Antioxidant activities of *Salvia fruticosa* and its effects on HT-29 cell line. 40th FEBS Congress (4-9 July 2015, Berlin, Germany). FEBS Journal 282 (Suppl. 1) (2015) 56–408, 269.

- Ahmet Altay, Mert Cem Didiş, Gulcin Sagdıcoglu Celep, Faruk Tahsin Bozoğlu. Antioxidant and GST inhibiton Activities of Cocoa Products and Their Bioactive Components. (22-25 June 2015, Glasgow, Scotland) ISSX Abstract Book P,65.

- Irtem Kartal D, Altay A, Yurteri G, Guray NT. Antioxidant activities of Propolis and Its Bioactive Components, and Their Effects on CYP1A1 Gene Expression in HT29 Adenocarcinoma Cell Line. 13th European ISSX Meeting (22-25 June 2015, Glasgow, Scotland) ISSX Abstract Book P,51.
- Ahmet Altay, Faruk Bozoglu, Gulcin Sagdıcoglu Celep, Antioxidant activitis of *Salvia fruticosa* and its inhibitory effects on Glutathione-S-transferase. FEBS EMBO, Paris, Book of Abstracts, 30 August-4 September, 2014.
- Altay A., Bozoglu F., Sagdıcoglu Celep G. Antioxidant Capacity and Polyphenolic Content of Grape Samples from Turkey. 13th International Conference on Antioxidants, Book of Abstracts, June, 2013,
- Sağdıçoğlu Celep AG, Altay A, Didiş MC, Övet B, Bozoğlu F. Dietary Intake of polyphenols and their health benefits, VI. International Conference on Polyphenols and Health, Book of Abstracts, 16-19 Ocober, 2013, Buenos Aires, Argentina.

Projects Participated

METU (ÖYP) 2010. "Changes in resveratrol Content of Different Fruits and Vegetables Upon Thermal And Non-Thermal Applications".