INVESTIGATION OF THE EFFECTS OF PLANT PHENOLIC OLEUROPEIN ON EXPRESSION LEVELS OF XENOBIOTIC METABOLIZING ENZYMES ALONG WITH POTENTIAL CYTOTOXIC AND GENOTOXIC IMPACT ON HUMAN COLON CANCER CELL LINE HT-29

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF PLANT PHENOLIC OLEUROPEIN ON EXPRESSION LEVELS OF XENOBIOTIC METABOLIZING ENZYMES ALONG WITH POTENTIAL CYTOTOXIC AND GENOTOXIC IMPACT ON HUMAN COLON CANCER CELL LINE HT-29

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Colon cancer is one of the major health problems causing malignancies and is frequently encountered in developed western countries. Today, in the treatment of colon cancer, several methods are used, mostly with adverse effects. In order to reduce the consequences of side effects usage of natural or artificial anti-cancer molecules is a serious topic in the cancer research area. Scientists began to perform research to find anti-carcinogenic phytochemicals and xenobiotics with anti-carcinogenic potentials. Until now, at least five thousands phenolic compounds have been described and oleuropein is one of the substantial phenolic compounds whose effects were determined on formation of cancer cells. In the light of this information, this study was aimed to identify potential oleuropein effects on colon cancer cell proliferation, DNA damage formation and protein expressions of CYP1A1, GSTM1 and NQO1 enzymes at *in vitr*o level.

In order to achieve goals of this study, HT-29 colon cancer cells were grown and treated with increasing oleuropein doses, and IC_{50} value was determined as 600 μM. Effects of oleuropein on DNA damage formation and protein expressions were studied by Comet assay and Western Blotting, respectively.

Oleuropein treatment caused decrease in protein expression levels of CYP1A1, GSTM1 and NQO1 enzymes. Oleuropein also caused increase in DNA damages of the colon cancer cells in a dose dependent manner.

In conclusion, the results of this study showed that oleuropein inhibits the proliferation of colon carcinoma cells by affecting the DNA damage formation and protein expression of CYP1A1, GSTM1 and NQO1 enzymes.

Keywords: Oleuropein, HT-29, CYP1A1, GSTM1, NQO1

ÖZ

BİTKİ FENOLİĞİ OLEUROPEİNİN HT-29 KOLON KARSİNOMA HÜCRE HATTI ÜZERİNE OLAN SİTOTOKSİK VE GENOTOKSİK ETKİLERİNİN KSENOBİYOTİK METABOLİZE EDEN ENZİMLERİN PROTEİN EKSPRESYONLARINA OLAN ETKİSİ İLE BİRLİKTE ARAŞTIRILMASI

Balkan, Ezgi
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Kolon kanseri malign gelişim ile sonuçlanabilen ve özellikle gelişmiş batılı ülkelerde sıkça rastlanan önemli bir sağlık sorunudur. Günümüzde kolon kanseri tedavisinde birçok yöntem kullanılmakta ve bu yöntemler pek çok yan etki doğurmaktadır. Bu yan etkilerin varlığını ortadan kaldırmak ya da azaltabilmek için daha az yan etkiye sahip doğal veya yapay kanser önleyici moleküller bulmak kanser araştırmalarında ciddi öneme sahip konular arasındadır. Bilim adamları bu moleküllere duyulan ilginin artması ile birlikte, fitokimyasal ve ksenobiyotiklerin metabolizmalarını, antioksidan, anti-kanserojen özelliklerini de araştırmaya başlamışlardır. Bitkilerde yaygın olarak bulunan en az beş bin tane fenolik madde tanımlanmış ve zeytin yaprağı ve meyvesinde bulunan oleuropein fenoliğinin kanser hücresi oluşumuna ve metastazına olan etkisi gösterilmiştir. Bu bilgiler doğrultusunda, bu çalışmanın amacı oleuropeinin HT-29 insan kolon kanseri hücrelerinde sitokrom P450 ve faz II enzim sistemleri üzerine olan olası etkileri ile sitotoksik ve genotoksik etkilerini *in vitro* düzeyde araştırmaktır.

Yapılan çalışmanın sonuçlarına göre, oleuropein muamelesi sonrası HT-29 kolon kanseri hücrelerinin CYP1A1, GSTM1 ve NQO1 protein ekspresyonlarında azalma

görülmüştür. Ayrıca, oleuropeinin artan dozları kolon kanseri hücrelerinde sitotoksisiteye ve DNA hasarında da artışa neden olmuştur.

Sonuç olarak, mevcut çalışma ile bir bitki fenoliği olan oleuropeinin kolon karsinom hücreleri üzerine olan sitotoksik ve genotoksik etkisi gösterilmiştir. Oleuropeinin kanser hücrelerine olan anti-proliferatif etkisini ksenobiyotik metabolize eden CYP1A1, GSTM1 ve NQO1 enzimlerinin protein ekpresyonlarını düzenleyerek gerçekleştirebileceği düşünülmektedir.

Anahtar Kelimeler: Oleuropein, HT-29, CYP1A1, GSTM1, NQO1

Dedicated to my family, for their love, endless support and encouragement

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LIST OF ABBREVIATIONS

AhR Aryl hydrocarbon receptor

APS Ammonium per sulfate

B[a]P Benzo[a]pyrene

BCIP 5-bromo 4-chloro 3-indoyl phosphate

BSA Bovine serum albumin

COMT Catechol O-methyl transferase

CYP Cytochrome P450

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

dNTP Deoxynucleoside triphosphate

dPBS Distilled phosphate buffered saline

ECL Enhanced chemiluminescence

EDTA Ethylenediaminetetraacetic acid

ERB Electronic running buffer

EtBr Ethidium bromide

FAD Flavin adenine dinucleotide

FBS Fetal bovine serum

FMN Flavin mononucleotide

FMO Flavin-containing monooxygenase

GSH Glutathione

GST Glutathione S-transferase

HAH Halogenated aromatic hydrocarbons

HRP Horseradish peroxide

IC50 Half maximal inhibitory concentration

kDa Kilo dalton

LDL Low Density Lipoprotein

LMPA Low melting point agarose

MAO Monoamine oxidases

MAP Mitogen-activated protein

mRNA Messenger RNA

MTT [4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NADH Nicotinamide adenine dinucleotide, reduced form

NADPH Nicotinamide adenine dinucleotide phosphate, reduced form

NAT N-acetyltransferases

NQO1 NAD(P)H-quinone oxidoreductase

OD Optical density

PAH Polycyclic aromatic hydrocarbon

PBS Phosphate buffered saline

Pen-Strep Penicillin-Streptomycin

PMSF Phenylmethylsulfonyl fluoride

PVDF Polyvinylidene fluoride

RNA Ribonucleic acid

ROS Reactive Oxygen Species

RPM Revolutions per minutes

SDB Sample dilution buffer

SDS Sodium dodecyl sulfate

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SULT Sulfotransferases

TBST Tris-buffered saline and Tween 20

TEMED Tetramethylethylenediamine

UGT UDP-glucuronosyltransferase

CHAPTER 1

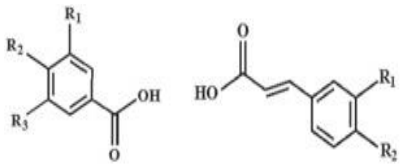
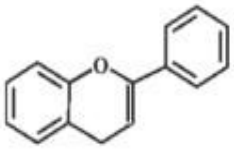
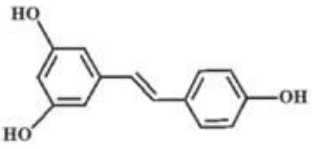
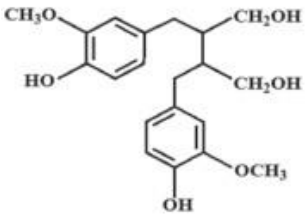
INTRODUCTION

1.1 . Polyphenolic Compounds

Polyphenolics are chemical compounds characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. They are heterogeneous group of secondary metabolites naturally found in higher plants. Biosynthesis of polyphenolic compounds occurs through shikimic acid and polyacetate pathways and they mainly include simple phenols, phenolic acids, stilbenes, coumarins, flavonoids, anthocyanins, catechins and tannins. They generally have biological activity in the plant host and play critical roles in growth of plants or in defense mechanisms against competitor, pathogens or predators. Normally, polyphenols are most commonly found antioxidants in plants. The antioxidant activities of these compounds appear to be related with their molecular structure. Because of having cyclic structure with double bounds and hydroxyl groups, polyphenols can transfer their electrons to free radicals, but their molecular structure prevents them from becoming a free radical compound. However, due to their electron transfer potentials, in the presence of transition metals such as iron and copper, increased concentrations of phenolic compounds may generate reactive oxygen species through Fenton's reactions. Number of polyphenols have chemopreventive and therapeutic effects against several diseases including cancer. In vitro and in vivo cancer studies have reported that polyphenols have anticancer and apoptosis-inducing properties (Yar Khan et. al., 2012). Polyphenols have been classified into different groups according to function and number of phenol rings which they contain in their structure and based on structural elements that bind these rings to one another (Pandey et.al., 2009). Classification of polyphenols can also be done on the basis of their source of origin, biological function, and chemical structures. In Table 1.1,

structures of the most common subclasses of polyphenols and their nutrient sources are represented.

Table 1.1 Structures of the most prevalent subclasses of polyphenolic compounds and their nutrient sources (Zamora-Ros et al., 2013, Pandey et al. 2009)

Subclass	**Prominent polyphenols**	**Food sources**
Phenolic Acids 	Protocatechuic acid Vanilic acid Coumaric acid Caffeic acid Gallic acid	Tea, coffee, garlic, olives, berries, onion, nuts and seeds
Flavonoids 	Apigenin Naringenin Catechin Genistein Quercetin Luteolin	Red wine, chocolate, grapes, onions, blueberries, soybeans, tea, broccoli, parsley
Stilbenoids 	Resveratrol	Grapes, red wine
Lignans 	Secoisolariciresinol Matairesinol	Linseed, beans, nuts

This study will be focalized on one of the most prominent polyphenol oleuropein, which takes part in secoiridoid polyphenols family.

1.1.1 Oleuropein

Oleuropein (methyl-4-[2-[2-(3,4-dihydroxyphenyl)_ethoxy]-2-oxoethyl]-5 ethylidene-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4H-pyran-3-carboxylate) belongs to the secoiridoid polyphenols family which are generated from the secondary metabolism of terpenes as a precursor of indole alkaloids. It is an ester form of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) and has the oleosidic skeleton that is common to the secoiridoid glucosides of *Oleaceae*, mainly in its aglycone form, which makes the sugar moiety insoluble in oil. Figure 1.1 represents the structure of oleuropein.

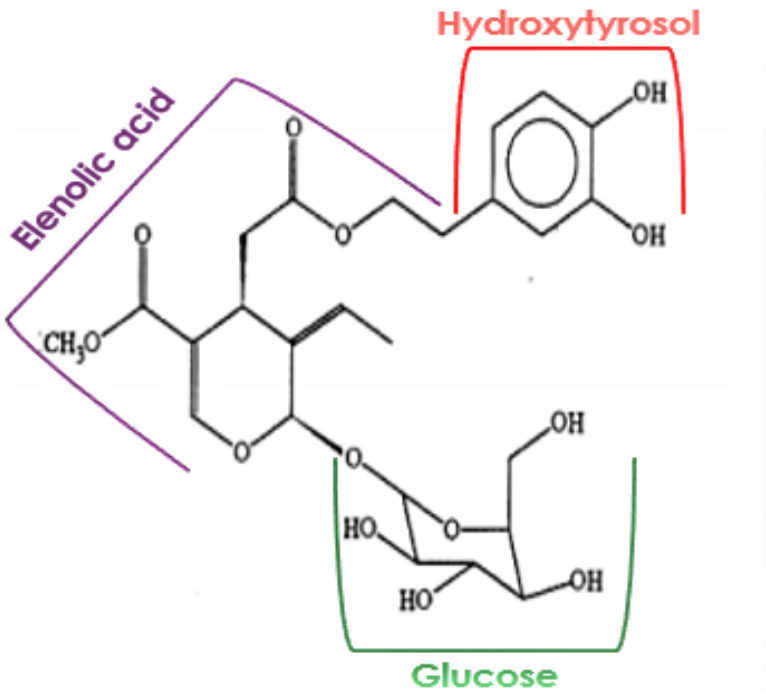


Figure 1.1 Structure of oleuropein (Omar, 2010)

Oleuropein is the major water-soluble phenolic compound in plants of the olive family which is botanically known as *Olea europaea* and it is present in many other plants like *Gentianaceae* and *Cornaleae* (Omar, 2010). Oleuropein is most abundant in the olive leaves (up to 264 mg/g of dry leaf) and its concentration in olive fruit

depends on maturity phases of fruit. In early growth phase, oleuropein accumulation occurs and its concentration reaches up to 14% of dry olive fruit. Its level starts decreasing in green maturation phase and in black maturation phase; oleuropein levels continue to fall while anthocyanins level increase (Amiot et al., 1986). It was also found that oleuropein is responsible for bitter and pungent taste of virgin olive and olive oil (Panizzi et al., 1960).

The benefits of Mediterranean diet have been executed previously and researchers revealed that those benefits are associated with phenolic compounds, which are plentiful in olive oil, oil fruit and olive leaves (Cicarele et al., 2010). Oleuropein was identified as one of the most important olive plant polyphenol and it possesses various pharmacological properties including antioxidant (Visioli et al., 2002), anti-inflammatory (Visioli et.al, 1998), anti-atherogenic (Carluccio et. al, 2003), anti-cancer (Owen et. al, 2000) and antimicrobial (Tripoli et. al, 2005). Particularly, its anti-cancer activities have been an issue of concern which have been discovered by some scientific researches in the recent years. It was shown that oleuropein is a remarkable agent in alleviating the initiation, promotion and progression of carcinogenesis. Some studies were carried out which demonstrate the protective role of oleuropein on leukemia, renal cell carcinoma, melanoma, colorectal cell carcinoma, brain and breast cancer cell lines (Hamdi et al., 2005, Menendez et. al, 2007). There are also two *in vivo* studies which show anti-breast-cancer and anti-skin-cancer effect of oleuropein in mice (Elamin et. al., 2017 and Kimura et. al., 2009). Those anti-carcinogenic effects may be result from one of the various mechanisms that oleuropein has been shown to be utilized on cancer cells. These mechanisms comprise; apoptotic, genotoxic, anti-oxidant and anti-inflammatory activities, cell cycle arrest, deactivation of cell proliferation and modulation of xenobiotic metabolizing Phase I and Phase II enzyme activities. According to other researches, oleuropein could also be a promising natural product for the prevention of crucial chemo-therapy drug induced kidney disease (Geyikoglu et. al, 2017).

Modulation of Phase I and Phase II enzymes with phytochemicals like oleuropein has been previously defined with some studies. For instance, it was reported that

oleuropein forms reactive metabolites that inhibits a CYP3A marker in human liver microsomes (Stupans et. al, 2001). This inhibition may clarify protected effects of oleuropein against LDL oxidation (Coni et. al, 2000). Also it was found that it is a weak inhibitor of CYP1A2 mediated 7-methoxyresorufin-*O*-deethylation (Stupans et. al, 2001). As another study showed, an oleuropein derivatives hydroxytyrosol upregulates the expression of endogenous human antioxidant genes (Heme Oxygenase 1 (HO-1), NAD(P)H-quinone oxidoreductase (NQO1), Glutathione (GSH)) (Zou et. al, 2012). Consequently, modulation of xenobiotic metabolizing enzymes activity by phenolic oleuropein may cause various biological changes in human physiology and metabolism.

1.2 Phase I and Phase II Xenobiotic Metabolizing Enzymes

Enzyme systems that catalyze the biotransformation of xenobiotic and drugs can be classified into two main classes according to their substrate activity and their sequence in the metabolic pathway; Phase I enzymes and Phase II enzymes. These enzymes have remarkable roles in the activation and deactivation of many environmental carcinogens, such as pesticides, air pollutants and polycyclic aromatic hydrocarbons (PAH). Phase I enzymes primarily include cytochrome enzymes which are responsible for oxidase activity to increase the hydrophilicity of lipophilic substrates, whereas Phase II enzymes carry out conjugation reaction for further metabolism of drugs and phase I enzyme products (Iyanagi et. al, 2007). Studies focused on polymorphism of these enzymes showed that cancer susceptibility, treatment efficacy, body's defense against cancer and clinical consequences of patients change in the case of some polymorphism (Swinney et al., 2006). Other researches also have represented that enzymatic activity levels and concentrations of these enzymes affect the cancer susceptibility and cancer prevention significantly (Mittal et. al, 2015, Sheweita et. al, 2003, Jana, 2009)

1.2.1 Phase I Xenobiotic Metabolizing Enzymes

Phase I enzymes are functional xenobiotic and drug metabolizing agents which convert lipophilic compounds into more freely hydrophilic products. This conversions include variety of oxidation, hydroxylation and reduction reactions which take place mainly in the liver. Most of the phase I enzyme substrates are activated to carcinogenic compounds or they are excreted efficiently to their increased hydrophilic forms after phase I enzyme interaction. Reactions, which contain phase I enzyme systems primarily work as detoxifying agents of body.

The most broadly studied phase I enzymes belong to the cytochrome P450 monooxygenases (CYPs) family. CYPs work in most tissues of the body except muscles and erythrocytes. They play a central role in the detoxification of xenobiotics and metabolism of endogenous compounds. CYPs are succeeding in attracting attention of researchers because they defend the body against toxic compounds and play a critical role in the metabolism of carcinogens leads to inaction of tumor proliferation whereas CYP-mediated biotransformation generates reactive metabolites which induce carcinogenic and toxic events (Arınç et al., 1991, 2000a, 2000b). In addition to the Cytochrome 450 family, flavine monooxygenases (FMO), monoamine oxidases (MAO), alcohol and aldehyde dehydrogenases, superoxide and aldo-keto dismutases are also involved in the Phase I enzyme system (Adalı et. al., 1998).

1.2.1.1 Cytochrome P450s

The cytochromes P450 (CYPs) represent the major enzyme family capable of catalyzing the oxidative biotransformation of drugs and other lipophilic xenobiotics and they are specific interested topic for clinical pharmacology. The first report which puts forward the presence of a CYP enzyme was published in 1958 (Klingenberg et. al., 1958). The CYP Nomenclature Committee has constituted the nomenclature for the CYP enzyme system that is still accepted (Nelson et al. 1996, 2004). Meaning of CYP term is haem-containing proteins which absorbs light

maximum at wavelength of 450 nm in the reduced state in the presence of carbon monoxide (Omura et. al, 1999). The CYP superfamily comprises over 13,000 genes that correspond 400 gene families and almost 57 different CYP genes and 58 pseudogenes have been reported in humans. However, in humans 18 different families and 44 subfamilies are known presently and the enzymes which belong to families 1-3 (CYP1A2, 2C9, 2C19, 2D6, 2E1 and 3A4) are the most functioning in the hepatic metabolism of xenobiotics while other CYP families are active in endogenous functions (Nelson, 2009). Table 1.2 represents the CYP450 families according to class of their substrates.

Table 1.2 Human P450s categorization according to class of substrates (Nelson, 2004).

Class of substrates	**CYP enzymes**
Sterols	1B1, 7A1, 7B1, 8B1, 11A1, 11B1, 11B2, 17A1, 19A1, 21A2, 27A1, 39A1, 46A1, 51A1
Xenobiotics	1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5, 3A7
Fatty acids	2J2, 4A11, 4B1, 4F12
Eicosanoids	4F2, 4F3, 4F8, 5A1, 8A1
Vitamins	2R1, 24A1, 26A1, 26B1, 26C1, 27B1
Unknown	2A7, 2S1, 2U1, 2W1, 3A43, 4A22, 4F11, 4F22, 4V2, 4X1, 4Z1, 20A1, 27C1

CYPs are assembled under two headings based on their roles; xenobiotics detoxification and endogenous compounds biosynthesis. They can be also classified according to membrane-bound forms that are generally seen in eukaryotes and soluble forms present in prokaryotes (Nelson 2005). Another classification was made based on CYPs subcellular localization and how they transfer electrons. Most of the CYPs are located in the endoplasmic reticulum of eukaryotic cells and these CYP

enzymes are reduced by a membrane bound FAD/FMN containing CYP reductase. In prokaryotes, electron transfer process is mediated by mitochondrial CYPs and they are reduced by a soluble two-component reductase system comprising FAD and iron-sulfur protein (McLean et. al, 2005).

Generally, CYP450 enzymes are expressed in hepatic cells in which detoxification occur, but are also found in other tissues, including lung, kidney, nasopharyngeal, and gastrointestinal tract tissue (Sheweita, 2000). Beside animals, CYPs also exist in plants, fungi, and bacteria. In hepatic tissues of mammals they have essential roles like bile acid biosynthesis, metabolism of drugs, carcinogen and environmental pollutants. In steroidogenic tissues CYPs are responsible for synthesis and degradation of endogenous steroid hormones. Vitamin metabolism, maintaining of cellular homeostasis, cholesterol biosynthesis and unsaturated fatty acid oxidation are among other remarkable functions of cytochrome P450 enzymes (Guengerich, et. al, 2016, Zanger et al. 2013).

During mono-oxygenation reactions mediated by CYPs, one oxygen atom is incorporated into the substrate and the other one is reduced to water (Isin et. al, 2007). Bond between the oxygen molecules are mainly broken down by NADPH cytochrome P450 reductase enzyme which transfers electron from NADPH through coenzymes, FAD and FMN. In the mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to CYP. When NADPH is absent, CYPs use another oxygen atom donors, such as hydroperoxides, peracids, perborate, percarbonate, periodate, chlorite, iodosobenzene and N-oxides. In catalytic cycle of CYP, substrate binds to the active site of CYP enzymes and then a water molecule is removed with the stimulation of Fe3+ ion state changing with the redox component. The ferric CYP-substrate complex is reduced by electron transfer from NADPH via the electron transfer chain. Then O2 binds to this complex to make a stable Fe3+-O2 - complex and this complex is reduced again to Fe3+-O_2^{2-} by a second electron transfer from NADPH. O-O bond is broken down by interaction of O_2^{2-} with two protons from the solvent in the environment.

After this breakage H2O and (Fe-O)3+ complex is formed which has an ability to cut down a H atom from the substrate to make FeOH3+-R•. FeOH3+-R• transfer its OH group is transferred to the substrate to produce a hydroxylated product. This hydroxylated product is liberated from the active site of CYP enzyme thus pure ferric ion and CYP could join other metabolic activities. The catalytic cycle of CYP is demonstrated in Fig. 1.2 (Manikandan et al., 2018).

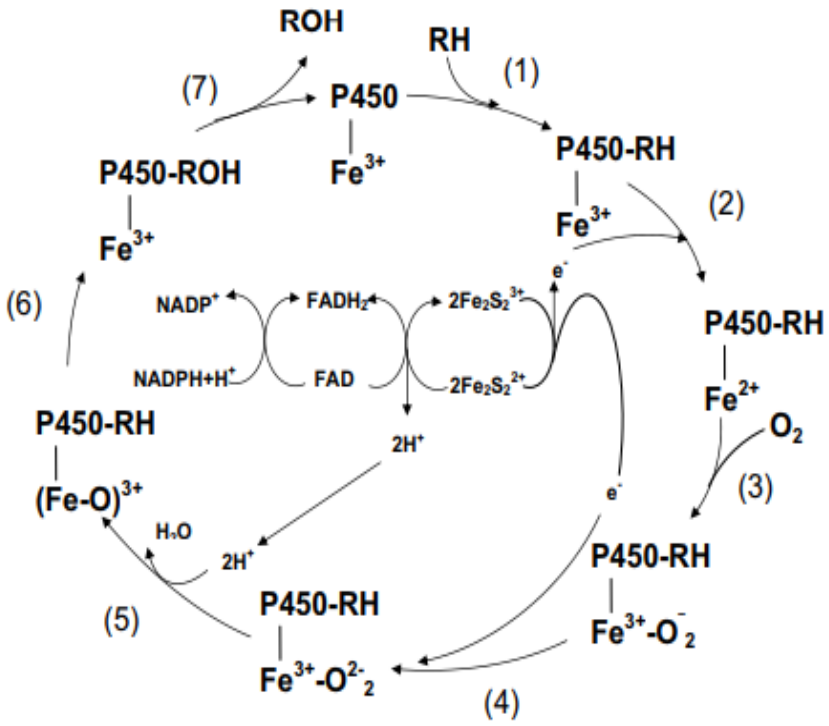


Figure 1.2 The catalytic cycle of cytochrome P450 (Manikandan et al., 2018)

1.2.1.1.1 CYP1A1

Cytochrome P450 1A1 is one of the three members of human CYP1A family. CYP1A1 gene (P1-450) also known as AHH (aryl hydrocarbon hydroxylase) is located at 15q22-q24 position, composed of seven exons and six introns and it contains 5810 base pairs. CYP1A1 is predominantly expressed in mitochondrial membrane and endoplasmic reticulum of extrahepatic tissues such as, epithelia of lung, gastrointestinal tract, cytosol of kidney and it is also found in placenta, fetus and embryo. Molecular weight of CYP1A1 is 59.23 kDa and it encodes 512 amino acids. CYP1A1 gene has 2608 bp mRNA length.

In order to establish structural elements of CYP1A1 and discover its ligand binding, its 2.6A structure in complex with inhibitor alpha-naphthoflavone had been reported (Walsh et. al., 2013). Crystal 3-D and global structure of CYP1A1 is displayed in Figure 1.3.

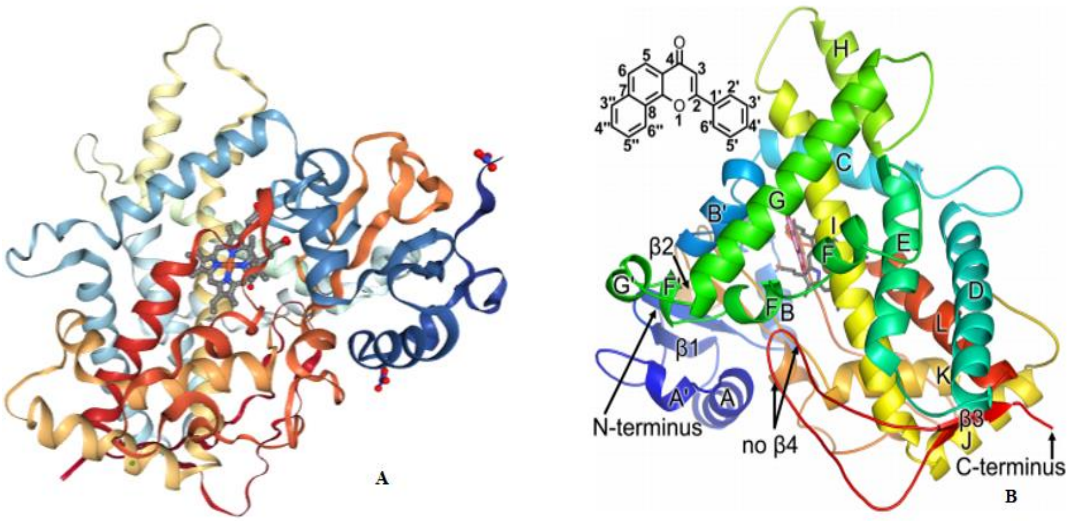


Figure 1.3 CYP1A1 3-D structure (A) and global structure of human Cytochrome P450 1A1 in complex with alpha-naphthoflavone (B) (Walsh et. al,2013).

Chemical pro-carcinogens in the environment are exposed to metabolic activation by CYP enzymes to more reactive carcinogen products. CYP1A1 is one of the cytochrome p450 enzyme which have a role in this activation process. Carcinogens are metabolized to epoxide intermediates by CYP1A1 and then those intermediates are activated to diol epoxides by enzyme epoxide hydrolase. In this regard, metabolic activation of Benzo[*a*]pyrene B[*a*]P and 7,12-dimethyl benzanthracene (7,12-DMBA) by CYP1A1 is one of the well-known process in which CYP1A1 play a trigger role. Figure 1.4 displays metabolic activation of (A) B[*a*]P and (B) 7,12-DMBA to the carcinogenic metabolites. CYP1A1 is also involved in the activation of tobacco related N-nitrosamines, carcinogenic mycotoxin aflatoxin B1, PAHs and HAHs (Shimada et. al, 2004).

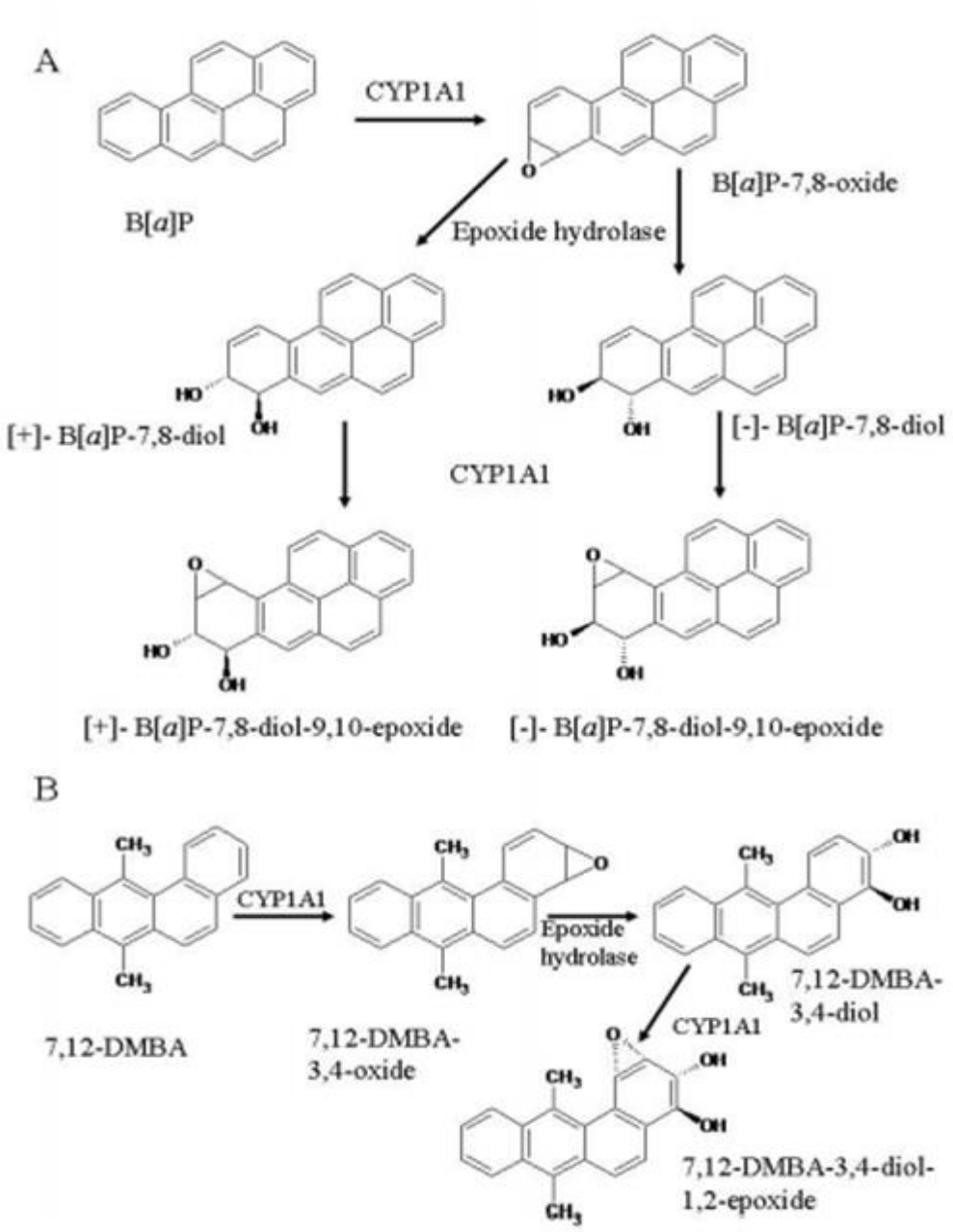


Figure 1.4 Metabolic activation of B[*a*]P and (B) 7,12-DMBA to the carcinogenic metabolites (Shimada et. al, 2004).

Studies have shown that some tumor types including colon cancer tumors overexpress CYP1A1 enzyme compared to their normal counterparts

(Androutsopoulos, et. al., 2013, Go et. al., 2015). These findings led researchers to test the hypothesis that CYP1A1 stimulate cancer progression and a study found that CYP1A1 stimulates breast cancer proliferation through suppression of AMPK signaling and inhibition of CYP1A1 activity may be a good strategy for breast cancer therapeutics (Rodriguez et. al., 2013). On the other hand, high expression of CYP1A1 enzymes within the tumour tissue serves many opportunities to develop new pro-drugs activated by CYP1A1 (McFadyen et al., 2004).

CYP1A1 activity can be induced by dietary constituents via aryl hydrocarbon nuclear receptor in cancer cell line models (Ciolino et. al, 1998 and 1999).On the contrary, certain dietary compounds including polyphenols can inhibit CYP1A1-metabolic activation and this inhibition prevents CYP1A1-mediated carcinogen production result in suppression of cancer progression (Ciolino et. al, 1999). As a result, Ciolino and their colleagues showed that different dietary constituents exert different effects on CYP1A1 activity in pharmacologically relevant concentrations. There has been also an *in vivo* study which suggests that CYP1A1 induction may cause carcinogen-detoxification, so in those cases, it can be considered as a potential cancer-preventive agent (Uno et. al, 2004, 2006). Studies which conduct research on the link between the cancer preventive properties of dietary compounds and CYP1A1 have shown that CYP1A1-mediated 7-ethoxyresorufin *O*-dealkylation is inhibited by flavone acacetin (Doostdar et al.,2000) and production of DNA damage by benzo[a]pyrene (BaP) is blocked by flavone apigenin via the inactivation of CYP1A1 (Lautraite et.al, 2002).

1.2.2 Phase II Xenobiotic Metabolizing Enzymes

Phase II enzymes play a remarkable role in the cellular biotransformation of endogenous xenobiotics and endobiotics to more rapidly excreted forms as well as inactivation of pharmacologically active compounds. Phase II biotransformation refers to the catalyzing the conjugation reactions such as glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. Conjugates which are formed at the end of these reactions are more hydrophilic than the parent

compounds. Primary enzymes involved in Phase II enzyme systems are ; UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases, (NATs), NAD(P)H quinone oxidoreductase I (NQO1), glutathione S-transferases (GSTs) and various methyltransferases (mainly thiopurine S-methyl transferase (TPMT) and catechol O-methyl transferase (COMT)). Although phase II enzyme systems mainly carry out detoxifying reactions in the body, they may be causal agent of drug toxicity as a result of their reduced capacity. Moreover, conjugates may activate the potentially carcinogenic compounds such as; benzylic alcohols, polycyclic aromatic hydrocarbons, aromatic hydroxylamines, hydroxamic acid and nitroalkanes by sulphotransferases. Several studies also show that absence or polymorphism of Phase II enzyme genes lead to many types of cancer formation and progression (Jancova et. al, 2010).

1.2.1.1 Glutathione S-Transferases

Glutathione S-transferases (GST's) are main Phase II detoxification enzymes which are present in the cytosol, mitochondria and microsome of the cell. They have a dimeric structure with subunits of 199-244 aminoacids (roughly 26 kDa) in length. They are primarily active in the liver, colon, kidney, testis and adrenal glands. GSTs catalyze several enzymatic reactions in which thioether conjugates is produced such as; nucleophilic aromatic substitution reactions, isomerations and reduction of hydroperoxides, conjugation of hydrophobic and electrophilic compounds with reduced glutathione. Moreover, epoxides that are derived from polycyclic aromatic hydrocarbons (PAHs) and alpha-beta unsaturated ketones are also detoxified by GSTs and they have roles in metabolization of prostaglandins and steroids. Human GSTs is divided in to three families: cytosolic GSTs, mitochondrial GSTs, and membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG family). The soluble and dimeric cytosolic GSTs are subdivided into eight classes designated based on their sequence similarity including Alpha, Kappa, Mu, Pi, Sigma, Theta, Zeta and Omega (Hayes et. al, 2005). Human GST enzymes are members of classes Alpha (A1-A4), Mu (M1-M5), Pi (P1), Kappa (K1) and Theta (T1, T2) with their subunit structure or isoenzyme type assigned by Arabic numerals.

GST mu class of enzymes are organized on chromosome 1p13.3 and they are highly polymorphic. Null mutations which occur in this mu class gene are associated with an increase in a number of cancers because of increased susceptibility to environmental toxins and carcinogens.

The most recent tertiary structure of GST enzyme is represented in 2012 and it is demonstrated in Figure 1.5 (Wu and Dong, 2012). Highly conserved Glutathione (GSH) binding site is called G site and it is linked to the domain 2 by a short linker sequence. Another substrate binding site contiguous to G site is called as H site and it is highly variable to make GST enzyme distinct from other enzymes in terms of size, shape and hydrophobicity.

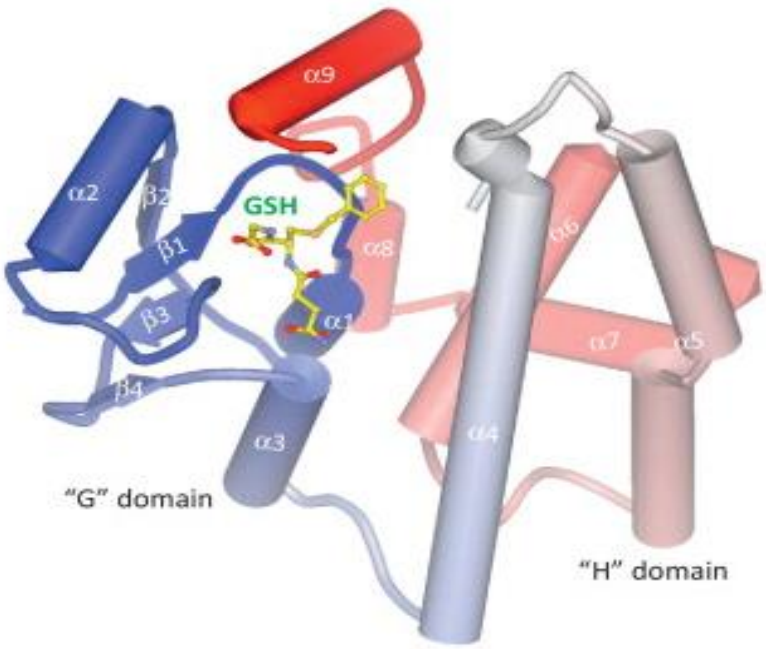


Figure 1.5 Tertiary structure of GST enzyme (Wu and Dong, 2012).

In detoxification pathway, glutathione thiolate is transferred to the electron deficient atom found in the hydrophobic and electrophilic xenobiotics by GSTs. While the hydrophobic H site of GSTs in charge of binding to the substrate, its hydrophilic G site is responsible for binding the GSH. As a result of conjugation reactions, a very hydrophilic metabolite that cannot pass through the cell membrane has been formed. Transportation of this hydrophilic metabolite through the cell membrane is facilitated

multi-drug resistance transport protein and final metabolite is delivered to the kidney eventually. Metabolite is composed of glycine, glutamic acid and cysteine and in the kidney, glycine and glutamate are removed and remaining part undergoes a further metabolism to excrete in urine. Figure 1.6 demonstrates a brief scheme of conjugation reaction catalyzed by GSTs (A) and some examples of reactions which are driven by GSTs.

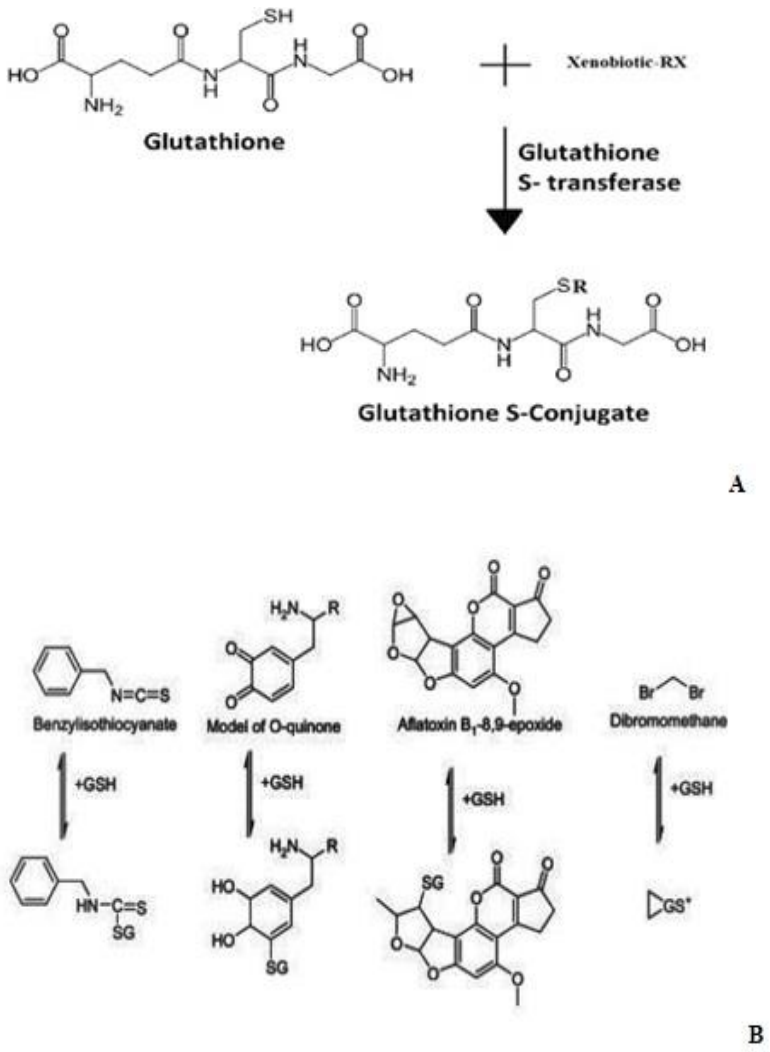


Figure 1.6 Basic conjugation reaction catalyzed by GSTs (A) and some examples of reactions catalyzed by GSTs (B) (Townsend et. al, 2003 and Smith, et.al, 2013).

GSTs have several therapeutic effects, including cell protection against oxidative stress and toxic compounds. For instance, they can defend genetic materials of the cells against oxidative damage which increases DNA mutations (Lin, Yi-Sheng et. al, 2009). It was also proven that GSTs are overexpressed in several type of cancer while they are expressed with very low level in normal and surrounding tissues so that GSTs are considered to be a good marker for carcinogenesis. In addition, a variety of anti-cancer drugs are direct substrates of GSTs. With some studies it was represented that GST was shown to be an endogenous inhibitor of JNK signaling pathway, a reaction that protects tumoral cells from apoptosis and be a regulator in the mitogen-activated protein (MAP) kinase pathway that associate with cellular survival and death signaling (Townsend, 2003). Multidrug resistance has been observed in the cell lines which express GSTs in high level. It was indicated that GSTs play important role increasing detoxification of anticancer drugs, even if not a direct determinant agents of resistance (Gate et.al, 2001). These studies promise that inhibition of GST enzymes by a natural or chemical agent has a potential to increase the effectiveness of anti-cancer drugs in certain effective doses. Considering the studies with GSTM1, similar results were obtained. It has been showed that some plant phenolic compounds inhibits GSTM1 expressions *in vitro* level and these natural products are considered as potent anti-cancer agents and chemo-preventive agents as well (Hayeshi et. al, 2007).

1.2.1.2 NAD(P)H: Quinone Oxidoreductase I

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a homodimeric flavoenzyme which belongs to Phase II enzyme family. NQO1 is a primarily cytosolic enzyme (90%) but it is also found in smaller amounts in mitochondria, endoplasmic reticulum and nucleus. The human NQO1 gene (DIA4) which is located on chromosome 16q22.1 encodes the NQO1 enzyme (Jaiswal et al., 1988). Molecular weight of the enzyme is 31kDa and each monomer which constitute NQO1 enzyme comprises 273 amino acids and is composed of two domains: a large N terminal catalytic domain (red and blue areas in Figure 1.7) and a small C-terminal domain (yellow and green areas in Figure 1.7) . Two FAD molecules are bound with catalytic domain of each monomer. (FAD molecules colored in red in Figure 1.7) The crystal structure of NQO1 enzyme

in complex with dicoumarol and FAD is represented in Figure 1.7 (Asher et al., 2006).

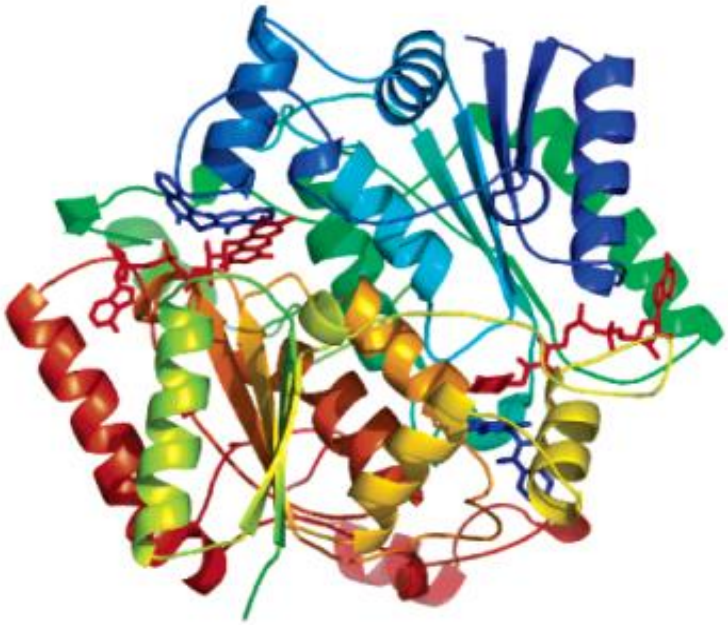


Figure 1.7 3-D Structure of NQO1 enzyme. Red and blue areas describe N-terminal domain, yellow and green areas defines C terminal domain (Asher et al., 2006).

As the name of the enzyme implies, NQO1 catalyzes two electron reduction of quinones with the usage of NADP(H) as an electron donor. It is a homodimer enzyme and performs through "ping-pong" mechanism. At the beginning, NAD(P)H binds to NQO1 and reduces FAD cofactor, then it is released to allow the binding of quinone substrate to the enzyme in order to be reduced. Due to binding locations of NAD(P)H and the quinone are overlapped in a large extent, NQO1 functions via ping-pong mechanism (Watanabe et. al, 2004). Beside of quinones, some other nitrogen containing xenobiotics such as dinitropyrenes and nitrobenzamides are reduced by NQO1 enzyme (Ross et. al, 2000).

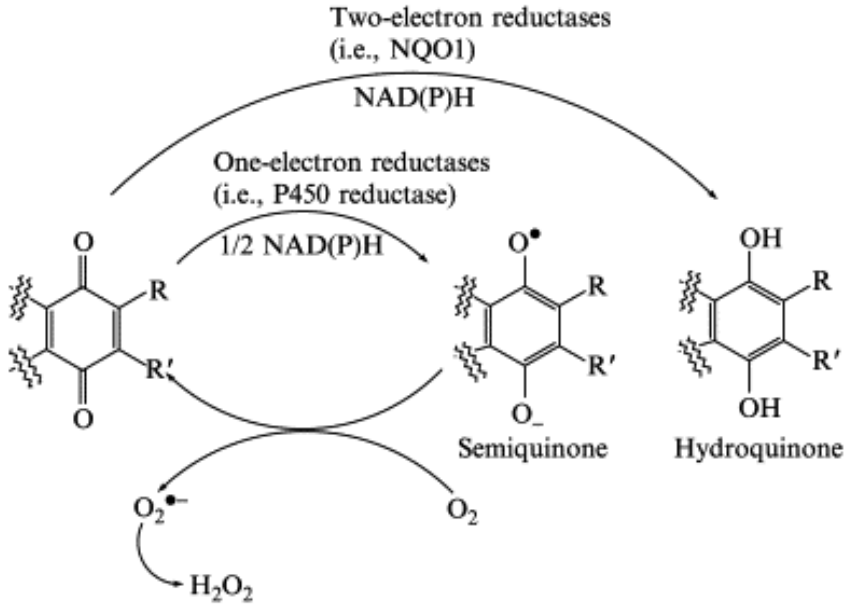


Figure 1.8 Quinone reduction by NQO1 (Watanabe et. al, 2004).

Quinonoid compounds have several harmful effects such as arylating nucleophiles and generating reactive oxygen species through redox cycling mechanisms thus removal of a quinone from a biological system by NQO1 has been regarded as a detoxification reaction (Ross et. al, 2004). It was demonstrated that induction of NQO1 may provide protection against the cytotoxic, mutagenic and carcinogenic effects of several chemical compounds. Studies related with NQO1 function have been introduced that it is an antioxidant enzyme and this crucial ability arises from endogenous quinones reduction. When quinones are reduced, cellular membranes are protected against oxidative damage and ROS generation is prevented, thus NQO1 serves as a chemopreventive agent (Siegel et. al, 1998, 2000). A preclinical study showed that NQO1 deficient mice are more susceptible to dimethylbenz[a]-anthracene-induced skin carcinogenesis (Moran et.al, 1999). Another researches also represented NQO1*2 polymorphism increases susceptibility to various type of cancer (Larson et. al, 1999) and later it was found that there is an interaction between p53 tumor suppressor protein and NQO1 enzyme which explains one possible mechanism underlying carcinogenesis due to lack of NQO1 (Anwar et. al, 2003). It was also showed that quinone activation by NQO1 may cause generations of some compounds that can alkylate the nucleophilic site of DNA and cause

apoptosis (Ross et. al 2000, Ceniene et. al, 2005). On the other hand, reduced form of quinones called hydroquinones, sometimes are not stable. This instability may result with formation of more active product which can produce ROS or generate alkylating species. For instance, toxicity of dinitropyrenes which is found in diesel has been arised by the activation of NQO1 (Hajos, et. al, 1991). Furthermore, NQO1 activity had been detected in increased level in some tumors such as breast, colon, liver and lung cancers (Schlager et. al, 1990 and Malkinson et. al, 1992). High NQO1 expression detection in those solid tumor types make NQO1 a viable target for designing personalized cancer therapy agents and NQO1 metabolizing anti-cancer drugs (Kelsey, 1997 and Huang et.al, 2012). Recently, a comprehensive *in vivo* study also found that there is a link between tumor-nqo1 expression and endurance of lung tumors (Madajewski et.al, 2015).

Modulation of NQO1 enzyme at different expression levels is possible by several types of natural compounds. Some studies have represented that NQO1 up-regulation with a natural dietary compounds could emerges good strategy for cancer prevention (Cornblatt et. al, 2007 and Surh, 2003). On the other hand, many studies showed that NQO1 activity in many cancers is significantly higher than in normal tissues (Yang et.al, 2014). Moreover, a biochemical study results have revealed that inhibition of NQO1 enzyme expression in transcriptional and translational levels with some phenolic compounds may modulate metabolism of carcinogens and so, inhibition of NQO1 may be a good strategy to withstand resistance of cancer cells against chemotherapeutic agents. (Karakurt et. al, 2015).

1.3 Aim of the Study

Natural compounds have been used in the treatment of several diseases for many years. Cancer has been the most curious disorder among diseases and people have used many plants in treatment of cancer without knowing exactly which components in those plants is a main anti-carcinogenic agent and how they affect cancer cells. Scientists have wondered the operating mechanism of such plants in cancer treatment, especially effects of plant polyphenols on cancer cell lines have been tried to investigate for decades. Studies have reported that phenolic compounds offer anti-oxidative, anti-proliferative, anti-carcinogenic and genotoxic effects. Major

component of Mediterranean type diet; oleuropein, is one of the best agent among these phenolic compounds. Investigating the possible working mechanism of oleuropein may reveal and strengthen its usage potential as an anti-cancer drug.

Modulation of Phase I and Phase II xenobiotic metabolizing enzymes with oleuropein may decrease the proliferation of cancer cells. Moreover, through regulation of these enzymes, dosage of chemotherapeutic drugs for cancer patient may be decreased with the use of oleuropein as a co-therapy agent, thereby side effects of anti-cancer drugs can be reduced.

In industrialized countries, dietetic factors have become the first cause of cancer and some dietetic factors are considered to have a preventive role against cancer development as well. Colorectal cancer is the 3rd most common cancer in the world and up to 80% of all colorectal cancer causes are based on diet. Because of these reasons, a metastatic colorectal cancer cell line, HT-29, serves as a good model to analyze the cytotoxic and genotoxic effects of oleuropein through the modulation of Phase I and Phase II xenobiotic metabolizing enzyme protein expression levels. After appropriate concentration of oleuropein determined with cell viability *in vitro* assays, cell lysates and protein were obtained from HT-29 cell culture. DNA damage capacity and protein expressions of CY1A1, NQO1 and GSTM1 were detected by Comet assay and Western Blot technique, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Line

In this study, protein expression of drug and carcinogen metabolizing enzymes CYP1A1, NQO1 and GSTM1 in human colorectal cell lines HT-29 (ATCC® HTB-38™) were analyzed. Studied cell line was a gift from Prof. Dr. Abdurrahim Koçyiğit, Medical Biochemistry Department, Bezmialem Vakıf University.

2.1.2 Chemicals and Materials

Oleuropein (O8889), Acrylamide (A9099), N,N′-bis-methylene-acrylamide (M7256), 2-mercaptoethanol (M3148), 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Trizma Base; T-1503), bovine serum albumin (A2153), ammonium persulfate (APS; A3678), ethylenediaminetetraacetic acid (EDT; E6758**),** Dimethyl sulfoxide (DMSO; D4540), ethidium bromide (EtBr; E7637), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; M5655), glycine (G8889), agarose (A9529), low-melting agarose (LMA; A9414), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; T9281), Trypsin-EDTA solution (T4049) were products of Sigma Aldrich, Inc. , St. Louis, Missouri, USA.

Methanol (106009), sodium dodecyl sulfate (SDS; 817034), triton X-100 (108643), tween-20 (822184), ethanol absolute (107017), isopropanol (818766), hydrochloric

acid (HCL; 100317), sodium chloride (NaCl; 106404), sodium hydroxide (NaOH; 106462) were purchased from Merck Corporate, Kenilworth, New Jersey, USA.

Freezing medium (Cryopreservation-medium; P07-90050) and Dulbecco's Phosphate Buffered Saline (DPBS; P04-36500) were products of Pan-Biotech Gmbh, Aidenbach, Bayern, Germany.

Mc-Coy's 5A,1X Medium (317-010-CL) was the product of Winsent Inc, Quebec, CANADA. Fetal bovine serum (FBS; 10270106) and Penicillin-Streptomycin (Pen-Strep; 150070063) were products of GIBCO life technologies, Waltham, Massachusetts, USA.

RIPA lysis buffer system involve 1X lysis buffer, PMSF, protease inhibitor cocktail, and sodium orthovanadate (sc-24948) was product of Santa Cruz Biotechnology, Inc.

CYP1A1 (sc-20772), NQO1 (sc-16464), and GSTM1 (sc-517262) primary antibodies and mouse anti-goat HRP secondary antibody (sc-2354) were purchased from Santa Cruz Biotechnology, Inc. Beta-tubulin primary antibody (2146S), anti-rabbit HRP-linked secondary antibody (7074S) and anti-mouse HRP-linked secondary antibody (7076S) were product of Cell Signaling Technology Inc. (CST, Leiden, Netherlands).

Blotting grade blocker (Non-fat dry milk; 1706404), Ouick-Start Bradford Protein Assay containing Quick-Start Bradford 1X dye reagent and Quick Start Bovine Serum Albumin Standard Set (2969), 2X Laemmli sample buffer (1610737), Clarity Western ECL Substrate (1705060) were purchased from Bio-Rad Laboratories Inc. Hercules, California, USA.

Page-Ruler Plus Pre-stained Protein Ladder (26619) was the product of Thermo Fisher Scientific, Waltham, Massachusetts, USA.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture Conditions

HT29 cell line was cultured in McCoy's 5A medium which contains 10 % fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) solution. Cultures were incubated at 37ºC with 5% carbon dioxide (CO_2) and 95% humidity in ESCO Cell Culture CO_2 incubator. The cell culture studies were carried out in Faster Safe Fast Classic 212 Cabinet. The growth medium of culture was renewed in 2-3 days for optimum growth conditions.

2.2.1.2 Cell Thawing

13 ml of growth medium pre-warmed to 37ºC was transferred in to T75 cell culture flask prior to thawing of the cells. Then, cryotubes were taken from the liquid nitrogen and the cells were defrosted at 37ºC water bath. After dissociation of the cells, they were transferred into 5 ml medium and then centrifuged for 5 min at 1000 x g to eliminate dimethylsulfoxide (DMSO) and 1 ml medium with pellet part which contains cells were transferred to T75 cell culture flask. Cells were incubated in CO_2 incubator at 37ºC. Following day from the cell thawing, medium had to be renewed to eliminate DMSO completely and again placed in to CO_2 incubator.

2.2.1.3 Subculturing the Cell Lines

When the cells were 70% confluent in the T75 flask, the medium was removed and cells were washed with 10 ml of 10 mM distilled phosphate buffered saline (dPBS) to inactivate fetal bovine serum (FBS) in the medium. 1:3 split of cell lines was performed by adding 2 ml of prewarmed trypsin to flask and placing the T75 flask in 37°C, CO2 incubator until cells were detached and 4 ml of pre-warmed growth medium was added to the flask to inactivate the trypsin and the 2 ml of this mixture was transferred into new T75 flask. Then 13 mL of growth medium was added to new T75 flask and the culture was placed in 37°C, CO2 incubator. This procedure was repeated in every 2-3 days.

2.2.1.4 Cell Freezing

When the cells were 80% confluent in the T75 flask, the medium was removed and cells were washed with 10 ml of PBS. 2 ml of pre-warmed trypsin was added to flask and placed in 37°C CO2 incubator for 5 minutes. After being sure of all the cells were detached, 2 ml of pre-warmed growth medium was added to the flask to inactivate the trypsin. The cells in the flask with trypsin and growth medium were transferred into a 15 mL falcon tube and centrifuged at 1000 x g for 5 minutes at room temperature. After centrifugation, supernatant was discarded and pellet was resuspended in 1 ml growth medium by pipetting. After that, the cell suspension was transferred to cryotube and 100 µl DMSO was added as a cryoprotectant. Cryotube was immediately placed in the -80°C freezer and in a week it was transferred into liquid nitrogen tank for longer term storage.

2.2.1.5 IC50 Determination for Oleuropein

In order to find IC50 value cells were inoculated to 96 well plate in 100µl at plating density 15.000 cells per well. After cell inoculation, microwell plates were incubated at 37°C, 5 % CO2, 95 % air and 100 % relative humidity for 24h before addition of oleuropein. After 24h, medium was replaced with 100µl oleuropein treated medium ranging from 100µM to 900µM oleuropein. Oleuropein treated medium was prepared by solving oleuropein in completed growth medium. Following oleuropein addition, the plates were incubated for an additional 48 h at 37°C, 5 % CO2, 95 % air and 100 % relative humidity. After 48 h, MTT Cell Viability Assay was performed to analyze cytotoxic effect of oleuropein on colon cancer cell lines. In order to perform this assay, oleuropein treated growth medium was discarded and cells were washed one times with 100 µl of 10mM distilled phosphate buffer saline (dPBS). Then MTT treated growth medium at a final concentration of 5 % was added to the wells and plate was returned to the incubator for approximately 4h. In this 4 time interval, plate was constantly checked for color change. When 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is added to the cell culture, NADP(H) dependent oxidoreductase enzyme in viable cells reduce yellow tetrazolium salt MTT to purple formazan crystals. After this incubation period,

purple formazan salt crystals are formed. These salt crystals were solubilized by adding 100 µl DMSO to each well and incubating the plate approximately 20 minutes at room temperature. The solubilized formazan product was spectrophotometrically quantified using Varioskan Flash (Thermo Scientific). An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase is directly correlated with the amount of purple formazan crystals formed, as monitored by the absorbance at 570 nm. The calculation of the percentage of cell proliferation inhibition (% Inhibition of cell proliferation) is as follows:

$$\%\ Inhibition\ of\ Cell\ Inhibiton = \frac{(ODcontrol - ODtreated)}{ODcontrol} \times 100$$

2.2.2 Protein Extraction

In order to accomplish protein extraction, cells were seeded to 6 well plates. After 24h, growth medium in the four of the dishes was replaced with growth medium, which contains 450 µM and 600 µM oleuropein (determined as IC50), and the other two wells were replaced with fresh growth medium as the control group. After the 48h oleuropein treatment, the procedure was performed identically for the control and the oleuropein treated cells. When cells were 80% confluent, growth medium in the dishes was removed and the cells were washed one times with PBS buffer and then cells were removed from flask with pre-warmed trypsin and put in to 2ml tubes. In order to remove trypsin from the medium cells were centrifuged at 1600 x g for 5 minutes at 4 ^{o}C, supernatant was removed and pellet was washed with PBS. This washing step was repeated 3 three times. 1ml RIPA lysis buffer includes 1 ml of 1X RIPA buffer, 20 µl PMSF, 10 µl Protease Inhibitor Cocktail and 10 µl sodium orthovanadate solution was prepared and 150 µl RIPA lysis buffer was added to each tubes to lysis of the cells. Tubes were vortexed 10 seconds and incubated on ice 10 minutes, these vortexing and incubation processes were done three times. After then, lysates of each tubes were centrifuged at 13000 x g at 4 ^{o}C for 10 minutes. Supernatants were taken gently and they are stored at -20^{o}C freezer.

2.2.3 Determination of Protein Concentration

Protein concentrations of cell lysates were measured by the Quick Start Bradford Assay (Bio-Rad) using bovine serum albumin as a standard (M. M. Bradford, 1976). The principle of this assay is the binding of protein molecules to Comassie Brilliant Blue G-250 under acidic conditions results in a color change from brown to a stable unprotonated blue form that absorbs light at a wavelength of 595 nm. This blue protein-dye form was detected at 595 nm by using a spectrophotometer reader (Varioskan Flash, Thermo Scientific).

Reagents

1x Dye Reagent:

1 L of dye solution containing methanol and phosphoric acid. One bottle of dye reagent is sufficient for 200 assays using the standard 5 ml procedure or 4,000 assays using the microplate procedure.

Bovine Serum Albumin Standard Set:

Set of 7 concentrations of BSA (2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 mg/ml) in 2 ml tubes.

Protein Sample:

Protein samples were diluted 5 times.1x dye reagent was removed from 4°C storage and warmed to ambient temperature. Each standard and unknown sample solution were pipetted into 96 microplate wells as 5 µl. Then 250 µl 1x dye reagent was added and incubated at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature. Varioskan Flash spectrophotometer was set to 595 nm and the absorbance of the standards and unknown were measured.

A standard curve was created by plotting the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). Determine the unknown sample concentration using the standard curve. If the samples were diluted, adjust the final concentration of the unknown samples by multiplying by the dilution factor used. Protein concentrations were calculated according to the following equation;

$$\text{Protein Concentration (mg/ml)} = \frac{[OD595nm]}{\textit{Slope of standarts}} \times \textit{Dilution}$$

2.2.4 Determination of Protein Expression

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

Protein expression of xenobiotic and carcinogen metabolizing enzymes; CYP1A1, NQO1 and GSTM1 in HT-29 cell line were analyzed by Western Blot method as described by Towbin et al. (1979). The first step of Western-blotting is protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 8 % stacking gel and 12 % separating gel in discontinuous buffer system as defined by Laemmli (1970). Separating and stacking gel solutions were prepared freshly. In Table 2.1 components of gel solutions were listed.

Table 2.1 Components of seperating and stacking gel solutions.

Components	**Separating Gel Solution**	**Stacking Gel Solution**
Monomer Concentration	% 12	% 8
Gel Solution	4000	650 µL
dH_2O	3500 µL	3050 µL
Separating Buffer	2500 µL	---
Stacking Buffer	---	1250 µL
10% APS	30 µL	25 µL
TEMED	10 µL	5 µL
Total Volume	10 mL	5 mL

Reagents:

Gel Solution:

30.0 g acrylamide and 0.8 g N, N′-bis-methylene-acrylamide was mixed in a total volume of 100 ml H2. Solution was filtered through a 0.45µm filter and stored at $4^{o}C$ in the dark.

Stacking Buffer: (0.5 M Tris-CL containing 0.4 % SDS, pH 6.8)

6.05 g Tris base was dissolved in 40 ml H_2O. pH was adjust to 6.8 with 3N HCL and then total volume was completed to 100 ml with H_2O. Solution was filtered through a 0.45- µm filter and 0.4g SDS was added. The pH should be checked again at the end.

Separating Buffer: (1.5 M Tris-Cl containing 0,4% SDS, pH 8.8)

91 g Tris base was dissolved in 300 ml H_2O. pH was adjust to 8.8 with 1N HCL. Total volume was completed to 500 ml with H_2O. Solution was filtered through a 0.45- µm filter then 2 g SDS was added. The pH should be checked again at the end.

Ammonium Persulfate-APS : (10%, Fresh)

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

Tetramethylenediamine-TEMED (Commercial)

Sample Dilution Buffer-Laemmli Sample Buffer

Laemmli sample buffer which bought commercially from Bio-Rad Laboratories, Inc. contains 31.5 mM Tris-HCl (pH 6.8), 10% glycerol, 0.005% Bromophenol Blue. 50 µl 2-mercaptoethanol was added per 950 µl Laemmli sample buffer as a reducing agent before use.

Electrophoretic Running Buffer-ERB:

0.25 M Tris, 1.92 M glycine (10x Stock, diluted to 1x before use by adding 0,1 % SDS).

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

Running buffer was prepared as 10x stock solution and it was diluted to 1x when it is used. 1g of SDS was added per liter of 1x before use.
SDS-PAGE was performed on 12% separating gel for CYP1A1, NQO1, GSTM1 in a discontinuous buffer systems. Vertical slab gel electrophoresis was conducted with Mini-PROTEAN tetra cell (Bio-Rad, Richmond, CA). After preparing separating gel solution (8%), it was immediately transferred in to sandwich unit up to 1 cm below the comb. In order to get a smooth gel surface, the top of the separating gel was covered with isopropanol. After the polymerization of separating gel, the isopropanol was taken off and stacking gel was transferred and finally the comb was placed as quickly as possible. When stacking gel was polymerized, the comb was removed. The wells were filled with 1x ERB and cleaned up by a Pasteur pipette to remove air bubbles and remaining gel particles.

To obtain the 1mg/ml (20 µg) concentration of protein, the proteins were diluted with distilled water according to the following formula;

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

V defines the volume of dH_2O to be added to dissolve 20 µL of the protein lysates.

1 part of sample was diluted with 1 part 2X Laemmli dilution buffer (SDB). After dilution, samples were incubated 10 minutes at 95ºC heat block for denaturation. Then, they were incubated in ice for 10 minutes and exposed to a quick centrifuge for 5 seconds. 30 µl sample was loaded on different wells of gel. 7 µl of protein ladder (Bio-Rad, Richmond, CA) was loaded as marker. After loading the sample, buffer tank was filled with running buffer and cell lid with power cables was trapped to tank. Then, tank was connected to the Bio-Rad power supply and electrophoresis

was run at 10mA-60 V at the beginning. When the samples were compacted and reached the same level, increase the voltage to 20mA-120V.

2.2.4.2 Western Blotting

Reagents:

Transfer Buffer: (25Mm Tris, 192mM Glycine, pH: 8.3) (10X)

30.3 g Tris-base and 144 g glycine was dissolved in 1 L distilled water. Then, 100 ml 10X transfer buffer was mixed with 200 ml methanol and the volume was completed to 1L with distilled water.

TBS: (150 mM NaCl, 10 mM Tris, pH: 7.4) (10X)

87.66 g NaCl and 12.11 g Tris was dissolved in 1 L distilled water. In order to arrange pH to 7.8 approximately 5 ml of HCL was used.

TBS-Tween (TBST):

100 ml 10X TBS buffer was mixed with 2 ml tween-20 and the volume was completed to 1L with dH_2O.

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

5 g non-fat dry milk was dissolved in 100 ml TBST.

Primary Antibody: 1/200 to 1/1000 dilutions

Dilution was made with 2.5% Non-Fat Dry Milk

Secondary Antibody: 1/500 to 1/5000 dilutions

Dilution was made with 2.5% Non-Fat Dry Milk.

Clarity Western ECL Substrate (Bio-Rad, Richmond, CA):

ECL substrate solution contain Clarity Western Peroxide Reagent and Western Luminol/Enhancer Reagent. 3ml peroxide solution and 3 ml of luminol enhancer solution were mixed and 6 ml of this mixture was used for each membrane.

In the first phase of transblotting, gel was removed from the glasses and placed in to transfer buffer for 10 minutes. PVDF membrane was cut as equal size with the gel. For a successful trans-blotting, hydrophobic PVDF membrane was activated with 100 % methanol for 30 seconds and it became more hydrophilic. Then, membrane was waited in distilled water for 5 minutes. Two filter papers also were wet with transfer buffer for a few seconds. After that, two filter paper, gel and PVDF membrane were placed in to transblot turbo system as shown in Figure 2.1. Transfer of the proteins from gel to polyvinylidene difluoride (PVDF) membrane was conducted with Trans-blot Turbo Blotting System (Bio-Rad, Richmond, CA) at 25V and 2.5 A for 10 minutes.

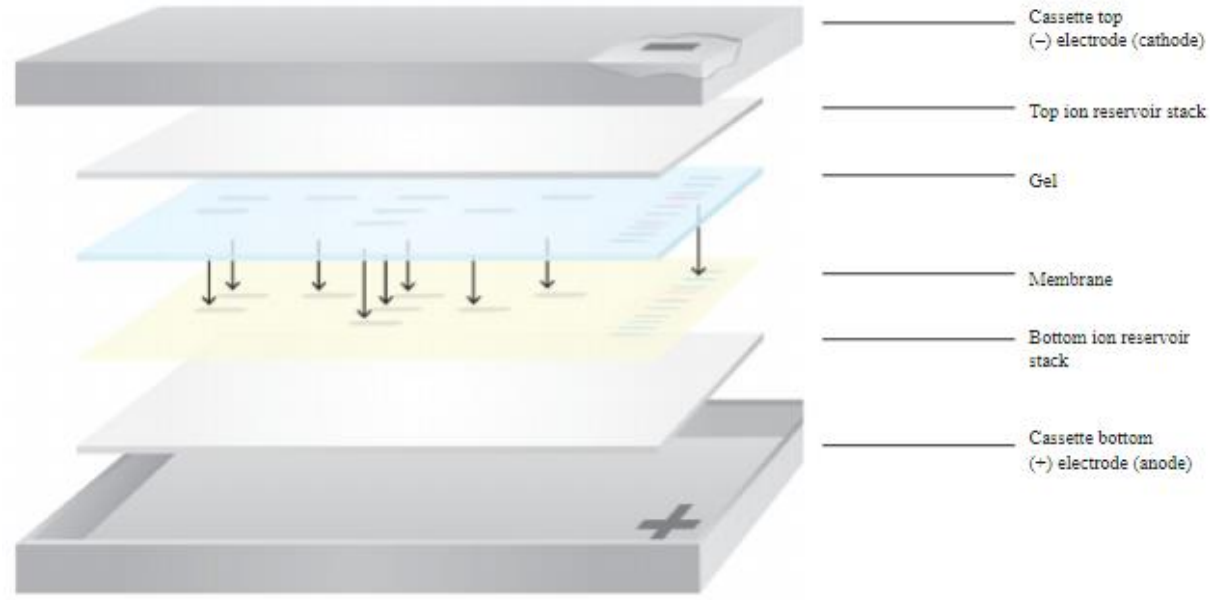


Figure 2.1 Assembly of the gel blot sandwich with the Trans-Blot Turbo system.

After transfer was completed, the membrane was washed with TBST for 5 minutes. Then membrane was incubated in to blocking solution at room temperature for an hour. After blocking step, membrane was washed with TBST for 5 minutes 5 times. Then, the membrane was incubated with 1/1000 dilutions of CYP1A1, NQO1 and GSTM1 overnight at 4^{o}C temperature by shaking. After primary incubation with primary antibodies, membrane was washed with TBST for 5 times each of which is 5

minutes in order to remove unbound primary antibody. Then, the membrane was incubated with 1/2000 dilutions of dilutions of horseradish peroxidase (HRP) conjugated secondary antibody**.** After that, membrane was washed again with TBST for 5 times each of which is 5 minutes in order to remove unbound secondary antibody. For visualization of HRP conjugated secondary antibody, membrane was incubated with ECL substrate 10 minutes then it was viewed under Fusion FX Chemi-Imaging System (Vilber Lourmat, France)**.** The band intensities were analyzed by Bio-profil-Bio-1D visualization software developed by Vilber Lourmat Ste.

2.2.5 The Single Cell Gel Electrophoresis / Comet Assay For Rapid Genotoxicity Assessment

2.2.5.1 Reagents

Normal Melting Agarose: (NMA, 1%)

1g Agarose was dissolved in 100 ml of 1X PBS

Low Melting Agarose (LMPA, 0.6%)

Low melting agarose (LMPA) was dissolved in 1X PBS

Stock Lysis Buffer (pH: 10)

2.5M NaCl, 100mM EDTA and 10mM Tris were dissolved in 1000 ml dH_2O. pH was arranged with 10M NaOH solution.

Lysis Buffer

10% DMSO and 1% Triton-X were added to stock lysis buffer. This mixture should be incubated at 4^oC before using and prepared freshly.

Neutralization Buffer (pH: 7.5)

0.4M Tris was dissolved in 1000 ml distilled water and pH was arranged to 7.5 with HCL solution.

Electrophoresis Buffer

10M NaOH was dissolved in 100 ml distilled water then 0.2M EDTA was poured slowly in to NaOH solution. After solution was dissolved completely, total volume was completed to 1000 ml.

Ethidium Bromide Solution

5 mg EtBr was dissolved in 50ml distilled water.

2.2.5.2 Cell culturing and removal prior to Comet Assay

Cells were seeded to 6 well cell culture plates (approximately 2×10^5 cells per well) and they were treated with oleuropein after 24 hours and when 48 hours incubation period with oleuropein ended, the media was removed and cells were washed with 10ml dPBS, after dPBS removal 0.005% Trypsin was added to the cells and the cells were incubated at 37^{o}C for 5 minutes to detach cells. Trypsin concentration was very low because higher concentrations increase DNA damage. Equal amount of medium was added (with FBS) to quench trypsin. Cells were centrifuged at 1600g for 10 minutes and wash with 1X PBS. This washing step should be repeated 3 times.

2.2.5.3 Preparation of Slides

After washing of cells, ~10.000 cells were mixed in 15 µl or less volume per 80 µl LMPA and these mixture were placed on 1% normal-melt agarose pre-coated slides.

2.2.5.4 Lysis of Cells

After agarose solidification, slides were immersed in lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) at 4 ° C overnight.

2.2.5.5 Electrophoresis of Cell Lysates

The slides were removed from the lysis solution and washed with cold PBS three times for 5 minutes, and placed side by side in a horizontal electrophoresis tank. After then, buffer reservoirs were filled with freshly made 4^{o}C temperature, pH>13

electrophoresis buffer until the liquid level completely covers the slides (avoid bubbles over the agarose). Firstly, slides were incubated in the alkaline buffer for 40 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage. The longer the exposure to alkali, the greater the expression of alkali-labile damage. Power supply was turned on to 25 volts (~0.72 V/cm) and adjust the current to 300 mA by raising or lowering the buffer level and samples on the slides were electrophoresed for 25 minutes at 4^0C.

2.2.5.6 Neutralization of Cell Lysates

The power was turned off. Slides were lifted gently from the buffer and place on a drain tray. Slides were rinsed with neutralization buffer and let sit for at least 5 minutes. This process was repeated three times, then slides were dehydrated with ethanol before staining.

2.2.5.7 Imaging and Analyzes of DNA Damage

The slides were stained with EtBr (2 μg/ml in distilled H2O, 50 ul/slide) and coated with a coverslip. Then, DNA damages were analyzed using an epifluorescence-equipped 200 × magnification fluorescent microscope (Leica DM 1000, Solms, Germany) equipped with a rhodamine filter (excitation wavelength of 546 nm and a barrier of 580 nm). Computerized image analysis system (Comet Assay IV; Perceptive Instruments) was used in order to examine tail intensity of tail in DNA. The percentage of DNA in the tail was used as the major criterion for DNA damage according to Hartmann et al., 2003.

2.2.6 Statistical Analysis

The results are presented as the mean ± standard deviation (Mean ± SD) of three replicates. Statistical analyses were done by using GraphPad Prism version 7 statistical software package for Windows and One-way ANOVA test was used. The p value <0.05 was considered as statistically significant. IC_{50} values of oleuropein over the cell lines were calculated by nonlinear regression analysis.

CHAPTER 3

RESULTS

3.1 Cell Culture

3.1.1 IC50 Determination for Oleuropein

Cytotoxic effects of oleuropein were analyzed on human metastatic colorectal cancer cell line HT-29. Cells were inoculated to 96 well-plate in 100 µl at plating density of 15.000 cells per well and incubated at 37° C, 5 % CO2, 95 % air and 100 % relative humidity for 24 h before oleuropein addition. After 24 hours, 5 µl oleuropein was added to wells. After 48 hours of treatment of cells with oleuropein ranging from 100 µM to 900µM, MTT Assay was performed as represented in method section in order to determine half maximal inhibitory concentration (IC50). Figure 3.1 demonstrates the color shift after exercising of MTT Assay.

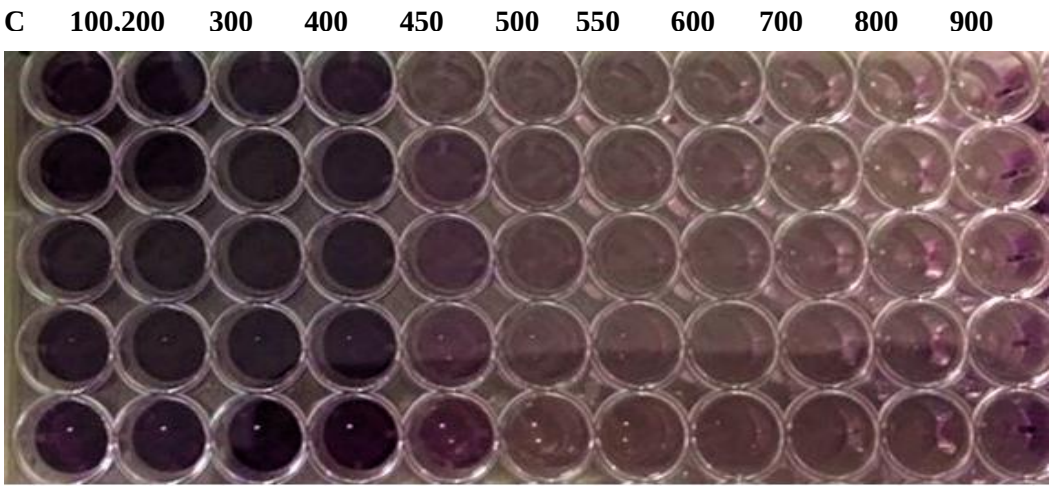


Figure 3.1 Color shift in wells after MTT assay following oleuropein treatment ranging from 100µM to 900µM.

After 4 hours incubation, purple precipitates were clearly visible under microscope and then they were dissolved with proper detergent. When color change was visible from purple to light pink, absorbance values were read by Varioskan

Spectrophotometer at 570 nm. MTT cell viability test was performed three times. Absorbance values which are lower than the control cells recommend a reduction in the cell proliferation. On the contrary, a higher absorbance rate indicates an increase in cell proliferation. Percentage of cell proliferation rate was calculated using the following formula;

$$Cell\ Proliferation\ Rate = (OD_{control} - OD_{treated}) \div (OD_{control}) \times 100$$

According to the percent survival values which are shown in Table 3.1, a cell viability graph was drawn and for IC50 determination, slope and equation of this graph was used. As a result of calculations, IC50 value of oleuropein for colorectal carcinoma cell line was detected as 600 μM. Figure 3.2 and Figure 3.3 demonstrate the % inhibition of cell proliferation and percent cell viability graphs for oleuropein treated HT-29 cell lines, respectively.

Table 3.1 Percent survival values of the cells following oleuropein treatment ranging from 100 to 900 μM.

Oleuropein Conc. (μM)	**% Inhibition of Cell Proliferation Rate**	**Percent Survival**
0	0	100
100	-3,8	103,8
200	2,6	97,4
300	19,8	80,1
400	22,1	77,9
450	26,5	73,5
500	34,3	65,7
550	46,5	53,5
600	48,5	51,5
700	49,3	40,7
800	79,6	20,4
900	90,9	9,1

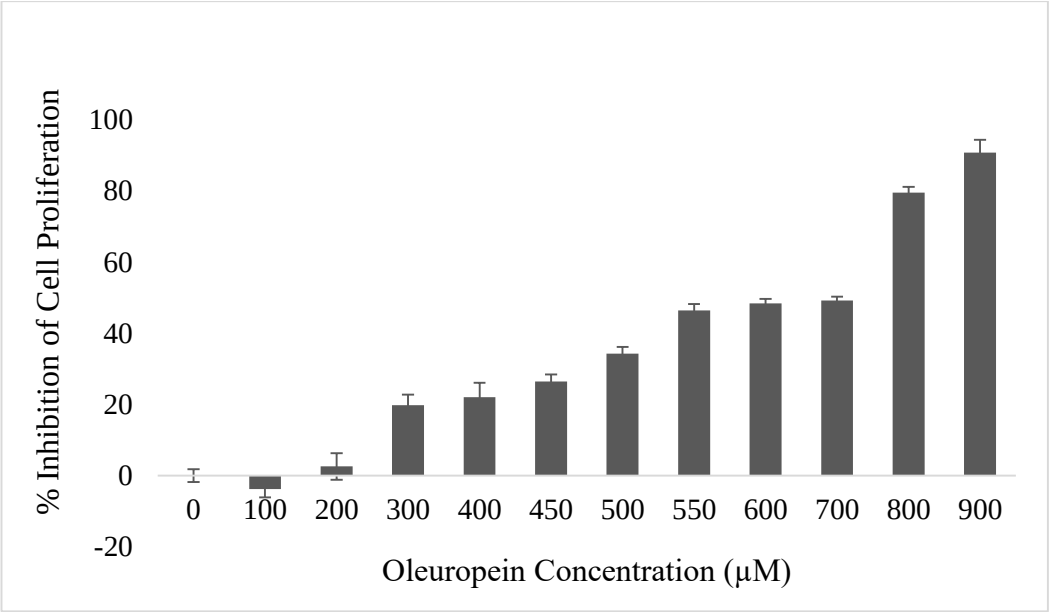


Figure 3.2 Cell proliferation rate graph for oleuropein (ranging from 100 to 900 µM) treated cells.

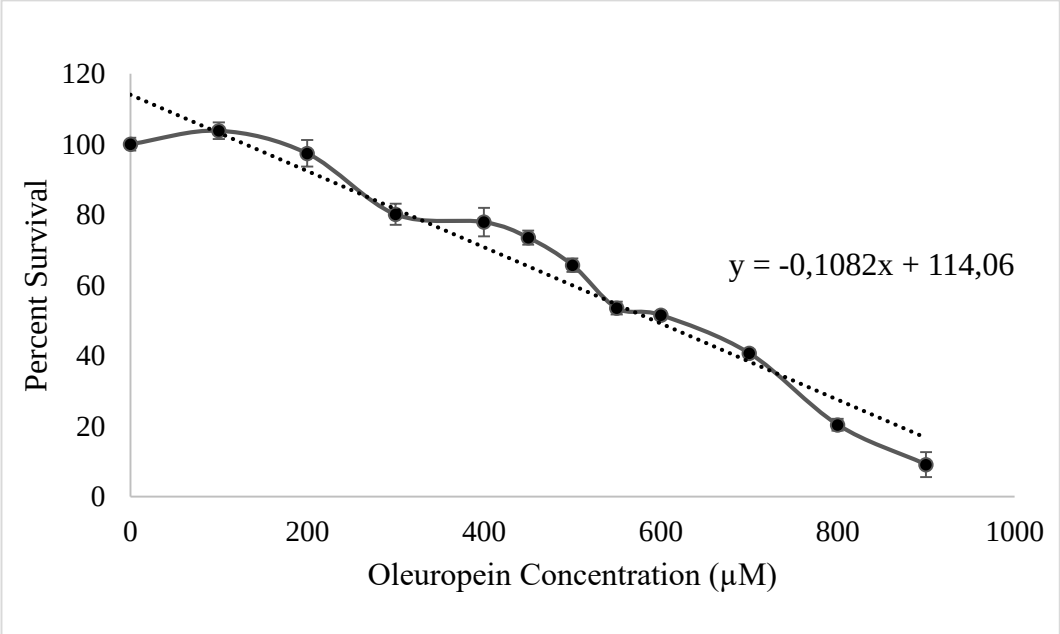


Figure 3.3 Percent survival graph for oleuropein treated cells

3.2 Protein Concentration of Cell Culture Lysates of the Control and Oleuropein Treated Cells

Effects of phenolic compound oleuropein on protein expressions of some xenobiotic metabolizing enzymes; CYP1A1, NQO1 and GSTM1 were accomplished by using HT-29 colon carcinoma cell line. Figure 3.4 shows the control and oleuropein treated plates microscopic images. Proteins were extracted from cells with RIPA buffer protocol and then protein concentrations in whole cell extracts were determined by Bradford Method as described before in methods part. Protein concentrations denominated in the mg/ml of control and oleuropein treated cells are given in Table 3.2.

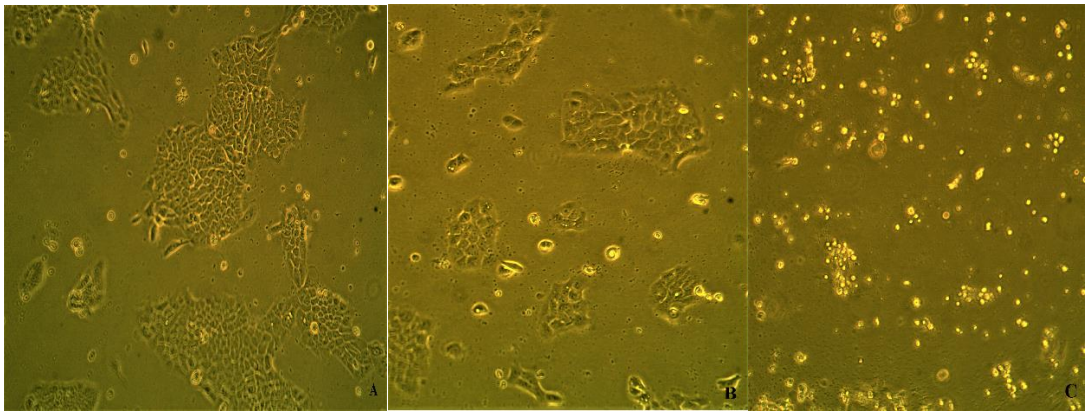


Figure 3.4 20X microscopic images of control (A), 450 µM oleuropein treated (B) and 600 µM oleuropein treated (IC50 value) (C) wells prior to protein extraction.

Table 3.2 Protein concentrations in the whole cell lysates of control and oleuropein treated HT-29 cells.

Cells	**Protein Concentration (mg/ml)**
Control	1.38±0.1
Treated (450µM)	0.89±0.037
Treated (600µM)	0.69±0.02

3.3 Protein Expression Analysis of CYP1A1, NQO1 AND GSTM1 Enzymes in HT-29 Cells

Phase I xenobiotic metabolizing enzyme, CYP1A1 and Phase II xenobiotic metabolizing enzymes, NQO1 and GSTM1 protein expressions in HT-29 metastatic colorectal carcinoma cells were analyzed by Western-Blot method. Protein lysates extracted from total cellular extracts of control and oleuropein treated cells were used in Western-blotting procedure. Immunochemical detection of expression levels was done by specific antibodies to corresponding proteins. β-tubulin (55kDa) was used as an internal standard.

3.3.1 CYP1A1 Protein Expression Levels in the Control and Oleuropein Treated Cells

CYP1A1 protein expression level in control and oleuropein treated cells was determined by Western-blotting. During immunochemical detection of CYP1A1 protein, primary rabbit monoclonal antibody (1/1500) dilution and horseradish peroxidase (HRP) conjugated anti-rabbit antibody (1/2000) were used. Western-blot results of CYP1A1 protein expressions in control and oleuropein treated HT-29 cell line extracts were shown in Figure 3.5. Bioprofil-Bio-1D software was used to quantify protein band intensities. Unpaired t-test was used to compare analysis of protein expressions of control and treated cells. Level of significance was selected as $p<0.05$. Relative protein expression results with statistical analyses were shown in Figure 3.6.

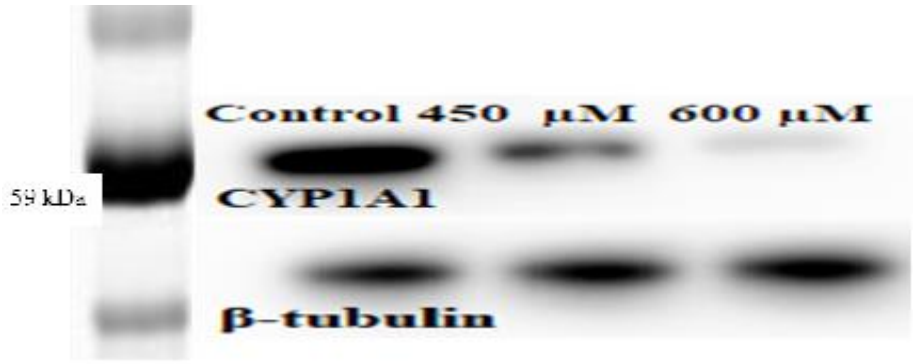


Figure 3.5 The CYP1A1 (59kDa) protein expression of control and oleuropein treated HT-29 colon carcinoma cells was determined with Western-blotting. β-tubulin (55 kDa) was used as internal standard. Each well was loaded with 20 µg protein.

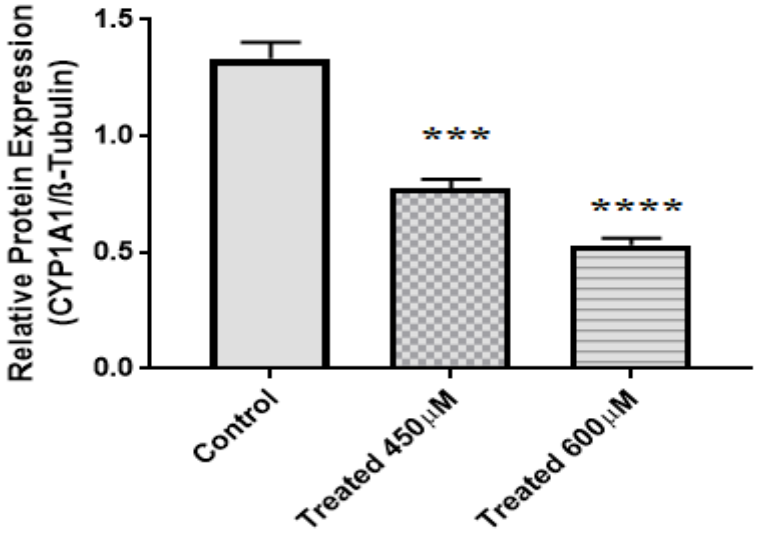


Figure 3.6 HT-29 cells were treated with two different concentrations of oleuropein to compare CYP1A1 protein expression of control and treated cells. Statistical tests were done by One-Way ANOVA test and significant differences according to the control were indicated by *** $p \leq 0.001$ and **** $p \leq 0.0001$. Band quantifications were presented as Mean ±SD and experiments were performed three times.

3.3.2 NQO1 Protein Expression in the Control and the Oleuropein Treated Cells

NQO1 protein expression level of control and oleuropein treated HT-29 cells was analyzed by Western-Blotting. Primary goat polyclonal anti-NQO1 antibody (1/1500 dilution) and a horseradish peroxidase (HRP) conjugated secondary rabbit anti-rabbit antibody (1/3500 dilution) were used in order to detect NQO1 protein expression in colon carcinoma cell extracts. The results of NQO1 protein expression levels in control and oleuropein treated HT-29 colon carcinoma cell extracts were shown in Figure 3.7. Quantification of band intensity was performed using Bioprofil-Bio-1D software. Figure 3.8 shows the comparison of NQO1 protein expressions between control and treated cells.

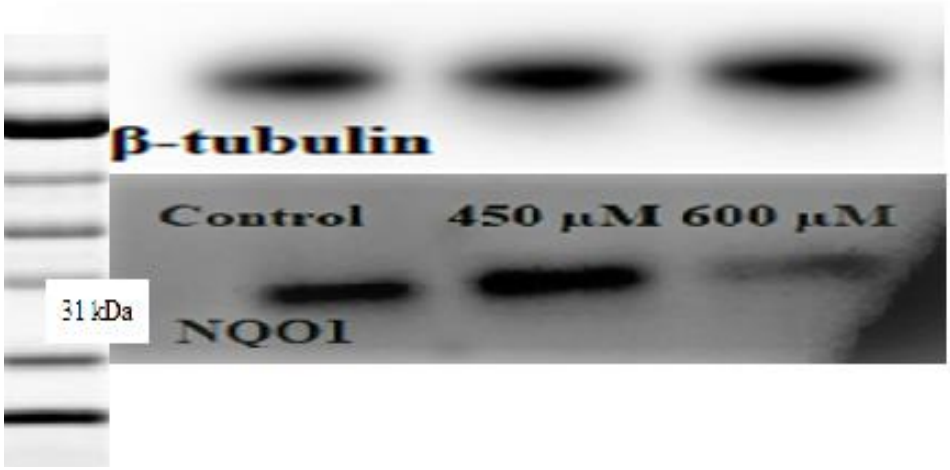


Figure 3.7 The NQO1 (31kDa) protein expression of control and oleuropein treated HT-29 colon carcinoma cells was determined with using Western-blotting. β-tubulin (55 kDa) was used as internal standard. Each well was loaded with 20 µg protein.

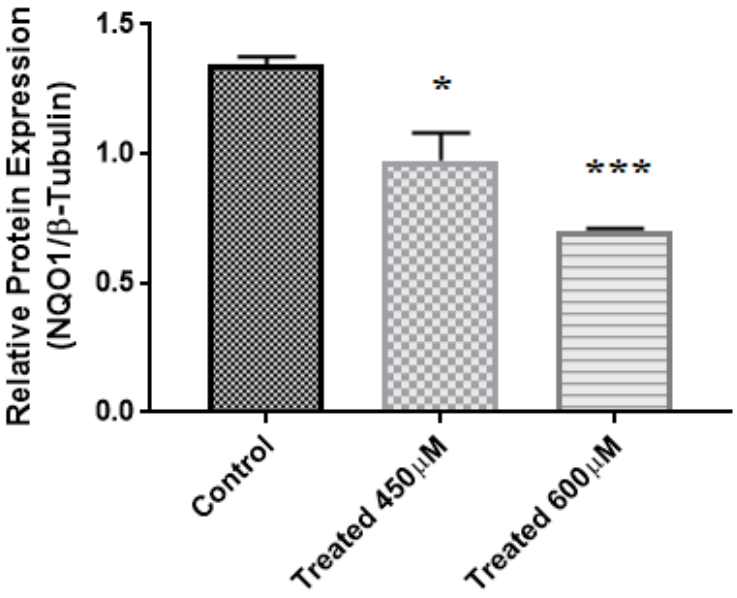


Figure 3.8 HT-29 cells were treated with two different concentrations of oleuropein to compare NQO1 protein expression of control and treated cells. Statistical tests were done by One-Way ANOVA test and significant differences according to the control were indicated by *$p<0.05$ and *** $p\leq0.001$. Band quantifications were presented as Mean ±SD and experiments were performed three times.

3.3.3 GSTM1 Protein Expression in the Control and Oleuropein Treated Cells

GSTM1 protein expression level of control and oleuropein treated HT-29 cells was analyzed by Western-Blotting. Primary rabbit monoclonal anti-GSTM1 antibody (1/1500 dilution) and a horseradish peroxidase (HRP) conjugated secondary anti-rabbit antibody (1/2000 dilution) were used in order to detect GSTM1 protein in colon carcinoma cells. The results of GSTM1 protein expression levels in control and oleuropein treated HT-29 colon carcinoma cell extracts were shown in Figure 3.9. Quantification of band intensity was done using Bioprofil-Bio-1D software. Figure 3.10 represents the comparison of GSTM1 protein expressions between control and treated cells.

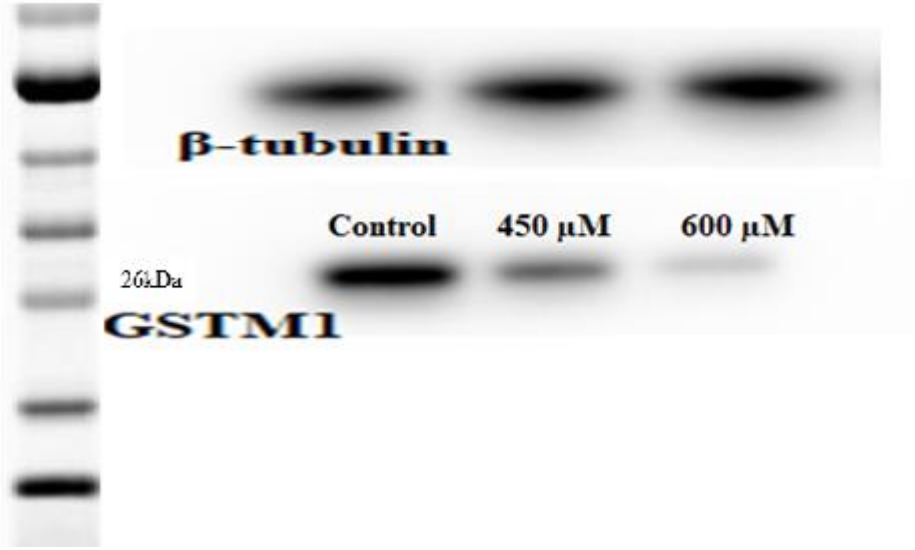


Figure 3.9 The GSTM1 (26kDa) protein expression of control and oleuropein treated HT-29 colon carcinoma cells was determined with Western-blotting. β-tubulin (55 kDa) was used as internal standard. Each well was loaded with 20 µg protein.

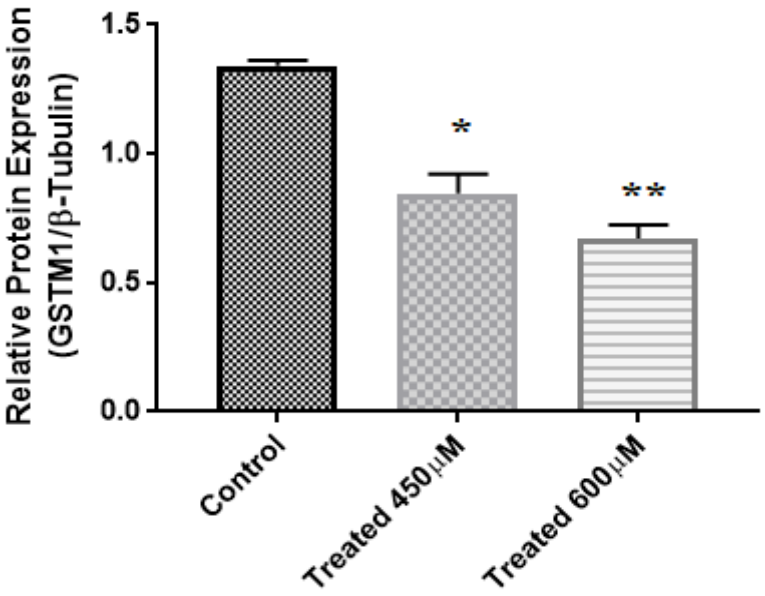


Figure 3.10 HT-29 cells were treated with two different concentrations of oleuropein to compare GSTM1 protein expression of control and treated cells. Statistical tests were done by One-Way ANOVA test and significant differences according to the control were indicated by *$p<0.05$, **$p\leq0.01$. Band quantifications were presented as Mean ±SD and experiments were performed three times.

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3.4 DNA Damage Analysis of Oleuropein Treated HT-29 Colon Carcinoma Cells by Comet Assay

The fundamental principle of the Comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. When cells are imaged under a microscope, a cell is seen to take the form of a comet, with a head (the nuclear region) and a tail which contain DNA fragments. Those fragments should migrate in the direction of the anode. In this research, for the analysis of genotoxic activity or DNA damage capacity of oleuropein on colon cancer cell line, cells were treated with 450 μM and 600 μM oleuropein for 48 hours and DNA damage in colon cancer cells was analyzed with the Comet Assay. Damaged DNA nuclei had a comet characteristic with a bright head and a tail, but nuclei with undamaged DNA appeared to be rounded without a tail. Analysis of DNA damages were done by an epifluorescence-equipped 200 × magnification fluorescent microscope. Each image represents a typical comet tail of the observed cells (at least 100 cells) and typical microscopic figures of Comet assays are shown in Figure 3.11. The percentage of DNA in the tail (tail intensity %) was as the major criterion for DNA damage analysis. In order to analyze tail intensity computerized image analysis system (Comet Assay IV; Perceptive Instruments) was used. Comparison graph of tail intensity percent between control and oleuropein treated cells is represented in Figure 3.12.

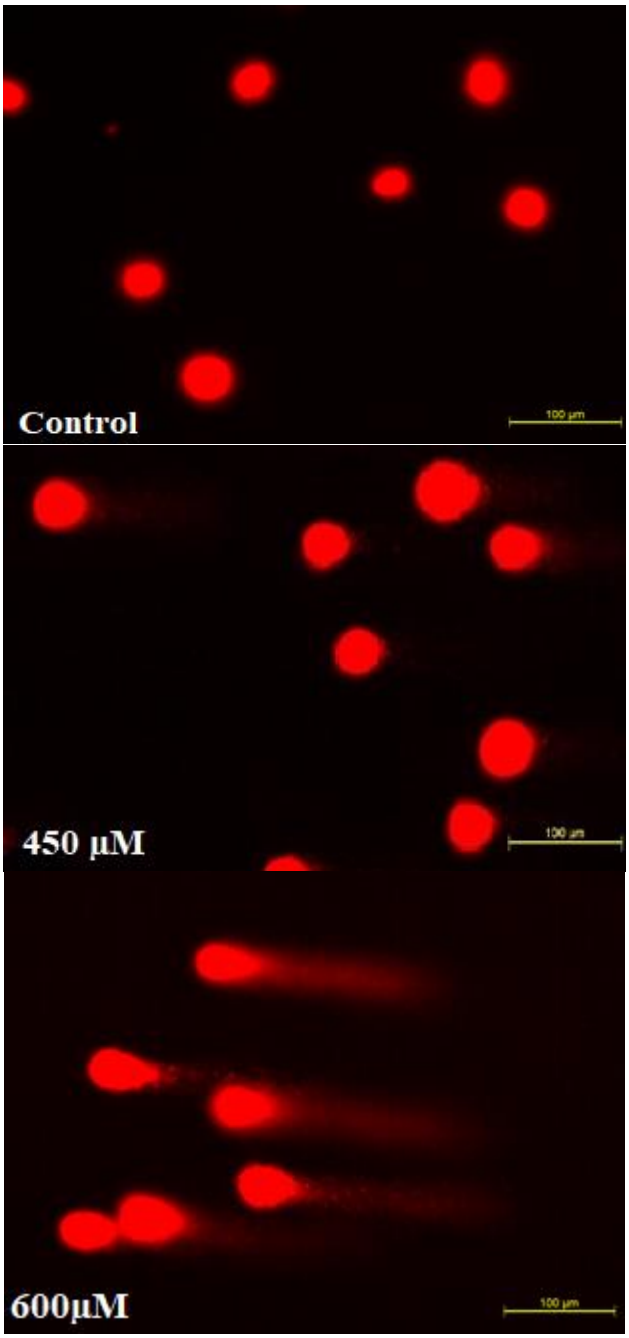

Figure 3.11 DNA damaging effect of different concentrations of oleuropein on HT-29 cells after 48 hours incubation. Comet formation pattern verifies that oleuropein induces DNA damage formation.

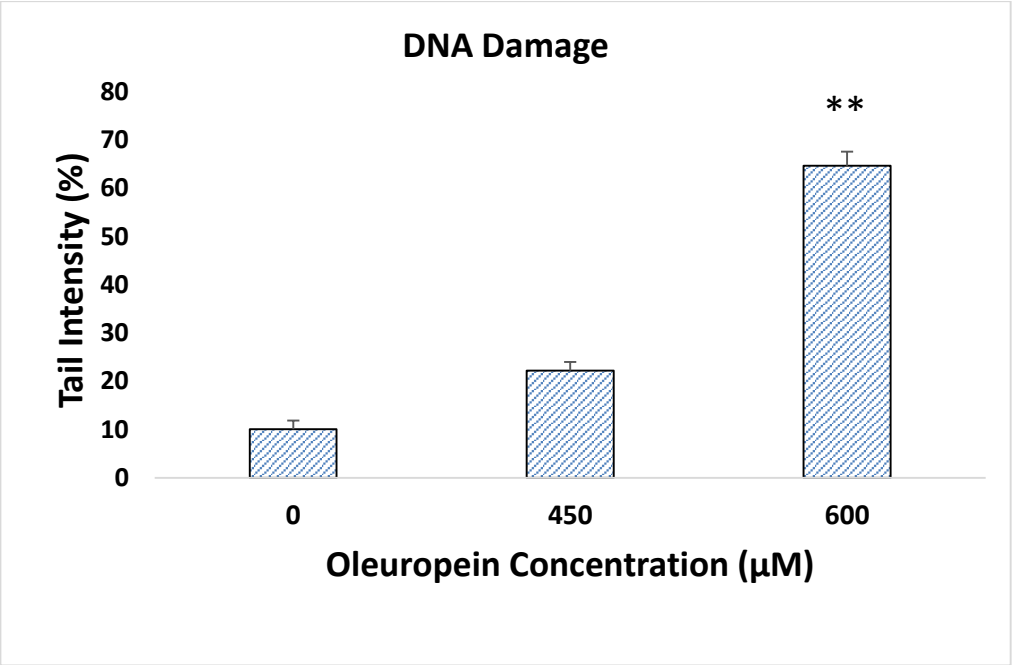

Figure 3.12 Oleuropein induces DNA damage in HT-29 colon cancer cell line. Cells were treated with two different concentrations of oleuropein for 48 hours and there were significant changes in the tail intensity % of DNA according to the control indicated by **$p \leq 0.01$

CHAPTER 4

DISCUSSION

Enzymes which catalyze the biotransformation of drugs and xenobiotics in to more readily excreted substances can be divided in to two major groups: oxidative or conjugative. The cytochrome P450 (CYP) enzymes are membrane-bound heme-containing proteins that play remarkable roles in the detoxification of xenobiotics, cellular metabolism and homeostasis. They catalyze several oxidative, peroxidative and reductive reactions including hydroxylations, epoxidations, N-dealklations, O-dealkylations and S-oxidations. CYPs are initiative enzymes of biotransformation in which lipophilic compounds are converted in to more freely hydrophilic products. Because of their critical roles in the metabolism of many therapeutic drugs, xenobiotics and exogenous chemicals, studies that investigate induction or inhibition of CYP enzymes have great importance. Especially, modulation of these enzymes is one of the most important mechanism that underlies carcinogenesis and drug-drug interactions. For instance, CYP1A1 activates polycyclic aromatic hydrocarbons (PAHs) into reactive intermediates which covalently bind to DNA and cause induction of carcinogenesis**.** Consequently, it could be stated that carcinogenic potential of PAHs or other carcinogens may be associated with the inhibition or induction of cytochrome P450 enzymes.

Phase II drug-metabolizing enzymes such as glutathione S-transferase conduct detoxification of drugs and xenobiotics through reduction and conjugation reactions. Substances which are previously metabolized by CYP enzymes are utilized to more rapidly excreted forms by GSTs. They have also many therapeutic effects including cell protection against oxidative stress and toxic compounds that cause damages in the genetic material of the cell (Lin, Yi-Sheng et. al, 2009). Another Phase II enzyme example is NQO1 which catalyze two electron reduction of quinones.

Removal of quinonoid compounds from biological system is a kind of detoxification reaction (Ross et. al, 2004) and NQO1 also activates some quinone based anti-cancer compounds (Simeone et al. 2003). When quinones are reduced, cellular membranes are protected against oxidative damage and generation of reactive oxygen species is prevented, thus NQO1 functions as a chemopreventive and an anti-cancer agent (Siegel et. al, 1998, 2000).

It is important to realize that doing scientific research about induction and inhibition of Phase I and Phase II enzymes involved in drug, xenobiotic and carcinogen metabolism provide many significant results in regard to their mechanism of action, especially their anti-cancer and chemo-preventive mechanisms. Regulation of these enzymes are executed at different molecular levels such as transcriptional, post-transcriptional, translational and post-translational. Consequently, modulation of those enzymes with a specific substance or complexes can reveal new mechanism underlie their anti-cancer effects. Phenolic compounds are mostly known and interested substances which have anti-proliferative and anti-metastatic effects on cancer cells. They can also change the rate of activation and detoxification of carcinogens by altering the activities of Phase I and Phase II enzymes (Carocho et. al, 2013).

The benefits of Mediterranean diet have been reported previously and researchers revealed that those benefits are associated with phenolic compounds which are plentiful in olive fruit, olive leaf and olive oil (Cicarele et al., 2010). Oleuropein has various pharmacological properties including antioxidant (Visioli et al., 2002), anti-inflammatory (Visioli et.al, 1998), anti-atherogenic (Carluccio et. al, 2003), anti-cancer (Owen et. al, 2000) and anti-microbial (Tripoli et. al, 2005). Particularly, its anti-cancer activity has been an issue of concern which have been discovered by some scientific researches in the recent years (Hamdi et al., 2005, Menendez et. al, 2007). Those anti-carcinogenic effects may be result from one of the several mechanisms that oleuropein has been shown to utilize on cancer cells. Modulation of xenobiotic metabolizing Phase I and Phase II enzymes by oleuropein is one of the possible mechanism underlying of its anti-cancer effect (Stupans et. al, 200, Zou et. al, 2012).

In first step of present study, cytotoxic effects of phenolic compound oleuropein on colon cancer cells had been analyzed. In order to investigate cytotoxicity impact of oleuropein, colon carcinoma cell line HT-29 was considered appropriate due to having a higher grade of its malignancy potential. HT-29 cells were seeded and they were treated and incubated with oleuropein for 48 h in order to determine its IC50 value. After MTT cell viability assay was performed, IC50 value of oleuropein on HT-29 cells was found to be 600 μM for 48h. According to result of other studies in recent years, IC50 dose of oleuropein changes in the range of 200 μM and 700 μM depending on the type of cancer and exposure times (Han et. al, 2009, Vanella, 2012, Cardeno et. al, 2013, Seçme et. al, 2016, Liman et al., 2017). When absorption and metabolism of oleuropein taking in the consideration, in order to avail one person of oleuropein in its 600 μM IC50 dose, approximately 250 kg dry olive leaf should be consumed daily, this consuming dose will increase for olive fruit and olive oil. Consequently, getting oleuropein in concentrated capsule or liquid form would make more sense than getting it from olive plant products directly (De Bock et. al, 2013) or it may be better administered it via injection than taking orally. These data may suggests that phenolic oleuropein which is found in olive leaf, olive fruit and olive oil may have a health protective role rather than a healing effect when it is used alone. Oleuropein has also a selective action on cancer cells; this was proved by a study which shows the cytotoxic effects of oleuropein on malignant and non-malignant cell lines (Vanella, 2012). Furthermore, oleuropein may be an efficient adjuvant when it is used in combination with a conventional chemotherapeutic drug. Adjuvant therapy is also another noticeable cancer research area because chemotherapy and radiotherapy bring with them many harmful side effects for patients. At this point, it is important to determine proper administration dose of oleuropein because it acts as an anti-oxidant agent even in low doses (< 50μm) (Saija et. al, 1998) and most of cancer chemotherapies based on the increase of oxidative stress and generation of ROS (Angsutararux et.al, 2015) Consequently, low oleuropein doses may decrease activity of chemotherapeutics via free radicals scavenge, but at higher doses phenols act as pro-oxidant agent (Fukumoto et. al, 2000) and they may increase effectiveness of chemotherapeutic agents by increasing ROS generation. During identify the effects of oleuropein administration as a co-

therapy agent, designating its modulation effects on Phase I and Phase II drug metabolizing enzymes would be seriously important. Even though, tumour suppressor effects of the oleuropein on colon adenocarcinoma cells was demonstrated with a cell viability test, doing further molecular based studies are needed to show potential mechanisms underlying its anti-cancer action.

After IC50 value determination, cells were treated with determined dose to examine the genotoxic activity of oleuropein on HT-29 cells with comet assay method (Fairbairn et.al, 1995). In this assay, DNA damage formation in colon cancer cell lines has been investigated in order to understand one of the possible mechanism underlying cytotoxic effect of oleuropein. Genotoxicity is a term that refers to the ability to interact with DNA or cell apparatus which regulates the fidelity of the genome causing damage in the genetic information within a cell and leads to mutagenicity or cytotoxicity but it doesn't mean necessarily that all cytotoxic agents affect the genome or all genotoxic agents cause mutagenicity. In the light of this information, determination of genotoxic potential of a cytotoxic agent can take its anti-cancer property a step further. Present study has showed that one of the possible working mechanism of natural compound oleuropein in preventing and blocking the development of colon cancer cells is its genotoxic activity. In oleuropein treated colon cancer cell lines, DNA damage increased by 54 %. In a lower dose of oleuropein, DNA damage increasing rate only reaches to 5 % with respect to control group. There are another supportive studies which showed the genotoxic effects of oleuropein and olive leaf extract in vitro and clinical levels (Liman et. al, 2017, Cabarkapa et. al, 2014) but their numbers are quite limited. It has been also showed that olive leaf extract had geno-protective effects on normal cells via the increase in the antioxidant capacity (Türkez et. al, 2011). Moreover, there is an approving letter which indicates that olive leaf extract is not genotoxic for normal body tissues either in the presence or absence of metabolic activation (EFSA, 2015). Consequently, genotoxic activity of oleuropein may have selectivity for cancer cells, but this implication should be supported with further studies in which normal cell types and other cancer types are examined. As it is well known, numerous genotoxins are

inactivated and detoxified by Phase I and Phase II metabolizing enzymes, thus discussion of relationship between oleuropein genotoxic activity and these enzymes would be one of the good outcome of this study.

In present study, effects of oleuropein on CYP1A1, GSTM1 and NQO1 protein expressions on human colon adenocarcinoma cell line HT-29 were studied for the first time. In order to perform protein expression analysis, cells were grown with 600 μM (determined IC50 dose) oleuropein before protein extraction. After 48 h treatment, different doses of oleuropein effects on these xenobiotic metabolizing enzyme was showed at translational level. (Table 4.1)

Table 4.1 Summary of the protein expression results of CYP1A1, GSTM1 and NQO1 enzymes from control and oleuropein treated cells.

	CYP1A1	**GSTM1**	**NQO1**
	Protein expression (% of control)	Protein expression (% of control)	Protein expression (% of control)
Oleuropein Treated- 600μM	% 43	%54	%52

According to the western-blot results, 600μM oleuropein (IC50 dose) cause 57 % decrease in CYP1A1 protein expressions of HT-29 colon carcinoma cell lines. As previously mentioned, CYP1A1 is one of the main cytochrome P450 enzyme which activates some carcinogenic compounds. When body is exposed to chemical and environmental carcinogens, CYP1A1 protein expression increases in non-hepatic tissues through the aryl hydrocarbon receptor (AhR) which regulates the CYP1A1 transcriptional activity and this elevated CYP1A1 activity is associated with higher cancer risk. Conversely, CYP1A1 also may play a role in detoxification of environmental carcinogen. As a consequence, role of CYP1A1 in cancer progression may depend on the balance between its pro-carcinogen activation and its

detoxification activity (Androutsopoulos, 2009). Additionally, it is known that, different dietary compounds exert different effects on CYP1A1 activity in their pharmacologically relevant doses (Ciolino et. al, 1999). Although there are limited number of studies which show oleuropein effects on CYP1A1 expression in carcinoma cells, some biochemical studies stated that phenolic oleuropein is an inhibitor of CYP1A2 (CYP1A isoforms like CYP1A1) enzyme or polyphenols including oleuropein inhibits CYP1A1 enzyme expressions in in vitro level (Stupans et. al, 2001 and Mutlu, 2015). In the light of previous studies, it is possible to say that the CYP1A1 protein expression is inhibited by oleuropein in dose dependent manner and these results are consistent with other previous studies and cytotoxic dose of oleuropein may be involved in prevention of cancer cells proliferation through inhibition of CYP1A1.

Experimental results have also showed that oleuropein treatment caused 46 % decrease in GSTM1 protein expression at its IC50 dose**.** As previously mentioned, GST enzymes are detoxification enzymes that protect the cell against oxidative stress and toxic compounds (Lin, Yi-Sheng et. al, 2009). Moreover, chemotherapeutic drug resistance has been observed in the cell lines which express GSTs in high level. It was also indicated that GSTs play important roles in detoxification of anticancer drugs, even if not a direct determinant agents of resistance (Gate et.al, 2001). These studies promise that inhibition of GST enzymes has a good potential to increase the effectiveness of anti-cancer drugs. Moreover, there is an *in vitro* study which shows the inhibitory effects of potent anti-cancer and chemo-preventive phenolic compounds on GTSM1 enzyme (Hayeshi et. al, 2007), but there has not been a completed study which shows the oleuropein effects on GSTM1 in colon carcinoma cells yet. The experimental findings of this study shows that oleuropein reduce the GSTM1 protein expression level in those dependent manner. These result support the some previous findings and also open a new aspect with respect to anti-cancer potential of oleuropein.

In present study, western-blot studies showed that plant phenolic oleuropein caused 48 % decrease in NQO1 protein expression at its IC50 dose. As previously mentioned, NQO1 enzyme is a kind of detoxification agent which remove the

quinones from the biological systems (Ross et. al, 2004). Quinone reduction protects cellular membranes against oxidative damage via preventing generation of reactive oxygen species (ROS), thus NQO1 may functions as a chemopreventive agent (Siegel et. al, 1998, 2000). On the other hand, reduced quinone forms, called hydroquinones, sometimes are not stable. This instability may result with formation of more active products which produce ROS or generation of alkylating species (Hajos, et. al, 1991). Furthermore, NQO1 activity has been detected in increased level in some tumors such as breast, colon, liver and lung cancers (Schlager et. al, 1990 and Malkinson et. al, 1992). High NQO1 expression detection in those solid tumor types make NQO1 a viable target for production of personalized cancer therapy agents and NQO1 metabolizing anti-cancer drugs (Kelsey, 1997 and Huang et.al, 2012). Recently, a comprehensive *in vivo* study has been completed that stated there is a link between tumor-nqo1 expression and endurance of lung tumors (Madajewski et.al, 2015). Quinone activation by NQO1 may also cause generations of some compounds that can alkylate the nucleophilic site of DNA and cause apoptosis (Ross et. al 2000, Ceniene et. al, 2005). In addition, according to the result of a biochemical study, inhibition of NQO1 enzyme expression in transcriptional and translational levels with phenolic compounds may modulate metabolism of several carcinogens. Furthermore, NQO1 inhibition may be a good strategy to withstand resistance of cancer cells against chemotherapeutic agents. (Karakurt et. al, 2015). This is the first study that has investigated oleuropein effects on NQO1 protein expression of colon cancer cell line and it could be realized from the experimental results; oleuropein can modulate NQO1 protein expression in spite of not being a quinoid compound. Its cytotoxic and genotoxic impacts may occur through down-regulation of NQO1 and these results are consistent with some other previous NQO1 enzyme studies.

In conclusion, the results of this study proposed that oleuropein may inhibit progression of colon adenocarcinoma cells through genotoxic activity and modulation of Phase I enzyme, CYP1A1 and Phase II enzymes, GSTM1 and NQO1. Nevertheless, in order to decide that oleuropein has substantial effects on colon cancer, it is necessarily make further studies with different colon carcinoma cells

with different characteristics because all three of these enzymes have different expression levels on different cell lines. In addition, effects of lower doses of oleuropein on carcinoma cells could be investigated because especially for polyphenolic compounds, dosage is very critical issue when evaluating their cancer preventive or cytotoxic potential. Beside of *in vitro* studies, *in vivo* studies with oleuropein are required in order to prove importance of its usage in cancer prevention, cancer treatment and adjuvant therapy.

CHAPTER 5

CONCLUSION

As mentioned in previous chapters, this is the first in vitro study to show the cytotoxic and genotoxic effects of plant phenolic oleuropein on HT-29 colon cancer line along with investigation of the effects of oleuropein on protein expressions of xenobiotic metabolizing Phase I enzyme, CYP1A1 and Phase II enzymes, GSTM1 and NQO1.

At the beginning of the experimental process of this study, cells were grown and then treated with 450 and 600µm (determined IC50) oleuropein before protein extraction and genotoxic activity determination. After cell culture studies were completed, genotoxic activity of oleuropein were studied with Comet assay and protein expression analysis of CYP1A1, GSTM1 and NQO1 in control and oleuropein treated HT-29 colon carcinoma cells were performed to understand the effects of oleuropein at translational level.

Oleuropein treatment of HT-29 colon cancer cells caused 57 % ($p<0.0001$) decrease in Phase I xenobiotic metabolizing enzyme; CYP1A1, protein expression. In addition, protein expression levels of GSTM1 and NQO1 phase II enzymes decreased 46 % ($p<0.01$) and 48 % ($p<0.001$) respectively by oleuropein treatment of cells. B-Tubulin was used as an internal standard. According to Comet assay results, oleuropein treatment caused 54 % increase in DNA damages ($p\leq0.01$) in HT-29 colon cancer cells.

In conclusion, the results of this study showed that plant phenolic compound oleuropein may inhibit progression of colon adenocarcinoma cells through genotoxic activity and inhibition of Phase I enzyme, CYP1A1 and Phase II enzymes, GSTM1 and NQO1. In order to understand that oleuropein has substantial effects on colon

cancer, it is necessarily make further studies with different colon carcinoma cells with different characteristics because all three enzymes have different expression levels on different cell lines. Beside of *in vitro* studies, *in viv*o studies with oleuropein are required in order to prove importance of its usage in cancer prevention, cancer treatment and adjuvant therapy. Nevertheless, this conducted research supports the hypothesis that oleuropein takes part in the inhibition of colon cancer cell progression, through genotoxic activity and causing the inhibition of xenobiotic metabolizing enzymes CYP1A1, GSTM1 and NQO1 which have important roles in pro-carcinogen activation. Revealing of a new perspective on the anti-cancer potential of olive plant family phenolic compound, oleuropein is another important outcome of this study.

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