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M.S. Thesis In Biology

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EFFECTS OF THE OLIVE EXTRACTS ON CANCER CELL LINES

by

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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June 2011

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Aysel KARAGÖZ

M. S. Thesis - Biology
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Supervisor: Assist. Prof. Dr. Sevim IŞIK

ABSTRACT

Mediterranean style diet has protective effects against degenerative diseases such as cardiovascular diseases, neurodegenerative diseases and cancer. This diet is characterized by high consumption of fruits, vegetables, and fiber, besides high intake of olive oil and olive products. Epidemiological studies have shown lower incidence of atherosclerosis, cardiovascular diseases and some type of cancers in Mediterranean countries compared to those in United States and Western countries. Previous studies into the beneficial effects of Mediterranean diet have shown due to the high and regular consumption of olive oil and olive products. Olive oil's beneficial affect is attributed to its phenolic content and fatty acid profile. Olive oil's phenolic content protect cells from reactive oxygen species attack via its antioxidant activity. Also monounsaturated fatty acid profile is preventive against coronary heart diseases.

In this study, effects of different types of olive extracts on cancer cell lines were evaluated. Proliferative, cytotoxic, apoptotic effects of olive extracts were investigated and gene expression profiles of cancer cells treated with olive extracts were analyzed. Topo IIα is essential for chromosome segregation in mitotic cells, so expression level of topo IIα can be used as marker of cell proliferation. Expression level of topo IIα was checked by using RT-PCR. Whenever these cells, treated with olive extracts, are proliferating cells, they express topo IIα. In addition, these effects on healthy human mesenchymal stem cells (hMSCs) were examined.

Results show diversity according to the cell types. Olive extracts have cytotoxic, anti-proliferative and apoptotic effects on gastric cancer cell lines at high concentration while colon cancer cell line and hMSCs were more resistant to extract treatment. RT-PCR results also confirm these results by expression of topo IIα in gastric cancer cell lines at low concentrations while no expression of topo IIα was observed at high concentration.

Keywords: Mediterranean diet, olive oil phenolics, cancer cell lines, apoptosis, proliferation, cytotoxicity.

ZEYTİN EKSTRAKTLARININ KANSER HÜCRELERİ ÜZERİNE ETKİSİ

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ÖZ

Akdeniz tipi beslenme kalp damar hastalıkları, beyin hasarına yol açan hastalıklar ve kanser gibi dejeneratif hastalıklara karşı koruyucu etkilere sahiptir. Bu beslenme tipi zeytin yağı ve zeytin ürünlerinin yüksek oranda alımı yanında meyve, sebze ve lif tüketiminin fazla olmasıyla karakterize edilir. Epidemiyolojik çalışmalar göstermiştir ki damar sertliği, kalp damar hastalıkları ve bazı tip kanserlerin görülme oranı Akdeniz ülkelerinde, Amerika Birleşik Devletleri ve Batı ülkeleri ile karşılaştırıldığında daha düşüktür. Önceki çalışmalar, Akdeniz diyetinin yararlı etkilerinin zeytin yağı ve zeytin ürünlerinin fazla ve düzenli tüketiminden olduğunu göstermiştir. Zeytin yağının faydalı etkileri onun fenolik içeriği ve yağ asidi profiline dayandırılır. Zeytin yağının fenolik içeriği antioksidan etkinliği aracılığı ile hücreleri reaktif oksijen türlerinin saldırısından korur. Ayrıca tekli doymamış yağ asit profili koroner kalp hastalıklarına karşı koruyucudur.

Bu çalışmada farklı çeşitlerdeki zeytin ekstraktlarının kanser hücreleri üzerine etkileri değerlendirilmiştir. Zeytin ekstraktlarının çoğalma, sitotoksisite, hücre ölümü üzerine etkileri araştırılmış ve zeytin ekstraktlarıyla muamele edilmiş kanser hücrelerinin gen ifade profili analiz edilmiştir. Topo IIα, mitotik hücrelerde homolog kromozomların birbirinden ayrılmasını sağlayan olayda temel enzimdir, bundan dolayı topo IIα ifade düzeyi RT-PCR tekniği ile incelenmiştir. Zeytin ekstraktlarıyla muamele edilen hücreler çoğalan hücreler ise, topo IIα' yı ifade ederler. Bunlara ek olarak bu etkilere sağlıklı insan kemik iliği kökenli mezenkimal kök hücreleri üzerinde de bakılmıştır.

Sonuçlar hücre tipine göre çeşitlilik göstermektedir; kolon kanseri hücre soyu ve insan kemik iliği kökenli mezenkimal kök hücreleri ekstrakt muamelesine daha dirençli iken, zeytin ekstraktları yüksek konsantrasyonda mide kanseri hücre soyları üzerinde sitotoksik, antiproliferatif ve apoptotik etki göstermektedir. Ayrıca RT-PCR sonuçları da bu sonuçları desteklemektedir; mide kanseri hücre soylarında yüksek

konsantrasyonlarda hiç topo IIα ifadesi gözlenmezken, düşük konsantrasyonlardaki yüksek topo IIα ifade düzeyi ile sonuçlar doğrulanmaktadır.

Anahtar Kelimeler: Akdeniz diyeti, zeytin yağı fenolik bileşikleri, kanser hücre soyları, apoptoz, proliferasyon, sitotoksisite.

To my beloved family

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL	ABBREVIATION
GI	Gastrointestinal
hMSC	Human mesenchymal stem cell
DMEM	Dulbecco’s modified Eagle’s medium
FBS	Fetal Bovine Serum
PBS	Phosphate buffer saline
EtOH	Ethanol
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
LDH	Lactate dehydrogenase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
RT-PCR	Reverse transcriptase polymerase chain reaction
TGF-β	Transforming growth factor beta
EGF	Epidermal growth factor
PDGF	Platelet-derived growth factor
Topo II	Topoisomerase II
Topo IIα	Topoisomerase II alpha
cDNA	Complementary DNA
ROS	Reactive oxygen species
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
LDL	Low density lipoprotein
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide
SD	Standard deviation

CHAPTER 1

INTRODUCTION

1.1 CANCER

Cancer is a group of diseases of higher multi-cellular organisms, characterized by abnormal proliferation of cells. In cancer progression oncogenes, tumor suppressor genes and DNA mismatch repair genes are important molecules as they modulate, either negatively or positively, cellular signaling cascades, transcription of target genes and cell cycle progression. Changes in these molecular networks influence the cellular behaviors, such as proliferation, differentiation, and apoptosis. Cancer leads to the alteration in multiple genes' expression. Changes in gene expression cause dysregulation of the normal cellular program for cell division and differentiation. This situation causes an imbalance between cell proliferation and cell death and leads to the growth of tumor cell population. Having ability to invade locally and metastasize to distant tissues and organs indicate the malignancy of tumor cell growth.

There are more than 100 types of cancer and also can be the organ specific tumors. In cancer cell genotypes some essential alterations and acquired capabilities during tumor progression are shared in common such as; self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hananhan and Weinberg, 2000).

1.1.1 Self-Sufficiency in Growth Signals

Normal cells require stimulatory signals such mitogenic growth signals in order to start proliferation. Cancer cells don't need this type of stimulatory signals, they can generate their own growth signals. Cancer cells can also provide their own growth factors which are made by another cells in order to stimulate proliferation like production of PDGF (platelet-derived growth factor) and EGF (epidermal growth factor) by glioblastomas (Hanahan and Weinberg , 2000).

During transition of a signal from extracellular area to intracellular there might be some alterations in signaling cascade molecules, they can have oncogenic potential such as RAS, RAF and MAP kinase pathways.

1.1.2 Insensitivity of Anti-Growth Signals

Antigrowth signals operate cellular quiescence (go into G_0) and homeostasis by acting as growth inhibitors and immobilized inhibitors (Hanahan and Weinberg , 2000). Antigrowth signals are controlled through retinoblastoma protein (pRb). pRb controls proliferation by effecting the activity of E2F transcription factors and cell cycle progression from G1 to S phase (Du and Pogoriler , 2006). Disruption of pRb cause releasing of E2F and allows cell proliferation and rendering cells insensitive to antigrowth signals. Antigrowth signals follow TGFβ signaling pathway. According to the types of cancer, pRb signaling pathway can be disrupted as a result of losing TGFβ responsiveness and mutated dysfunctional receptors (Hanahan and Weinberg, 2000).

1.1.3 Evading Apoptosis

Apoptosis or programmed cell death is an important event for the normal development and homeostasis. There is a balance between proliferating cells and dying cells in the organism. Excess amount of cell death cause degenerative diseases of the heart and nervous system and too little cell death can result in autoimmune diseases or cancer. Nearly all types of cancers are resistant to apoptosis.

There are two main well-characterized pathways of apoptosis, intrinsic and extrinsic apoptotic pathways. Apoptotic cell death through intrinsic pathway occurs activation or inhibition of mitochondrial pro-apoptotic (Bax, Bak) and anti-apoptotic

proteins (Bcl-2). Mitochondrial pathway controls the permeabilization of outer mitochondrial membrane in response to death inducing events in the cell (Brunella and Letai, 2009).

Extrinsic pathway involves death receptors and their ligands on the cell surface. When a death signal stimulates the cell, death receptors (located on the surface of target cell) bind to its ligands (located on the surface of T lymphocytes or natural killer cells) and death signals pass through inside the cell. Caspases are important molecules for both intrinsic and extrinsic pathways.

In cancer progression these pathways are defective. Overexpression of Bcl-2 anti-apoptotic protein, mutations and epigenetic silencing in pro-apoptotic genes' expression, decreased or completely absent cell surface expression of death receptors are characteristic features of many tumors (Ashkenazi and Dixit, 1998).

1.1.4 Limitless Replicative Potential

Probably all types of mammalian cells have an autonomous program that limits cell proliferation. This program proceeds independently of the cell to cell signaling. In normal conditions, cells have a finite replicative potential (~50 mitosis). After the progression through a certain number of doublings they stop growing termed senescence. During tumor progression, tumor cells appear to be immortalized, suggesting that limitless replication potential, acquired *in vivo* is essential for malignancy (Hayflick, 1997).

In the light of the discovering of telomeres (ends of the chromosomes), it was understandable for tumor cells to have limitless replicative potential. Telomeres composed of a short six base pairs sequence repeats. During cell cycle 50-100 base pairs telomeric DNA is lost. This shortening progression cause inability of DNA polymerase to replicate the ends of the chromosomes. Telomeres protect chromosomes from degradation, fusion and recombination. These short sequences are added at the end of the chromosomes by an enzyme called telomerase. Increased telomerase activity can increase the replication rate and this might be the reason of cancer formation (Hanahan and Weinberg, 2000).

1.1.5 Sustained Angiogenesis

Tumor cells can penetrate blood or lymphatic vessels, circulate through the intravascular system and then proliferate at another site; metastasis. For metastatic activity of a cancer tissue, growth of vascular network is important. The process which blood vessels form is called angiogenesis. It is an essential process for supplying nutrients, oxygen and immune cells as well as a continuous removal of cytotoxic metabolic waste products (Nishida *et al*., 2006).

In the absence of vascularization, tumor cells may become necrotic or apoptotic. Angiogenic factors' activity and inhibitory molecules of angiogenesis effect this process. As an angiogenic factor, more than a dozen different proteins such as VEGFR, bEGF, IL-8 and also several smaller molecules such as adenosin have been identified. For a successfully occured process inhibitors of angiogenesis may need to be downregulated including angiostatin, endostatin, interferon (Nishida *et al*., 2006).

1.1.6 Tissue Invasion and Metastasis

As a selective difference between benign and malignant tumors, invasion and metastasis refer invading the adjacent tissues, destroying them and leaving from the primary tumor, travelling through circulation to a distant tissue and form a secondary tumor. To obtain characteristic malignant tumor there are some steps needed to be involved;

1. Disconnection of intercellular adhesions and separation of single cells from solid tumor tissue.
2. Escape from anoikis.
3. Proteolysis of extracellular matrix.
4. Locomotion of tumor cells in the extracellular matrix.
5. Invasion of lymph and blood vessels.
6. Immunologic escape in the circulation.
7. Adhesion to endothelial cells.
8. Extravasation from lymph and blood vessels.
9. Proliferation and induction of angiogenesis (Bohle and Kalthoff, 1999).

In each of these steps there are lots of mechanisms for facilitating invasion. Such as down-regulation of integrins that bind to extracellular matrix, matrix degradating enzymes that control degradation of extracellular matrix and remodeling it. Matrix metalloproteases (MMPs) are also important molecules for increasing the cancer cell growth, migration, invasion-metastasis and angiogenesis. In cancer cells protease genes are up-regulated and protein inhibitor genes are down-regulated (Leber and Efferth, 2009).

1.1.7 Cancer Incidence and Mortality Worldwide

Cancer deaths are increasing in both economically developed and developing countries. Globally, lung cancer is the most common cancer type, followed by breast cancer, stomach cancer, colon cancer and rectum cancer (The Global Burden of Disease: 2004, 2008). Cancer incidence differs according to some factors such as age, sex, dietary habits, physical activities and the place of people. China and North America are the places that is most commonly seen new cancer cases. The majority of cancers such developed countries are those associated with Western lifestyle and related cancers such as colon and rectum, breast and prostate. In developing countries liver, stomach and esophagus cancers are commonly seen. Incidence rate of cancer cases in Mediterranean countries is lower than in Western countries. In most of the cancers such as lung and breast, North America and European countries come first while Asia and some African regions are low risk countries (Parkin *et al*., 2005 ; Jemal *et al*., 2011).

For colon and rectum cancers, incidence rates are also high in North America, Australia/New Zealand, Europe and in men especially Japan. Stomach cancer high risk areas include China, Japan, and Eastern Europe. Although as a high risk country, survival for stomach cancer is good in Japan. This situation is based on the presence of advanced diagnostic methods such as mass screening by photofluoroscopy. Survival is also high in North America due to the early diagnosis and endoscopic examinations performed for gastric disorders. Risk is increased for stomach cancer by dietary habits such as salted preserved foods, fatty acid composition of foods, consumption pattern of meat and is decreased by high consumption of fruit, vegetable, fiber, and high and regular intake of olive products. Colon and rectum cancer risk is associated with physical inactivity, obesity, and dietary habits (Parkin *et al*., 2005). Figure 1.1 shows

the incidence and mortality rates of cancers in developed and developing countries both in men and women.

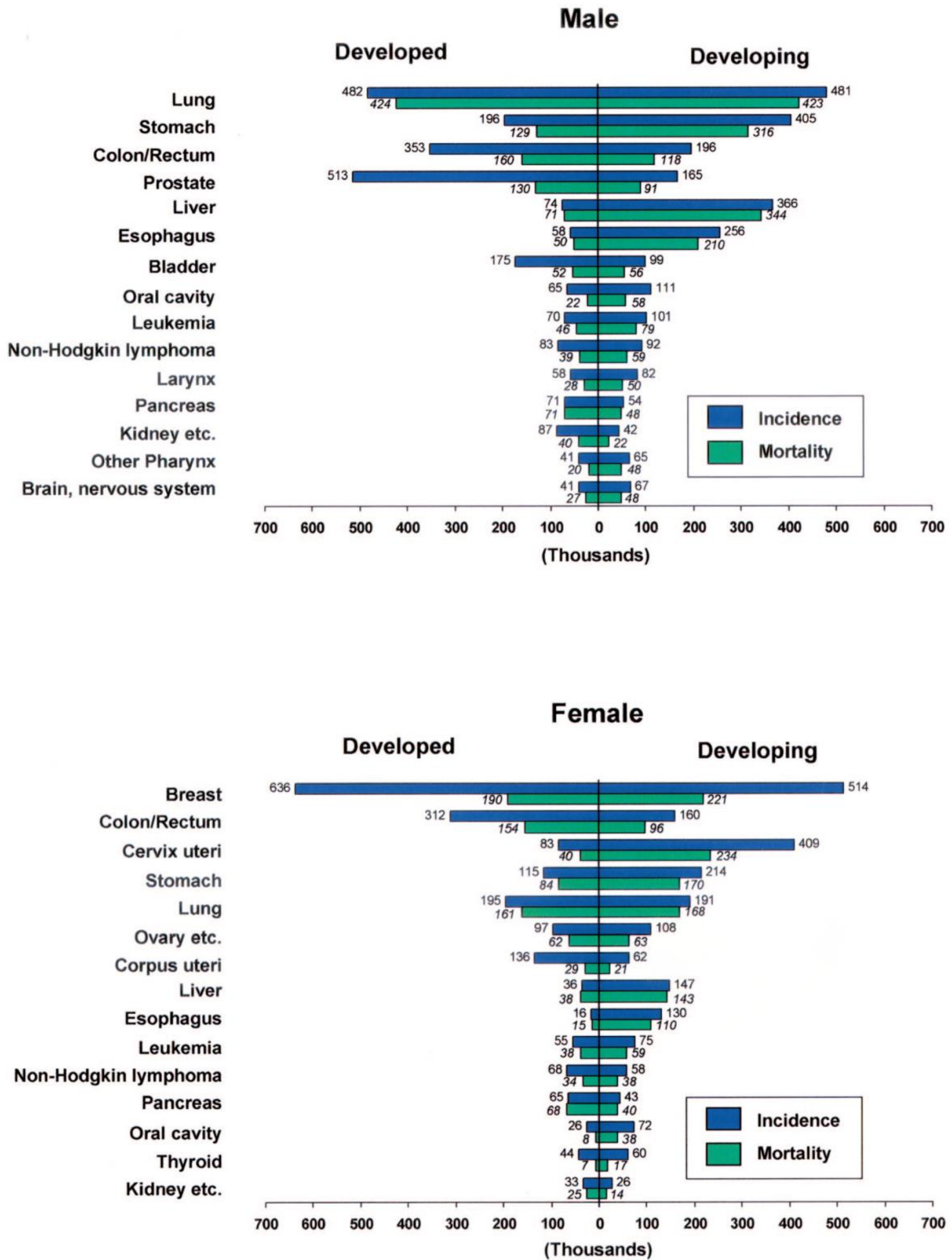


Fig 1.1 Numbers of new cancer cases and deaths in 2002 for thousands in developed and developing countries (Parkin *et al*., 2002).

1.2 CAUSES OF CANCER

Cancer incidence rates continue to increase in both developed and developing countries as a result of cancer causing behaviors such as smoking, Western type diet, environmental conditions and infections result in cancer. Both genetic and non-genetic risk factors play an important role in cancer development. Genetic susceptibility, chemical and physical agents, radiation and some family members of RNA viruses can cause the development of malignant tumors. Also changes in cancer incidence at different populations show that, environmental and lifestyle exposures are major determinants of human cancer risk (Parkin, 2004; Lichtenstein *et al*., 2000).

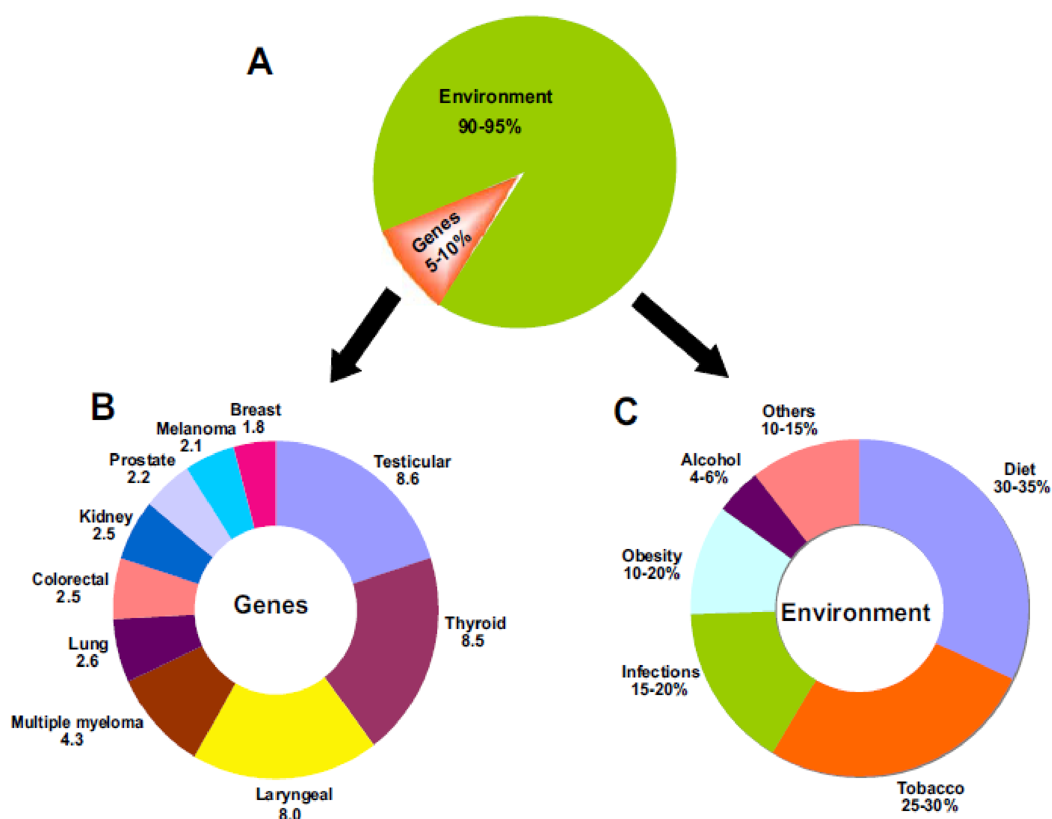


Figure 1.2 The role of environmental conditions and genes in the cancer progression (Anand *et al.*, 2008)

According to the studies not only our genes but also environmental and lifestyle factors are identical for most chronic diseases. Figure 1.2 demonstrates that environmental conditions are important factors for cancer progression as well as hereditary factors. All cancers start with multiple mutations in the cell and the main

sources of these mutations are environmental factors. Thus, lifestyle and environmental conditions account for 90-95% of chronic illnesses.

Although hereditary factors cannot be changed, environmental factors and lifestyle can be modified for prevention of cancer. As an example for modified lifestyle, tobacco usage can be given. The rate of tobacco smoking in developing countries increase in recent years. And the altering effect of smoking in many cellular signaling pathways and relation between cigarette smoke and inflammation are known (Anand *et al.*, 2008). As a specific example, tobacco smoke can induce activation of NFκB which is a transcription factor and an inflammatory marker and all of the inflammatory gene products are regulated by NFκB. Consumption of fruit and vegetables, resulting in taking of several natural phytochemicals, can inhibit the NFκB activation induced by some carcinogens found in tobacco (Ichikawa *et al*., 2007).

As serving a healthy or unhealthy lifestyle, dietary factors are thought to be the second preventable cause of cancer after tobacco and it is accounted for about 30% of cancers. There have been lots of investigations about the association of dietary habits and cancer in various populations in developing and developed countries about the intake incidence and type of fat, meat, consumption of fruit, vegetables and alcohol (Key *et al*., 2002). Populations who have diets with high intake of animal products, fat and sugar, have increased risk of oesophagus, colorectum, breast, endometrium and prostate cancers. High alcohol consumption cause cancers of the oral cavity, pharnyx, larynx, oesophagus and of course liver. On the other hand regular and high consumption of fruit and vegetables, cause lower risk for some types of cancers especially gastrointestinal (GI) tract cancers (Key *et al*., 2002). People's dietary habits depend on their living country thus, changing lifestyle affects the incidence risk of cancer and other degenerative diseases. Unhealthy lifestyle with reduction in physical activity can also trigger this risk. Table 1.1 refers us the possible effects of dietary factors and other risk factors on some types of cancers.

Table 1.1 Dietary risk factors, probable dietary protective factors, and other risk factors for the common cancers (Key *et al.,* 2002 with permission).

Cancer	Dietary and diet related risk factors	Dietary protective factors	Other risk factors
Oral cavity, Pharynx, Oesophagus	Alcohol, Very hot drinks, obesity, Chinese style salted fish	Fruit and vegetables	Smoking
Stomach	Probably high intake of salt preserved foods and salt	Fruit and vegetables	Infection by *Helicobacter pylori*
Colorectum	Obesity, possibly red and processed meat	Fruit and vegetables	Sedentary lifestyle
Liver	High alcohol intake, foods contaminated with aflatoxins	None established	Hepatitis viruses
Pancreas	None established	None established	Smoking
Larynx	Alcohol	None established	Smoking
Lung	None established	Fruit and vegetables	Smoking
Breast	Obesity after menopause, Alcohol	None established	Reproductive and hormonal factors
Endometrium	Obesity	None established	Low parity
Cervix	None established	None established	Human papillomavirus
Prostate	None established	None established	None established
Kidney	Obesity	None established	None established

As seen in the table 1.1, smoking is an important risk factor for most of the human cancers, mainly for lung cancer and other upper aero-digestive tract. It accounts for about 25-30% of all deaths from cancer and 87% of deaths from lung cancer (Anand *et al.*, 2008). Because tobacco smoke contains at least 50 carcinogens act as mutagens such as polycyclic aromatic hydrocarbons (PAH) and nitrosamins.

Alcohol act as a co-carcinogen which activate or enhance the activation of carcinogens. But overall its action is not clear yet. Specifically metabolizing of ethanol can cause generation of free radicals result in binding of DNA and proteins and causing hyper-proliferation (Anand *et al.*, 2008).

Researches have shown that dietary factors are linked to various types of cancers and may account of 30-35% of cancer deaths. Figure 1.3 shows the relation between dietary factors and various types of cancers (Anand *et al.*, 2008).

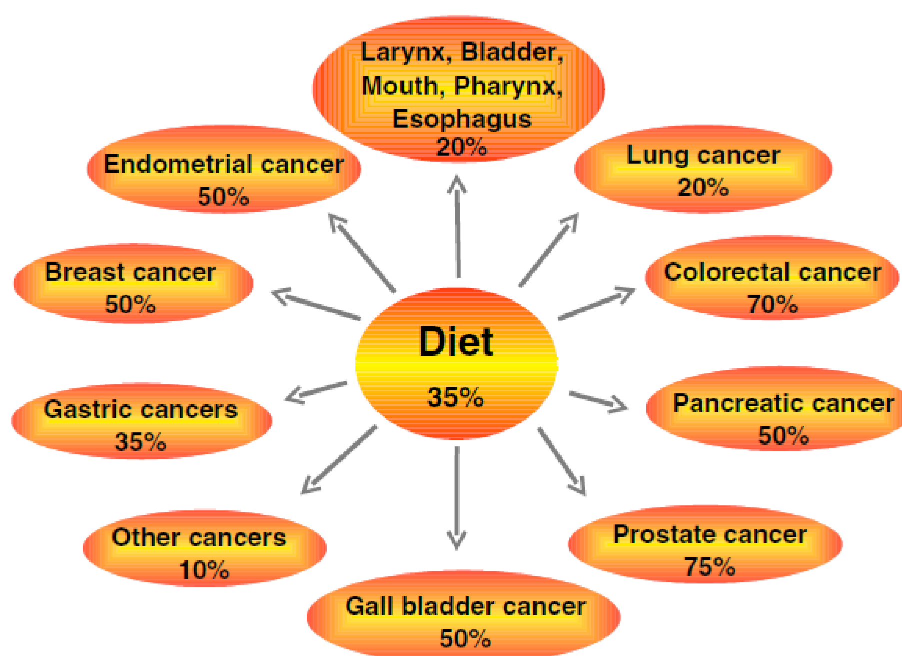


Figure 1.3 Cancer deaths associated with diet (Anand *et al.*,2008)

As seen in the figure 1.3 diet is an effective factor for colorectal cancer progression (70%) and other types of cancers.

In the light of these studies it can be said that in food intake imbalance may be the risk factor for some cancers. Rich in calories and animal fat (means rich in poly-unsaturated fatty acids (PUFA)) and low in fiber, vegetables and fruits can increase oxidative stress in the cell and cause formation of mutagenic free radicals which are harmful for the organelles of the cell. Diets rich in fiber, vegetable and fruit serve a healthy lifestyle by protecting cells from the effects of mutagenic free radicals with their natural antioxidant contents (Irigaray *et al*., 2007).

As a result, a cleaner environment and modified lifestyle would be helpful in prevention of cancer and other related diseases.

1.3. The Mediterranean Diet and Health Benefit Effects

Ongoing studies associated with diet and cancer revolves around the Mediterranean style diet which represents a healthy lifestyle with its high and regular consumption of fruits, vegetables, fiber, fish and olive oil (Trichopoulou *et al*., 2000).

Mediterranean style diet has a great potential to prevent the diseases associated with oxidative damage such as cardiovascular diseases and cancer. Olive oil is the major component of Mediterranean diet and consumption rate of olive oil in Mediterranean countries is 25-50mL per day (Corono *et al*., 2009). We can calculate the intake rate of phenols from the concentration of 180mg/kg phenolic content in 50g. of olive oil consumption per day. This refers the 9mg phenolic intake per day in this consumption conditions (Vissers *et al*., 2004). In addition to this, Mediterranean diet is characterized by the consumption of legumes, grapes and their derivative products (Hashim *et al*., 2005).

Overall the traditional Mediterranean diet is thought to have eight major properties;

1. High monounsaturated:saturated fat ratio,
2. Limited ethanol consumption,
3. High consumption of legumes,
4. High consumption of cereals,
5. High consumption of fruits,
6. High consumption of vegetables,
7. Low consumption of meat and meat products,
8. Moderate consumption of milk and dairy products (Trichopoulou *et al*., 2000).

The health beneficial effects of Mediterranean diet is attributed to not only high ratio between unsaturated and saturated fatty acids but also to the phenolic contents and antioxidant potential of olive oil.

Olive and olive oil which are the main food group in the Mediterranean diet explain the high ratio of monounsaturated fatty acids to saturated fatty acids. Olive oil contains a large number of substances such as monounsaturated fatty acids (MUFA)

(eg.oleic acid), hydrocarbon squalene, tocopherols, aroma component, lignans, secoiridoids and phenolic compounds (Owen *et al*., 2000). Mainly this phenolic antioxidant content can be divided into three classes, simple phenols, secoiridoids and lignans, squalene (Owen *et al*., 2000). Hydroxytyrosol and tyrosol are simple phenolic compounds while secoiridoids oleuropein are complex compounds. Tocopherols, phenolic acids, phenolic alcohols and flavonoits are present in many fruit and vegetables but secoiridoits are found in Olearaceae family plants (Tripoli *et al*., 2005). These phenolic compounds and flavonoits have antioxidant activity via their aromatic ring structure with one or more -OH groups and the number of -OH groups and their positions are important for antioxidant activity because of forming hydrogen bound between hydroxyl groups and harmful phenoxylic radicals (Evans, 1996; Vissers *et al*., 2004). In general, studies related with the protective effects of olive oil focus on mainly three major phenolics; hydroxytyrosol, tyrosol and oleuropein. Figure 1.4 demonstrate the structural properties of phenolic compounds and number of changeable -OH groups (Vissers *et al*., 2004).

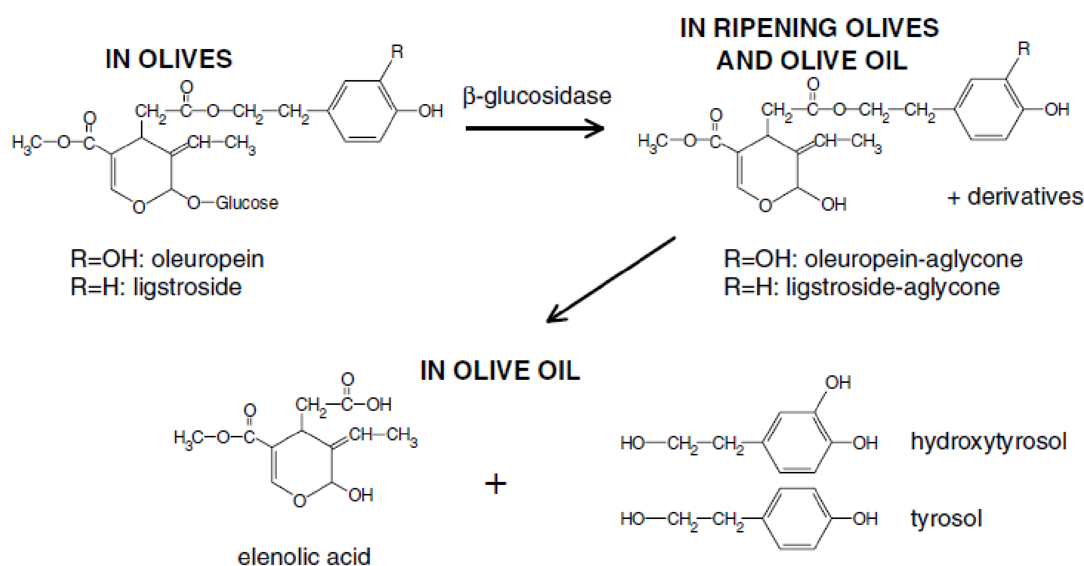


Figure 1.4 Structural properties of phenolic compounds found in olive oil (Vissers *et al.,* 2004)

1.3.1 Absorption of Polyphenols

It is essential to understand whether olive oil phenolics are absorbed in the intestine, absorption rate and distribution to the organism and possible effects on *in vivo* and *in vitro* models. There had been some *in vivo* studies but results aren't reliable.

By developing a technique called as 'an intestinal perfusion technique *in situ*' give opportunities in this area for applying both in iso-osmotic and hypotonic luminal conditions (Edgecombe, 2000). In iso-osmotic conditions oleuropein was absorbed. The absorption mechanism was not clear but it may be via transcellular transport (carrier Na^{+}-dependent glucose transporter 1) or paracellular movement. In hypotonic conditions, opening of the paracellular junctions facilitate paracellular movement and the permeability of oleuropein get higher than in iso-osmotic conditions.

Also as a polar large phenolic, oleuropein glycoside can probably use a glucose carrier. Two of these glucose carriers function by facilitated diffusion. Another of them use Na^{+}-dependent glucose transporter 1, by carrying glucose across the concentration gradient (Takata, 1996).

There are also studies on the absorption properties of hydroxytyrosol and tyrosol, performed in rat. A dose dependent plasma concentration of hydroxytyrosol was obtained in 5-10 minutes in maximum levels in the experiments (Bai *et al*., 1998).

Studies with the radiolabelled (^{14}C) hydroxytyrosol on transport kinetics performed in Caco-2 colon adenocarcinoma cells have demonstrated that passive diffusion was the reason of transport (Manna *et al*., 2000).

Also *in vivo* experiments with radiolabelled hydroxytyrosol and tyrosol which are polar phenolics were performed in rat both mouth and intravenously. In this case hydroxytyrosol was observed in the plasma at maximum levels as soon as 10 minutes after oral administration. After elimination from the plasma, excreted to the urine and bound to glucuronic acid as a free substance (D'Angelo, *et al.*, 2001). Excretion amount of hydroxytyrosol in the urine is important for understanding the absorption rate and time eagerly.

Other studies performed *in vivo* in human subjects looked for too the intestinal absorption and urinary excretion of hydroxytyrosol and tyrosol. Results were similar with previous studies, the absorption occurred in dose dependent manner and urinary excretion mostly occurred by bounding of glucuronic acid (Visioli *et al*., 2000).

Findings demonstrate that ~40-95% of phenolics (especially hydroxytyrosol and tyrosol) are absorbed in humans (Vissers *et al.*, 2002).

1.3.2 The Health Benefit Effects of Polyphenols Found in Olive Oil

The reactive oxygen species (ROS) are continuously formed in the cell as a result of normal cellular processes. Excess amount of ROS production cause cellular damage and lead to the development of degenerative diseases such as atherosclerosis, cancer, diabetes and inflammatory diseases. Also studies suggest that production rate of ROS in cancerous tissues is higher than in normal tissues (Owen *et al.*, 2000). Antioxidants found in fruits and vegetables prevent food oxidation (especially lipid oxidation) by acting as ROS scavengers and protect against degenerative diseases.

As mentioned before the most important dietary antioxidants are vitamins such as ascorbic acid, tocopherols, carotenes and phenolic compounds which are found in olive oil as the major food group of Mediterranean diet.

Phenolic compounds can function as an antioxidant in various ways. The most important one is breaking the chain of reactions catalyzed by free radicals via its free radical scavenging ability. Also having low content of PUFA makes olive oil resistant to oxidative degradation.

Phenolic compounds of olive oil also have ability to interact with important enzymatic systems in the cell (Benkhalti *et al.*, 2002). Studies demonstrate that olive oil phenols have functions in upregulation of immune system by inhibiting platelet aggregation in the blood serum, reduction in pro-inflammatory agents such as thromboxane B_2 and leucotriene B_4 and inhibiting oxygen usage in neutrophils (Tripoli *et al.*, 2005).

As an important simple polyphenol, caffeic acid has cyto-protective effects on endothelial cells by blocking the increase concentration of intracellular Ca^{2+} in response to lipoprotein oxidation (Vieira *et al.*, 1998) and can have protective activity on LDL oxidation (Tripoli *et al.*, 2005).

The antioxidant potential of the oil polyphenols have been studied in erythrocytes and intestinal cells (Caco-2). Human erythrocytes are especially chosen cells because of having function to carry O_2 and the main target of H_2O_2 is hemoglobin (Hb) (Van Dyke *et al.*, 1996). Exposure of cells to the oxidative stress (treatment with H_2O_2) result in lipid peroxidation and alteration in protein structure cause haemolysis.

Under these conditions ROS are quickly removed by enzymatic or non-enzymatic activity. If excess amount of ROS is produced or less antioxidant activity occurred, oxidative damage is formed in the cell. Erythrocytes pre-treated with phenols extracted from olive oil are more resistant to lipid oxidation and haemolysis after treatment with H_2O_2 (Manna *et al*., 2002).

As mentioned before excess amount of ROS production cause cellular oxidative injuries and lead to the development of age related diseases. Especially mitochondrial membranes are more sensitive against oxidative stress because of having double bound carbon-carbon in the lipid tails of their phospholipids which exposure of free radical attack cause developing neurodegenerative diseases. According to investigations Mediterranean diet with its high MUFA content also associate with some age-related neurodegenerative diseases such as Alzheimer's disease, vascular dementia, Morbus Parkinson and cognitive decline. This effect is thought to be due to the preservation of neural membranes structural integrity by MUFA (Solfrizzi and Panza, 1999; Panza *et al*., 2004; Omar, 2010). Figure 1.5 shows us the biological activities of olive oil phenolics.

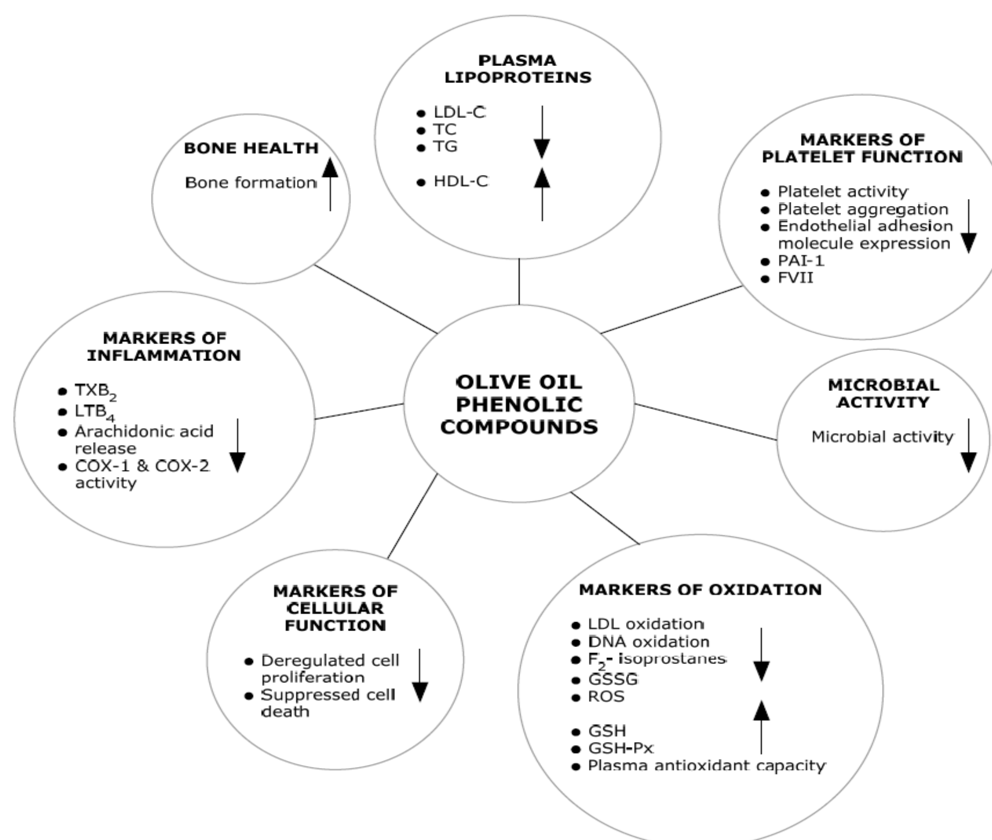


Figure 1.5 Biological activities of olive oil phenolics (Cicerale S. *et al.,* 2010)

1.3.2.1 Protective Activity of Phenolic Compounds Against Atherosclerosis

High levels of total cholesterol (TC), low density lipoprotein (LDL) and LDL oxidation are risk factors for atherosclerosis while high density lipoprotein (HDL) cholesterol is thought to have protective effects against coronary heart diseases (Cicerale *et al.*, 2010). This type of diseases are effected by plasma LDL concentration which is associated with consumption rate of saturated fatty acids and MUFA. It is thought to be low incidence of coronary heart diseases is attributed to the high consumption of MUFA (Vissers *et al.*, 2004; Katan *et al.*, 1995). Oleic acid as an olive oil phenolic inhibit lipid peroxidation. LDL oxidation stimulates carrying LDL particles by macrophages to inside the arterial wall and cause vascular damage. Olive oil phenols attach to the LDL particles in the plasma and prevent carrying by macrophages and vascular injury (Vissers, 2004).

As a preventing or triggering agent for cardiovascular diseases blood lipid profile (plasma lipoproteins) is an important factor. Studies in this area in human and animal models demonstrate that the consumption rate of olive oil is associated with increasing rate of HDL cholesterol in the blood. Also high intake of olive oil cause decreasing of triglyceride, TC and LDL levels in the plasma (Cicerale, 2010).

1.3.2.2 Anti-inflammatory Activity of Phenolic Compounds and Other Components of Olive and Olive Oil

Fatty acids might have functions in immune system cells such as changing the fatty acid composition of immune cell membrane phospholipids, this alteration may influence the synthesis of major immunoregulatory molecules such as eicosanoids and fatty acids may interact with target molecules in the cell which are important for gene expression (Escrich *et al.*, 2007). Via these effects dietary lipids can modulate the immune response. Effects of dietary PUFA on lymphocyte proliferation, natural killer cell function and expression of adhesion molecules have been investigated and findings demonstrate that PUFA suppress immune response (Hwang, 2000).

Phenolic concentration of olive oil effects the platelet aggregation (related to cardiovascular risk) in the blood serum by decreasing the pro-inflammatory agents. Increasing of platelet aggregation by plasma thromboxane B2 (TXB2) and triggering

the leukotriene B4 (LTB4) activity for directing neutrophils to the damage cells are enhanced by olive oil phenolics (Bogani *et al.*,2007).

Lipid peroxidation and free radical forming occurs as a result of lipo-oxygenase and cyclo-oxygenase activity. There are some studies suggest that the olive oil phenolic compounds have inhibitory effects against these molecules related to inflammatory process (Martinez-Dominguez, E., R. *et al.*, 2001).

1.3.2.3 Phenolic Compounds with Antimicrobial Activity

Olive oil phenolics especially oleuropein, hydroxytyrosol and tyrosol have wide range of antimicrobial activity responsible for respiratory and intestinal infections such as inhibition of the bacterial activity, preventing the development and germination of spores and acting as mycotoxin (Tripoli *et al.*, 2005; Medina *et al.*, 2006; Tunçel G., Nergiz C., 1993).

1.3.2.4 Phenolic Compounds and Prevention of Cancer

According to the epidemiological and case control studies, olive oil may be preventable for some types of cancers (Owen *et al.*, 2004). Olive oil phenolic compounds inhibit the activity of ROS, that produce during fatty acid processing and normal cellular metabolism. In general lung cancer is the most frequently cancer, seen in men followed by prostate, stomach, colon and rectum. Breast cancer and *cervix uteri* are common cancers in women beside colon and rectum, lung and stomach (Jemal *et al.*, 2011). The diverse effects of dietary polyphenols against cancer have been shown in studies such as protection from oxidative damage, influence on signaling pathways, associated with cell proliferation and apoptosis. (Escrich *et al.*, 2007; López *et al.*, 2004 ; Ramos, 2008).

Carcinogenesis consists of three critical stages; initiation, promotion and progression. Chemopreventive agents have functions in these stages such as inhibiting the initiation or arresting the promotion and progression of cancer. The high and regular consumption of olive oil is suggested to be the chemopreventive agent for cancer progression. As an interesting investigation Budiyanto *et al.*, 2000 and Ichihashi *et al.*, 2000, have shown that olive oil phenolics were effective in damaged tissues in animal models. In response to UV radiation, skin cancer initiation, progression and processing

were observed in mice, pre-treated and post-treated with extra virgin olive oil and refined olive oil (no phenolic content) in addition to mice post-treated with extra virgin olive oil. Post-treatment with extra virgin olive oil put off the initiation of cancer and reduce cancer risk while pre-treatment with extra virgin olive oil and post-treatment with refined olive oil have no inhibitory effect on cancer progression.

A recent study on different colorectal carcinogenesis demonstrated that olive extract have anti-genotoxic (reduction in DNA damage) impact, inhibitory effect on invasion and metastasis and increasing effect on barrier function (decreased barrier function resulting in formation of tumor-like polyps), with dose and time dependent manner (Gill *et al.*, 2005). Another study on HT-29 colon cancer cell line was interested in the effects of olive fruit extracts on proliferation and apoptosis. Findings demonstrated that olive fruit extract inhibits cell proliferation without cytotoxicity and induce apoptosis by intrinsic pathway of apoptosis which was determined by the detection of superoxide radicals in the mitochondria (Juan *et al.*, 2006). The protective effect of olive oil polyphenols against oxidative injury on Caco-2 cells was investigated also. In response to enzymatic oxidative stress markers protective effect of olive phenolic content was demonstrated as detecting the cell viability with different assay system (Manna *et al.*, 1997). Hydroxytyrosol as a phenolic antioxidant found in olive demonstrated anti-proliferative and apoptotic effects on HL-60 human promyelocitic cell line while no effect was observed on Caco-2 and HT-29 colon adenocarcinoma cell lines (Ragione *et al.*, 2000). Also cell cycle blocking effect of hydroxytyrosol was demonstrated in HL-60 cells. Treatment with hydroxytyrosol cause G_0 /G_1 phase cell cycle blocking and decreasing cell number in S and G_2/M phase (Fabiani *et al.*, 2002). In addition, this olive oil polyphenols have inhibitory effects on proliferation in colonic cancer cells by induction of cell cycle blocking in G_2/M phase. Downregulation of COX-2 expression which is over-expressed in colonic cells, by a mechanism involving p38 inhibition and downstream inhibition of the transcription factor CREB underlies this cell cycle blocking inhibitory effect (Corona *et al.*, 2007). Similar studies also have done with different phenolic compounds derived from olive in colon cancer models (Juan *et al.*, 2008 ; Llor *et al.*, 2003; Obied *et al.*, 2009).

Oleuropein and hydroxytyrosol which are the major phenolic compounds of olive oil, cause reduction in cell viability, inhibition of proliferation, promoting the apoptotic cell death, and blocking the G_1/S transition in MCF-7 breast cancer cell lines (Han *et al.*, 2009). Another study demonstrate the direct anti-tumor activity of

oleuropein *in vitro* in human advanced grade tumor cell lines and *in vivo* with animal studies. It was shown that oleuropein inhibits cell growth, motility and invasiveness and disrupts the actin cytoskeleton of living cells (Hamdi *et al*., 2005). Maslinic acid, a triterpene from *Olea europea* represent anti-proliferative and p53 independent apoptotic effects selectively on colon cancer cell lines HT-29 and Caco-2 whereas no effects was observed in non-tumoral mammalian intestine cell lines IEC-6 and IEC-18 (Reyes *et al*., 2006). As a first such study to date, antitumor activity of four pentacyclic triterpenes (erythrodiol, uvaol, oleanolic acid, maslinic acid) found in olive skin against MCF-7 breast cancer cell line was investigated and data suggests that these triterpenes reduce malignancy and inhibit new generation of cancer cells (Allouche *et al*., 2011). All findings are dose and chemical structure depending and support the previous studies doing with another olive polyphenols.

Studies at the level of gene expression was also done. The differentiation and apoptotic ability of hydroxytyrosol was investigated in HL-60 human promyelocytic leukemia cells. To determine whether hydroxytyrosol is able to modify the expression of proteins related with cell cycle, apoptosis and differentiation, cyclin D3, CDK6, CDKi $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ gene expressions were checked. Results show that hydroxytyrosol induce overexpression of cyclin D3, $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ and a decrease of CDK6 and these findings show the inhibitory effects on G1/S transition and differentiation of HL-60 cells into monocytes and granulocytes (Corona *et al*., 2007). Another study indicate that hydroxytyrosol induce cell cycle block by inhibiting the ERK1/2 phosphorylation and downstream inhibition of cyclin D1, rather than by the inhibition of p38 phosphorylation/activation and COX-2 expression (Corona *et al*., 2009).

A recent study examined the effects of methanol extract from olive drupes, on cell proliferation, apoptosis and inflammation at protein and mRNA level through the regulation of IL-8 and ICAM-1 in AGS stomach adenocarcinoma cell line. Olive extract inhibits AGS cell proliferation and induce apoptosis in a dose dependent manner. In addition to this, extract treatment cause downregulation of IL-8 and ICAM-1 which are highly expressed in inflammatory conditions in the stomach (Kountouri *et al*., 2009).

CHAPTER 2

MATERIALS & METHODS

2.1 CELL CULTURE

GI tract is the the major site that is thought to be expose directly to olive oil polyphenols. Thus, gastric adenocarcinoma and colon adenocarcinoma cell lines were chosen for our *in vitro* studies. AGS, HGC-27, 23132/87 and Caco-2 cells were our GI tract cells while human mesenchymal stem cells (hMSCs) were used for analyzing the effects of olive extracts on healthy human cells.

2.1.1 Thawing of GI Tract Cell Lines (AGS, 23132/87, HGC-27 and Caco-2)

Before thawing process, 10 ml of pre-warmed Dulbecco's Modified Eagle's Medium with low glucose (DMEM-LG, Gibco) at 37ºC was transferred to the 15 ml falcon tubes. Then cryovial tubes were taken from the liquid nitrogen tank (-196ºC), transferred into the 37ºC water bath and shaked gently until melting. Liquid content in the cryovial was transferred to the 15 ml falcon tube containing medium (DMEM) as quickly as possible and centrifuged at 2000 rpm for 3 min. The supernatant was discarded to get rid of DMSO (Dimethyl Sulfoxide, Applichem) and other unwanted cellular products, leaving pellet in medium. Pellet was resuspended by finger mixing. Then cells were seeded into a 25 cm^2 tissue culture flask (Greiner) in 10 ml DMEM including 20% Fetal Bovine Serum (FBS, Biochrom) and 1% Penicillin/Streptomycin (Biochrom). Cells were incubated in 37°C, 5% CO_2 incubator and the screw cap of the flask was kept loose in order to allow circulation of CO_2 into the flask and the next day medium was refreshed in order to get rid of dead cells.

2.1.2 Subculture of Cells

After seeding of cells into culture flasks, cells attached to the surface of flasks and started to proliferate and colony formation. When cells became 80-90% confluence they were subcultured. DMEM, FBS, Phosphate Buffered Saline (PBS, Biochrom) and trypsin were prewarmed to 37°C at water bath. Medium in the flask was removed by a sterile pipette and then cells were washed with 5 ml of PBS with calcium and magnesium in the flask. After removal of PBS, cells were trypsinized with 4 ml of pre-warmed 0.25% Trypsin/EDTA (Gibco) at 37 ^{o}C in the incubator for 5-10 min. Then cells were observed under inverted microscope. If most of the cells were detached from the surface of the flask, 1 ml of FBS was added to the flask to inactivate the activity of trypsin. The cells in the flask with trypsin and FBS were transferred into a new 15 ml falcon tube and centrifuged at 2000 rpm for 3 min at room temperature. After centrifugation, supernatant was discarded by leaving about 0.5 ml of the cell suspension at the bottom of the tube. Pellet was finger mixed and volume was up to 10 ml with DMEM medium in order to remove the remaining any trypsin. Centrifugation step was repeated once more and then cells were counted in hemocytometer. After counting, cells were seeded at a density of 1×10^{4} cells/cm^{2} with DMEM containing 10% FBS for expansion. Subculture of cells was repeated at about 3-4 days intervals according to the cell types and doubling times.

2.1.3 Freezing of GI Tract Cells

After subculture, cells were resuspended in FBS at density of 1-2x10^{6} cells/ml. Cryovial tubes were placed on ice and 950 µl cell suspension was transferred into each tube. 50 µl DMSO (Dimethyl Sulfoxide, Applichem) was added into each tube drop by drop and after each drop cell suspension was mixed by pipetting. Tubes were left at -20°C for 1 h and then kept at -80 ^{o}C overnight . The next day cryovial tubes were transferred to the liquid nitrogen tank (-196 ^{o}C) for long term preservation.

2.1.4 Seeding and Subculture of hMSCs

Bone marrow derived hMSCs were obtained from healthy volunteer donors and all participants signed an informed written consent approved by Ethics Committee of Fatih University Medical School. hMSCs were seeded at a density of 1500 cells/cm^2 with 10% hMSCs qualified FBS containing DMEM for expansion. Subculture of cells was repeated at about 5-6 days intervals and medium was refreshed once in between two passages.

2.2 DETERMINATION OF OPTIMUM CELL SEEDING NUMBERS AND EXTRACT CONCENTRATIONS

2.2.1 Determination of Optimum Cell Seeding Number

First of all growth curve analysis was done for determining the optimum cell growth at 24 h for extract treatment. Real time cell electronic sensing system (RT-CES) known as xCELLigence (Roche), was used to determine the optimum cell seeding number to the wells and the optimum time point for addition of extracts for this process. This system monitors cellular events in real time with incorporation of labels by measuring the cell-electrode impedance across interdigitated microelectrodes integrated on the bottom of tissue culture E-plates. The impedance measurement provides quantitative information about the biological status of cells, including cell number, viability and morphology. Figure 2.1 shows the mechanism of RT-CES system. The E-Plates are integrated with interdigitated gold microelectrodes. At time zero (t_0), the baseline impedance (Z_0) represents the impedance of the gold microelectrodes in the absence of adherent cells seeded on the sensors. Attachment of cells to the sensor microelectrodes, leads to changes in impedance signal at time t_1 (Z_{cellt1}) that is directly suitable to the number of cells seeded on the sensors and is displayed as the cell index. The cell index value changes according to the time (Z_{cellt2}) and reflects the morphology, adhesion, and number of cells inside the well (Abassi *et al.*, 2009). Cells were seeded to the 96 E-plate at an initial cell number of 1x10^5 cells/well. We have done our experiments in triplicate for each cell seeding number and by serial dilutions different cell seeding numbers were tried for determining the optimum growth curve in 96 well plate.

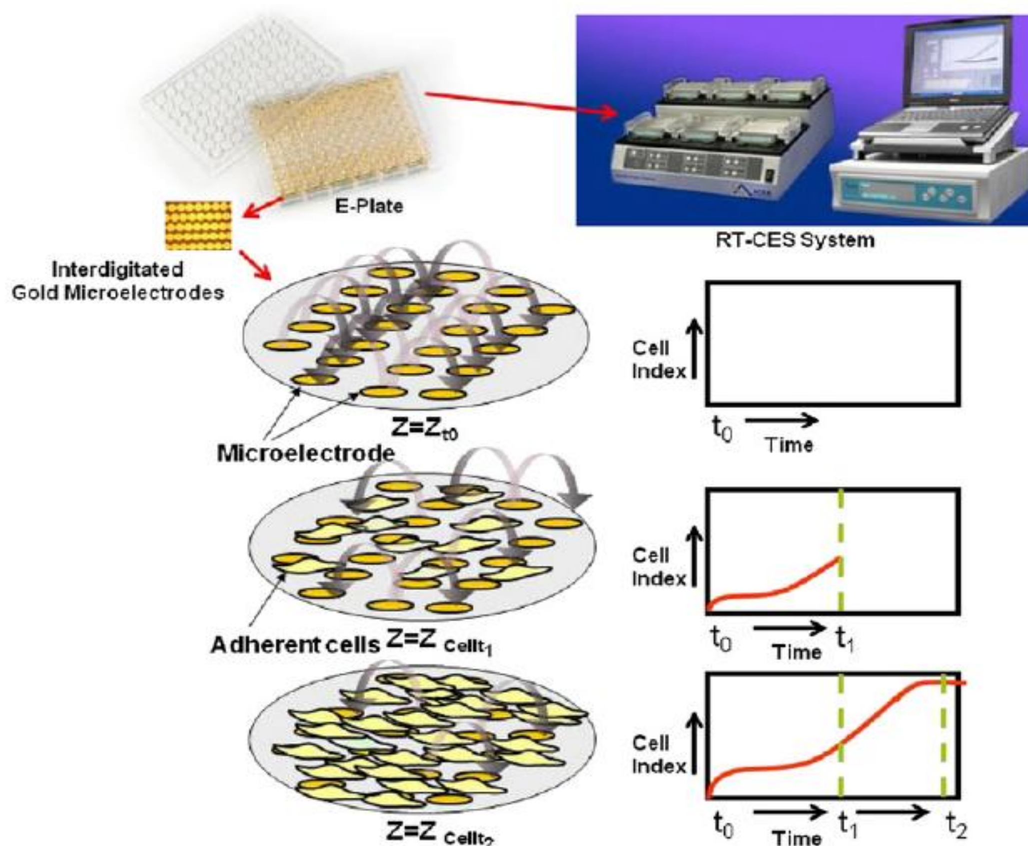


Figure 2.1 Schematic representation of microelectrodes and RT-CES system (Abassi *et al*., 2009).

2.2.2 Determination of Optimum Extract Application

Olive samples were acquired from Olive Research Institute, Bornova, İzmir/Turkey. Xcelligence system was used to determine the optimum extract concentrations. Extracts were dissolved in 50% EtOH (Ethanol). According to the EtOH contents of diluted samples, controls with EtOH and DMEM with 10% FBS were also used in addition to the controls with untreated cells. The initial concentration was $1x10^5$ µg/ml dilution with final concentration of 2.5% EtOH and other concentrations were kept on decreasing EtOH concentration by serial dilutions with a multichannel pipette. According to the cell index, extract concentrations for cells were chosen correspondingly.

2.2.3 Cell Treatment with Olive Extracts (Ayvalık, Domat, Uslu)

Cells were seeded into the wells at $1.2x10^4$ cells/well for cancer cell lines and $7x10^3$ cells/well for hMSCs. The stock concentrations of olive fruit extracts were $2x10^6$ µg/ml (2mg/ml). Four different concentrations were used for the experiments, three of them were low concentrations while one of them was the highest concentration. Samples were diluted at 1/600, 1/300, 1/150, 1/20 and the final EtOH concentration at $1x10^5$ µg/ml did not exceed 2.5%. According to the stock concentration of olive extracts ($2x10^6$ µg/ml) the amount of extracts at chosen concentrations were calculated as $3.33x10^3$ µg/ml (1/600), $6.66x10^3$ µg/ml (1/300), $1.33x10^4$ µg/ml (1/150), and $1x10^5$ µg/ml (1/20).

2.3 CYTOTOXICITY ASSAY

AGS, 23132/87, HGC-27, Caco-2 and hMSC cells were seeded in triplicate at $1.2x10^4$ cells/well and $7x10^3$ cells/well for hMSCs to the 96 well plates in 100 µl medium and then pre-incubated for 24 h before the treatment. Following exposure to the determinant extract concentrations, cytotoxicity for 23132/87, Caco-2 and hMSC cells at 24, 48 and 72 h were analyzed with the lactate dehydrogenase (LDH) leakage assay (Roche), while Xcelligence system was used for AGS and HGC-27 cell lines. LDH cytotoxicity assay is based on the measurement of cytoplasmic enzyme lactate dehydrogenase (LDH), released from damaged cells into the culture supernatant. LDH is a cytoplasmic enzyme present in all cells and release quickly upon the damage of plasma membrane. In this assay an increase in the amount of dead and membrane damaged cells result an increase in the amount of LDH release in culture supernatant and indicate the cytotoxicity of extract application. Cytotoxicity assay was performed according to the manufacturer’s instructions. Afterwards, absorbance was measured at 490 nm using the ELISA reader at three different time points.

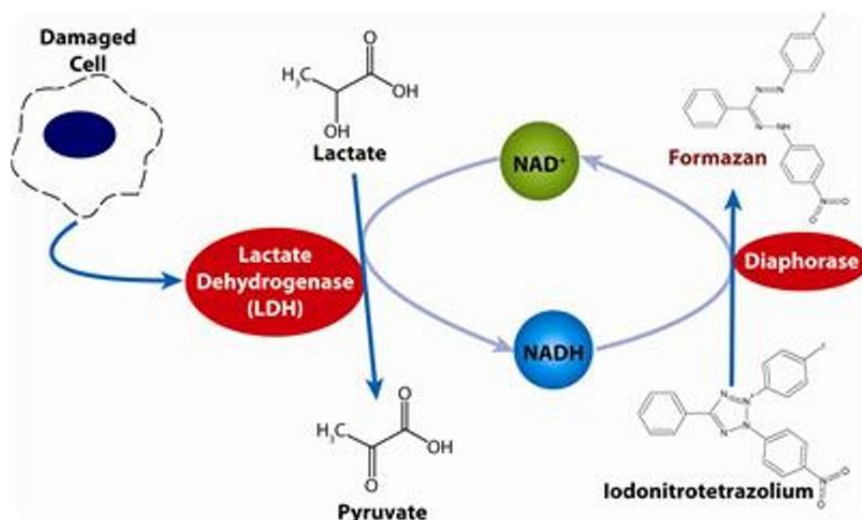


Figure 2.2 Schematic representation of LDH assay. In the first step LDH, released from damaged cells, reduce NAD^+ to $NADH^+$ by oxidation of lactate to pyruvate. Next step include transferring of $2H^+$ from $NADH^+H^+$ to the yellow tetrazolium salt by a catalyst (CytoScan ™ LDH Cytotoxicity Assay).

2.4 CELL PROLIFERATION ASSAY

AGS, 23132/87, HGC-27, Caco-2 cell lines and hMSC cells were seeded in triplicate at $1.2x10^4$ cells/well and 7000 cells/well for hMSCs to the 96 well plates in 100 µl medium and pre-incubated for 24 h before the treatment. Following exposure to the determinant extract concentrations, cell proliferation rate for 23132/87, Caco-2 and hMSCs at 24, 48 and 72 h were determined with the WST-1 cell proliferation assay (Roche). on the other hand, Xcelligence system was used for AGS and HGC-27 cell lines. WST-1 cell proliferation assay have become available for analyzing the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. The tetrazolium salts are cleaved to the formazan by the cellular enzymes. Increasing number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. Increasing enzyme activity causes formazan formation correlate with the number of metabolically active cells in the culture. WST-1 assay was performed according to the manufacturer's instructions. Afterwards, absorbance was measured at 450 nm using the ELISA reader at these time points.

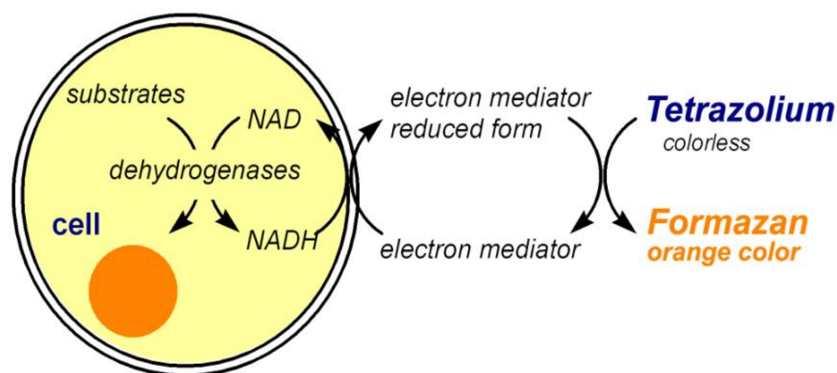


Figure 2.3 Cleavage of tetrazolium salts to the formazan (minecraftaccountsfree.com)

2.5 APOPTOSIS ASSAY (ANNEXIN-V DETECTION)

Annexin-V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). Thus, detection of cell-surface PS with annexin-V serves as a marker for apoptotic cells.

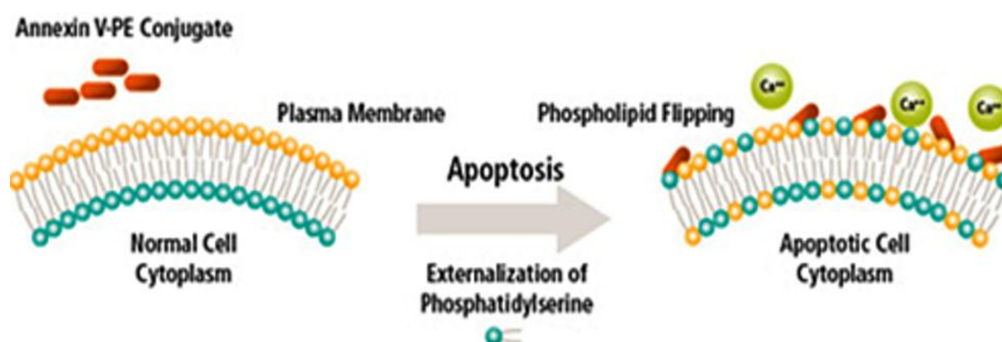


Figure 2.4 Schematic representation of Annexin-V detection (BD™ Biosciences). As an early indication of apoptosis, translocation of the membrane phospholipid phosphatdylserine (PS) from inner to the outher plasma membrane cause becoming available of binding sites on PS for Annexin-V.

Apoptotic induction potential of olive extract was detected by annexin-V staining in AGS gastric adenocarcinoma, Caco-2 colon adenocarcinoma and hMSCs after 24 h exposure of the Domat olive extract at determined concentrations as used before. In addition to the extract treated samples, negative (cells in complete medium without treatment) and positive controls (cell treat with camptothecin) were used. Camptothecin, which is a potent inhibitor of topoisomerase I, was used as an apoptosis

inducer whereas untreated negative control was used for observing the cells in normal conditions. Briefly after treatment with indicated concentrations of extract, cells were washed with PBS and resuspended in 103 µl of staining solution (including annexin-V fluorescein and buffer, containing 10mM Hepes/NaOH, 140 mM NaCl, 5mM $CaCl_2$ at pH 7.4). After incubation at room temperature DAPI and sytox green staining were performed to visualize the nucleus and necrotic cells and then cells were analyzed under the fluorescent microscope (Nikon).

2.6 DNA FRAGMENTATION ASSAY (TUNEL; Terminal Deoxynucletidyl Transferase dUTP Nick End Labeling)

DNA fragmentation is an indicator for detecting the late apoptosis. TUNEL (Roche) is an assay that is composed of an enzyme solution and labeling solution which detects the nicks (single strand breaks) in DNA and binds to free 3'-OH ends. Adding of dUTPs to 3'-OH ends by terminal deoxynucleotidyl transferase cause labeling of DNA. Fluorescently labeled ends were detected by using confocal microscopy (Leica). AGS, Caco-2 and hMSCs were used for this experiment as in annexin-V staining. $2x10^4$ µg/ml, $4x10^4$ µg/ml, $1x10^5$ µg/ml dilutions were used in addition to untreated control and control treated with camptothecin. Following treatment with olive extract, at 24h cells were harvested with trypsin, permeabilized with hypotonic solution and fixed with 3:1 and 2:1 acetic acid-methanol solution. Slides were washed with PBS for 5 min more than 2 times before incubating with TUNEL reaction mixture. After 1 h incubation at 37 °C in the incubator, DAPI (Abbott) was added to the slides and visualized.

2.7 REVERSE TRANSCRIPTION PCR

2.7.1 RNA Isolation

RNA was extracted by using RNeasy kit (Qiagen). $2.5x10^5$ cells were seeded into 60 mm in diameter tissue culture dish. Procedure was performed for AGS cell line at 24 and 48 h following extract treatments. Cells were disrupted by adding Buffer RLT. Buffer RLT was added in the appropriate volume (see Table 2.1) and vortex mixing was done for 1 min.

Table 2.1 Volumes of buffer RLT for lysing pelleted cells

Number of cells	Volume of buffer RLT (µl)
$<5x10^6$	350
$5x10^6 – 1x10^7$	600

EtOH (70%) was added 1 volume to the homogenize lysate, and mixed well by pipetting. The sample was transfered up to 700 µl, into an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15s at 8000 x g. Buffer RW1 was added in 700 µl to the RNeasy spin column and centrifuged for 15s at 8000 x g. 500 µl of buffer RPE was added into RNeasy spin column. The spin column membrane was centrifuged for 15s at 8000 x g for washing. 500 µl of buffer RPE was added into the RNeasy spin column and centrifuged for 2 min at 8000 x g. The RNeasy spin column was placed in a new 1.5 ml collection tube. 30 µl of RNase-free water was added into the spin column membrane directly and centrifuged for 1min at 8000 x g to elute the RNA. For quantifying the amount of RNA sample, tubes were set as follows, with the components as described in Table 2.2.

Quant-iT working solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. 200 µl of working solution was required for each sample and standard. Assay tubes were prepared according to the table and read in Qubit fluorometer.

Table 2.2 RNA Quantification Kit ingredients and amounts (µl) required for assay

	Standard Assay Tubes	**User Sample Assay Tubes**
Volume of working solution to add	190 µl	180-199 µl
Volume of standard to add	10 µl	—
Volume of sample to add	—	1–20 µl
Total volume in each assay tube	200 µl	200 µl

2.7.2 cDNA Synthesis

RNA was reverse transcribed to obtain cDNA by quantitect reverse transcription kit (Qiagen). Template RNA, gDNA wipeout buffer, quantiscript reverse transcriptase (RT), quantiscript RT buffer, RT primer mix, and RNase-free water were thawed at room temperature. The genomic DNA elimination reaction was prepared on ice according to Table 2.3 and mixed well and then stored on ice.

Table 2.3 Genomic DNA elimination reaction components

Component	**Volume/Reaction**	**Final Concentration**
g DNA wipeout buffer,7x	2 µl	1x
Template RNA (0.1µg)	variable	
RNase-free water	variable	
Total volume	14 µl	

Reaction mixture was incubated for 2 min at 42°C and then placed on ice. Then, the reverse-transcription master mix was prepared on ice, according to Table 2.4.

Table 2.4 Reverse-transcription reaction components

Component	Volume/Reaction	Final Concentration
Quantiscript-reverse transcriptase	1 µl	
Quantiscript-rt buffer,5x	4µl	1x
RT primer mix	1 µl	
Entire genomic DNA elimination reaction	14 µl	
Total	20 µl	

Template RNA was added (14 µl) to each tube containing reverse-transcription master mix and stored on ice. Master mix was incubated for 15 min at 42°C and 3 min at 95°C to inactivate quantiscript reverse transcriptase and stored at -20°C.

2.7.3 RT-PCR

As a constitutively active gene in all cells, the expression level of β actin was checked, in addition to topo IIα expression as a cell proliferation marker, in AGS cells treated with Domat olive extract at mRNA level with polymerase chain reaction.

Table 2.5 PCR solutions and their initial and final concentrations with final volumes.

Reagent	**Initial Concentration**	**Final Concentration**	**Final Volume**
Taq buffer	10X	1X	2.5 µl
dNTP	10 mM	0.2mM	0.5 µl
$MgCl_2$	25 mM	2 mM	2 µl
Primers (x2)	12.5 pmol/µl	12.5 pmol	2 µl
ddH2O	-	-	16.8 µl
Taq DNA polymerase	5 U/µl	1 U	0.2 µl
cDNA			1 µl
Total reaction volume			25 µl

Table 2.6 List of the Primers used for quantitative RT-PCR

Primers	**Denaturation temp. / time**	**Annealing temp./ time**	**Extension temp./time**	**Number of cycle**
Actin	94°C 30s	60°C 45s	72°C 1min	30
Topo IIα	94°C 30s	54°C 45s	72°C 1min	35

2.7.3.1 Agarose Gel Electrophoresis

2% of agarose gel was prepared by adding 2.6 g of powdered agarose into 130 ml of 0.5xTBE buffer (Fluka) and boiled until the agarose was completely dissolved in the buffer solution. 40 µl of safe DNA staining dye was added when the boiled solution began to cool down. Solution was mixed homogenously by making hand-shaking. It was directly poured into horizontal agarose gel platform and 20 well comb was placed at one side of the gel. Gel was let to solidify for at least 10 min before loading of samples.

2.7.3.2 Loading and Visualization of the Gel

12.5 µl PCR products were loaded in each slot. 3.5 µl of 100 bp DNA Ladder (Bioron) was mixed with 2 µl bromophenolblue (Fermentes). Then 5.5 µl of this mixture was put into the first slot as a molecular size marker. The gel was run at 110 V in 0.5X TBE buffer for 45 min. Bands were detected under UV transilluminator (BioRad).

2.8 STATISTICAL ANALYSIS

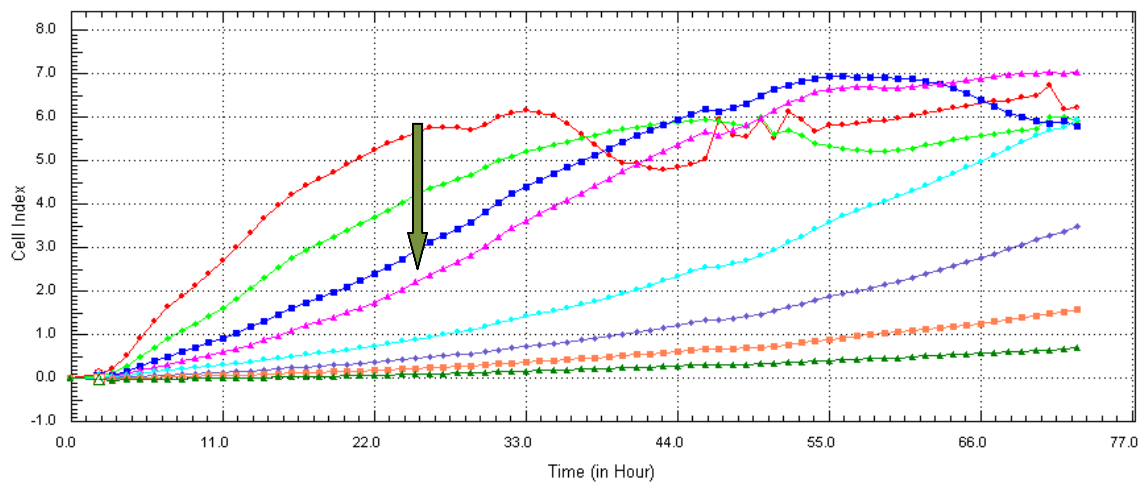
All statistical analysis was performed by using the SPSS 18 program. Data were evaluated by T-test, independent samples Mann-Whitney test and one way ANOVA. Statistical differences between time, dose and extract were analyzed. A $p < 0.05$ level was taken as significant.

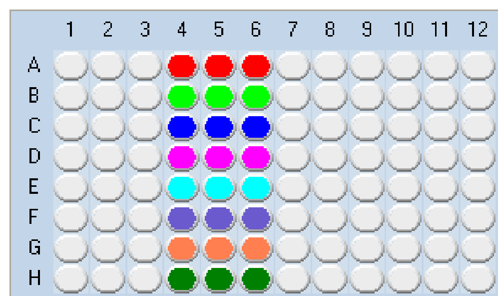
CHAPTER 3

RESULTS

3.1 Determination of Optimum Cell Seeding Number

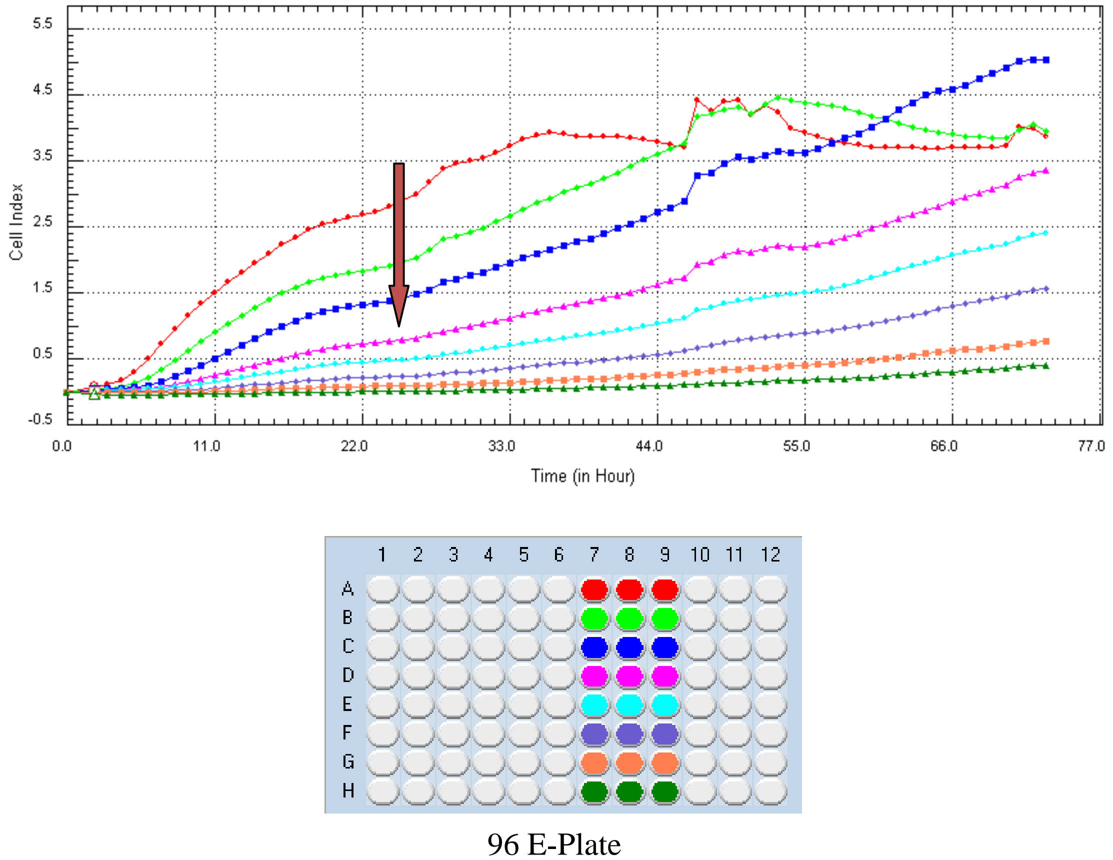
Deciding the number of cells, seeded to the wells is very important for determination of the exposure time and effective doses of olive extracts. Xcelligence system is a good advantage for the real time measurement and monitoring of cellular index. In the following figures each color refers one of the cell number trials and in all of the figures related with this experiment, cells were seeded to the 96 E-plate at decreasing numbers from $1x10^5$ to approximately $7x10^2$ cells/well, means each cell number was half of its previous one through the plate.





96 E-Plate

Figure 3.1 Determination of optimum cell number in AGS gastric adenocarcinoma cell line. AGS cells with an initial cell number of $1x10^5$ cells/well (red) in the plate and decreasing cell numbers towards down in the plate ($1x10^5$; $5x10^4$; $2.5x10^4$; $1.25x10^4$; $6.25x10^3$; $3.125x10^3$; $1.5x10^3$; $7.5x10^2$). The optimum cell number determined at 24 h was $1.2x10^4$ (pink). Cells, seeded at $1.2x10^4$ refers a stable, linear rise. Thus this number was chosen as an optimum cell number for seeding in the further experiments.



96 E-Plate

Figure 3.2 Determination of optimum cell number in HGC-27 gastric adenocarcinoma cell line. The optimum cell growth was observed in $1.2x10^4$ cells/well at 24 h, as it was determined for AGS cell line, and this cell number was decided to use in the future experiments.

These cell numbers, determined for both AGS and HGC-27 were also used for 23132/87 and Caco-2 cells. For hMSCs, $7x10^3$ cells/well were seeded at 50-60% confluency.

3.2 Determining the Optimum Concentrations of Olive Extracts

Here is the schematic representation of experimental set up, used for determining the effective concentrations of olive extracts on AGS and HGC-27 cells. As it is seen in the diagram, several extract concentrations were applied. In the following graphs each color represent a different concentration.

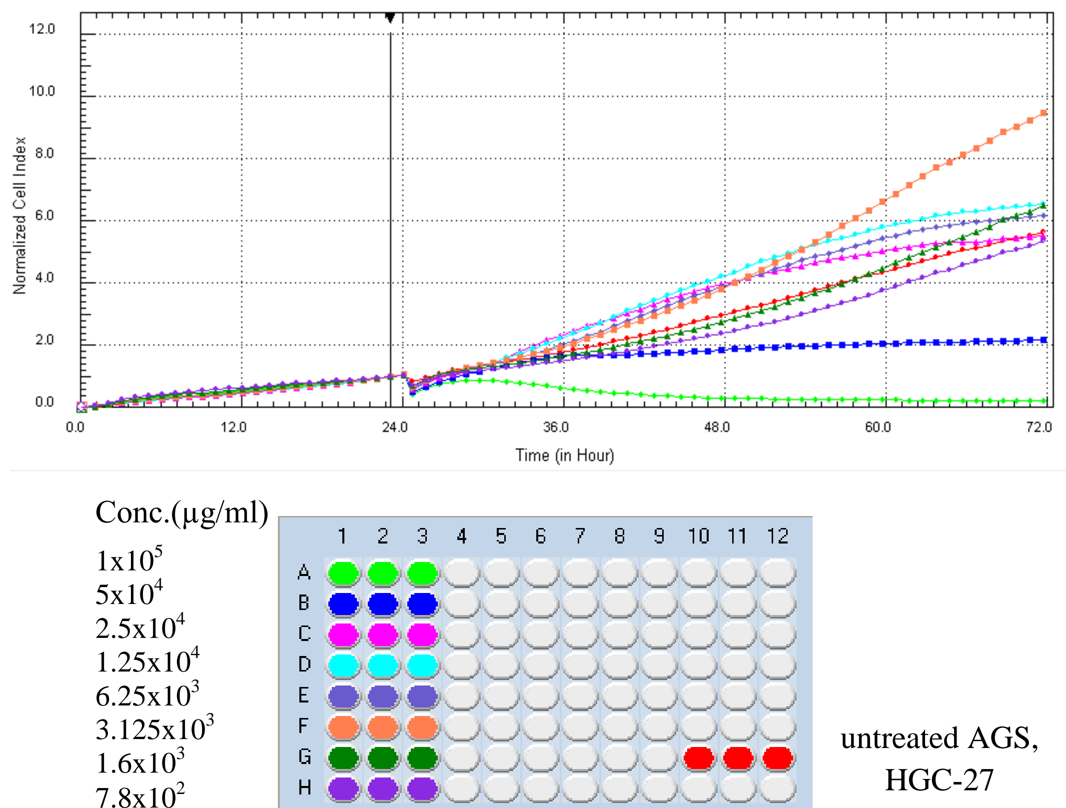


Figure 3.3 Treatment of AGS cells with Ayvalık olive extract at 24 and 48 h.

According to the graph, concentrations can be divided into three groups. Red wells were our untreated control cells. Highest concentration, $1x10^5$ µg/ml, referred to green color, have cytotoxic and anti-proliferative effects, while the next three of them, located between controls and highest dose, caused inhibition of cell proliferation, last group increased the cell growth rate which are above the control in the graph.

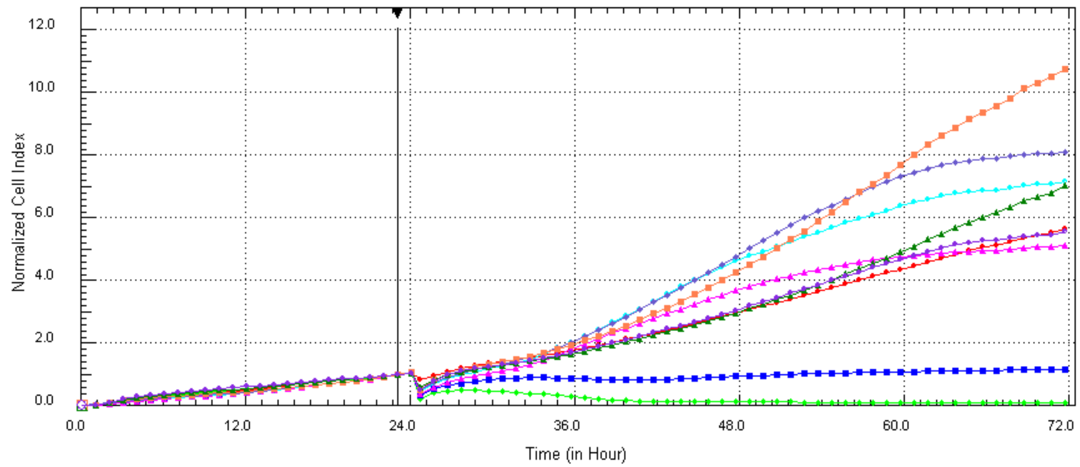


Figure 3.4 Treatment of AGS cells with Domat olive extract at 24 and 48 h.

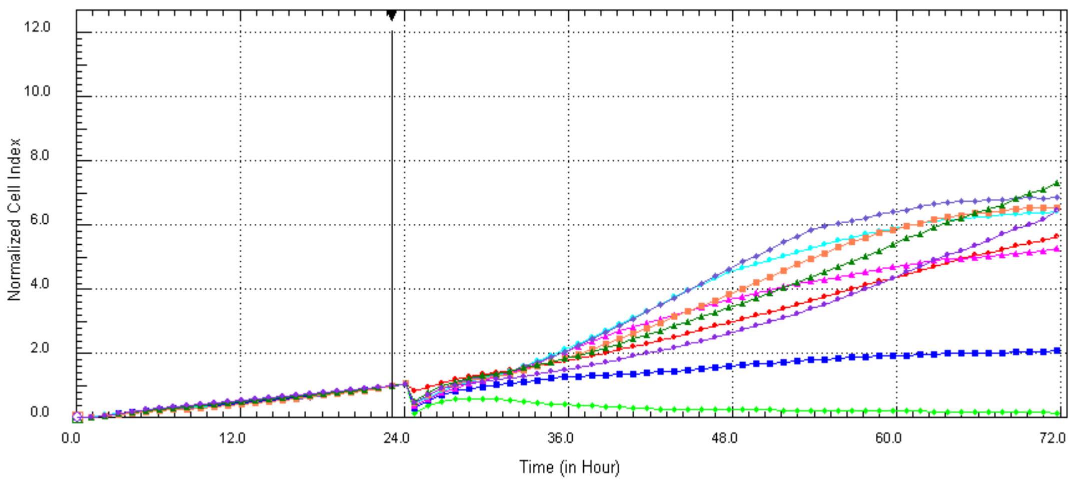


Figure 3.5 Treatment of AGS cells with Uslu olive extract at 24 and 48 h.

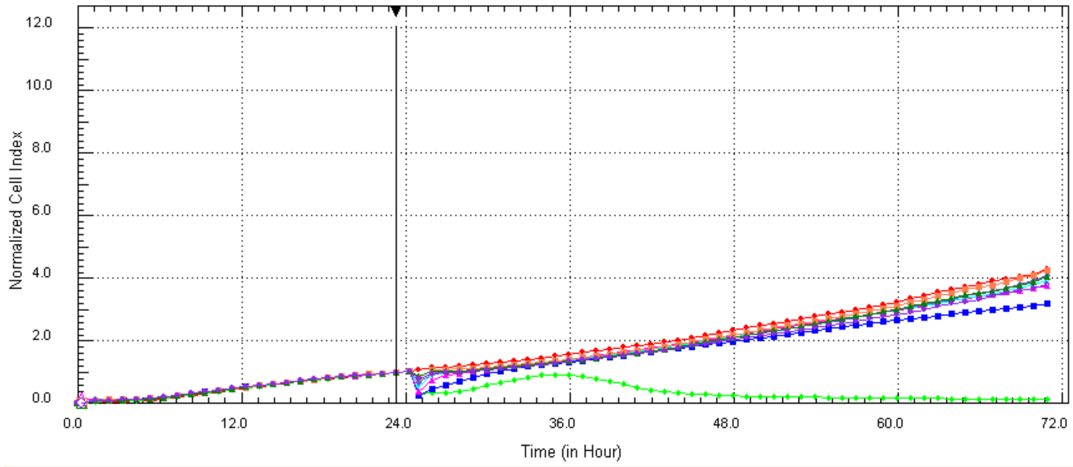


Figure 3.6 Treatment of HGC-27 cells with Ayvalık olive extract at 24 and 48 h.

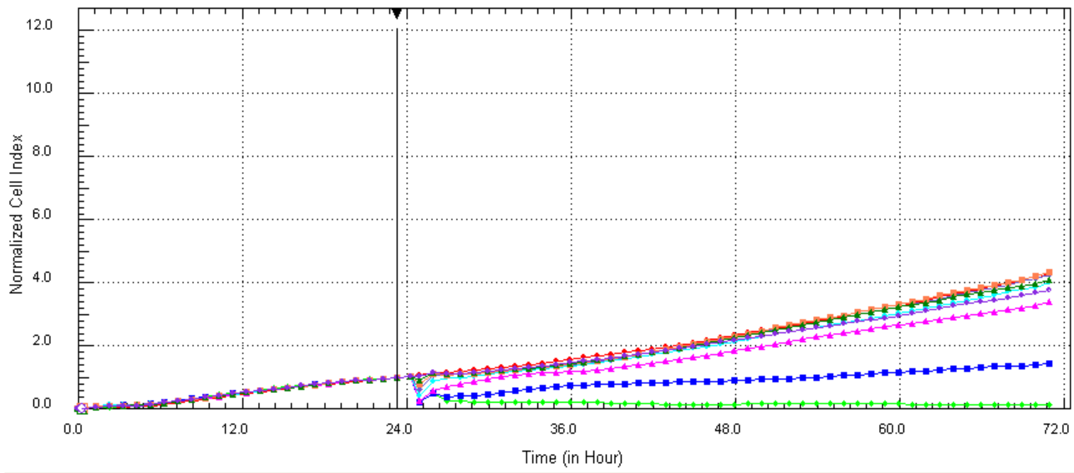


Figure 3.7 Treatment of HGC-27 cells with Domat olive extract at 24 and 48 h.

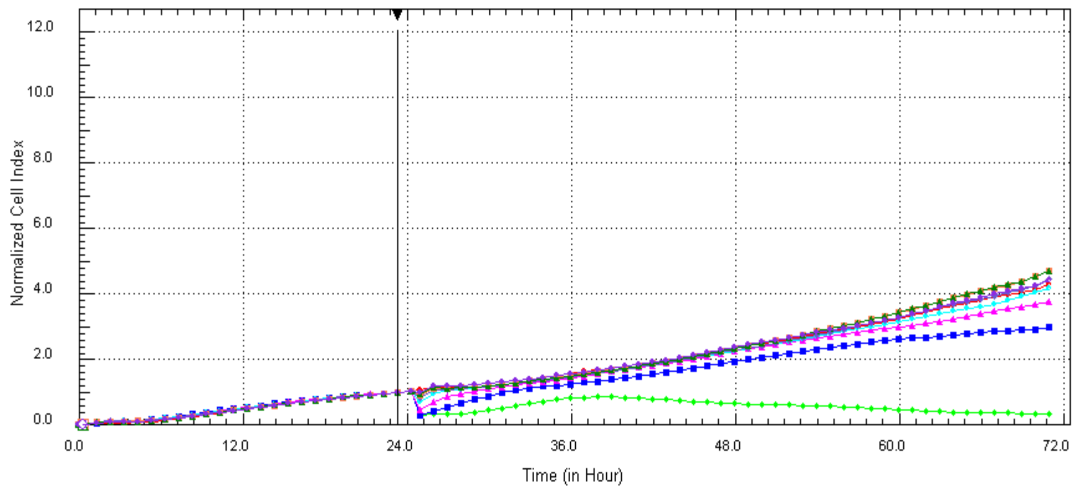


Figure 3.8 Treatment of HGC-27 cells with Uslu olive extract at 24 and 48 h.

As seen in the figures, three different types of olive extracts had similar effects on AGS cells. The same experimental conditions were used for HGC-27 cells. Graphs show the effects of olive extracts on HGC-27 cells. Similar effects as seen in AGS cells were observed in HGC-27.

Since a significant reduction in cell index was observed at high concentrations in RTCA system, to investigate whether this effect was cytotoxic or proliferative, cytotoxicity and cell proliferation assays were performed for the further experiments.

3.3 Cytotoxicity and Proliferation Results

Following treatment of cancer cells with Ayvalık, Domat and Uslu extracts at determined concentrations, cytotoxicities for 23132/87, Caco-2 and hMSCs at 24, 48 and 72 h were analyzed with the LDH leakage assay (Roche) which is based on the detection of lactate dehydrogenase enzyme in the culture media, released from damaged cells.

For detection of viable cells in response to extract treatment, WST-1 cell proliferation assay was used. We established similar experimental set up with LDH assay to compare the results.

To see whether effects of olive extracts were specific to cancer cell lines, healthy human cells, hMSCs were also treated with olive extracts by using the same experimental set up.

Following graphs represent the effects of three types of olive extracts on GI tract cell lines and hMSCs. In all of experiments, values are mean ± standard deviation (SD) of experiments. Results between untreated controls and extract treated cells were statistically significant ($p<0.05$).

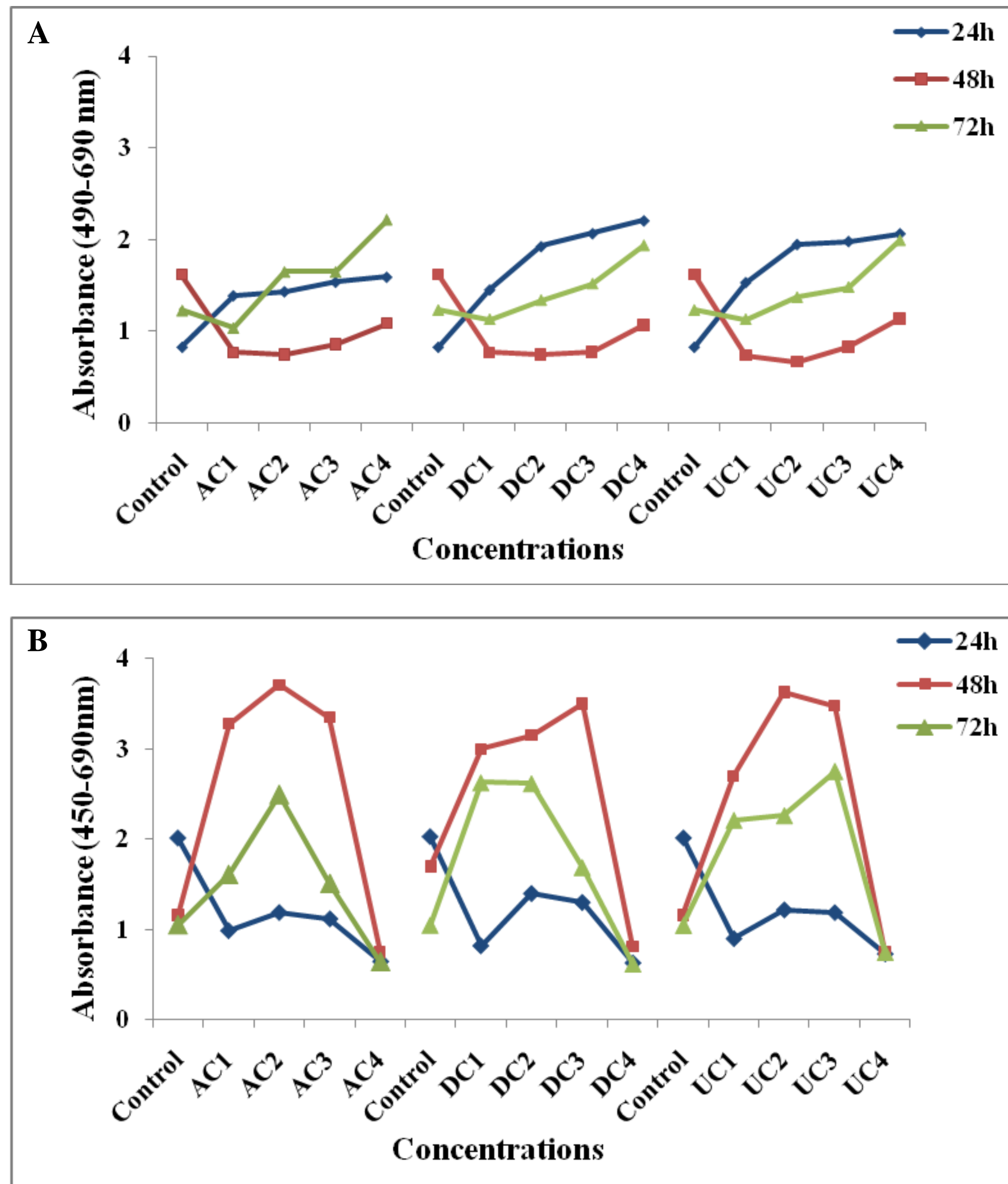


Figure 3.9 A) LDH release in 23132/87 cell line at 24, 48 and 72 h in response to three types of olive extract treatments, **B)** Cell proliferation in 23132/87 cell line treated with three types of olive extract in determined concentrations. C1; 3.33×10^3 µg/ml, C2; 6.66×10^3 µg/ml, C3; 1.33×10^4 µg/ml, C4; 1×10^5 µg/ml. Values = mean ± SD.

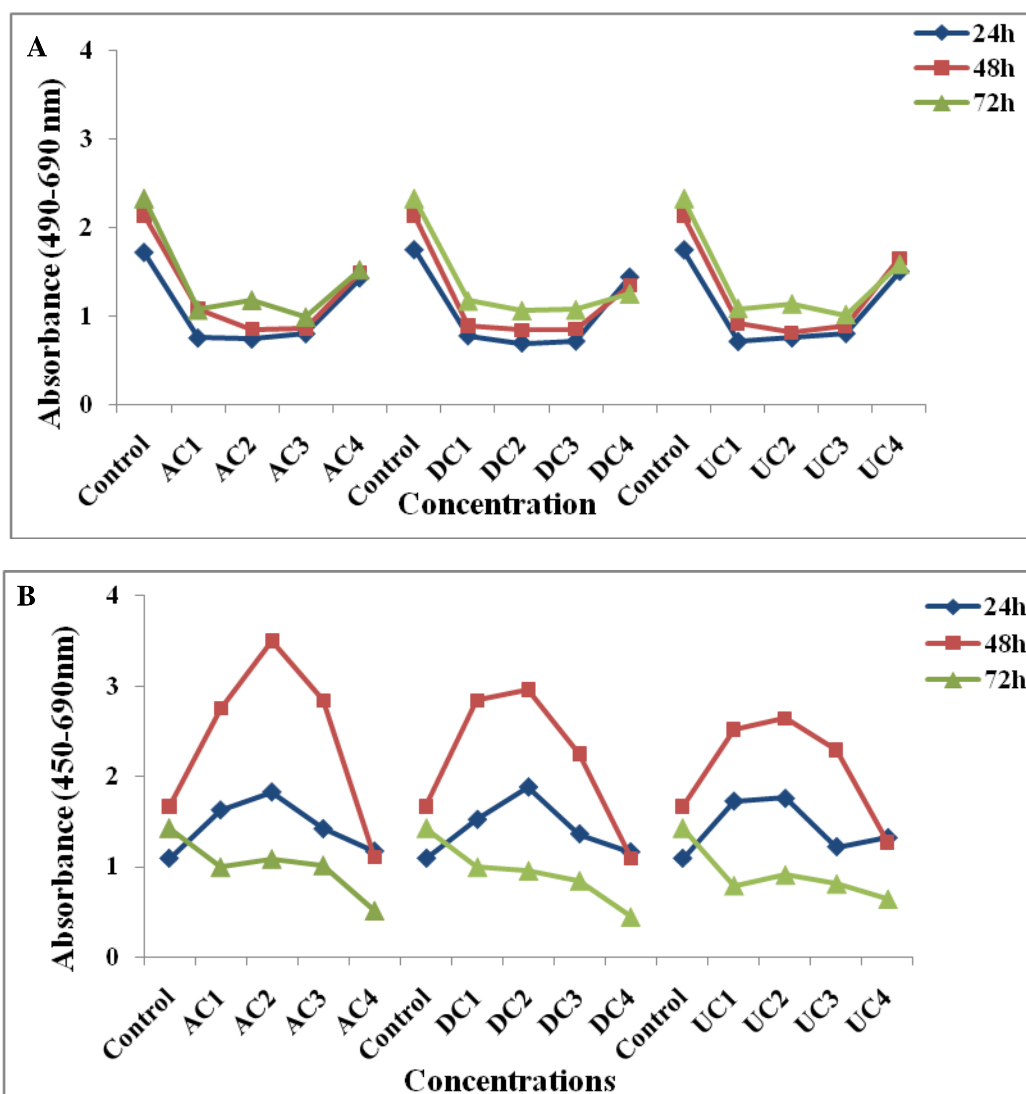


Figure 3.10 A) LDH release in Caco-2 cell line at 24, 48 and 72 h in response to three types of olive extracts treatment, **B)** Cell proliferation in Caco-2 cell line treated with three types of olive extract in determined concentrations. C1; $3.33x10^3$ µg/ml, C2; $6.66x10^3$ µg/ml, C3;$1.33x10^4$ µg/ml, C4; $1x10^5$ µg/ml. Values = mean ± SD.

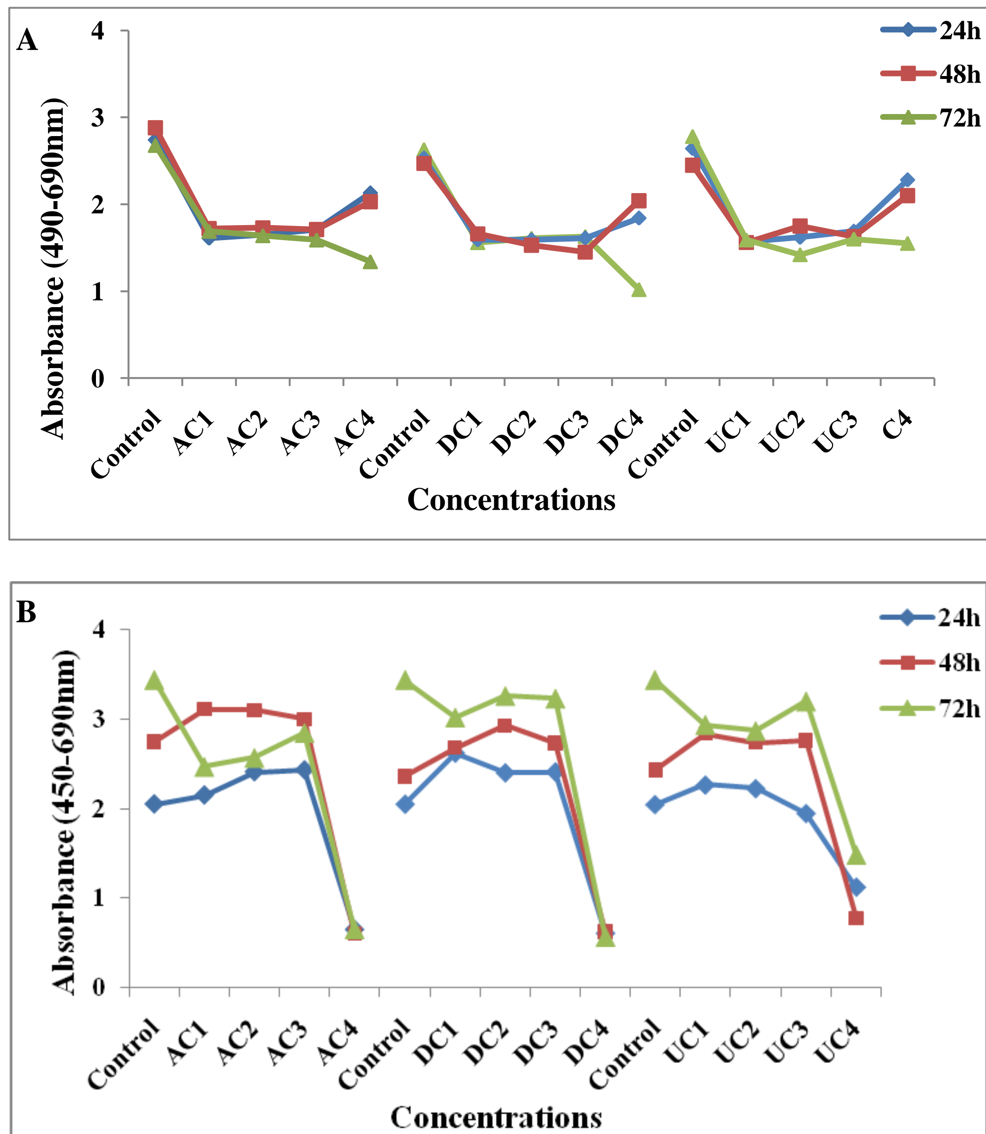


Figure 3.11 A) LDH release in hMSCs at 24, 48 and 72 h in response to three types of olive extract treatments, **B)** Cell proliferation in hMSCs treated with three types of olive extract in determined concentrations. C1; $3.33x10^3$ µg/ml, C2; $6.66x10^3$ µg/ml, C3;$1.33x10^4$ µg/ml, C4; $1x10^5$ µg/ml. Values = mean ± SD.

Table 3.1 Cytotoxic effects (LDH release) of the olive extracts on 23132/87 cell lines.

Sample and Treatment	**24h**	**48h**	**72h**
Control	0.83 ± 0.15	1.61 ± 0.16	1.23 ± 0.02
Ayvalık /C1	1.39 ± 0.1	0.75 ± 0.03	1.04 ± 0.17
Ayvalık /C2	1.43 ± 0.18	0.77 ± 0.02	1.65 ± 0.18
Ayvalık /C3	1.54 ±0	0.86 ± 0.14	1.64 ±0.09
Ayvalık /C4	1.59 ±0	1.09 ± 0.01	2.22 ± 0.11
Control	0.83 ± 0.15	1.61 ± 0.16	1.23 ± 0.02
Domat/C1	1.45 ± 0.07	0.77 ±0.02	2.22 ± 0.11
Domat /C2	1.92 ± 0.26	0.75 ± 0.04	1.13 ± 0.06
Domat /C3	2.07 ± 0.01	0.77 ± 0.008	1.52 ± 0.11
Domat /C4	2.21±0.01	1.07±0.02	1.94±0.005
Control	0.83 ± 0.15	1.61± 0.16	1.23± 0.02
Uslu/C1	1.53 ± 0.53	0.73± 0.04	1.13± 0.06
Uslu/C2	1.94 ± 0.18	0.67± 0.05	1.37± 0.07
Uslu/C3	1.97 ± 0.10	0.83± 0.05	1.48± 0.11
Uslu/C4	2.06 ± 0.19	1.14± 0.07	1.99± 0.05
Values = mean ± SD, ***p* < 0.05 versus untreated control**			

Table 3.2 Cell proliferation in 23132/87 cell lines treated with olive extracts.

Sample and Treatment	**24h**	**48h**	**72h**
Control	2.01 ± 0.006	1.16 ± 0.26	1.05 ±0.009
Ayvalık /C1	0.99 ± 0.21	3.28 ± 0.03	1.61 ± 0.03
Ayvalık /C2	1.19 ± 0.27	3.71 ± 0.22	2.53 ± 0.26
Ayvalık /C3	1.12 ± 0.05	3.35 ± 0.66	1.51 ± 0.37
Ayvalık /C4	0.65 ± 0.04	0.75 ± 0.08	0.64 ± 0.04
Control	2.03 ± 0.08	1.16 ± 0.26	1.05±0.009
Domat/C1	0.82 ± 0.35	2.98 ± 0.27	2.63 ± 0.18
Domat /C2	1.40 ± 0.06	3.15 ± 0.3	2.62 ± 0.14
Domat /C3	1.3 ± 0.12	3.5 ± 0.2	1.69 ± 0.19
Domat /C4	0.63± 0.1	0.81±0.02	0.62±0.02
Control	2.03 ± 0.08	1.16 ± 0.26	1.05 ±0.009
Uslu/C1	0.89 ± 0.36	2.7 ± 0.28	2.21 ± 0.64
Uslu/C2	1.22 ± 0.26	3.6 ± 0.19	2.26 ± 0.75
Uslu/C3	1.19 ± 0.09	3.48 ± 0.24	2.75 ± 0.54
Uslu/C4	0.73 ± 0.12	0.75 ± 0.06	0.75±0.006
Values = mean ± SD, ***p*<0.05 versus untreated control**			

Table 3.3 Cytotoxic effects (LDH release) of the olive extracts on Caco-2 cell lines.

Sample and Treatment	24h	48h	72h
Control	1.71 ± 0.15	2.1 ± 0.02	2.32 ± 0.08
Ayvalık /C1	0.75 ± 0.02	1.08 ± 0.37	1.07 ± 0.01
Ayvalık /C2	0.74 ± 0.04	0.85 ± 0.11	1.18 ± 0.25
Ayvalık /C3	0.8 ± 0.09	0.86 ± 0.04	0.99 ± 0.04
Ayvalık /C4	1.43 ± 0.07	1.48 ± 0.06	1.52 ± 0.14
Control	1.75 ± 0.11	2.13 ± 0.02	2.32 ± 0.08
Domat/C1	0.77 ± 0.08	0.9 ± 0.08	1.17 ± 0.11
Domat /C2	0.69 ± 0.01	0.83 ± 0.06	1.06 ± 0.06
Domat /C3	0.71 ± 0.09	0.85 ± 0.05	1.07 ± 0.008
Domat /C4	1.53± 0.02	1.34± 0.08	1.25± 0.01
Control	1.44 ± 0.07	1.34 ± 0.08	1.25 ± 0.01
Uslu/C1	1.74 ± 0.05	2.13 ± 0.02	2.32 ± 0.08
Uslu/C2	0.71 ± 0.05	0.91 ± 0.06	1.08 ± 0.04
Uslu/C3	0.75 ± 0.05	0.8 ± 0.04	1.13 ± 0.2
Uslu/C4	1.5 ± 0.14	1.65 ± 0.12	1.58 ± 0.24
Values = mean ± SD, *p* < 0.05 versus untreated control			

Table 3.4 Cell proliferation in Caco-2 cell lines treated with olive extracts.

Sample and Treatment	24h	48h	72h
Control	1.09 ± 0.04	1.67 ± 0.1	1.43 ± 0.02
Ayvalık /C1	1.63 ± 0.17	2.75± 0.4	0.95 ± 0.12
Ayvalık /C2	1.83 ± 0.03	3.5 ± 0.4	1.09 ± 0.06
Ayvalık /C3	1.42 ± 0.07	2.84 ± 0.7	1.02 ± 0.17
Ayvalık /C4	1.17 ± 0.004	1.11 ± 0.01	0.52 ± 0.007
Control	1.09 ± 0.04	1.67 ± 0.1	1.43 ± 0.02
Domat/C1	1.52 ± 0.35	2.84 ± 0.4	0.96 ± 0.3
Domat /C2	1.88 ± 0.07	2.96 ± 0.3	0.96 ± 0.2
Domat /C3	1.36 ± 0.08	2.25 ± 0.24	0.85 ± 0.31
Domat /C4	1.16 ± 0.01	1.09 ± 0.008	0.45 ± 0.01
Control	1.09 ± 0.04	1.67 ± 0.1	1.43 ± 0.02
Uslu/C1	1.73 ± 0.38	2.52 ± 0.1	0.79 ± 0.11
Uslu/C2	1.76 ± 0.2	2.64± 0.3	0.91 ± 0.2
Uslu/C3	1.22 ± 0.006	2.29 ±0.2	0.81 ± 0.09
Uslu/C4	1.32± 0.15	1.27 ± 0.13	0.64 ± 0.01
Values = mean ± SD, ***p* < 0.05 versus untreated control**			

Table 3.5 Cytotoxic effects (LDH release) of the olive extracts on hMSCs.

Sample and Treatment	**24h**	**48h**	**72h**
Control	2.74 ±0.08	2.88 ± 0.11	2.68 ± 0.06
Ayvalık /C1	1.61 ± 0.008	1.72 ±0.13	1.69 ± 0.1
Ayvalık /C2	1.65 ± 0.004	1.73 ± 0.19	1.64 ± 0.1
Ayvalık /C3	1.71 ± 0.04	1.71 ± 0.22	1.59 ± 0.09
Ayvalık /C4	2.13 ± 0.04	2.03 ± 0.16	1.34 ± 0.09
Control	2.6 ± 0.06	2.53 ± 0.08	2.47 ± 0.05
Domat/C1	1.56 ± 0.03	1.59 ± 0.02	1.66 ± 0.09
Domat /C2	1.61 ± 0.03	1.59 ± 0.01	1.53 ± 0.05
Domat /C3	1.63 ± 0.01	1.61 ± 0.01	1.45 ± 0.05
Domat /C4	1.02 ± 0.05	1.84 ± 0.02	2.04 ± 0.53
Control	2.64 ± 0.08	2.45 ± 0.13	2.78 ± 0.04
Uslu/C1	1.57 ± 0.01	1.56 ± 0.04	1.59 ± 0.04
Uslu/C2	1.62 ± 0.02	1.75 ± 0.29	1.42 ± 0.12
Uslu/C3	1.69 ± 0.07	1.63 ± 0.04	1.6 ± 0.13
Uslu/C4	2.28 ± 0.03	2.1 ± 0.007	1.55 ± 0.04
Values = mean ± SD, ***p* < 0.05 versus untreated control**			

Table 3.6 Cell proliferation in hMSCs treated with olive extracts.

Sample and Treatment	**24h**	**48h**	**72h**
Control	2.05 ± 0.02	2.75 ± 0.19	3.44 ± 0.11
Ayvalık /C1	2.15 ± 0.06	3.11 ± 0.06	2.47 ± 0.17
Ayvalık /C2	2.41 ± 0.07	3.1 ± 0.02	2.57 ± 0.12
Ayvalık /C3	2.43 ± 0.09	3 ± 0.007	2.85 ± 0.04
Ayvalık /C4	0.64 ± 0.01	0.6 ± 0.007	0.66 ± 0.05
Control	2.05 ± 0.02	2.36 ± 0.15	3.44 ± 0.11
Domat/C1	2.62 ± 0.16	2.68 ± 0.02	3.02 ± 0.05
Domat /C2	2.4 ± 0.14	2.93 ± 0.19	3.26 ± 0.01
Domat /C3	2.41 ± 0.1	2.73 ± 0.04	3.23 ± 0.01
Domat /C4	0.61 ± 0.04	0.62±0.007	0.55 ± 0.01
Control	2.05 ± 0.02	2.43 ± 0.08	3.44 ± 0.11
Uslu/C1	2.27 ± 0.01	2.84 ± 0.03	2.94 ± 0.18
Uslu/C2	2.23 ± 0.03	2.74 ± 0.04	2.87 ± 0.35
Uslu/C3	1.95 ± 0.05	2.76 ± 0.03	3.2 ± 0.13
Uslu/C4	1.12 ± 0.1	0.78 ± 0.09	1.49 ± 0.19
Values = mean ± SD, ***p* < 0.05 versus untreated control**			

According to the findings, it can be said that olive extracts have cytotoxic activity on 23132/87 gastric adenocarcinoma cell line in time and dose dependent manner. Caco-2 colon adenocarcinoma cell line was too resistant against olive extract treatment. Statistically significant results show that olive extracts haven't got cytotoxic activity on colon adenocarcinoma cell lines while a decrease in cell proliferation is observed in a time dependent manner. For hMSCs high dose cause decrease in cell proliferation without cytotoxicity.

Following figures show the effects of olive extracts morphologically on cancer cell lines.

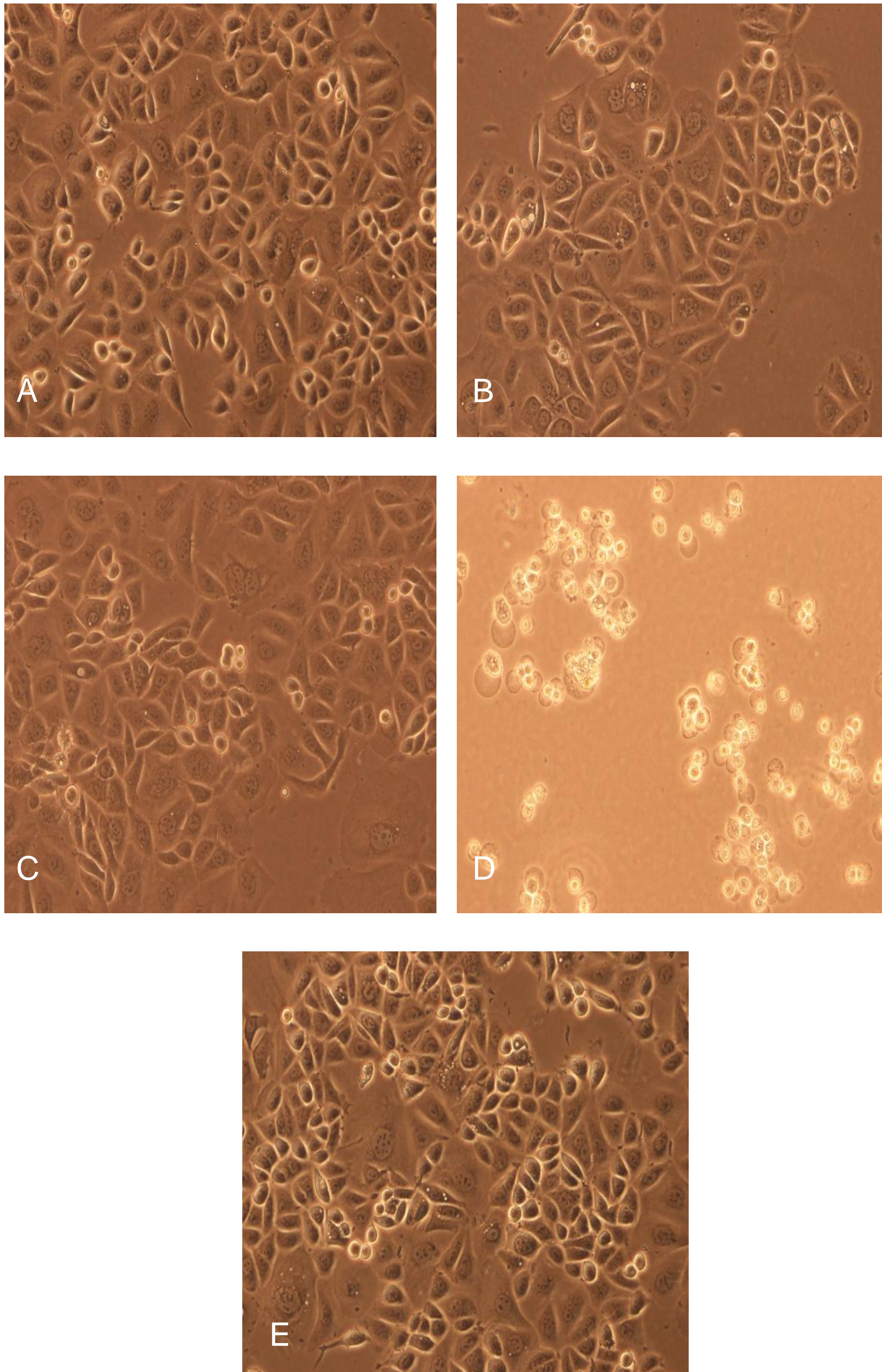


Figure 3.12 AGS cell line treated with different concentration of Domat extract (A, B, C, D) and untreated (E) control cells (Cells+DMEM with 10%FBS) at 10X magnification under inverted microscope at 24 h, A) $3.33x10^3$ µg/ml, B) $6.66x10^3$ µg/ml, C) $1.33x10^4$ µg/ml, D) $1x10^5$ µg/ml dilutions.

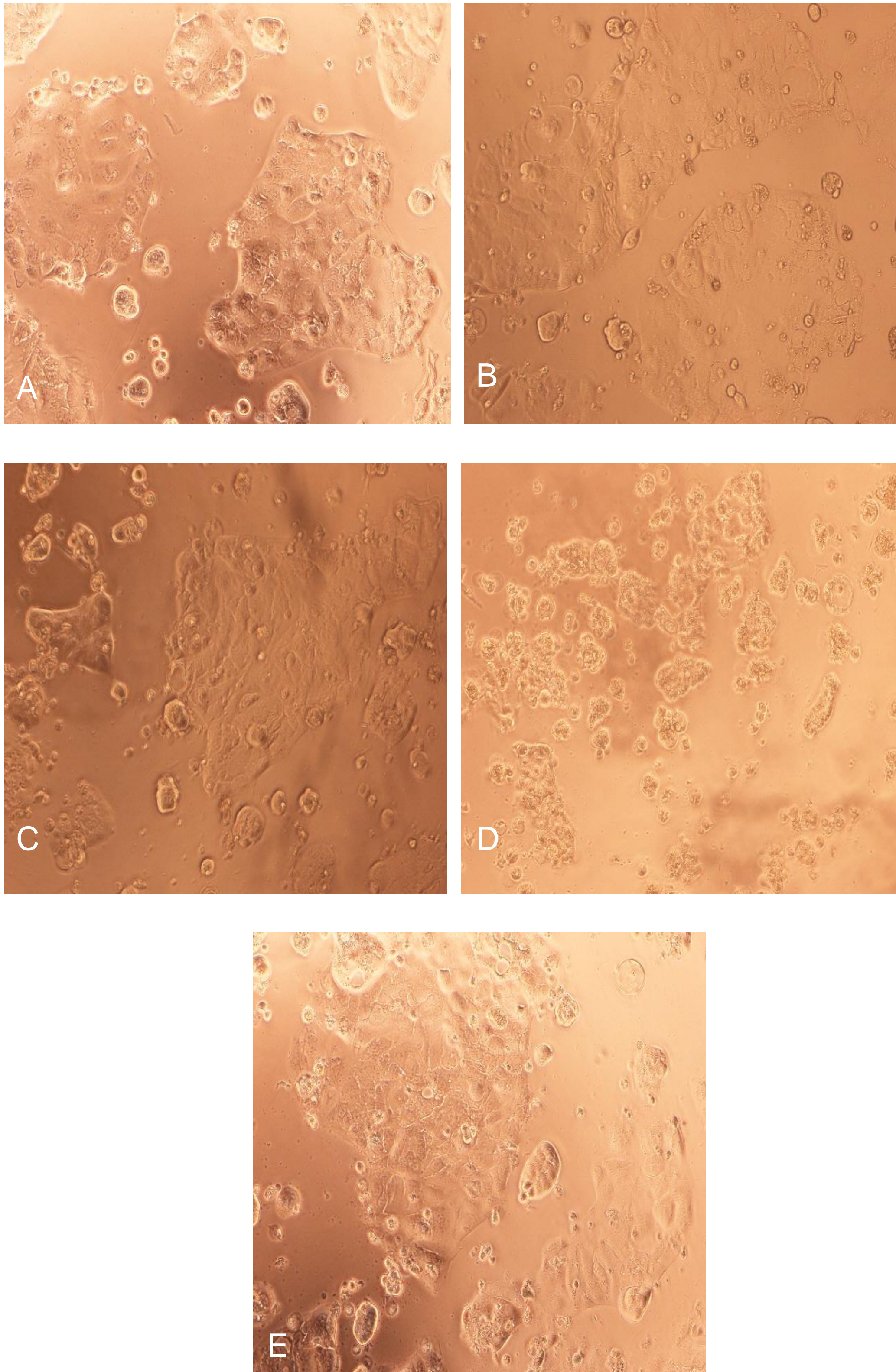


Figure 3.13 Caco-2 cell line treated with different concentration of Ayvalık extract (A, B, C, D) and untreated (E) control cells (Cells+DMEM with 10%FBS) at 10X magnification under inverted microscope at 24 h, A) $3.33x10^3$ µg/ml, B) $6.66x10^3$ µg/ml, C) $1.33x10^4$ µg/ml, D) $1x10^5$ µg/ml dilutions.

As seen in the figures, both AGS and Caco-2 cell lines have similar response against different types of olive extracts. Cell attachment and growing at low concentrations is close to the control cells while almost all of the cells at $1x10^5$ µg/ml concentration were detached from the surface as a result of olive extract treatment.

Since all types of extracts had very similar effects on the cell lines, we decided to use one type of olive extract in the following experiments.

3.4 Detection of Apoptotic Cells

The distruption of the cell membrane and the translocation of phosphotidylserine, which are indicators of apoptosis were detected with Annexin-V Alexa 568 assay kit (Roche). The results were observed under fluorescence microscopy (Nikon).

Camptothecin is a chemical that inhibits the activity of DNA topoisomerase I, was used as a positive control in addition to untreated negative control, $3.33x10^3$ µg/ml, $6.66x10^3$ µg/ml, $4x10^4$ µg/ml, and $1x10^5$ µg/ml diluted samples.

AGS, gastric adenocarcinoma, Caco-2 colon adenocarcinoma cells and hMSCs were used for this experiment in order to investigate the apoptotic effects of olive extracts on these cell lines.

Following results show the apoptotic induction potential of Domat olive extract.

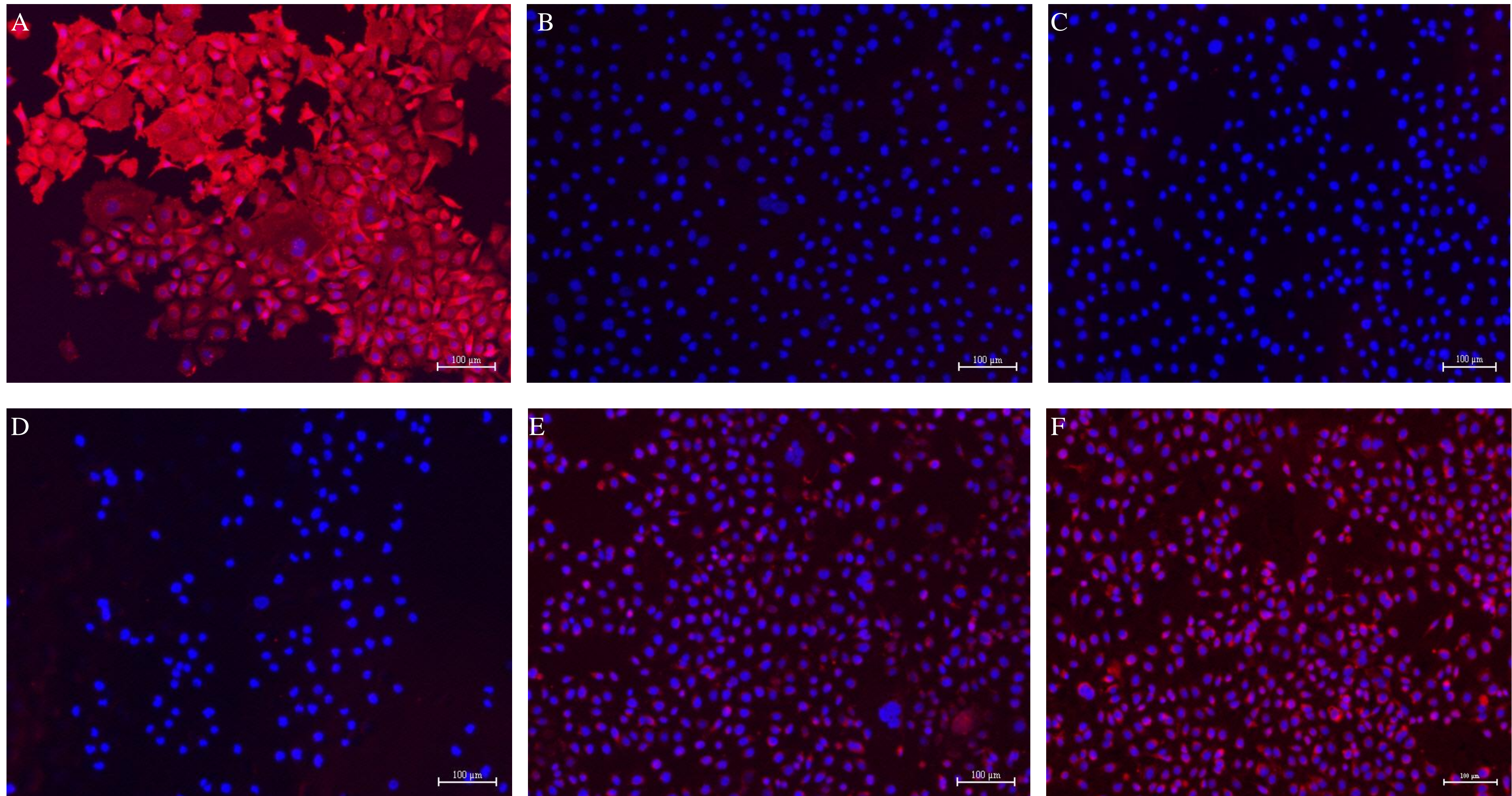


Figure 3.14 Apoptotic induction potential of Domat olive extract on AGS gastric adenocarcinoma cells (images under fluorescence microscopy at 10X). A) Camptothecin, B) Untreated control, C) $3.33x10^{3}$ μg/ml, D) $6.66x10^{3}$ μg/ml, E) $4x10^{4}$ μg/ml, and F) $1x10^{5}$ μg/ml.

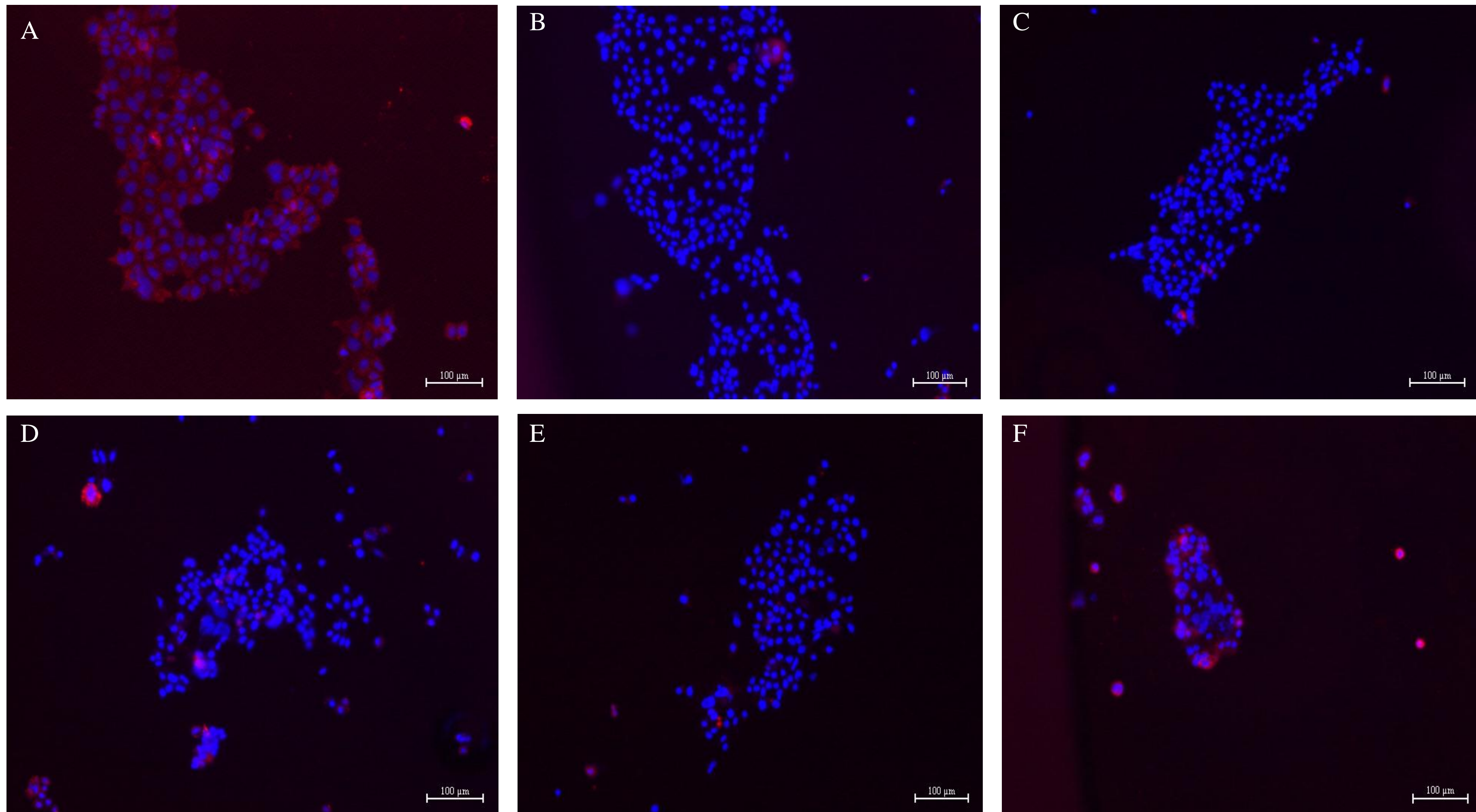


Figure 3.15 Apoptotic induction potential of Domat olive extract on Caco-2 colon adenocarcinoma cells, images under fluorescence microscopy (10X). A) Camptothecin, B) Untreated control, C) 3.33×10^3 µg/ml, D) 6.66×10^3 µg/ml, E) 4×10^4 µg/ml, and F) 1×10^5 µg/ml.

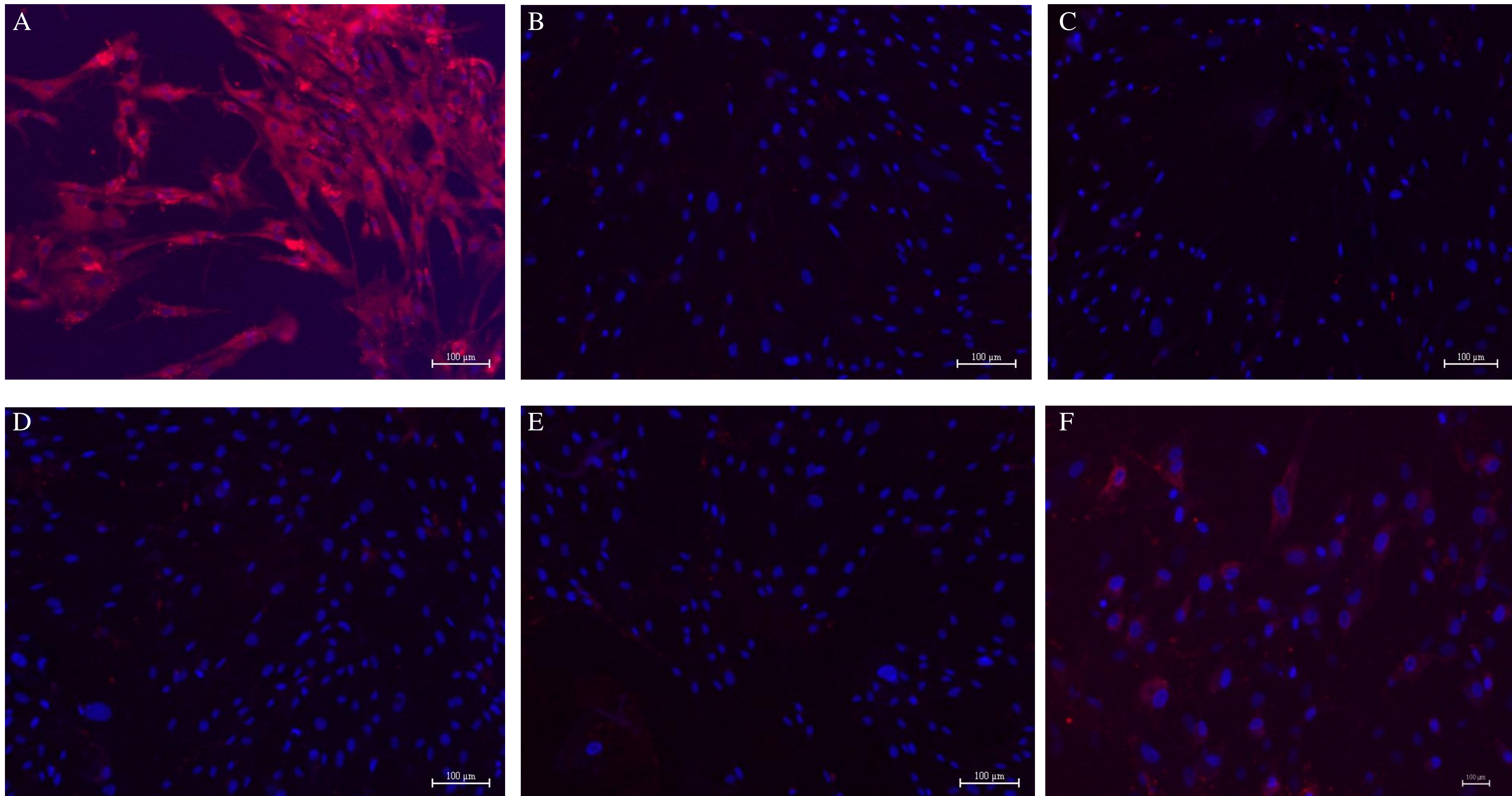


Figure 3.16 Apoptotic induction potential of Domat olive extract on hMSCs, images under fluorescence microscopy (10X). A) Camptothecin, B) Untreated control, C) $3.33x10^3$ µg/ml, D) $6.66x10^3$ µg/ml, E) $4x10^4$ µg/ml, and F) $1x10^5$ µg/ml.

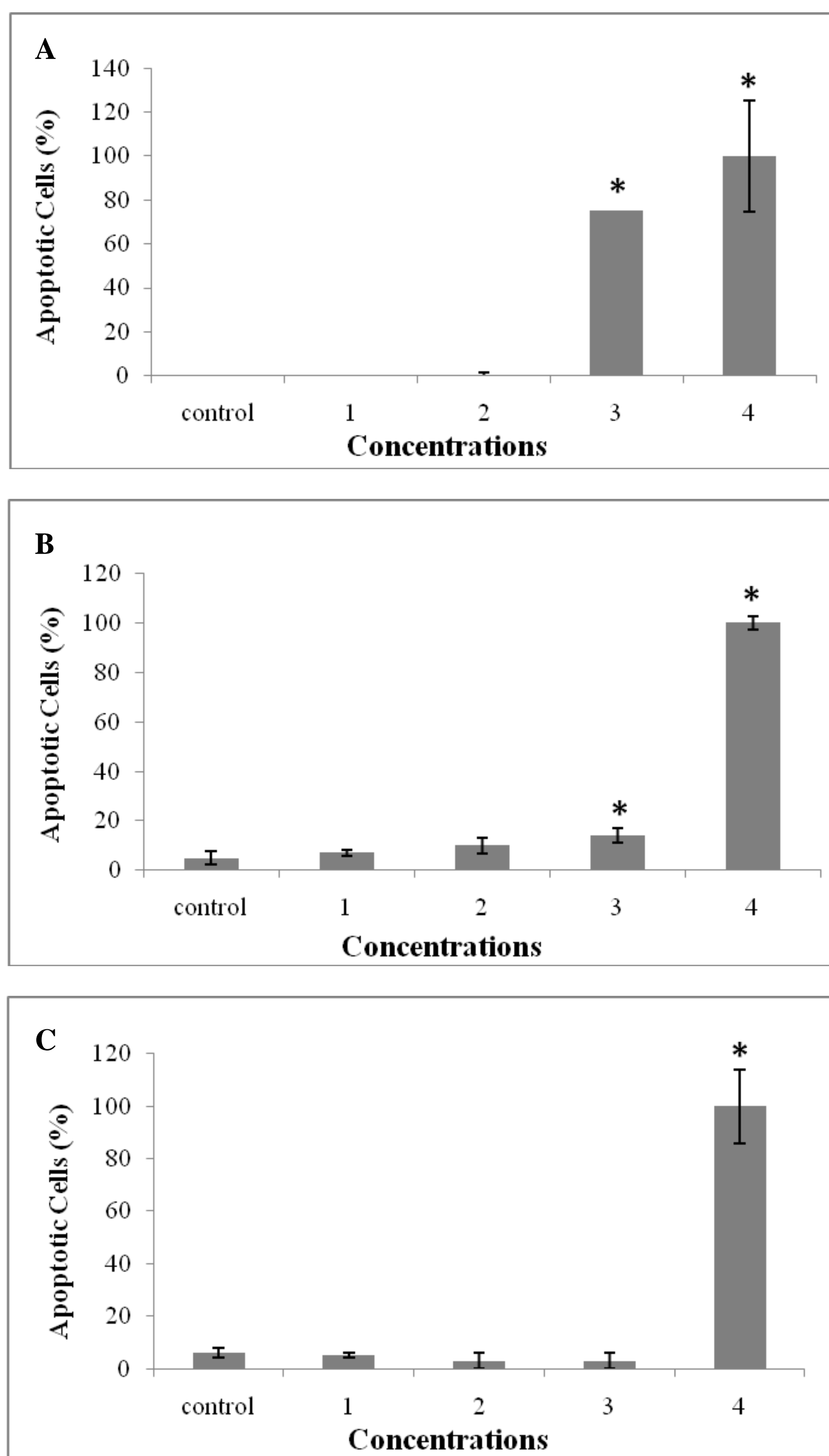


Figure 3.17 Schematic representation of the rate of apoptotic cells treated with Domat olive extracts. A) AGS, B) Caco-2, C) hMSCs. *Statistically significant results (p<0.05) ANOVA, post-hoc test LSD. 1) 3.33×10^3 µg/ml, 2) 6.66×10^3 µg/ml, 3) 4×10^4 µg/ml, and 4) 1×10^5 µg/ml.

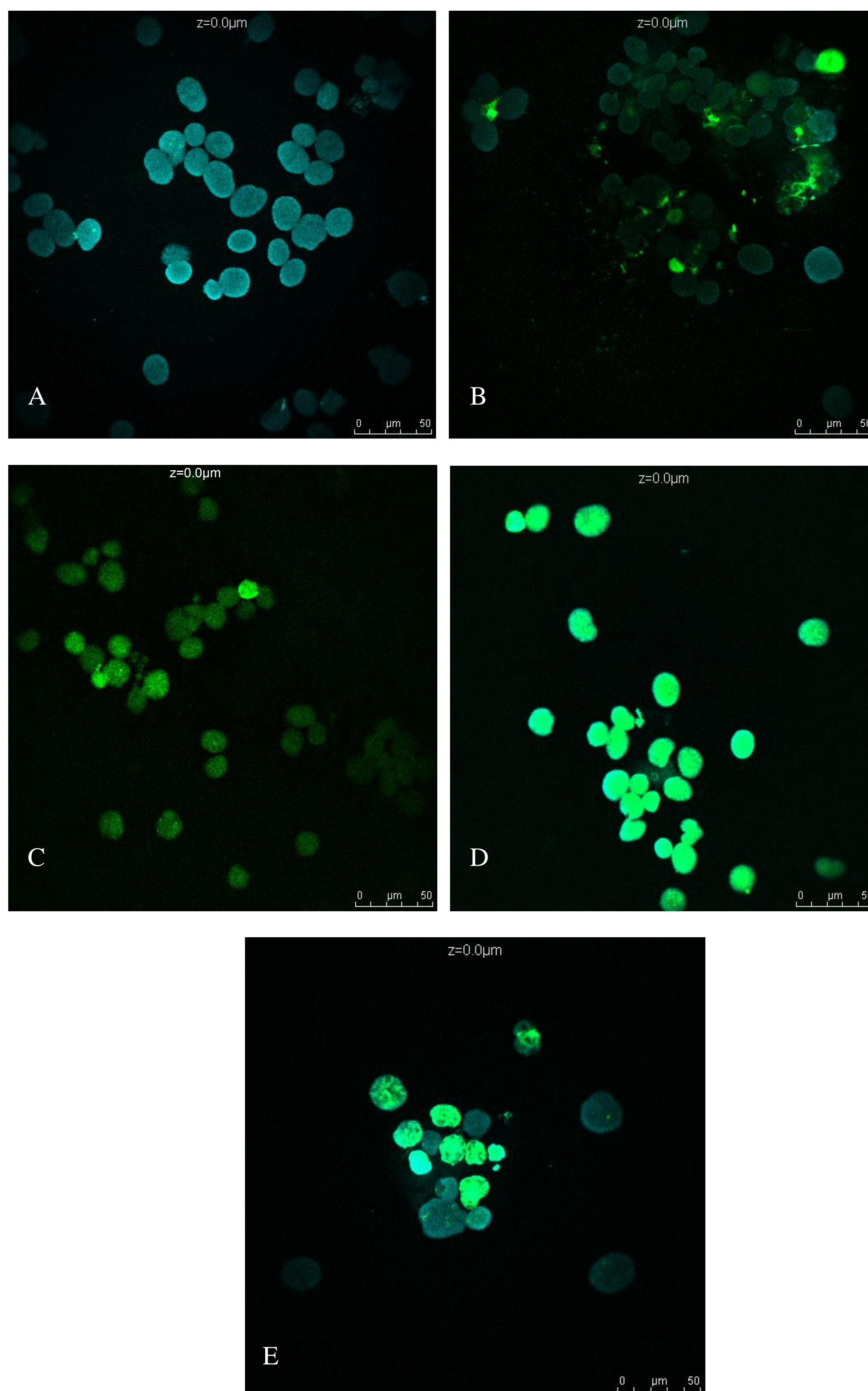


Figure 3.18 TUNEL assay images, under confocal microscopy at 40X in AGS cells treated with Domat olive extract. A) Untreated control, B) Camptothecin, C) $2x10^4$ µg/ml, D)$4x10^4$ µg/ml, E)$1x10^5$ µg/ml.

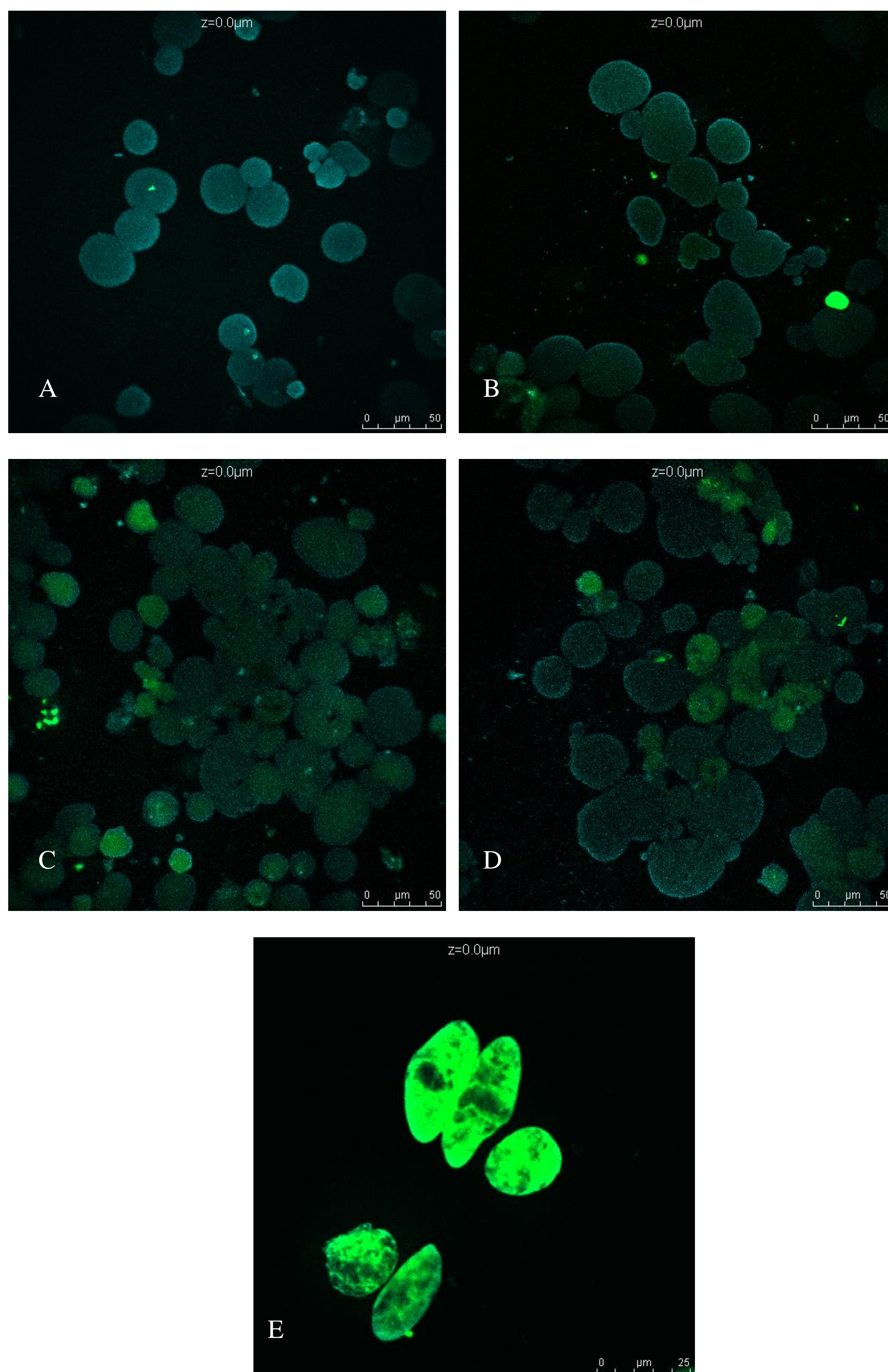


Figure 3.19 TUNEL assay images, under confocal microscopy at 40X in Caco-2 cells treated with Domat olive extract. A) Untreated control, B) Camptothecin, C) $2x10^4$ µg/ml, D) $4x10^4$ µg/ml, E)$1x10^5$ µg/ml.

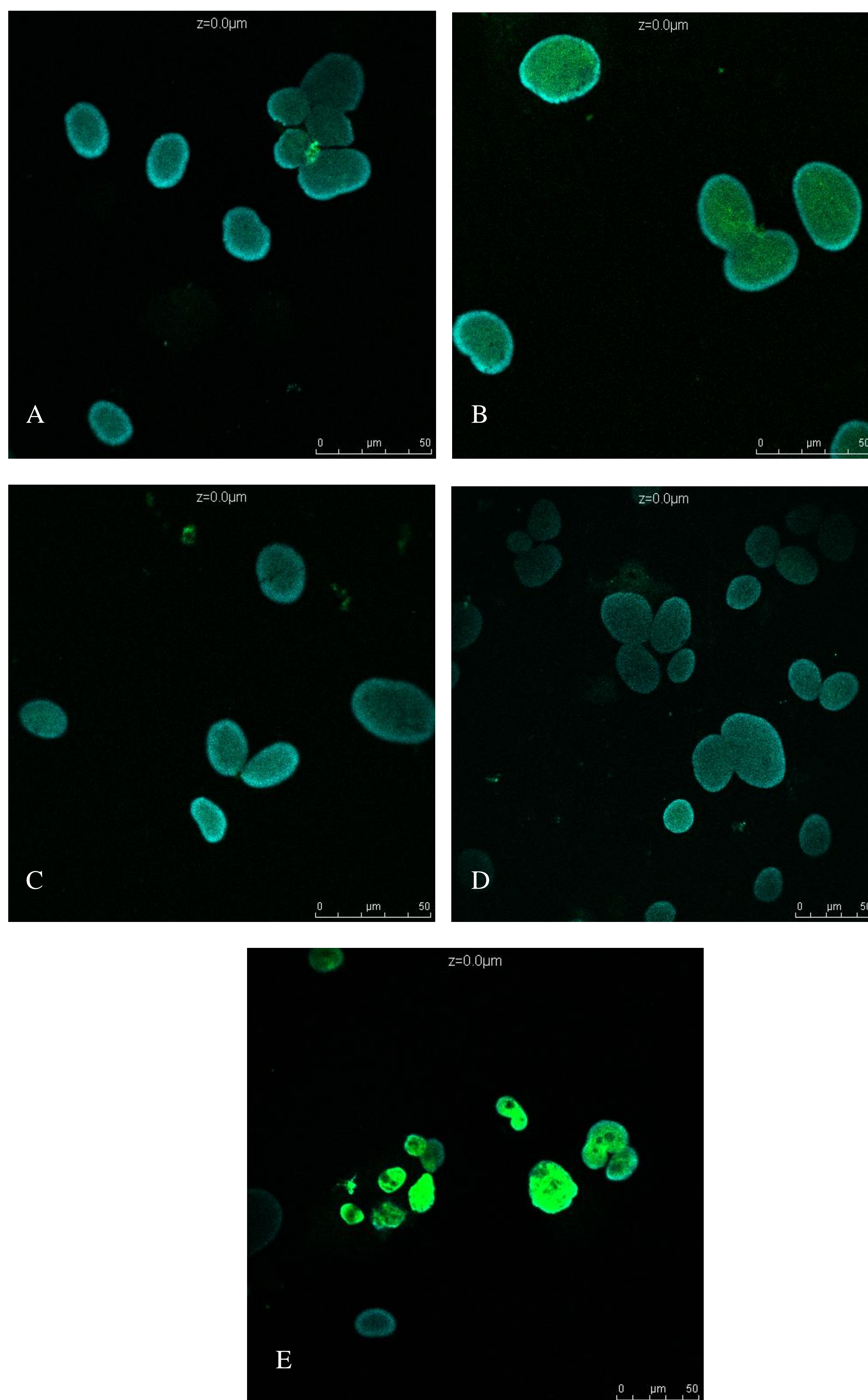


Figure 3.20 TUNEL assay images, under confocal microscopy at 40X in hMSCs treated with Domat olive extract. A) Untreated control, B) Camptothecin, C) $2x10^4$ µg/ml, D) $4x10^4$ µg/ml, E) $1x10^5$ µg/ml.

Since no apoptotic cells were detected in annexin-V staining at low concentrations, $3.33x10^3$ µg/ml (1/600 dilution), $6.66x10^3$ µg/ml (1/300 dilution), we decided to use more concentrated sample for TUNEL assay. So $2x10^4$ µg/mlwas also used. Results show that olive extract was effective at this concentration in addition to $4x10^4$ µg/ml and $1x10^5$ µg/ml in both Caco-2 and hMSCs. But it can be said that hMSCs were more resistant against olive extract treatment compared with Caco-2 and AGS cell lines. Apoptotic induction potential of olive extract was confirmed by the TUNEL assay.

3.5 Determination of Topo IIα Gene Expression at mRNA Level by RT-PCR Analysis

Topo IIα plays a role in mitotic processes and only is present in proliferating tissues such as tumors. As a result of this situation in cancer research, there is an interest in topo IIα. While cell proliferation rate increases, concentration of topo IIα also increases. So it can be used as a proliferation marker. On the other hand, β actin is constitutively expressed gene and it was used as control in this study. β actin and topo II α expression levels in control and extract treated samples were compared.

Cells were preincubated in 60mm tissue culture plates for 24 h before adding the extracts. Samples were prepared at 24 and 48 h of treatment for RT-PCR. Total RNA was extracted with RNeasy kit (Qiagen) according to the manufacturer's protocol, then cDNA synthesis protocol was performed using quantitect reverse transcription kit (Qiagen).

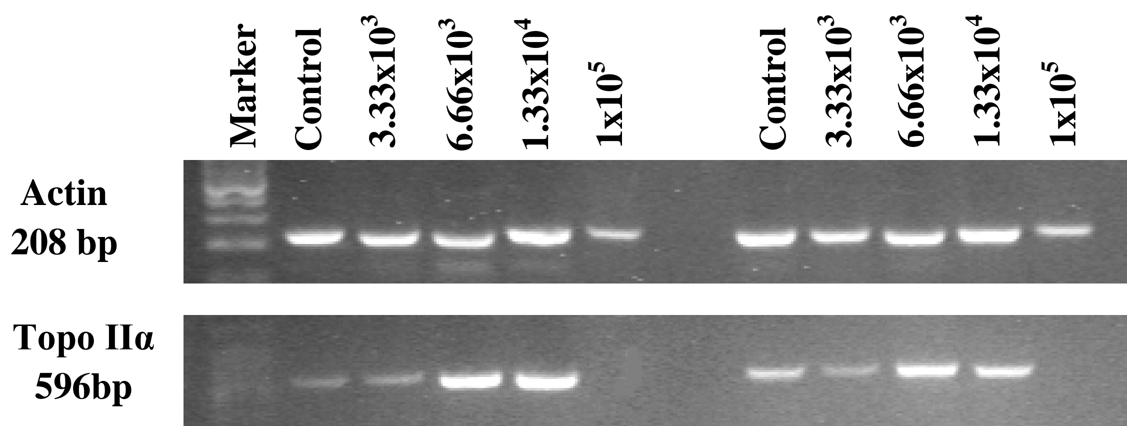


Figure 3.21 β actin (208bp) and topo IIα (596 bp) expressions in AGS cell line in response to Domat olive extract treatment at different time points (24, 48 h). Low and high doses refer the concentrations (μg/ml) used in previous experiments ($3.33x10^3$ μg/ml, $6.66x10^3$ μg/ml, $1.33x10^4$ μg/ml, $1x10^5$ μg/ml).

RT-PCR results confirm the previous implications of the study. As it is expected β actin expressions at low doses were not different to each other meaning that β actin is a constitutively active gene and its expression level did not change according to the olive extract application, except high dose. Exposure of high dose caused cell death and blocked gene expressions.

As mentioned before topo IIα gene expression level is an indicator of cell proliferation. Figure 3.21 represents the expression level of this gene in AGS cell line treated with olive extract and at low doses, topo IIα gene expression level was high when it was compared with control cells. Expression of topo IIα in high dose was not observed.

CHAPTER 4

CONCLUSION & DISCUSSION

Lifestyle related factors like dietary habits influence the incidence rate of diseases such as cardiovascular, neurodegenerative diseases and cancer. Dietary habits depend on the region that people live. Epidemiological studies demonstrate that the incidence rate of cancer and another related diseases is lower in Mediterranean countries compared with United States and Western countries (Parkin, *et al*., 2001). These dietary influences differs by cancer type. Mutagens, found in foods can cause DNA damage and dietary factors can influence this process by inhibiting the enzymes related with activation of these mutagens and also affect the signaling pathways associated with DNA repair either promoting or inhibiting the cell proliferation, cell viability and cell death (apoptosis).

As a major food group in Mediterranean style diet, olive and olive oil consumption is thought to be the main reason of these protective effects (Owen *et al*., 2004). In this study, expectation is to demonstrate the possible effects of olive extracts on several GI tract cell lines *in vitro*. In addition the same experimental procedure was performed for healthy human cells.

Previous studies have demonstrated that olive and olive oil consumption cause high intake of polyphenols which are thought to be the major effective molecules found in olive and have excitatory/inhibitory effects on cell proliferation, cell viability, cell death and another dysregulated cellular processes on some carcinoma models, mainly colon carcinomas. But results of these studies are controversial (Gill *et al*., 2005; Manna *et al*., 1997). Olive extract concentrations used in the studies are in a narrow range (Juan *et al*., 2006), and mostly single type of phenolic compounds are tested on cell lines (Reyes *et al*., 2006).

In addition there are very few studies correlating the relation between these compounds and the effects on gastric adenocarcinomas. In this respect, this study aimed to contribute to this area with new knowledge by comparing the effects of total phenolic extracts in olive on both colon and gastric adenocarcinoma cell lines in a broad range of extract concentrations.

In this study we have focused on the cytotoxic, proliferative and apoptotic effects of olive extracts on different cancer cell lines. Previous studies have demonstrated the anti-proliferative and apoptotic effects of olive phenolic compounds on breast and colon cancer cell lines in time and dose dependent manner (Corona *et al.*, 2009; Allouche *et al.*, 2011). In our study, Caco-2 colon adenocarcinoma cells were treated with three types of olive extracts at increasing concentrations from $3.33x10^3$ µg/ml to $1x10^5$ µg/ml. While high doses ($4x10^4$ µg/ml, $1x10^5$µg/ml) had apoptotic effect (except highest one, $1x10^5$ µg/ml, since it was cytotoxic for the cells), low concentrations ($3.33x10^3$µg/ml, $6.66x10^3$µg/ml, $1.33x10^4$µg/ml) increased cell proliferation of all type of cells used in the study in a time dependent manner with decreased cytotoxicity. At high concentration cell proliferation is significantly inhibited without cytotoxicity in Caco-2 cells ($p<0.05$). So, we divided the extract concentrations into three groups according their effects on the cell line: highest dose (cytotoxic dose), high dose (apoptotic dose) and low dose (proliferative dose). The same experimental set up was used for three different types of gastric adenocarcinoma cell lines, AGS, HGC-27 and 23132/87 cells. In the study of Kountori *et al.* (2009), olive extract inhibited AGS gastric adenocarcinoma proliferation, in addition to IL-8 and ICAM-1 expression whose expression is responsible for gastric inflammation and carcinogenesis. In our study we observed the three groups of effective doses on cellular index in this gastric adenocarcinoma cell lines as well as HGC-27 and 23132/87 cell lines as it was concluded in Caco-2 colon carcinoma cells. First group includes highest dose with their inhibitory effects on cell proliferation by blocking the proliferation or induction of cell death. Second group triggers cell proliferation with increasing rates and the third group causes downregulation of cell proliferation. And it is determined that olive extracts have both effective apoptotic doses for gastric adenocarcinoma cell lines and nontoxic helpful doses for increased proliferation of these cells. Then inhibitory and increasing effects of the extracts on cell proliferation of the cells were confirmed at these effective doses (cytotoxic, apoptotic and proliferative) in one of these gastric cancer cell lines, AGS, by RT-PCR results. At low concentrations, as a proliferation marker topo IIα

gene expression level was checked and results demonstrated that gene expression level of topo IIα was increased at nontoxic low concentrations while no expression was observed at high concentration which blocks proliferation. According to these results it can be said that olive extracts are more effective on gastric regions.

This study also includes the investigation of selective effects of olive extracts on cancer cells. In the literature, effects of olive polyphenols were studied on primary cells such as normal human gingival GN61 fibroblasts and normal human neutrophils and polyphenols were shown to be nontoxic for these cell lines in dose dependent manner (Babich and Visioli, 2003). For investigation of this type of effect, hMSCs were used as healthy human cells. The same experimental processes were performed for hMSCs for comparing the effects on cancer cells. Results were convenient to the literature knowledge. It was not observed any cytotoxic effect at high dose, besides this olive fruit extracts have inhibitory effects on cell proliferation in time and dose dependent manner.

Analysis of the apoptotic induction potential of olive extracts on carcinoma models and healthy hMSCs is done by indicating the DNA fragmentation and quantifying the apoptotic cells with annexin-V FICT staining. Findings demonstrate that olive extract induce apoptosis in both gastric and colon carcinoma cells, as well as hMSCs. Apoptotic effect of olive extract on gastric carcinoma cells is more significant while colon carcinoma cells and hMSCs are more resistant against extract treatment. In the study of Reyes *et al*., (2006) findings have demonstrated that in non-tumoral intestinal cells, induction of apoptosis was not detected which are compatible with our results.

As a result of this study, gastrointestinal tract as a place where direct exposure of cancerous agents is seen, is more sensitive to treatment of olive extracts compared with colon carcinoma models and healthy human cells. In gastric cancer cell lines both cytotoxic, anti-proliferative and apoptotic effects were observed besides the gene expression analysis of cell proliferation markers. We hope these findings lead to improve new ideas, aspects, investigations and interests in relation between gastric carcinoma models and consumption of olive and olive products.

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