IN-VITRO EVALUATION CYTOTOXIC POTENTIAL OF NOVEL ISOINDOLE DERIVATIVES ON VARIOUS CANCER CELL LINES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE

in Chemistry

by
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July 2021

İZMİR

ACKNOWLEDGEMENTS

First of all, I would like to express my endless thanks and gratitude to Prof. Dr. Gülşah ŞANLI MOHAMED for giving me a great opportunity by including me in the biochemistry research group and their projects. I cannot express how lucky I am to be working with such a wonderful Professor for her confidence, support, motivation, and broadening my horizons.

I would like to thank Prof Dr. Yunus Kara, for contributed to my project by including me in the Tübitak project and allowing me to try experimental materials.

Also, I would like to thank warmly to Dr. Özlem Gündoğdu for her politely helps.

Besides, I would like to thank Özgür OKUR, Murat DELMAN, and Dane RUSÇUKLU, specialists of Biotechnology and Bioengineering Research Center, for their kind help.

I am grateful to Derya METE, for contributed a lot to this project and helped me not only in science but also in life, reminding me that I can overcome anything with her smiling face, patience, and positive energy. I want to thank Biochemistry Laboratory members Sinem ŞAHİN and Dorukhan ÖZEN for their support.

Finally, my special thanks go to my mother Funda EŞKİN, my father Haluk YEMEZTAŞLICA, my sister Şevval Deniz YEMEZTAŞLICA and my lovely husband Ali Erkin YETİŞKİN. They provided the greatest support in my education life, who never spared their love and support and always encouraged me. It would not have happened without them.

This study was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, 119Z097).

ABSTRACT

IN-VITRO EVALUATION CYTOTOXIC POTENTIAL OF NOVEL ISOINDOLE DERIVATIVES ON VARIOUS CANCER CELL LINES

Cancer, which is the disease of our age, arises because of a very complex set of mechanisms. Especially with the proliferation of cancer disease and the increase in cancer-related deaths, it has a great impact on the development of drug studies by improving existing treatments or researching new treatment methods.

Cantharidine and its analogs are natural anhydrides with an inhibitory effect on protein phosphatases. However, they are not included in cancer therapies due to their toxicity. In recent studies, it has been found that derivatives of cantharidin as isoindole-1,3-dione and its derivatives have anticancer effects.

The main purpose of this study to investigate the effects of four different drugs, which are newly synthesized isoindole derivatives for use in cancer treatment, on different cancer cells.

The cytotoxic effects of drugs on A549 (human lung adenocarcinoma), HeLa (human cervical carcinoma), PC3 (human prostate carcinoma), MCF-7 (human breast carcinoma), and Caco-2 (human colorectal carcinoma) cell lines were investigated by the MTT assay method, and the optimum incubation time was determined, then IC_{50} values were calculated. Then, the IC_{50} concentrations of the drugs were applied at 48 hours, which is the optimum incubation period, and apoptotic stages and cell cycle stages were compared using flow cytometry to understand whether the drugs have a suppressive function in cancer development. Scratch assay was performed to investigate the migration effect of drugs on cells.

The results showed that the drugs are suppressive to cancer cells and can be used for therapeutic purposes in the future.

ÖZET

ÇEŞİTLİ KANSER HÜCRE DİZİLERİNDE YENİ İZOİNDOL TÜREVLERİNİN SİTOTOKSİK POTANSİYELİNİN İN-VİTRO DEĞERLENDİRİLMESİ

Erken tanı ve teşhis olmadan tedavisinin zor olmasıyla bilinen kanser, normal hücrelerin bir dizi kompleks mekanizma sonucu mutasyon geçirerek anormal hücrelere dönüşmesiyle oluşmaktadır. Tedavi yöntemlerinde kullanılan ilaçların önemi büyüktür ve bu da ilaç araştırmalarının hız kazanmasına sebep olmaktadır.

Protein fosfatazlara karşı inhibitor etkisi olan kantaridin çok eski yıllarda halk ilacı olarak kullanılmıştır. Fakat yüksek toksisitelerinden dolayı kanser tedavilerine dahil edilmemiştir. Yapılan çalışmalarla birlikte kantaridin türevlerinden biri olan isoindol-1,3-dion türevleri sentezlenerek anti-kanser etkilere sahip olduğu belirlenmiştir.

Bu çalışmanın temel amacı bir kantaridin türevi olarak yeni sentezlenmiş epoksi alkollerin beş farklı kanser hücresi üzerindeki etkisini araştırmaktır.

İlk olarak ilaçların A549 (akciğer kanseri), HeLa (rahim kanseri), PC3 (prostat kanseri), MCF-7 (meme kanseri) ve Caco-2 (kolon kanseri) hücre hatlarında ilaçların sitotoksik aktiviteleri MTT testi ile araştırılarak IC_{50} değerleri hesaplanmıştır ve hücrelerdeki etkisine bağlı olarak optimum inkübasyon süresi belirlenmiştir. Yeni sentezlenen epoksi alkol türevlerinin IC_{50} dozları kullanılarak hücre hatları üzerindeki apoptotik etkisi ve hücre siklusundaki etkisini belirlemek için Akış Sitometrisi kullanılmıştır. Ayrıca ilaçların hücreler üzerindeki göç etkisini belirlemek için yara çizik deneyi yapılmıştır.

Sonuçlara göre yeni sentezlenen epoksi alkol bileşiklerinin kanser hücrelerinde büyüme ve gelişmeyi durdurarak öldürücü etki göstermesiyle, gelecekte bir ilaç türü olarak kullanılabileceğini işaret etmektedir.

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CHAPTER 1

INTRODUCTION

1.1. Cancer

The basic building blocks of human beings are cells, and these cells, which have a defined order, grow, develop, and die in a controlled way. Although the DNA in a normal cell is damaged in the life cycle, it either repairs itself or dies. However, DNA damage that causes mutation creates abnormal cells and cancer cells are formed, and these cancer cells, unlike normal cells, begin to multiply uncontrollably and damage tissues and organs. Metastasis is the spread of cancer cells from the area where they were formed to different tissues and organs of the body through the blood and lymphatic vessels (Poste and Fidler 1980). This condition, which is caused by cancer cells, can be fatal. There are many complex steps related to each other such as angiogenesis, invasion, migration-motility, extravasation, and proliferation for metastasis to occur (Pezzuto, Kosmeder et al. 2005).

Although carcinoma, sarcoma, melanoma, lymphoma, and leukemia are the main types of cancer, it is known that there are more than 200 types of cancer caused by them. According to the 2020 data of the World Health Organization, the cancer types with the highest cause of death are lung, colon and rectum, liver, stomach, and breast, respectively.

Though there are so many types of cancer, and the cause of death is high, there are various types of treatment such as surgery, chemotherapy, radiation therapy, immunotherapy, targeted therapy, hormone therapy, stem cell or bone marrow transplantation, and treatment types are being developed day by day.

Pharmaceutical studies also play an important role in the development of these treatment methods. Because although most drugs currently used for cancer treatment can treat, they also have many more aggressive side effects such as hair loss, nausea and

vomiting, easy bruising and bleeding, anemia, and infection. However, developing a drug, especially in cancer, may require a very long process because first of all, the chemical synthesis and identification of the drug, and then the pre-clinical and post-clinical evaluations should be investigated for which type and treatment it may be for.

1.1.1. Lung Cancer and A549 Cell Line

Lung cancer begins when structurally normal lung cells multiply out of need and out of control, forming a tumor in the lung. The mass formed here first grows in its environment, and in advanced stages, it spreads to the surrounding tissues or organs such as liver, bone, brain, etc., causing damage.

Although lung cancer is a very common type of cancer, it accounts for 12-16 percent of all cancers and 17-28% of cancer-related deaths and ranks first in cancer-related deaths in both men and women (Bray, Ferlay et al. 2018).

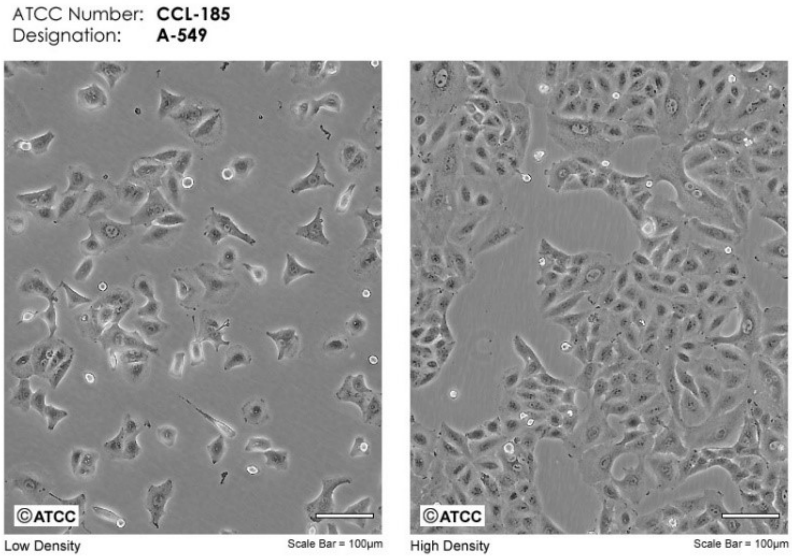


Figure 1. Morphology of A549 cell monolayer

(Source: ATCC Global Biological Materials Resource)

There are two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC types have a higher rate of metastasis than NSCLC and therefore cases with SCLC cells can be fatal within a few weeks unless promptly treated.

A549 cells are a type of non-small lung cancer cell and are adenocarcinoma human alveolar basal epithelial cells.

1.1.2. Cervical Cancer and HeLa Cell Line

There are two different parts in the cervix: the endocervix, which is covered with glandular cells, and the exocervix, which is covered with squamous cells, which reveals the common type of cancer. Types 16 and 18 of HPV, known as the human papillomavirus, cause 70% of cervical cancer, according to the World Health Organization.

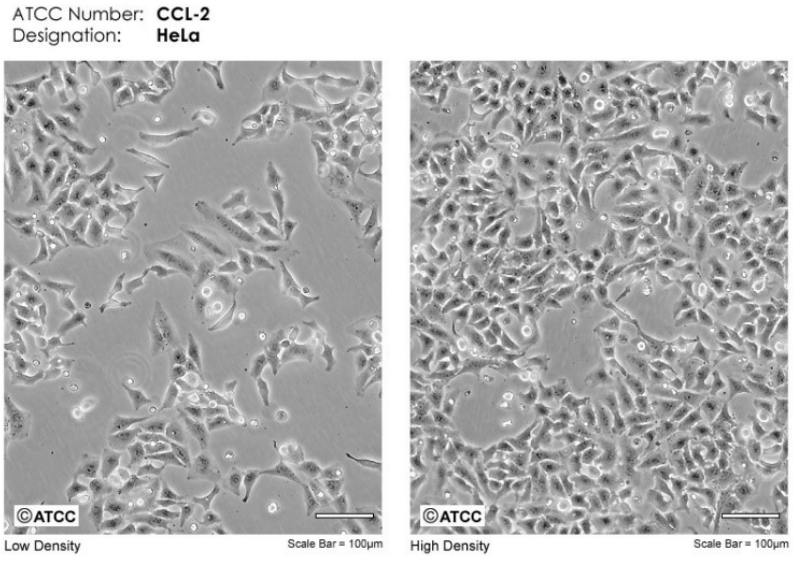


Figure 2. Morphology of HeLa cell monolayer

(Source: ATCC Global Biological Materials Resource)

The first immortal cell line from cervical cancer samples is HeLa (human cervix adenocarcinoma cells). The reason why it is defined as immortal unlike other cells is its ability to divide indefinitely.

1.1.3. Prostate Cancer and PC3 Cell Line

Prostate cancer is the second most common tumor in the male genitourinary system. Prostate cancer, which is in the class of glandular cancer or adenocarcinoma, is formed by the transformation of semen-secreting cells into cancer cells in the prostate gland in the male reproductive system. It has a high rate of metastasis to organs such as lymph nodes, bones, bladder, and rectum.

PC3, the human prostate cancer cell line, has acidic phosphate activity and low testosterone-5-alpha reductase.

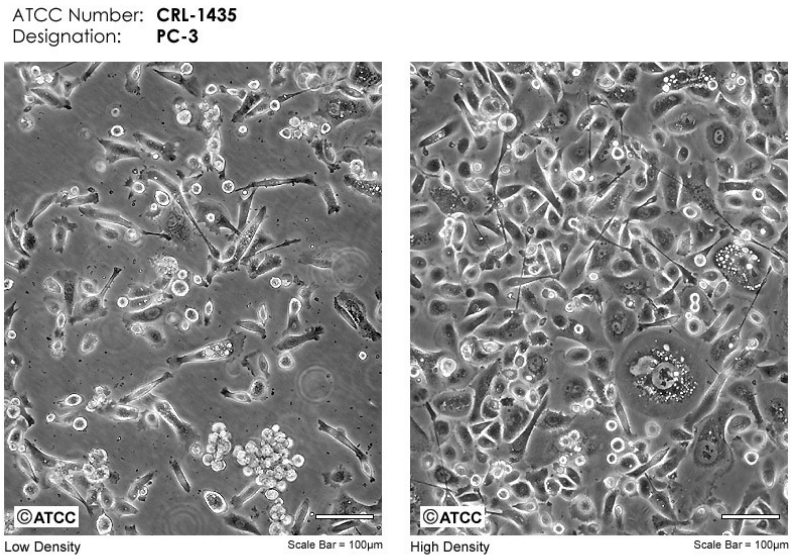


Figure 3. Morphology of PC3 cell monolayer

(Source: ATCC Global Biological Materials Resource)

1.1.4. Breast Cancer and MCF-7 Cell Line

Although 5±10% of breast cancer is due to mutation in genes (mutation in BRCA1 and BRCA 2 genes), it is seen in one out of every ten women (Easton and Peto 1990, Pisani, Parkin et al. 1999). It is caused by mutations in the BRCA1 and BRCA2 genes (Easton, Ford et al. 1995). In recent years, biomarkers that can detect breast cancer have been used by using intraepithelial neoplasia (Boone and Kelloff 1993), mammographic breast density (Atkinson, Warren et al. 1999), insulin growth factor-1 (Hankinson, Willett et al. 1998) and serum estrogen and testosterone (Cauley, Lucas et al. 1999). Although there are various approaches to prevent breast cancer today, they have not been proven to reduce deaths from this disease yet.

MCF-7, a breast cancer cell line, is a cell line with helical mutations in the PIK3CA gene and low AKT activation (Vasudevan, Barbie et al. 2009). Omega-3 and 6 TNF inhibit the development and growth of alpha MCF-7 cells (Mansara, Deshpande et al. 2015).

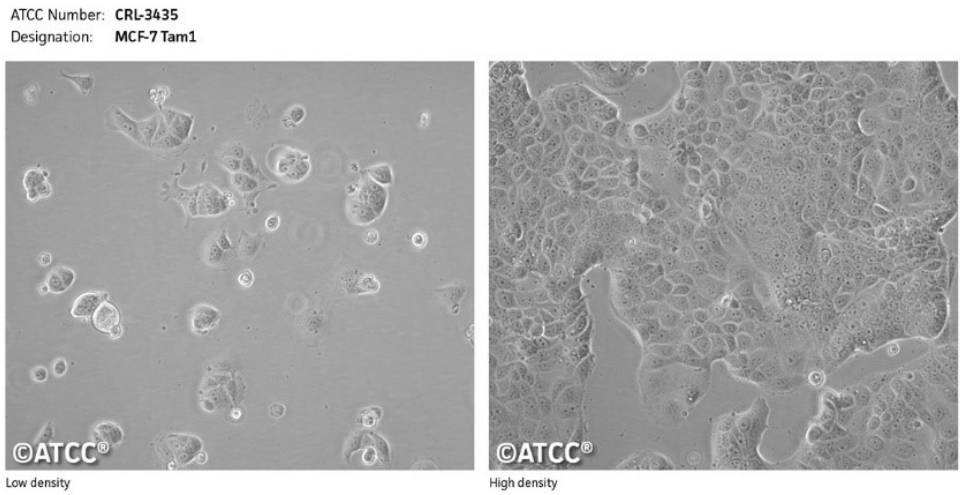


Figure 4. Morphology of MCF-7 cell monolayer

(Source: ATCC Global Biological Materials Resource)

1.1.5. Colorectal Cancer and Caco-2 Cell Line

Colorectal cancer occurs when noncancerous polyps begin to develop on the lining of the colon and turn into cancer (Haggar and Boushey 2009). It takes about 10 years for these polyps to turn into cancer, which is of vital importance for the prevention of cancer development by non-surgical interventions. In people with inflammatory bowel diseases, the build-up of abnormal cells in the lining of the colon and rectum is called dysplasia. Dysplasia is evaluated according to the appearance of the cell. In mild dysplasia, the cell is more similar to the normal cell, while in severe dysplasia, changes similar to cancer cells are observed in the cell.

Cancer coli-2 or Caco-2 cells is a cancer cell type that grows as a monolayer, is heterogeneous, and forms from a human colorectal with many different morphologies. Due to the heterogeneous and different morphologies, the results may be different even if experiments are carried out under similar conditions (Sambuy, De Angelis et al. 2005).

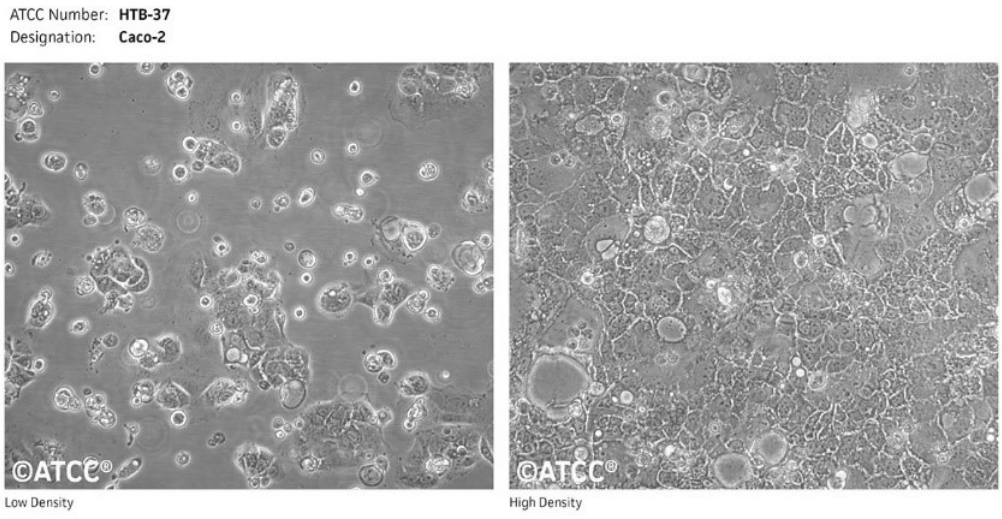
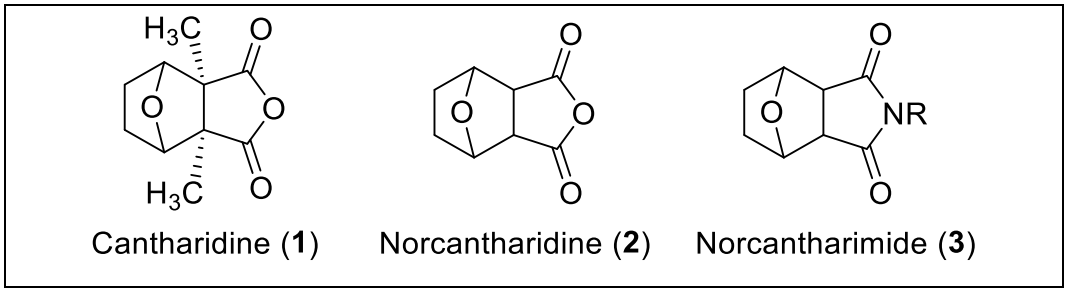


Figure 5. Morphology of Caco-2 cell monolayer

(Source: ATCC Global Biological Materials Resource)

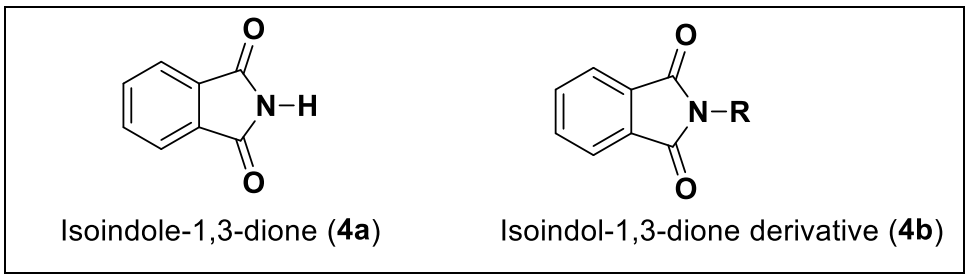
1.2. Isoindole Derivatives

Cantharidin, a natural anhydride obtained from Spanish flies, has been known for over 2000 years. Especially cantharidin was used as folk medicine by the Chinese (Wang 1989). The inhibitory effects of cantharidine and its analogs against protein phosphatases (PP1 and PP2A) are known (Zhao, Wu et al. 2008, Liu and Chen 2009) (Scheme 1). However, it is not included in basic oncology due to its toxicity (Tagwireyi, Ball et al. 2000). The anticancer effects of norcantharidine and norcantharimides (isoindol-1,3-dione derivatives), known as cantharidin analogs, were also investigated and it was stated that they have anti-cancer effects. For example, N-methylnorcantarimide containing isoindole-1,3-dione skeletal structure has been reported to act as a cancer inhibitor in animals (Zhou, Li et al. 2016) (Scheme 2).



Scheme 1. Chemical structure of cantharidine and its derivatives

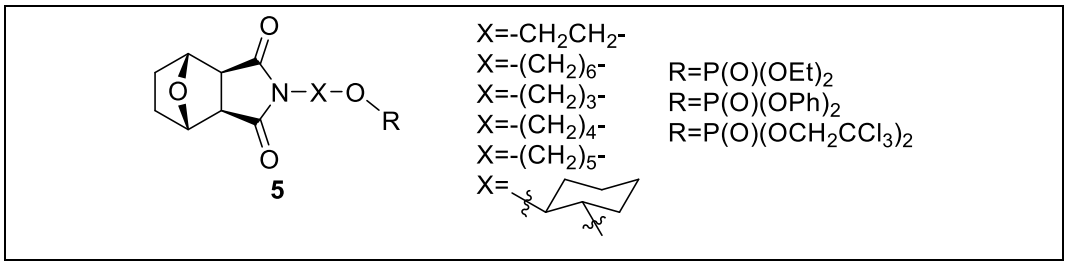
(Source: Tan, Koç et al. 2016)



Scheme 2. The structure of isoindole-1,3-dione

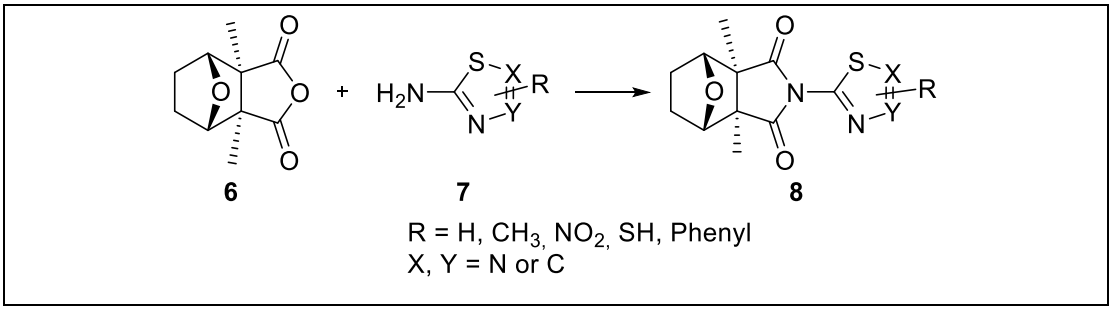
(Source: Zhao, Ma et al. 2012)

The important advantage of isoindole-1,3-dione derivatives over cantharidine is their low toxicity. Robertson and his group synthesized modified norcantarimide (isoindole-1,3-dione) analogs and investigated their cytotoxicity against different cancer types and obtained favorable results. In cytotoxic studies on eight cell lines, they found that a cantharidine derivative as a norcantharidine, and its derivatives increased the anti-proliferative activity five-fold (Robertson, Gordon et al. 2011) (Scheme 3).



Scheme 3. Chemical structure and its functional group of norcantarimide (isoindole-1,3 dione) derivatives

(Source: Robertson, Gordon et al. 2011)



Scheme 4. Chemical structure and its aliphatic, aryl, and pyridyl groups of norcantarimide analogs

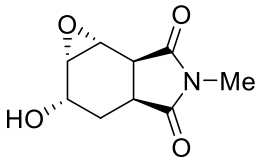
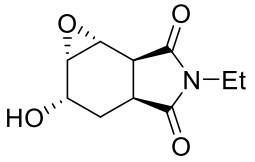
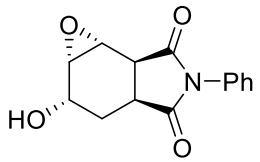
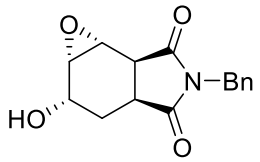
(Source: Pen-Yuan, Sheng-Jie et al. 2000)

Lin et al. studied the synthesis and cancer activities of norcantarimide analogs containing aliphatic, aryl and pyridyl groups. The IC_{50} values of synthesized cantharidin derivatives and cantharidin were compared in cytotoxicity studies performed with

Hep3B and SK-Hep1 cell lines during 48 hours of incubation. The IC_{50} value of cantharidin was 2 µM for the Hep3B cell line, 0.4 µM for its derivative, and the IC_{50} value of cantharidin for the SK-Hep1 cell line was 4 µM, while it was 1.25 µM for its derivative (Lin, Shi et al. 1998, Pen-Yuan, Sheng-Jie et al. 2000) (Scheme 4).

1.3. Chemical Structure of Drugs

The molecules as potential anticancer drugs given in Scheme 5 were synthesized by Prof. Yunus Kara in the Organic Chemistry Laboratory of the Department of Chemistry of Atatürk University.

	Chemical Structure	Chemical Formula	Moleculer Weight (Da)
OG1(9)		$C_9H_{11}NO_4$	197,19
OG2 (10)		$C_{10}H_{13}NO_4$	211,22
OG3 (11)		$C_{14}H_{13}NO_4$	259,26
OG4 (12)		$C_{15}H_{15}NO_4$	273,29

Scheme 5. Chemical structure of newly synthesized potential anticancer drugs

1.4. Cell-Based Methods

Determining and developing the therapeutic efficacy of any drug and treatment method for cancer is a challenging process. In-vitro tests are measurement methods performed in cell culture to evaluate substances whose toxic profile is being investigated or newly synthesized drugs. There are various cell-based tests to determine therapeutic efficacy and cell viability, proliferation assays, colony formation assays, colorimetric assays, binding assays, cell apoptosis assays, cell cycle arrest assays are some of them (Gordon, Brown et al. 2018).

1.4.1. Cell Viability and Proliferation Assays

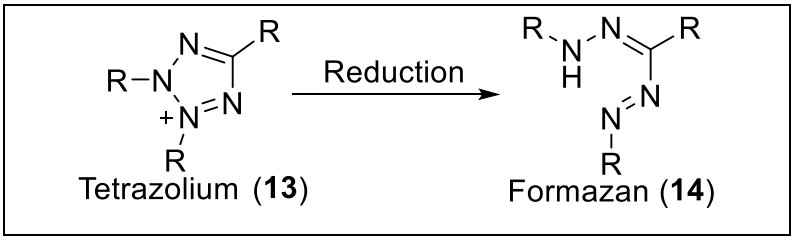
Cytotoxicity studies are conducted to determine whether a substance has a cytotoxic potential. Cell-based cytotoxicity studies have emerged as an alternative to animal experiments and have become frequently preferred in toxicology laboratories due to their ease of application and compatibility with data obtained from in vivo studies (Riss and Moravec 2004). There are various tests to determine the cytotoxicity of substances to be used on the cell. Cytotoxicity detection methods are generally colorimetric, luminescent, and enzymatic. The method is important to be consistent about what to measure, how to measure it, what is the limitation of the chemicals used.

Lactate Dehydrogenase (LDH) Release Cytotoxicity Assays is an enzymatic cytotoxicity detection method is to determine the lactate dehydrogenase (LDH) activity released from damaged/dead cells to the media (Korzeniewski and Callewaert 1983, Decker and Lohmann-Matthes 1988). Lactate dehydrogenase is a cytoplasmic enzyme found in all cells. When cells are exposed to toxic effects, their plasma membrane integrity is disrupted and the LDH enzyme leaks from the cells and enters the medium. Thus, cell damage can be evaluated by measuring LDH enzyme activity after exposure (Lappalainen, Jääskeläinen et al. 1994, Riss and Moravec 2006).

The ATP Bioluminescence Test is an example of a luminescence detection method. Following cell death, the cell's ability to synthesize ATP is lost and endogenous ATPases rapidly degrade the available ATP. For this reason, intracellular ATP content

has been qualified as the main indicator of cell viability and has taken its place among viability determination methods.

Tetrazolium Reduction Cell Viability Assays is the most common cell viability test and is a calorimetric measurement method. The reduction of tetrazolium salts by gaining electrons enables them to turn into formazan, resulting in a color change (Scheme 6). The tetrazolium ring can only be broken by active mitochondria so that only living cells can produce the color reaction (Mosmann 1983). If dead cells lose their ability to reduce tetrazolium compounds and do not cause any color change (Riss and Moravec 2006).



Scheme 6. The chemical reaction of reduced to formazan

(Source: Kregiel 2012)

Viability tests with tetrazolium compounds are carried out in three stages. In the first stage, cells are exposed to a toxic substance for a certain period. In the second step, the toxic substance is removed and the tetrazolium compound is added to the medium and incubated for an average of 1-4 hours. Meanwhile, living cells reduce the related compounds and convert them to formazan, and they change color. At the last stage, the number of live/dead cells is determined by measuring the color change with the spectrophotometric method (Mosmann 1983, Riss and Moravec 2004).

There are various tetrazolium compounds with different properties such as MTT, XTT, MTS, WST-1, WST-8.

1.4.2. Cell Apoptosis

While new cells are formed in living things, some of the existing cells are eliminated by cell death, thus maintaining a stable balance. These existing cells are destroyed by various types of cell death, such as physiological, programmed cell death, which is apoptosis, necrosis, and autophagy which is pathological cell death (Ellis, Yuan et al. 1991, Bellamy, Malcolmson et al. 1995).

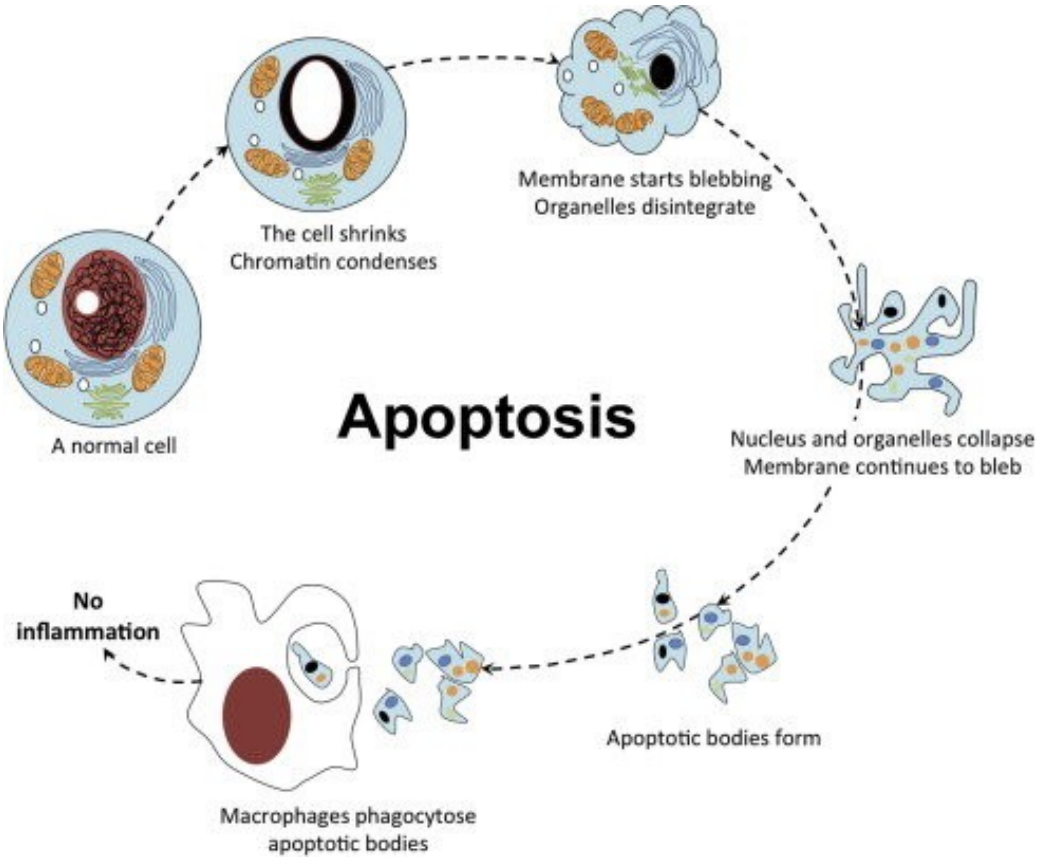


Figure 6. Apoptosis pathway

(Source: Abou-Ghali and Stiban 2015)

Apoptosis is an event in which the cell engages a series of metabolic and physiological processes to self-destruct. The cell that receives apoptosis stimulus moves

away from its environment, breaks its connection with neighboring cells, and shrinks, chromatin condenses and takes a pycnotic appearance. Its DNA is cut from its nucleosomes and takes the typical ladder band appearance in gel electrophoresis. However, cell organelles maintain their structural integrity. Phosphatidylserine in the cell membrane structure is translocated from the inner surface of the cell membrane to the outer surface. The core shrinks breaks into pieces. The cell breaks off into membrane-bound buds and separates into apoptotic bodies. Apoptotic bodies are recognized and phagocytosed by macrophages, but inflammation is not seen. (Ellis, Yuan et al. 1991, Bellamy, Malcolmson et al. 1995).

In general, molecules such as calcium, ceramide, Bcl-2 family, proteins such as p53, caspases, cytochrome-c, and mitochondria play a role in the regulation of apoptosis. During the apoptotic process, there is a constant influx of calcium into the cell. Calcium ions; Involved in endonuclease, protease, and transglutaminase activation, gene regulation, and cytoskeletal organization.

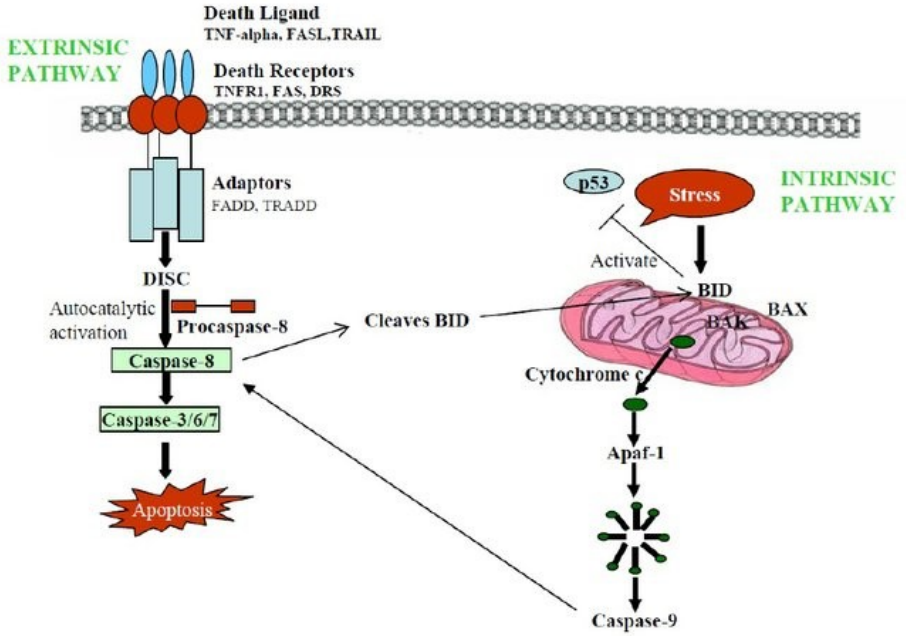


Figure 7. Extrinsic and Intrinsic pathway of apoptosis

(Source: Rampal, Khanna et al. 2012)

The mechanism of apoptosis occurs in two ways. Intracellular signals triggering apoptosis; DNA damage, increase in intracellular calcium level, decrease in pH, metabolic and/or cell cycle disorders, and hypoxia. Extracellular signals are deficiency of growth and reproduction factors, activation of death receptors, cytotoxic T lymphocytes, and external factors. Caspases are involved in both signaling pathways. Intracellular signals activate the intrinsic apoptosis pathway, while extracellular signals induce apoptosis via the extrinsic pathway (Smaili, Hsu et al. 2000, Danial and Korsmeyer 2004, Kroemer, Galluzzi et al. 2007).

Apoptosis is an important pathway that prevents mutant cell proliferation as well as normal cell death.

Necrosis is a random process that cannot be controlled by genes. The most common cause is hypoxia. Toxic substances such as arsenic, cyanide, insecticides, and heavy metals cause necrosis. During necrosis, mitochondrial ROS production increases, nonapoptotic proteases are activated, ATP production decreases, and Ca++ channels are opened (Nicotera and Melino 2004, Golstein and Kroemer 2007).

Autophagic cell death is a mechanism that occurs by the fusion of intracellular macromolecules and organelles enclosed in a vacuole with primary lysosomes and fragmentation. In this way, anabolic and catabolic cell functions are balanced and unwanted unnecessary organelles are eliminated. Digested organelle components are recycled and used for cell growth and development. Part of the cytoplasm or an organelle is first surrounded by the extracellular membrane of the endoplasmic reticulum. Primary lysosomes combine with this structure and secondary lysosome, that is, autophagic vacuole (autosome = autophagosome) is formed and is broken down by hydrolytic enzymes (Gozuacik and Kimchi 2007). Unlike apoptosis, nuclear condensation occurs much later. DNA breaks and apoptotic body formation are not observed (Gozuacik and Kimchi 2007).

1.4.3. Cell Cycle

Three important processes determine the total number of cells in an organism. These; cell division provided by the formation of two daughter cells from one cell,

programmed cell death required to destroy damaged cells, and activation of differentiated cells. Homeostasis is maintained by cell proliferation, cell growth arrest, and apoptosis (Bellamy 1997).

The process by which a cell grows and then divides is called the cell cycle. In continuously dividing cells, after mitosis, the cycle is repeated as G1, S, and G2 (interphase) and M (mitosis). In this process, cell stimulation and growth occur or as long as they do not receive a division signal, they remain in the resting phase G0 (Bellamy 1997, Vermeulen, Berneman et al. 2003). G1, S, G2 phases (Interphase) cover 90% of the cell cycle and last for 16-24 hours. Mitosis takes 1-2 hours. Cell growth is coordinated by the restriction point (R point) in the G1 phase. At the limiting point, the cell will either stop or complete the cell cycle (Vermeulen, Berneman et al. 2003). In the G1 phase, cells control their environment, receive signals and induce growth. In this phase, preparations are made for DNA synthesis (replication). RNA and protein synthesis occurs. In the S phase, after DNA synthesis, the cell continues to grow in the G2 phase, at the same time RNA synthesis, protein synthesis takes place and the cell is prepared for mitosis. Mitosis (M phase); It consists of prophase, metaphase, anaphase and telophase. In telophase, cytoplasmic division is completed and two new cells are formed with the same genetic material (Vermeulen, Berneman et al. 2003, Vermeulen, Van Bockstaele et al. 2003).

A large number of proteins play dual roles in the cell cycle and apoptosis. DNA damage caused by environmental factors disrupts cell cycle control mechanisms. Mutations in cell cycle checkpoints have been identified in many cancer types (Bellamy 1997). Growth arrest, DNA repair, and inhibition of apoptosis are critical pathways in cancer development. Mutations in tumor suppressor genes cause cell cycle progression of damaged cells and tumor development (Bellamy 1997). The p53 protein, which is also defined as the guardian of the genome, is a protein with complex activities that suppresses the cell cycle. p53 is a transcription factor that regulates the cell cycle. It is a very important protein that has a role in suppressing cancer in many organisms. p53 protein is also involved in cell growth arrest, programmed cell death, cell differentiation, and initiation of DNA repair mechanism. p53 mutant plays an important role in protecting the genome against cell proliferation (Bellamy 1997)

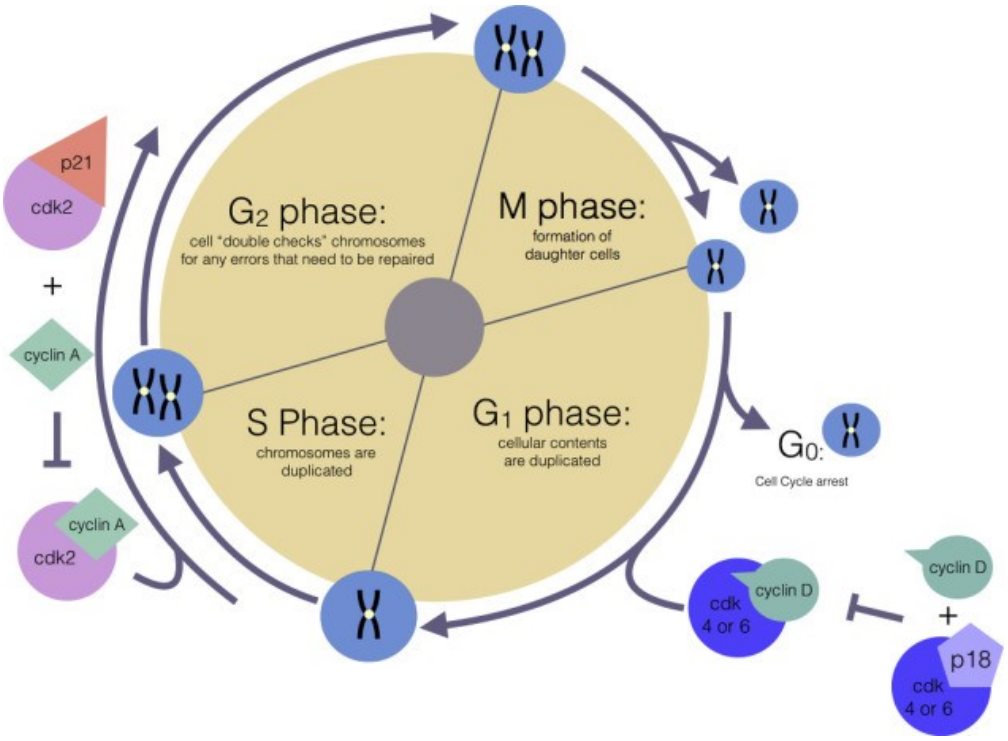


Figure 8. Cell cycle stages

(Source: Dupre, Sharp et al. 2018)

1.4.4. Cell Migration

Cell migration is an important function in sustaining the development of multicellular organisms. In addition, if cancer and atherosclerosis, abnormal cell migration is also a pathological disorder (Friedl, Sahai et al. 2012, Li, He et al. 2013). Cell migration consists of clustered cells or a single cell moving from one place to another (Grada, Otero-Vinas et al. 2017). There are two different types of migration. One is a single cell migration and the other is a collective cell migration. If more than one cell sticks to each other, exhibits collective polarization and colony skeletal activity and acts in a coordinated manner, it means that collective cell migration is taking place (Friedl, Sahai et al. 2012). In a scratch experiment, a wound is created that causes cells to migrate, and a cell monolayer is observed by monitoring the recolonization of the wound (Liang, Park et al. 2007). In in vitro tests; due to the simplicity of the experimental design and low cost, the wound healing assay is the preferred method for

analyzing cell migration (Liang, Park et al. 2007, Kramer, Walzl et al. 2013). In the wound migration assay, many different quantitative methods are used to evaluate cell migration, such as percentage of wound width at different time points (Walter, Wright et al. 2010, Ranzato, Martinotti et al. 2011), wound width or area change (Masuzzo, Van Troys et al. 2016, Grada, Otero-Vinas et al. 2017), and wound width formed at specific time points (Büth, Buttigieg et al. 2007, Topman, Sharabani-Yosef et al. 2012).

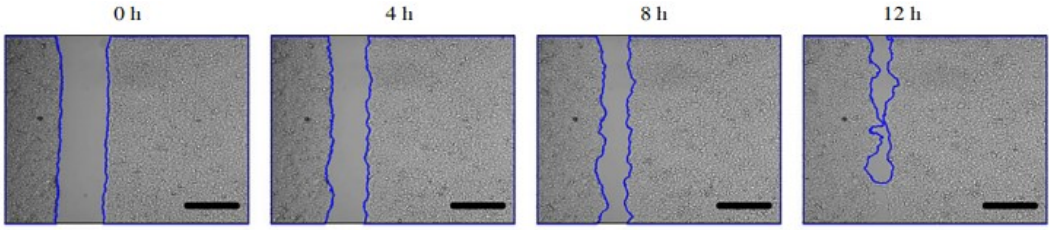


Figure 9. Images of cell migration

(Source: Bobadilla, Arévalo et al. 2019)

In the study of Bobadilla et al., a new quantitative method was discovered by a scratch assay using the human renal carcinoma cell line. The transition in scratch analysis in this method determined the rate of cell migration by characterizing it over time with a series of linear approximations to the cell velocity field (Bobadilla, Arévalo et al. 2019).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell Lines

A549 (human lung adenocarcinoma), HeLa (human cervical carcinoma), PC3 (human prostate carcinoma), and MCF-7 (human breast carcinoma) cell lines were obtained from the Biotechnology and Bioengineering Research Center. Caco-2 (human colorectal carcinoma) was provided by Izmir International Biomedicine and Genome Institute.

2.1.2. Culture Medias

The medium for HeLa and MCF-7 cell lines was Roswell Park Memorial Institute – 1640 (RPMI 1640 containing 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, and 1% L-glutamine. For A549, PC3, and Caco-2 cells, 10% of all volume Fetal Bovine Serum, 1% Penicillin-Streptomycin, and 1% L-glutamine were modified into Dulbecco's Eagle medium F12 (DMEM F12).

Mediums are not directly provided to cells they require specific additives. L-glutamine is an amino acid required for cell culture. L-glutamine participates in the formation of purine and pyrimidine nucleotides, amino sugars, glutathione, L-glutamate, and other amino acids, as well as in protein synthesis and glucose production. It is used as an auxiliary energy source. FBS contains growth factors to boost cellular growth and buffer components. It can provide the cell health, maintenance, and viability critical for

optimal performance and cell growth in a culture medium. The antibiotics penicillin and streptomycin are used to prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria.

2.1.3. Drugs

Drugs were supplied from Prof. Yunus Kara in Ataturk University Department of Chemistry, Organic Chemistry Laboratory. Stock solutions were prepared with dimethyl sulfoxide (DMSO) by using the powder form of drugs.

2.1.4. Chemicals, Solutions, and Reagents

All chemicals, reagents, and solutions used were presented in Appendix A

2.2. Methods

2.2.1. Thawing the Frozen Cells

Cells frozen at -80°C in a 2 mL cryotube were taken from the storage and thawed by shaking in a 37°C water bath for 1-2 minutes. Approximately 4 mL of cell medium was used for dilution and transferred to 15 mL of a sterile falcon. After, the cell suspension was centrifuged at 800 rpm for 5 minutes at +4°C to remove the DMSO contained. The supernatant was removed and cells were re-suspended with 5mL of fresh medium then transferred to a 25 cm^2 cell culture flask. The cells were incubated at 37°C, 5% CO_2 incubator.

2.2.2. Passaging the Cells

First of all the biosafety cabinet (class II) was UV sterilized for 15 minutes and cleaned using 70% ethanol solution. All solutions and medias to be used before cell passaging were warmed at 37°C water bath. Cells at 80% confluence were taken from the incubator and passaging. The old medium in the 75 cm^2 cell flask was removed and washed gently with 9 mL of sterile PBS (3 mL per 25cm^2 surface area). Then 3 mL of trypsin (1 mL per 25cm^2 surface area) was added and put in the 37°C, 5% CO_2 incubator for 2-3 min to activate and separate the cells from the flask surface. When all the cells were removed from the surface, 9 mL of fresh medium (3 mL per 25cm^2 surface area) was added and washed. Then the cell suspension was transferred to the 15 mL sterile falcon and centrifuged at 800 rpm for 5 minutes. After centrifugation, the supernatant was removed. The cell pellet was resuspended in a fresh medium (depending on the cell type and cell density) and 1 mL was taken and transferred to a new cell flask. The cells were then placed in a 5% CO_2 at 37°C incubator for growth.

2.2.3. Freezing the Cells

After the cell pellet was re-suspended in 15 mL falcon during cell passaging, 10% of dimethyl sulfoxide (DMSO) and 10% of FBS were added to the total suspension volume by gentle pipetting for homogenized and transferred to 2 mL cryotubes. Then cells in the stocked cryotube were stored at -80°C to ensure cell continuity.

2.2.4. Counting the Cells

After centrifugation during the cell passage, 100 µL of cells re-suspended with the new medium were taken and placed in a 2 mL eppendorf. 100 µL of 0.4% trypan blue solution was added to the cell suspension and mixed well by pipetting. A small amount was taken from the mixed suspension on the hemocytometer for counting. Then

living cells that appeared opaque in the squares on the hemocytometer were counted under the microscope. The following equation was used to calculate the number of cells in 1 mL.

Number of cells per mL = average counted of cell number per square x dilution factor x 10^4

2.2.5. Cell Viability Assay (MTT Test)

The cytotoxicity of the newly synthesized isoindole derivatives evaluated by the colorimetric MTT assay. 3×10^3 of A549, HeLa and MCF-7 cells, 5×10^3 of PC-3 cells and 8×10^3 of Caco-2 cells were seeded into a 96-well plate at 95 µl in each well and placed in a 5% CO_2 at 37°C incubator for 24 hours for cells to grow. All compounds were dissolved in sterile dimethyl sulfoxide (DMSO) and diluted to the appropriate concentrations with culture medium (1000-500-250-100-50-25 µM). The final DMSO concentration was 1% in all wells. Cells from the 96-well plate kept in the incubator for 24 hours to adhere to the surface were taken from the incubator and 5 µl of the drug at the appropriate concentration was added to each well. Drug-treated cells were incubated for 24, 48, and 72 hours to determine cytotoxic effects. At the end of each incubation period, 10 µL of MTT dye solution (5 mg / mL PBS) was added to each well and placed in the 5% CO_2 at 37°C incubator to reduce the tetrazolium salt. After 3.5 hours of incubation, the 96-well plate was centrifuged at 1800 rpm for 10 minutes and the supernatant was removed. Then the formazan crystals were dissolved in 100 µl of DMSO and shaked for 15 min to homogenize. Optical density was determined at 540 nm (Varioscan Flash).

2.2.6. Apoptosis Analysis

FITC Annexin V Apoptosis Detection Kit containing PI on A549, HeLa, PC3, MCF-7, and Caco-2 cell lines was used to determine the apoptotic effects of the compounds. $5x10^5$ cells in 1980 µL were seeded in a 6-well plate and incubated to

adhere for 24 hours at 37°C 5% CO_2. After the incubation period, test compounds dissolved in sterile DMSO and added to 20 µL of in each well at their IC_{50} concentrations. The treated cells were incubated for 48 hours in a CO_2 incubator at 37°C. Then all media were collected in their tubes and washed with 1 mL of phosphate-buffered saline (PBS). Cells were harvested by 250 µL trypsin and all collected cells were again put in their tubes and centrifuged at 800 rpm for 5 minutes. The pellet was dissolved in 5 mL of PBS and centrifuged again. Then the pellet was re-suspended in 200 µL of binding buffer and 2 µL of Annexin V-FITC and 5 µL of propidium iodide were added respectively and tubes were incubated for 15 min at room temperature in the dark. Finally, tubes were analyzed to determine the apoptotic effect of compounds on cells by flow cytometer (FACSCanto, BD) (Çakmak 2011).

2.2.7. Cell Cycle Analysis

1980 mL A549, HeLa, PC3, MCF-7, and Caco-2 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well and incubated at 37°C, 5% CO_2 for 24 hours. The next day, cells adhered to the surface were treated with 20 µL of IC_{50} concentrations of test compounds and incubated at 37°C in a CO_2 incubator for 48 hours. After incubation time, cells harvested by 250 µL of trypsin and suspensions were collected in their tube. Tubes were centrifuged at 800 rpm for 5 minutes. The pellet was resuspended by adding 1 mL cold PBS on ice and 4 mL of -20 ° C ethanol, respectively, and incubated at -20°C for at least 24 hours for the fixed cell. After, the tubes were centrifuged at 1200 rpm for 10 minutes for the remove PBS and ethanol. Pellet was re-suspended with 5 mL of PBS and centrifuged at 1200 rpm for 10 minutes again. The supernatant was removed carefully and the pellet was re-suspended in 200 µl 0.1% Triton X-100 in PBS and 20 µL RNase A (200 µg/mL) was added. Cell suspensions were incubated for 30 minutes at 37°C. Then, 20 µL PI (1 mg/mL) was added and incubated at room temperature for 15 minutes. Tubes were analyzed by flow cytometry (BD FACSCanto) (Çakmak 2011).

2.2.8. Scratch Assay (Wound Healing Assay)

Glass lamellas were washed with 100% methanol and autoclaved then has been placed in 6-well plates. 1980 µL of A549, HeLa, PC3, MCF-7, and Caco-2 cells were cultured in 6-well plates with $5x10^5$ cells/well and waited for 24 hours to attach the surface of the lamellas. When the cell confluence was approximately 90%, three parallel lines were created by using a 200 µL pipette tip with a gap of approximately 1 mm. The old medium was removed and washed with 1 mL of PBS in each well and a 1980 µL fresh medium was added. All compounds were dissolved in DMSO according to IC_{50} concentration, and 20 µL were added to each well (final DMSO concentration was 1% in a well). The same procedure was applied without the drug as a negative control. The migration was monitored for 0 and 48 hours under the microscope (Zeiss Z1 Inverted Fluorescence Microscope).

2.2.9. Statistical Analysis

Each analysis was performed in at least three replicates. Cell viability, apoptosis, cell cycle composition, and scratch assay gap closure rates, averages, and standard error means in the experiments performed were analyzed using Graphpad Prism 8 (GraphPad Software Inc., USA) using the nonlinear regression (curve fit) dose-response-inhibition method compared to the control groups. The visual graphs of the results were drawn using Origin 2016 (OriginLab Corporation, USA).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cell Viability Assay

The cytotoxic effects of newly synthesized OG1, OG2, OG3, and OG4 compounds were evaluated on A549, HeLa, PC3, MCF-7, and Caco-2 cell lines at 24, 48, and 72 hours incubation time by using the MTT test. Six different concentrations (1000-500-250-100-50-25 μM) were selected for each drug. IC_{50} concentrations, which inhibited 50% of the cells at each incubation time, were calculated using GraphPad Prism 8 (GraphPad Software Inc., USA).

For the A549 cell line treated with OG1 drug, low significant dose-dependent cytotoxic effect was observed on cell viability at the end of 24 hours incubation, while a dose-dependent cytotoxic effect was observed at the end of 48 hours. In the 72 hour treatment, most cell deaths occurred, and the viability was between 11% and 66% (Figure 10-a). Considering the effect of the OG2 drug, although dose-dependent cell viability was linear at 24 hours, the effect of the drug on cell viability was greater at the end of 48 hours, and a significant decrease was observed in cytotoxicity, especially at a concentration of 250 μM and above. At the end of the 72 hour incubation period, the viability decreased to 11% (Figure 10-b). When the cytotoxic effect of the OG3 drug was examined, it was observed that the viability was between 41% and 70% in 24 hours, while it decreased to 54% even at low concentrations in 72 hours of treatment (Figure 10-c). While a decrease in viability was observed depending on the incubation time and dose for the A549 cell line treated with OG4 drug, it was determined that the cytotoxic effect was the highest at 72 hours (Figure 16-d). According to IC_{50} values, it was determined that OG4 was the most effective drug in A549 cells (Table 1).

The cell viability rate of the OG1 drug, which was treated in the HeLa cell line, decreased depending on the incubation time, especially after 250 μM and above (Figure

11-a). At the end of 24 hours, the effect of OG2 on viability was between 24% and 64%, while it decreased to 9% in 72 hours (Figure 11-b). On the other hand, in OG3, similar cytotoxic effects were observed at low concentrations in all incubation periods, while at higher doses (1000-500-250 µM) the viability decreased to 8% (Figure 11-c). At the end of 48 hours, OG4 was the most effective in the dose-dependent decrease in cell viability. The cell viability rate remained between 9% and 54% in 72 hour treatment (Figure 11-d).

The inhibition rate of OG1 in the PC3 cell line decreased depending on the incubation period (Figure 12-a). For the OG2 drug, the viability of the 25-50-100 µM concentration in the 24-hour incubation was very high compared to the other doses, while it showed its cytotoxic effect in the 72-hour treatment (Figure 12-b). While there were similar results for OG3 at all incubation times, a significant decrease in cell viability was observed at 250 and 500 µM (Figure 12-c). It was determined that OG4 was the most effective drug for reducing cell viability for the PC3 cell line. Even at the low concentration of 25 µM, the viability decreased from 75% to 53% (Figure 12-d).

While the viability was between 42% and 68% in treatment with the OG1 drug on the MCF-7 cell line, it decreased between 26% and 55% in 72 hours (Figure 13-a). Cell viability was similar between 24 and 48 hours incubation period in cells treated with OG2 drug, and the effect on cytotoxicity at 250-500-1000 µM concentrations at the end of 72 hours was almost the same (Figure 13-b). In MCF-7 cells exposed to OG3, while there was a dose-dependent decrease, it was determined that it was not very dependent on the incubation period (Figure 13-c). OG4 showed its effect by reducing the viability rate below 37% at 250 µM concentration in 72 hours (Figure 13-d).

It was determined that the OG1 drug, whose effect on the Caco-2 cell line was investigated, had a low dependence on the treatment time at 25-50-100-250 µM (Figure 14-a). At 25 µM, which is the low concentration for the OG2 drug, the cell viability rate is still high as the incubation time increases, while it shows its significant effect at 250 µM (Figure 14-b). In the treatment with OG3 drug, a decrease in cell viability is clear especially at 250 µM depending on the exposure time to the drug, and it has a lethal effect at high concentrations of 1000 and 500 µM (Figure 14-c). In cell viability studies with drugs for the Caco-2 cell line, it was determined that the most effective drug was OG4. At the end of the 72 hour incubation period, the cell viability did not change much

depending on the time but decreased to 19% for the 50 μM and upper doses (Figure 14-d).

In the comparison of cell viability tests performed on 48-hour cell lines, the optimum incubation time for use in other experiments was determined as 48 hours. Especially in the tests performed with HeLa, PC3, MCF-7, and Caco-2 in 24-hour incubation, the low difference in viability showed that 24 hours was not enough for the drug to dissolve in the medium and be transferred to the cell.

Cell viability tests were performed using various concentrations on A549 and MCF-7 cell lines treated with two newly synthesized isoindole derivatives, 8a, and 8b. Results showed 39% metabolic activity for the A549 cell line and 55% for MCF-7 at 50 μM concentration of compound 8a in 24 hours incubation period. In the study performed under the same conditions as 8b, cell viability was found to be 53% for A549 and 64% for MCF-7 cell lines, and the cytotoxic effects of the compounds increased depending on the incubation time. The cell viability analyzes performed are supported by these results (Köse, Bal et al. 2017).

Bhatia et al. determined the IC_{50} values in his study in HeLa cells with newly synthesized isoindole compounds as a cantharidin derivative. The low IC_{50} values of 0.100μM indicated that these compounds have high cytotoxicity (Kaur Bhatia 2017).

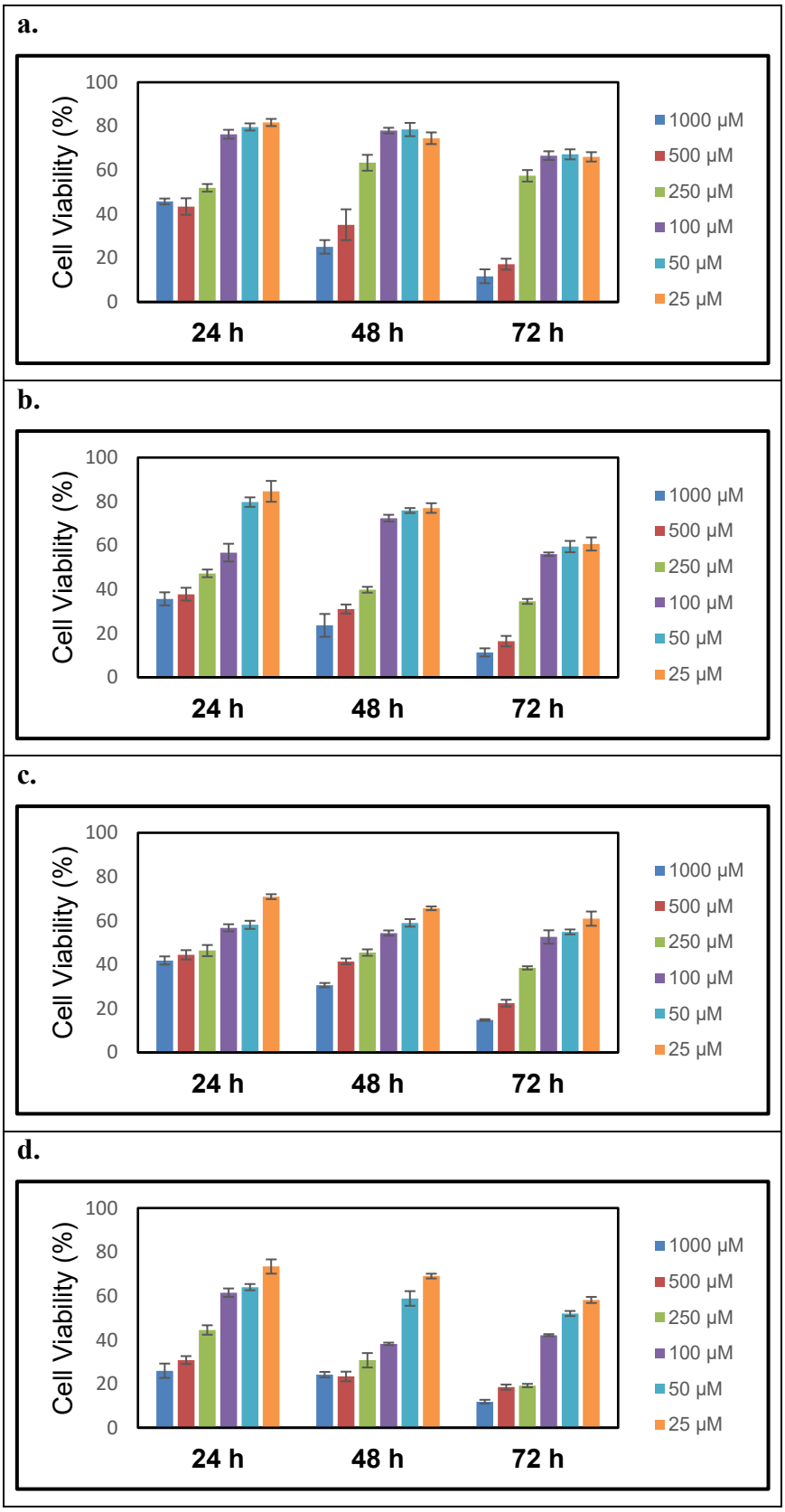


Figure 10. Cell viability rate results of (a) OG1, (b) OG2, (c) OG3, (d) OG4 on A549 cell at 24, 48, and 72 hours incubation period

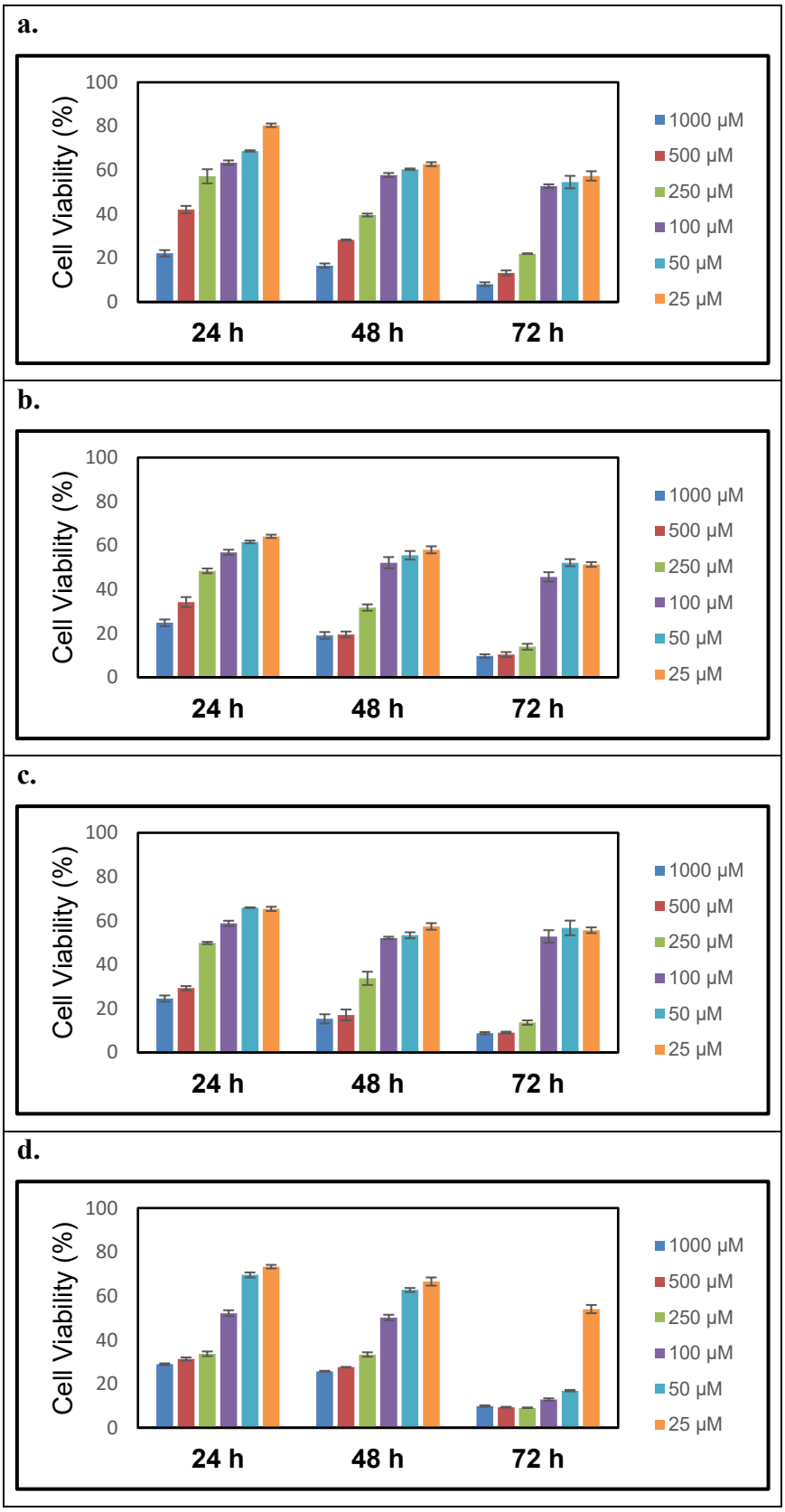


Figure 11. Cell viability rate results of (a) OG1, (b) OG2, (c) OG3, (d) OG4 on HeLa cell at 24, 48, and 72 hours incubation period

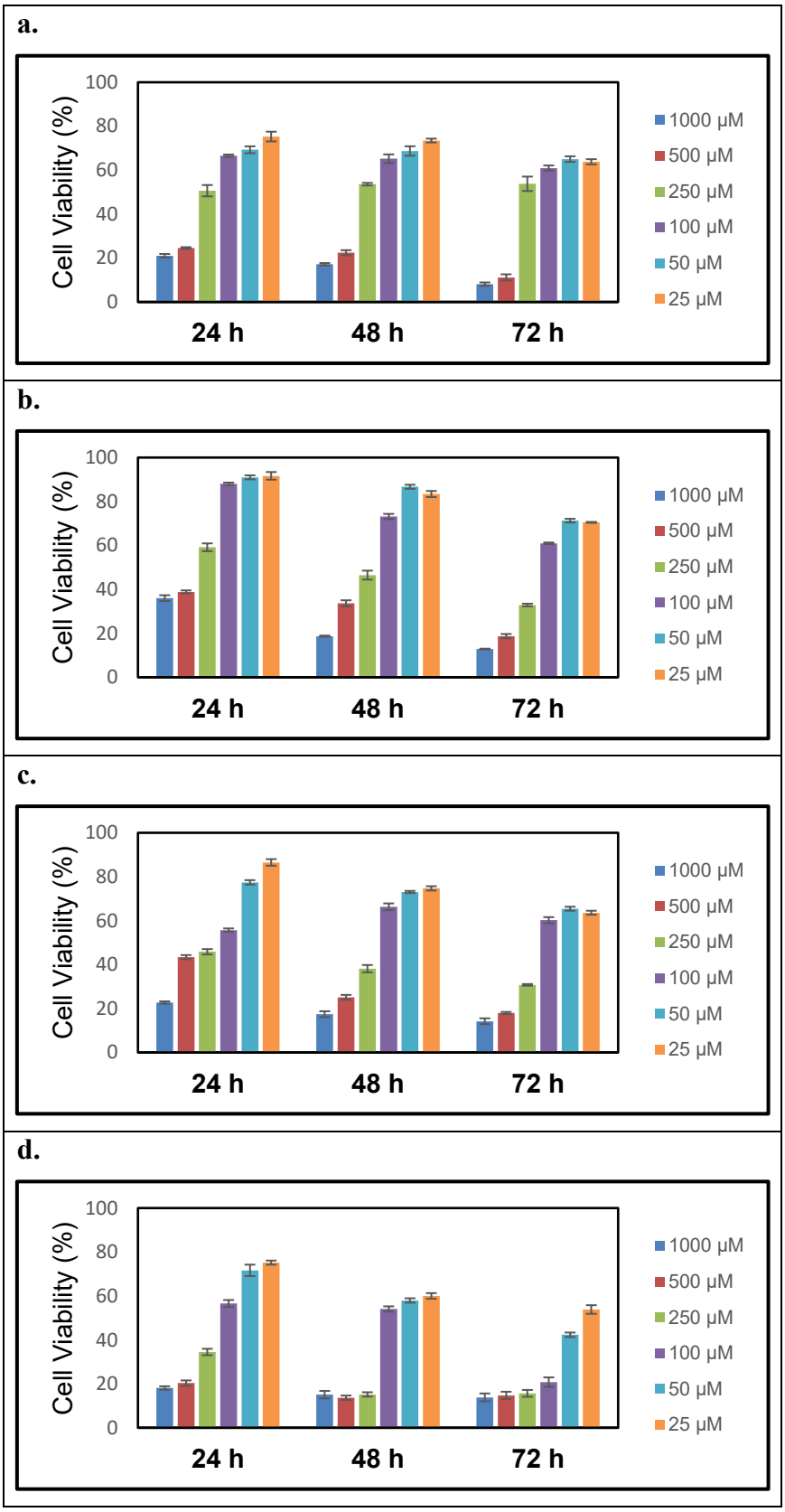


Figure 12. Cell viability rate results of (a) OG1, (b) OG2, (c) OG3, (d) OG4 on PC3 cell at 24, 48, and 72 hours incubation period

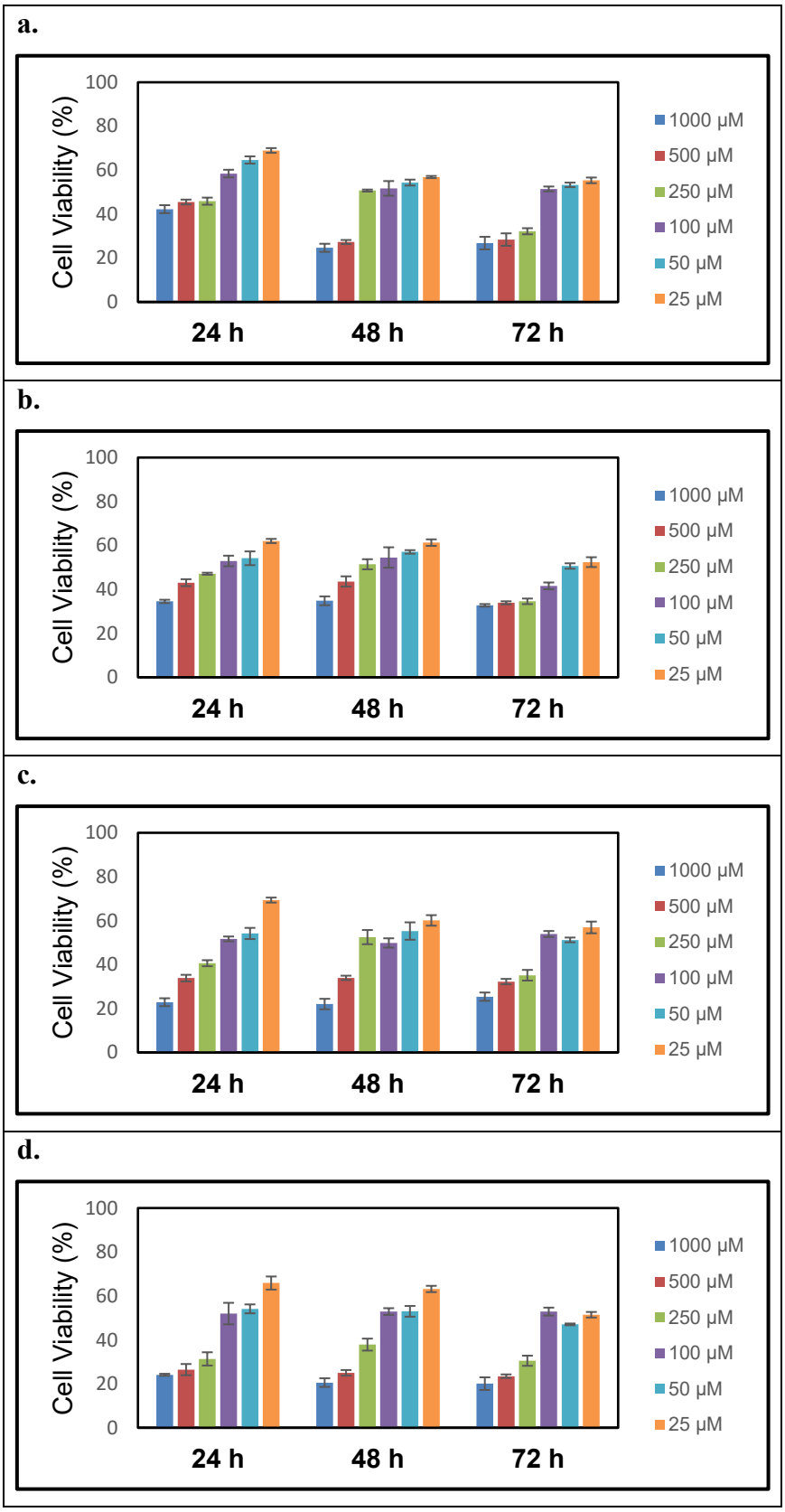


Figure 13. Cell viability rate results of (a) OG1, (b) OG2, (c) OG3, (d) OG4 on MCF-7 cell at 24, 48, and 72 hours incubation period

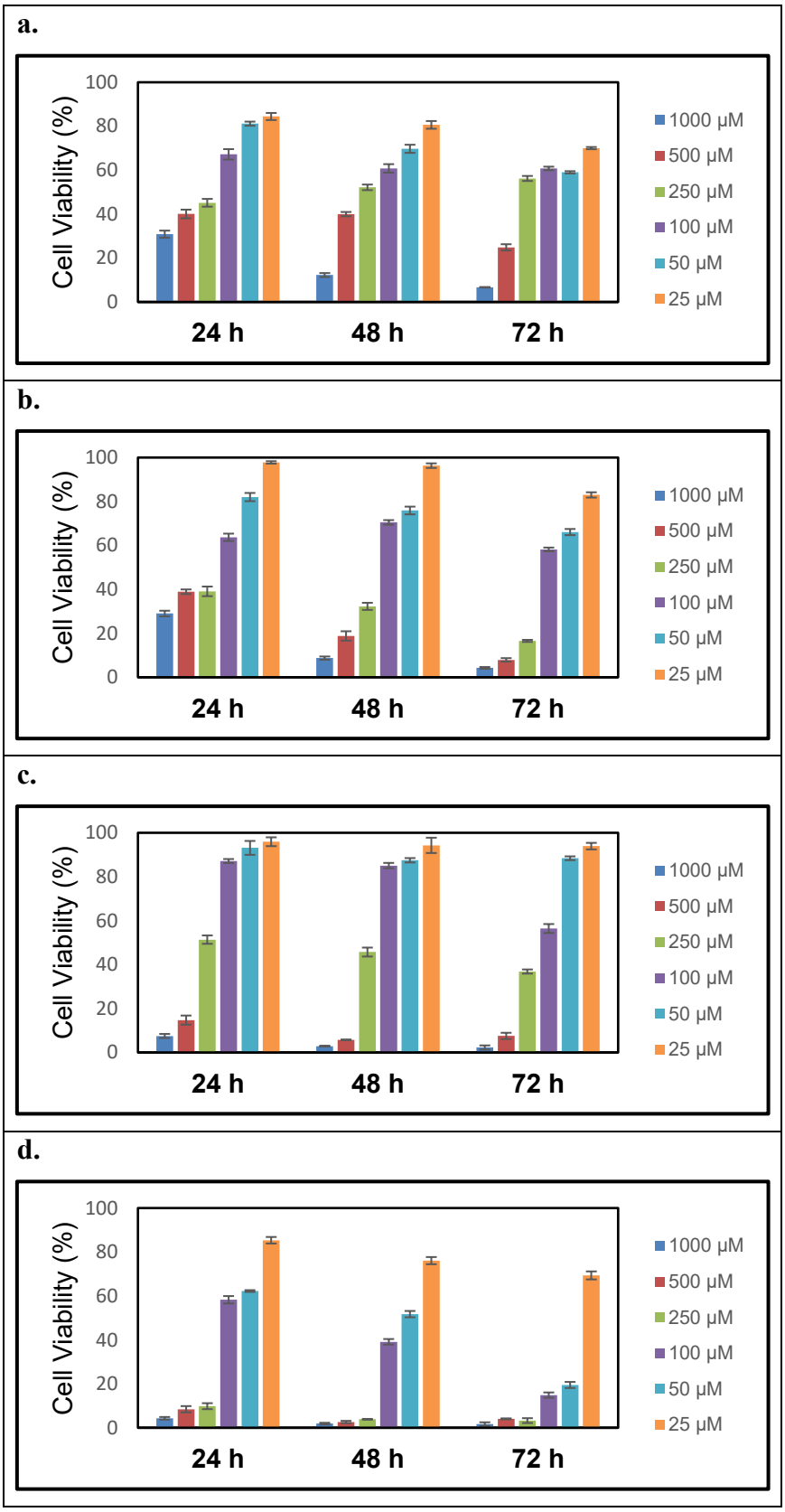


Figure 14. Cell viability rate results of (a) OG1, (b) OG2, (c) OG3, (d) OG4 on Caco-2 cell at 24, 48 and 72 hours incubation period

Table 1. IC_{50} values of A549 cells treated with OG1, OG2, OG3, and OG4 at 24, 48, and 72 hours incubation period. Results are expressed as means ± SEM

	OG1, µM	OG2, µM	OG3, µM	OG4, µM
24h	250,3 ± 44,6 (R^2 = 0,98)	233,8 ± 99,5 (R^2 = 0,81)	234,2 ± 59,1 (R^2 = 0,82)	215,6 ± 44,9 (R^2 = 0,98)
48h	307,8 ± 72,4 (R^2 = 0,90)	161,0 ± 45,8 (R^2 = 0,95)	192,3 ± 62,7 (R^2 = 0,90)	69,65 ± 12,8 (R^2 = 0,99)
72h	333,5 ± 47,4 (R^2 = 0,99)	135,6 ± 13,8 (R^2 = 0,99)	163,9 ± 14,3 (R^2 = 0,99)	67,35 ± 17,7 (R^2 = 0,95)

Table 2. IC_{50} values of HeLa cells treated with OG1, OG2, OG3, and OG4 at 24, 48, and 72 hours incubation period. Results are expressed as means ± SEM

	OG1, µM	OG2, µM	OG3, µM	OG4, µM
24h	353,3 ± 52,1 (R^2 = 0,91)	247,6 ± 95,7 (R^2 = 0,95)	246,6 ± 63,6 (R^2 = 0,97)	116,8 ± 39.5 (R^2 = 0,95)
48h	148,7 ± 86,5 (R^2 = 0,91)	155,2 ± 26,7 (R^2 = 0,96)	179,3 ± 69,0 (R^2 = 0,96)	99,81 ± 15,1 (R^2 = 0,99)
72h	130,3 ± 31,8 (R^2 = 0,98)	92,8 ± 10,6 (R^2 = 0,94)	135,3 ± 23,7 (R^2 = 0,98)	32,09 ± 6,1 (R^2 = 0,97)

Table 3. IC_{50} values of PC3 cells treated with OG1, OG2, OG3, and OG4 at 24, 48, and 72 hours incubation period. Results are expressed as means ± SEM

	OG1, µM	OG2, µM	OG3, µM	OG4, µM
24h	250,2 ± 96,7 (R^2 = 0,93)	265,0 ± 36,0 (R^2 = 0,94)	192,9 ± 50,1 (R^2 = 0,87)	177,1 ± 52,6 (R^2 = 0,89)
48h	338,8 ± 80,9 (R^2 = 0,92)	242,0 ± 48,9 (R^2 = 0,92)	204,7 ± 33,4 (R^2 = 0,99)	110,5 ±37,2 (R^2 = 0,97)
72h	299,8 ± 70,4 (R^2 = 0,84)	156,4 ± 36,4 (R^2 = 0,89)	119,6 ± 18,3 (R^2 = 0,84)	32,35 ± 15,2 (R^2 = 0,82)

Table 4. IC_{50} values of MCF-7 cells treated with OG1, OG2, OG3, and OG4 at 24, 48, and 72 hours incubation period. Results are expressed as means ± SEM

	OG1, µM	OG2, µM	OG3, µM	OG4, µM
24h	132,4 ± 25,5 (R^2 = 0,95)	212,6 ± 43,9 (R^2 = 0,88)	102,6 ± 24,1 (R^2 = 0,88)	113,8 ± 28,3 (R^2 = 0,91)
48h	263,3 ± 48,9 (R^2 = 0,98)	274,6 ± 51,4 (R^2 = 0,91)	302,1 ± 40,5 (R^2 = 0,97)	225,1 ± 20,1 (R^2 = 0,94)
72h	170,3 ± 50,5 (R^2 = 0,87)	96,4 ± 17,1 (R^2 = 0,93)	195,2 ± 38,1 (R^2 = 0,90)	144,9 ± 26,5 (R^2 = 0,95)

Table 5. IC_{50} values of Caco-2 cells treated with OG1, OG2, OG3, and OG4 at 24, 48, and 72 hours incubation period. Results are expressed as means ± SEM

	OG1, µM	OG2, µM	OG3, µM	OG4, µM
24h	183,0 ± 22,4 (R^2 = 0,91)	135,4 ± 48,4 (R^2 = 0,92)	246,2 ± 72,8 (R^2 = 0,83)	122,8 ± 32,1 (R^2 = 0,86)
48h	295,3 ± 96,6 (R^2 = 0,91)	136,3 ± 28,7 (R^2 = 0,94)	208,5 ± 42,0 (R^2 = 0,93)	77,41 ± 22,3 (R^2 = 0,95)
72h	235,7 ± 56,6 (R^2 = 0,90)	115,3 ± 29,3 (R^2 = 0,90)	190,9 ± 59,2 (R^2 = 0,86)	38,28 ± 11,4 (R^2 = 0,92)

3.2. Apoptosis Analysis

The apoptotic effects of OG1, OG2, OG3, and OG4 drugs, which are newly synthesized isoindole derivatives, on A549, HeLa, PC3, MCF-7, and Caco-2 cells were performed using FITC Annexin V Apoptosis Detection Kit containing PI. Cell lines treated with IC_{50} concentrations of drugs were analyzed by Flow Cytometry to determine their apoptotic composition versus control groups at the end of the 48 hour incubation period. The number of dead cells in each sample of 10,000 events was measured for the determination of the total rate. The untreated cells were used as the control group.

For the A549 cell line treated with IC_{50} doses of OG1, OG2, OG3, and OG4, the rate of late apoptosis of OG4 was higher than the other drugs, although the rates of

necrosis were similar for all drugs. This result was associated with OG4 having a greater cytotoxic effect on cell viability results of A549 (Figure 21).

It was determined that drugs for the HeLa cell line caused early, and late apoptosis compared to the control group. Among them, OG4 had the highest early apoptosis with 8%, while OG3 had the highest late apoptosis with 19% (Figure 22).

When the effects of OG1, OG2, OG3, and OG4 on PC3 cells are examined, it has been determined that both drugs cause apoptosis and while the early apoptosis rates caused by all drugs are similar, OG1 has the highest late apoptosis rate with 15% (Figure 23).

In MCF-7 cells, it was observed that the drugs did not cause direct cell death, necrosis, but generally increased apoptosis rates and decreased the number of viable cells (Figure 24).

OG4 had the lowest percentage of viable cells in the Caco-2 cell line (21%). This may be supported by the fact that OG4 has the highest cytotoxicity in Caco-2 in MTT assay results (Figure 25). When the results of apoptosis analysis in 48 hours incubation time are evaluated in general, the OG4 drug is more effective than other drugs in terms of apoptotic rates.

In the study of Sharma et al. in 2012, the biological activities of 34 newly synthesized diindolylmethane-containing compounds on HeLa, A549, and MCF-7 cancer cell lines were investigated. It caused 7d, an indole derivative, to arrest in the G1 phase in the HeLa cell line, and according to the results, it was determined that the effect of indole derivatives was less sensitive in the MCF-7 cell line compared to the HeLa and A549 cell line (Sharma, Rah et al. 2012).

Two novel compounds of Isoindole derivatives 5a and 5g were synthesized and their apoptotic effect on HepG2 (human liver) and MCF-7 (breast cancer) cell line were evaluated. The percentage of apoptotic cells increased from 1.65% to 21.25% for the HepG2 cell treated with 5a and the MCF7 cell line, the apoptosis rates of cells exposed with compound 5g increased from 1.92% to 18.26%. Ultimately, these two compounds are effective for apoptotic programmed cell death (Philoppes and Lamie 2019).

Diana et al. investigated the effect of Caspase-3 activation on programmed cell death in the Jurkat cell line, which they treated with 3-Methoxy-5H-isoindolo[2,1-

a]quinoxalin-6-one, and investigated that the compound led the cell line to apoptosis. Explained the cause of apoptosis with its ability to induce the activation of Caspase-3, as well as by activating Caspase-9 (Diana, Martorana et al. 2011).

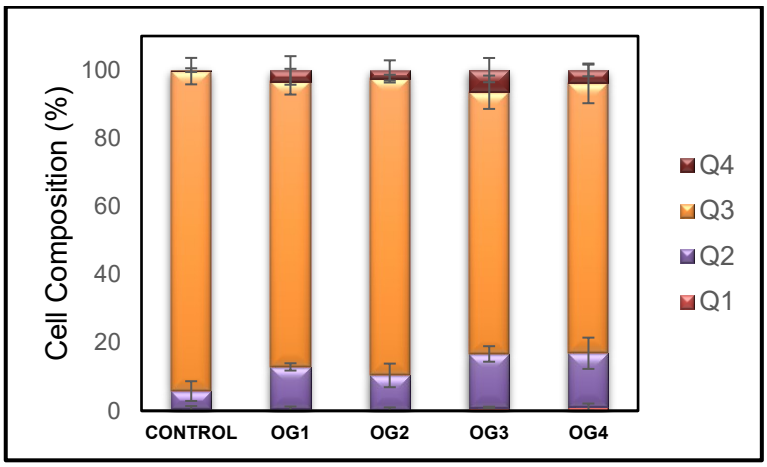


Figure 15. Apoptosis rates of A549 cells treated with IC_{50} doses of OG1, OG2, OG3, and OG4 at 48 hour incubation period. (Q1: necrosis, Q2: late apoptosis, Q3: live cell, Q4: early apoptosis)

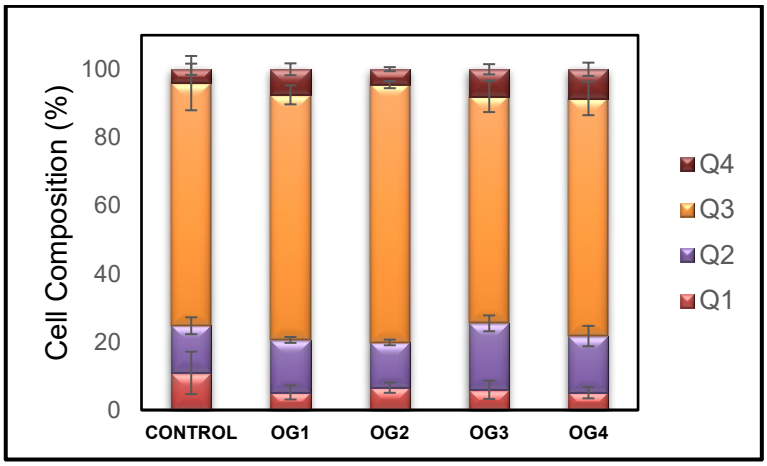


Figure 16. Apoptosis rates of HeLa cells treated with IC_{50} doses of OG1, OG2, OG3, and OG4 at 48 hour incubation period. (Q1: necrosis, Q2: late apoptosis, Q3: live cell, Q4: early apoptosis)

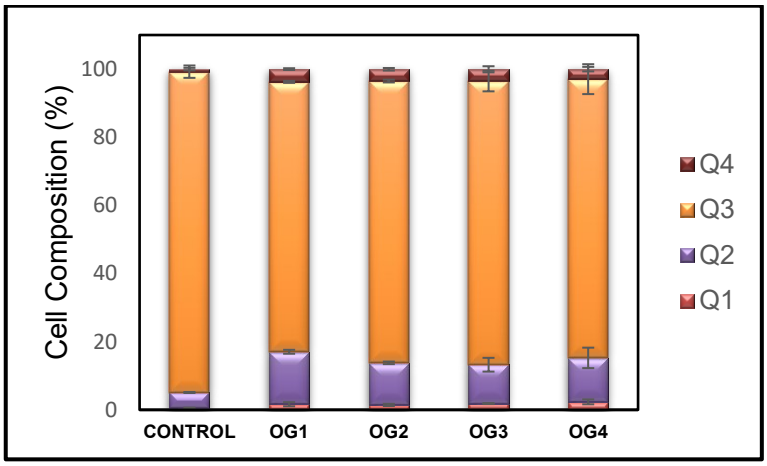


Figure 17. Apoptosis rates of PC3 cells treated with IC_{50} doses of OG1, OG2, OG3, and OG4 at 48 hour incubation period. (Q1: necrosis, Q2: late apoptosis, Q3: live cell, Q4: early apoptosis)

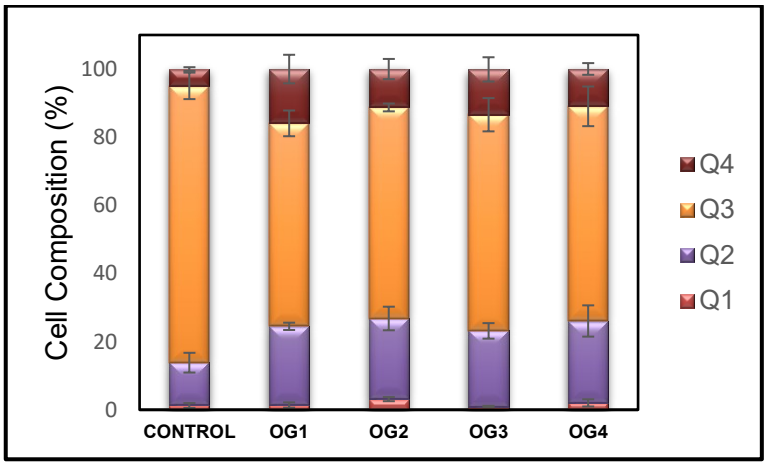


Figure 18. Apoptosis rates of MCF-7 cells treated with IC_{50} doses of OG1, OG2, OG3, and OG4 at 48 hour incubation period. (Q1: necrosis, Q2: late apoptosis, Q3: live cell, Q4: early apoptosis)

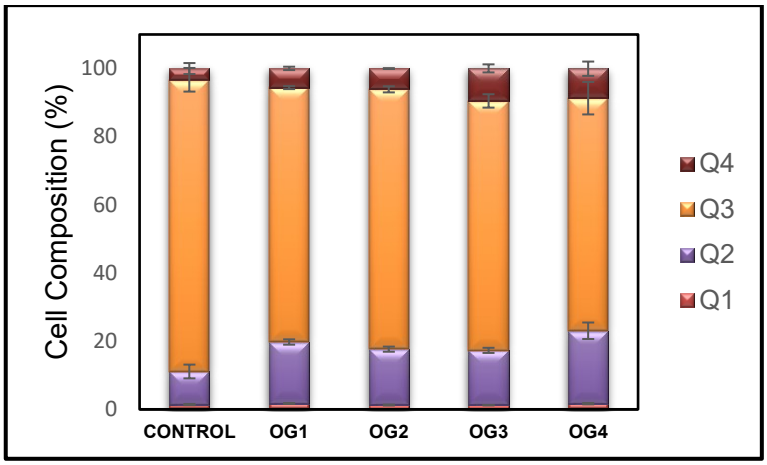


Figure 19. Apoptosis rates of Caco-2 cells treated with IC_{50} doses of OG1, OG2, OG3, and OG4 at 48 hour incubation period. (Q1: necrosis, Q2: late apoptosis, Q3: live cell, Q4: early apoptosis)

3.3. Cell Cycle Analysis

A549 was treated with IC_{50} doses of HeLa, PC3, MCF-7, and Caco-2 cell lines OG1, OG2, OG3, and OG4 for 48 hours and cell cycle analysis were performed and G1, G2, and S phase ratios were determined. Propidium iodide staining was used in cell cycle analysis, and it was calculated as 10000 events for each drug in the flow cytometer. The control group consists of untreated cells**.**

In the cell cycle analysis performed on the A549 cell line, OG1 and OG2 drugs had similar cell distribution, while OG3 and OG4 caused G2 phase arrest. In particular, the G2 phase ratio of OG4 increased by 17% and DNA duplicates were prevented (Figure 26).

For the HeLa cell line, all drugs were found to cause G2 arrest. G2 phase ratios are 5% for OG1, 6% for OG2, 9% for OG3, and 14% for OG4 (Figure 27).

For the PC3 cell line treated with IC_{50} doses of OG1, OG2, OG3, and OG4 drugs, the G1 phase was reduced at a similar rate in OG1 and OG2. In OG3 and OG4, the G2 phase increased almost at the same rate (Figure 28).

According to the phase ratio results of cell cycle analysis performed with MCF-7, it was determined that OG2 and OG3 drugs increased the G2 phase population by 13% and 12% respectively compared to the other drugs, thus preventing the division of cancer cells (Figure 29).

In the study performed on the Caco-2 cell line, it was determined that the drugs significantly increased the S phase, and also caused the G2 phase, especially in OG2 more (20%) (Figure 30).

Cell cycle analysis of compounds 5a and 5g synthesized as a cantharidin derivative was investigated using HepG2 and MCF-7 cell lines. The percentages in the G1 phase were found to be 55.37% for 5a and 48.22% for 5g, and 31.22% and 33.54% for 5a and 5g in the G2/M phase, respectively. The increase in the G1 phase showed that the synthesized compounds affected the growth mechanism of the cell (Philoppes and Lamie 2019).

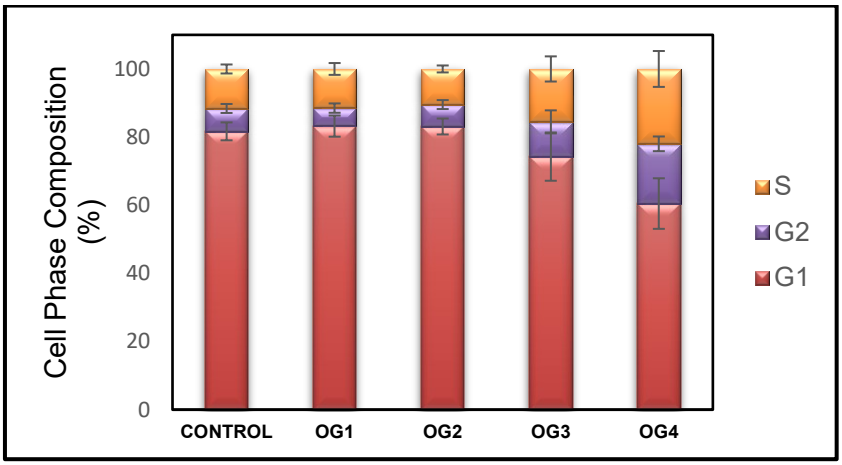


Figure 20. Percent cell phase distribution in G1, S, and G2 phases by treatment of A549 cells with IC_{50} doses of OG1, OG2, OG3, and OG4 for 48 hours

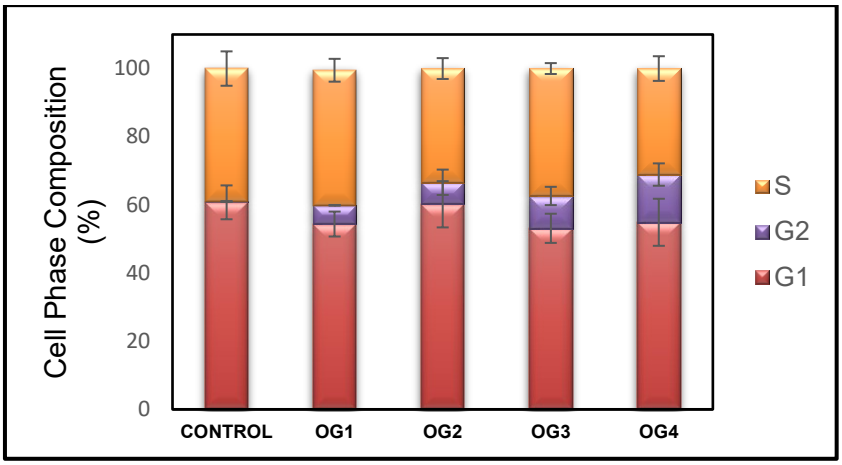


Figure 21. Percent cell phase distribution in G1, S, and G2 phases by treatment of HeLa cells with IC_{50} doses of OG1, OG2, OG3, and OG4 for 48 hours

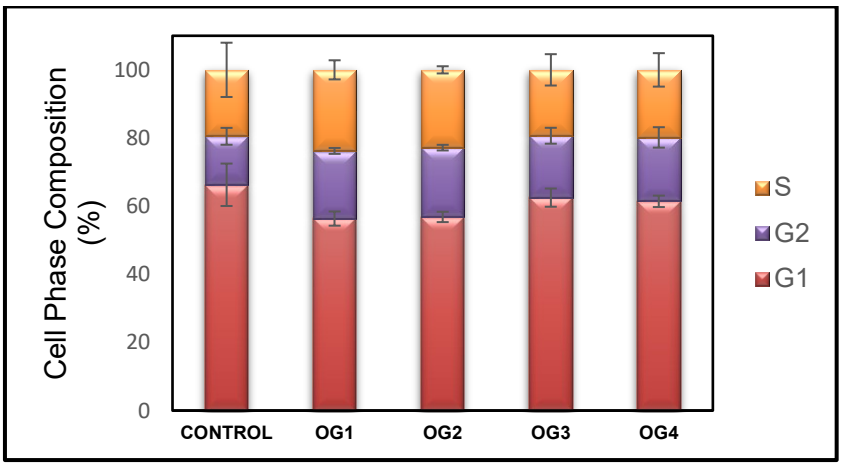


Figure 22. Percent cell phase distribution in G1, S, and G2 phases by treatment of PC3 cells with IC_{50} doses of OG1, OG2, OG3, and OG4 for 48 hours

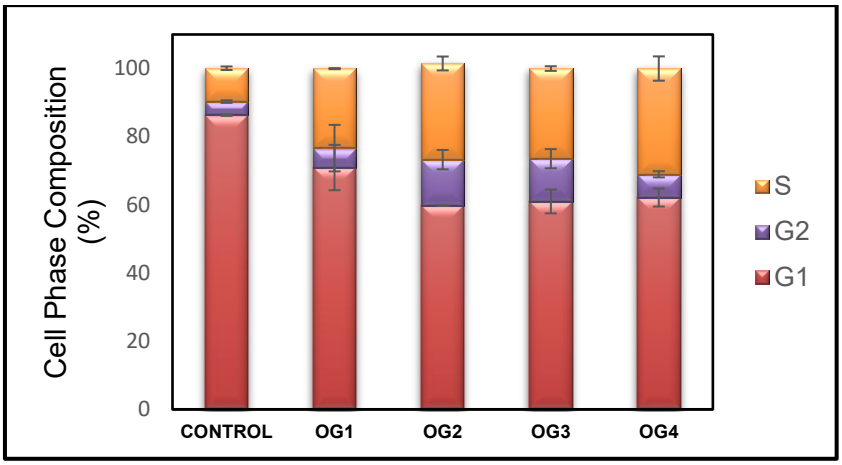


Figure 23. Percent cell phase distribution in G1, S, and G2 phases by treatment of MCF-7 cells with IC_{50} doses of OG1, OG2, OG3, and OG4 for 48 hours

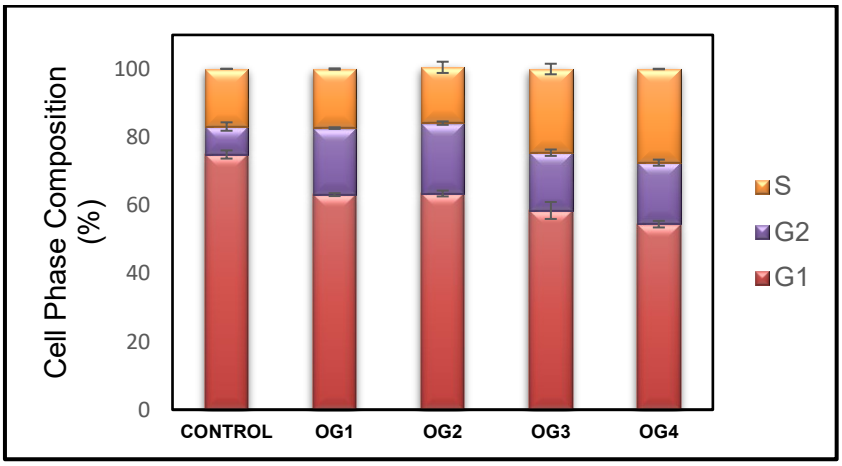


Figure 24. Percent cell phase distribution in G1, S, and G2 phases by treatment of Caco-2 cells with IC_{50} doses of OG1, OG2, OG3, and OG4 for 48 hours

3.4. Scratch Assay

0 and 48-hour migration effects of A549, HeLa, PC3, MCF-7, and Caco-2 cells exposed to IC_{50} dose of OG1, OG2, OG3, and OG4 drugs for scratch assay were

visualized using Zeiss Z1 Inverted Fluorescence Microscope with 200 μm scale. Also, closing gap areas were calculated using ImageJ software. Untreated cells were used as negative controls.

At the end of 48 hours of the scratch assay with A549 cell, it was determined that OG2 reduced migration the most according to gap closure percentages (Figure 36). However, when looking at the microscope images, it is seen that the number of dead cells increased in OG3 and OG4, and 15% and 14% closure was realized, respectively (Figure 31).

For the HeLa cell line, it was determined that OG3 had a greater effect on migration compared to other drugs. Then, OG4 had a significant effect in terms of gap closure with a rate of 21% (Figure 37).

OG4 (9%) showed the most reduction of the migration in terms of wound healing in the PC3 cell line, followed by OG3 (12%), OG2 (17%), and OG1 (22%), respectively. This result is supported by the cytotoxic effect of OG4 on PC3 (Figure 38).

In the scratch assay analysis performed on MCF-7, the lethal effect of the drugs on the cells is seen in the microscope images compared to the control. In addition, the highest inhibition of migration by drugs was detected in this cell line, and the gap closure rate was below 1% (Figure 39).

Among the OG1, OG2, OG3, and OG4 drugs used to heal the Caco-2 cell, the gap closure rate was the lowest at 7% and the most effective drug was OG4. The low migration rates compared to the control group is an indication of the loss of movement of the cells (Figure 40).

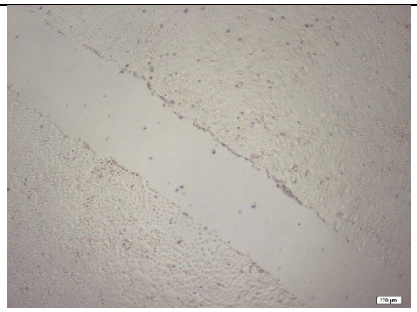
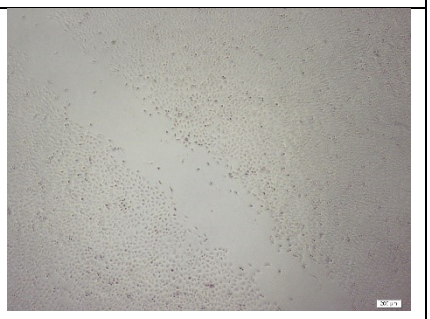
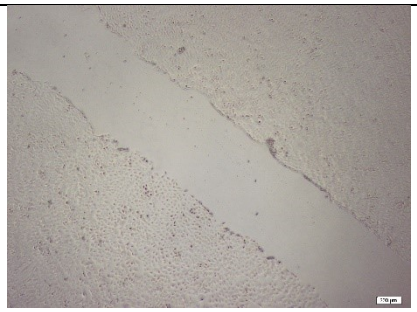
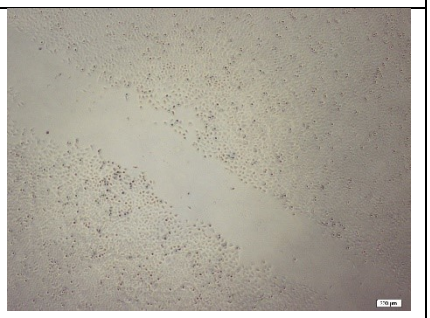
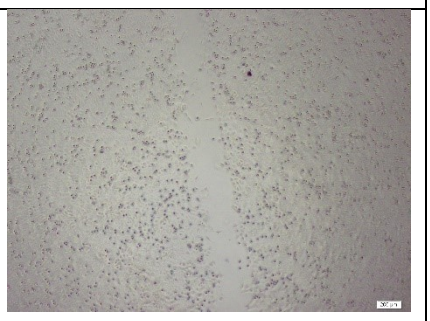
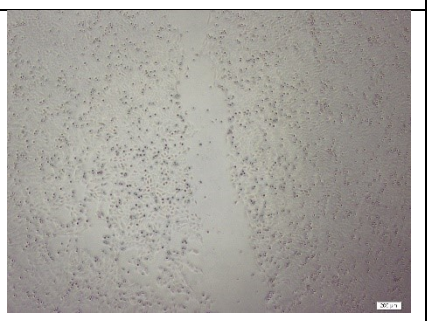
	0 h	48 h
Control		
OG1		
OG2		
OG3		
OG4		

Figure 25. Images of Cell migration on A549 monolayer in control and IC_{50} concentrations treated of OG1, OG2, OG3, and OG4 at 0 h and 48 h incubation

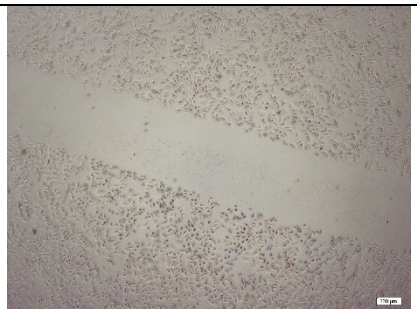
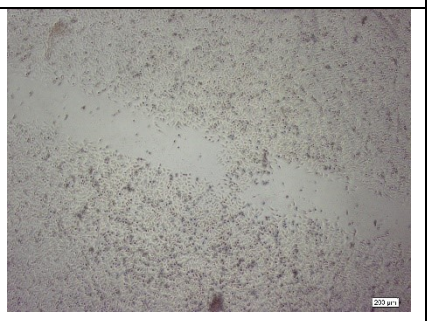
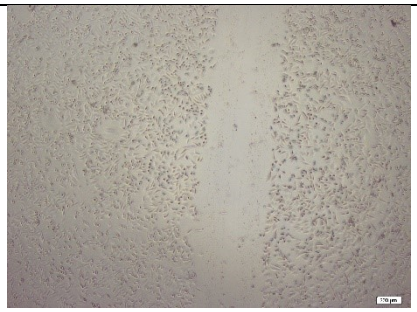
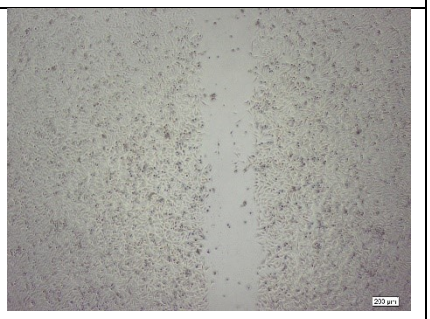
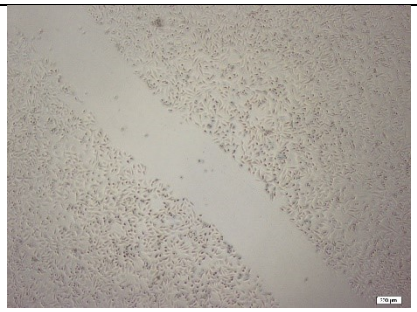
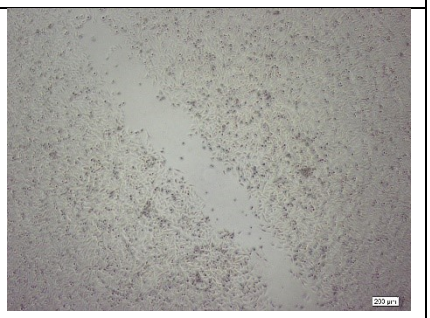
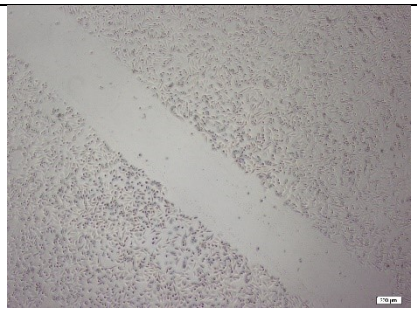
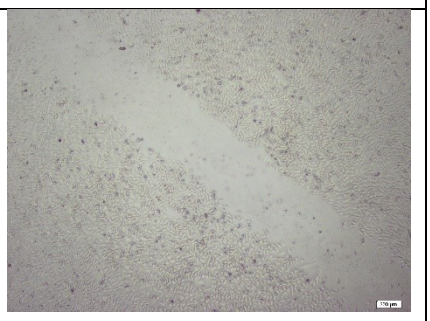
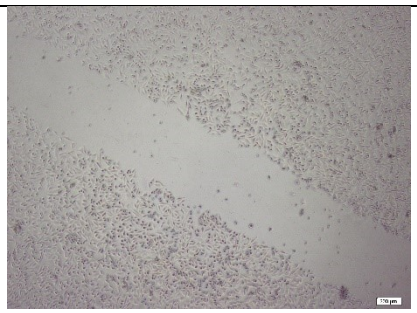
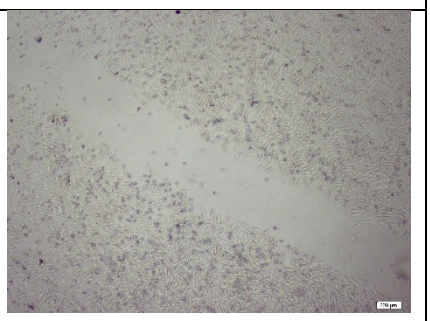
	0 h	48 h
Control		
OG1		
OG2		
OG3		
OG4		

Figure 26. Images of Cell migration on HeLa monolayer in control and IC_{50} concentrations treated of OG1, OG2, OG3, and OG4 at 0 h and 48 h incubation

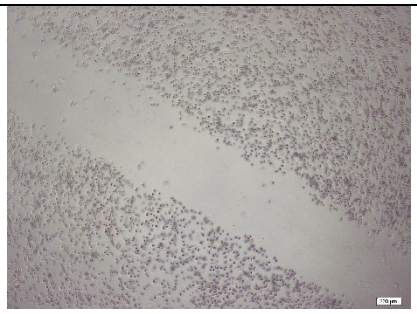
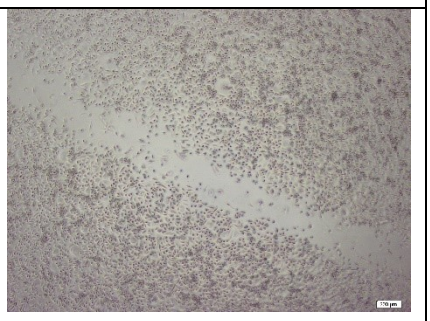
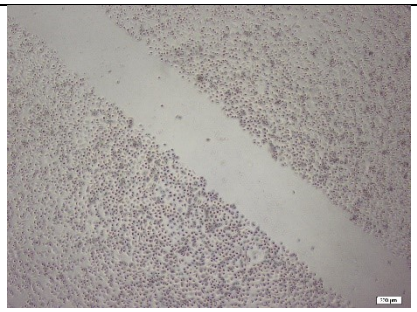
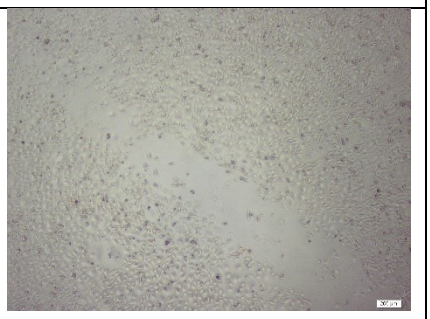
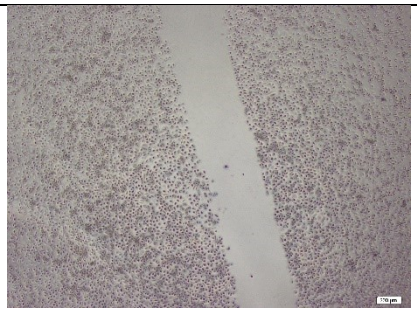
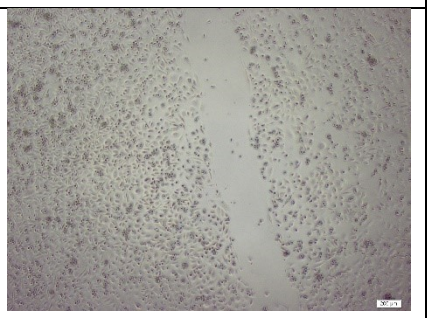
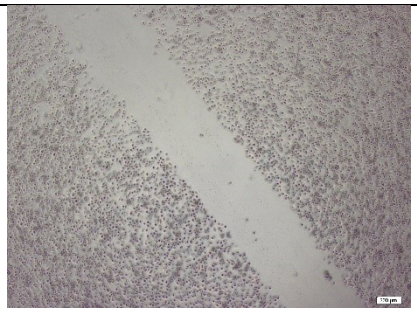
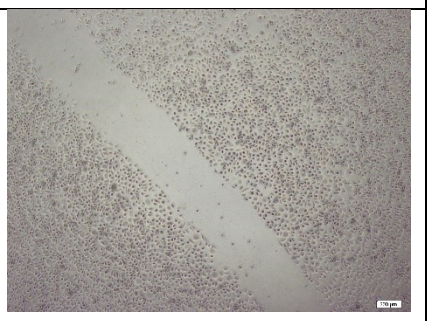
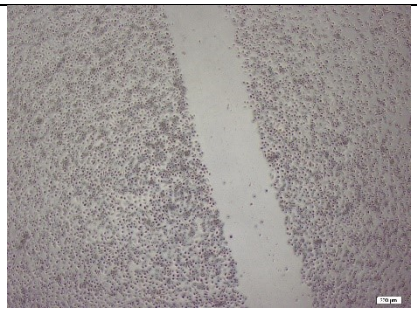
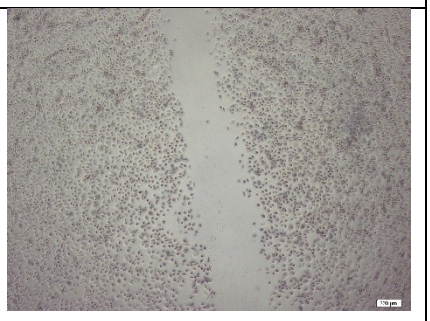
	0 h	48 h
Control		
OG1		
OG2		
OG3		
OG4		

Figure 27. Images of Cell migration on PC3 monolayer in control and IC_{50} concentrations treated of OG1, OG2, OG3, and OG4 at 0 h and 48 h incubation

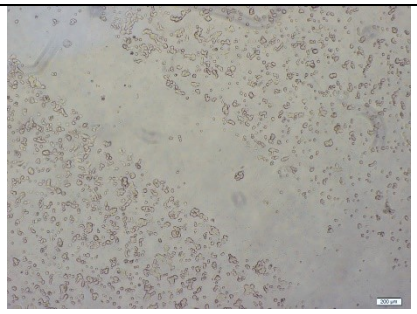
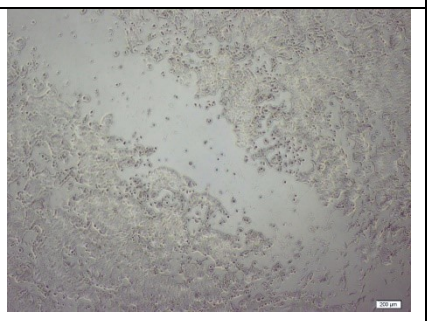
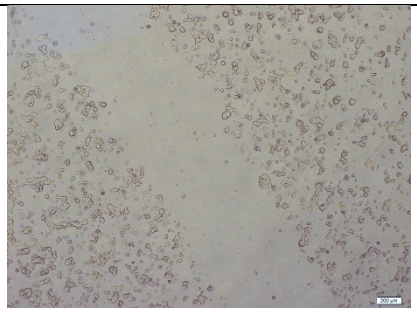
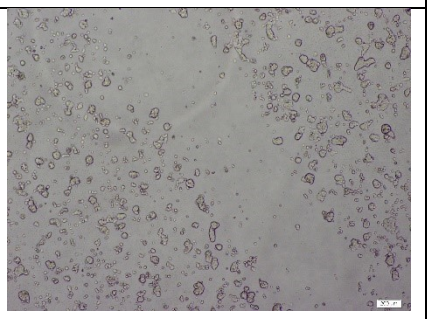
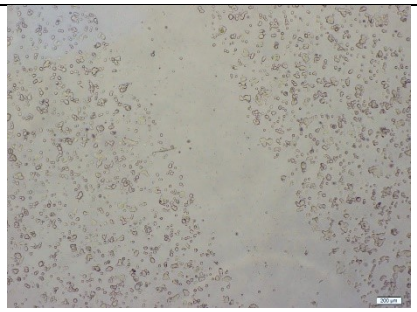
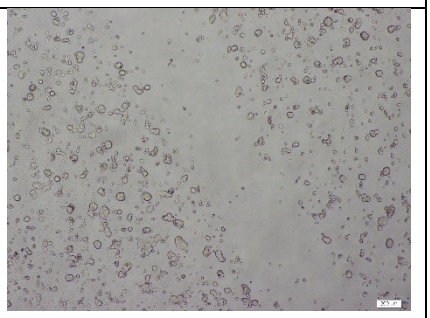
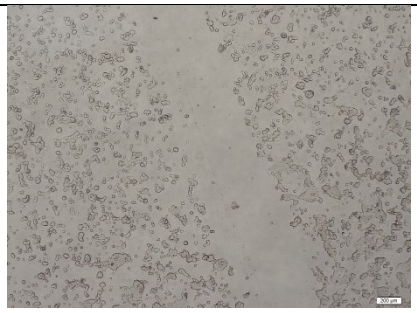
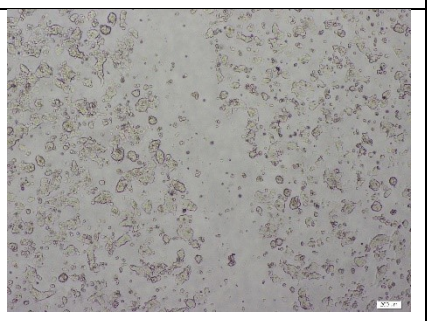
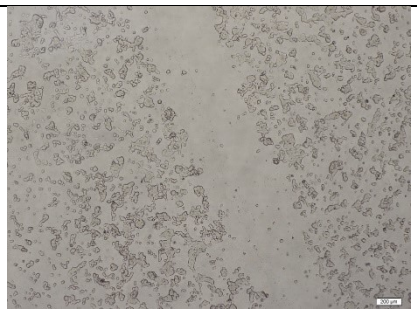
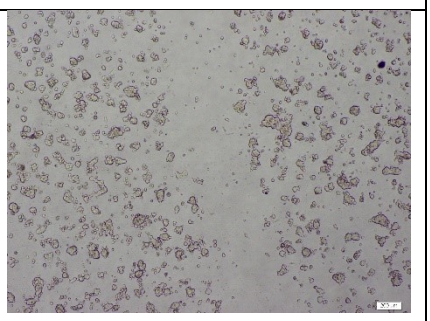
	0 h	48 h
Control		
OG1		
OG2		
OG3		
OG4		

Figure 28. Images of Cell migration on MCF-7 monolayer in control and IC_{50} concentrations treated of OG1, OG2, OG3, and OG4 at 0 h and 48 h incubation

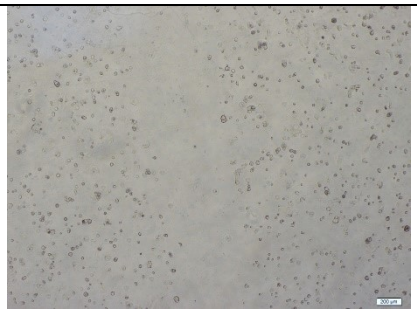
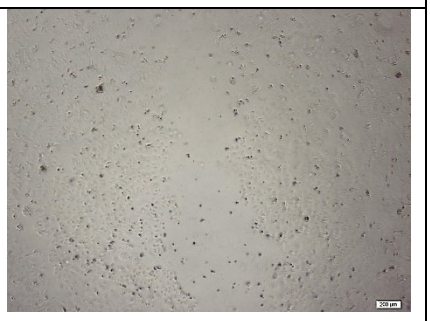
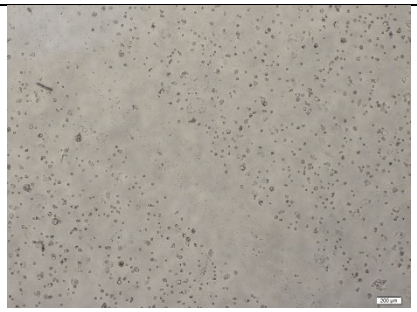
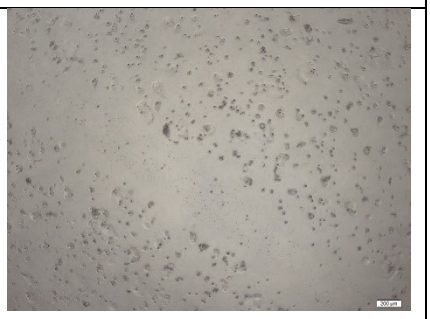
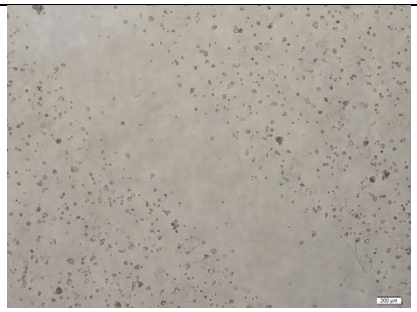
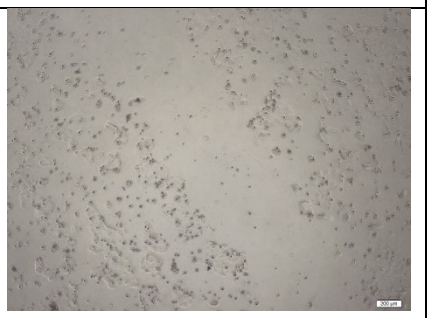
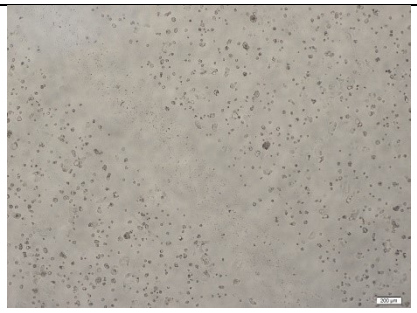
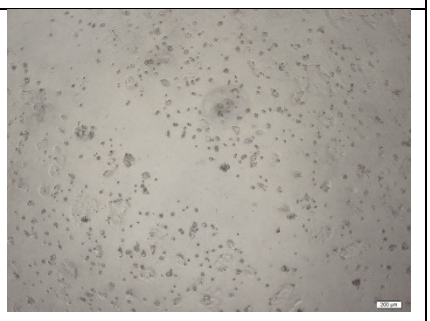
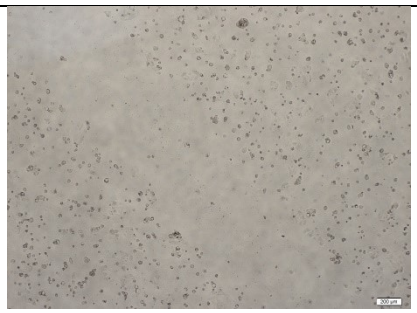
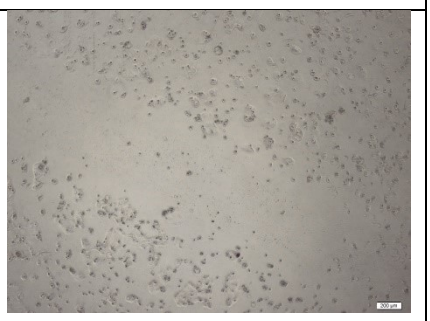
	0 h	48 h
Control		
OG1		
OG2		
OG3		
OG4		

Figure 29. Images of Cell migration on Caco-2 monolayer in control and IC_{50} concentrations treated of OG1, OG2, OG3, and OG4 at 0 h and 48 h incubation

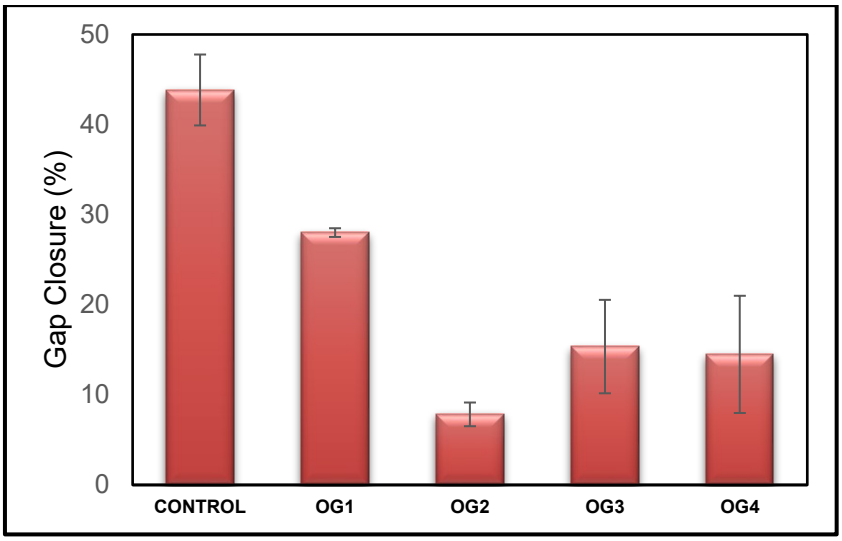


Figure 30. Graphical representation of filled gap in A549 monolayer in control and IC_{50} doses of OG1, OG2, OG3, and OG4 treated groups

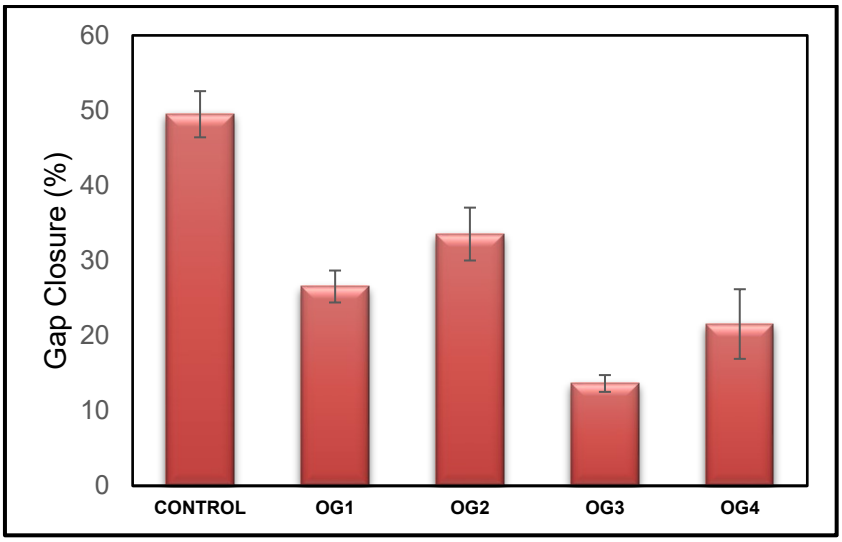


Figure 31. Graphical representation of filled gap in HeLa monolayer in control and IC_{50} doses of OG1, OG2, OG3, and OG4 treated groups

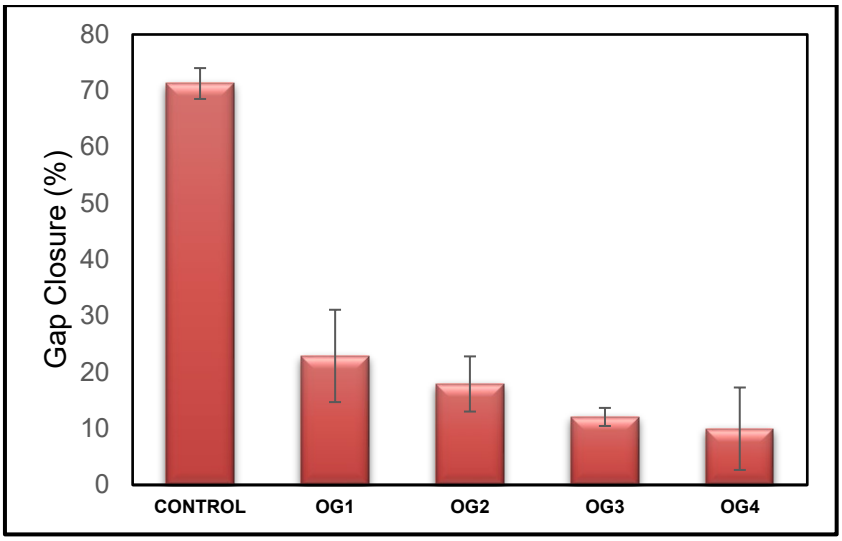


Figure 32. Graphical representation of filled gap in PC3 monolayer in control and IC_{50} doses of OG1, OG2, OG3, and OG4 treated groups

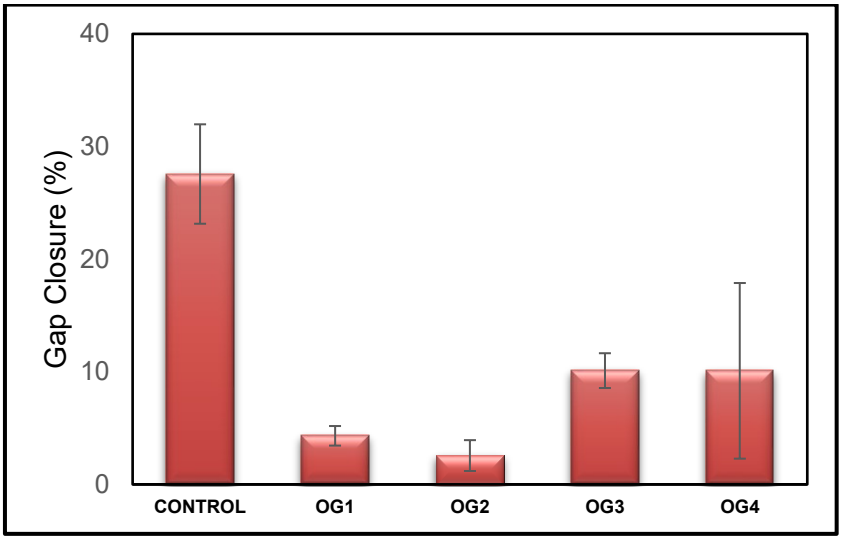


Figure 33. Graphical representation of filled gap in MCF-7 monolayer in control and IC_{50} doses of OG1, OG2, OG3, and OG4 treated groups

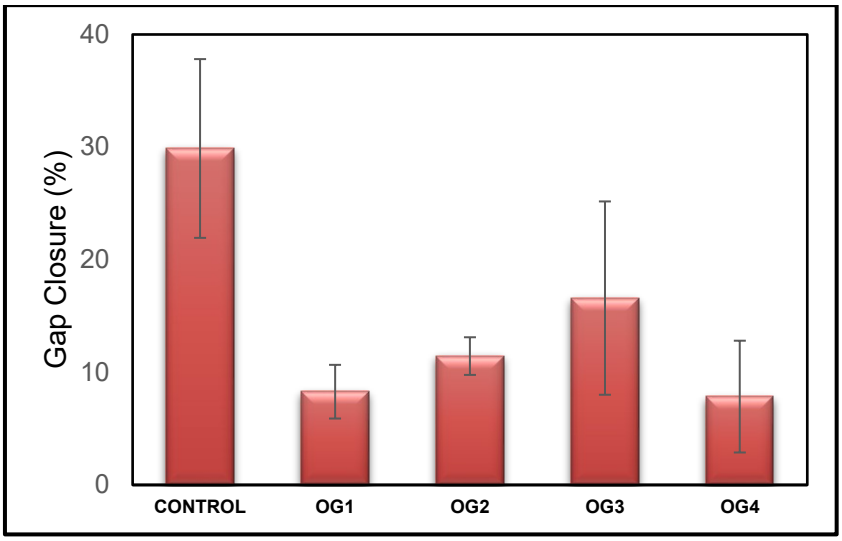


Figure 34. Graphical representation of filled gap in Caco-2 monolayer in control and IC_{50} doses of OG1, OG2, OG3, and OG4 treated group

CHAPTER 4

CONCLUSION

Side effects of the drugs used in cancer therapy, studies to develop less aggressive drugs have gained momentum. For this reason, Prof. Yunus Kara and his research group synthesized four different isoindole derivative drugs as potential anticancer drugs.

In this study, five different cell lines were used to investigate the biological activity of these four different new molecules synthesized in cancer types and their inhibitions of MTT assay, apoptosis, cell cycle, and cell migration were investigated.

To investigate the cytotoxic effects of the compounds, A549, HeLa, PC3, MCF-7, and Caco-2 cells were exposed to six different concentrations of drugs at 24, 48, and 72 hours incubation times.

According to the results of the cell viability assay, it was determined that the cell viability decreased as the drug dose and incubation time increased and calculated IC_{50} values, the compound with the highest cytotoxic effect on cells is OG4. In addition, the cell line most inhibited by OG4 was Caco-2. According to MTT results, the optimum incubation time was determined as 48 hours. On the other hand, the A549 cell line treated with OG3, and the Caco-2 cell line treated with OG1 was determined that the cytotoxic effect was the lowest at 24 hours.

Cells were exposed to IC_{50} concentrations of drugs for 48 hours in apoptosis, cell cycle and cell migration analyze. According to the results, the drug that provides the most apoptosis rate is OG3 for A549 and HeLa, OG1 for PC3 and MCF-7 cell lines, and OG4 for Caco-2 cell lines. In the cell cycle results, it was determined that for the A549 and HeLa cell lines OG4 and the PC3, MCF-7, and Caco-2 cell lines OG2 caused G2 phase arrest and prevented the transition to the mitosis phase. In scratch assay results, the highest inhibition rate was OG2 for A549 and MCF-7, OG3 for HeLa, and

OG4 for PC3 and Caco-2. When looking at the microscope images, it was determined that the drugs not only inhibited the activities in the cells but also killed them.

Each of these synthesized isoindole derivatives showed effective biological activity in different cell lines. Indole and isoindole derivatives are known to be potential tyrosine kinase inhibitors and maybe a good treatment modality in cancer treatment because they inhibit cell growth.

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APPENDIX A

CHEMICALS, REAGENTS, AND SOLUTIONS

Table A.1. Chemicals and reagents

Chemical Name	**Company**
Annexin-V Apoptosis Detection Kit with PI	Biolegend
DMEM F12	Biological Industries
Dimethyl Sulfoxide (DMSO)	Carlo Erba
Ethanol	Amresco
Fetal Bovine Serum	Biological Industries
HCl	Merck
KCl	Sigma
KH_2PO_4	Merck
L-glutamine	Biological Industries
Methanol	Sigma
MTT	Amresco
Na_2HPO_4	Merck
NaCl	Applichem
NaOH	Carlo Erba
Penicillin-Streptomycin	Biological Industries
RNase	Applichem
RPMI 1640	Lonza
Triton X-100	Amresco
Trypan Blue	Sigma
Trypsin	Sigma

Phosphate Buffered Saline Solution (1x PBS)

To prepare 1 liter of 1X sterile PBS solution, 800 mL of distilled water, 8g of NaCl, 200mg KCl, 1.44g Na_2HPO_4, and 245mg KH_2PO_4 were put into the glass bottle and the salts were dissolved on the stirrer. Then the pH was adjusted to 7.4 using HCl or NaOH with a pH meter and the total volume was brought to 1 liter with distilled water. Autoclaved for sterilization and stored at 4°C.

MTT Reaction Solution

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder dye was dissolved in sterile phosphate-buffered saline (PBS) at a concentration of 5 mg/mL, protected from light. Then, the solution was sterilized using a 0.45 μm sterile filter and transferred to the eppendorf for use in cell viability tests. (Stored for 6 months at -20°C, 4 weeks at 4 ° C)