TURKISH REPUBLIC OF NORTHERN CYPRUS NEAR EAST UNIVERSITY INSTITUTE OF HEALTH SCIENCES

EVALUATION OF ANTICANCER POTENTIAL OF ESSENTIAL OILS FROM LAMIACEAE

Manal Salah Ali

MASTER THESIS

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Prof. Dr. Nedime Serakıncı

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Evaluation of anticancer potential of essential oils from Lamiaceae

By

Manal Salah Ali

Master of Science in Medical Biology and Genetics

Supervisor

Prof. Dr. Nedime Serakıncı

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APPROVAL

A thesis submitted to the Institute of Health Sciences of Near East University in partial fulfillment of the requirement for the degree of Master of Science in Medical Biology and Genetics.

Thesis Committee;

Chair of the committee: Prof. Dr. Nedime Serakinci
Near East University
……

Supervisor: Prof. Dr. Nedime Serakinci
Near East University
…….....

Members: Assoc. Prof. Dr. Dudu Özkum Yavuz

Assoc. Prof. Dr. Tufan Cankaya

…….....

Approved by:

.....

STATEMENT

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis.

Manal Salah Ali

signature

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ABSTRACT

EVALUATION OF ANTICANCER POTENTIAL OF ESSENTIAL OILS FROM LAMIACEAE

Manal Salah Ali

Department of Medical Biology and Genetics

Thesis Supervisor: Prof. Dr. Nedime Serakıncı

Aim:

This study aimed to evaluate the anticancer potential of medical essential oils from family Lamiaceae.

Material:

Chemicals:

Medium (DMEM, Sigma-Aldrich) which is supplemented with 10% fetal bovine serum (FBS, SigmaAldrich), streptomycin (0.1 mg/ml), penicillin (64 µg/ml), L-glutamine, Fetal Calf Serum, phosphate-buffered saline (PBS) and Trypsin/EDTA Solution.

Kits:

Cell viability/ Cell Proliferation Kit - (MTT), Roche, and Cat. No.11465007001.

β-galactosidase assay kit.

Plant extract:

Thymus capitatus and origanum dubium essential oils extracted from lamiaceae family in Cyprus**.**

Cell lines:

telomerase-immortalized human mesenchymal stem cells (hMSC -telo1) and their tumorigenic counterpart (tumorigenic hMSC-telo1 cells)

Instrumentations:

Biological safety cabinet, humidified incubator (at 37 °C, 5% CO2), Freezer, Water bath, centrifuge, microscope, Hemocytometer (counting chamber), Cryotubes, falcon tubes, pipettes, micropipettes, Pipette controller, Cell culture T-75 flasks, Cell culture T-25 flasks, 96-well plates, 6-well plates.

Method:

hMSC-telo1 and tumorigenic hMSC-telo1 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) which is supplemented with 10% fetal bovine serum (FBS, SigmaAldrich), L-glutamine, penicillin (64 µg/ml), and streptomycin (0.1 mg/ml) at 5% CO2 and 37°C. Cells were treated with various concentrations (0.05% v/v, and 0.005% v/v) of *Thymus capitatus* and origanum dubium essential oils. According to the literature, the oils concentrations has been converted into weight/volume, based on the amount of their main contents which is (corresponding 3 µg/ml, 0.3 µg/ml thymol in final concentration of *Thymus capitatus*), and (corresponding 4 µg/ml, 0.4 µg/ml carvacrol in final concentration of origanum dubium). The cells were incubated for 24 hours a and a new medium was added after they washed with PBS. Cell viability/ Cytotoxic activity of *Thymus capitatus* and origanum essential oils were evaluated by using MTT Assay for Cell Viability and Proliferation according to manufacturers' given protocol for Cell Proliferation Kit I (MTT), Roche, Cat. No.11465007001. The long-term proliferation of hMSC-telo1 and tumorigenic hMSC-telo1 cell lines determined by calculating the population doubling level (PD) by using the initial seeding number (N-start) and cell number harvested at 80% confluence (N-finish) with

the formula PD = ln (N-Finish/ N-Start)/ln2. The cell senescence was evaluated by Beta-galactosidase assay by the detection of blue dye produced only in the senescent cells, stained cells were counted under the microscope, percent of β-gal-positive cells from the total number was calculated.

Results:

***Thymus capitatus*:** After exposing hMSC-telo1 and tumorigenic hMSC-telo1 cell lines to different concentrations of *Thymus capitatus* essential oil (corresponding 3 µg/ml, 0.3 µg/ml thymol in final concentration) for 24 hr., morphologically, it was observed that both cell lines started to gain round look and shrank into a small spindle shape as a possibility of cytotoxic effect. The cell viability was also evaluated and compared to untreated (control) cells as 100%. At 3 µg/ml concentration of thymus essential oil, the cell survival of hMSC-telo1 was (1.36%), and for the tumorigenic hMSC-telo1 cell lines, the viability was (2.8%). However**,** at 0.3 µg/ml concentration of thymus essential oil, the cell viability of hMSC-telo1 cells was higher than control (101.56 %), while it was (48.3%) for the tumorigenic hMSC-telo1 cells.

Origanum dubium: Under the microscopic examination, the cells have been modified into small spindle appearance; the cells shrunk and took round shape after their exposure to different concentrations (4 µg/ml, 0.4 µg/ml) of origanum dubium essential oil based on carvacrol in final concentration for 24 hr. Furthermore, the cell viability was evaluated, and at 4 µg/ml the viability of hMSC-telo1 was 1.51%, and in tumorigenic hMSC-telo1 cell lines was 4.52. while at concentration 0.4 µg/ml, the viable cells of hMSC-telo1 cell lines was calculated as (4.52%), and in the tumorigenic hMSC-telo1 cells it was (16.37%).

The population doubling level was calculated to investigate the proliferation activity of hMSC-telo1 cells and tumorigenic hMSC-telo1 cell lines, as for the

untreated (control) cells, the proliferation rate of tumorigenic hMSC-telo1 (population doubling level =1.67) cells is higher than non-tumorigenic hMSC-telo1 (population doubling level = 0.88).

The senescence of the hMSC-telo1 and tumorigenic hMSC-telo1 cells after their treatment with *Thymus capitatus* and origanum dubium Essential oils were evaluated by Beta-galactosidase assay, both hMSC-telo1 cell lines have not shown an increase in the number of stained cells compared to the untreated control.

Conclusion

The findings of this study indicate that *Thymus capitatus* essential oil could be used as an effective cancer therapy in the future. We also indicated that origanum dubium essential oil is not sufficient enough as a cancer treatment independently; however, it can be used with modifications as an enhancer of standard chemotherapy; however, it may need further investigations.

Keywords: Anticancer, Lamiaceae family, cytotoxicity, Essential oils, *β*-gal, senescence.

TABLE OF CONTENTS Page

Chapter One

Introduction

Chapter Two

Materials and Method

Chapter Three

Results

Chapter Four

Discussion and Conclusion

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

µl.	Microliter
µg	Microgram
ml	Milliliter
mg	Milligram
DMEM	Dulbecco's Modified Eagle Medium
EOs	Essential oils
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FID	Flame Ionization Detector
GC	Gas Chromatograph
GC/MS	Gas chromatography/Mass Spectrometry
hMSC's	human mesenchymal stem cells
IARC	International Agency for Research on Cancer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffer saline
PDL	Population doubling level
RRI	Relative Retention Index

X-gal	5-Bromo-4-chloro-3-Indolyl β -D-galactopyranos

Chapter One

1.Introduction

1.1 General information

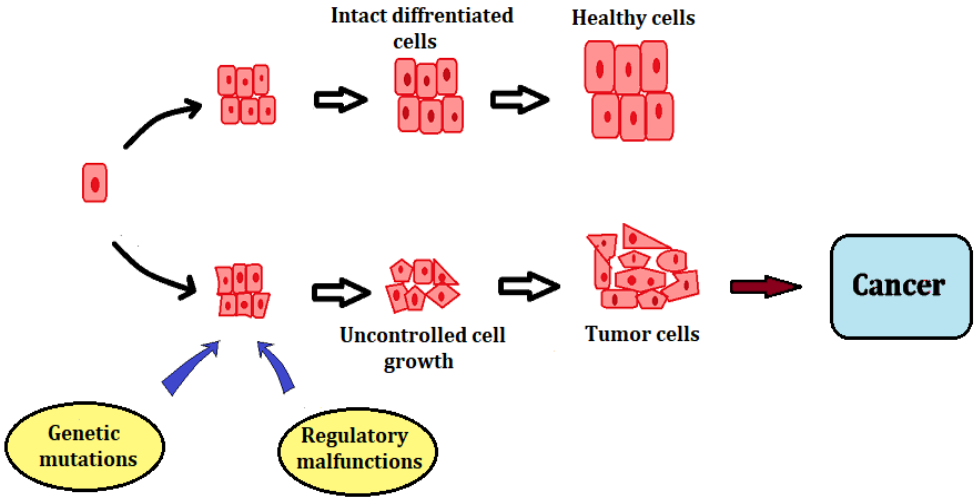
1.1.1 Cancer

Cancer is one of the most complicated genetic diseases which has been evolved over the last decades, in present Cancer has become the most important leading cause of death. The International Agency for Research on Cancer (IARC) has reported about more than 17 million cancer cases in 2018, and the deaths of cancer has reached around 9.5 million (without mentioning the nonmelanoma skin cancer cases) all around the world. Cancer is an outflow cells disease, the normal cells in the body manage to control their growth by regulating their division and apoptotic induction mechanisms to maintain the balance of the organism, during the cell cycle each cell type has its biological clock, the cell undergoes through many phases during the cell division and formation of new cells, when the new cell in its G1 period it produces RNA as well as protein. The cell starts the DNA synthesis when it enters the S period which is the longest period in the lifespan of the cell. During G2 period the DNA and protein are in active time to prepare for the cell division (mitosis). Finally, the cell goes through G0 period when it sits and waits for an action.

However in cancer, regulatory malfunctions and genetic mutations occur during this process and the cells lose their normal relationship with the whole organism, they may look like they are acting as normal cells but in fact, they change biologically, morphologically and functionally and start to grow abnormally and reproduce in different rates to form a tumor that sometimes proliferates and spread into other surrounding tissues (Figure 1).cancer is a

group of diseases that target the body cells, it can involve various tissues of the human body in various forms. Most of the cancer development includes two main stages, growth transformation (tumorigenesis) and the metastasis stage

Figure 1: Cancer progression



1.1.1.1 Growth transformation (Tumorigenesis)

Unlike the normal cells, cancer cells reproduce faster, they could have more than one nucleus or do not, cancer cells have undefined membranes and hard to be recognized as living cells they don't stop and start growing over other cells. In this stage, the cell undergoes an initial genetic or epigenetic alteration and environmental carcinogens that can lead to uncontrollable cell division until the cells accumulate further genetic mutations of the genes that responsible of the regulation of the cell division (proliferation), cell death (apoptosis) and DNA

repair (such as oncogenes and tumor suppressor genes) to form a population of tumor cells.

Proto-Oncogenes

They are the genes that regulate the growth and division of the cells when they mutate under various conditions, they become mutated genes that are capable of causing cancer called oncogenes (such as growth factors c-Sis, Regulatory GTPases Ras) these oncogenes are activated by gene duplications or rearrangement of the chromosome.

Tumor suppressor genes

They are the genes that order the cells to go apoptotic or slow the cell growth (such as p53, p21, pRB), these genes when they are inactivated, they cause uncontrollable cell growth which lead to cancer.

Genomic instability

The genomic instability is a very common in most of cancer cells. The tumor cells have high susceptibility to the genomic alterations during their division. The genomic instability in cancer can be manifested by many defects or abnormalities in the chromosome such as translocation, deletion, duplication, DNA amplification, and aneuploidy.

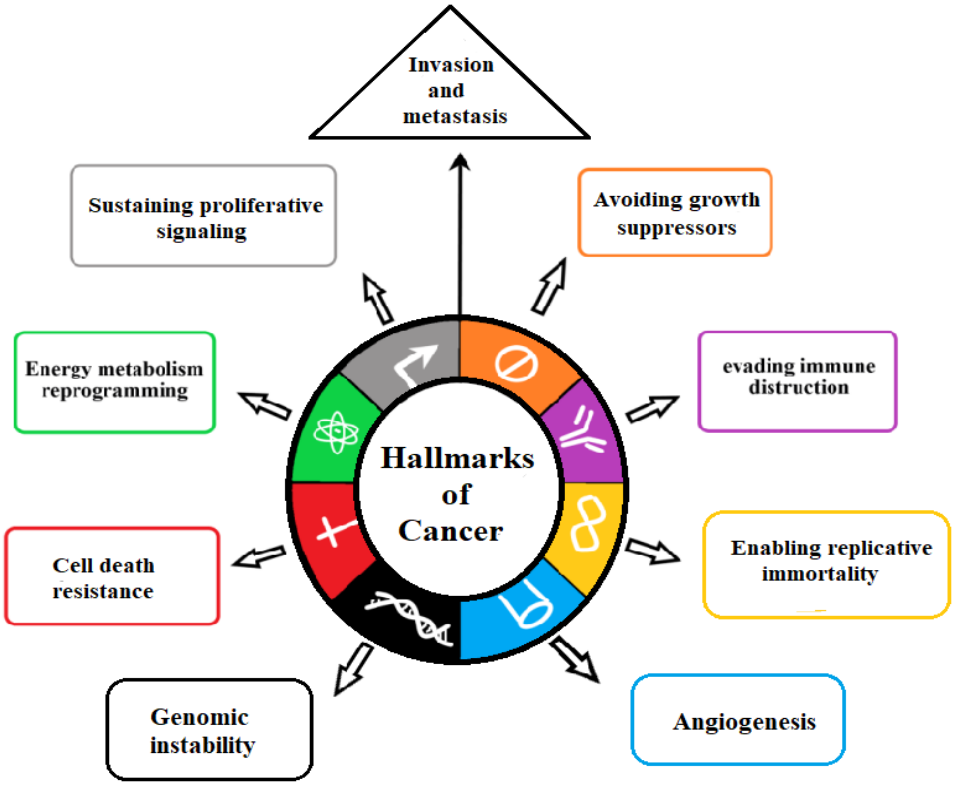
1.1.1.2 Metastasis

In the metastasis stage, the tumor cells continue to accumulate as a result of their perpetual ability to grow and divide until they can spread from their localized area (benign tumor) to invade the surrounding tissues and metastasize (malignant tumor). Matrix metalloproteases (MMP) are enzymes that secreted

by the proteins in cancer cells, these enzymes have the ability to cut through the proteins that prevent the cancer cells from the migration into the surrounding tissues. Once they cross the basal lamina, they enter the bloodstream pressing between the cells that comprise the blood vessels. The tumor cells undergo angiogenesis to contain their microenvironment which includes blood vessels, fibroblasts, immune cells, extracellular matrix, and signaling molecules to support their growth and metastasis.

Several characteristic hallmarks obtained by normal cells during their transformation into tumorigenic cells such as cell death resistance, sustaining proliferative signaling, avoiding growth suppressors, angiogenesis, and metastasis, enabling replicative immortality, evading immune destruction and energy metabolism reprogramming (Hanahan and Weinberg, 2011) (Figure 2). Recently, numerous researches and a lot of costs have been carried out to develop that cancer-targeting therapy by modifying some fatal malignancies into manageable chronic conditions, however, the problem of curing cancer remains, a profound understanding of the hallmarks can be helpful to further improve better cure of cancer.

Figure 2: Hallmarks of cancer



1.1.2 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent cells that foremostly exist in adult human bone marrow and also can differentiate into myoblasts, adipocytes, osteoblasts, and chondrocytes and in addition to endothelial cells and fibroblasts. However, research has found that mesenchymal stem cells have several various sources such as umbilical cord tissue, molar teeth and body fat.

1.1.2.1 Potential of Mesenchymal stem cells (MSCs)

Mesenchymal stem cells have numerous noteworthy properties. They play a unique role in maintaining the function as well as the structure of the body connective tissues, as well as hematopoiesis. They have a high ability for tissue repair and they secret different immunomodulatory molecules which provide regeneration of damaged tissues, in addition to suppression of the immune reactions and allogeneic transplants. Therefore, MSCs have been widely investigated for their potential and implementations in regenerative medicine, genetic diseases, and debilitating diseases in addition to cancer pathogenesis.

1.1.3 Essential Oils

Medicinal plants and their preparation have been used as effective therapies in many different cultures all over the world for thousands of years. The plants' primary and secondary metabolites comprise a broad range of functions, these metabolites were exploited due to their benefits, the alteration of their chemical structure depends on several factors as the techniques of extraction, botanic origin, bacterial endophytes, geographical location, and genetics.

Essential oils are natural products they are mostly synthesized as secondary metabolites by many organs (leaves, roots, flowers, buds, fruits, wood, and stem) of highly volatile aromatic plants that are applied traditionally as medicinal therapies. their constituents have been classified based on their chemical structure into terpenes as in Monoterpenes (C10), sesquiterpenes

(C15), diterpenes (C20) and the oxygenated sedatives, and phenylpropanoids as in (phenol and phenol esters). These constituents have been utilized for functional and potential applications (Sawamura, 2000; Gianni et al.,2005).

1.1.4 Essential Oils Therapeutic potentials

The essential oils are highly valued for their biological activities such as sedatives, anti-inflammatory, antiseptic, antibacterial and antioxidants which justified that 80% of the populations still use these oils as the main care for health. Recent research papers show that the essential oils can bind different cellular receptors that can cure inherited and infectious diseases and have therapeutic value, more importantly, the essential oils mode of action can lead to metabolic and cellular responses make them able to be a provenance of anticancer therapeutic strategies. It is thought that the essential oils have a multicellular target, because of their complex and large range compounds.

Furthermore, their numerous functional groups each complex combination starting different effects in the cells through their main components. The essential oils have been used in overall health improvement and treating particular diseases across many countries and different cultures around the world due to their different potentials as antibacterial, antifungal, spasmolytic, antimicrobial, anti-inflammatory, antiseptic and antioxidant, this indicates that the essential oils have a promising future in the competition in this field.

1.1.5 Anticancer properties of essential oils

Treatment of cancer is one of the most difficult challenges. The basic ways of cancer treatment involve radiation, surgery, and drugs.

Chemotherapeutic agents can prolong the cancer patient's lifespan and relieve the symptoms temporarily but their side effects can't be ignored, successful anticancer therapy should completely cure the disease with minimum unpleasant effects and without harming the intact cells.

There is a tendency to use traditional and herbal medicines in cancer treatment. Essential oils have been demonstrated to possess antifungal, spasmolytic and antibacterial activities, recently, their anticancer effect has been reported. There is emerging literature on herbal products that have anticancer potential that supports the traditional medical approach with fewer side effects. The antiproliferative and cytotoxic activities of the essential oils are investigated to evaluate these oils as integral or alternative treatments for cancer. Plants molecules are very effective against cancerous cell proliferation; a part of the conventional chemotherapies is from plant origin, chemically modified forms of photoproducts directly derived from plants. The essential oils showed several anticancer potentials in preliminary studies in different mechanisms, such as interaction with the microenvironment, acting on the established tumor cell and cancer preventative mechanisms such as apoptosis induction.

The essential oils are still in the level of the evaluation as therapeutic agents or as a complementation to standard therapy they can be potentially preventive for cancer therapy, to confirm their potential of the essential oils as a cure for cancer, in vitro assays through systematic studies and clinical investigations must be done.

1.1.6 The Lamiaceae family plants

The Lamiaceae family plants are famous aromatic flowering plants and one of very important essential oil-bearing families, it is also known as the mint

family and contains about 236 genera and 6,900 – 7,200 species. Some of the Lamiaceae family members are cultivated widely due to their ease of cultivation in addition to their high aromatic qualities This family can be obtained in various regions all over the world; some of them are broadly used to improve food products. As they are the main source of phytochemical compounds that have a beneficial effect on health and have a significant role in the enhancement of diseases. Besides the biological activities of Lamiaceae family essential oils, lately their anticancer properties were evaluated and it is also considered as a source of antitumorigenic drugs (Mesquita, et al 2019). They also have potential anti-proliferative effects such as apoptosis, cell cycle arrest, and mechanisms of DNA repair that can be used in support of traditional cancer treatments.

1.1.7 *Thymus capitatus*

Thyme is one of the largest genera of Lamiaceae family it has almost (200) different species and each with different specifications. For thousands of years, Thyme oil was used medicinally and traditionally as a treatment for many different diseases such as respiratory diseases. Animal studies have shown spasmolytic activities of thyme components more specifically it has been recommended for hundreds of indications, based on antimicrobial, antioxidant other biological activities. Up to now, there are many clinical experiments, that support *Thymus capitatus* essential oil for therapeutic use. Based on the historical application and clinical trials, it has been showed that *Thymus capitatus* leaves and flowers are safe in limited medicinal use such as treating numerous symptoms including sore throat, arthritis, diarrhea and stomach ache. However, it still has some harmful effects for some people such as headache, digestive system upset or irritation if applied to the skin that couldn't be explained.

1.1.8 Cyprus Thyme

Thyme is one of the most popular endemic species of Lamiaceae family in Cyprus which grows in (rocky hillsides) and Akamas area and common on Troodos range. Thyme has a history in the medical field, it considered one of the most important herbs in Cyprus. In addition to its use as an enhancer of food flavors, it also facilitates the digestion of the fats. Cypriot thyme has different potentials such as antiseptic, anti-bacterial and anti-pyretic. Also, it can be used as a protector and as a treatment for various conditions including respiratory tract diseases such as bronchitis, coughs and sore throats. And it lowers cholesterol levels and blood pressure. Moreover, previously, it has shown that cyprus *Thymus capitatus* essential oil has an antiproliferative activity (Yavuz, D. Ö, et al. 2017).

1.1.9 *Origanum dubium*

Origanum dubium is one of the most important multipurpose genera of the family Lamiaceae medicinally it includes 42 species distributed in wide areas of North Africa and Eurasia. Also found in parts of the Mediterranean mountains of Asia and Europe. The essential oil of origanum plant was used in many products as flavors, diuretic, and anti-asthmatic and anti-paralytic drugs, it is also used in herbals and their antimicrobial activity and antioxidant activity have been investigated. Furthermore, some species of this genus appear to possess anticancer properties as well (Johnson et al.,2002; Leung et al., 2003).

1.1.10 Cyprus *Origanum dubium*

Origanum dubium is an aromatic plant that grows up to 100 cm in the rocky areas, has spade-shaped and thick leaves and it can be used for the

treatment of the respiratory ailments' inflammations. It is useful for stomach disturbances, insomnia and also can be used as anti-microbial, analgesic, anti-fungal and sedatives. Previously, the potential antioxidant activity, and the antimicrobial activity and of cyprus *Origanum dubium* has been investigated, furthermore, it was found to play an important role in scavenging O2- (Karioti A. 2006).

1.2 Aim of the study:

This study aims evaluate the antiproliferative as well as anticancer potential of essential oils from the Lamiaceae family.

1.3 Study design

The current study was designed in order to investigate the anticancer potential of two essential oils (*Thymus capitatus* and *Origanum dubium*) extracted from Lamiaceae family that grow widely in Cyprus. The oils are tested for their potential therapeutic roles by using previously published immortalized-mesenchymal stem cells (hMSC-telo1) and their tumorigenic counterpart (tumorigenic hMSC-telo1) due to their proliferative capacity and their potential such as the ability to differentiate into different tissues.

1.3.1 Sitting

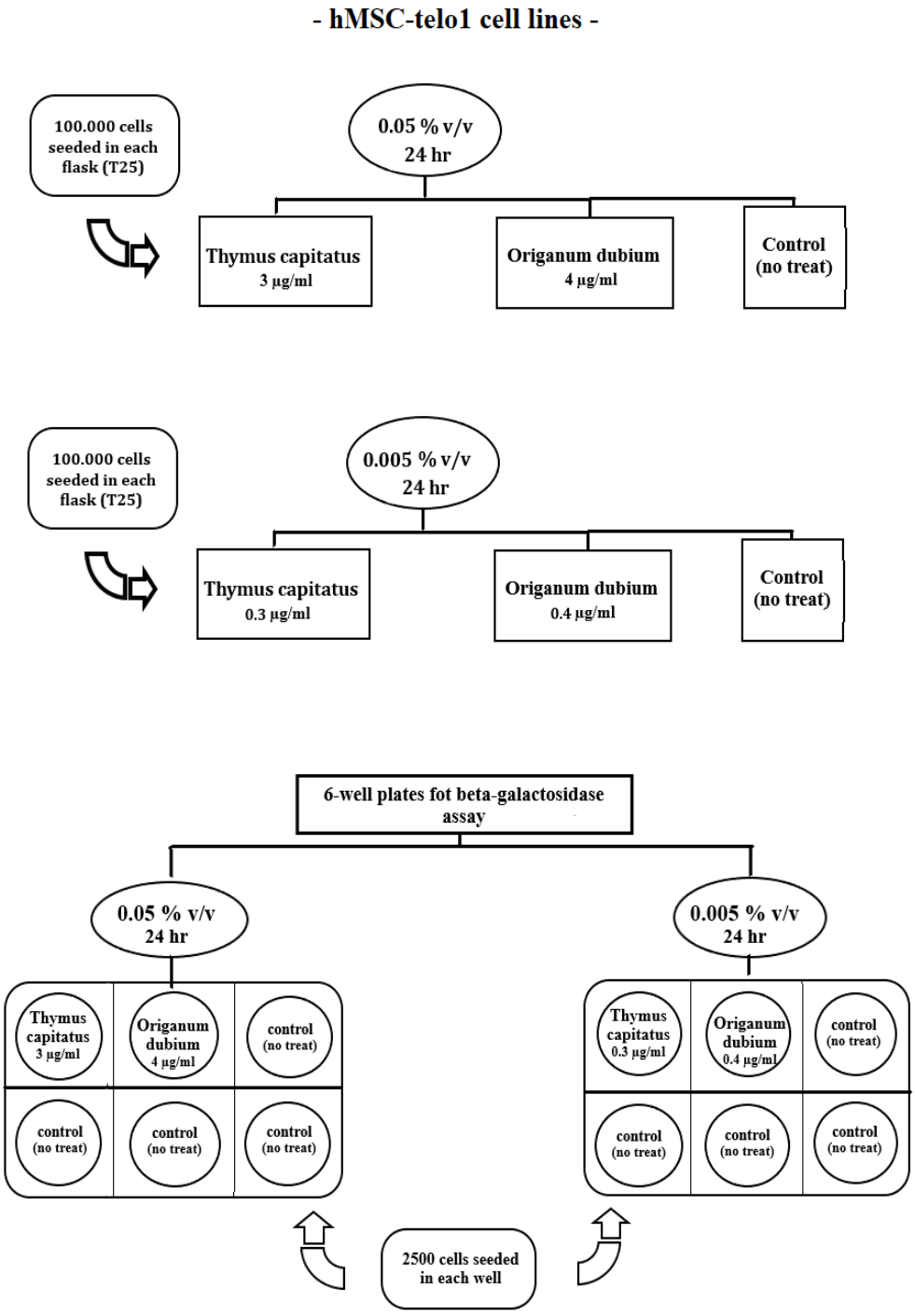
The experiment was conducted at the Near East University Hospital Medical Genetics Department in the Genetics and Cancer Diagnosis and Research Centre, Nicosia, Cyprus. It is the only genetic and cancer diagnosis

research that founded in North Cyprus 2011. the Centre is joining the diagnostic laboratories that competing some of the centers in Europe by joining Molecular genetics, Cytogenetics, Molecular cytogenetics, and Pre-implantation genetic diagnosis laboratories.

1.3.2 Implementation

Each of the (hMSC-telo1) and (tumorigenic hMSC-telo1) cell lines were exposed to various concentrations (0.05 % v/v, 0.005 % v/v) of each oil for 24 hrs., and were compared to untreated (control) cells. However, according to the literature where it has been mentioned the concentrations of (thymol and carvacrol) the chemical contents of the essential oils, the concentrations of the essential oils in our study were converted from volume/volume into weight/volume, based on the amount of their main contents which is (corresponding 3 µg/ml, 0.3 µg/ml thymol in final concentration of *Thymus capitatus*), and (corresponding 4 µg/ml, 0.4 µg/ml carvacrol in final concentration of *Origanum dubium*). Once cells are subjected to oils, cellular morphology will be examined under the light microscope, and the cell viability, proliferation rate, and senescence will be investigated (figure 3).

Figure 3: Overveiw of study implementation



1.4 The Intended Outcome of The Thesis /Significance

The results of this study may lead the health care providers who are specialists in cancer cases to understand and improve the biological mechanisms of these essential oils from Lamiaceae family to allow their as anticancer drugs or support to conventional treatments, thus, help to plan the best and the right management for cancer patients. Furthermore, the results of our study can be integrated into laboratory practices as well as medical curricula in order to assist the researchers and students to understand the nature of this disease and its seriousness and to be able to do more scientific researches to develop this field and reduce cancer mortality.

Chapter Two

2.Materials and method

Ethical approval was granted by the Near East University Institutional Review Board (YDU/2019/75-920).

2.1 MATERIALS

2.1.1 Chemicals

Medium (DMEM, Sigma-Aldrich) which is supplemented with 10% fetal bovine serum (FBS, SigmaAldrich), streptomycin (0.1 mg/ml), penicillin (64 μg/ml), L-glutamine, Fetal Calf Serum, phosphate-buffered saline (PBS) and Trypsin/EDTA Solution.

2.1.2 Kits

Cell viability/ Cell Proliferation Kit - (MTT), Roche, and Cat. No.11465007001. is used to determine the antiproliferation effect and viability of the cells. It uses tetrazolium salt (MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

β-galactosidase assay kit, is used to determine the cell senescence by using the organic compound (x-gal, sigma) 5-Bromo-4-chloro-3-Indolyl *β*-D-galactopyranoside.

2.1.3 Instrumentation

Class II Type AC Biological safety cabinet (MAXISAFE, Germany)

Humidified incubator (at 37 °C, 5% CO2), (Thermo Hera cell, Germany

Freezer (Thermo, Ultima II, Germany)

Water bath (Thermo, SWB 25, Germany)

Centrifuge (Thermo, Germany)

Microscope (Leica DMIL LED, CMS GmbH, Germany)

Falcon tubes (Corning, Mexico)

Cryotubes (Corning, Mexico)

Pipettes (Eschau, Germany)

Micropipettes (Gilson, France)

Pipette controller (Gilson, France)

Hemocytometer (counting chambers, MARIENFELD, Germany)

Cell culture T-75 flasks (Corning, Mexico)

Cell culture T-25 flasks (Corning, Mexico)

96-well plates (NEST, Jiangsu, China)

6-well plates (CELLSTAR, Grenier, Austria)

2.1.4 Cell lines

Previously published telomerase-immortalized human mesenchymal stem cells (hMSC -telo1) and their tumorigenic counterpart (tumorigenic hMSC-telo1 cells) (Serakinci et al. 2007). The cell lines exist in different tissues of the body and proliferate extensively in vivo and in Vitro. Moreover, they serve remarkable roles in malignancy and considered significant components of the microenvironment of tumors. Many of the tissues of mesenchymal vessel's walls, which subsequently lead to an uncontrollable proliferation of cells and metastasis and give rise to cancer. Accordingly, human mesenchymal stem cells present a great model system for our study.

2.2 METHOD

2.2.1 Essential oils

The essential oils (*Thymus capitatus* and *Origanum dubium)* were kindly provided by Assoc Prof. Dudu Özkum Yavuz (Hanoğlu et al., 2017; Karadağlıoğlu et al., 2019)

2.2.2 Thawing cell lines

The cell lines were frozen in cryotubes within DMSO (Dimethyl sulfoxide (%5) in (-) 80 °C freezer. The cells were kept in water bath at 37 °C until they thawed. The cells transferred into centrifuge tubes supplemented with 10 ml of culture medium and centrifuged (rpm 1400, time 3min., temperature 4°C), after discharging the supernatant of the tubes, the cells seeded in T-25 flasks with culture medium to grow.

2.2.3 Cell culture

hMSC -telo1 and tumorigenic hMSC-telo1 cell lines were sustained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) which is supplemented with 10% fetal bovine serum (FBS, SigmaAldrich), L-glutamine, penicillin (64 µg/ml), and streptomycin (0.1 mg/ml). Cells were kept at 37˚C in (T25) plastic cell culture flasks as monolayers in a humidified atmosphere contains 5% CO2 (Serakıncı, N., 2017).

2.2.4 Subjecting the cells with Essential Oils:

Cells were treated with concentrations (0.05% v/v, and 0.005% v/v) of *Thymus capitatus* essential oil (corresponding to 3 µg/ml, 0.3 µg/ml thymol in final concentration), and the same concentrations (0.05% v/v, and 0.005% v/v) of *Origanum dubium* essential oil which (corresponding to 4 µg/ml, 0.4 µg/ml carvacrol in final concentration) and incubated for 24 hours. After the incubation period cells were washed with PBS and a new medium was added, cells were incubated for 24 hours before the final count.

2.2.5 MTT Assay for Cell Viability and Proliferation

MTT assay was used to determine the cell viability and effect of *Thymus capitatus* and *Origanum dubium* essential oils on proliferation of non-tumorigenic hMSC-telo1 and tumorigenic hMSC-telo1cells. MTT assay applied according to the protocol of the manufacturer for Cell Proliferation Kit - (MTT), Roche, and Cat. No.11465007001.the method measures the activity of the mitochondrial dehydrogenase of the viable cells which cleave the MTT tetrazolium ring and produce formazan.

Cells were seeded in 100µl culture medium with different concentrations 0.05% (v/v), 0.005% (v/v) of *Thymus capitatus* and *Origanum dubium* essential oils into microplates then Cells were incubated at 5% CO2 and +37°C for 4 days2. then in each well was added 10µl of the MTT labeling reagent. The microplate was incubated for 4 hours. after the incubation period in each well 100µl of the Solubilization solution was added. The plate was kept in the incubator overnight in (37°C, 5% CO2) humidified atmosphere.

2.2.6 Cell proliferation study

Cells were grown through continuous sub-cultivations in 10% FCS with DMEM supplemented with streptomycin (0.1 mg/ml), L-glutamine and penicillin (64µg/ml). The long-term proliferation of hMSC-telo1 and tumorigenic hMSC-telo1 cell lines in vitro was determined by calculating the population doubling level (PD) by using the initial seeding number (N-start) and cell number harvested at 80% confluence (N-finish) with the formula PD = ln (N-Finish/ N-Start)/ln2. Thus, the cumulative population doubling level is the sum of PD.

2.2.7 β-galactosidase staining

, Beta-galactosidase assay was used for the investigation of the cell senescence by detection of blue dye produced due to the cleavage of the organic compound (x-gal) by senescence-associated beta-galactosidase enzyme which is only present in the senescent cells, hMSC-telo1 cells were seeded in six-well plates and treated with concentration (0.5 % v/v) of *Thymus capitatus* and

Origanum dubium essential oils for 24 hours. after the incubation Cells were washed twice in PBS and fixed by fixing solution consists of (2% glutaraldehyde and 2% formaldehyde in PBS) for 4-5 minutes at room temperature, then washed with PBS after washing; 2ml of β-galactosidase (βgal) solution was added to the cells. The cells incubated in dark at 37°C (no CO2) overnight.

Chapter Three

3. Results

3.1 Effect of thymus capitatus EO

3.1.1 Morphology

hMSC-telo1 cells have a fibroblastic appearance with large spindle-shaped. After their exposure to various concentrations of *Thymus capitatus* essential oil based on (3 µg/ml, 0.3 µg/ml thymol in final concentration) for 24 hours, the cells were examined under the light microscope, and it has been observed that the cells start to gain round look as a possibility of cytotoxic activity of thymus essential oil. The cells shrunk and modified into small spindle-shaped (Figure 4).

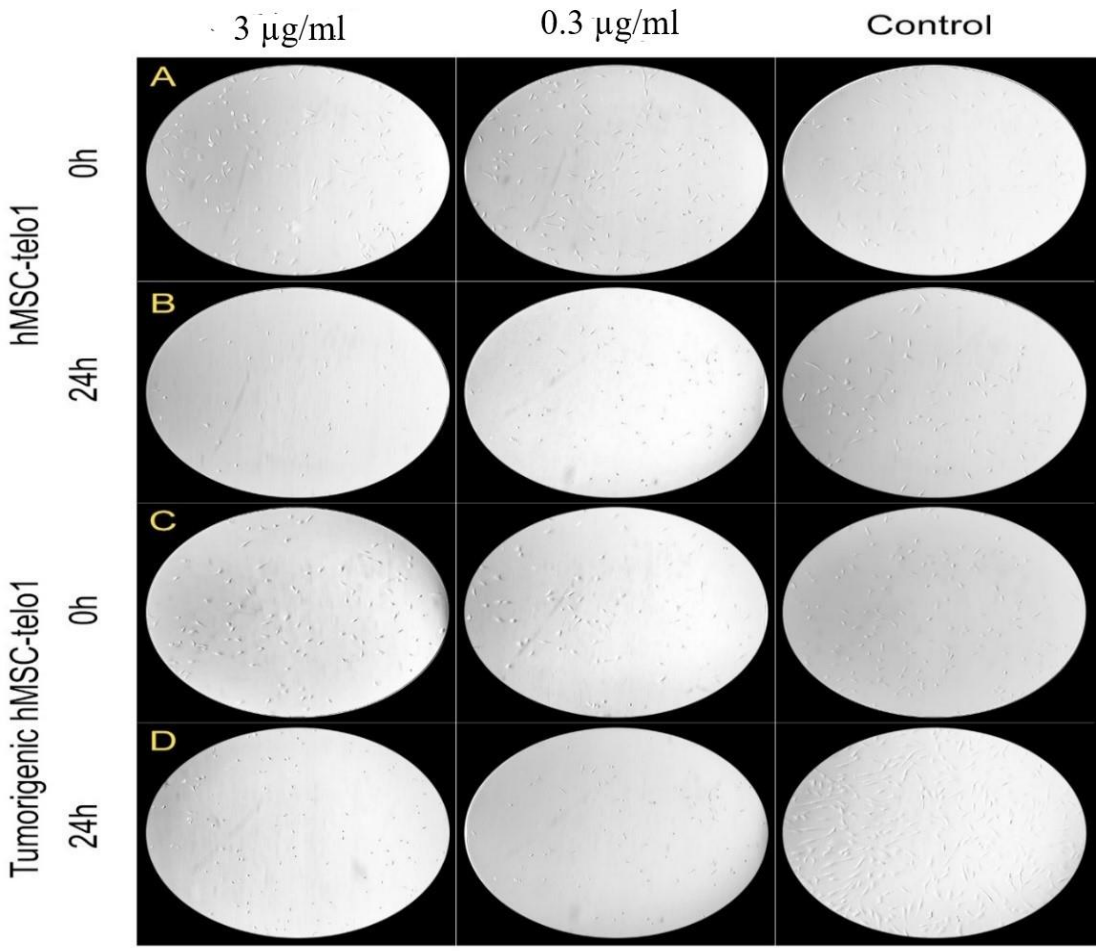


Fig.4 Morphology of hMSC-telo1 cells and tumorigenic hMSC-telo1 cells after their exposure to various concentrations (3 µg/ml first column, 0.3 µg/µl middle column), of *Thymus capitatus* **based on thymol content in the final concentration for 24 hours. (A, B first and second rows) showed the morphological alterations of hMSC-telo1 cells at 0hr and 24hr compared with control (not treated, last column). (C, D third and fourth rows) showed the morphological alterations of tumorigenic hMSC-tel1 cells at 0 h and 24 h compared with control (not treated) cells, there was a reduction in the input amount of the cells. In both cell lines, the cells had a sizeable spindle-shaped appearance (at 0 h), and they start to gain round or small spindle shape (at 24 h).**

3.1.2 Proliferation and cell viability

The population doubling level was calculated to investigate the proliferation activity of hMSC-telo1 cells and tumorigenic hMSC-telo1 cell lines, as for the untreated (control) cells, the proliferation rate of tumorigenic hMSC-telo1 (population doubling level =1.67) cells is higher than non-tumorigenic hMSC-telo1 (population doubling level = 0.88). Furthermore, hMSC-telo1 cells and tumorigenic hMSC-telo1 cells have been subjected to different concentrations of *Thymus capitatus* essential oil (corresponding 3 µg/ml, 0.3 µg/ml thymol in final concentration) for 24 hours, the cytotoxicity has been evaluated by the investigation of cell viability by using MTT assay (Figure 5).

At 3 µg/ml concentration, *Thymus capitatus* essential oil showed 2.8% viability in the tumorigenic hMSC-telo1 cells when compared to non-treated control cells as 100%. However, hMSC-telo1 cells had 1.36% viability. At (0.3 µg/ml) of *Thymus capitatus* essential oil, the viability of the tumorigenic hMSC-telo1 was (48.3%), while hMSC-telo1 cells had 101.5% viability compared to the untreated cells (control). Overall, *Thymus capitatus* essential oil has shown high cytotoxicity at high concentrations. Surprisingly, non-tumorigenic hMSC-telo1 cells had higher proliferation when compared to tumorigenic hMSC-telo1 cells at decreased concentrations, which will mean that *Thymus capitatus* essential oil has a selective effect.

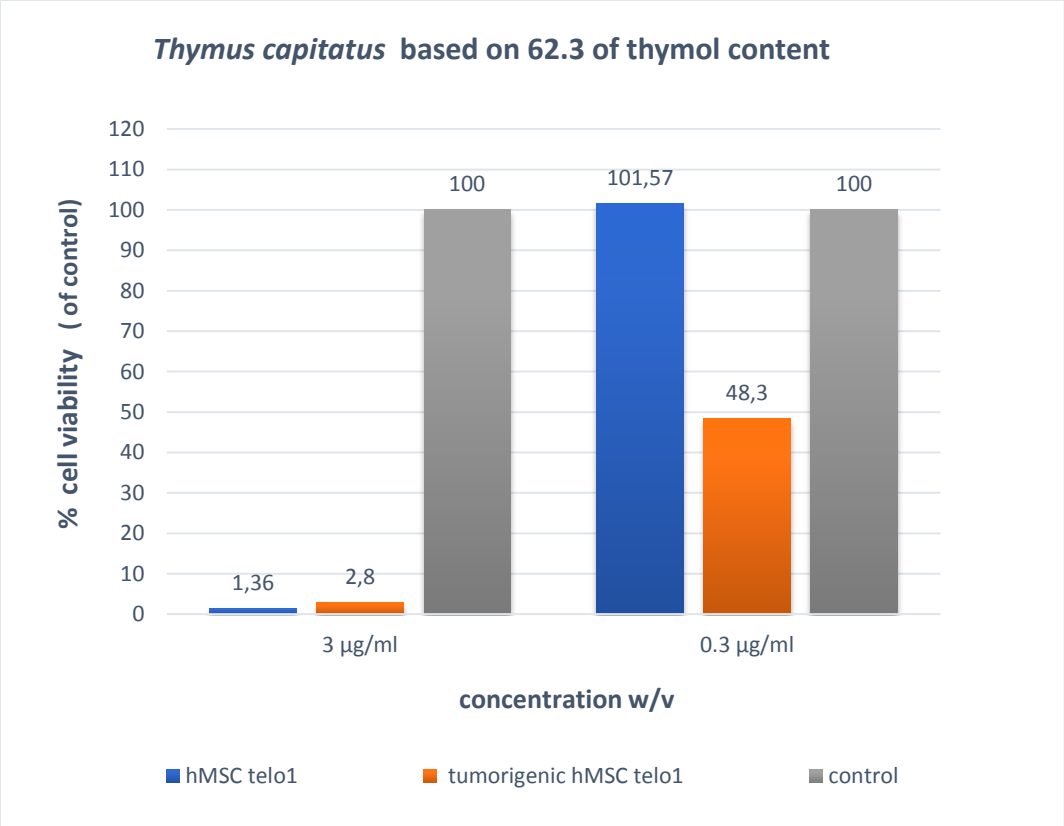


Fig.5 Cell viability determined by the MTT assay, the viability of hMSC-telo1 cells ■ and tumorigenic hMSC-telo1 cells ■ was evaluated at two different concentrations of *Thymus capitatus* essential oil (corresponding 3 µg/ml, 0.3 µg/ml thymol in final concentration) for 24 h of exposure, the number of survival cells was compared to that of untreated (control) cells ■ as 100%. At 3 µg/ml concentration of *Thymus capitatus* essential oil, the cell survival of hMSC-telo1 was (1.36%), and for the tumorigenic hMSC-telo1 cell lines, the viability was (2.8%). At 0.3 µg/ml, the viability of both hMSC-telo1 cell lines was less than 5%. At 0.3 mg/ml, the viability of the tumorigenic hMSC-telo1 cells was (48.3%); however, in hMSC-telo1 cells, it was higher than the control (101.5%).

3.1.3 Investigation of cell senescence:

hMSC-telo1 and tumorigenic hMSC-telo1 cell lines have been subjected to 0.5% v/v of *Thymus capitatus* Essential oil (corresponding 30 µg/ml of thymol in final concentration). The beta-galactosidase assay has been used for the investigation of the cell senescence by detection of blue dye produced due to the cleavage of the organic compound (x-gal) by a senescence-associated beta-galactosidase enzyme which is only present in the senescent cells. After 24 hours of exposure, both hMSC-telo1 cell lines have not shown an increase in the number of stained cells, compared to the untreated control, which had (17%) of senescence cells.

3.2 Effect of *Origanum dubium* EO

3.2.1 Morphology

hMSC-telo1 cells were subjected to various concentrations (4 µg/ml, 0.4 µg/ml) of *Origanum dubium* essential oil based on its main chemical content (carvacrol). hMSC-telo1 cells have a fibroblastic appearance with large spindle-shaped. After 24-hour exposure, the cells were examined under the light microscope it was significantly shown that the cells shrunk and have been modified to a small spindle-shaped with a rough membrane, as a result of a possible cytotoxic effect the cells took a round shape (Figure 6).

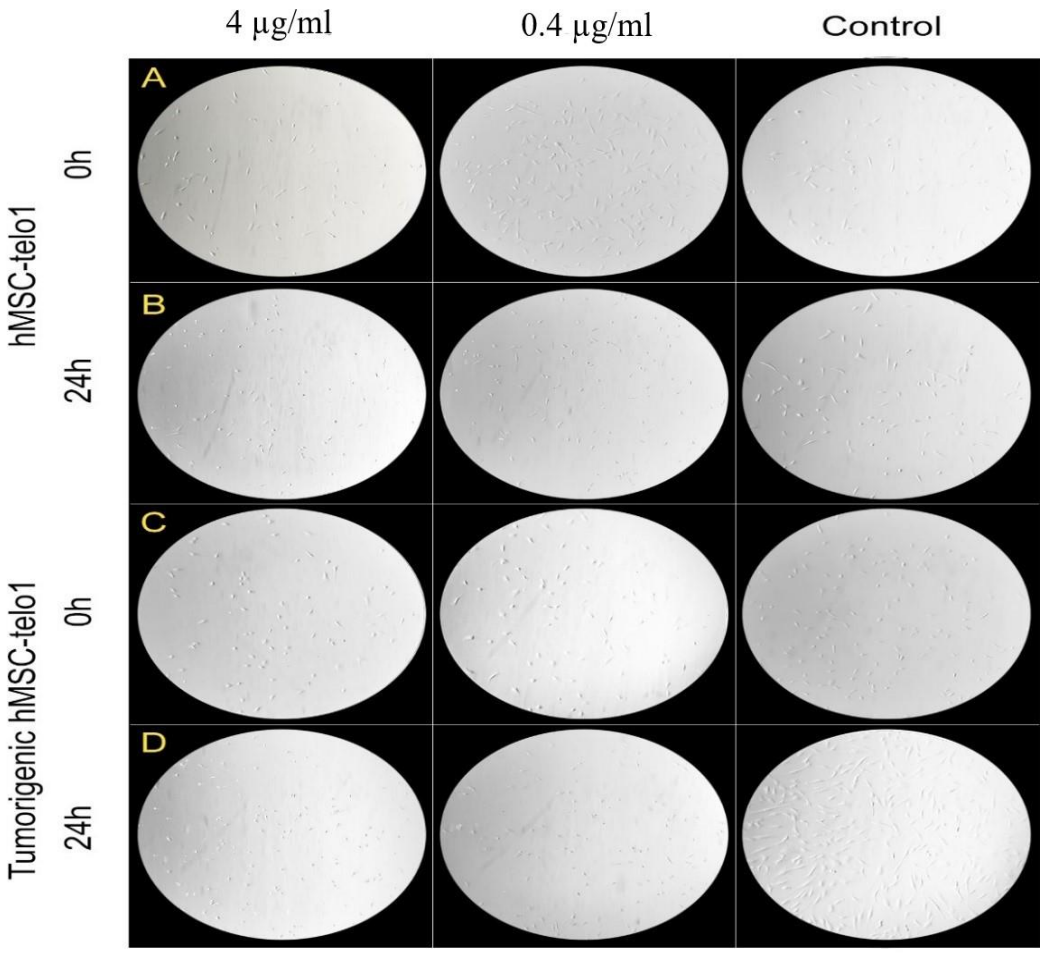


Fig.6 Morphology of hMSC-telo1 cells and tumorigenic hMSC-telo1 cells after their exposure to various concentrations (4 µg/ml first column, 0.4 µg/ml middle column) of *Origanum dubium* based on carvacrol content for 24 hours and compared to the non-treated (control last column). (A, B first and second rows) showed the morphological alterations of hMSC-telo1 cells at 0hr and 24hr compared with control (not treated) cells. (C, D third and fourth rows) morphological alterations of tumorigenic hMSC-tel1 cells at 0 h and 24 h compared with control (not treated) cells, there was a reduction in the input amount of the cells. Both hMSC-tel1 cell lines have been modified from large fibroblastic spindle-shaped (at 0 h) into small spindle appearance with and took round shape with a rough cellular membrane (at 24 h).

3.2.2 Proliferation and cell viability

The proliferation activity of hMSC-telo1 and tumorigenic hMSC-telo1 cells was evaluated after their exposure to different concentrations of *Origanum dubium* essential oil (corresponding to 4 µg/ml, 0.4 µg/ml carvacrol in final concentration) for 24 hours. It was observed that for the untreated (control) cells, the proliferation rate of tumorigenic hMSC-telo1 cells (PDL =1.67) is higher than non-tumorigenic hMSC-telo1 cells (PDL =0. 88).

Moreover, the cell viability of hMSC-telo1 and tumorigenic hMSC-telo1 cell lines was determined by MTT assay in order to evaluate the cytotoxic effect of *Origanum dubium* essential oil (Figure 7). Generally, *Origanum dubium* essential oil showed a significant cytotoxic effect on both hMSC-telo1 and tumorigenic hMSC-telo1 cell lines. After cells were exposed to 4 µg/ml of *Origanum dubium* essential oil, the viability in the tumorigenic hMSC-telo1 was 4.52% compared to that of untreated (control) cells as 100%, while it was 1.51% in the non-tumorigenic hMSC-telo1 cells. However, at 0.4 µg/ml, *Origanum dubium* essential oil showed (16.37%, 4.52%) viability in the tumorigenic hMSC-telo1 and the non-tumorigenic hMSC-telo1 cells, respectively, this determines that the cytotoxic effect of *Origanum dubium* essential oil in the non- tumorigenic hMSC-telo1 cells is significantly higher compared to the tumorigenic hMSC-telo1 cells.

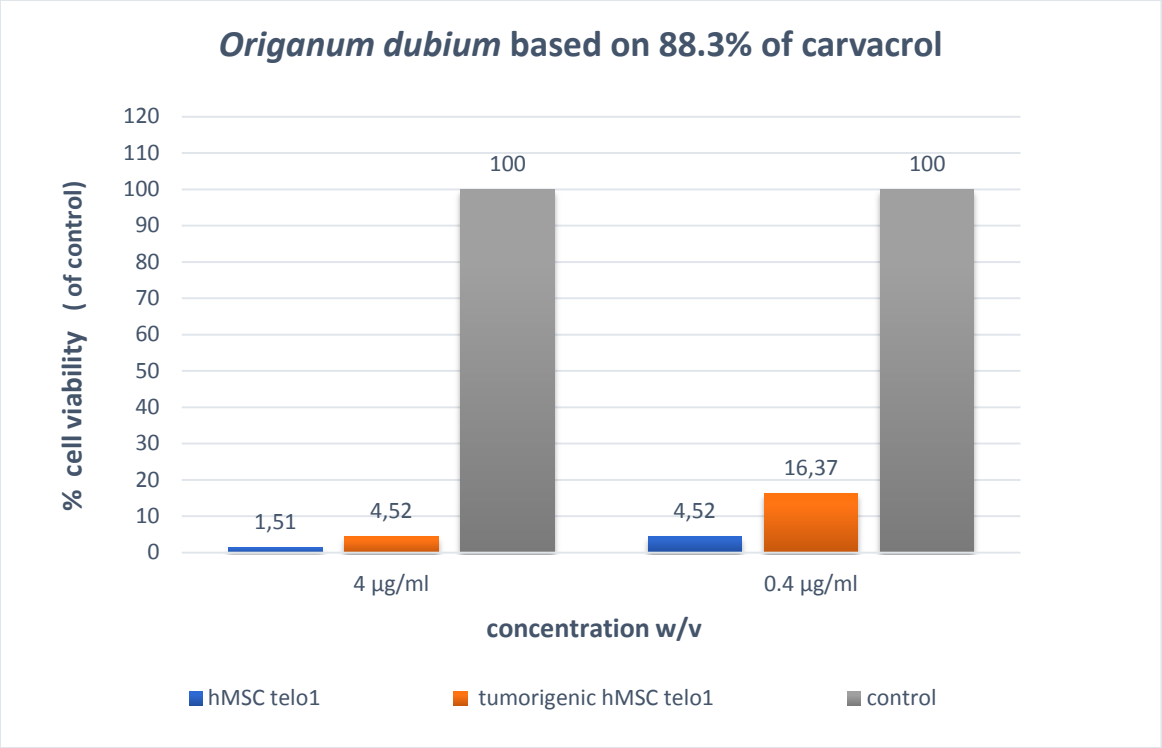


Fig.7 Cell viability determined by the MTT assay, the viability of hMSC-telo1 cells ■ and tumorigenic hMSC-telo1 cells ■ was evaluated under different concentrations (4 µg/ml, 0.4 µg/ml) of *Origanum dubium* essential oil based on carvacrol amount in final concentration, at 24 h of exposure the number of survival cells was comparable to that of untreated (control) cells ■ as 100%. It was shown that at 4 µg/ml, the viability hMSC-telo1 was 1.51%, and in tumorigenic hMSC-telo1 cell lines was 4.52. while at concentration 0.4 µg/ml, the viability of hMSC-telo1 cells was (4.52%), and in the tumorigenic hMSC-telo1 cells was (16.37%).

3.2.3 Investigation of cell senescence:

As it was mentioned previously in thyme essential oil beta-galactosidase assay was used for the evaluation of the cell senescence after exposing the hMSC-telo1 and tumorigenic hMSC-telo1 cell lines to 0.5% v/v of *Origanum*

dubium Essential Oil (corresponding 40 µg/ml of carvacrol in final concentration) for 24 h. Both cell lines have not shown an increase in the number of stained cells when compared to the untreated control. The percentage of senescence cells in the untreated control was (17%).

Chapter Four

4. Discussion

Cancer has become a tremendous term in society, that evokes a range of scientific, emotional, and economic responses. The prevalence of cancer as the leading cause of death increased its severity and the importance of spending more effort to obtain the best treatment with fewer side effects. Scientists and researchers in different medical fields all around the world have done researches and experiments to find a better cure. The essential oils were one of the most concerned, which attracted the focus of the researchers in this field as a natural source due to their antioxidant and antimicrobial activity, recently, essential oils suggested to have anticancer activity as well. The antiproliferative and cytotoxic activity of these essential oils is being investigated for their evaluation as a cancer treatment. Lamiaceae family is one of the most used as a medicinal therapy for many diseases. Among all the essential oils of the Lamiaceae family, *Thyme* and origanum essential oils are considered the most critical and widespread species of Lamiaceae family that grow wild in Cyprus and have been used in various properties in the medicinal field. *Thyme* and Origanum oils which can be extracted from various species of plants have a great attentiveness due to their pharmacological characteristics such as antimicrobials (Boskovic, M et al. 2015; Nzeako, B. C. et al. 2006), anti-inflammatory (Ismaili, H., et al. 2001; Khouya, T et al. 2015; Ocana-Fuentes, A., et al. 2010; Shen, D et al. 2010); antioxidants (Amarowicz, R et al. 2009; Kulisic, T., A. et al. 2005; Lagouri, V et al. 1993; Ceker, S et al. 2012), neuroprotective (Qneibi, M, et al. 2019; Taghouti, M, et al. 2018).

Several studies have been done for the investigation of the effect of thyme in various cancer cell lines. Ait M'Barek et al. observed the essential oils cytotoxic activity against tumor cells and its chemo-resistant counterparts. Interestingly, the study showed that the thyme essential oil has a promising antitumor effect after developing tumors by grafting the fragments of tumors from P815 (murin mastocytoma cell line) subcutaneously, the fragments were injected in the DBA-2/P815 (H2d) mouse model. The development of the tumor significantly reduced after the repeated intra-tumoral injection of EO, associated with a marked extension of the mouse life span. All cancer cell lines were differently sensitive to the cytotoxic effect of the essential oil (Ait M'Barek et al. 2007).

Recently, the antiproliferative effect of *Origanum vulgare* against human breast adenocarcinoma (MCF-7) and human colon adenocarcinoma (HT-29) was investigated (Begnini, K. et al. 2014) suggested anticancer potential. Later on, the first indication of the biological activity of *Origanum vulgare* in human dermal fibroblasts was provided by Han, Xuesheng, and his colleague in 2017. Moreover, in each of the previous studies, thyme and origanum were extracted from different species of plants; in that case, cytotoxic activity and the results determined in each study are different. Although many studies have been conducted to detect the antitumor effect of these essential oils from different species of Lamiaceae family, several laboratory experiments must be done to determine the best concentrations in order to evaluate the anticancer potential of these oils so they can be used as a successful treatment for cancer in the future.

In accordance with the above-mentioned studies, current work designed to determine the cytotoxic effect of different concentrations of two extracted essential oils of Lamiaceae family (*Thymus capitatus* and *Origanum dubium*) that grow wild in Cyprus, on two types of cells tumorigenic hMSC-telo1 cells

and non-tumorigenic hMSC-telo1 cell lines in vitro by the investigation of the cell viability, cellular morphology, the proliferation rate, and senescence in order to evaluate the anticancer potential of these oils. As it was observed in the cellular microscopic examination of our cells, the morphological alterations could be due to the loss of ability to divide and grow as a result of the cytotoxic activity. Furthermore, the great reduction in the input cells might also refer to a possibility of apoptosis (as a result of a possible cytotoxic effect of the essential oils), which is a physiological process of programmed cell death. It is characterized by numerous representative morphological features such as cell shrinkage; also, the apoptotic cell manifested as a round mass or in an elliptic appearance with dark cytoplasm. A recent study in Northern Cyprus strongly supports the result of our study. The cellular responses of immortalized stem cells (hMSC-telo1) and radiation-induced tumorigenic cell line (IR hMSC-telo1) after their exposure to increasing concentrations of *Thymus capitatus* essential oil were investigated, and the most significant effect of thymus essential oil was demonstrated after 24 hr. the cells gained pre-apoptotic look and shrunk, and the cellular membrane got rough as a result of cytotoxic activity, they also demonstrated that *Thymus capitatus* essential oil may have cytotoxic potential effect due to the significant reduction of the input cells (Yavuz, D.Ö, et al. 2017).

The cellular proliferation relies on the cell division and death rate, while the difference in the cellular response depends on many factors, such as the chemical constituents of the essential oils, concentration, time exposure, and drug resistance. It was also reported previously that the various cytotoxicity assays could give different results depending on the agent that has been tested and the employed cytotoxicity assay (Weyermann et al., 2005). Previously, ten essential oils, including thyme, were examined for the investigation of their

cytotoxic effect, Cell viability of three cancer cells were determined by the MTT assay (Zu et al., 2010). in another study, MTT assay was also used to evaluate the potential activity of four different extracts from native plants of (Lamiaceae) family in Morocco Aristolochia baetica L. and *Origanum compactum* on human breast cancer (Chaouki, W et al. 2010). moreover, *Thymus linearis*, and *Thyme serbyllum* showed notable antiproliferative activity against MCF-7, LNCaP, and NIH-3T3 cell lines, the cell viability was also assessed by MTT assay (Hussain et al. 2013). In accordance with these previous studies, our result indicated the cytotoxicity, as well as the antiproliferative effect of the *Thymus capitatus* and *Origanum dubium* essential oils on hMSC-telo1 cell and the tumorigenic hMSC-telo1 cells by using MTT assay, which is a colorimetric assay that measures the metabolic activity of the cell to determine the cell viability, and it was the most cytotoxic assay that used in the literature.

In our study, the cytotoxicity results indicate that *Thymus capitatus* and *Origanum dubium* essential oils inhibit cell proliferation in a time and dose-dependent fashion. It was the same for Cetinus et al. paper even though they used different cytotoxicity and proliferation assay (they used xCELLigence system while we used MTT assay), the value of (IC50) 0.347 mg/mL was achieved after 24 hours of treatment with thyme oil, they showed that thyme essential oil has a concentration-dependent cytotoxic effect on DLD-1 CRC cell. (Çetinus, Er. et al. (2013).

Moreover, in line with the literature, cell viability decreased at the highest concentrations. (Özkan, A., & Erdoğan, A. 2011) evaluated the cell viability of hepatoma G2 cell with the CellTiter-Blue® Cell Viability Assay. After 24-h treatment of increasing concentrations between 20-170 of *origanum onites* essential oil, carvacrol, and thymol, they also indicated that Hep G2 cells viability decreased at higher concentrations. Which supports our findings even

though they used different concentrations, under our experimental setting, according to the cell viability, we determined that *Thymus capitatus* and *Origanum dubium* decrease the viability at high concentrations (based on their main components thymol and carvacrol 3 and 4 µg/ml) respectively. Previous studies and investigations also supported our results., (Rao, S. 2014) *Origanum marjorana* ethanol extract showed anticancer potentials on fibrosarcoma (HT-1080) cell line by using MTT assay. They indicated that the cell viability of the treated fibrosarcoma cells significantly decreased at the highest concentration of 120 µg/mL.

According to our findings, we indicated that *Thymus capitatus* essential oil has a selective effect. Strikingly, an unexpected observed effect was that low-dose of the thyme essential oil stimulated proliferation and viability of non-tumorigenic cells 101.3% compared to the untreated control cells, while decreased the cell viability of the tumorigenic cells to almost half. Thyme essential oil seems to be toxic efficient if we decrease the dose; it will not kill the intact cells but the cancer cells, which is in line with a previous paper. Despite using XTT assay to determine the cytotoxicity of thyme essential oil on the human UMSCC1 head and neck squamous cell carcinoma (HNSCC) cells, Sertel and his colleagues supported our findings, they also found an unexpected effect, that subtoxic concentrations of *Thymus capitatus* essential oil increased the cell viability and stimulated proliferation. Compared to the untreated control, the curve of dose-response showed a constant rise in viability to 127.4% (Sertel et al. 2011).

Cell senescence is considered as a potential mechanism for a cell to evade malignancy; it is a natural barrier to uncontrollable cell proliferation. Many signaling pathways can lead to cell senescence, including cellular stress such as loss of tumor suppressor, hyperactivation of oncogenes, telomere

shortening, and chemotherapy. Furthermore, due to the unpleasant side effects of the cancer therapies the represent different strategies to kill the cancer cells, the scientists established that the chemical drugs and low-dose radiotherapy could be effective with minimum side effects by the senescence promotion in tumor cells. In our finding, the by Beta-galactosidase assay showed that *Thymus capitatus* and *Origanum dubium* essential oils have no significant effect on the senescence of hMSC-telo1 cell lines which may indicate that these essential oils do not affect the cell senescence.

5, Conclusion

In conclusion, our study showed that *Thymus capitatus* essential oil could be used as an effective cancer therapy in the future, which is in line with the literature, as we have mentioned previously. We also showed that *Origanum dubium* essential oil is not good enough as a treatment independently; however, it can be used with modifications as an enhancer of standard chemotherapy; moreover, it may need further investigations.

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