REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOENGINEERING

INVESTIGATION OF POTENTIAL INHIBITORY EFFECTS OF NATURAL COMPOUNDS FROM MELISSA OFFICINALIS L. IN DRUG RESISTANT NON SMALL CELL LUNG CARCINOMA

MELİKE DOĞUKANLI

MASTER OF SCIENCE

ADANA 2022

REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOENGINEERING

INVESTIGATION OF POTENTIAL INHIBITORY EFFECTS OF NATURAL COMPOUNDS FROM MELISSA OFFICINALIS L. IN DRUG RESISTANT NON SMALL CELL LUNG CARCINOMA

MELİKE DOĞUKANLI
MASTER OF SCIENCE

SUPERVISOR
ASSIST. PROF. DR. ÖZGÜR CEM ERKİN

ADANA 2022

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

[Signature]

Melike DOĞUKANLI

ABSTRACT

INVESTIGATION OF POTENTIAL INHIBITORY EFFECTS OF NATURAL COMPOUNDS FROM MELISSA OFFICINALIS L. IN DRUG RESISTANT NON-SMALL CELL LUNG CARCINOMA

Melike DOĞUKANLI
Department of Biongineering
Supervisor: Assist. Prof. Dr. Özgür Cem ERKİN
December 2022, 69 pages

In cancer treatment, the development of new generation drugs and innovations in surgery have significantly extended the life expectancy of patients. However, despite all these developments, the main obstacle in the treatment of lung cancer is the recurrence of the disease over time due to intrinsic or extrinsic resistance to the chemotherapeutic drugs used in the treatment. Alternative methods and new therapeutics are needed to cope with the development of chemoresistance. Due to their low cytotoxicity and good tolerability in the human body, natural plant compounds are very important sources that can be used to obtain drug active ingredients that have been used in the treatment of many diseases, including cancer, for many years. In this study, the anticarcinogenic effects of *Melissa officinalis* L., a well-known medicinal plant from the *Lamiaceae* family, widely grown in Turkey and known as lemongrass, on the non-small cell lung cancer cell line A549 and the erlotinib resistant A549 sub-cell line and its parallel parental sub cell-line were evaluated.

In our study, four different extracts were obtained from the leaf parts of *Melissa officinalis* L. plant with different solvents. The cytotoxic effects of these extracts on each cell line were determined by MTT cell viability analysis. As a result, although each type of extract was cytotoxic on each sub-cell line, there was no significant difference between their inhibitory intensities. The remainder of the study was then continued with the total methanolic fraction, which includes all of the substance groups in the plant leaf. Afterwards, the effects of this extract on the migration of different sub-cell lines were determined by the wound healing assay, whereas tumorigenic capacities were determined by the soft agar colony formation test. Finally

the effects of the total methanolic extract on the cell cycle progression were determined by flow cytometry analysis.

According to the results, increasing concentration of total MeOH extract of *Melissa officinalis* L. decreases cell viability in a concentration dependent manner and prevents both migration capacity and colony formation in all types of sub-cell lines. A decrease was observed in the S-phase cell populations, where DNA replication takes place, compared to cells exposed to control in all three sub-cell lines. In the light of these findings, it is thought that *Melissa officinalis* L. plant may represent an alternative source of novel therapeutic compounds for treatment of non-small cell lung cancer.

Keywords: *Melissa officinalis* L. , natural compounds, A549, anticancer , drug resistance

ÖZET

MELISSA OFFICINALIS L. DOĞAL BİLEŞİKLERİNİN İLAÇ DİRENÇLİ KÜÇÜK HÜCRELİ DIŞI AKCİĞER KANSERİNDE POTANSİYEL İNHİBİTÖR ETKİLERİNİN ARAŞTIRILMASI

Melike DOĞUKANLI
Biyomühendislik Anabilim Dalı
Danışman: Dr. Öğr. Üyesi Özgür Cem ERKİN
ARALIK 2022, 69 sayfa

Kanser tedavisinde yeni nesil ilaçların geliştirilmesi ve cerrahideki yenilikler hastaların yaşam beklentisini önemli ölçüde uzatmıştır. Ancak tüm bu gelişmelere rağmen akciğer kanseri tedavisindeki temel engel, tedavide kullanılan kemoterapötik ilaçlara karşı içsel veya dışsal direnç nedeniyle hastalığın zamanla tekrarlamasıdır. Kemorezistansın gelişmesiyle başa çıkmak için alternatif yöntemlere ve yeni terapötiklere ihtiyaç vardır. Doğal bitki bileşikleri, düşük sitotoksisiteleri ve insan vücudunda iyi tolere edilebilirlikleri nedeniyle kanser dahil birçok hastalığın tedavisinde uzun yıllardır kullanılan ilaç etken maddelerinin elde edilmesinde kullanılabilecek çok önemli kaynaklardır. Türkiye'de yaygın olarak yetiştirilen ve limon otu olarak bilinen *Lamiaceae* familyasından iyi bilinen bir tıbbi bitki olan *Melissa officinalis* L.'nin küçük hücreli dışı akciğer kanseri hücre hattı A549 ve erlotinib dirençli A549 alt hücre hattı ile ilaç hariç paralel koşullarda geliştirilmiş parental alt hücre hattı üzerindeki antikanserojenik etkileri değerlendirilmiştir.

Çalışmamızda *Melissa officinalis* L. bitkisinin yaprak kısımlarından farklı çözgenlerle dört farklı ekstrakt elde edilmiştir. Tüm ekstraktların farklı A549 alt hücre hatları üzerindeki sitotoksik etkileri MTT hücre canlılık testi ile belirlenmiştir. Bu analizde farklı çözgenlerle üretilen özütlerin herbirinin tüm alt hücre hatlarında etkili oldukları görülmekle birlikte aralarında önemli farklılıklar bulunmadığı görülmüştür. Bundan dolayı bitkideki tüm madde gruplarını içeren total metanolik fraksiyon ile çalışma devam ettirilmiştir. Daha sonra total metanolik yaprak fraksiyonunun tüm alt hücre hatlarında hücre migrasyonu üzerindeki etkileri yara iyileştirme testi ile, hücrelerin koloni oluşturma ve tümörijenik kapasiteleri üzerindeki etkileri yumuşak agar koloni oluşturma testi ile belirlenmiştir. Son olarak aynı yaprak fraksiyonunu kanser hücre döngüsü ilerleyişi üzerindeki etkileri de akış sitometrisi analizi ile belirlenmiştir.

Analiz sonuçlarına göre, total metanolik yaprak fraksiyonunun artan konsantrasyonun, konsantrasyona bağlı biçimde hücre canlılığını azalttığı ve üç alt hücre hattında da göç kapasitesini ve koloni oluşumunu önlediği, ayrıca DNA replikasyonunun gerçekleştiği S fazındaki hücre popülasyonunu, kontrol bileşiğine maruz bırakılan hücrelere göre azalttığı belirlenmiştir. Bu bulgular ışığında *Melissa officinalis* L. bitkisinin küçük hücreli dışı akciğer kanseri tedavisi için yeni bir alternatif terapötik kaynağı olabileceği düşünülmektedir.

Anahtar Kelimeler: *Melissa officinalis* L., doğal bileşikler, A549, anti-kanser , ilaç direnci

ACKNOWLEDGEMENTS

I would like to acknowledge and express my gratitude to my advisor, Asst. Prof. Dr. Özgür Cem ERKİN, for his patience, guidance, and immense knowledge during my study.

Besides my advisor, I would like to thank the rest of my thesis committee, Prof. Dr. Mehmet Bertan YILMAZ and Asst. Prof. Dr. Merve ÇAPKIN YURTSEVER for their encouragement and advice.

I would also like to give special thanks to my family as a whole for their continuous support during my research and writing my thesis.

Finally, I would like to thank TÜBİTAK Directorate of Science Fellowships and Grant Programmes (BİDEB) for financially supporting my thesis within the scope of 2210-C National MSc/MA Scholarship Program in the Priority Fields in Science and Technology.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

Akt : Protein Kinase B

ALK : Anaplastic Lymphoma Kinase

BRAF : Proto-oncogene B-Raf

CDH1 : E-cadherin

cDNA : complementary Deoxyribonucleic acid

DMEM : Dulbecco's Modified Eagle Medium

EDTA : Etilendiamin Tetraasetic Acid

EGFR : Epidermal Growth Factor Receptor

EGFR-TKI : Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor

EML4 : Echinoderm Microtubule-associated Protein-like 4

EMT : Epithelial to Mesenchymal Transition

ER : Erlotinib Resistant Subline

FBS : Fetal Bovine Serum

FDA : Food and Drug Administration

KRAS : Kirsten Rat Sarcoma Viral Proto-oncogene

MAPK : Mitogen-Activated Protein Kinase

mTOR : mammalian Target of Rapamycin

MTT : 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

NSCLC : Non-Small Cell Lung Cancer

PBS : Phosphate Buffered Saline

PI3K : Phosphatidylinositol 3-Kinase

PS : Penicilin Streptomycin Solution

PT : Parental Subline

qRT-PCR : Quantitative Reverse Transcription Polimerase Chain Reaction

qPCR : Quantitative Polimerase Chain Reaction

SCLC : Small Cell Lung Cancer

STAT3 : Signal Transducer and Activator of Transcription 3

VEGF : Vascular Endothelial Growth Factor

1. INTRODUCTION

Lung cancer is one of the most common and fatal types of cancer worldwide after breast cancer. It is the main cause of cancer-related deaths in men, while it is in the second place after breast cancer in women (WHO, 2020). Lung cancer is categorized histologically in 2 main groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Inamura, 2017). NSCLC is further subdivided into adenocarcinoma, squamous cell carcinoma (SqCC), and large cell carcinoma (Travis et al., 2015). About 80-85% of the encountered lung cancers are NSCLC and makes it the most commonly diagnosed type of the disesase in the clinic (Molina, Yang, Cassivi, Schild, and Adjei, 2008).

Various reasons can cause lung cancer. Smoking and having relatives with lung cancer increases the risk of developing the disease (Wangari-Talbot and Hopper-Borge, 2013a). However, lung cancer can occur at people who never smoked or who is not a second hand-smoker. The way of treating lung cancer is determined by the stage of the disease. Options such as surgery, radiotherapy and chemotherapy (Molina et al., 2008) have been applied individually or in different combinations (Mustafa et al., 2016).

Surgical operations have been identified as the best option in non-small cell lung cancer treatment. However, the disease is generally noticed at the advanced levels~~,~~ and surgery can be applied to only less than 25% of patients. This leaves chemotherapy as the sole treatment option for this kind of patients.But chemotherapy can also fail due to development of chemoresistance (Xiao and He, 2010). Classic chemotherapy drugs, target cancer cells that can multiply rapidly. But these cells develop resistance to the drugs by changing their genetic structure and activating stress mechanisms (Sosa Iglesias, Giuranno, Dubois, Theys, and Vooijs, 2018)

Drug resistance may appear through two main mechanisms: intrinsic (or *de novo*) or acquired drug resistance (Du and Shim, 2016). Intrinsic drug resistance occurs under the influence of number of factors before treatment. In this case, genetic and epigenetic factors are thought to have an effect. Acquired resistance is the resistance that occurs during or after treatment. It can be associated with the mutations that may occur due to chemical exposure or spontaneous changes in genetic structure in cancer cells. In this case, the patient initially responds to treatment, but later develops drug resistance (Holohan, Van Schaeybroeck, Longley, and Johnston, 2013).

In the clinical treatment of cancer patients, drug resistance is a major concern due to low survival rates. Therefore, new therapeutic drugs are needed to overcome drug resistance. Natural plant compounds provide an important alternative resource for the discovery of new therapeutics due to their low toxicity and good tolerance in the human body. Accordingly, it has been determined that the use of many plants rich in phytochemical content together with chemotherapeutic agents increases the effectiveness of the drug and reduces the side effects in cancer patients.

Melissa officinalis L., which is accepted as a medicinal plant, spreads in the Mediterranean region and has reported antidepressant, antioxidant, antimicrobial and most importantly anticancer effects in human body.

The main purpose of this study is to investigate the potential anti carcinogenic effects of *Melissa officinalis* L. plant extracts especially on drug resistant NSCLC cells. After examination of their chemical contents, plant extracts were used to determine their effects on the cell viability, migration,colony forming capacity and cell cycle progression of drug resistant NSCLC cells by various *in vitro* methods.

2. LITERATURE REVIEW

2.1. Lung Cancer

2.1.1. Epidemiology and etiology of lung cancer

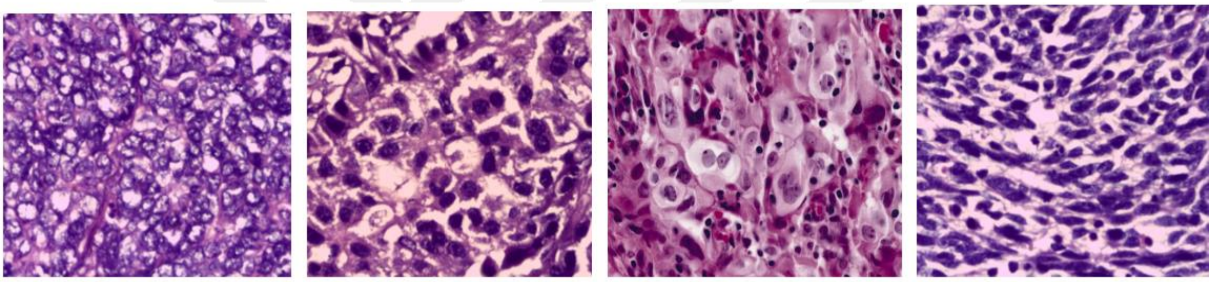
Lung cancer is the formation of a malignant tumor mass in the human lungs due to the uncontrolled proliferation of cells in normal tissue. While lung cancer is the most typical reason behind cancer-related deaths in men worldwide, it ranks second after breast cancer in women. Parallel to the prevalence of cases, the main reason for the increase in mortality rates is that the disease can only be diagnosed in advanced stages and even the most complicated treatment methods are ineffective in curing the disease (Pathak et al., 2004). Lung cancer is the second most common type of cancer in 2020, with over 2.2 million new cancer cases. In addition, lung cancer constitutes 17.6% (41.264) of 233,834 cancer cases diagnosed in our country and ranks first in cancer-related deaths according to World Health Organization data (WHO, 2020).
Smoking is one altogether the foremost important cause of lung carcinoma. Furthermore, it is responsible for 80-90% of lung cancer-related deaths. The danger of developing lung carcinoma is 20-30 times higher in smokers (Burcu, 2012). Smoking is the most typical risk, but other factors such as air pollution, asbestos or radon gas exposure, and passive smoking are also involved in lung cancer formation. Lung cancer also can be inherited, such that someone with a family history of carcinoma, is about four-fold more likely to have lung cancer (Wangari-Talbot and Hopper-Borge, 2013a).

2.1.2. Classification of lung cancer

Histopathological classification of lung cancer plays a particularly important role in the progression of the disease and the determination of the appropriate treatment method. Lung cancer is divided into two basic classes depending on the size of the affected cells when viewed with the aid of a microscope (Arkan, 2016).

Small cell lung cancers account~~s~~ for about 15% of all lung cancers. They are fast-growing and quickly metastasizing cancers(Abeloff, Eggleston, Mendelsohn, Ettinger, and Baylin, 1979). On the other hand, NSCLCs account~~s~~ for 85% of detected lung cancers and further divided into three histological types according to the classification system proposed by the World Health Organization (Travis et al., 2015). These are squamous cell carcinomas, adenocarcinomas and large cell carcinomas (Zappa and Mousa, 2016). Correctly determining the histological subtype is crucial in choosing the most appropriate treatment (Petrella, 2015).

Adenocarcinomas account for almost 40% of all lung cancers. It is more common in non-smokers, especially women. If the tumor tissue is examined, it appears to have a heterogeneous structure, usually on the lung's surface parts. Due to its early metastatic feature, it is considered to have a poor prognosis. Squamous cell carcinomas account for about 30% of all lung cancers and are the most common type after adenocarcinomas. It is closely related to smoking and is more common in men. In squamous cell carcinoma, the masses are located in the central parts of the lungs, close to the primary airway. Large cell carcinomas account~~s~~ for 10% of lung cancers. They mostly consist of large masses located peripherally and can occur in any part of the lungs. Their development is closely related to smoking and they have a poor prognosis due to early metastasis and rapid growth (Arkan, 2016; Ergelen, 2013; Travis, 2011).



(a) Squamous cell carcinoma (b) Adenocarcinoma (c) Large cell carcinoma (d) Small cell carcinoma

Figure 2.1 Representative pathological specimens from four classes of typical lung cancers. Adopted from: (Gao, Yue, Chen, and Wang, 2014).

2.1.3. Non-small cell lung cancer treatment methods

The strategy to be followed for treating lung cancer patients varies depending on the stage of the disease, which is determined by the size of the tumor and the degree of tissue penetration (Table 2.1). American Joint Committee on Cancer (AJCC) TNM system is used for staging lung cancer. In this system, the features of the tumor (T), its spread to the lymph nodes (N), and the conditions of metastasis (M) are examined. Numbers and letters are used to obtain more detail after the letters T, N, and M (Edge and American Joint Committee on Cancer, 2010).

Table 2.1 Lung Cancer-Staging Chart

STAGES		TNM SUBGROUPS
STAGE 1	IA1 IA2 IA3 IB	T1(mi,a) N0 M0 T1(b) N0 M0 T1(c) N0 M0 T2(a) N0 M0
STAGE 2	IIA IIB	T2(b) N0 M0 T1(a,b,c) N1 M0 T2(a,b) N1 M0 T3 N0 M0
STAGE 3	IIIA	T1(a,b,c) N2 M0 T2(a,b) N2 M0 T3 N1 M0 T4 N(0,1) M0
	IIIB	T1(a, b, c) N3 M0 T2(a, b) N3 M0 T3 N2 M0 T4 N2 M0
	IIIC	T3 N3 M0 T4 N3 M0
STAGE 4	IVA IVB	Any T Any N M1(a, b) Any T Any N Mc

Source: (American Cancer Society, 2015b; Ergelen, 2013; Turhan, 2018)

Surgery is the primary treatment for Stage I, where the tumor is localized in the lungs, and for Stage II, where the tumor has spread to the lymph nodes around the lung. In addition, radiotherapy treatment is applied to patients who cannot be operated on or who do not accept surgery (Scott, Howington, Feigenberg, Movsas, and Pisters, 2007). In stage II lung cancer patients, if the tumor tissue is completely resected, adjuvant chemotherapy is suggested to scale back the chance of cancer recurrence (Lemjabbar-Alaoui, Hassan, Yang, and Buchanan, 2015). Stage IIIA is one of the early stages of lung cancer, such as stages I and II. At this stage, suitable patients undergo surgical resection after neo-adjuvant chemotherapy, while chemotherapy treatment is applied simultaneously with radiotherapy in patients with tumors which cannot be operated (Depierre et al., 2016; Paraschiv et al., 2016). In stage IIIB, one of the advanced and metastatic stages, the tumor has spread to the lymph nodes in the lungs or neck. Surgical resection can be performed at this stage if the tumor shrinks with platinum-based chemotherapy. However, if surgery is not possible, the treatment regimen is determined as chemotherapy simultaneously with radiotherapy (Crinò, Weder, Meerbeeck, and Felip, 2010). Chemotherapy is the only treatment option for advanced metastatic stage IV. The mixture of medications like cisplatin and carboplatin with other conventional drugs like paclitaxel, vinorelbine, gemcitabine, and docetaxel, should be preferred within the treatment protocol to be determined (Wangari-Talbot and Hopper-Borge, 2013b). At this stage, radiotherapy treatment can also be palliative (Alberts, 2007).

The aim of chemotherapy treatment in advanced NSCLC is to increase the quality of life and life expectancy of the patients (Tsvetkova and Goss, 2012). In line with a better understanding of molecular mechanisms of the disease, targeted drugs have begun to be developed for NSCLC treatment. Mutations and amplifications in EGFR, translocations in EML4-ALK, and mutations in genes such as BRAF and KRAS are some of the biomarkers identified and targeted in lung cancer (Wangari-Talbot and Hopper-Borge, 2013b).

EGFR, one of these biomarkers, has a critical role in the development and progression of many human cancers, including NSCLC, due to its effects on cell cycle, apoptosis, cell migration, and angiogenesis (Zheng, 2017). Overexpression of EGFR is observed in approximately 60% of patients with metastatic NSCLC and it is related to poor prognosis (Nurwidya et al., 2012). Also, overexpression causes many signaling pathways such as PI3K/AKT, MAPK, and STAT to remain constantly activated and can result in uncontrolled proliferation of cancer cells (Ono and Kuwano, 2006; Sharma, Bell, Settleman, and Haber, 2007). For such reasons, the EGFR protein has been a frequently preferred target for new studies in treating the disease.

Gefitinib and erlotinib are first-generation EGFR tyrosine kinase inhibitors approved by the American Food and Drug Administration (FDA). As a result of clinical studies, their use as chemotherapeutic agents has been found promising. These small molecule inhibitors competitively bind to the ATP binding site of the EGFR receptor and selectively and reversibly block EGFR phosphorylation, preventing the activation of signaling pathways related to cell proliferation (Abouzid and Shouman, 2008; Krause and Van Etten, 2005; Tong, Wu, Loong, Cho, and To, 2017). However, chemoresistance (both intrinsic and acquired) decreases the success rate of treatment in most patients. And therefore, second and third-generation tyrosine kinase inhibitors have been developed.

Afatinib, for instance, is a second-generation EGFR tyrosine kinase inhibitor. It irreversibly binds to the enzyme-active inner part of EGFR and blocks intracellular growth signal transduction pathways (Nan, Xie, Yu, and Liu, 2017). In treatments performed on patients, afatinib has increased progression-free survival compared to gemcitabine and cisplatin treatments (Wu et al., 2018). Similarly, osimertinib, a third-generation tyrosine kinase inhibitor, is used to slow the progression of the disease and stop its spread by targeting metastatic EGFR T790M mutation-positive NSCLC (M. Singh and Jadhav, 2018). Studies have shown that osimertinib improves progression-free survival in patients with advanced NSCLC compared to standard TKIs (gefitinib or erlotinib) (Soria et al., 2018).

Despite the clinical benefits of EGFR TKIs, most NSCLC patients develop drug resistance sometime after initiation of treatment and it possesses great challenges in targeted cancer therapy.

2.2. Drug Resistance

The first is natural, intrinsic drug resistance, in which factors that cause resistance in the body are pre-existing before the patient is treated with medication. Intrinsic resistance may arise due to inherent genetic mutations that limit the ability of cancer cells to respond, the presence of heterogeneous tumors in non-drug-sensitive tissues, and internal mechanisms that activate against environmental factors such as anti-cancer drugs. The other is acquired resistance which can develop after exposure to the drug .The acquired drug resistance is the failure of initially sensitive cells to respond to therapy due to mutations in cells during treatment, increased expression of therapeutic targets, and activation of alternative signaling pathways(Wang, Zhang, and Chen, 2019)

Several complex mechanisms may regulate chemoresistance. Projected mechanisms include drug inactivations, drug effluxes, drug target changes, involvement of DNA damage repair genes, gene amplifications, and epithelial-mesenchymal transition (EMT) (Housman et al., 2014; Zheng, 2017). Understanding these regulatory mechanisms plays an essential role in increasing the effectiveness of chemotherapy and overcoming drug resistance (Wangari-Talbot and Hopper-Borge, 2013b).

2.2.1. Role of drug inactivation in drug resistance

The effectiveness and activities of anticancer drugs are regulated by complex mechanisms. Interactions of drugs with different proteins can alter, disrupt, complicate or cause activation of drugs. In addition, cancer cells can become resistant by reducing the activity of drugs (Druker et al., 2001).

Cancer cells can suppress drug activity by interacting with several sulfur-containing molecules. One of these molecules is metallothionein, a protein-rich in cysteine, with a low molecular weight, and localized in the Golgi apparatus. Metallothioneins (MTs) can bind to metal ions such as zinc, copper, selenium, mercury, cadmium, and platinum. Synthesis of metallothioneins increases during stress when protection against cell cytotoxicity or DNA damage is required (Shanker, Willcutts, Roth, and Ramesh, 2010; Thirumoorthy, Manisenthil Kumar, Shyam Sundar, Panayappan, and Chatterjee, 2007).

Metallothioneins can also be linked to chemotherapeutics used in cancer therapy, such as platinum-based cisplatin. Cisplatins interfere with DNA repair mechanisms, causing DNA damage and triggering programmed cell death or apoptosis in cancer cells. Studies have shown that cisplatin in the cell induces MT expression, and MTs protect cell DNA from the toxic effects of cisplatin. In a study by Matsumoto et al., after chemotherapy treatment was applied to non-small cell lung cancer patients, an increase in MT expression was observed, which was thought to be related to drug resistance (Matsumoto et al., 1997).

2.2.2. Role of carrier pumps in drug resistance

Adenosine triphosphate (ATP)-binding cassette (ABC) transport proteins are families that transport drugs, amino acids, peptides, sugars, and many substrates through cell membranes via ATPase carriers or channel proteins (Altenberg, 2004; Zheng, 2017). Members of this family are involved in many mechanisms responsible for drug resistance. Overexpression of ABC proteins reduces the accumulation of anticancer drugs in the cell, resulting in drug resistance and allowing cancer cells to survive.

The most important members of the ABC family known to play a role in drug resistance are P-glycoprotein (P-gp), multiple drug resistance-related proteins (MRP), and breast cancer resistance protein (BCRP) (Sodani, Patel, Kathawala, and Chen, 2012). P-glycoprotein, which is an outflow pump works with ATP hydrolysis, is the first identified member of the ABC family proteins(J. H. Lin and Yamazaki, 2003). It has been determined that P-gp overexpression causes multiple drug resistance by preventing the accumulation of drugs used in cancer treatment (such as leukemia, ovarian, and breast cancer) in the cell (Balayssac, Authier, Cayre, and Coudore, 2005). However, no relation has been established between P-gp and multiple drug resistance in lung cancer (Lai et al., 1989; Oka et al., 1997).

Multidrug-Resistance Protein 1 (MRP1) has been identified as an exhalation pump responsible for the multidrug-resistant phenotype in small cell lung cancer cells (Munoz, Henderson, Haber, and Norris, 2007). MRP1 expression has been shown to be a major cause of failed chemotherapy and reduced overall survival in NSCLC (Hsia, Lin, Wang, Ho, and Kao, 2002; Munoz et al., 2007). MRP7, another member of this family, is a protein produced in all cells. Its overexpression has been detected in many types of cancer. While MRP7 is rarely expressed in normal lung cells, it has been found to be very high in NSCLC specimens (Wangari-Talbot and Hopper-Borge, 2013a). Another study revealed that MRP7 gene expression could be used as a biomarker for preliminary diagnosis in paclitaxel-resistant lung cancer cells (Oguri et al., 2008).

Breast cancer resistance protein (BCRP) is the last identified member of the ABC family. Its over-expression causes drug resistance by decreasing the intracellular accumulation of the drug. In a normal lung cell, the expression of BCRP is so low compared to MRP1 and MRP7. But strong BCRP expression has been identified in lung tumor cells (Kawabata et al., 2001). In NSCLC cells, BCRP expression is associated with resistance to tyrosine kinase inhibitors. The low concentration of TKIs induces ATP hydrolysis, while their high concentrations prevent BCRP from carrying solutes (Shukla, Chen, and Ambudkar, 2012).

2.2.3. Role of drug target change in drug resistance

Their molecular targets influence ~~s~~ the effect of chemotherapeutic agents. Some changes occurring at the target can cause drug resistance. These changes may be expression differences or mutations at the target (Mansoori, Mohammadi, Davudian, Shirjang, and Baradaran, 2017).

2.2.3.1. Epidermal growth factor receptor (EGFR)

Recent research has revealed that EGFR's overexpression and mutations play a crucial role in the chemoresistance of NSCLC. Many tyrosine kinase inhibitors (TKIs) that target EGFR have been tested to prevent this resistance and to slow down the cancer progression (Sridhar, Seymour, and Shepherd, 2003).

As mentioned in section 2.1.3, the first-generation reversible EGFR-tyrosine kinase inhibitors approved by the FDA for the treatment of metastatic NSCLC are gefitinib and erlotinib (M. Singh and Jadhav, 2018). However, patients gain resistance to these drugs within about one year from the start of treatment (Huang and Fu, 2015). The most prominent and frequently studied resistance mechanism is an acquired T790M point mutation, which occurs when methionine is replaced by threonine at amino acid position 790 in exon 20 at the ATP binding site of EGFR (Tan, Gilligan, and Pacey, 2015). As a result of this mutation, a higher affinity for binding to ATP is observed, and EGFR-related signal transduction pathways are activated and promote tumor growth. Because of the conformational change resulting from the T790M mutation in EGFR prevents competitive binding of gefitinib or erlotinib, cancer cells develop resistance to inhibition by these drugs (Gonzales et al., 2008). Since treatment with first-generation tyrosine kinase inhibitors has failed due to drug resistance, second-generation drugs that irreversibly bind ~~s~~ to the ATP binding site with higher affinity, have been developed (Tong et al., 2017). Likewise, strong EGFR inhibitory effects against T790M mutations were tried to be obtained with third-generation drugs (Russo et al., 2017).

Other common mutations in EGFR are missense mutation (L858R) in Exon 21 and deletion in Exon 19. These are known as classical activating mutations, resulting in increased EGFR kinase activity and thus increased signaling. In addition to these EGFR-activating mutations in lung cancer cells, other genetic changes can cause resistance to EGFR inhibitors. These can be increased PI3K/AKT signal, IGFR1 crosstalk, and KRAS mutations (Wangari-Talbot and Hopper-Borge, 2013b).

PI3K/AKT/mTOR is an important signaling pathway involved in the regulation of the cell cycle. Activation of PI3K localizes it to the plasma membrane, and consequently, AKT serine/threonine-specific protein kinase is phosphorylated. AKT is associated with cellular silence, proliferation, transcription, cell migration, and cancer (Wangari-Talbot and Hopper-Borge, 2013a). Loss of tumor suppressor PTEN, which plays a role in regulating the PI3K/AKT/mTOR signaling pathway, causes the signal to increase. The increase in phosphorylated AKT has been associated with intrinsic resistance to EGFR inhibitors (L. Lin and Bivona, 2012). In fact, it has been found that preventing loss of PTEN in H1650 lung cancer cells that are resistant to erlotinib makes the cells susceptible to erlotinib again (Sos et al., 2009).

IGFR1 is a hormone with a similar molecular structure as insulin. This hormone plays a role in childhood growth and has anabolic effects in adults. Crosstalk between EGFR and IGFR1 also stimulates intrinsic resistance in EGFR-targeted treatments (Cortot et al., 2013).

The *KRAS* proto-oncogene encodes the K-ras protein acting in the RAS/MAPK signaling pathway. This protein sends signals to the cell nucleus, allowing it to divide, grow, or differentiate. Point mutations occurring in codons 12, 13 and 61 of the *KRAS* gene were found to have a negative effect on the treatment of NSCLC with EGFR tyrosine kinase inhibitors (Slebos et al., 1990). The effects of gefitinib and erlotinib on 60 lung cancer cells were investigated in a study.Tumor cells with normal *KRAS* gene responded to these drugs whereas those with abnormal *KRAS* did not (Pao et al., 2005).

2.2.3.2. Echinoderm Microtubule-Associated Protein-Like 4 – Anaplastic Lymphoma Kinase (EML4- ALK)

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor. ALK receptor gene combines with the Echinoderm Microtubule-Associated protein-like 4 (EML4) gene to form a fusion gene after a chromosomal inversion in 3-5% of lung adenocarcinomas. (Soda et al., 2007; Takeuchi et al., 2012). A study observed that patients with ALK-EML4 fusion could not benefit from EGFR tyrosine kinase inhibitors (Koivunen et al., 2008; Shaw et al., 2009). Patients with this rearrangement respond to specific ALK inhibitors, the most promising of which is crizotinib (U. McDermott et al., 2008). However, it was determined that some patients developed resistance to crizotinib in about one year (Choi et al., 2010). C1156Y and L1196M mutations in the kinase domain of ALK play a role in resistance development (Choi et al., 2010). Treatment is continued with tyrosine kinase inhibitors such as ceritinib, alectinib, or brigatinib in crizotinib resistant tumors (Jain and Chen, 2017).

2.2.4. Role of DNA damage repair genes in drug resistance

Chemotherapeutic agents damage directly or indirectly by targeting the DNA of cancer cells. Increasing the effectiveness of chemotherapeutic agents depends on the inhibition of DNA repair systems. Inhibition of DNA repair systems makes cancer cells more susceptible to such drugs (Mansoori et al., 2017).

Cisplatin is a drug used in many cancer regimens and undergoes a process called aquation. In this process, two chlorine molecules in cisplatin are replaced by water molecules, and by this way, cisplatin becomes activated and ready to bind various molecules. The effect of cisplatin on cell destruction is based on the formation of a DNA-platinum.Some of these bindings form cross-covalent bonds between DNA chains and inhibit replication (Dasari and Tchounwou, 2014).

ERCC1 is a protein that functions in the DNA repair mechanism, and it forms a complex with XPF protein which is also involved in the process, binding together to damaged DNA.Although ERCC1 provides DNA repair in the recombination of processes by breaking crosslinks between DNA, it also destroys cisplatin-DNA bonds and causes cisplatin resistance (Lord et al., 2002).

One study demonstrated that NSCLC patients whose tumors are completely resected and ERCC1-negative can benefit from cisplatin treatment where ERCC1-positive patients cannot(Olaussen et al., 2006).

ERCC1 mRNA level is determined as a prognostic factor for survival in NSCLC patients whose tumor tissue is completely removed and has not received chemotherapy or radiotherapy (Deng et al., 2014).

2.2.5. Role of gene amplifications in drug resistance

Gene amplification can be expressed as an increase in the number of gene copies on a chromosome and has a very important role in producing genetic material (Albertson, 2006). Gene amplification was first demonstrated in 1978, where cultured murine sarcoma cells with methotrexate resistance were found to express high levels of the dihydrofolate reductase gene (Schimke, Kaufman, Alt, and Kellems, 1978).

The dihydrofolate reductase gene in almost all organisms catalyzes the reduction of dihydrofolates to tetrahydrofolates, which are used in synthesizing purines and, thymidylates which have a very important role in cell growth and proliferation. Methotrexate, a chemotherapy agent and immune suppressor, inhibits the activity of the dihydrofolate reductase gene, thereby helping to stop DNA replication and exert cytotoxicity (Schimke et al., 1978).

The MET proto-oncogene ~~is~~ also known as the c-Met or hepatocyte growth factor receptor, was first described in 1984. The hepatocyte growth factor (HBF) is the only known ligand of this receptor, which has tyrosine kinase activity. Tyrosine kinase activation by binding of HBF to the MET receptor enables activation in many signaling pathways such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, signal transducer and transcription protein activator (STAT) (Bottaro et al., 1991). Many studies have shown that abnormal MET signaling plays an important role in tumor growth, progression, and invasion in different cancers.

In addition, amplifying the MET gene plays an important role in the resistance to many EGFR inhibitors used in cancer treatment. In a study, the NSCLC cell line with EGFR exon 19 mutation was treated with increasing concentrations of gefitinib to investigate mechanisms of resistance developed against this EGFR-TKI, and clones with gefitinib resistance were obtained after six months. Unlike the parental cell line, the resistant cell line has been shown to maintain

ERBB3 and AKT phosphorylation in the presence of gefitinib. After the copy analysis between the parental and resistant lines, it was determined that the chromosome region where the MET gene is located in the resistant cell line is 5-10 times amplified. To determine whether the increase in the MET gene was associated with immunity against gefitinib, the MET gene was inhibited, and it was found that ERBB3 and ECT expression were completely suppressed in gefitinib-resistant cell lines (Engelman et al., 2007).

2.2.6. Role of epithelial-mesenchymal transition in drug resistance

Epithelial-mesenchymal transition (EMT) can be explained as transforming epithelial cells into mesenchymal phenotype by losing intercellular connections and cell polarization, and gaining invasion as well as migration abilities (Housman et al., 2014). This transformation is determined by decreased expressions of the epithelial cell markers such as E-cadherin, γ-catenin, occludins, claudins, desmoplakin, and increased expressions of the mesenchymal cell markers such as vimentin, fibronectin, N-cadherin, in addition to increased activity of matrix metalloproteinases (MMPs) associated with the invasive phenotype (Du and Shim, 2016).

The link between EMT and drug resistance was established long time ago, but its detailed mechanisms governing chemoresistance have not been fully understood. Recent research has shown that EMT aids in the development of resistance in cancer cells to conventional therapeutics and EGFR inhibitors (Byers et al., 2013; Suda et al., 2011). For example, it has been observed that resistant epithelial ovarian cancer cells obtained from continuous administration of a chemotherapeutic, paclitaxel, made sudden and multiple metastases. It was determined that the cells showed mobile and invasive EMT-like behaviors (Kajiyama et al., 2007). In another study, transcription factor Slug, known to be involved in the development of EMT, was shown to provide gefitinib resistance by suppressing the expression of proapoptotic protein Bim and preventing the activation of apoptosis initiator caspase 9 in NSCLC (Chang et al., 2011). Again, in a study by Xie et al., the overexpression of the Notch-1 gene has shown to be contributed to the formation of the EMT phenotype in an acquired resistance model in which variable expressions of Notch-1 protected EGFR-mutant cells from gefitinib-induced apoptosis (Xie, He, Wei, and Zhang, 2013).

2.3. Modeling Drug Resistance *in vitro*

Drug resistance is one of the main reasons for the failure of chemotherapy treatment in cancer. Therefore, investigating the detailed molecular mechanisms that cause drug resistance is essential in delaying recurrence and preventing early death of cancer patients. Many methods have been developed to examine mechanisms of drug resistance *in vivo* and *in vitro* (Jo et al., 2018). Since it is not usually possible to collect tissue samples from actual patients, artificially established cell lines are often preferred for investigating acquired drug resistance mechanisms. However, developing a drug-resistant cell line is a highly challenging process that can take months or even years (Lee, Choi, Kim, and Shin, 2016).

Two different methodologies are used for *in vitro* modeling of drug resistance. These are clinically relevant and high-level laboratory models (M. McDermott et al., 2014). In clinically-relevant models, it is aimed to imitate the chemotherapy treatment regimens of cancer patients. Thus, the clinically acceptable dose of the drug is applied to the cell lines as sudden drug pulses, and then the drug is removed from the environment, and the cells are left for healing in a drug-free environment (Breen, Murphy, Keenan, and Clynes, 2008). Unlike clinically-relevant models, high-level laboratory models show more stable resistance in which molecular changes occurring during resistance development are more significant and easier to analyze. In this model, cell lines are exposed to increased drug concentrations over prolonged periods without any dose limitation (Clynes, Redmond, Moran, and Gilvarry, 1992). However, as these models reach incredibly high levels of resistance, they may deviate from the clinically acceptable drug concentration range (McDermott et al., 2014). Certain critical decisions are made during the development of a clinically-relevant or high-level laboratory model. These include selecting the parental cell line, determining the drug dose and treatment intervals. After these steps, candidate resistant cells are harvested and molecularly compared to the parental cell line.

Likewise, many experimental models are used to understand cancer's pathobiology, to identify new diagnostic and prognostic markers in cancer progression, and to find a new therapeutic agent for overcoming drug resistance. As it is known, cell characteristics are affected mainly by the architecture of the cell population. Therefore, molecular modeling of a cancer cell is very important in understanding drug resistance mechanisms and plays an essential role in discovering a new drug to overcome this obstacle. Until now, two-dimensional cell cultures remained the most widely used *in vitro* methods for therapeutic screening due to their ease, reproducibility, and low cost (Ryan et al., 2016). However, it was shown several times that in two-dimensional cultures, cells that were constantly growing lost their tumor heterogeneity and became a homogeneous cell population.

Moreover, cells in two-dimensional cultures fail to represent a typical tumor's genetic makeup and physiology. They either fully express existing or acquired resistance to the therapeutics used (Langhans, 2018). Since two-dimensional cell cultures do not reflect the properties of normal cancer cell populations properly, they may end up with inaccurate results and cause unnecessary use of experimental animals, prolongation of the process, and increase in cost of *in vivo* analyses to be carried out at advanced stages. On the other hand, three-dimensional cell cultures provide more suitable options for drug screening purposes because they show similar characteristics to cancer cells *in vivo* in terms of cell-cell and cell-matrix interactions. Also, they better represent the gene expression, signaling, and drug resistance profiles of solid tumors *in vivo* (Antoni, Burckel, Josset, and Noel, 2015; Nunes, Barros, Costa, Moreira, and Correia, 2019). Three-dimensional cell culture methodologies can be produced in scaffold-based or non-scaffolded forms. They can be used as an intermediate step between two-dimensional cultures and animal experiments in drug screening.

Intratumoral heterogeneity is a feature that has variable properties in different cancer types and influences the morphology, proliferation rate, metastasis capacity, dependence on growth factors, and tumor-forming capacity of cancer cells. Also, the tumor's heterogeneous structure and microenvironment conditions play crucial roles in determining development of drug resistance and its metastatic properties (Kaushik, Yakisich, Kulkarni, Azad, and Iyer, 2018). For this reason, spherical cell layers consisting of a single cell type called spheroids are used to mimic better a tumor's actual cell layers and heterogeneity (Costa et al., 2016).

As it is mentioned earlier, there are two main methodologies to transform cells into ~~3~~ three-dimensional structures or spheroids that can be used in cancer research(Joseph, Malindisa, and Ntwasa, 2018). Scaffold-based methods include a scaffold that mimics the natural extracellular matrix (ECM) structure and biological function with appropriate mechanical and biological properties. Three-dimensional scaffolds provide structural support to cancer cells, allowing them to multiply and migrate (Langhans, 2018; Stadler et al., 2015). Hydrogels are the most preferred scaffolds in three-dimensional cell cultures because of their injectable structures and the ability to mimic the extracellular matrix of normal tissue. Hydrogels can be produced from natural or synthetic sources. Natural materials used in hydrogel production can be listed as collagen, gelatin, alginate, fibrin, hyaluronic acid, agarose, chitosan and laminin (Hoarau-Véchot, Rafii, Touboul, and Pasquier, 2018; Stadler et al., 2015).

In non-scaffold-based methods, cancer cells reproduce independently of anchorage in serum-containing media. An artificial scaffold is not created in these methods. Cells are expected to become cellular aggregates by producing their own extracellular matrix (Langhans, 2018). Methods such as hanging drop plates, low adhesion plates, bioreactors, magnetic levitation, and microfluidic devices are routinely used to produce spheroids independently from the scaffold (Joseph et al., 2018; Langhans, 2018; Lv, Hu, Lu, Lu, and Xu, 2017).

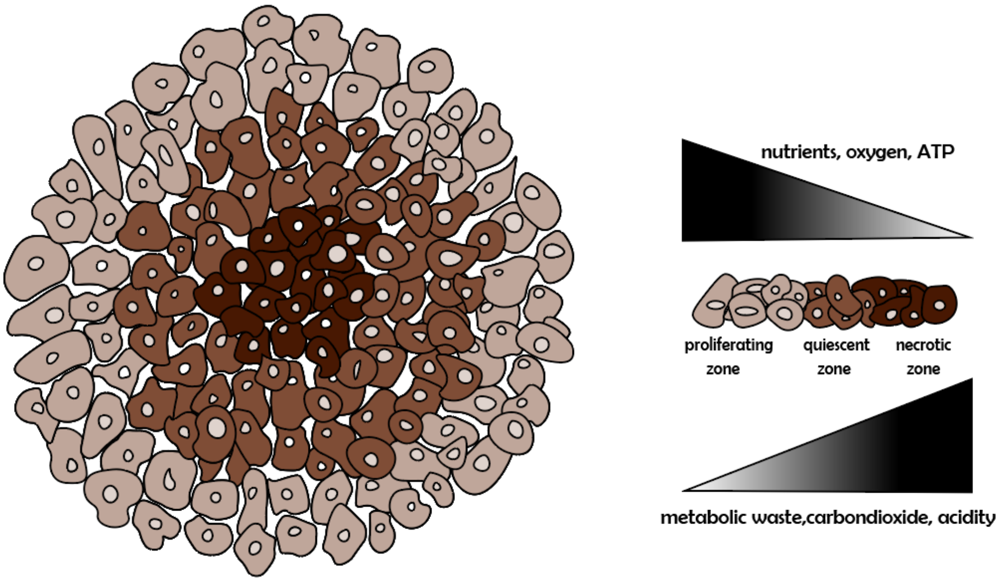


Figure 2.2 A schematic presentation of three-dimensionally grown spheroids.

An ideal three-dimensional spheroid simulates an environment where cells can proliferate and differentiate. A spheroid is composed of three main layers, considering differences in nutrients, oxygen, and pH gradients (Figure 2.2). The outermost layer, called the proliferative zone, can reach enough oxygen and food to grow. As it progresses to the inner layers of the spheroid, quiescent and necrotic regions begin to form. Cells in the quiescent region can survive but do not multiply. The innermost cells die due to the accumulation of waste and insufficient supply of oxygen and form the necrotic region (Kaushik et al., 2018; Pinto, Henriques, Silva, and Bousbaa, 2020). Since this cellular heterogeneity is similar to the *in vivo* tissue environment, it results in very similar cellular processes and creates an excellent three-dimensional model in drug studies (Nath and Devi, 2016).

2.4. Overcoming Drug Resistance

Currently, many chemotherapeutic agents used in cancer treatment consist of chemicals that do not selectively target cancer cells and cause toxicity in normal cells. Similarly, existing drugs used in lung cancer treatment have limited potential due to their relatively high toxicity, inefficiency, cost, and, most importantly the development of resistance against them (Tsao, Kim, and Hong, 2004). As stated in the previous sections, intrinsic or acquired drug resistance causes the failure of chemotherapy agents used in treatment. Therefore, overcoming drug resistance is of great importance in cancer management.

Overcoming drug resistance is a topic that has been studied frequently. For example, nano-medicine, which is a novel technology, is aimed to overcome drug resistance through utilization of nanoparticles (J. L. Markman, Rekechenetskiy, Holler, and Ljubimova, 2013). This system has the potential to increase drug uptake into tumor cells, suppress proteins such as P-gp, which plays a role in drug resistance, and induce apoptosis (Majidinia et al., 2020). Along with cutting age technologies, using herbs and plant-derived compounds is rather a classical strategy to develop alternative solutions to drug resistance.

Chemo-preventive and drug resistance modulating effects of many herbal products has been shown by several *in vitro* and *in vivo* studies (Nobili et al., 2009; Surh, 2003). It is hypothesized that plant products can be used as ideal drug candidates for reversing drug resistance since they can target critical cancer signaling pathways without triggering significant side effects(Isaoglu, Güllüce, and Karadayi, 2020; P. Wang, Yang, Yang, Wang, and Lee, 2015).

2.5. Role of Plants in Chemoprevention of Cancer

Herbs have been the most popular form of traditional medicine since ancient times and are widely used in many parts of the world. A plant that contains therapeutically active substance in at least one of its body parts is defined as a medicinal plant which is a significant healing source in traditional medicine (Sofowora, Ogunbodede, and Onayade, 2013). There are about half a million plant species worldwide, of which about 70,000 were studied for their medicinal use (Schippmann, Leaman, and Cunningham, 2006). Medicinal plants can have a synergistic effect by interacting with each other. They can be used as drug support and protective properties (Hassan, 2012). In addition, it has been proven that plant components can prevent the emergence of some diseases and are very effective in reducing the side effects of treatment with chemical drugs after the disease occurs.

Medicinal plants also provide a significant chemical depository in developing anticancer drugs, such that 60% of anticancer drugs currently in use and determined to be clinically valuable are known to be of natural origin (Cragg, Newman, and Snader, 1997). Table 2.2 lists six plant-derived anticancer drugs that have received U.S. Food and Drug Administration (FDA) approval for commercial production.

Table 2.2 Six plant-derived anticancer drugs that received FDA approval

Drug	**Plant Source**	**Clinical Use**	**Reference**
Taxol	*Taxus brevifolia*	Ovary and breast cancer	(Zhang, Yang, Wang, and Dong, 2014)
Vinblastine, Vincristine	*Catharanthus roseus*	Leukemia, bladder and testicular cancer	(Barrales-Cureño et al., 2019; Ngan, Bellman, Hill, Wilson, and Jordan, 2001)
Irinotecan	*Camptotheca acuminata*	Metastatic colorectal cancer	(Thirumaran, Prendergast, and Gilman, 2007)
Etoposide and Teniposide	*Podophyllum peltatum*	Small-cell lung, testicle cancer and lymphoma	(Thirumaran et al., 2007)

Considering the abundance of medicinal plants in nature, their low toxicity and easy tolerability in the human body, and also the successful anticancer drugs that have been developed with natural products obtained from plants so far; It is clear that more potential anticancer compounds exist in nature and are discoverable with an appropriate strategy, effort, and technological advances (David, Wolfender, and Dias, 2015).

2.5.1. Cancer and plant natural compounds

Natural products are chemical products found in nature, produced by plants, fungi, and microorganisms or marine creatures. Historically, natural products dating back to ancient times have often been used as medicine, elixir, and oil to treat many diseases. Furthermore, many bioactive natural products remain unidentified (Dias, Urban, and Roessner, 2012).

As it is known, all organisms take certain substances and energy from outside to live, grow and reproduce and use them to synthesize organic molecules that will continue their vital processes. All chemical reactions carried out by living things for this purpose are called metabolism, and intermediate and primary products formed due to metabolism are called metabolites. Plant metabolites are generally divided into two classes, primary and secondary. Primary metabolites are essential for forming viability and participate in basic metabolic processes such as growth, development, and differentiation. Secondary metabolites help plants adapt to environmental conditions, defense, survival, and regulate their relationships with the ecosystem. Natural product compounds are also called secondary metabolites. These secondary metabolites are terpenes, phenolic compounds, N-containing compounds, or S-containing compounds (Jamwal, Bhattacharya, and Puri, 2018).

The largest and most diverse class of secondary metabolites, terpenes, are produced by a variety of plants and some animals. Terpenes are composed of 5-carbon isoprene (C5H8) building blocks linked together to form a linear or circular structure. They are called hemiterpene, monoterpene, sesquiterpene, diterpene, sesterterpene, triterpene, respectively, according to the number of isoprene units forming their structures (Paduch, Kandefer-Szerszeń, Trytek, and Fiedurek, 2007). Many terpenes exist in nature as hydrocarbons or have oxygen compounds such as hydroxyl, carbonyl, and ketone. Modified forms of terpenes containing oxygen are called terpenoids. As previously stated, terpenoids can be obtained from plants and are easily accessible and frequently consumed in products such as vegetables, fruits, cereals, and tea (Cox-Georgian, Ramadoss, Dona, and Basu, 2019).

In epidemiological studies, it has been determined that terpenoids inhibit the progression of tumorigenesis by inducing cell cycle arrest and apoptosis in the early stages and succeed in suppressing angiogenesis, invasion, and metastasis by regulating various signaling pathways in advanced stages (Ansari and Akhtar, 2019a).

Another class of secondary metabolites is phenolic compounds. *Phenolics* are compounds that have a benzene ring to which one or more hydroxyl groups are attached (Anantharaju, Gowda, Vimalambike, and Madhunapantula, 2016).The amount of hydroxyl group and benzene ring is divided into two subheadings: simple phenols (phenolic acid and coumarins) and polyphenols (flavanoid, tannins, stilbenes, lignans). Phenolic compounds are common components of products such as fruit, vegetables, tea, beer, wine, coffee, etc. In addition to their fragrance and color formation to some plants; They play a major role in protecting plants against parasites and predators (Ansari and Akhtar, 2019a). Up to now, about 8000 phenolic substances have been identified, most of which are flavonoids (R. Tsao, 2010).

As is known, reactive oxygen species cause damage to DNA. When damaged DNA is replicated before it is repaired, this leads to the accumulation of mutations in cells and cancer formation (Chatterjee and Walker, 2017). As a result of the studies, it has been determined that phenolic compounds play a role in diseases related to oxidative stress and show anti-cancer activity. The anti-cancer effects of phenolic compounds are mainly due to their ability to induce cell cycle arrest and the action of tumor suppressors such as p53, inhibit oncogenic signaling pathways that control cell proliferation, angiogenesis, and apoptosis, and regulate ROS levels (Ansari and Akhtar, 2019b).

2.5.2. *Melissa officinalis* L.

The *Lamiaceae* plant family ranks 6th in size among plant families, with over 250 genera and over 7000 species. The species belonging to the *Lamiaceae* family are spread all over the world, especially in the United States and the Mediterranean region, and are generally found in the form of grass, rarely shrubs and trees (Bramley, Harley, and Paton, 2009; Naghiloo, Khodaverdi, Siahkolaee, and Dadpour, 2013; S. Singh, Batish, Kohli, and Singh, 2015).The most important features of these family plants are that they have a four-cornered stem, an array of annular leaves, and essential oils (Carović-Stanko et al., 2016). Plants are frequently used in the cosmetics, food, and pharmaceutical industries due to the essential oils found in their structure (Özkan, 2008).

Melissa officinalis L., belonging to the *Lamiaceae* family, is a widely used aromatic- medicinal plant species. Although it has a herbaceous structure, it is perennial and has a height that can reach from 30 cm to 90 cm. Also, it has lush green leaves with toothed and hairy edges in all seasons, and it blooms towards the end of summer with bluish and yellowish-white flowers (Ceylan, 1997). The plant likes moist, sunny, and warm places and grows well in sandy soils rich in nutrients. Hence, it originates from West Asia and Europe. In our country, it is generally distributed in the Marmara, Mediterranean, and Aegean regions along the coastline (Baytop, 1984). The lemon balm has three subspecies, *officinalis*, *altissima*, and *inodora* (Edmondson, Mill, and Tan, 1982).

2.5.2.1. Phytochemistry of Melissa officinalis L.

As a result of the phytochemical analysis of *the Melissa officinalis* L. plant, terpenes (monoterpene, sesquiterpene, and triterpene) and phenolic components (phenolic acids, flavonoids, and tannins) were determined. The main active ingredients of *Melissa officinalis* L. are volatile compounds, triterpenes, and phenolics (Moradkhani et al., 2010). Table 2.3 summarizes the main phytochemicals isolated from the *Melissa officinalis* L. plant in more detail.

Melissa officinalis L. essential oil has a wide range of uses in the medicine and food industry, herbal teas, cosmetics, and even as an additive to ornaments. The essential oil can be obtained from the plant's fresh or dried flowers, leaves, or branches. Many different methods can be used to obtain the essential oil. These methods include water distillation, steam distillation, vacuum distillation, solvent extraction, supercritical fluid extraction, microwave-assisted extraction, pressurized solvent extraction, and solid-phase microextraction (Kiliç, 2008).

The amount of essential oil in the plant varies between 0.01% and 0.30%, and the main components are citral isomers (geranial and neral), citronellal, and geraniol, which are oxygenated monoterpenes. The chemical components of the essential oil obtained from the plant may vary depending on the climate, season, geographical conditions, harvest time, and the distillation technique applied (Shakeri, Sahebkar, and Javadi, 2016).For instance, in the study conducted by Carnat et al., It was determined that the essential oil obtained from the *Melissa officinalis* L plant collected from France had a high content of citral (neral and geranial; 48%) and citronellal (40%) (Carnat, Carnat, Fraisse, and Lamaison, 1998). In another study, the composition of the essential oil obtained from *Melissa officinalis* L. harvested in Turkey, β-cubebene (15.41%) and the β-caryophyllene (14.24%) was determined to be dominant. Similarly, the main components of essential oil obtained from *Melissa officinalis* L collected from Vojvodina (Serbia) are geranial (23.4%), neral (16.5%), and citronellal (13.7%) (Mimica-Dukic, Bozin, Sokovic, and Simin, 2004). Abdellatif and Hassani studied the effect of different distillation methods on the amount of essential oil obtained using traditional hydrodistillation, steam distillation, organic solvent extraction, and microwave-assisted hydrodistillation methods. And predominantly, neral (24.04%, 24.06%, 38.18% and 18.86%) and geranial (33.79%, 37.91%, 35.57% and 27.79%) was determined (F Abdellatif and Hassani, 2015).

The phenolic acids mentioned in the previous section are the main components of the *Melissa officinalis* L. plant. It can be said that the antioxidant activity of the *Melissa officinalis* L. plant is caused by the phenolic acids in its structure, especially rosmaniric acid. The studies investigated phenolic compounds in the *Melissa officinalis* L. plant's content. And these compounds are designated as Rosmarinic acid, 3- (3,4-dihydroxyphenyl)-Lactic acid, Caffeic acid, Salvianolic acid F, Salvianolic acid A, Salvianolic acid C, Salvianolic acid B, Sagerinic acid, Yunnaneic acid F, Gallic Acid (Barros et al., 2013; Caniova and Brandsteterova, 2001; Cunha et al., 2016).

Likewise, flavonoids, which are a subgroup of phenolic compounds obtained from the *Melissa officinalis* L. plant, are divided into four subgroups. These can be listed as flavones (nine compounds), flavanones (five compounds), flavonols (three compounds), and flavonols (two compounds) (Shakeri et al., 2016). As a result of a study, two flavone glycosides named luteolin7-O-glucoside and apigenin 7-O-glucoside, a flavonol glycoside named isoquercitrin, and a flavonol named rhamnocitrin were isolated from the *Melissa officinalis* L. plant (Mulkens and Kapetanidis, 1987). Likewise, flavanone glycosides such as hesperidin, hesperetin, and naringin were isolated from a 45% ethanol extract of the *Melissa officinalis* L. plant (Dastmalchi et al., 2008).

And finally, triterpenes, one of the most important secondary metabolites obtained from the *Melissa officinalis* L.. plant, are ursolic acid and oleanolic acid (Mencherini, Picerno, Scesa, and Aquino, 2007).

2.5.2.2. Anticancer effects of Melissa officinalis L.

As mentioned before, plants have been used in general medicine for many years. Side effects caused by synthetic drugs have been an important factor in the preference for natural compounds derived from plants in treatment. Plants can show many biological activities, including anticancer and anti-tumor, through phytochemicals in their structure. Phytochemicals of the *Melissa officinalis* L. plant examined in the previous section also play a role in many biological activities, and many of these components have anticancer effects. Table 2.4 summarizes in more detail the anticancer effects of the main phytochemicals isolated from the *Melissa officinalis* L. plant.

For example, one study has shown that citral, which forms a large part of *Melissa officinalis* L. essential oil and is a mixture of neral and geranial, inhibits Src-TK activation, resulting in the suppression of STAT3 activity, which plays a role in the initiation and regulation of the important mechanism in cancer, EMT (Maruoka et al., 2018; Wendt, Balanis, Carlin, and Schiemann, 2014).In addition, other studies demonstrated that citral reduces cell proliferation and induces apoptosis in HeLa and ME-180 cell lines (Ghosh, 2013) and plays a role in inducing the glutathione-S-transferase class enzyme that protects healthy parenchymal cells in the chemotherapy of liver cancer (Nakamura et al., 2003).

Citronellal is a monoterpenoid aldehyde that gives a lemon scent to citronella oil. This component is found in very high amounts in *Melissa officinalis* L. essential oil. As a result of a study, it mediates p38 MAPK phosphorylation in hepatocellular carcinoma cells and supports the reduction of cell proliferation (Massberg et al., 2014).

β-caryophyllene (BCP), a bicyclic sesquiterpene, and its oxidation derivative, beta-caryophyllene oxide(BCPO), are also compounds with anticancer effects found in the essential oil content of *Melissa officinalis* L. In one study, BCP inhibited motility and invasion in the human colon cancer cell line HCT116 (Dahham et al., 2015). In addition, in a study by Park et al., it has been observed that Beta-caryophyllene oxide suppresses cell proliferation in a dose-dependent manner in the PC-3 prostate cancer cell line and the MCF-7 breast cancer cell line and induces inhibition of the MAPK and PI3K/AKT signaling pathway, which has important roles in tumor survival and proliferation (Park et al., 2011).

Geraniol and geranyl acetate are two important monoterpenes found in *Melissa officinalis* L. essential oil. As a result of a study, it was determined that these two monoterpenes have significant anticancer activity like other components. Thus, both components induce apoptosis via the mitochondrial pathway by decreasing the expression of BCL-2 proteins that inhibit apoptosis while increasing the expression of Bax2 protein that promotes apoptosis in Colo-205 colon cancer cells (Qi et al., 2018).

Rosmaniric acid is a phenolic ester found in *Melissa officinalis* L. extract. This ingredient is also found in many other herbs and hase biological effects such as anti-inflammatory, anti-diabetic and anticancer (Amoah, Sandjo, Kratz, and Biavatti, 2016). Recent studies have shown that rosmaniric acid also has apoptosis-inducing and anti-metastatic effects. Indeed, Xu et al. Demonstrated that rosmaniric acid inhibited the bone metastasis caused by breast cancer in a dose-dependent manner(Xu, Jiang, Ji, and Liu, 2010) and also inhibited the metastasis of Ls174-T human colon carcinoma cells by suppressing MMP-2 and MMP-9 activities (Xu, Xu, Liu, Xu, and Liu, 2010).Likewise, in a study conducted on colorectal cancer cells, it was shown that this component induces cell cycle arrest and apoptosis and also inhibits metastatic abilities such as EMT and migration through AMPK activation (Han, Kee, and Hong, 2018).

Luteolin-7-O-Glucoside is a glycosidic form of Luteolin, which contains less in *Melissa officinalis* L. extract than other ingredients. As a result of the studies, it has been observed that Luteolin prevents cell proliferation and cell migration in ovarian (Liu et al., 2017), melanoma (Yao, Jiang, Yu, and Yan, 2019), and gastric (Pu et al., 2018) cancer cells by suppressing MMP-2 and MMP-9 expression. Likewise, its glycosidic form, Luteolin-7-O-Glucoside, has significantly reduced oral cancer metastasis by suppressing MMP-2 expression (Velmurugan et al., 2020).

Another component that is less in the extract is isoquercitrin. Studies have determined that this chemical component also has an anticancer effect. So that as a result of a study by Chen et al., isoquercitrin has been observed to significantly reduce the expression level of PKC, which is thought to support the proliferation, differentiation, and migration of cancer cells in bladder cancer cells (Chen et al., 2016).

Table 2.3 *Melissa officinalis* L. leaf essential oil and chemical extract contents

Main Compound	Extraction Method	Analysis of Essential Oil and Extract	Compound Amount in Percentage	References
Citronellal	Hydrodistillation	GC-MS	36.62 - 43.78%	(Cosge, İpek, and Gürbüz, 2009)
(E)-citral (neral)	Hydrodistillation	GC	0.78-45.65 %	(Kittler et al., 2018)
(Z)-citral (geranial)	Hydrodistillation	GC	0.50 - 34.27%	(Kittler et al., 2018)
β-Citronellol	Microdistillation	GC-MS	9.48 %	(Popova, Mihaylova, Alexieva, Deseva, and Dalemska, 2016)
Geraniol	Hydrodistillation	LSC/GLC/GC-MS	0.38 %	(Sarer and Kökdil, 1991)
Geranyl Acetate	Hydrodistillation	GC-MS/GC-FID	0.5% - 3.0%	(Nurzyńska-Wierdak, Bogucka-Kocka, and Szymczak, 2014)
β-caryophyllene	Hydrodistillation	GC-MS/GC-FID	1.3%	(Fahima Abdellatif, Hadjira, Zitouni, and Hassani, 2014)
Caryophyllene Oxide	Hydrodistillation	GC-MS/GC-FID	1.3%	(Fahima Abdellatif et al., 2014)
Germacrene-D	Hydrodistillation	HS-SPME/GC-MS	8.3%	(Rehman et al., 2017)
***β*-pinene**	Hydrodistillation	GC/GC-MS	6.4–18.2%	(Basta, Tzakou, and Couladis, 2005)
α-Humulene	Hydrodistillation	GC/GC-MS	0.43 %	(Adinee, Piri, and Karami, n.d.)

Table 2.3 continued

Main Compound	Extraction Method	Analysis of Essential Oil and Extract	Compound Amount in Percentage	References
Quercetin	Leaf (99.9% ethanol)	HPLC-DAD	4.13%	(Cunha et al., 2016)
Rosmaniric acid	Leaf (50% aqueous ethanol)	HPLC	3.67 - 7.55%	(Kittler et al., 2018)
Luteolin-7-o-glucoside	Plant (boiling double distilled water (ddH2O)	GC/MS, HPLC/DAD,LC/MS	2.6%	(Skotti et al., 2019)
Isoquercitrin	Leaf (99.9% ethanol)	HPLC-DAD	2.35%	(Cunha et al., 2016)
Hesperidin	Plant (50% ethanol)	HPLC-PDA	1.73%	(Dwivedi, Kumar, and Trivedi, 2017)
Caffeic Acid	Plant (boiling double distilled water (ddH$_2$O)	GC/MS, HPLC/DAD,LC/MS	7.6%	(Skotti et al., 2019)
Ferulic Acid	Plant (aqueous solution)	HPLC-MS	0.498%	(Ordaz et al., 2018)
Rutin	Leaf (99.9% ethanol)	HPLC-DAD	9.26%	(Cunha et al., 2016)
Hesperetin	Plant (50% ethanol)	HPLC-PDA	6.24%	(Dwivedi et al., 2017)
Apigenin	Plant (aqueous solution)	HPCL-MS	5.234%	(Ordaz et al., 2018)
Gallic Acid	Leaf (99.9% ethanol)	HPLC-DAD	3.76%	(Cunha et al., 2016)
Myricitrin	Plant (boiling double distilled water (ddH$_2$O)	GC/MS, HPLC/DAD,LC/MS	4.6%	(Skotti et al., 2019)
Naringenin	Plant (50% ethanol)	HPLC-PDA	7.70%	(Dwivedi et al., 2017)

Table 2.4 Anti-cancer effects of chemicals from *Melissa officinalis* L. leaf essential oil and extract

Main Compound	**Extraction Method**	**Analysis of Essential Oil and Extract**	**Compound Amount in Percentage**	**References**
Citral	LU134AM, LU135,LU165 (Small-cell lung cancer)	IC_{50} : LU134AM 68.31 ±1.43 µM LU135 62.24 ±2.73 µM	Src/Stat3 activity and suppress the proliferation/survival of small-cell lung cancer cells	(Maruoka et al., 2018)
	ME-180, HeLa (Cervical Cancer)	IC_{50} : ME-180 300 µg/ml HeLa 500 µg/ml	Reduces cell proliferation and induces apoptosis	(Ghosh, 2013)
Citronellal	Huh7 (Hepatocellular Carcinoma)	Huh7 500 µM	Mediates p38 MAPK phosphorylation and supports the reduction of cell proliferation	(Massberg et al., 2014)
β-caryophyllene	HCT-116 (Colon Cancer)	IC_{50} : HCT-116 19 µM	Inhibits motility and invasion	(Dahham et al., 2015)
β - Caryophyllene oxide	PC-3,MCF-7 (Prostate and Breast Cancer)	PC-3 / MCF-7 50 µM	Inhibits growth and induces apoptosis via suppression of PI3K/AKT/mTOR/S6K1 and MAPKs signaling pathways	(Park et al., 2011)
Geraniol and geranyl acetate	Colo-205 (Colon Cancer)	IC_{50} : Colo-205 20-30 µM	Induces apoptosis, DNA damage and cell cycle arrest	(Qi et al., 2018)
Rosmaniric acid	Ls174-T (Colon Cancer)	Ls174-T 150 µg/ml	Inhibits the metastasis by suppressing MMP-2 and MMP9 activities	(Xu, Xu, et al., 2010)
	CT26 , HCT116 (Colorectal Cancer)	CT26 / HCT116 50, 100, and 200 µM	Induces cell cycle arrest and apoptosis, and also inhibits metastatic abilities such as EMT and migration through AMPK activation	(Han et al., 2018)
Luteolin-7-O-Glucoside	FaDu,HSC-3,CA9-22(Oral Cancer)	FaDu/HSC-3 /CA9-22 0, 10, 20, and 40 µM	Reduce metastasis by suppressing MMP-2 expression	(Velmurugan et al., 2020)

Table 2.4 continued

Main Compound	Extraction Method	Analysis of Essential Oil and Extract	Compound Amount in Percentage	References
Isoquercitrin	5637, T24 (Bladder Cancer)	5637 / T24 0, 100, 200, 400 and 800 µM	Inhibits cancer progression via inhibiting the PI3K / Akt phosphorylation and decreasing the PKC phosphorylation.	(Chen et al., 2016)
Hesperidin	A549 (Lung Cancer)	IC_{50}: A549 12.5-25 µM	Inhibites the cell proliferation through down regulation of c-myc, β-catenin and induces apoptosis.	(Kamaraj et al., 2019)
Caffeic Acid	HeLa, ME-180 (Cervical Cancer)	HeLa / ME-180 30, 40, 50 µg/ml	Reduces cell proliferation, and triggers cell death by altering mitochondrial membrane potential and apoptosis.	(Kanimozhi and Prasad, 2015)
Rutin	A549 (Lung Cancer) HT29/Caco-2 (Colon Cancer)	IC_{50}: HT29 388.33±4 µM A549 559.83 ±3.5 µM Caco-2 710.59 ± 3.5 µM	Inhibits cancer cell viability and effects adhesion and migration.	(Ben Sghaier et al., 2016)
Hesperetin	PC-3 (Prostate Cancer)	IC_{50} : PC-3 450 Mm	Allows cancer cells to be reduced significantly, without increasing apoptosis.	(Shirzad, Beshkar, and Heidarian, n.d.)
Apigenin	A549(Lung Cancer)	IC_{50}: A549 60 µmol/l	Downregulates the expression of Akt, and suppresses the migration and invasion of A549 cells.	(Zhou et al., 2017)
Gallic Acid	TSGH-8301 (Bladder Cancer)	TSGH-8301 0, 50, 100, 150 µM	inhibits cell growth, invasion and migration through inhibition of fatty acid synthase and through p-AKT/MMP-2 signaling pathway.	(Liao, Chen, Huang, and Wang, 2018)
Naringenin	MDA-MB-231 /MCF-10A (Breast Cancer)	MDA-MB-231/ MCF-10A 6.25, 12.5, 25, 50, 100 µg/ml	Inhibits cell proliferation in a concentration-dependent manner and regulates cell cycle arrest and apoptosis in the G0/G1 phase.	(Zhao, Jin, Ge, and Guo, 2019)

2.5.2.3. *Effects of chemicals from Melissa officinalis L. on drug resistance in different cancer types*

Drug resistance is one of the most inhibitory issues in cancer treatment. Therefore, scientists examine the properties of many natural compounds derived from plants to reverse drug resistance in cancer treatment. Section 2.5.2.2., studies examining the anticancer effects of compounds obtained from the essential oil and extract of the *Melissa officinalis* L. plant are listed. Likewise, it has been supported by studies that the plant's chemical components, as mentioned above, reverse drug resistance through many mechanisms.

For instance, a study by Nigjeh et al. found that citral, an oxygenated monoterpene from the plant's essential oil, inhibits growth in spheroids from a breast cancer cell line MDA-MB-231. Then using tamoxifen as a positive control, evaluated drug resistance and determined that citral also reduces the self-renewal capacity and induces apoptosis in drug-resistant spheroids (Nigjeh et al., 2018). Another compound in the essential oil content, apigenin, inhibits the STAT-3 signaling pathway in the adriamycin-resistant breast cancer cell line (MCF-7 / ADR), thereby suppressing breast cancer development, drug resistance, and reducing the expression of the drug-resistant related protein, P-gp (Seo et al., 2017). Likewise, in another study, using colon cancer cell lines that highly express efflux pumps and doxorubicin-resistant leukemia cell lines, beta-caryophyllene and beta-caryophyllene oxide were shown to be agents that can restore drug sensitivity and inhibit efflux pumps (DI Giacomo, DI Sotto, Mazzanti, and Wink, 2017).

The drug resistance reversal activity of caffeic acid found in *Melissa officinalis* L. extract was studied by Teng et al. In the study; it was shown that the combination of caffeic acid with drugs used in chemotherapy decreased the IC_{50} value in human P-gp stable expression cells (ABCB1 / Flp-InTM-293) and multidrug-resistant human cervical cancer cell line (KB / VIN). It was also determined that caffeic acid inhibits P-gp efflux function through rhodamine123 and doxorubicin (Teng, Wang, Liao, Lan, and Hung, 2020).In another study, Rosmarinic acid, another component found to be abundantly found in *Melissa officinalis* L. extract, increases the chemosensitivity to 5-Fu in Fluorouracil-resistant gastric carcinoma cell line by downregulating the expression of miR-6785-5p and miR-642a-3p and promoting FOXO4 expression (C. Yu et al., 2019). In addition, it was observed that rosmarinic acid inhibits cell growth by stopping the cell cycle in cisplatin-resistant non-small cell lung cancer cell line and inhibits P-gp and MDR1 expression by inducing apoptosis via MAPK (X.-Z. Liao et al., 2020). Considering these results, it has been suggested that Rosmarinic acid can be used as an agent to reverse drug resistance.

A study examining how 40 dietary phytochemicals are related to the p-gp transmembrane domain demonstrated that the flavonoids such as quercetin and rutin, which are found in the *Melissa officinalis* L. extract and have better connections with the p-gp domains, decrease paclitaxel resistance and p-gp protein levels in multidrug resistance endocervical adenocarcinoma cell lines (Mohana et al., 2016). Likewise, a study by Iriti et al., has shown that rutin stops the cell cycle and induces programmed cell death in human breast cancer cell lines, and decreases the activity of p-glycoprotein and BCRP (breast cancer resistance protein), thereby increasing the sensitivity of drugs such as CYC and MTX (Iriti et al., 2017).

It has been shown that gallic acid, another component in the *Melissa officinalis* L. extract, increases cisplatin activity through the ROS-mediated mitochondrial apoptotic pathway in the non-small cell lung cancer cell line, which is the most aggressive type of lung cancer. These results show that gallic acid can be used as an agent to help reduce cisplatin-induced multidrug resistance (Wang et al., 2016).

Another clinical study investigating the effects of hesperetin on drug resistance showed that the compound increase cisplatin sensitivity and decreases p-gp expression by inhibiting NF-κB signaling-related proteins in cisplatin-resistant non-small cell lung cancer cells (Kong et al., 2020). In addition, in Doxurubicin-resistant breast cancer cells, hesperidin, another compound tested to overcome resistance, has been shown to reduce p-gp expression by showing a synergistic effect with doxorubicin (Febriansah et al., 2014). Finally, naringenin, one of the compounds found in high amounts in *Melissa officinalis* L. extract, inhibited the efflux of doxorubicin in MRP-expressing cancer cells such as A549 and MCF-7 and thus increased the accumulation of doxorubicin (Zhang et al., 2009). Considering the results, it has been suggested that combinations of naringenin and chemotherapeutic agents may be a new treatment option, especially in MRP-regulated intrinsic resistance.

The components of the *Melissa officinalis* L. plant also show inhibiting activities of epithelial-mesenchymal transition, which is one of the drug resistance mechanisms. For instance, in a study, it was determined that the apigenin in the MO extract inhibits an important EMT transcription factor, Snail, and also suppresses migration, invasion, and epithelial-mesenchymal transition in colon cancer cells by inhibiting the NF-κB / Snail pathway (J. Tong et al., 2019). Similarly, caffeic acid suppressed the expression of the mesenchymal transcription factor SNAI1 in human cervical squamous carcinoma cells and increased the expression of epithelial markers such as E-cadherin and occludin (Tyszka-Czochara et al., 2018).

Transforming growth factor-β (TGF-β) signaling is one of the most important regulators of EMT. In a study by Lou et al., EMT was induced by exposing pancreatic cancer cells to TGF-β1, and metastasis and drug resistance inhibitory effect of naringenin, a component abundant in MO extract, was investigated. As a result, naringenin inhibited the TGF-β1 / Smad3 signaling pathway and decreased the expression of mesenchymal markers such as vimentin, N-cadherin, MMP2, and MMP9, preventing metastasis, invasion, and reversing Gemcitabine resistance (Lou et al., 2012). In another study investigating the inhibition of rosmarinic acid's metastatic properties of colorectal cancer cells via AMP-activated protein kinase (AMPK), rosmarinic acid increased the expression of epithelial markers such as E-cadherin while decreasing the expression of mesenchymal markers such as N-cadherin, vimentin, MMP2, and MMP9. Also, It has been observed that these expression changes are due to AMPK activation (Han et al., 2018).

Finally, in a study investigating the mechanisms underlying invasion and epithelial-mesenchymal transmission in pancreatic cancer cells, it was determined that quercetin blocks the STAT3 signaling pathway and inhibits EMT, invasion, and metastasis (D. Yu et al., 2017).

3. MATERIALS AND METHODS

3.1. Materials

Roswell Park Memorial Institute-1640 medium (RPMI-1640, Biowest, Nuaille, France),fetal bovine serum (FBS, Biowest, Nuaille, France), phosphate-buffered saline (PBS, Biowest, Nuaille, France), 0.25 % Trypsin- Ethylenediaminetetraacetic acid (Trypsin-EDTA, Gibco, MA, USA), penicillin-streptomycin (PS, Gibco, MA, USA), Erlotinib hydrochloride (Santa Cruz Biotechnology, Inc., Hiedelberg, Germany), Dimethylsulphoxide (DMSO, Merck, NJ, USA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, 1 % aqueous crystal violet solution, powder agar, agarose (Sigma Aldrich, MO, USA), propidium iodide, and RNAase A were purchased from commercial resources.

3.2. Methods

3.2.1. Plant material

Melissa officinalis L. was obtained from the organic production certificated, *Temmuz Çiftliği* and plant was diagnosed by Prof. Dr. İpek Süntar, who works at Gazi University Pharmacognosy Department, and used in the experiments.

3.2.2. Extraction and fractionation of plant samples

The plant material was subjected to gradual extraction to separate the plant's non-polar and polar substance groups. For this, fractionation of the samples was carried out with three different solvents (n-hexane, ethyl acetate, methanol) from non-polar to polar, respectively.

In the first step of the extraction process, *Melissa officinalis* L. leaf parts were dried and ground into medium-sized powder, and 50.134 g of the powdered drug of *Melissa officinalis* L. was extracted using n-hexane. After the extract was filtered, the solvent used was removed using an evaporator at 40ºC under low pressure to obtain only the extract of the plant material. In the next step, the plant parts above the strainer were taken into a new container, and another solvent, ethyl acetate, was added. The steps in the first solvent were repeated in ethyl acetate. Finally, the plant parts remaining on the strainer after obtaining the ethyl acetate extract were taken into another container, and methanol solvent was added. After filtration and solvent removal processes were carried out the same way, n-hexane, ethyl acetate, and methanolic extracts were obtained. Also, *Melissa officinalis* L. leaf parts were directly extracted with methanol to obtain all substance groups in the plant. As a result, the obtained extracts were stored at +4 °C for further applications.

Table 3.1 Efficiency of yields of leaf extracts from *Melissa officinalis* L.

Name of the Extract	Amount of Extract	Efficiency
n-hexane (M.O* hexane)	1,4808 gr	% 2,95
Ethyl acetate (M.O EtOAc)	3,096 gr	% 6,18
Methanol (M.O MeOH)	4,2413 gr	% 8,46
Total Methanol (M.O Total MeOH)	1,8695 gr	% 18,64

**Melissa officinalis* L.

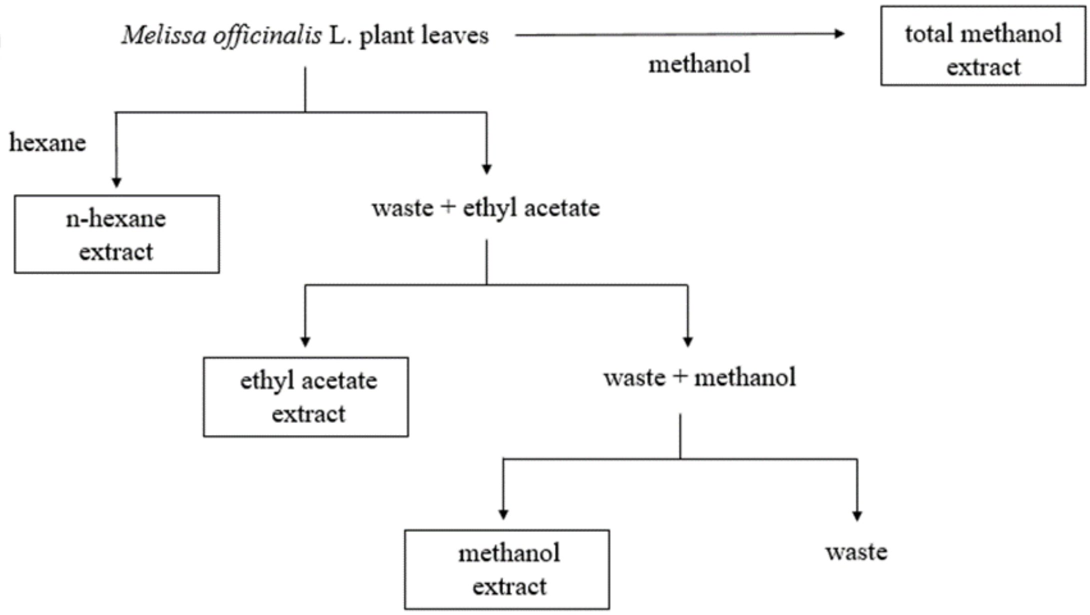


Figure 3.1 Extraction process of leaves of *Melissa officinalis* L.

3.2.3. SPME-GC-MS analysis and chemical composition of plant material

Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS) analysis was performed to identify and quantify the natural components of the *Melissa officinalis* L. plant. For this, 1 g of leaf sample was placed in a 20 ml vial of SPME. For analysis, 2 cm long 50/30 u PDMS/DVB-CAR fiber was used. First, the SPME vial was kept in the headspace heater block for 10 minutes at 40°C before incubation. Extraction was then performed at 40 °C for 60 minutes. After fiber extraction, it was inserted into the injector port of the GC-MS system, wherein the metabolites were transferred directly to the analytical capillary column (DB-WAX column) by thermally desorbing in the splitless mode for 6 min at 250 °C.

Identification and quantification of volatiles were performed using “Agilent 7890B” gas chromatography (USA) and the associated mass spectrometer “Agilent 6430 MSD” (USA). Volatiles were separated using a DB-WAX capillary column (60 m x 0.25 mm x 0.25 µm).The oven temperature was programmed to start at 60°C, after waiting for 3 minutes, to increase by 2°C/min to 220°C and then to increase by 3°C/min to reach 245°C and remain constant at this temperature for 20 minutes. Helium was used as carrier gas (99.99% purity, flow rate: 1.5 mL/min). The ionization energy of the mass spectrometer is 70 eV, and the injector and detector temperatures are 250°C. Identification was carried out in scanning mode. Volatile compounds were identified based on retention index, reference flavor compounds, and mass spectra using a commercial spectrum database (Wiley 7, Flavor 2L, NIST-98). These compounds were calculated using the 2-octanol internal standard (41.5 µg/kg).

3.2.4. Cell lines and culture conditions

The A549 cell line used in the study was obtained from the laboratory of Dr. Ece Şimşek of Akdeniz University, Antalya. The characteristics of A549 non-small cell lung cancer cells shown in Table 3.2. Cells were cultured in RPMI-1640 growth medium prepared by adding 10% fetal bovine serum (FBS) and 1% penicilin streptomycin (PS). The cells were cultured in an incubator at 37 °C, containing 5% CO_2, and the medium was changed every other day. When cells reached 80% density, the sub-culturing protocol was applied. Sub-culturing carried out by removing the medium, washing the cells with phosphate buffered saline (PBS), and adding Trypsin-EDTA to dissolve the cells from the flask. The removed cells passaged into the new culture medium at desired cell density.

Table 3.2 Characteristics of A549 non-small cell lung cancer cells

A549 (ATCC® CCL-185™)	
Organism	*Homo sapiens*, human
Tissue	Lung
Morphology	Epithelial-like
Culture Properties	Adherent
Disease	Carcinoma
Age	58 years
Gender	Male

3.2.5. Effects of different plant extracts on A549 cell viability

The effect of each of the different extracts of *Melissa officinalis* L. on the viability of parental A549 cells was determined by a colorimetric-based test, MTT. This method is based on converting yellow-colored 3- (4,5-dimethylthiazol2-yl) -2,5-diphenyltetrazolium bromide salt into purple-colored formazan crystals by the activity of dehydrogenases in mitochondria. The resulting color intensity is measured colorimetrically in the SPECTROstar Nano microplate reader at 570 nm reference range.

For MTT analysis, A549 cells were seeded in 96-well plates ($1x10^4$ cells/per well) at a volume of 200 µL. Plates were incubated for 24 hours at 37 °C in incubation conditions containing 5% CO_2. Cells were then treated with various concentrations (µg/ml) of plant extract dissolved in DMSO. Working solutions of each extract of *Melissa officinalis* L. were determined as 8 mg/mL, and final concentrations were adjusted to be 1 mg/mL, 500 µg /mL, 250 µg /mL, 125 µg/mL, and 62,5 µg/mL using RPMI-1640. As the control group, the amount of solvent DMSO at the highest extract concentration was determined and the cells were treated with DMSO-containing medium with a total volume of 200 µL. After 24 hours of incubation, extracts were removed, and 100 µL of the MTT solution prepared was added to the wells. Four hours after the MTT application, 150 µL DMSO was added to each well to dissolve formazan crystals formed in the wells. Afterward, DMSO added plates were shaken with a plate shaker for 15 minutes and centrifuged at 400xg for 5 minutes. In order to read the absorbance values of the cells, the DMSO solutions in the centrifuged plates were transferred to a new plate, and their absorbance was measured with the SPECTROstar Nano microplate reader. The control cell viability was not treated with the test substance assumed as 100% and the viability rates of the test cells as a percentage expressed.

% Cell viability = (the absorbance of the test well / the absorbance of the control well) x 100

3.2.6. Establishment of Erlotinib resistant A549 cell line

In order to obtain an erlotinib resistant A549 cell sub-line, a clinically relevant model was utilized (Gündüz, 2019). For this, cells were exposed to increasing drug concentrations (3, 6, 9 and 12 µM) for 2 weeks. Cells were then treated with the highest concentration for an additional 1 month to stabilize the drug resistant phenotype (A549-ER).At the same time , in order to compare the morphological and molecular differences occurs as a result of erlotinib treatment the cells are cultured without any drug treatment for the same time (A549-PT).

3.2.7. Wound healing assay

A wound healing assay was made to determine the effect of total MeOH extract on the migration ability of A549, A549-ER, and A549-PT cells. For this, cells were separated into single cells using trypsin. Cell suspensions containing 1.5×10^5 cells/mL were added to a 6-well plate and incubated for 48 hours to allow cell attachment to occur. Using the tip of a 200 ml pipette, a wound was made on each well of the 6-wells. The media was carefully rinsed with PBS, total MeOH with 1 mg/mL, 500 µL/mL, 250 µL/mL, 125 µL/mL, and 62,5 µL/mL concentrations were added to the wells, and plates were placed in the incubator. Images of the wounds were captured just above each line using the Leica DM IL LED inverted microscope 24-48-72 hours after wound onset. The wound healing areas were analyzed using ImageJ software to calculate the migration rate of the cells.

3.2.8. Soft agar colony formation assay

The Soft Agar Colony Formation Assay was used to determine the effect of total MeOH extract of *Melissa officinalis* L. plant on the colony-forming ability of A549, A549-ER and A549-PT cell lines. For this, 0.5% agar solution was first prepared and placed in a 56°C water bath to be used after being melted in the microwave for 3 minutes. The agar solution was held at 56 °C for 15 minutes and mixed with a heated RPMI-1640 growth medium, then 6-well plates were coated with this mixture.

The top agarose layer was obtained by mixing 1×10^5 cell/per well in PBS with RPMI-1640 growth medium and 0.3% agarose. The the mixture containing the cells transferred to the wells without damaging the agar base. After 24 hours, cells were fed every other day with RPMI-1640 growth medium with total MeOH extract of *Melissa officinalis* L. Colonies formed were stained with 0.1% crystal violet dye and photographed with a Leica EZ50 dissection microscope and identified by Image J software.

3.2.9. Cell cycle analysis with flow cytometry

Cell cycle analysis was performed using RNAse-A and propidium iodide (PI) to examine the effects of total MeOH extract obtained from *Melissa officinalis* L. plant on cell cycle progressions in A549, A549-PT, and A549-ER cell lines.

For this analysis, firstly, cells were seeded as $1x10^6$ cells/75 cm^2 petri and treated with 250 µL/mL of total MeOH extract for 48 hours. The cells were washed with cold PBS and fixed with 70% ethanol for 3 hours. After the supernatant was removed, RNAase-A and PI were added, and the cells were incubated in the dark for 40 minutes. After centrifugation, the supernatant was removed. Finally, each cell suspension sample was placed in 1.5 ml centrifuge tubes and analyzed by FACSCalibur flow cytometry device and ModFit LT 6.0 cell cycle analysis program.

4. RESULTS

4.1. SPME-GC-MS Analysis of *Melissa officinalis* L. Leaves

The GC-MS analysis of *Melissa officinalis* L. was made using the leaves of the plant. As a result of the analysis, twenty-three components were identified, five of which were 2-Octanone (7.7%), 1-octen-3-ol, (5%), Citronellal (26%), Nerolidol (13.4%), Isopulegol (6.2%) is over 5%.

Table 4.1 Chemical composition of *Melissa officinalis* L. by GC/MS

	Component Name	**%**
1	p-Cymene	4,6
2	2-Octanone	7,7
3	6-Methyl-5-hepten-2-one	3,4
4	2-octanol	0,0
5	1-octen-3-ol	5,0
6	beta-Caryophyllene	1,7
7	Citronellal	26,0
8	Benzaldehyde	3,6
9	3,4:5,6-Dibenzocarbazole 7H-Dibenzo[c,g]carbazole;	2,0
10	Terpinolene	3,1
11	Nerolidol	13,4
12	Isopulegol	6,2
13	Camphene	1,4
14	(E)-.beta.-farnesene	3,9
15	Acetophenone	1,0
16	4,7-Dimethylbenzofuran	1,4
17	Citral	4,0
18	1S,CIS-CALAMENENE	2,2
19	Phenol	3,2
20	m-Cresol	2,5
21	p-Methylanisole	2,5
22	Carvacrol	0,6
23	1,6-dimethyl-4-(1-methylethyl)-naphthalene	0,3
24	2,4-Di-tert-butylphenol	0,2

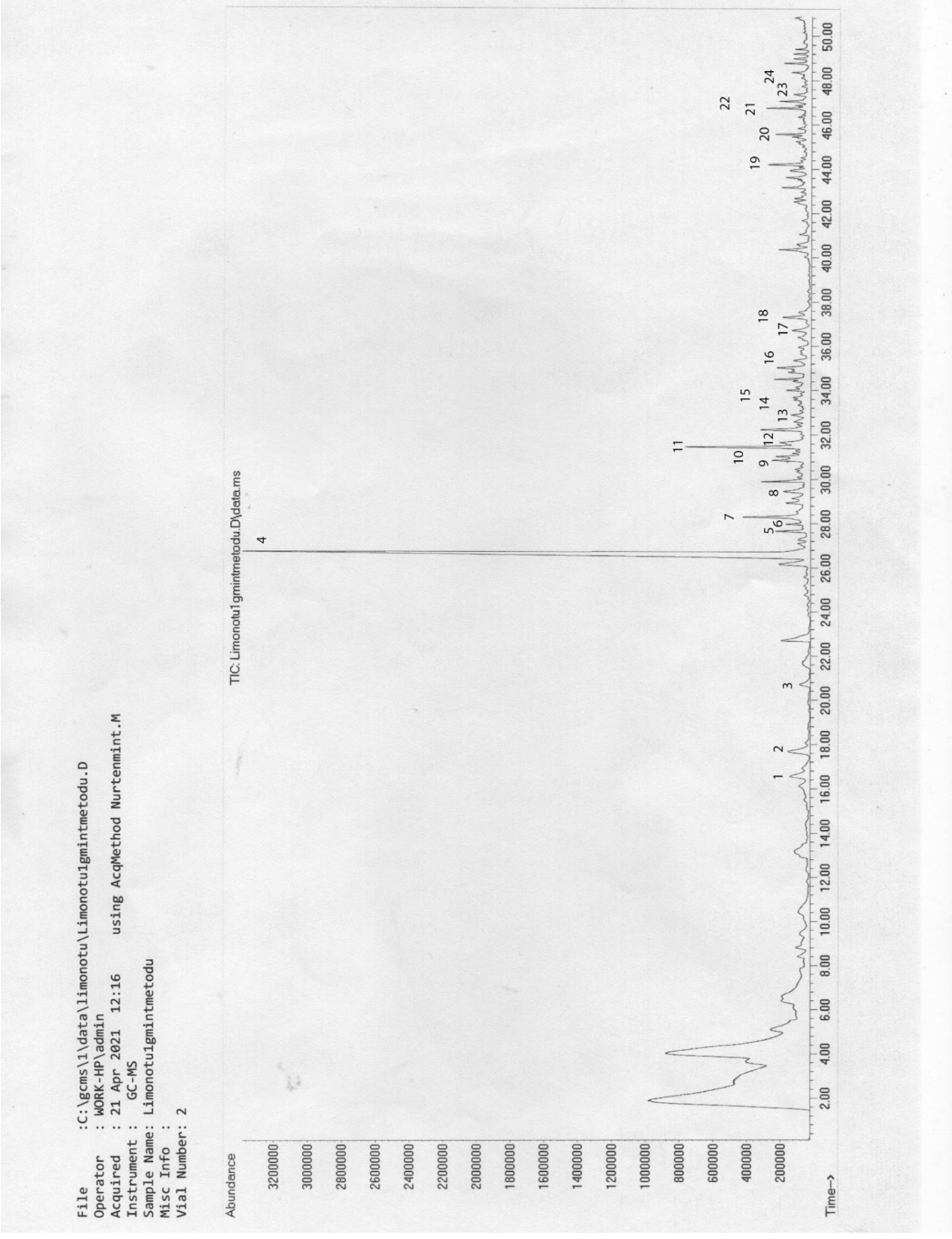


Figure 4.1 Mass spectra of *Melissa officinalis* L. leaves

4.2. Sensitivity of A549 Sub-Cell Lines to Different Leaf Extracts

The effect of four different fractions obtained from the leaf parts of *Melissa officinalis* L. plant on the viability of A549 cells was determined by MTT analysis. The extracts were applied to A549 cell line for 24 hours at final concentrations of 3 mg/mL, 1.5 mg/mL, 0.75 mg/mL 0.375 mg/mL and 0.1875 mg/mL.

As a result of MTT analysis, it was determined that all extracts obtained from the *Melissa officinalis* L. plant had an effect on the viability of the A549 cell line. The IC_{50} value of the n-hexane extract of the plant was determined as 761.149 µg/ml, the IC_{50} value of the EtOAc extract was 476.05 µg/ml, the IC_{50} value of the total MeOH extract was 372.271 µg/ml, and the IC_{50} value of the MeOH extract was determined as 493.957 µg/ml in A549 cell line, respectively. Since there was no significant difference between the extracts and the IC_{50} value at 24 hours was lower than the other extracts, the study was continued with the total methanolic fraction containing all the substance groups in the plant.

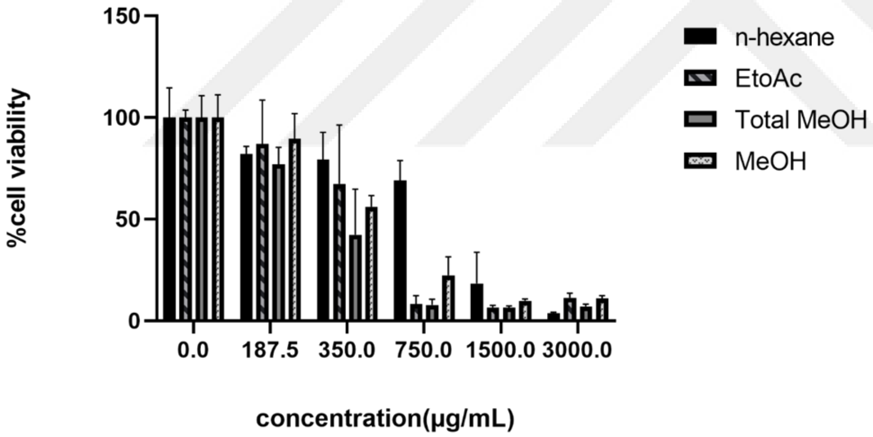


Figure 4.2 The inhibitory effects of different fractions *Melissa officinalis* L. Cell viability assessment of different extracts of *Melissa officinalis* L. (0.1875 , 0.375 , 0.75 , 1.5 , 3 mg/mL) in A549 cells at 24 h post stimulation by means of MTT assay. The results are expressed as a cell viability percentage (%) normalized to cells stimulated with DMSO.

Final concentrations of 1000, 500, 250,125, 62.5, µg/ml of *Melissa officinalis* L. total MeOH extract were applied to A549, A549-PT and A549-ER cell lines for 24, 48, and 72 hours. And the IC_{50} values of the total MeOH extract, respectively, were determined as 352.235 µg/ml, 188.764 µg/ml and 244.176 µg/ml in 48 hours according to the MTT test.

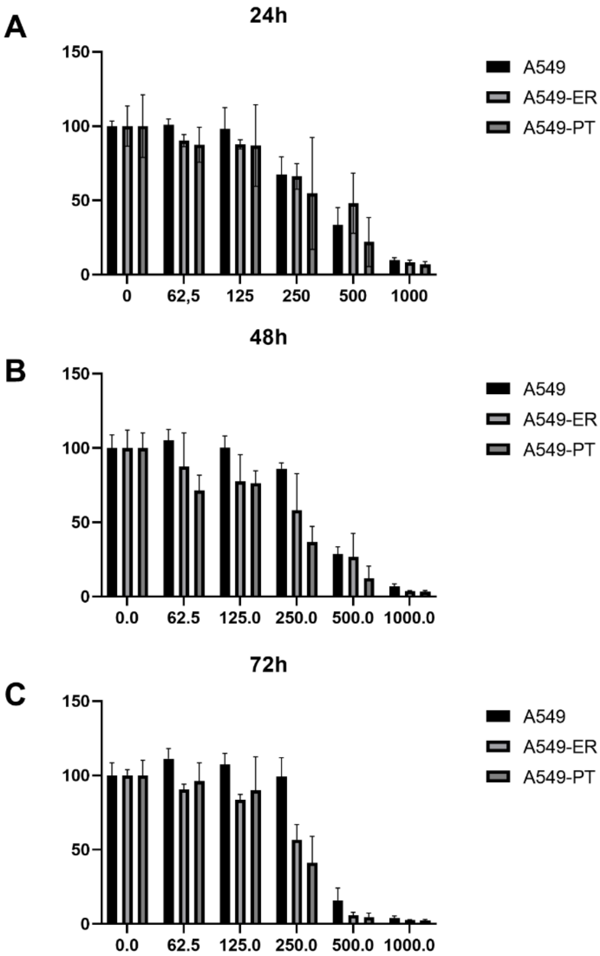


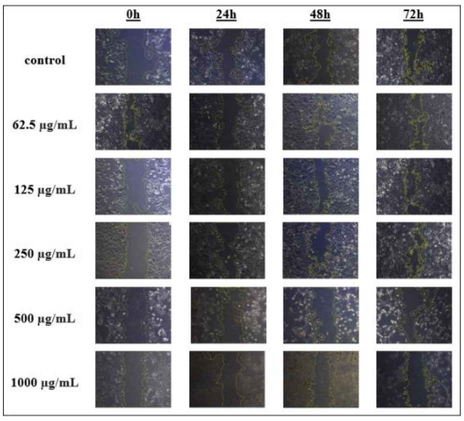
Figure 4.3 Time dependent effects of *Melissa officinalis* L. total MeOH extracts on erlotinib resistant and parental A549 cell sublines. Cell viability versus extract concentration after a)24 hours, b)48 hours and c) 72 hours in the presence of plant methanolic extract

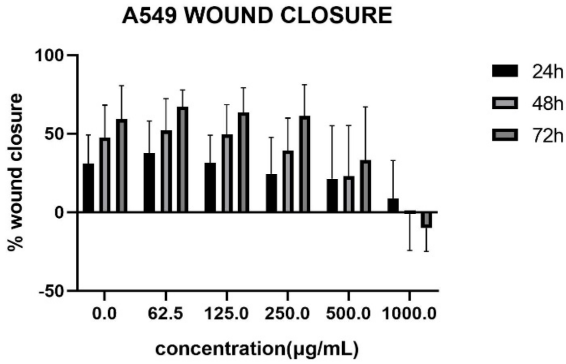
4.3. Cell Migration Ability of A549 Sub-cell Lines

The effect of total MeOH extract on the wound healing capacity of A549, A549-PT, and A549-ER cell lines was evaluated by a wound closure test. For this, cells were exposed to total MeOH extract at determined concentrations, and images were taken with a microscope at 24, 48 and 72 hours. As seen in Figure 4.3A, wound closure areas were calculated with the help of ImageJ software, and wound closure percentages were plotted over time in Figure 4.3B. As a result of the analysis, it was observed that total MeOH extract inhibited cell migration in A549, A549-PT, and A549-ER cell lines in a concentration-dependent manner. In fact, at the highest concentration in 3 cell lines, the extract highly killed the cells, and no wound closure was observed.

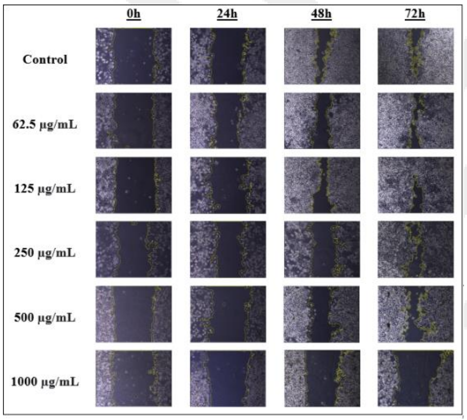
A. **B.**

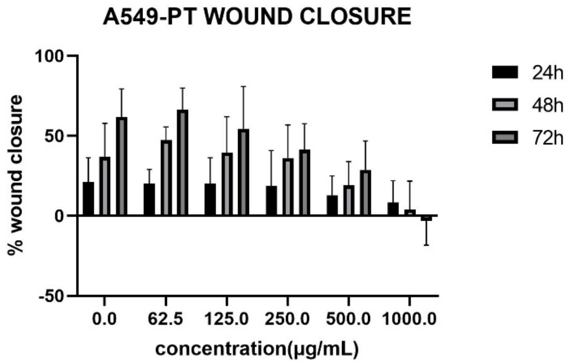
<u>A549</u>



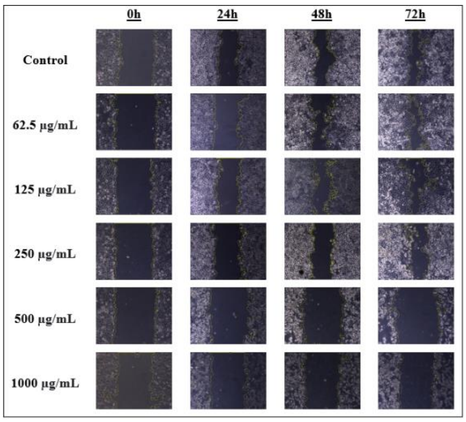


<u>A549-PT</u>





<u>A549-ER</u>



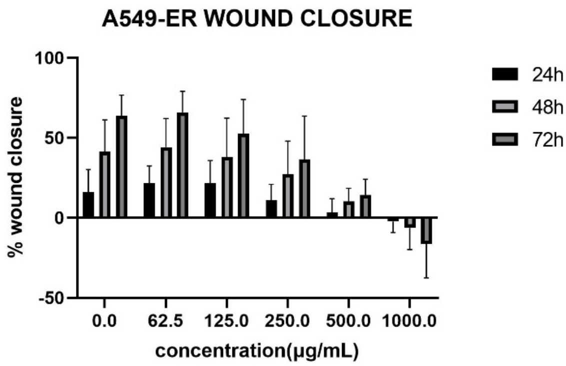


Figure 4.4 The effect of total MeOH on cell migration ability and wound healing capacity of A549,A549-PT and A549-ER A) The speed of wound closure was monitored by taking pictures with inverted microscope at 24, 48, and 72 hours after total MeOH treatment. Concentrations of the extract were included on the left. B) Wound healing area was calculated by using Image J programme. The percentage of wound closure of the increased concentration of total MeOH over time was graphed.

4.4. The Effect of total Methanolic Leaf Extract on Tumor Formation Potential of A549 Sub-cell Lines

A soft agar colony formation assay was designed to evaluate the effect of total MeOH extract on the ability of a single cell to transform into a colony. For this, cells suspended in a medium containing agar were treated with total MeOH extract at concentrations of 1 mg/mL, 500 µL/mL, 250 µL/mL, 125 µL/mL, 62.5 µL/mL, and after three weeks, the cells were stained with crystal violet dye to determine the colony numbers.

As a result of the analysis, it was observed that *Melissa officinalis* L. total MeOH extract inhibited the colony count in a concentration-dependent manner in all three cell lines(Figure 4.5). This result indicates that total MeOH extract has an anti-proliferative effect in normal and drug-resistant lung cancer cell lines.

4.5. The Effect of total Methanolic leaf extract on A549 Sub-cell Lines Cell Cycle Progression

The cell cycle is one of the most fundamental processes resulting in eukaryotic cell growth and division. The regularity of the cell cycle is directly related to the repair of genetic damages that may occur and the stopping of uncontrolled dividing cells that will cause cancer (Mercadante and Kasi, 2021). Therefore, analyzing the cell cycle plays a very important role in understanding the anticancer effects of natural compounds.

In this study, A549, A549-ER, and A549-PT cells were trypsinized after 48 hours of incubation with 250 µL/mL of total MeOH. Afterward, PI staining was performed, and the distribution of cell cycle progression of the marked cells was determined by flow cytometry. The effect of total MeOH extract on the percentages of cell populations in the G0/G1, S, and G2/M cell cycle phases in all three cell lines is shown in Figure 4.6**.**

Compared with the control, total MeOH treatment increases G2/M phase cell population from 13.67% (control) to 17.23% in A549 cells , from 15,49% (control) to 16.77% in A549-PT cells and from 15,015 (control) to 18.06% in A549-ER cells, respectively. Also, total MeOH treatment decreases the S phase cell population from 20.24% (control) to 16.79% in A549 cells , from 15,91% (control) to 11,38167% in A549-PT and from 16.36% (control) to 11.05% in A549-ER cells , respectively.

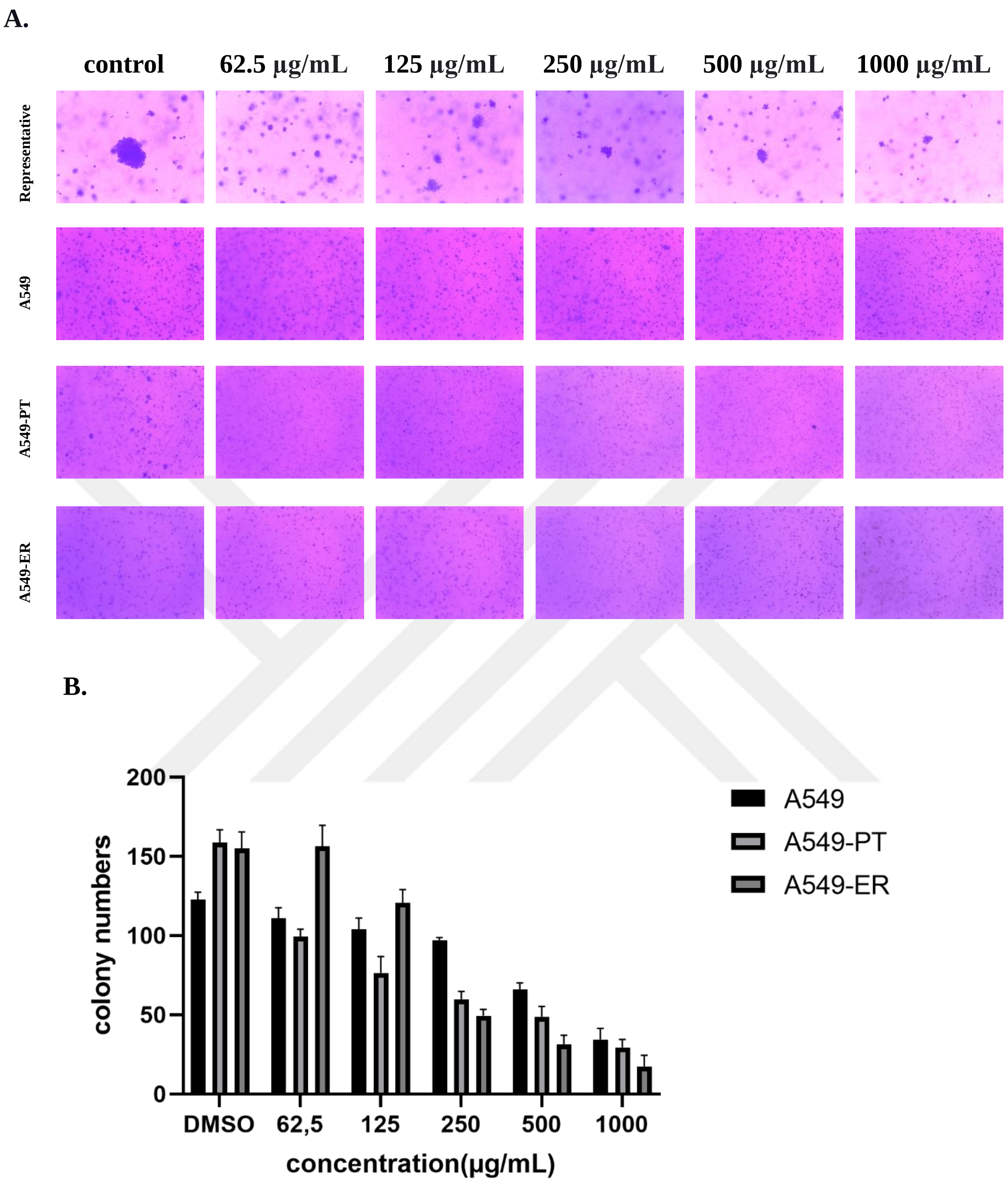


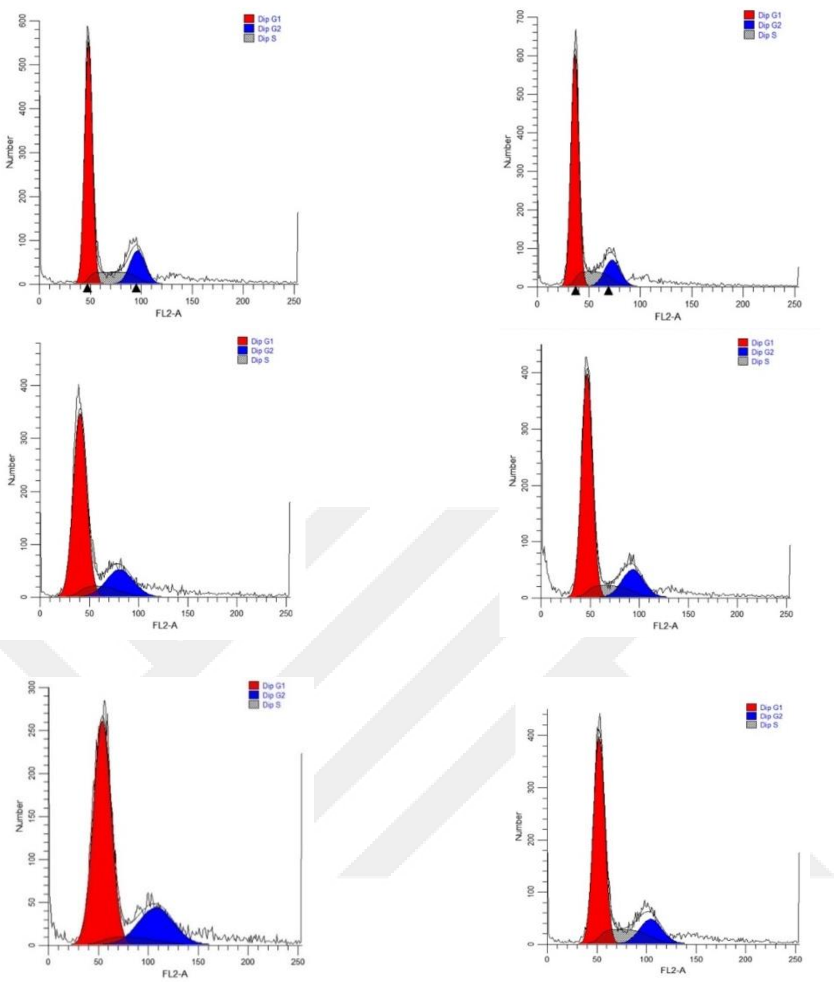
Figure 4.5 The effect of total MeOH on tumor formation capacity of A549,A549-PT and A549-ER cell lines. Colony formation assay was performed to evaluate the effect of total MeOH extract of *Melissa officinalis* plant on colony formation in A549, A549-ER, A549-PT cells. A) Colonies were photographed under dissecting microscope after crystal violet staining. B) Colonies > 0.2 mm were counted on these images by using Image J software.

A. **Treated with total MeOH** **DMSO Control**

A549

A549-PT

A549-ER



B.

	G0/G1 (%)	S (%)	G2/M (%)
A549-DMSO	66,081	20,246	13,671
A549-TM	65,971	16,796	17,233
A549/PT-DMSO	68,591	15,913	15,493
A549/PT-TM	71,845	11,381	16,771
A549/ER-DMSO	68,618	16,366	15,015
A549/ER-TM	70,886	11,051	18,063

C.

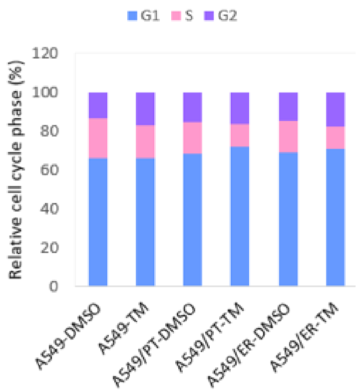


Figure 4.6 The effect of Total MeOH extract on cell cycle distributions of A549, A549-PT and A549-ER The flow cytometric analysis was performed after treating cells with total MeOH extract (250 µg/ml) for 48 hours. **A)** Distribution of A549, A549-ER and A549-PT cells in different cell cycle stages by ModFit LT analysis program**.** **B)** Evaluation of area under curve The percent distribution of different cell cycle stages after total MeOH or DMSO Treatment. **C)** Graphical representation of cell cycle distribution data. TM: total methanolic extract

5. DISCUSSION

While lung cancer was a rare disease at the beginning of the 20th century, it has become the deadliest cancer in the world today, according to the data of the World Health Organization, due to the increase in smoking and industrialization (WHO, 2020). Most types of cancer, including lung cancer, can be treated with surgery, chemotherapy, or radiotherapy. However, intrinsic or acquired resistance to chemotherapeutic drugs reduces survival rates in NSCLC. Since cancer cells resist the current treatment, finding alternative treatment methods is necessary (Holohan et al., 2013) has become necessary. In this context, it is possible to say that natural compounds obtained from plants are an essential resource in discovering new chemotherapeutic agents due to their toxicity and tolerability in the human body (David et al., 2015). These natural compounds have been used in cancer treatments for several years, and many cancer drugs originate from natural compounds. For example, Taxol, a natural diterpene alkaloid, has been isolated from the bark of the *Taxus brevifolia* tree. And it's used as a paclitaxel in treating breast and lung cancer (M. Markman and Mekhail, 2002).

From this point of view, this study tried to reveal whether the extracts obtained from the *Melissa officinalis* L. plant, which is widely distributed in the European region, will have an anti-carcinogenic effect in normal and drug-resistant cell lines by using various in vitro methods. In the study, the antiproliferative effect was analyzed by the MTT test, the effect on migration by wound healing analysis, the effect on colony formation by colony formation analysis, and finally, the effect on cell cycle stages with the cell cycle test. The antiproliferative effect of 4 different extracts obtained from the *Melissa officinalis* L. plant was determined by the MTT test. However, only the total MeOH extract with the lowest IC_{50} value in 24 hours was used in the continuation of the study.

In the first part of the study, extracts were obtained from the leaves of the *Melissa officinalis* L. plant using three different solvents (n-hexane, ethyl acetate, and methanol). Also, as a result of the weighing process, the efficiency of the extracts was calculated, and it was determined that the total MeOH extract had the highest efficiency.

It is known that natural compounds obtained from plants are used in the treatment of various diseases, including cancer.For example, in a study, it was determined that the citronellal component obtained from the plant *Melissa officinalis* L. prevents cell proliferation by mediating MAPK phosphorylation in hepatocellular carcinoma(Massberg et al., 2014). Another component of the plant, beta-caryophyllene, exhibited anti-cancer properties by inhibiting motility and invasion in HCT-116 cells(Dahham et al., 2015). And in this study, GC-MS analysis was performed using the *Melissa officinalis* L. plant leaves. As a result of this analysis, the presence of compounds such as citronellal, citral, and beta-Caryophyllene was determined. And this result shows that similar components are obtained compared with the literature (Fahima Abdellatif et al., 2014; Kittler et al., 2018).

In this study, n-hexane, ethyl acetate, and methanol extracts obtained from the *Melissa officinalis* L. plants were expected to inhibit the growth of A549 cells. In a study by Darzi et al., which examined the cytotoxic effect on K562 and Jurkat cell lines, it was determined that n-hexane extract showed a powerful inhibitory effect. And IC_{50} doses were determined as 57.6 and 33.90 (µg/ml) at 48 hours for the two cell lines (Ebrahimnezhad Darzi and Amirghofran, 2013a). In another study performed with ethyl-acetate extract of *Melissa officinalis* L, the cytotoxic effect on MCF-7 and HeLa cells was investigated. As a result, it was determined that the extract had an equal cytotoxic effect with approximately 0.2 mg/ml in both cell lines (Canadanovic-Brunet et al., 2008). In another study by Bitgen et al., how *Melissa officinalis* L. affects the death pathways of MCF-7 breast cancer cells was examined. For this, a 70% methanolic extract was prepared using the aerial parts of the plant, and the extract was applied to the cell line at different rates; the IC_{50} value in 24 hours was determined as 20 µg/ml by MTT analysis (Bitgen et al., 2022).

In this study, n-hexane, ethyl acetate (EtOAc), methanol (MeOH), and total methanol (total MeOH) extracts obtained from the leaves of *Melissa officinalis* L. plant were applied to the A549 cell line in a dose-dependent manner (3-0.1875 mg/ml). As a result of the 24-hours treatment, the total MeOH extract was determined as the most cytotoxic extract with an IC_{50} dose of 372,271 µg/ml. After the decision to use total MeOH extract in the continuation of the study, the cytotoxic effect of the extract on the erlotinib-resistant A549 cell line was investigated for the first time in the literature. Depending on the dose and time, the extract was applied to the cell line, and the IC_{50} value was determined as 352,235 µg/ml, 188,764 µg/ml, and 244,176 µg/ml in the A549, A549-PT, and A549-ER cell lines, respectively, at 48 hours. This result shows that *Melissa officinalis* L. total MeOH extract prevents proliferation in resistant A549 cells by reversing the acquired resistance.

The ability of cancer cells to migrate from one place to another in the body is one of the most important steps in tumor development and metastasis. At this stage, it is thought that the small population of cells that do not die with chemotherapy treatment and that have gained drug resistance can lead to metastasis. A study determined that the increase in mitochondrial membrane potential of A549 cells with cisplatin resistance increased the migration and invasion of cells compared to parent cells (Jeon et al., 2016). In another study by Gündüz et al., erlotinib-resistant A549 cells showed a higher migration capacity than parental cells (Gündüz, 2019).

In the literature, the anti-migration effect of the ethanolic extract obtained from the leaf and stem of *Melissa officinalis* L. in MDA-MB-231 cells was investigated by scratch assay. And at the lowest concentrations (20, 100, 250 µg/ml), it has been determined that it has an inhibitory effect on migration(Moacă et al., 2018)m. In the present study, the effect of total MeOH extract obtained from *Melissa officinalis* L. plant on migration in erlotinib-resistant and parental A549 cells was investigated by wound healing analysis. And as a result, it was determined that the extract inhibited migration in resistant and parental A549 cells in a concentration-dependent manner.

The colony-forming potential is associated with metastasis in cancer cells. And drug-resistant cancer cells tend to form colonies. In a study by Xie et al., a soft agar colony formation experiment was performed. As a result, it was observed that normal HCC287 cells formed colonies, but colonies disappeared after 1µm of erlotinib treatment. In comparison, colonies of erlotinib-resistant HCC287 cells grew larger after 1µm erlotinib treatment (Xie et al., 2016).

In this study the effect of total MeOH extract of *Melissa officinalis* L. plant on colony formation in standard and erlotinib resistant A549 cell lines was eveluated. For this, a soft agar colony formation test was performed. In a study using the water extract of the *Melissa officinalis* L. plant, the cells were first treated with the extract for 48 hours to determine the long-term effect of the extract. Afterward, colony formation was observed by planting 250 cells per well. As a result, it was determined that increasing extract concentration prevented colony formation (Kuo et al., 2020). In our study, in parallel with the literature, it was determined that increasing the concentration of total MeOH extract prevented colony formation in both standard and erlotinib-resistant A549 cells.

The order of the cell cycle has a very important place in cancer cells that can multiply abnormally. Many studies have proven that the extracts obtained from the Melissa offinalis L. plant have cell cycle blocking effects. In one study, *Melissa officinalis* L. water extract increased the cyclin b1 level, which has a vital role in the cell cycle, and inhibited the G2/M phase (Kuo et al., 2020b), hydroethanolic extract caused the cells to accumulate in the G2/M phase in the HT-29 cell line (Weidner et al., 2015). In another study, the dichloromethane fraction of the plant was found to be It has been determined to inhibit the G2/M phase (Ebrahimnezhad Darzi and Amirghofran, 2013b).

This study determined the effect of *Melissa officinalis* L. total MeOH extract on the cell cycle in normal and drug-resistant A549 cell lines. Cell percentages at cell cycle stages were calculated in three cell lines treated with total MeOH at the determined concentrations. According to the data obtained as a result of the analysis, a decrease was observed in the cell population in the S phase, while an increase in the cell population in the G2/M phase was observed in the A549, A549-PT and A549-ER cells compared to the control groups.

6. REFERENCES

Abdellatif, F, and Hassani, A. (2015). Chemical composition of the essential oils from leaves of Melissa officinalis extracted by hydrodistillation, steam distillation, organic solvent and microwave hydrodistillation. 7.

Abdellatif, Fahima, Hadjira, B., Zitouni, A., and Hassani, A. (2014). Chemical composition and antimicrobial activity of the essential oil from leaves of Algerian Melissa officinalis L. EXCLI Journal, 13, 772–781. https://doi.org/10.17877/DE290R-6927

Abeloff, M. D., Eggleston, J. C., Mendelsohn, G., Ettinger, D. S., and Baylin, S. B. (1979). Changes in morphologic and biochemical characteristics of small cell carcinoma of the lung: A clinicopathologic study. The American Journal of Medicine, 66(5), 757–764. https://doi.org/10.1016/0002-9343(79)91113-6

Abouzid, K., and Shouman, S. (2008). Design, synthesis and in vitro antitumor activity of 4-aminoquinoline and 4-aminoquinazoline derivatives targeting EGFR tyrosine kinase. Bioorganic & Medicinal Chemistry, 16(16), 7543–7551. https://doi.org/10.1016/j.bmc.2008.07.038

Adinee, J., Piri, K., and Karami, O. (n.d.). Essential Oil Composition of Lemon Balm (Melissa officinalis L.) Leaves Grown in Hamadan Province, Iran. 3.

Amoah, S. K. S., Sandjo, L. P., Kratz, J. M., and Biavatti, M. W. (2016). Rosmarinic Acid—Pharmaceutical and Clinical Aspects. Planta Medica, 82(5), 388–406. https://doi.org/10.1055/s-0035-1568274

Anantharaju, P. G., Gowda, P. C., Vimalambike, M. G., and Madhunapantula, S. V. (2016). An overview on the role of dietary phenolics for the treatment of cancers. Nutrition Journal, 15. https://doi.org/10.1186/s12937-016-0217-2

Ansari, I., and Akhtar, M. S. (2019a). Current Insights on the Role of Terpenoids as Anticancer Agents: A Perspective on Cancer Prevention and Treatment. https://doi.org/10.1007/978-981-13-7205-6_3

Ansari, I., and Akhtar, M. S. (2019b). Recent Insights on the Anticancer Properties of Flavonoids: Prospective Candidates for Cancer Chemoprevention and Therapy. https://doi.org/10.1007/978-981-13-7154-7_13

Antoni, D., Burckel, H., Josset, E., and Noel, G. (2015). Three-dimensional cell culture: A breakthrough in vivo. International Journal of Molecular Sciences, 16(3), 5517–5527. https://doi.org/10.3390/ijms16035517

Balayssac, D., Authier, N., Cayre, A., and Coudore, F. (2005). Does inhibition of P-glycoprotein lead to drug-drug interactions? Toxicology Letters, 156(3), 319–329. https://doi.org/10.1016/j.toxlet.2004.12.008

Barrales-Cureño, H. J., Reyes, C. R., García, I. V., Valdez, L. G. L., Jesús, A. G. D., Ruíz, J. A. C., … Montoya, J. M. (2019). Alkaloids of Pharmacological Importance in Catharanthus roseus. Alkaloids - Their Importance in Nature and Human Life. https://doi.org/10.5772/intechopen.82006

Barros, L., Dueñas, M., Dias, M. I., Sousa, M. J., Santos-Buelga, C., and Ferreira, I. C. F. R. (2013). Phenolic profiles of cultivated, in vitro cultured and commercial samples of Melissa officinalis L. infusions. Food Chemistry, 136(1), 1–8. https://doi.org/10.1016/j.foodchem.2012.07.107

Basta, A., Tzakou, O., and Couladis, M. (2005). Composition of the leaves essential oil of Melissa officinalis s. L. From Greece. Flavour and Fragrance Journal, 20(6), 642–644. https://doi.org/10.1002/ffj.1518

Ben Sghaier, M., Pagano, A., Mousslim, M., Ammari, Y., Kovacic, H., and Luis, J. (2016). Rutin inhibits proliferation, attenuates superoxide production and decreases adhesion and migration of human cancerous cells. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie, 84, 1972–1978. https://doi.org/10.1016/j.biopha.2016.11.001

Bitgen, N., Baran, M., Önder, G. Ö., Suna, P. A., Gürbüz, P., and Yay, A. (2022). Effect of Melissa officinalis L. on human breast cancer cell line via apoptosis and autophagy. Cukurova Medical Journal, 47(2), 765–775. https://doi.org/10.17826/cumj.1090930

Bramley, G., Harley, R., and Paton, A. (2009). Neotropical Lamiaceae. Retrieved 9 September 2020, from In: Milliken, W., Klitgård, B. & Baracat, A. (2009 onwards), Neotropikey—Interactive key and information resources for flowering plants of the Neotropics. Www.kew.org/neotropikey (accessed Sept 9, 2020]). website: https://www.kew.org/science/tropamerica/neotropikey/families/Lamiaceae.htm

Breen, L., Murphy, L., Keenan, J., and Clynes, M. (2008). Development of taxane resistance in a panel of human lung cancer cell lines. Toxicology in Vitro, 22(5), 1234–1241. https://doi.org/10.1016/j.tiv.2008.04.005

Burcu, D. (2012). (Functional Assessment of Cancer Therapy–Lung) YAŞAM KALİTESİ ÖLÇEĞİNİN TÜRKÇE SÜRÜMÜNÜN PSİKOMETRİK ÖZELLİKLERİNİN (Geçerlilik ve Güvenilirlik) VE KLİNİKTEKİ. 77.

Canadanovic-Brunet, J., Cetkovic, G., Djilas, S., Tumbas, V., Bogdanovic, G., Mandic, A., … Canadanovic, V. (2008). Radical scavenging, antibacterial, and antiproliferative activities of Melissa officinalis L. extracts. Journal of Medicinal Food, 11(1), 133–144.

Caniova, A., and Brandsteterova, E. (2001). Hplc Analysis of Phenolic Acids in Melissa Officinalis. Journal of Liquid Chromatography & Related Technologies, 24(17), 2647–2659. https://doi.org/10.1081/JLC-100106092

Carnat, A. P., Carnat, A., Fraisse, D., and Lamaison, J. L. (1998). The aromatic and polyphenolic composition of lemon balm (Melissa officinalis L. subsp. Officinalis) tea.

Pharmaceutica Acta Helvetiae, 72(5), 301–305. https://doi.org/10.1016/S0031-6865(97)00026-5

Chatterjee, N., and Walker, G. C. (2017). Mechanisms of DNA damage, repair and mutagenesis. Environmental and Molecular Mutagenesis, 58(5), 235–263. https://doi.org/10.1002/em.22087

Chen, F., Chen, X., Yang, D., Che, X., Wang, J., Li, X., … Song, X. (2016). Isoquercitrin inhibits bladder cancer progression in vivo and in vitro by regulating the PI3K/Akt and PKC signaling pathways. Oncology Reports, 36(1), 165–172. https://doi.org/10.3892/or.2016.4794

Clynes, M., Redmond, A., Moran, E., and Gilvarry, U. (1992). Multiple drug-resistance in variant of a human non-small cell lung carcinoma cell line, DLKP-A. Cytotechnology, 10(1), 75–89. https://doi.org/10.1007/BF00376102

Cosge, B., İpek, A., and Gürbüz, B. (2009). GC/MS Analysis of Herbage Essential Oil from Lemon Balms (Melissa officinalis L.) Grown in Turkey. Journal of Applied Biological Sciences 3(2), 149–152.

Cox-Georgian, D., Ramadoss, N., Dona, C., and Basu, C. (2019). Therapeutic and Medicinal Uses of Terpenes. Medicinal Plants, 333–359. https://doi.org/10.1007/978-3-030-31269-5_15

Cragg, G. M., Newman, D. J., and Snader, K. M. (1997). Natural products in drug discovery and development. Journal of Natural Products, 60(1), 52–60. https://doi.org/10.1021/np9604893

Crinò, L., Weder, W., Meerbeeck, J. van, and Felip, E. (2010). Early stage and locally advanced (non-metastatic) non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology, 21, v103–v115. https://doi.org/10.1093/annonc/mdq207

Cunha, F., Tintino, S. R., Figueredo, F., Barros, L., Duarte, A. E., Vega Gomez, M. C., … Franco, J. (2016). HPLC-DAD phenolic profile, cytotoxic and anti-kinetoplastidae activity of Melissa officinalis. Pharmaceutical Biology, 54(9), 1664–1670. https://doi.org/10.3109/13880209.2015.1120320

Dahham, S. S., Tabana, Y. M., Iqbal, M. A., Ahamed, M. B. K., Ezzat, M. O., Majid, A. S. A., and Majid, A. M. S. A. (2015). The Anticancer, Antioxidant and Antimicrobial Properties of the Sesquiterpene β-Caryophyllene from the Essential Oil of Aquilaria crassna. Molecules (Basel, Switzerland), 20(7), 11808–11829. https://doi.org/10.3390/molecules200711808

Dasari, S., and Tchounwou, P. B. (2014). Cisplatin in cancer therapy: Molecular mechanisms of action. European Journal of Pharmacology, 0, 364–378. https://doi.org/10.1016/j.ejphar.2014.07.025

David, B., Wolfender, J.-L., and Dias, D. A. (2015). The pharmaceutical industry and natural products: Historical status and new trends. Phytochemistry Reviews, 14(2), 299–315. https://doi.org/10.1007/s11101-014-9367-z

Deng, Q., Yang, H., Lin, Y., Qiu, Y., Gu, X., He, P., … Zhou, J. X. (2014). Prognostic value of ERCC1 mRNA expression in non-small cell lung cancer, breast cancer, and gastric cancer in patients from Southern China. International Journal of Clinical and Experimental Pathology, 7(12), 8312–8321.

DI Giacomo, S., DI Sotto, A., Mazzanti, G., and Wink, M. (2017). Chemosensitizing Properties of β-Caryophyllene and β-Caryophyllene Oxide in Combination with Doxorubicin in Human Cancer Cells. Anticancer Research, 37(3), 1191–1196. https://doi.org/10.21873/anticanres.11433

Dias, D. A., Urban, S., and Roessner, U. (2012). A Historical Overview of Natural Products in Drug Discovery. Metabolites, 2(2), 303–336. https://doi.org/10.3390/metabo2020303

Du, B., and Shim, J. S. (2016). Targeting Epithelial–Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. Molecules, 21(7). https://doi.org/10.3390/molecules21070965

Dwivedi, S., Kumar, M., and Trivedi, S. P. (2017). Mitigating potential of Melissa officinale against As3+-induced cytotoxicity and transcriptional alterations of Hsp70 and Hsp27 in fish, Channa punctatus (Bloch). Environmental Monitoring and Assessment, 189(7), 306. https://doi.org/10.1007/s10661-017-6002-7

Ebrahimnezhad Darzi, S., and Amirghofran, Z. (2013a). Dichloromethane fraction of Melissa officinalis induces apoptosis by activation of intrinsic and extrinsic pathways in human leukemia cell lines. Immunopharmacology and Immunotoxicology, 35(3), 313–320. https://doi.org/10.3109/08923973.2013.768268

Ebrahimnezhad Darzi, S., and Amirghofran, Z. (2013b). Dichloromethane fraction of Melissa officinalis induces apoptosis by activation of intrinsic and extrinsic pathways in human leukemia cell lines. Immunopharmacology and Immunotoxicology, 35(3), 313–320. https://doi.org/10.3109/08923973.2013.768268

Edge, S. B., and American Joint Committee on Cancer (Eds.). (2010). AJCC cancer staging manual (7th ed). New York: Springer.

Edmondson, J. R., Mill, R. R., and Tan, K. (1982). Melissa L. In: Davis PH, editor. Flora of Turkey and the East Aegean Islands. Edinburgh University Press.

Gao, Jianling, Yue, S., Chen, J., and Wang, H. (2014). Classification of normal and cancerous lung tissues by electrical impendence tomography. Bio-Medical Materials and Engineering. https://doi.org/10.3233/BME-141035

Ghosh, K. (2013). Anticancer effect of lemongrass oil and citral on cervical cancer cell lines. Pharmacognosy Communications, 3, 41–48. https://doi.org/10.5530/pc.2013.4.6

Gündüz, A. (2019). Establishment of drug-resistant cell lines in non-small cell lung cancer (Master of Science Thesis, Fen Bilimleri Enstitüsü). Fen Bilimleri Enstitüsü. Retrieved from https://acikbilim.yok.gov.tr/handle/20.500.12812/581661

Han, Y.-H., Kee, J.-Y., and Hong, S.-H. (2018). Rosmarinic Acid Activates AMPK to Inhibit Metastasis of Colorectal Cancer. Frontiers in Pharmacology, 9, 68. https://doi.org/10.3389/fphar.2018.00068

Hoarau-Véchot, J., Rafii, A., Touboul, C., and Pasquier, J. (2018). Halfway between 2D and Animal Models: Are 3D Cultures the Ideal Tool to Study Cancer-Microenvironment Interactions? International Journal of Molecular Sciences, 19(1). https://doi.org/10.3390/ijms19010181

Holohan, C., Van Schaeybroeck, S., Longley, D. B., and Johnston, P. G. (2013). Cancer drug resistance: An evolving paradigm. Nature Reviews Cancer, 13(10), 714–726. https://doi.org/10.1038/nrc3599

Hsia, T.-C., Lin, C.-C., Wang, J.-J., Ho, S.-T., and Kao, A. (2002). Relationship between chemotherapy response of small cell lung cancer and P-glycoprotein or multidrug resistance-related protein expression. Lung, 180(3), 173–179. https://doi.org/10.1007/s004080000091

Huang, L., and Fu, L. (2015). Mechanisms of resistance to EGFR tyrosine kinase inhibitors. Acta Pharmaceutica Sinica. B, 5(5), 390–401. https://doi.org/10.1016/j.apsb.2015.07.001

Inamura, K. (2017). Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification. Frontiers in Oncology, 7. https://doi.org/10.3389/fonc.2017.00193

Isaoglu, M., Güllüce, M., and Karadayi, M. (2020). Plant-Derived Natural Products as Multidrug Resistance Modulators in Cancer Therapy. Anatolian Journal of Biology, 1(2), 1–51.

Jain, R. K., and Chen, H. (2017). Spotlight on brigatinib and its potential in the treatment of patients with metastatic ALK-positive non-small cell lung cancer who are resistant or intolerant to crizotinib. Lung Cancer (Auckland, N.Z.), 8, 169–177. https://doi.org/10.2147/LCTT.S126507

Jamwal, K., Bhattacharya, S., and Puri, S. (2018). Plant growth regulator mediated consequences of secondary metabolites in medicinal plants. Journal of Applied Research on Medicinal and Aromatic Plants, 9. https://doi.org/10.1016/j.jarmap.2017.12.003

Joseph, J. S., Malindisa, S. T., and Ntwasa, M. (2018). Two-Dimensional (2D) and Three-Dimensional (3D) Cell Culturing in Drug Discovery. https://doi.org/10.5772/intechopen.81552

Kamaraj, S., Anandakumar, P., Jagan, S., Ramakrishnan, G., Periyasamy, P., Asokkumar, S., … Devaki, T. (2019). Hesperidin inhibits cell proliferation and induces mitochondrial-mediated apoptosis in human lung cancer cells through down regulation of β-catenin/c-myc. Biocatalysis and Agricultural Biotechnology, 18, 101065. https://doi.org/10.1016/j.bcab.2019.101065

Kanimozhi, G., and Prasad, N. R. (2015). Chapter 73—Anticancer Effect of Caffeic Acid on Human Cervical Cancer Cells. In V. R. Preedy (Ed.), Coffee in Health and Disease Prevention (pp. 655–661). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-409517-5.00073-5

Kaushik, V., Yakisich, J. S., Kulkarni, Y., Azad, N., and Iyer, A. K. V. (2018). Chemoresistance of Lung Cancer Cells: 2D and 3D In Vitro Models for Anticancer Drug Screening. Lung Cancer - Strategies for Diagnosis and Treatment. https://doi.org/10.5772/intechopen.78946

Kittler, J., Krüger, H., Ulrich, D., Zeiger, B., Schütze, W., Böttcher, Ch., … Marthe, F. (2018). Content and composition of essential oil and content of rosmarinic acid in lemon balm and balm genotypes (Melissa officinalis). Genetic Resources and Crop Evolution, 65(5), 1517–1527. https://doi.org/10.1007/s10722-018-0635-4

Krause, D. S., and Van Etten, R. A. (2005). Tyrosine kinases as targets for cancer therapy. The New England Journal of Medicine, 353(2), 172–187. https://doi.org/10.1056/NEJMra044389

Kuo, T.-T., Chang, H.-Y., Chen, T.-Y., Liu, B.-C., Chen, H.-Y., Hsiung, Y.-C., … Huang, T.-C. (2020). Melissa officinalis Extract Induces Apoptosis and Inhibits Migration in Human Colorectal Cancer Cells. ACS Omega, 5(49), 31792–31800. https://doi.org/10.1021/acsomega.0c04489

Langhans, S. A. (2018). Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. Frontiers in Pharmacology, 9. https://doi.org/10.3389/fphar.2018.00006

Lee, Y., Choi, Y.-R., Kim, K.-Y., and Shin, D. H. (2016). The impact of intermittent versus continuous exposure to EGFR tyrosine kinase inhibitor on selection of EGFR T790M-mutant drug-resistant clones in a lung cancer cell line carrying activating EGFR mutation. Oncotarget, 7(28), 43315–43323. https://doi.org/10.18632/oncotarget.9703

Lemjabbar-Alaoui, H., Hassan, O., Yang, Y.-W., and Buchanan, P. (2015). Lung cancer: Biology and treatment options. Biochimica et Biophysica Acta, 1856(2), 189–210. https://doi.org/10.1016/j.bbcan.2015.08.002

Liao, C.-C., Chen, S.-C., Huang, H.-P., and Wang, C.-J. (2018). Gallic acid inhibits bladder cancer cell proliferation and migration via regulating fatty acid synthase (FAS). Journal of Food and Drug Analysis, 26(2), 620–627. https://doi.org/10.1016/j.jfda.2017.06.006

Lin, J. H., and Yamazaki, M. (2003). Role of P-glycoprotein in pharmacokinetics: Clinical implications. Clinical Pharmacokinetics, 42(1), 59–98. https://doi.org/10.2165/00003088-200342010-00003

Lin, L., and Bivona, T. G. (2012). Mechanisms of Resistance to Epidermal Growth Factor Receptor Inhibitors and Novel Therapeutic Strategies to Overcome Resistance in NSCLC Patients. Chemotherapy Research and Practice, 2012. https://doi.org/10.1155/2012/817297

Lv, D., Hu, Z., Lu, L., Lu, H., and Xu, X. (2017). Three-dimensional cell culture: A powerful tool in tumor research and drug discovery. Oncology Letters, 14(6), 6999–7010. https://doi.org/10.3892/ol.2017.7134

Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S., and Baradaran, B. (2017). The Different Mechanisms of Cancer Drug Resistance: A Brief Review. Advanced Pharmaceutical Bulletin, 7(3), 339–348. https://doi.org/10.15171/apb.2017.041

Markman, J. L., Rekechenetskiy, A., Holler, E., and Ljubimova, J. Y. (2013). Nanomedicine therapeutic approaches to overcome cancer drug resistance. Advanced Drug Delivery Reviews, 65(13–14), 1866–1879. https://doi.org/10.1016/j.addr.2013.09.019

Markman, M., and Mekhail, T. M. (2002). Paclitaxel in cancer therapy. Expert Opinion on Pharmacotherapy, 3(6), 755–766. https://doi.org/10.1517/14656566.3.6.755

Maruoka, T., Kitanaka, A., Kubota, Y., Yamaoka, G., Kameda, T., Imataki, O., … Tanaka, T. (2018). Lemongrass essential oil and citral inhibit Src/Stat3 activity and suppress the proliferation/survival of small-cell lung cancer cells, alone or in combination with chemotherapeutic agents. International Journal of Oncology, 52(5), 1738–1748. https://doi.org/10.3892/ijo.2018.4314

Massberg, D., Simon, A., Häussinger, D., Keitel, V., Gisselmann, G., Conrad, H., and Hatt, H. (2014). Monoterpene (−)-citronellal affects hepatocarcinoma cell signaling via an olfactory receptor. Archives of Biochemistry and Biophysics, 566. https://doi.org/10.1016/j.abb.2014.12.004

Mencherini, T., Picerno, P., Scesa, C., and Aquino, R. (2007). Triterpene, antioxidant, and antimicrobial compounds from Melissa officinalis. Journal of Natural Products, 70(12), 1889–1894. https://doi.org/10.1021/np070351s

Mercadante, A. A., and Kasi, A. (2021). Genetics, Cancer Cell Cycle Phases. In StatPearls [Internet]. StatPearls Publishing. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK563158/

Mimica-Dukic, N., Bozin, B., Sokovic, M., and Simin, N. (2004). Antimicrobial and Antioxidant Activities of Melissa officinalis L. (Lamiaceae) Essential Oil. Journal of Agricultural and Food Chemistry, 52(9), 2485–2489. https://doi.org/10.1021/jf030698a

Moacă, E.-A., Farcaş, C., Ghiţu, A., Coricovac, D., Popovici, R., Cărăba-Meiţă, N.-L., … Avram, Ş. (2018). A Comparative Study of Melissa officinalis Leaves and Stems Ethanolic Extracts in terms of Antioxidant, Cytotoxic, and Antiproliferative Potential. Evidence-Based Complementary and Alternative Medicine : ECAM, 2018, 7860456. https://doi.org/10.1155/2018/7860456

Molina, J. R., Yang, P., Cassivi, S. D., Schild, S. E., and Adjei, A. A. (2008). Non–Small Cell Lung Cancer: Epidemiology, Risk Factors, Treatment, and Survivorship. Mayo Clinic Proceedings. Mayo Clinic, 83(5), 584–594.

Mulkens, A., and Kapetanidis, I. (1987). [Flavonoids of the leaves of Melissa officinalis L. (Lamiaceae)]. Pharmaceutica Acta Helvetiae, 62(1), 19–22.

Munoz, M., Henderson, M., Haber, M., and Norris, M. (2007). Role of the MRP1/ABCC1 Multidrug Transporter Protein in Cancer. IUBMB Life, 59(12), 752–757. https://doi.org/10.1080/15216540701736285

Mustafa, M., Azizi, J., IIIzam, E., Azizan, N., Sharifa, S., and Abbas, S. (2016). Lung Cancer: Risk Factors, Management, And Prognosis. IOSR Journal of Dental and Medical Sciences, 15, 94–101. https://doi.org/10.9790/0853-15100494101

Naghiloo, S., Khodaverdi, M., Siahkolaee, S. N., and Dadpour, M. R. (2013). Comparative floral development in Lamioideae (Lamiaceae): Marrubium, Phlomis, and Stachys. Plant Systematics and Evolution. https://doi.org/10.1007/s00606-013-0960-1

Nan, X., Xie, C., Yu, X., and Liu, J. (2017). EGFR TKI as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer. Oncotarget, 8(43), 75712–75726. https://doi.org/10.18632/oncotarget.20095

Nath, S., and Devi, G. R. (2016). Three-Dimensional Culture Systems in Cancer Research: Focus on Tumor Spheroid Model. Pharmacology & Therapeutics, 163, 94–108. https://doi.org/10.1016/j.pharmthera.2016.03.013

Ngan, V. K., Bellman, K., Hill, B. T., Wilson, L., and Jordan, M. A. (2001). Mechanism of Mitotic Block and Inhibition of Cell Proliferation by the Semisynthetic Vinca Alkaloids Vinorelbine and Its Newer Derivative Vinflunine. Molecular Pharmacology, 60(1), 225–232. https://doi.org/10.1124/mol.60.1.225

Nunes, A. S., Barros, A. S., Costa, E. C., Moreira, A. F., and Correia, I. J. (2019). 3D tumor spheroids as in vitro models to mimic in vivo human solid tumors resistance to therapeutic drugs. Biotechnology and Bioengineering, 116(1), 206–226. https://doi.org/10.1002/bit.26845

Nurzyńska-Wierdak, R., Bogucka-Kocka, A., and Szymczak, G. (2014). Volatile constituents of Melissa officinalis leaves determined by plant age. Natural Product Communications, 9(5), 703–706.

Ono, M., and Kuwano, M. (2006). Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. Clinical

Cancer Research: An Official Journal of the American Association for Cancer Research, 12(24), 7242–7251. https://doi.org/10.1158/1078-0432.CCR-06-0646

Ordaz, J. J., Hernández, J. M., Ramirez, J., Castañeda, A., gonzález-olivares, L., and Lopez, E. (2018). Bioactive compounds in aqueous extracts of lemon balm (Melissa officinalis) cultivated in Mexico. Archivos Latinoamericanos de Nutricion, 68, 268–279.

Paduch, R., Kandefer-Szerszeń, M., Trytek, M., and Fiedurek, J. (2007). Terpenes: Substances useful in human healthcare. Archivum Immunologiae Et Therapiae Experimentalis, 55(5), 315–327. https://doi.org/10.1007/s00005-007-0039-1

Park, K.-R., Nam, D., Yun, H.-M., Lee, S.-G., Jang, H.-J., Sethi, G., … Ahn, K. S. (2011). β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Letters, 312(2), 178–188. https://doi.org/10.1016/j.canlet.2011.08.001

Pinto, B., Henriques, A. C., Silva, P. M. A., and Bousbaa, H. (2020). Three-Dimensional Spheroids as In Vitro Preclinical Models for Cancer Research. Pharmaceutics, 12(12). https://doi.org/10.3390/pharmaceutics12121186

Popova, A., Mihaylova, D., Alexieva, I., Deseva, I., and Dalemska. (2016). Melissa officinalis L.- GC Profile and Antioxidant Activity. International Journal of Pharmacognosy and Phytochemical Research, 8, 634–638.

Qi, F., Yan, Q., Zheng, Z., Liu, J., Chen, Y., and Zhang, G. (2018). Geraniol and geranyl acetate induce potent anticancer effects in colon cancer Colo-205 cells by inducing apoptosis, DNA damage and cell cycle arrest. Journal of B.U.ON.: Official Journal of the Balkan Union of Oncology, 23(2), 346–352.

Rehman, S.-, Latief, R., Bhat, K. A., Khuroo, M. A., Shawl, A. S., and Chandra, S. (2017). Comparative analysis of the aroma chemicals of Melissa officinalis using hydrodistillation and HS-SPME techniques. Arabian Journal of Chemistry, 10, S2485–S2490. https://doi.org/10.1016/j.arabjc.2013.09.015

Sarer, E., and Kökdil, G. (1991). Constituents of the Essential Oil from Melissa officinalis. Planta Medica, 57(1), 89–90. https://doi.org/10.1055/s-2006-960032

Schimke, R. T., Kaufman, R. J., Alt, F. W., and Kellems, R. F. (1978). Gene amplification and drug resistance in cultured murine cells. Science, 202(4372), 1051–1055. https://doi.org/10.1126/science.715457

Schippmann, U., Leaman, D., and Cunningham, A. B. (2006). A comparison of cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. Frontis, 75–95.

Scott, W. J., Howington, J., Feigenberg, S., Movsas, B., and Pisters, K. (2007). Treatment of Non-small Cell Lung Cancer Stage I and Stage II. Chest, 132(3), 234S-242S. https://doi.org/10.1378/chest.07-1378

Shakeri, A., Sahebkar, A., and Javadi, B. (2016). Melissa officinalis L. – A review of its traditional uses, phytochemistry and pharmacology. Journal of Ethnopharmacology, 188, 204–228. https://doi.org/10.1016/j.jep.2016.05.010

Shanker, M., Willcutts, D., Roth, J. A., and Ramesh, R. (2010). Drug resistance in lung cancer. Lung Cancer: Targets and Therapy, 1, 23–36.

Sharma, S. V., Bell, D. W., Settleman, J., and Haber, D. A. (2007). Epidermal growth factor receptor mutations in lung cancer. Nature Reviews. Cancer, 7(3), 169–181. https://doi.org/10.1038/nrc2088

Shirzad, M., Beshkar, P., and Heidarian, E. (n.d.). The effects of hesperetin on apoptosis induction and inhibition of cell proliferation in the prostate cancer PC3 cells. 4.

Shukla, S., Chen, Z.-S., and Ambudkar, S. V. (2012). Tyrosine kinase inhibitors as modulators of ABC transporter-mediated drug resistance. Drug Resistance Updates: Reviews and

Commentaries in Antimicrobial and Anticancer Chemotherapy, 15(1–2), 70–80. https://doi.org/10.1016/j.drup.2012.01.005

Singh, M., and Jadhav, H. R. (2018). Targeting non-small cell lung cancer with small-molecule EGFR tyrosine kinase inhibitors. Drug Discovery Today, 23(3), 745–753. https://doi.org/10.1016/j.drudis.2017.10.004

Singh, S., Batish, D., Kohli, R. K., and Singh, H. P. (2015). Singh, H.P. An Evaluation of the Antioxidant Properties of Some Oil Yielding Lamiaceous Plants from Morni Hills (Haryana, India).

Skotti, E., Sotiropoulou, N. S., Lappa, I., Kaiafa, M., Tsitsigiannis, D., and Tarantilis, P. (2019). Screening of Lemon Balm Extracts for Anti-Aflatoxigenic, Antioxidant and Other Biological Activities. https://doi.org/10.20944/preprints201907.0005.v1

Sodani, K., Patel, A., Kathawala, R. J., and Chen, Z.-S. (2012). Multidrug resistance associated proteins in multidrug resistance. Chinese Journal of Cancer, 31(2), 58–72. https://doi.org/10.5732/cjc.011.10329

Sofowora, A., Ogunbodede, E., and Onayade, A. (2013). The Role and Place of Medicinal Plants in the Strategies for Disease Prevention. African Journal of Traditional, Complementary, and Alternative Medicines, 10(5), 210–229.

Sosa Iglesias, V., Giuranno, L., Dubois, L. J., Theys, J., and Vooijs, M. (2018). Drug Resistance in Non-Small Cell Lung Cancer: A Potential for NOTCH Targeting? Frontiers in Oncology, 8. https://doi.org/10.3389/fonc.2018.00267

Sridhar, S. S., Seymour, L., and Shepherd, F. A. (2003). Inhibitors of epidermal-growth-factor receptors: A review of clinical research with a focus on non-small-cell lung cancer. The Lancet. Oncology, 4(7), 397–406. https://doi.org/10.1016/s1470-2045(03)01137-9

Stadler, M., Walter, S., Walzl, A., Kramer, N., Unger, C., Scherzer, M., … Dolznig, H. (2015). Increased complexity in carcinomas: Analyzing and modeling the interaction of human cancer cells with their microenvironment. Seminars in Cancer Biology, 35, 107–124. https://doi.org/10.1016/j.semcancer.2015.08.007

Tan, C.-S., Gilligan, D., and Pacey, S. (2015). Treatment approaches for EGFR-inhibitor-resistant patients with non-small-cell lung cancer. The Lancet. Oncology, 16(9), e447–e459. https://doi.org/10.1016/S1470-2045(15)00246-6

Teng, Y.-N., Wang, C. C. N., Liao, W.-C., Lan, Y.-H., and Hung, C.-C. (2020). Caffeic Acid Attenuates Multi-Drug Resistance in Cancer Cells by Inhibiting Efflux Function of Human P-glycoprotein. Molecules (Basel, Switzerland), 25(2). https://doi.org/10.3390/molecules25020247

Thirumaran, R., Prendergast, G. C., and Gilman, P. B. (2007). Cytotoxic Chemotherapy in Clinical Treatment of Cancer. In Cancer Immunotherapy (pp. 101–116). Elsevier. https://doi.org/10.1016/B978-012372551-6/50071-7

Thirumoorthy, N., Manisenthil Kumar, K., Shyam Sundar, A., Panayappan, L., and Chatterjee, M. (2007). Metallothionein: An overview. World Journal of Gastroenterology : WJG, 13(7), 993–996. https://doi.org/10.3748/wjg.v13.i7.993

Tong, C. W. S., Wu, W. K. K., Loong, H. H. F., Cho, W. C. S., and To, K. K. W. (2017). Drug combination approach to overcome resistance to EGFR tyrosine kinase inhibitors in lung cancer. Cancer Letters, 405, 100–110. https://doi.org/10.1016/j.canlet.2017.07.023

Travis, W. D., Brambilla, E., Nicholson, A. G., Yatabe, Y., Austin, J. H. M., Beasley, M. B., … WHO Panel. (2015). The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification. Journal of Thoracic Oncology: Official Publication of the International Association for the Study of Lung Cancer, 10(9), 1243–1260. https://doi.org/10.1097/JTO.0000000000000630

Tsao, A. S., Kim, E. S., and Hong, W. K. (2004). Chemoprevention of cancer. CA: A Cancer Journal for Clinicians, 54(3), 150–180. https://doi.org/10.3322/canjclin.54.3.150

Tsvetkova, E., and Goss, G. D. (2012). Drug resistance and its significance for treatment decisions in non-small-cell lung cancer. Current Oncology, 19(Suppl 1), S45–S51. https://doi.org/10.3747/co.19.1113

Velmurugan, B. K., Lin, J.-T., Mahalakshmi, B., Chuang, Y.-C., Lin, C.-C., Lo, Y.-S., … Chen, M.-K. (2020). Luteolin-7-O-Glucoside Inhibits Oral Cancer Cell Migration and Invasion by Regulating Matrix Metalloproteinase-2 Expression and Extracellular Signal-Regulated Kinase Pathway. Biomolecules, 10(4). https://doi.org/10.3390/biom10040502

Wang, P., Yang, H. L., Yang, Y. J., Wang, L., and Lee, S. C. (2015). Overcome Cancer Cell Drug Resistance Using Natural Products. Evidence-Based Complementary and Alternative Medicine, 2015, e767136. https://doi.org/10.1155/2015/767136

Wang, X., Zhang, H., and Chen, X. (2019). Drug resistance and combating drug resistance in cancer. Cancer Drug Resistance, 2, 141–160. https://doi.org/10.20517/cdr.2019.10

Wangari-Talbot, J., and Hopper-Borge, E. (2013a). Drug Resistance Mechanisms in Non-Small Cell Lung Carcinoma. Journal of Cancer Research Updates, 2(4), 265–282. https://doi.org/10.6000/1929-2279.2013.02.04.5

Wangari-Talbot, J., and Hopper-Borge, E. (2013b). Drug Resistance Mechanisms in Non-Small Cell Lung Carcinoma. Journal of Cancer Research Updates, 2(4), 265–282. https://doi.org/10.6000/1929-2279.2013.02.04.5

Weidner, C., Rousseau, M., Plauth, A., Wowro, S. J., Fischer, C., Abdel-Aziz, H., and Sauer, S. (2015). Melissa officinalis extract induces apoptosis and inhibits proliferation in colon cancer cells through formation of reactive oxygen species. Phytomedicine, 22(2), 262–270. https://doi.org/10.1016/j.phymed.2014.12.008

Wendt, M. K., Balanis, N., Carlin, C. R., and Schiemann, W. P. (2014). STAT3 and epithelial–mesenchymal transitions in carcinomas. JAK-STAT, 3(2). https://doi.org/10.4161/jkst.28975

WHO. (2020). Cancer today. Retrieved 11 May 2021, from http://gco.iarc.fr/today/home

Xiao, D., and He, J. (2010). Epithelial mesenchymal transition and lung cancer. Journal of Thoracic Disease, 2(3), 154–159. https://doi.org/10.3978/j.issn.2072-1439.2010.02.03.7

Xie, M., He, C.-S., Wei, S.-H., and Zhang, L. (2013). Notch-1 contributes to epidermal growth factor receptor tyrosine kinase inhibitor acquired resistance in non-small cell lung cancer in vitro and in vivo. European Journal of Cancer (Oxford, England: 1990), 49(16), 3559–3572. https://doi.org/10.1016/j.ejca.2013.07.007

Xu, Y., Jiang, Z., Ji, G., and Liu, J. (2010). Inhibition of bone metastasis from breast carcinoma by rosmarinic acid. Planta Medica, 76(10), 956–962. https://doi.org/10.1055/s-0029-1240893

Xu, Y., Xu, G., Liu, L., Xu, D., and Liu, J. (2010). Anti-invasion effect of rosmarinic acid via the extracellular signal-regulated kinase and oxidation-reduction pathway in Ls174-T cells. Journal of Cellular Biochemistry, 111(2), 370–379. https://doi.org/10.1002/jcb.22708

Yao, X., Jiang, W., Yu, D., and Yan, Z. (2019). Luteolin inhibits proliferation and induces apoptosis of human melanoma cells in vivo and in vitro by suppressing MMP-2 and MMP-9 through the PI3K/AKT pathway. Food & Function, 10(2), 703–712. https://doi.org/10.1039/c8fo02013b

Zappa, C., and Mousa, S. A. (2016). Non-small cell lung cancer: Current treatment and future advances. Translational Lung Cancer Research, 5(3), 288–300. https://doi.org/10.21037/tlcr.2016.06.07

Zhang, D., Yang, R., Wang, S., and Dong, Z. (2014). Paclitaxel: New uses for an old drug. Drug Design, Development and Therapy, 8, 279–284. https://doi.org/10.2147/DDDT.S56801

Zhao, Z., Jin, G., Ge, Y., and Guo, Z. (2019). Naringenin inhibits migration of breast cancer cells via inflammatory and apoptosis cell signaling pathways. Inflammopharmacology, 27(5), 1021–1036. https://doi.org/10.1007/s10787-018-00556-3

Zhou, Z., Tang, M., Liu, Y., Zhang, Z., Lu, R., and Lu, J. (2017). Apigenin inhibits cell proliferation, migration, and invasion by targeting Akt in the A549 human lung cancer cell line. Anti-Cancer Drugs, 28(4), 446–456. https://doi.org/10.1097/CAD.0000000000000479