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MASTER OF SCIENCE (MSc) DEGREE THESIS

**EFFECT OF OLEUROPEIN ADMINISTRATION ON miR-146a-5p
EXPRESSION LEVEL IN MICE WITH EHRLICH ASCITES TUMOR**

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ÖZET

Yüksek Lisans Tezi

EHRLICH ASİT TÜMÖRLÜ FARELERE OLEUROPEİN UYGULAMASININ miR-146a-5p EKSPRESYON SEVİYESİNE ETKİSİ

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Kanser, her yıl dünya çapında yaklaşık 8.2 milyon ölüme neden olmaktadır. Meme kanseri, dünyada özellikle kadınlar arasında en sık görülen kanser türüdür. Kanser araştırmalarında *in vitro* hücre modelleri ve *in vivo* tümör modelleri sıklıkla kullanılmaktadır. Bu modeller arasında Ehrlich asit tümörü (EAT) yaygın olarak kullanılan sıvı bir tümör modelidir. Polifenoller meyvelerde bulunan ve insan beslenmesinde önemli olan antioksidanlardır. Oleuropein (OL), zeytin ve zeytinyağına acı tat veren fenolik bir bileşiktir. Bu bileşik, antioksidan, vazodilatör, antiaritmik, antispazmodik, hipotansif ve antimikrobiyal özelliklere sahiptir. Bu araştırmanın amacı, EAT taşıyan farelere oleuropein uygulamasının, EAT hücrelerinde miR-146a-5p ekspresyonu üzerindeki etkisini araştırmaktır. Çalışmaya toplam 42 adet BALB/c fare dahil edildi. Tüm farelere EAT hücresi enjekte edilerek ilki kontrol olmak üzere 4 ana deney grubu oluşturuldu. Kontrol grubuna herhangi bir tedavi uygulanmadı. Koruyucu etki grubundaki farelere EAT enjeksiyonundan 7 gün önce OL (150mg/kg/gün) verilmeye başlandı ve toplam 15 gün OL uygulamasına devam edildi. Tümör inisiyasyonu grubunda EAT enjeksiyonu ile aynı gün başlayarak farelere toplam 8 gün OL verildi. Tümör büyümesi üzerindeki etkinin araştırıldığı grupta fareler 3 alt gruba ayrıldı. Birinci alt gruba 6 gün OL, ikinci alt gruba tek doz 5- Fluorourasil (5-FU, 20 mg/kg), üçüncü alt gruba ise bu ikisinin kombinasyonu uygulandı. Meme kanseri tedavisinde kullanılan bir kemoterapötik ajan olan 5-FU, EAT hücrelerinde miR-146a-5p ekspresyonunu anlamlı şekilde arttırdı. 6, 8 ve 15 gün sürelerle OL uygulaması sonucunda miR-146a-5p ekspresyonu, tedavi edilmemiş kontrol grubundaki ile benzer bulundu. Üstelik pozitif kontrol olan 5-FU ile karşılaştırıldığında ekspresyonda anlamlı bir azalış tespit edildi. Elde edilen veriler, (i) miR-146a-5p'nin EAT modelinde tümör baskılayıcı olarak rol oynadığını, (ii) 6, 8 veya 15 gün süreyle tek başına oleuropein uygulamasının bu miRNA'nın ekspresyonunu etkilemediğini, (iii) ancak kombine uygulamada 5-FU'nin etkisini baskıladığını ortaya çıkarmıştır. Bulgularımız, literatürdeki pek çok *in vitro* çalışmanın aksine, OL'in meme kanseri olan bireylerde hastalığın seyrini olumsuz yönde etkileyebileceğini göstermiştir. Sonuç olarak, meme kanseri ve tüm kanserleri önlemek veya tedavi etmek için fitokimyasalların kullanılmasında daha dikkatli olunması gerektiği düşünülmektedir.

ANAHTAR KELİMELER: Ehrlich Asit Tümörü, mikroRNA, Oleuropein, meme kanseri, miR-146a-

ABSTRACT

MSc Thesis

EFFECT OF OLEUROPEIN ADMINISTRATION ON miR-146a-5p EXPRESSION LEVEL IN MICE WITH EHRLICH ASCITES TUMOR

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Cancer causes approximately 8.2 million deaths worldwide each year. Breast cancer is the most common cancer around the globe, particularly among women. In cancer research, in vitro cell models, and in vivo tumor models are frequently used. Among these models, Ehrlich ascites tumor (EAT) is a liquid tumor model that is commonly used. Polyphenols are antioxidants found in fruits and are essential in human nutrition. Oleuropein is a phenolic compound that imparts a bitter flavor to olives and olive oil. This compound has antioxidant, vasodilator, antiarrhythmic, antispasmodic, hypotensive, and antimicrobial properties. The objective of this investigation was to explore the influence of oleuropein administration to EAT-bearing-mice on miR-146a-5p expression in EAT cells. A total of 42 BALB/c mice were included in the study. All mice were injected with EAT cells, and four main experimental groups were formed, the first of which was the control. No treatment was applied to the control group. Mice in the protective effect group were started to give OL (150mg/kg/day) 7 days before the EAT injection, and OL was continued for 15 days. Starting on the same day as the EAT injection in the tumor initiation group, mice were given OL for a total of 8 days. Mice were divided into three subgroups in the group where the effect on tumor growth was investigated. The first subgroup received OL for six days, the second received a single dose of 5-Fluorouracil (5-FU, 20 mg/kg), and the third received a combination of these two. 5-FU, a chemotherapeutic agent used in breast cancer treatment, significantly increased miR-146a-5p expression in EAT cells. As a result of OL application for 6, 8, and 15 days, miR-146a-5p expression was found to be similar to that in the untreated control group. Moreover, a significant decrease in expression was detected compared to the positive control, 5-FU. The data obtained show that (i) miR-146a-5p acts as a tumor suppressor in the EAT model, (ii) oleuropein administration alone for 6, 8, or 15 days does not affect the expression of this miRNA, (iii) but in combination application 5 It has been revealed that it suppresses the effect of FU. Our findings showed that, contrary to many in vitro studies in the literature, OL might adversely affect the course of the disease in individuals with breast cancer. As a result, it is thought that should be more cautious taken in using phytochemicals to prevent or treat breast cancer and all cancers.

KEYWORDS: Ehrlich Ascites Tumor, microRNA, Oleuropein, breast cancer, miR-146a-5p

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LIST OF SYMBOLS and ABBREVIATIONS

5-FU	5-Flourourasil
ALND	Axillary lymph node dissection
ATP	Adenosine triphosphate
EAT	Ehrlich Ascites Tumor
EGF	Epidermal Growth Factor
IARC	International Agency for Research on Cancer
OL	Oleuropein
PET	Positron emission tomography
Rb	Retinoblastoma
ROS	Reactive oxygen species
SLN	Sentinel lymph node
TSG	Tumor suppressor gene
TSTA	Tumor specific transplantation materials

1. INTRODUCTION

Cancer is a leading cause of mortality globally, accounting for more than 10 million deaths per year. Cancer therapies now available include surgery, radiation, and chemotherapy, which can cause harm to healthy cells and toxicity in individuals. As a consequence, researchers are investigating methods that specifically target cancerous cells. The primary hurdle to successful cancer therapy is the heterogeneity of malignant cells inside the tumor. However, better knowledge of the molecular basis of tumors as well as the development of novel diagnostic technologies may benefit the treatment of a variety of malignancies. Thus, it is critical to research epigenetic alterations, cancer cell gene expression, and the use of techniques that enable us to reverse or restrict these changes (Zaimy et al., 2017).

There are 1.38 million new instances of breast cancer each year worldwide, making it the most common disease among women (23 % of all malignancies) (Ferlay, 2008). Modern breast Strategies for cancer therapy include surgery, radiation, adjuvant chemotherapy, and internal secretory therapies; nonetheless, the vast majority of patients with advanced breast cancer do not have a cure at present time. According to recent research uncovered new techniques, biomarkers, and drugs that seem to be beneficial in the therapy of breast cancer (Cazzaniga and Bonanni, 2012). The current study examines the current state of knowledge on natural substances having anti-breast cancer effects. Natural compounds including curcumin, ursolic acid, lycopene, genipin, capsaicin, and oleuropein are all being investigated for their anti-breast cancer activity. These natural substances are anti-inflammatory, anti-metastatic, and anti-cancer (Conney et al.,1991).

MicroRNAs have stayed intact since their discovery in (Lee et al.,1993). Studies in eukaryotes have demonstrated that microRNAs play a vital role in controlling gene expression throughout a wide range of biological events. As a consequence, misexpression of microRNAs may have an influence on target gene expression, changing normal cell processes and resulting to a number of different conditions, including cancer. MicroRNAs have been shown to exist in cancer, namely in chronic

lymphocytic leukemia. According to the study, MicroRNAs have a role in the start and advancement of several cancers, most notably breast cancer (Bhaskaran and Mohan, 2014).

Bioactive chemicals produced from natural products serve an essential role in the development of innovative anticancer treatments by acting as crucial leads (Bishayee and Sethi, 2016). These naturally occurring chemicals operate on a variety of targets, some of these are growth factor receptors; transcription factors; cytokines; adhesion molecules; (also known as growth factors), and inflammatory enzymes (Shanmugam et al., 2018). Numerous epidemiological investigations have been conducted already shown that olive oil intake has anti-cancer qualities and may greatly minimize the risk of contracting cancer (Pelucchi et al., 2010). Previous research has also shown that consuming a lot of olive oil lowers the risk of breast cancer (Psaltopoulou et al., 2011). The most frequent phenolic component in olives is oleuropein, which is the glucosidic ester of hydroxytyrosol and elenolic acid. Both in vivo and in vitro investigations have shown oleuropein to be an effective anticancer drug with high specificity (Shamshoum et al., 2017). Oleuropein was discovered to suppress the multiplication of breast cancer cells in another investigation (Han et al., 2009). However, the susceptibility of various tumor cell types to the same dose of oleuropein can vary dramatically (Elamin et al., 2013).

In past years, there has been a growing recognition for the significance of post-transcriptional gene regulation in the initiation and development of cancer. While transcriptional activation and repression have a significant influence on the amount of messenger RNA (mRNA) in cells, it is eventually up to post-transcriptional processes to determine whether or not they are translated into protein. These include RNA-binding proteins' control of RNA stability and microRNAs' negative regulation of microRNAs (miRNAs). miRNAs are a kind of noncoding RNA distinguished by their short length, generally 18–22 nucleotides. Experts believe that miRNAs control the vast majority of protein-coding genes (Friedman et al., 2009). Numerous studies on miR-146a's role in inflammation and immune function have been published. Breast cancer has the highest number of papers demonstrating a function for miR-146a.

Additionally, it is the malignancy with the most contradictory evidence about the molecular role of miRNAs (Labbaye and Testa, 2012).

Additionally, some evidence indicates that miR-146a has oncogenic properties in breast cancer cells. In mammary epithelial cells, BRCA1 is a critical tumor suppressor, was found in 2011 by spectacular research (Roy et al., 2011). In these cells, miR-146a acts as a direct target. After transfection of cell lines with synthetic miR-146a, BRCA1 expression was lowered, which was associated with increased cell proliferation and a decreased homologous recombination rate. miR-146a expression was found to be greater in basal-like and triple negative breast cancer cells. (Garcia et al., 2011).

The purpose of this research was to determine how oleuropein influences the mir146a-5p expression on Ehrlich Ascites Tumor cells in mice.

2. LITERATURE REVIEW

2.1. What is Cancer

Cancer encompasses a wide range of conditions that occurs when cellular alterations promote unregulated cell growth and division. Some kinds of cancers cause cells to grow and divide quickly. Most cells in the body have defined functions and lifespans. In addition, cell death is actually a natural and healthy process termed apoptosis. Cancerous cells can create tumors, immune system dysfunction, and other alterations that prevent the body from functioning correctly. Cancerous cells could begin in one location and spread throughout the lymph nodes. (Rachel, 2020).

2.2. Hallmark of Cancer

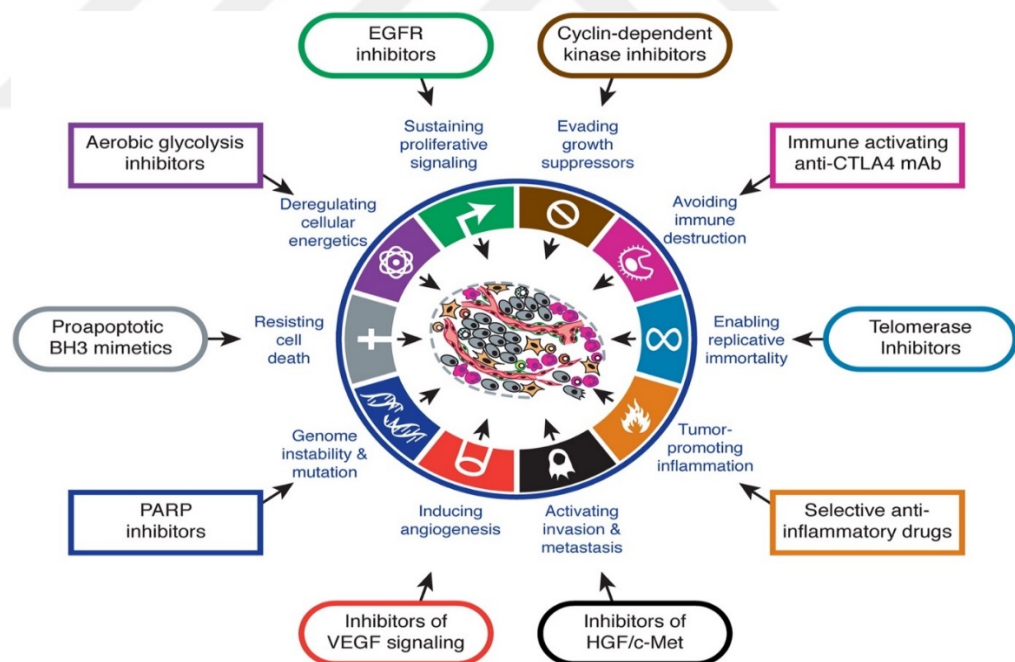


Figure 2.1. Hallmarks of cancer (hanhan and Weinberg, 2011)

2.2.1. Hallmark 1: sustaining proliferative signaling

Cancer is defined by persistent, aberrant cell proliferation, which is caused by cellular restriction networks that coordinate (transitory) cell proliferation throughout the body during embryonic development, physiological growth, and tissue balance maintenance. Signals (inductive and repressive) govern cell division and proliferation. As a consequence, Female daughter cells are generated by a series of complicated inductive signals that drive the cell growth and division cycle from the beginning to the end. In the case of cancer, such stimulatory impulses are triggered, and, unlike in other contexts, where proliferative signalling is transient, the signals persist throughout time. The most well-known and infrequently applied approach for maintaining proliferative signals is the mutation of genes inside cancer cells, which converts these genes into active cell proliferation drivers. Oncogenes are activated genes that contribute to the persistence of previously transient proliferation-promoting signals. Oncogenes are typically cypher proteins that are structurally, functionally, or abundantly different from their traditional cellular counterparts, and They receive proliferative signals from living things and transmit them to the cell's restricted circuits. For example, the nucleolar cell division machinery receives growth-stimulatory signals from the EGF receptor and downstream signal transducers in the KRAS – RAF – MKO – MAPK pathway. EGFR and related receptors, aminoalkanoic acid kinases such as HER2 and ALK, and downstream signal transducers such as KRAS, BRAF, and MEK are all chronically activated in many kinds of human cancer. The activation of this major mitogenic pathway in cancer cells is not dependent on genetic alterations that occur throughout the illness. There may be growth-promoting signals in cancer cells if epigenetic modification of autocrine and paracrine communication pathways occurs without underlying physiological changes (Hanhan and Weinberg., 2011).

2.2.2. Hallmark 2: evading growth suppressors

Proliferative impulses are countered by braking systems in normal cells that override or fully block cell division. These supermolecules are deciphered by TSCs (TSGs). The most common inhibitors of cell growth and division include malignant

tumor protein (pRb) and other "cyclin-dependent" kinases. Controlling the physiology of tissues and coordinating their erratic growth This molecular brake mechanism is regularly controlled by extracellular pro- and antigrowth signals provided by cell surface receptors. They also serve as biosensors for the cell's health. The p53 supermolecule's internal monitoring mechanism ensures that cells grow and divide only when physiologically permitted. This leads p53 to classify unrepaired order damage as serious physiological dysregulations that hinder normal DNA duplication and cell division. Cell stress triggers P53 to activate cell-cycle inhibitors. Instead of preventing cell proliferation, p53 and its partners induce programmed cell death when serious genetic damage or physiological aberrations occur. Many TSGs in the Rb and p53 pathways are lost function due to deletion or intragenic mutations; alternative mechanisms could achieve the same result by silencing the expression of these genes via epigenetic mechanisms, particularly those involving desoxyribonucleic acid and simple protein methylation. As a result, roughly 40% of all human cancers contain p53 mutations, and many tumors with wild-type p53 have genetic or epigenetic changes that impair p53 signaling. The majority of human malignancies are produced by genetic or epigenetic changes in the Rb and p53 tumor-suppressor pathways. TSGs in these pathways have been firmly established as significant inhibitors of cancer occurrence in both neoplastic cells and mouse models of tumor start, development, and progression. As a result, growth suppressor evasion is obviously distinct, and cancer cell proliferation is essential. As a result, the brake mechanisms that ordinarily limit cell growth to preserve tissue homeostasis are unaffected (Hanahan and Weinberg, 2011).

2.2.3. Hallmark 3: resisting cell death

The mechanisms that detect abnormalities in cancer cells may be altered, inhibiting correct signaling and activation of programmed cell death. Cancer cells may potentially induce abnormalities in downstream signaling or apoptosis-related proteins, preventing proper apoptosis. Apoptosis is also important in Hallmark Evading Growth Suppressors; however, this is apoptosis that is triggered by external cues. This Hallmark refers to intrinsic apoptosis (Elmore, 2017).

2.2.4. Hallmark 4: enabling replicative immortality

Telomeres, located at the ends of chromosomes, maintain track of how many cell generations a cell lineage has gone through, progressively shortening with each biological cycle. Telomeres are composed of thousands of bicycle-built-for-two copies of a particular hexanucleotide DNA sequence that are connected to a specialized collection of DNA-binding proteins and placed at the ends of each chromosome. These protein complexes cooperate to protect body ends from End-to-end fusions generated by blank DNA ends are detected and prevented by DNA repair machinery. After a specific number of end repeats, a tripwire activates, causing cell cycle arrest or apoptosis. The p53 tumor suppressor protein controls this by recognizing DNA damage. Although p53 has antiproliferative properties, cancer cells with geologic process telomeres will continue to grow even if the p53 gene is mutated, but only for a limited length of time. The lack of protective protein caps that shelter the body DNA ends combined with continued telomeric DNA erosion results in end-to-end body fusions, breaking, fusion, and bridge cycles throughout mitosis, as well as karyotypic abnormality, which culminates in necrobiosis rather than cell division. Several fully established cancers circumvent both the proliferative barrier demonstrated by end erosion and the acute mitotic catastrophe produced by telomere malfunction by activating a telomere maintenance and extension mechanism. Telomerase, an enzyme that lengthens telomeres, is expressed throughout this process. They maintain telomere length less often by using an unusual intrachromosomal recombination process. As a consequence, cancer cells acquire the ability to retain their telomeres, overcoming the barrier of unacceptably short telomeres and enabling the infinite replicative capacity, also known as cellular immortality, essential for the continuing proliferation of neoplastic cell populations (Hanhan and Weinberg, 2011).

2.2.5. Hallmark 5: inducing angiogenesis

Due to the vital importance of oxygen and nutrients delivered by the circulatory system for cell function and survival, practically all cells in tissue must be within 100 microns of a capillary artery. Coordinated arterial and parenchymal growth produces this proximity throughout organ development. Angiogenesis is the process by which

new blood vessels are produced. It is a straightforward and exact process. Due to this need on nearby capillaries, it is reasonable to presume that tissue cell growth may produce blood vessels on its own. However, the evidence points in the other direction. At initially, cells within aberrant proliferative lesions lack angiogenic capacity, severely reducing their growth potential. To get larger, the neoplasm's early stages must contain angiogenic potential (Bouck, 1996).

2.2.6. Hallmark 6: activating invasion and metastasis

The majority of human cancers produce pioneer cells, which spread, infiltrate surrounding tissue, and ultimately travel to distant regions in search of new colonies. 90% of all cancer fatalities are caused by metastasis, these vast clumps of cancer cells. Cancer cells that have the capacity to invade and metastasis may leave the main tumor and colonize new territory in the body, where nutrients and space are not constrained, at least initially. Cancer cells and normal host tissue support cells are recruited to produce newly developed metastases. For efficient invasion and metastasis, the five unique talents acquired, as well as the development of the initial tumor mass, are essential. Invasion and metastasis are very complicated processes that have unknown genetic and pharmacological origins. These are mechanistically similar behaviors, which supports their designation as cancer cells' only general competence. Both scenarios take advantage of alterations in cells' physical connection to their surroundings as well as the activation of extracellular proteases (Sporn, 1996).

2.2.7. Hallmark 7: deregulating cellular energetics and metabolism

Even in the presence of chemical elements that normally favour aerophilic phosphorylation, Otto Warburg discovered nearly 92 years ago that bound cultured cancer cells have a greater uptake of glucose, which is metabolized via metastasis, establishing the concept that cancer cells alter their energy utilization, particularly glucose, to support proliferation. Strange because glycolysis produces inefficient ATP, the basic energy currency of living organisms. It is currently believed that in addition to ATP, Warburg's "aerobic glycolysis" produces some of the macromolecules essential for cell growth and division. Cancer cells have a metabolism similar to that

of rapidly reproducing normal cells. A cancer malignancy's glycolysis rate might change depending on the time, subregion, and tissue microenvironment the cancer is in. PET scans with radiolabelled analog tracers are often used to assess aerobic glycolysis. As glucose absorption is elevated in glycolytic malignancies, PET using [18 F]-fluorodeoxyglucose is widely employed to scan them. A crucial bloodborne source of energy and precursor to lipids and amino acids, amino acids are replacing glucose in many cancer cells. When it comes to providing energy and nutrients to cancer cells during growth and proliferation, glutamine typically complements and increases glucose (Daye and Wellen, 2012).

T-nursing may function as a third metabolic energy source. Lactate, formerly thought to be a harmful consequence of aerobic and anaerobic glycolysis, is now shown to promote tumor growth. Some cancer cells, particularly those with aldohexose insufficiency, routinely absorb wet nurses and use them to build ATP and biomaterials. Some cancer-associated fibroblasts may wet-nurse. Because of this, metabolic symbioses between glucose-exporting/glucose-importing cells are becoming more common (Dhup et al., 2012).

As the last point, we'd want to emphasize that this hallmark's restrictive mechanisms are still being debated, as is whether or not it is congruently regulated by the other hallmark qualities. [18 F] PET FDG is utilized to scan glycolytic cancers because it increases glucose absorption and expression of aldohexose transporters. A crucial bloodborne source of energy and precursor to lipids and amino acids, amino acids are replacing glucose in many cancer cells. When it comes to providing energy and nutrients to cancer cells during growth and proliferation, glutamine typically complements and increases glucose (Ward and Thompson., 2012).

2.2.8. Hallmark 8: avoiding immune destruction

The eight features have been brewing for decades. Originally intended to teach people how to avoid system-wide active monitoring, which would otherwise kill premalignant cells. Oncogenic virus-induced human malignancies and very small tumours in mice models have shown immune monitoring of early cancer as a barrier

to neoplastic growth. As a result of this tolerance, the overwhelming majority of self-antigens generated by *ad libitum* growing cancer cells are disregarded. Antigens derived from genetic mutations, such as embryonic antigens, are valued by certain cancer cells and have been the subject of research into effective tumor resistance. Oncogenic viruses produce immune responses that destroy virus-infected precancer cells, eradicating early neoplasia. Tumor viruses may have evolved immune-evasive traits that may be chosen by virus-transformed cancer cells, as shown by the ability of virus-transformed cancer cells to evade immunological elimination. But increased cancer rates among immune compromised people, such organ transplant recipients and AIDS patients, imply the immune system is important in preventing virus-induced cancer. Even though immunodeficiency does not increase the incidence of non-virus-induced human cancers, implying that immune surveillance of early neoplasia is lacking in the remaining 80% of human cancers, evidence suggests that certain tumor types should manage immune recognition and attack during later stages of tumor progression and thus acquire immune-evasive strategies. Human tumor tissue histopathology revealed possible drug assault and evasion involvement. Surgery-removed large intestine carcinomas with dense infiltrating cytotoxic T lymphocytes showed a considerably better prognosis than those with less dense infiltrating CTLs (Fridman et al., 2012).

2.3. Breast Cancer

It is a kind of cancer that develops in breast tissue and spreads throughout the body. Breast cancer symptoms include a lump, a change in the form of the breasts, dimpling of the skin, and an unsuitable nipple, a nipple that has been inverted, or a red area of skin. Disease disseminated from afar causes bone pain, swelling body fluid nodes, shortness of breath, and yellow complexion. Being a woman, obesity, physical activity, alcohol, internal secretion replacement medical aid throughout menopause, ionizing radiation, possessing children later in life or not at all, being older, have a history of breast cancer, and having a case history of cancer are all variables that increase one's chance of developing breast cancer. In approximately 5–10% of cases, a genetic predisposition of BRCA1 and BRCA2 which is passed down from one's parents is to blame. Breast cancer is now most often seen in the cells that line milk

ducts and, as a result, in the lobules that provide milk to these channels. An age range of fifty to seventy-four should be tested every two years, according to the women's organization. In order to keep breast cancer at bay in women at high risk, two frequent treatments are estrogen antagonists and raloxifene. Surgical removal of both breasts is an option for women who are very self-conscious about their appearance. There are a variety of cancer treatments available, including surgical resections and various forms of radiation and chemotherapy administered intravenously or intravenously (Frank et al., 2001).

2.3.1. Etiology and epidemiology

Breast cancer is a disease with numerous traits, consequences, and variances. Contrary to popular belief, most women who get breast cancer have no known predisposing factors such BRCA1 or BRCA2 gene mutations, despite their significant and well-documented impact.

Only 5–10% of all breast tumors are caused by genetic alterations with a significant penetrance. Age, family history, fertility, and secretory variables (early menarche, late pregnancy, few pregnancies with brief or no lactation periods, and later menopause), all of which have been linked to an increased risk of breast cancer in women (Chang-Claude, 2001).

2.3.2. Reproductive factors and hormones

The childbearing years of a woman are estimated by subtracting her menarche age from her menopause age. Throughout this time, the ovary generates steroid hormones that impact the development and appearance of the breasts (Collaborative Group on Hormonal Factors in Breast Cancer, 2012). a potential source of hazard in premenopausal and postmenopausal women is increased by long-term stimulation of high endogenous estrogen levels. and related pathways that are implicated in breast cancer risk hazard. a breast cancer Even though ER/PR tumors may have non-hormonal origins, cancers of the good grade progesterone receptor (ER/PR) are more common (Hamajima et al., 2002).

2.3.3. Age of pregnancy

Late births have been connected to breast cancer, which has been linked to every socioeconomic improvement and more effective contraception (Rosner and Colditz, 1996).

The mother's gestational age and the interval between births appear to have an influence on the degree and timing of the elevated risk associated with the majority of breast cancers, showing that the relationships between reproductive data and breast cancer risk are complex. In addition, the predictive value of breast cancer incidence over the previous two years may have been impacted by changes in reproductive patterns (older age and later births) (Lee et al., 2003).

2.3.4. Breastfeeding

Breastfeeding and the risk of contracting cancer have been contentious issues. Meanwhile, breastfeeding seems to decrease the hazard of developing breast cancer, according to a growing body of data. (Hamajima et al., 2002) Women who have ever breastfed have a 39% lower chance of getting breast cancer than women who have never breastfed. This is based on a meta-analysis of 27 studies that looked at 13,907 breast cancer patients (Zhou et al., 2015).

2.3.5. Exogenous hormones

The International Agency for Research on Cancer (IARC) discovered considerable evidence of the causal impact of combination estrogen-progestin (OC) and estrogen-progestin (OC) menopausal therapies on breast cancer when comparing prescription drugs. as a human carcinogen Women with certain breast, mutations are significantly more likely to develop breast cancer and the majority of ovarian malignancies. Breast cancer is linked to two genes: BRCA1 and BRCA2. One or both of these genes are being replaced as technology advances. Breast cancer has also been related to a mutation in the TP53 gene. Your risks of developing breast cancer rise if you have a close relative who has the disease (Grosse et al., 2009).

2.3.6. Alcohol consumption and tobacco smoking

The International Agency for Research on Cancer (IARC) has identified alcohol usage as a risk factor for breast cancer in females. found a dose-response association between alcohol use and breast cancer risk. The risk of breast cancer was 46 percent higher in women who drank 45 grams or more of alcohol per day than in women who did not drink, and the risk increased by 7.1% for every additional 10 grams consumed daily, regardless of whether the women smoked or not. Drinking more alcohol each day increased the risk of cancer in women by 6.3 percent and in males, by 8.1 percent, during the same period. Due to a lack of data, the International Agency for Research on Cancer (IARC) categorized tobacco smoking as a low-risk agent for breast cancer (Cogliano et al., 2011).

2.4. Therapeutic Strategies for Breast Cancer

More than 90 percent of breast tumors are not metastatic when detected. For patients without metastatic illness, the aims are tumor elimination and recurrence prevention. Breast cancer with stage 1 triple-negative is more likely to survive than cancers with hormone receptor-positive or ERBB2-positive. Locally advanced nonmetastatic breast cancer patients should have surgical resection. The goal of metastatic breast cancer therapy is to extend survival time and relieve symptoms (Adriance et al., 2005).

2.4.1. Principe of therapy

Preventing recurrence of nonmetastatic breast cancer is the main goal of nonmetastatic breast cancer treatment. Localized breast cancer is treated with surgical excision, node of lymph nodes biopsy or removal, and postoperative radiation. After (adjuvant) surgery, or both, systemic therapy may be given before (neoadjuvant). Chemotherapy alone or in conjunction with endocrine treatment (if concurrent HR positive) is the main systemic therapy for triple-negative breast cancer. Metastatic breast cancer treatment aims to prolong life and ease symptoms (Adriance et al., 2005).

2.4.2. Local Therapy for Nonmetastatic Breast Cancer

In recent decades, Breast cancer surgery has made remarkable achievements in terms of limiting the aesthetic and functional consequences of local therapy in the long run. According to decades of study, the traditional treatment is either a total mastectomy or an excision with radiation, providing those clean margins are available. When administered regularly, these two regimens have been proven to be similar in terms of relapse-free and overall survival (1) the presence on mammography of diffuse suspicious microcalcifications. (2) Post-lumpectomy positivity of tumor margins. (3) Diseases that, with the exception of a small number of individuals, cannot be adequately addressed by removing A single strand of breast tissue area. In the following cases, conservative surgery should be avoided: (4) Particular collagen-vascular illnesses, such as scleroderma; and (5) previous breast irradiation (Rinaa et al., 2007).

Apart from breast surgery, axillary node surgery should be prioritized. Dissection of lymph nodes is performed for diagnostic and therapeutic purposes (to ascertain the anatomic extent of breast cancer) (removal of malignant cells) (removal of cancerous cells). Lymph node exclusion at the time of diagnosis, as well as the presence or absence of neoadjuvant systemic treatment, govern surgical options. A sentinel lymph node (SLN) biopsy was performed on all females who had ALND and were then converted to ALND only if the SLN was positive. There was a 5-10% incidence of "false-negative" SLN biopsy in patients of mild disease and surgical as initial therapy, showing that leaving some axillary disease in situ does not significantly damage long-term prognosis (Rinaa et al., 2007).

2.4.3. Systemic Therapy for Metastatic Breast Cancer

In the United States, around 150 000 women have been diagnosed with metastatic breast cancer. Metastatic disease is virtually always the cause of death for every year 41,000 women are diagnosed with breast cancer. Patients with visceral metastases, CNS metastases, and many metastatic sites are likely to have a bad prognosis, whereas those with bone-only metastatic illness are more likely to have a

good prognosis with more time between diagnosis and the development of metastatic recurrence (Seema et al., 2002).

2.5. Natural Compounds for Breast Cancer

Breast cancer is the primary cause of cancer death among women, and it is a serious public health concern around the globe (Jemal et al., 2011).

Due to better detection algorithms, early identification is now feasible. On the other side, those diagnosed with breast cancer are dying at a greater rate. For the great majority of patients with advanced breast cancer, there is currently no treatment. In spite of the wide range of breast options available today. These options include surgery, radiation, adjuvant chemotherapy, and internal secretory therapies. Recent research has revealed innovative approaches, biomarkers, and medications that appear to be efficient in the therapy of breast cancer (Cazzaniga and Bonanni, 2012).

There is an urgent need for breast cancer medicines that diminish the occurrence of the illness in people at high risk. As a result, a new research says that a bar should be built. It is possible to use natural or synthetic chemicals in chemoprevention to delay, diminish or even stop the progression of malignant neoplastic illness. Chemoprevention may be attractive and realistic for advanced breast cancer patients. Frequent development of natural prescription drugs is vital for lowering unwanted effects, boosting selectivity, decreasing toxicity, and increasing neoplastic cell death (Khazaei et al., 2015).

2.5.1. Sauchinone

In MCF-7 human cancer cells, *Saururus Chinensis*. has antitumorigenic action. SC inhibits angiogenesis, cell proliferation, and anti-apoptotic activity by inhibiting the production of VEGF, cyclin D1, and Bcl-2 factor products (Kim et al., 2019).

SC activates caspase-3 in breast cancer cell lines, which is necessary for apoptosis produced by SC and may be employed as a chemotherapeutic agent. SC is mostly comprised of sauchinone, a compound derived from the source. Auchinone is an antipyretic, diuretic, and anti-inflammatory agent. Sauchinone has been used in

traditional Korean medicine for millennia to treat jaundice, edema, fever, and inflammatory illnesses (Chung and Shin, 1990).

Sauchinone has been shown to decrease mortality rates and halt bone deterioration. It inhibits cancer cell spread by inhibiting VEGF, cyclin D1, Bcl-2, caspase-3, and the ERK signaling pathway (Bae et al., 2010).

NF-B activity causes anti-apoptotic proteins to be produced via the ERK signaling pathway, which causes enhanced cell proclivity and tumor development (Escárcega et al., 2006).

TNF production, the ERK pathway, and related NF-B activation have all been linked to the use of sauchinone as a pharmaceutical flavoring agent (Bae et al., 2010).

2.5.2. Lycopene

Tomatoes, like many other fruits and vegetables, contain the antioxidant lycopene (Kucuk et al., 2001).

It has been shown that it inhibits tumor development in a variety of human malignant tumors. Men are at an increased risk of developing two forms of cancer: prostate and breast. In human cartilaginous tube animal tissue cells, lycopene protein metabolites stimulate Nrf2-mediated production of clinical trial detoxifying/antioxidant enzymes (Ono et al., 2015).

Lycopene reduces proliferation in triple-negative breast cancers, which estrogen receptor deficient, lipo-Lutin receptor, and HER2 protein expression. Carotenoid induces apoptosis via decreasing Akt phosphorylation, a protein involved in necrosis and cell survival and a downstream target of mTOR. Carotenoid prevents the Akt/mTOR signaling pathway in triple-negative breast cancer cells, resulting in cell death. (Takeshima et al., 2014)

2.5.3. Ursolic acid

Ursolic acid (UA) is a naturally occurring pentacyclic triterpenoid chemical obtained from plants (Li et al, 2015).

In several human malignancies, its anti-cancer, anti-invasive, (Kim ES, Moon A, 2015) anti-metastatic, (Xiang et al., 2015) anti-proliferation, (Zhang J, et al., 2015). and anti-angiogenesis properties, it also induces apoptosis. Also, ursolic acid aids in the prevention of the beginning of the soft liver disease. Ursolic acid derivatives have recently been created using a variety of cost-effective processes. However, we've chosen to describe facts from the natural molecule clearly here (Yang et al., 2015).

In breast cancer cells from humans. Ursolic acid contains cancer-fighting effects. Apoptotic factors and signaling pathways have been linked to UA (Wang et al., 2012).

Ursolic acid usage induces necrosis and impairs cell growth. FoxM1 is required for cell growth and cell cycle progression, while CyclinD1/CDK4 is involved in cell cycle progression and cancer development. Through FoxM1 regulation, UA-induced cell death decreases cyclinD1/CDK4 expression in MCF-7 human cancer cells (Lamb et al., 2013). Ursolic acid induces programmed cell death by increasing Bax, a pro-apoptosis factor, and decreasing Bcl-2, an anti-apoptotic factor (Kassi et al., 2009).

By releasing cytochrome and inhibiting migration and invasion, ursolic acid promotes apoptosis in a highly pathogenic cancer cell line. A cancerous cell, MDA-MB 231, may be present in the sample. MMP-2 and urokinase-type peptidase expression is inhibited by ursolic acid, which inhibits NF-B and Jun N-terminal kinase. In metastatic breast cancer, ursolic acid is often used as a chemopreventive drug (Kim et al., 2011).

2.6. Oleuropein

Oleuropein is most recognized for its ability to decrease force per unit area. Oleuropein dramatically lowers pulsation and pulsation blood pressure in animal models once given intraperitoneal or endovenous injections. The capability of oleuropein to cut back blood pressure would possibly support the utilization of olive leaf within the treatment of gentle to moderate cardiovascular disease in the past. Our current analysis sheds light-weight on the mechanism through that oleuropein lowers

blood pressure. Oleuropein protects the neural structure from aerophilous stress by increasing mitochondrial activity via the Nrf2-mediated communication pathway, in step with our findings (Sun, 2017).

These effects are shown whether or not oleuropein is taken before or when the beginning of hypertension, indicating that it'd be accustomed each forestall and cure high blood pressure. As well as decreasing blood pressure, studies have shown that oleuropein has a number of other beneficial effects on the human body. These include anti-inflammatory and antioxidant effects as well as anticancer, anti-angiogenic, and neuroprotective effects. To protect elderly rats' neurons against the most common kind of Parkinson's disease neurodegeneration, oleuropein has been shown to be an effective supplement. oleuropein prevents the amyloid beta and tau supermolecules from aggregating, both of which are implicated in the development of Alzheimer's disease (Luccarini, 2014).

Oleuropein may be a powerful substance of human dermal protein receptor 2, a protein that's normally induce the production in breast cancer, cells, that has relevancy to cancer (Fayyaz, 2016).

In mice, it can facilitate forestall large intestine cancer caused by redness by acting as a chemopreventative (Giner E, 2016).

Autophagy and suppression of the class target of rapamycin (mTOR) are involved within the protecting edges of oleuropein, in step with mechanistic studies (Rigacci S, 2015).

Despite the assorted advantages of oleuropein, it's necessary to stay in mind that sure folks could be adversely affected. Oleuropein has been shown to irritate low blood pressure in folks that have already got it. Oleuropein may act with alternative medicine medicines that are accustomed to treating high blood pressure or diabetes. To prevent potentially harmful medication interactions and side effects, patients ought to consult their medical practitioner before usage and keep their physician privy to any medications they're taking. Oleuropein's surprising edges and distinct traits justify its

continuing use and analysis within the Mediterranean diet to boost human health. Future study is required to own a stronger understanding of the cellular networks concerned in the varied protecting effects of oleuropein. carcinoma is fatal owing to distant metastases, that are caused by tumor cells manufacturing Matrix Metalloproteinase (MMP) enzymes, which permit invasion (Kohrmann et al., 2009).

Upregulation of MMPs in tumor cells has been associated with metastasis, and also the expression of MMP members of the family is absolutely connected with tumor growth (Overall CM and Lopez-Otin., 2002). The TIMPS family of tissue inhibitors of metalloproteinases (TIMPS) induces programmed cell death and is down-regulated in a very vary of human neoplastic cell types. TIMP overexpression inhibits malignant melanoma metastasis within the laboratory. TIMP-1 production delays the progression of cancer (BUCK et al., 1999). TIMP-2 may be a supermolecule that promotes cancer growth and metastasis (Stetler-Stevenson and Seo., 2005).

In prostate cells and tumor tissues, it's downregulated (Pulukuri et al., 2007). TIMP-3 overexpression causes apoptosis in a carcinoma cell line. MMP-1, -2, -3, -9 expressions and suppression of TIMP-1, -2 were higher in tumor cells than in inflammatory cells in breast cancer (Baker et al., 2002). oil has been discovered as a potent inducer, promoter, and progressor of a period of time carcinogenesis. European olive tree, the olive tree, produces a good vary of business items. owing to the phenoplast compounds found altogether parts of the olive plant, the medicinal qualities of olives are essential in medicine (Visioli et al., 2002).

Oleuropein is that the most overabundant phenolic part in the dicot genus *Europaea* and has varied pharmacological effects, as well as anti-inflammatory drug capabilities (Visioli et al., 1988). Oleuropein reduces the viability of carcinoma cells (Mendendez et al., 2007). In HER2-overexpressing breast carcinomas, it's a considerable tumoricidal impact (Mendendez, 2008).

Oleuropein (200 g/mL) inhibits cell growth and induces cell death, reducing the viability of MCF-7 cells and reducing the quantity of MCF-7 cells. Human breast carcinoma (MCF-7), human vesica breast cancer, and bovine brain capillary

epithelium cells are all inhibited by crude extracts containing oleuropein because of the main ingredient. the present work was aimed to judge the influence of Oleuropein on the MMPs and TIMPS genes, in search of prospective molecular targets to limit carcinoma metastasis, supported this premise and with the goal of higher understanding (Goulas, 2009).

Oleuropein-containing crude extracts have been proven to prevent the growth of human breast cancer (MCF-7), human vesica carcinoma, and bovine brain capillary epithelial cells in animal studies. Oleuropein was studied to see whether it affected the MMPs and TIMPS genes, in an attempt to find molecular targets for inhibiting cancer spread. Over the last fifteen years, survival rates for women with early breast cancer have constantly improved in the United Kingdom, and thus in the United States, as a result of discoveries in and greater access to early detection, as well as increased efficiency of medical treatment. On the other hand, future advancements will need the creation of novel preventative and treatment options, demanding a bigger data set of human carcinoma's biological science (Peto, 2000).

2.7. Experimental Models for Breast Cancer

2.7.1. Syngeneic and genetically engineered mouse model

The number of animal models available on the market has recently expanded due to the emergence of syngeneic mice neoplasm models (Shi et al., 2001).

However, since mice and their tumors have a biology that is markedly different from that of people and human carcinoma, the greater part extensively used animal models have a limited function in cancer research. The differences between mouse and human biological process programs appear in a variety of ways, with size being one of the most obvious. As a result, the number of cellular targets for growth transformation changes between mouse and human tissues, as does the degree of maturation and differentiation. Pregnancy is required for full organ development in eutherian animals; however, this is not the case in humans. This has important consequences for the existence or absence of robust stem cells, as well as their function in exocrine gland

cancer. Because mice have a shorter life span than humans, detectable malignancies should progress quickly, as mice may acquire exceedingly malignant tumors with several genetic changes in a relatively short period of time (6–18 months). Despite the fact that each species' mutation rates are similar, rodent cells are easier to convert in vitro by cistron transfection or chemical carcinogens. Less efficient polymer repair, poor genetic stability management, and/or altered control of organic phenomena via processes comparable to DNA methylation are all plausible explanations for the faster transition (Holliday., 1996).

Another distinction is immortalization, which might be a critical innovation in cancer progression (Hanahan and Weinberg, 2000).

2.7.2. Xenograft models

Isaacson and Cattanaach demonstrated for the first time many human breast cancer cell types. to develop tumors in immunocompromised mice (Isaacson and Cattanaach,1962). However, because to the intricacy of the treatments necessary to make immunodeficient wild-type mice, which entailed a combined surgical procedure, radiation, and medication therapy, this approach was not often employed until the development of the mutant naked mouse. In graft research, the most often utilized models are naked (Foxn1) mice and severe combined immunological disease (SCID) mice, both of which have single-cistron mutations affecting their immune systems. Nudes have a recessive chromosome eleven mutation that results in hair loss and other abnormalities, as well as dysgenesis of the thymic animal tissue, which results in T-cell Diamond (Kindred, 1971).

A natural mutation inactivates the deoxyribonucleic acid macromolecule enzyme in SCID mice, resulting in a paucity of functional T and B cells (Bosma and Carroli,1991: Nakajima and Bosma, 2002). Different species, such as the rat, have produced immune-deficient strains, although these aren't frequently used. breast cancer, like naked mice and SCID mice, is one of the most difficult malignancies to transplant into experimental animals. Invasive human breast cancer has a documented success rate of 7–20 % (take rates) (Mehta et al., 1993). with variances explained by

implant placement (orthotopic is preferable), animal age and type, and secretory supplementation (nude mice have low steroid levels, compared with humans). Serially transplantable xenografts are substantially scarcer. Surprisingly, samples of preinvasive cancer (Gandhi et al., 2000). According to a recent investigation, subgroups of immunophenotypically different (CD44+/CD24-) cells found inside primary breast neoplasms had a higher percentage of transplant acceptance (Al-Hajj et al., 2003). It wasn't until the early 1990s when directly manufactured breast cancer xenograft lines with the ability to expand were established (Hurst et al., 1993).

Despite the fact that some established in vitro lines are transformed to xenograft cultures before to and after this, a subset of these lines may invade or spread locally, typically as a consequence of later genetic tinkering to produce a more aggressive phenotype. Numerous human transplant models are presently available for carcinoma research, with the majority derived from existing cancer cell lines and conventional breast animal tissue cells that were spontaneously or genetically immortalized. The MCF10AT and MCF-7 systems are among the most often used because to their ease of usage and the wealth of information available on these lines from previous in vitro studies. On the other hand, employing existing cancer lines as xenograft models introduces a variety of complications. Primary breast neoplasm cells are projected to have very distinct environmental needs than culture-adapted cancer cells. In vitro institution is a very uncommon occurrence, occurring in fewer than 1% of first malignancies (O'Hare, 1991).

This will very definitely entail the choosing of an 'establishment' constitution in the future. Thus, cell selection during continuous culture line conversion, alterations in subsequent generations of cell lines, infection by an infectious agent or a true bacterial infection, mislabeling of individual cell lines, and doubts about their true neoplasm source all affect the validity of such models. Primary tumor engraftment, on the other hand, has gotten far less study. According to the current study, athymic mice may be capable of producing histomorphological intact primary human breast lesions and cancers. Unrelated cells from surgical cancer tissues were blended with living thing matrices and implanted into naked mice as part of an experimental model system.

These transplanted cells develop in the same way as their original phenotypic, making them a lot of realistic models for working in vivo on human primary breast lesions and cancers. On the other hand, models produced directly from clinical specimens are limited by their intrinsic characteristics. Xenografts often include fewer stroma, and those that do present are murine-derived, leading in a chimera neoplasm (Yang et al., 2000).

2.7.3. Ehrlich ascites carcinoma

Ehrlich ascites carcinoma [EAC, (Syn: Ehrlich ascites tumor, EAT)] is one of the most well-liked types of experimental cancers used in modeling. EAC is a generally undifferentiated carcinoma that is initially hyperdiploid, has a strong proclivity for migration, lacks regression, proliferates rapidly, has a shorter life expectancy, is 100 percent malignant, and lacks tumor-specific transplanting materials (TSTA). Tumor virulence frequently increases with time as a result of recurring passages, while the pace at which such tumors multiply advances gradually. However, the differentiation diminishes with time as the cells establish free growth management systems, improving hetero-transplant ability, and finally become ascites-like. Because EAC is undifferentiated and grows rapidly, it is similar to human cancers, which are the most treatable. The employment of natural sources as another cancer medical assistance is thought to provide an excessive value for cancer management and program destruction at this time, with ideal medications being ineffective or only somewhat beneficial for traditional cells. A lot of research on mobile malignancies has been done in the last two to a few decades. The study's intended goal was to promote innovative treatments, particularly for experimental malignancies in animals, that were based on recent developments in cancer therapy. EAC is one of the most frequently utilized experimental tumor models. It first manifested itself as spontaneous breast cancer in a very feminine mouse during a highly feminine mouse (Aktaş, 996). Ehrlich and Apolant (1905) utilized the tumor as a model tumor, subcutaneously transferring tumor tissues from mouse to mouse. The liquid type was isolated from the mouse's serous membrane and was dubbed "Ehrlich ascites carcinoma" owing to the pathologic liquid and carcinoma cells. Their study during WWII enabled not only the capture of this tumor but also its conversion into a test system suited for qualitative and

quantitative cancer analysis. When EAC cells were discovered in 1948, they swiftly spread to research facilities all over the world. EAC is a relatively undifferentiated malignant neoplastic illness that is hyperdiploid, has a high mobile capacity, no regression, rapid proliferation, a short life span, and is 100% malignant. It also lacks information on tumor-specific transplantation (TSTA) (Kaleoğlu and İşli, 1977).

Inheritable a tetraploid chromosomal sub-clone, but subsequent years saw research on diploid, hypertetraploid, and tetraploid chromosomes. It was decided to make sub-clones. Colchicine-resistant tumor clones and Sholz clones, as well as polyose (+) and glycogen Ehrlich clones, were obtained (Aktaş, 1996). Because it contains growth cells that proliferate once tumor cells are introduced into the serous membrane cavity, the effusion is called "ascites." As a result of recurring passages, tumor pathogenicity frequently increases with time, albeit the rate at which such tumors multiply slow down. But, when the cells acquire free growth management systems, improve hetero-transplant ability, and finally become ascites-like, differentiation disappears (Kaleoğlu and İşli, 1977). Every 0.1 cc of pathology liquid comprises 10 million neoplastic cells and is gray-white or occasionally includes a light-weight scarlet viscose liquid (Aktaş, 1996; Kaleoğlu and İşli, 1977). Since the creation of the Ehrlich pathologic type, this has been the preferred method in many research. The suspension comprises undifferentiated free tumor cells from the Ehrlich ascites tumor, making it a possible sure quantitative tumor cell transplantable into another mouse (Klein, 1951).

As a consequence, ordinary basic counter techniques may be used to assess not only the tumor cell count but also the rising tumor size (Ekinci, 2000). Because of these objectives, EAC is obtained in two forms: ascites and solid. The ascites form is obtained if the ascites fluid does not include the tumor cell that injects; however, if it does, the solid type is obtained (Okay, 1998; Zeybek, 1996). In vitro, EAC cells grow in suspension inside the mouse body cavity and do not attach to an artificial surface (Aktaş, 1996; Song et al, 1993; Vinuel et al., 1991). The pathologic fluid is prepared 4–6 days after passage, and a total of 7–12 cc ascites fluid is deposited (Gümüştan, 2002).

3. MATERIALS and METHOD

3.1. Materials

Animal cages
 Oleuropein
 5-Fluorouracil
 Gastric gavage canule
 Total RNA isolation kit (Qiagen)
 cDNA conversion kit (Qiagen)
 SYBR Green enzyme mix

3.2. Methods

3.2.1. Animal experiment:

Fourty-two mice were used in the present study. The animals were housed (4 rats/cage) in a standard laboratory condition according to standard animal guide care (12 h light: 12 h dark photoperiod and 22 ± 4 °C). Each mouse had free access to tap water and laboratory food during the experiment. The study was carried out in the Animal Experiment Application and Research Center, Harran University (Sanliurfa, Türkiye) from the period of September to the end of November, 2020. The cancer model was formed by inoculation of 3×10^5 Ehrlich ascites tumor cell line into the mice periton. Some groups of mice were also given Oleuropein or/and balanced salt solution (BSS). The detailed mice groups were divided as mentioned in the following

Table 3.1. The groups that were used for experiments

Groups		Regime	Number of mice
EAT (untreated control)		3×10^5 EAT cells were inoculated intraperitoneally (i.p) on day 0	7
Tumor growth	EAT + OL (6d)	3×10^5 EAT cells were inoculated (i.p.) on day 0 + oleuropein (OL, 150 mg/kg/day) was given orally, day 2-7 (for 6 days)	7

Table 3.1. (Continue)

Tumor initiation	EAT + 5-FU (sd)	3×10 ⁵ EAT cells were inoculated (i.p) on day 0 + 5-fluorouracil (5-FU) was injected into periton on day 2 (single dose)	7
	EAT + 5-FU (sd) + OL (6d)	3×10 ⁵ EAT cells were inoculated (i.p) on day 0 + 5-fluorouracil (5-FU) injected into periton on day 2 (single dose) + OL (150 mg/kg/day) was given orally, day 2-7 (for 6 days)	7
	EAT + OL (8d)	3×10 ⁵ EAT cells were inoculated (i.p.) on day 0 + OL (150 mg/kg/day) was given orally, day 0-7 (for 8 days)	7
	Protective effect EAT + OL (15d)	OL (150 mg/kg/day) was given orally, day 0-14 (for 15 days) + 3×10 ⁵ EAT cells were inoculated (i.p.) on day 7	7

EAT: Ehrlich ascites tumor, OL: Oleuropein, 5-FU: 5-Fluorouracil, i.p.: intraperitoneally, sd: single dose, 6d: six days, 8d: eight days, 15d: fifteen days

3.2.2. Molecular analysis

EAT cells obtained from the peritoneum after decapitation from mice was stored in liquid RNA later solution in liquid nitrogen until the study was performed. Molecular analyzes were carried out in the following steps:

- a. miRNA isolation
- b. cDNA converts
- c. real time PCR (qPCR)

3.2.2.1. miRNA isolation

The miRNeasy mini kit (Qiagen) was used for the isolation of total RNA from homogenized cell samples. The miRNAeasy mini kit isolates total RNA containing miRNAs. For this purpose, the kit procedure steps were applied as follows:

1. Thawing the cell for several second after take 50µl from the cell after centrifuge for 3 minutes in 1500 RPM.
2. Add 700µl QiAzol to the tube (to break all of the part of cell except DNA)
3. Add 140µl chloroform to the tube (for separation RNA face)
4. Centrifuging the tube for 15 minutes in 1400 RPM. After centrifuge collecting the upper phase of the tube

5. Add ethanol 1.5 time more than sample. (To precipitate the miRNA to the bottom of the tube)
6. After centrifuging for 15 second in 10000 RPM.
7. Add 700µl RWT (buffer) to the tube (for cleaning the RNA)
8. Centrifuging for 15 second in 10000RPM.
9. Add 500µl RPE to the tube.
10. Centrifuging for 15 second in 10000 RPM.
11. Repeat step 9, add 500µl RPE to the tube.
12. Centrifuging 2 minute in 10000 RPM.
13. Transfer to new tube after centrifuging for 1 minute in 10000 RPM. (This step is important because we clean all wastes).
14. Transfer tube into Eppendorf tube after add 30-50µl free water.
15. Centrifuging for 1 minute in 10000 RPM.

After isolation miRNA measuring miRNA concentration by using (nano drop spectrophotometer (MultiskanIt spectrophotometer, Thermo). The samples integrity was controlled by agarose gel electrophoresis.

3.2.2.2. cDNA converts

Total RNA was translated into cDNA using the miRCURY LNA II RT(Qiagen), according to kit procedure. First, the mix from kit components was prepared, then the samples and kit components were incubated for 60 minutes at 42°C, and inactivated the Reverse Transcriptase Enzyme by incubating for 5 minutes at 95°C.

3.2.2.3. Real-time PCR

The qPCR analysis was made by using miRCURY LNA SYBR green kit (Qiagen) according to manufacturer's protocol guide. Primer's name was miR-146a-5p, and SNORD 65 was used as housekeeping gene.

4. RESULTS and DISCUSSION

4.1. Results

EAT cells were obtained from Ehrlich ascites tumor-bearing mice given Oleuropein, and then total RNA was isolated as described in Chapter 3.3.1. The amount and quality of total RNAs containing miRNAs were determined and studied with samples with an OD of 260/280 (Optical density) of ~2. The integrity of the samples was checked by agarose gel electrophoresis (Figure 4.1.).

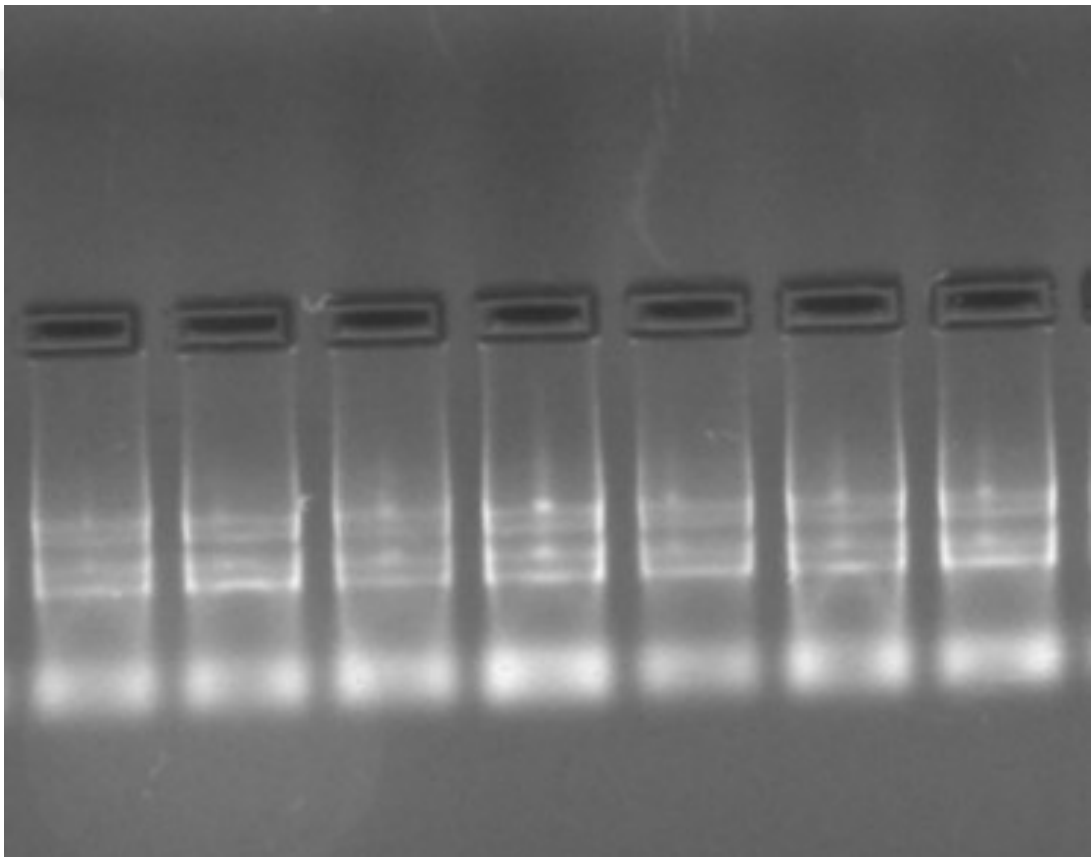


Figure 4.1. Gel image of total RNAs isolated from EAT cell

4.1.1. Evaluation of protective effect

In the group investigated whether oleuropein has a protective effect against breast cancer, OL (150 mg/kg/day) was administered orally by using a gastric gavage canule for a total of 15 days. EAT cell (3×10^5) was injected into the peritoneal region of mice on the 8th day of the schedule and the animals were sacrificed on the 16th day

(as explained in chapter 3). As shown in Table 4.1. and Figure 4.2., it was determined that miR146a-5p expression in EAT cells obtained from this group increased slightly compared to the control group that did not receive any treatment, but the difference was not statistically significant ($p>0.05$).

Table 4.1. The effect of Oleuropein administration for a total of 15 days in the group in which the protective effect of OL was investigated on miR-146a-5p expression in EAT cells

Groups	Relative expression value of miR-146a-5p (FC)
EAT (untreated control)	1.00 ± 0.36
EAT + OL(15d)	1.47 ± 0.26

EAT: Ehrlich ascites tumor, OL: Oleuropein, 15d: fifteen days, FC: fold change.

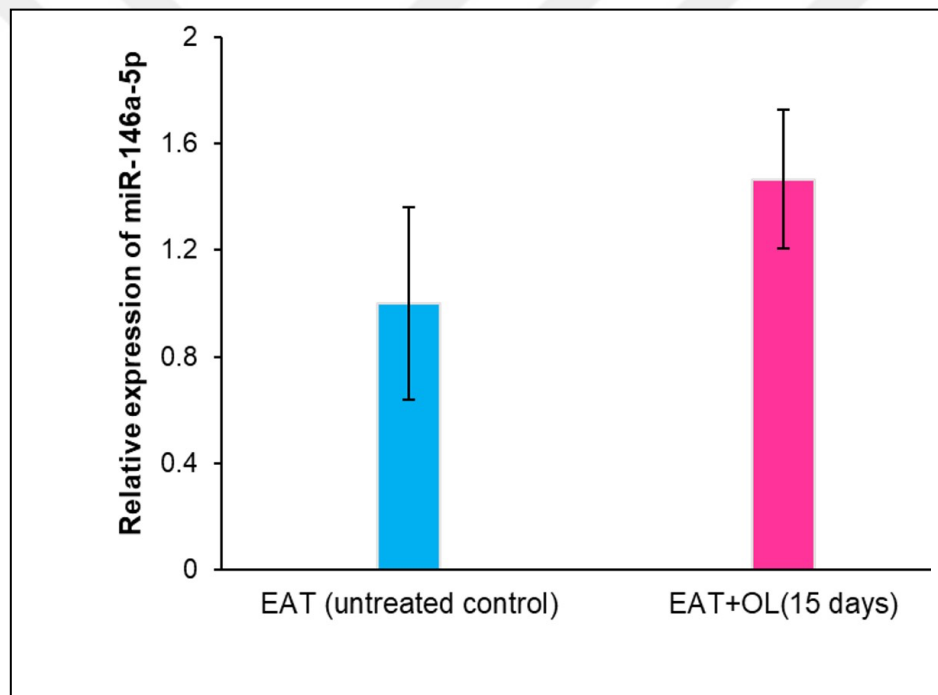


Figure 4.2. The effect of oleuropein administration on miR-146a-5p expression for a total of 15 days in the group in which the protective effect of oleuropein was investigated in the EAT breast cancer model

4.1.2. Effect on tumor initiation

In order to determine the effect of Oleuropein on tumor initiation, animals were given OL (150 mg/kg/day) for a total of 8 days starting on the day of tumor cell injection into the peritoneal region, and animals were sacrificed on the 9th day. It was observed that miR146a-5p expression increased in EAT cells obtained from this group.

However, this increase was not found to be statistically significant ($p>0.05$) when compared to the untreated control (EAT) group (Table 4.2., Figure 4.3.).

Table 4.2. The effect of Oleuropein administration for 8 days starting on the same day as the EAT cell injection intraperitoneally to mice on miR-146a-5p expression in EAT cells

Groups	Relative expression value of miR-146a-5p (FC)
EAT (untreated control)	1.00 ± 0.36
EAT + OL(8d)	1.43 ± 0.15

EAT: Ehrlich ascites tumor, OL: Oleuropein, 8d: eight days, FC: fold change.

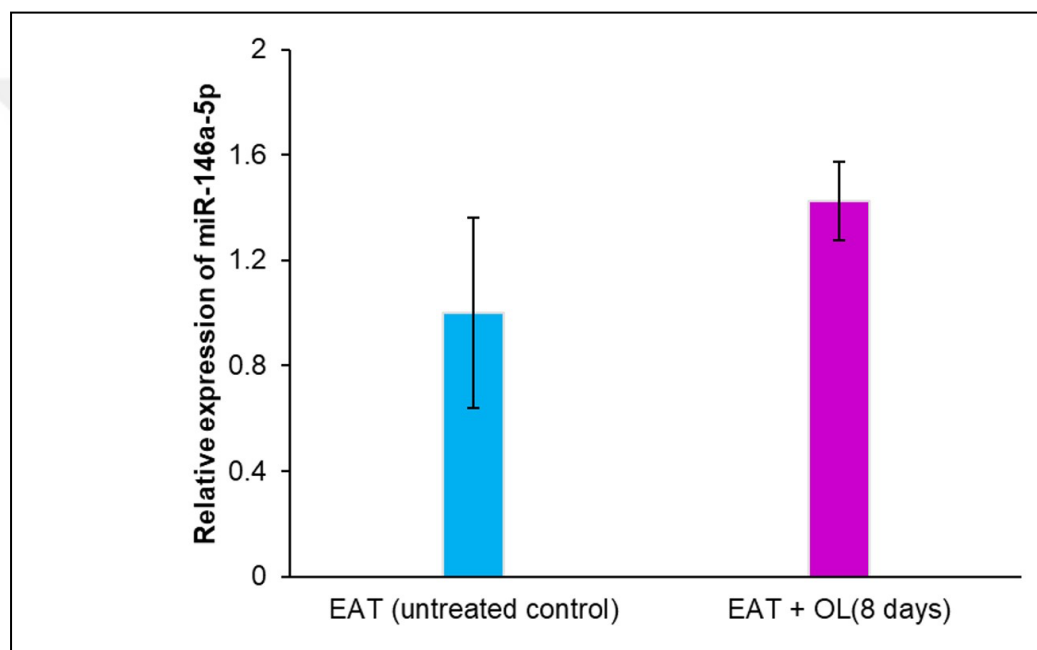


Figure 4.3. The effect of oleuropein administration (150mg/kg/day, 8 days) starting on the same day as the EAT cell injection intraperitoneally on miR-146a-5p expression, in the group in which investigating the effect of oleuropein on tumor initiation

4.1.3. Effect on tumor growth

The values obtained from the OL-treated (150mg/kg/day, for 6 days) groups were compared with the untreated control group (EAT). Accordingly, it was observed that there was no statistically significant change in miR-146a-5p expression between these two groups.

A significant ($p < 0.05$) increase in miR-146a-5p expression was observed in group 5-FU in which 5-Fluorouracil (5FU), a chemotherapeutic agent used for breast cancer treatment, was administered in a single dose (20 mg/kg) (Table 4.3., Figure 4.4.). It was observed a slight increase in miR-146a-5p expression in group 5-FU+OL in which was treated with 5-FU (single dose) and OL (150mg/kg/day, 6 days) compared to the untreated control (EAT). However, this increase was not as high as in group 5-FU. There were statistically significant differences ($p < 0.05$) between group 5-FU and others. Furthermore, the fact that the expression of miR-146a-5p in the group given OL together with 5FU was lower than the group in which 5FU was administered alone indicated that OL might have suppressed the expression of tumor suppressor miR-146a-5p.

Table 4.3. The effect of Oleuropein administration for 6 days after tumor detected on miR-146a-5p expression in EAT cells

Groups	Relative expression value of miR-146a-5p (FC)
EAT (untreated control)	$1.00 \pm 0.36^*$
EAT + OL(6d)	$0.93 \pm 0.18^*$
EAT + 5FU(sd)	4.89 ± 0.27
EAT + 5FU(sd) + OL(6d)	$1.97 \pm 0.15^*$

EAT: Ehrlich ascites tumor, OL: Oleuropein, 5-FU: 5-Fluorouracil, sd: single dose, 6d: six days, FC: fold change.

*: Statistically significant difference ($p < 0.05$) compared to EAT + 5FU(sd) group.

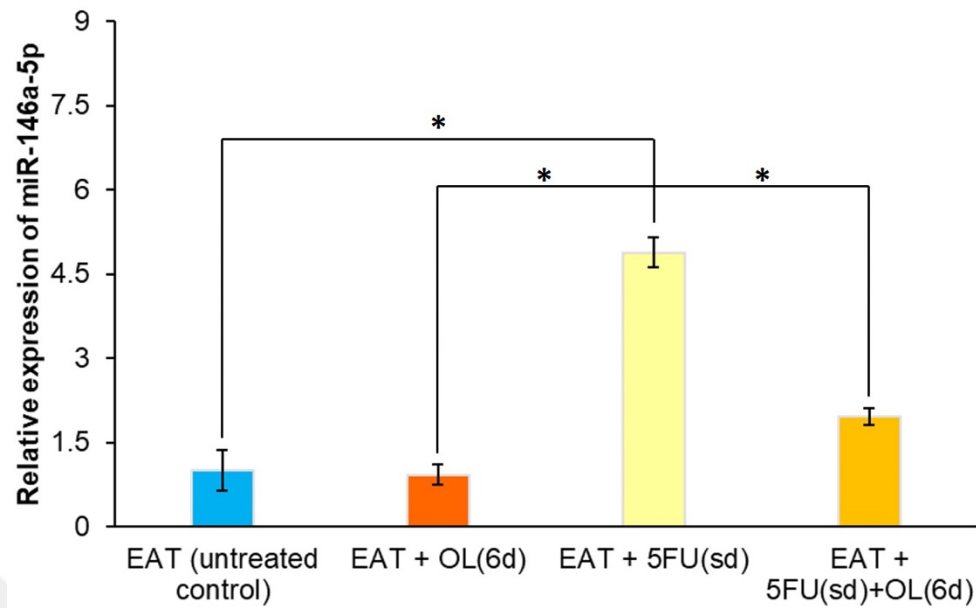


Figure 4.4. The effect of Oleuropein (OL) administration on miR-146a-5p expression for 6 days after tumor detection in the group in which the effect on tumor growth was investigated in the EAT breast cancer model

*: Statistically significant difference ($p < 0.05$) compared to [EAT+5-FU(sd)] group

4.1.4. Effect of Oleuropein treatment duration

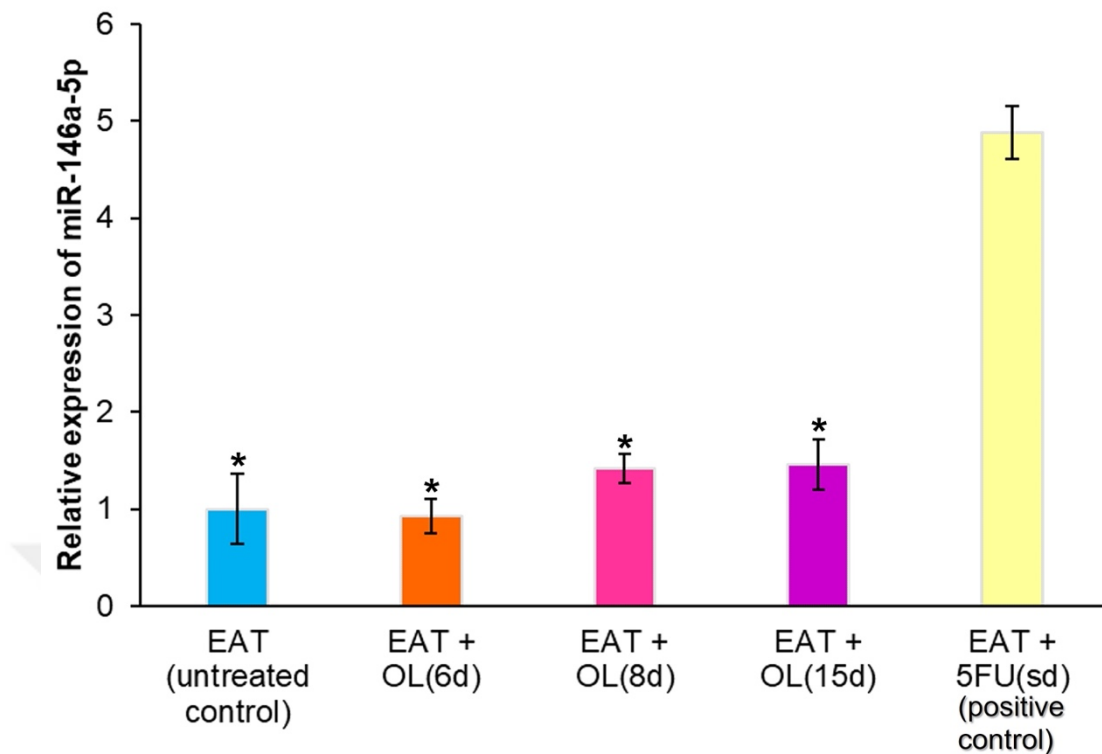
When the effects of oleuropein on mir-146a-5p expression were compared in terms of the different durations (6, 8 and 15 days) of the OL administration, it was determined that there was no significant difference between them and the untreated control (EAT) group.

Table 4.4. The effect of Oleuropein treatment duration on miR-146a-5p expression in EAT cells

Groups	Relative expression value of miR-155-5p (FC)
EAT (untreated control)	$1.00 \pm 0.36^*$
EAT + 5FU(sd) (positive control)	4.89 ± 0.27
EAT + OL(6d)	$0.93 \pm 0.18^*$
EAT + OL(8d)	$1.43 \pm 0.15^*$
EAT + OL(15d)	$1.47 \pm 0.26^*$

EAT: Ehrlich ascites tumor, OL: Oleuropein, 5-FU: 5-Fluorouracil, sd: single dose, 6d: six days, 8d: eight days, 15d: fifteen days.

*: Statistically significant difference ($p < 0.05$) compared to positive control [EAT+5FU(sd)] group.



*: Statistically significant difference ($p < 0.05$) compared to positive control [EAT+5FU(sd)] group

Figure 4.5. Effect of Oleuropein treatment duration on miR-146a-5p expression in an EAT breast cancer model

4.2. Discussion

Treatment for cancer, which is a remarkable cause of death, employs a variety of techniques. There is yet to be found an alternative to chemotherapy, which occupies a unique position among these treatment options. Despite the fact that various chemical and herbal chemotherapeutic agents are utilized in chemotherapy, a chemotherapeutic agent has yet to be discovered that provides completely successful results while minimizing side effects. As a result, the international quest for novel chemotherapeutic drugs derived from chemical and plant sources continues apace.

Triple-negative breast tumors, which account for 10 percent to 15 percent of all breast cancer in women, have gained substantial attention in recent years (Arnedos et al., 2012). Additionally, the involvement of microRNAs (miRNAs) in the epigenetic control of a broad variety of cellular processes is quickly gaining recognized as a critical tool for fine-tuning gene expression (Liu, 2012).

Kumaraswamy et al., (2014) showed that miR-146a is substantially overexpressed in triple-negative breast cancers. Another group of researchers determined that miR-146a had no effect on BRCA1 expression in MDA-MB-231 and MCF7 cells. On the other hand, miR-146a has been shown to inhibit BRCA1 expression in triple-negative sporadic breast tumors (Garcia et al., 2011). This disparity could be attributed to the various cell lines chosen for the exogenous expression experiments, (MDA-MB-468, MDA- MB-157 and MDA-MB-436). Another research demonstrated that miR-146a can prevent a highly metastatic human breast cancer cell line from invading and spreading (Bhaumik et al., 2008). These results revealed that miR-146a and BRCA1 have a complicated feedback loop that is probably different in various situations (Fkir et al., 2015).

In a study, Jagnathan et al. (2010) measured the vital of EAC-bearing animals, on certain days, in the same way, every time. Every experimental animal's body weight rose by more on day 12 than it did on day 1. The only exception was the 5-FU treatment, which saw a drop. 125 mg/kg doses of eugenol, on the other hand, killed animals, it was found. It was clear that Sample C and Eugenol had an influence on the body weights of animals that had been given them when they were weighed on day 12 (Jagnathan et al., 2010).

Jaganathan and Mandal (2010) treated EAC-bearing mice with eugenol and honey. Eugenol was given in three different doses at random: 80, 100, and 125 mg/kg. They found that animals were poisoned by a dose of 125mg/kg. With Ehrlich ascites, a dose of 100 mg/kg was found to help, with a 29% anticancer effect. To treat the Ehrlich solid tumor, the same amount of medicine (100 mg/kg) was used. Honey didn't work very well against Ehrlich solid tumors, but eugenol showed that there was a big decrease in the formation of solid tumors (antitumor efficacy of 24.35 percent) (Jaganathan and Mandal, 2010).

Cisplatin was the first anticancer treatment licensed by the Food and drug administration; nonetheless, nephrotoxicity, which may manifest as oxidative stress, inflammation, is a significant disadvantage of cisplatin therapy (Lippert et al., 1999).

At the moment, new drugs are being explored to mitigate the adverse effects of chemotherapy. In a study, it was demonstrated that OLE dramatically reduced cisplatin's genotoxicity in kidney cells. Indeed, when kidneys treated with OLE were compared to those treated with cisplatin, there was a significant decrease in cell damage. This protective effect seems to be related to OLE's ability to inhibit the generation of (ROS) and so ameliorate renal impairment (Basu et al., 2016). Polyphenols are a class of bioactive chemicals that exhibit a range of beneficial qualities. Flavonoids are capable of scavenging free radicals when ingested. Both flavonoids and their metabolites have antioxidant properties in vivo (Bouaziz et al., 2005).

Another study's results indicated that combining renal treatment with OLE exposure might enhance the antioxidant status and balance oxidative stress in chemotherapy patients. Although no differences in oxidative factors were seen between rats treated with chemotherapeutics and healthy rats, OLE therapy significantly raised TAS and lowered TOS levels and altered the levels of these variables in cisplatin-treated rats, bringing them into line with healthy rats. Because of the oxidative stress caused by cisplatin, tissues have shown a large rise in TAS. This rise may be attributable to the antioxidant enzyme glutathione scavenging superoxide ions. Antioxidant deficiency and higher oxidative stress in the body are connected with an increased risk of cancer (Xu et al., 2016).

miRNA is a non-coding short single-stranded RNA with a length of 20–24 nucleotides that plays a role in gene transcription and expression (Schanzaet et al., 2017). Although miRNA is not a protein, it is capable of directly degrading or impeding the translation of the target mRNA by creating a complete or partial complementary pair with the target mRNA (Kagiya, 2016). Numerous studies have shown that miRNA influences oncogenesis, migration, and other associated genes, implying that it has a role in tumor growth, metastasis, and angiogenesis. (Jansson and Lund, 2012).

A study by Meshkat et al., (2016) has established that miR146a-5p expression is much greater in breast cancer tissue than in non-cancerous tissue. It was confirmed in this work using RTqPCR, and it was also observed that miR-146a-5p expression was dramatically elevated in the breast cancer cell line MCF-7 compared to control cells. miR-146a-5p expression at high levels may increase proliferation in MCF-7, whilst low levels may inhibit it. Using bioinformatics prediction and fluorescent reporter gene detection, we identified BRCA1 as the miR-146a-5p target gene (Meshkat et al., 2016).

BRCA1 is a tumor suppressor gene that is typically altered in hereditary breast cancer families. DNA repair, cell cycle regulation, transcriptional activation, and ubiquitination are all activities in which BRCA1 is involved. MCF-7 cells' proliferation was increased by high levels of miR-146a-5p in this study, whereas it was reduced by low levels of miR-146a-5p. Tumor cells' higher proliferation may have been aided by decreased BRCA1 expression, which elevated miR-146a-5p expression. Tumors were suppressed and their proliferation capacity was reduced as a consequence of the upregulation of BRCA1 when miR-146a-5p was downregulated (Schayek et al., 2018).

It is also possible that increased expression of miR-146a-5p regulates MCF-7 proliferation through altering BRCA1 expression (Drost and Jonkers, 2014).

5. CONCLUSIONS and RECOMMENDATIONS

In this thesis, 150 mg/kg oleuropein was administered to male mice with Balb/c Ehrlich ascites tumor (EAT), and the difference in miRNA-146-5p expression as a result of changes in mechanisms at the molecular level due to the protective effect of oleuropein ingestion was investigated. As a result of our thesis study, the following data were obtained:

We have a significant increase ($p < 0.05$) in miR-146a-5p expression was observed in the 5-FU group in which 5-Fluorouracil (5FU), a chemotherapeutic agent used in the treatment of breast cancer, was administered in a single dose (20 mg/day). It showed a negative effect by masking the effect of 5-FU on tumor growth in the 5-FU+OL group treated with 5-FU (single dose) and OL (150mg/kg/day, 6 days). In addition, the expression of miR-146a-5p in the group given OL with 5FU was lower than the group given with 5FU alone, suggesting that OL may have suppressed the expression of the tumor suppressor miR-146a-5p.

As a result, it has been shown in our study that the Oleuropein compound has a negative effect on EAT cells at the molecular level, and even reduces the curative effect of the drugs used in the treatment. For this reason, since it is thought that the use of OL as a stand-alone treatment in breast cancer individuals may adversely affect the course of the disease rather than its curative effect, the use of Oleuropein compound during cancer treatment or chemotherapy cannot give positive results. Therefore, after the tumor is detected, the application of oleuropein alone or in combination with a chemotherapeutic agent may increase the growth of breast cancer, so caution should be taken in the oncological treatment process

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