



**T.C.
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SCIENCE AND ENGINEERING**



Ph.D. THESIS

**EFFECTS OF EXOSOME ENCAPSULATED ANTICANCER DRUG
LAPATINIB ON BREAST CANCER CELLS**

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LIST OF SYMBOLS ABBREVIATIONS

Abbreviation	Explanation
μg	: Microgram
μL	: Microliter
μM	: Micro Molar
$^{\circ}\text{C}$	: Degree centigrade
AFM	: Atomic force microscopy
ATCC	: American Type Culture Collection
ATPS	: Aqueous two-phase system
BBB	: Blood brain barrier
BRCA 1	: Breast cancer sensitivity gene 1
BSA	: Bovine serum albumin
cDNA	: Complementary DNA
CHEK-2	: Checkpoint kinase 2
CO₂	: Carbon dioxide
DEX	: Dextran
DLS	: Dynamic light scattering
DMEM	: Dulbecco's modified eagle medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
ECM	: Extracellular matrix
EGFR	: Epidermal Growth Factor Receptor
EMT	: Epithelial mesenchymal transition
ER	: Estrogen receptor
ERP	: Enhanced permeability and retention
ESCRT	: Endosomal sorting complexes required for transport
EV	: Extracellular vesicles
Exo	: Exosome
FBS	: Fetal bovine serum
FDA	: U.S. Food and Drug Administration
GLOBOCAN	: Global Cancer Observatory

GFR	: Growth factor receptor
HER2	: Human Epidermal Growth Factor Receptor 2
HIF	: Hypoxia-inducible factor
HSP	: Heat shock protein
IC₅₀	: Half maximal inhibitory concentration
ILC	: Invasive lobular carcinoma
JAK	: Janus kinase
Lap	: Lapatinib
LapExo	: Lapatinib- loaded exosome
MAPK	: Mitogen-activated protein kinase
MHC	: Major histocompatibility complex
miRNA	: microRNA
MRI	: Magnetic resonance imaging
MTS	: 3-(4,5-dimethyl-thiazol-2)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium)
MVB	: Multivesicular body
NGS	: Next generation sequencing
NST	: Non-specific ductal carcinoma
NTA	: Nanoparticle tracking analysis
PBS	: Phosphate Buffered Saline
PD-1	: Programmed death-1 receptor
PEG	: Poly(ethylene glycol)
PET	: Positron emission tomography
PI	: Propidium Iodide
PI3K	: Phosphoinositide 3- kinase
PR	: Progesteron receptor
PSA	: Penicillin/Streptomycin/Amphotericin
PSA	: Prostate specific antigen
qPCR	: Quantitative polymerase chain reaction
RTK	: Receptor tyrosine kinase
SDS-PAGE	: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SPECT	: Single phane emission computed tomography
STAT	: Signal transducer and activator of transcription protein
TIL	: Tumor-infiltrating lymphocytes
TNBC	: Triple negative breast cancer

VEGF : Vascular endothelial growth factor



ÖZET

EKSOZOM İLE KAPSÜLE EDİLMİŞ KEMOTERAPÖTİK İLAÇ OLAN *Lapatinib*’in MEME KANSERİ HÜCRELERİ ÜZERİNDE ETKİLERİ

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Meme kanserinin alt tiplerinden biri olan HER2 pozitif meme kanseri, diğer tiplere göre daha agresif özellik göstermektedir. HER2 tedavisinde kullanılan kemoterapötik ilaçlardan biri Lapatinibdir. Lapatinib, bir tirozin kinaz inhibitörü olarak HER2 ve EGF gibi hücre büyümesini sağlayan reseptörlerin hücre içi kinaz alanlarına bağlanarak hücre büyümesini inhibe etmektedir. Yapılan çalışmalar, kan dolaşımında düşük çözünürlüğe sahip olan lapatinibin nano taşıyıcılar ile birlikte uygulandığında aktivitesini arttırdığını göstermiştir. Son yıllarda yapılan çalışmalar eksozomların ilaç taşıyıcı olarak kullanılabilirliğini ortaya koymaktadır. Eksozomlar 20-200 nm boyutunda hücrelerden salınan, hücreler arası trafikte rol alan küçük yapılı veziküllerdir. Çift lipid tabakalı membran yapısına sahip bu eksozomların içinde DNA, RNA ve proteinleri bulunmaktadır. Doğal nanopartikül olan eksozomların sentetik nanopartiküllere kıyasla yan etkilerinin bulunmadığı bildirilmektedir.

Bu çalışmada sağlıklı meme hücrelerinden elde edilen eksozomlar nano taşıyıcı olarak kullanılmıştır. Lapatinib, elektroporasyon yöntemi uygulanarak eksozomlar ile kapsüle edilmiş ve yaklaşık olarak %40-45 oranında başarılı bir kapsülasyon gerçekleştirilmiştir. HER2 pozitif meme kanseri hücre hattı (SKBR-3) ve sağlıklı meme epitel hücre hattı (MCF10 A) üzerinde, serbest lapatinib, eksozom ile kapsüle edilmiş lapatinib ve boş

eksozomun sitotoksik etkileri değerlendirilmiştir. Serbest lapatinibin IC₅₀ değeri, lapatinibin eksozom ile kapsüle edilmesiyle doza ve zamana bağlı olarak düşürülmüştür. Boş eksozomların ise hücre proliferasyonu üzerinde herhangi bir etkisinin olmadığı görülmüştür. Eksozomlar, ilaç taşıma da yalnızca aracı olarak kullanılmıştır. Eksozom ile kapsüle edilen Lapatinib' in düşük konsantrasyonda SKBR-3 hücreleri üzerinde daha etkili sitotoksik etki gösterdiği, ancak sağlıklı MCF10 A hücreleri üzerinde herhangi bir sitotoksik etkisinin olmadığı belirlenmiştir. Annexin V ile yapılan apoptotik analizler eksozom ile kapsüle edilmiş lapatinibin, 0,1 µM konsantrasyonda ve 48. saatte, SKBR-3 hücreleri üzerinde serbest Lapatinibe kıyasla apoptozu daha fazla arttırdığını, MCF10 A hücreleri üzerinde ise apoptotik etkisinin daha düşük olduğunu göstermiştir. Western blot ve RT-qPCR analizleri lapatinib ve eksozom ile kapsüle edilmiş lapatinibin SKBR-3 ve MCF10 A hücrelerine ait ERBB1, ERBB2, AKT, MAPK, caspase 3, caspase 9, BCL-2, TP53 genlerin ve HER2, EGFR, ERK 1/2 (p44/42 MAPK), AKT, pAKT, P53 ve caspase 3 proteinlerin ekspresyonları üzerinde benzer etkilere sahip olduğu gösterilmiştir.

Sonuç olarak bu tez kapsamında hidrofobik bir ilaç olan lapatinibin çözünürlüğü ve etkinliği eksozom ile kapsüle edilerek meme kanseri hücreleri üzerindeki etkileri arttırılmıştır. Sağlıklı epitel meme hücrelerinden (MCF10 A) türetilen eksozomlar, ilk kez bu tez kapsamında lapatinib taşıyıcısı olarak kullanılmıştır. Lapatinib elektroporasyon yöntemi kullanılarak eksozomlarla kapsüle edilmiştir. Yapılan bu çalışma HER2 pozitif meme kanseri tedavisinde kullanılabilecek yeni bir strateji olabilir.

Haziran 2022, 133 sayfa.

Anahtar kelimeler: Meme kanseri, kemoterapi, lapatinib, ilaç taşıma sistemi, eksozomlar

SUMMARY

EFFECTS OF EXOSOME ENCAPSULATED ANTI-CANCER DRUG LAPATINIB ON BREAST CANCER CELLS

Ph.D. THESIS

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HER2 positive breast cancer, one of the subtypes of breast cancer, is more aggressive than other types. One of the chemotherapeutic drugs used in the treatment of HER2 is Lapatinib. As a tyrosine kinase inhibitor, lapatinib inhibits cell growth by binding to the intracellular kinase domains of cell growth receptors such as HER2 and EGF. Studies have shown that lapatinib, which has low solubility in the bloodstream, increases its activity when applied with nanocarriers. Studies conducted in recent years reveal that exosomes can be used as drug carriers. Exosomes are small vesicles of 20-200 nm, released from cells and involved in intercellular traffic. These exosomes, which have a double lipid layered membrane structure, contain DNA, RNA, and proteins. It is reported that exosomes, which are natural nanoparticles, do not have side effects compared to synthetic nanoparticles.

This study used exosomes obtained from healthy breast cells as nanocarriers. Lapatinib was encapsulated with exosomes by applying the electroporation method, and a successful encapsulation rate of approximately 40-45% was achieved. The cytotoxic effects of free Lapatinib, exosome-encapsulated Lapatinib, and empty exosome were evaluated on HER2

positive breast cancer cell line (SKBR-3) and healthy breast epithelial cell line (MCF10 A). The IC₅₀ of free Lapatinib was dose- and time-dependently reduced by encapsulating Lapatinib with the exosome. Empty exosomes were found not to affect cell proliferation. Exosomes have been used only as mediators for drug delivery. It was determined that Lapatinib encapsulated with exosome had a more effective cytotoxic effect on SKBR-3 cells at low concentrations. However, it did not have any cytotoxic effect on healthy MCF10 A cells. Apoptotic analyzes with Annexin V showed that exosome-encapsulated Lapatinib at a concentration of 0.1 μ M and 48 hours increased apoptosis more on SKBR-3 cells than free Lapatinib but had a lower apoptotic effect on MCF10 A cells. Western blot and RT-qPCR analyses of Lapatinib and exosome-encapsulated Lapatinib of ERBB1, ERBB2, AKT, MAPK, caspase 3, caspase 9, BCL-2, TP53 genes and HER2, EGFR, ERK 1/ 2 (p44/42 MAPK), AKT, pAKT, p53 and caspase 3 have been shown to have similar effects on the expression of proteins.

As a result, within the scope of this thesis, the solubility and effectiveness of lapatinib, a hydrophobic drug, were encapsulated with exosomes, and its effects on breast cancer cells were increased. Exosomes derived from healthy epithelial mammary cells (MCF10 A) were used as lapatinib carriers for the first time in this thesis. Lapatinib was encapsulated with exosomes using the electroporation method. This study may be a new strategy that can be used to treat HER2 positive breast cancer.

June 2022, 133 pages.

Keywords: Breast cancer, chemotherapy, lapatinib, drug delivery system, exosomes

1. INTRODUCTION

1.1. CANCER

Cancer is a severe disease that can affect most people and is one of the most common cause of death. According to GLOBOCAN (Global Cancer Observatory), 18,1 million newly diagnosed cancer patients and 9.6 million cancer deaths worldwide in 2018 (Korgaonkar *et al.*, 2019; Tao *et al.*, 2020). Cancer is a disease of diversity that occurs at the tissue level. This diversity represents a significant challenge for disease diagnosis and the effectiveness of treatment. They are the most common types of prostate, bladder, colon, and lung cancer in the male population. In women, breast, lung, colon, uterus, and thyroid cancers are the most common cancers. In children, blood, brain, and lymph cancers are among the most common cancers (Hassanpour *et al.*, 2017).

Cancer occurs as a result of mutations in genes, and these mutations change the function of the cell. Chemical compounds are effective in the formation of cancer cells. Radiation rays and microorganisms such as bacteria and viruses are other factors that cause mutations in the gene and cause cancer (Parkin, 2006; Aizawa *et al.*, 2016). Cancer is the genetic instability of somatic cells. This genomic evolution creates genetic and epigenetic equality (Ben-David and Beroukhi *et al.*, 2019). DNA methylation, nucleosome position, and histone modification, which play an essential role in cancer formation, are epigenetic modifications (Portela and Esteller, 2010). Genetic disorders that cause cancer include chromosomal translocation, point mutation, deletion, amplification, and insertion (Thomas *et al.*, 2007). Under normal conditions, P53 is involved in cell division, differentiation, senescence, death, and DNA metabolism. Mutation in this gene causes cancer by causing abnormality of these biological events, and it is known that 60% of cancer cases have P53 abnormality (Taylor and Stark, 2001). The body also has a control system used when needed to grow and divide a cell. Uncontrolled division and aberrant proliferation of cells cause a mass called a tumor. The tumor is divided into two parts, benign and malignant. The benign tumor grows slowly, and tumor mass can be removed by operation so they do not spread to the tissues. On the other hand, malignant tumors grow invasively (Korgaonkar *et al.*, 2019; Khanmohammadi *et al.*, 2020). The development of secondary tumors far from the primary cancer site is known as

metastasis. Most of the deaths caused by cancer are the metastatic spread of malignant cells. Metastasis is the most critical feature of cancer biology. As stated by Hanahan and Weinberg, “activating invasion and metastasis” is the most basic distinguishing hallmark of cancer (Figure 1.1)(Hanahan *et al.*, 2011). Metastasized cancer cells leave the bloodstream's primary site, escape from the immune cells, and migrate to the secondary cell environment. Furthermore, distant metastases and invasion of nearby tissues remain an essential feature of cancer malignancy.

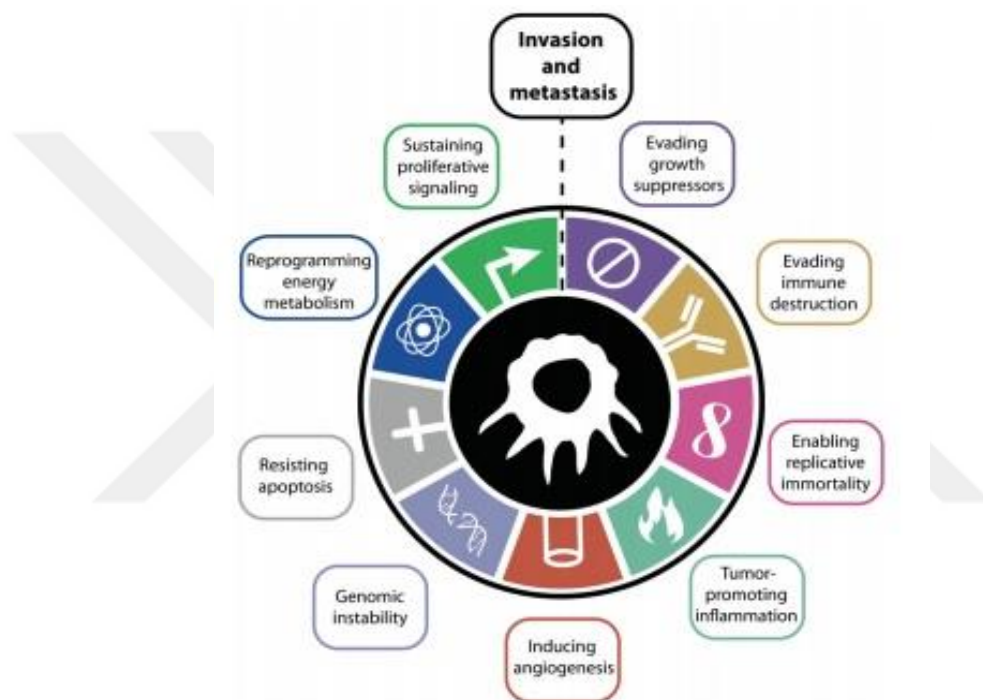


Figure 1.1: Hallmark of cancer with invasion and metastasis (Hanahan and Weinberg, 2011)

The factors that trigger the activation of invasion and metastasis are epigenetic factors caused by environmental factors, signals from extracellular matrix (ECM) components such as collagen and fibrin, cell-cell interactions, growth factors, and cytokines (Figure 1.2) (Fares *et al.*, 2020).

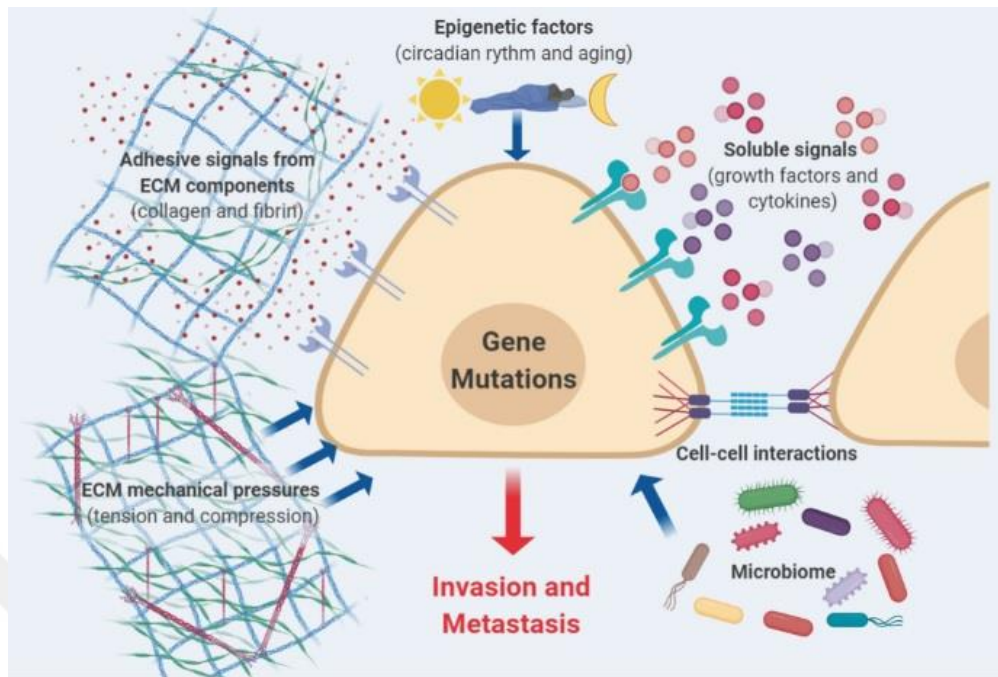


Figure 1.2: Factors activating invasion and metastasis (Fares *et al.*, 2020)

Epithelial-mesenchymal transition (EMT) is a regulator that organizes invasive and metastasis events (Figure 1.3) (Fares *et al.*, 2020). EMT is defined as disruption of apical basal polarity by deregulation of epithelial proteins like E-cadherin and stimulation of mesenchymal markers like Snail and Twist to resemble mesenchymal phenotype. Integrins are important cellular transmembrane receptors which are cell surface proteins that are found in almost every stage of cancer process from beginning of the tumor to metastasis. Moreover, integrin expression level is altered in tumors and they have roles in supporting oncogenic growth factor receptor (GFR) signaling and GFR-dependent cancer cell migration and invasion (Jiang *et al.*, 2016; Tian *et al.*, 2021)

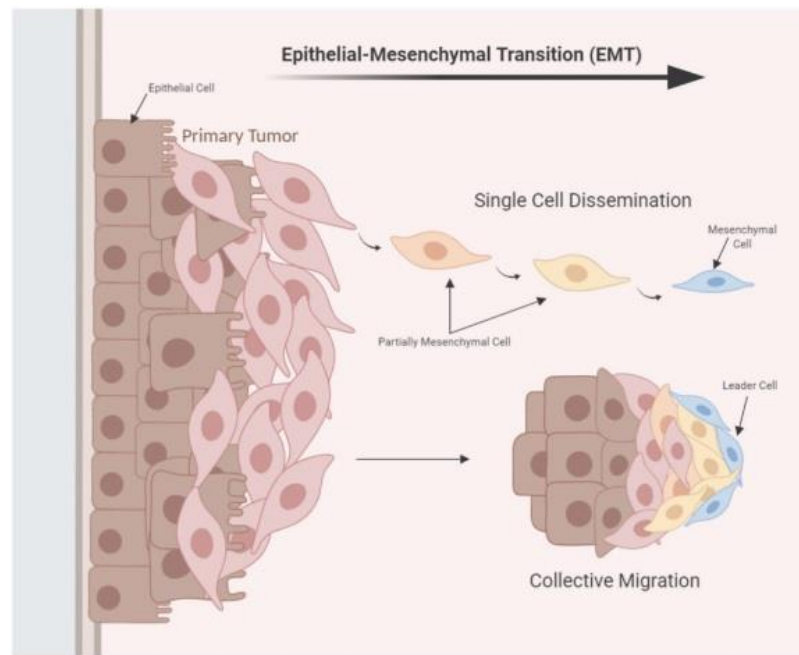


Figure 1.3: Epithelial–mesenchymal transition (EMT) occurs through the migration of a single cell or collective cell (Fares *et al.*, 2020).

1.1.1. Metabolism of Cancer

Cancer cells need to choose a different metabolic pathway than normal cells to metastasize and invade. During the formation and spread of cancer, cancer cells are exposed to various metabolic stresses. Tumor microenvironments are hypoxic and acidic, and cancer cells have different nutrient compounds than non-tumor tissues. In this case, cancer cells adapt to this environment to grow and spread in the tumor microenvironment. In addition, for cancer cells to enter the blood vessels and survive there, they must rearrange and reprogram their metabolic states. When cancer cells spread to other organs, they must adapt to different metabolic environments than the primary site. At each stage of cancer progression, cancer cells must reprogram their metabolic states. Finally, metabolic reprogramming is one of the essential hallmarks of cancer (Ward and Thompson, 2012; Schild *et al.*, 2018). Tumor tissues show an altered and different metabolism compared to normal tissues. The metabolism of tumor tissues changes with various intrinsic and extrinsic factors (Figure 1.4) (Nagarajan *et al.*, 2016; Ohshima and Morii, 2021). The most classic and well-known intrinsic factor is the Warburg effect. As Warburg observed the destruction of sugar in tumor tissues of patients with cancer, researchers focused on the cellular metabolism of cancer. Besides, the Warburg effect is aerobic glycolysis, which is defined as an increase in glycolysis and lactate even in the presence of oxygen in the metabolic pathway of cancer cells (Ohshima and Morii, 2021).

Cancer cells transform the pyruvate into lactate and indicate the transition from oxidative phosphorylation to glycolysis for primary ATP production. Moreover, glycolytic metabolism helps primarily to canalize carbon sources to biosynthetic pathways, producing nucleic acids, lipids, and proteins for cell division. Oncogenic mutations in cancer cells allow independent nutrient intake for growth factors and re-metabolic programming against the anabolic pathway—hypoxia, extracellular acidification, and nutrient conditions that cause the abnormal metabolic appearance. Rapamycin (mTOR) complex are activated with up-regulation of phosphoinositide 3-kinase (PI3K)-Akt signaling and downregulation of hypoxia-inducible factor (HIF) cellular reprogramming that causes the transition of glycolysis of cancer cells. In normoxic conditions, the cell temporarily carries the hypoxic transcription factors. In hypoxic conditions, HIFs are stayed permanently and promote transcription genes related to cell survival. Hypoxia and PI3K-Akt signaling cause HIFs to be overexpressed because mutations commonly upregulate tyrosine kinases or the protein regulator p53 becomes dysfunctional, and these are accountable for the regulation of proliferation and glucose metabolism (Allison *et al.*, 2017).

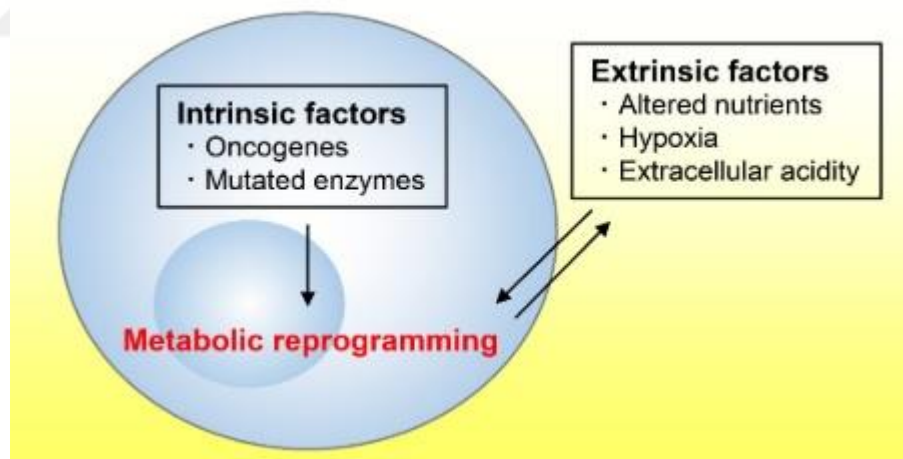


Figure 1.4: The factors that induce the reprogramming of cancer cells are intrinsic and extrinsic (Nagarajan *et al.*, 2016; Ohshima and Morii, 2021)

1.1.2. The Immune Control of Cancer

The immune system removes tumor cells and controls the growth of the tumor. Tumor-infiltrating lymphocytes (TILs) consist of lymphocytes and plasma cells and promote survival and proliferation, generating a response to tumors. However, TILs are favorable prognostic factors, especially for solid tumors, suggesting that lymphocytes can reduce tumor growth.

TIL levels significantly increased the sensitivity and efficacy of neoadjuvant chemotherapy in patients with breast cancer. They were positively associated with survival outcomes in primary cutaneous melanoma, non-small cell lung cancer, or breast cancer patients. It has been shown in previous studies that the TILs, such as CD3⁺ and CD8⁺ T cells with an immunologic response, that has been promoted the effects of chemotherapy and radiotherapy in cancer patients (Balermipas *et al.*, 2014).

The metabolic programming of cancer cells has changed and has become different from healthy cells. Moreover, cancer cells' increasing abnormal processes and suppression of catabolic pathways are affected by altered signaling, hypoxia, and mutations in oncogenic genes. Furthermore, the tumor microenvironment is suppressed with cancer cells which inhibit T cells (Tian *et al.*, 2021). Cancer cells change the expression level of receptors such as programmed death-1 receptor (PD-1) and its PD-L1 ligand, immune checkpoints to have developed strategies to escape the immune system. PD-1 is associated with ligands PD-L1 and PD-L2, which are T-cell inhibitory receptors. However, PD-1 and PD-L1 are overexpressed by cancer cells to disrupt T cells according to micro-environmental conditions. Notably, the effect of immunotherapies and metabolic targeting agents is related to both immune response and cancer cells and metabolic changes and immune evade. Therefore, novel treatments developed to break down the metabolic activity of cancer cells can disrupt tumor immune responses (Allison *et al.*, 2017).

1.1.3. Cancer Diagnosis

Cancer is a disease that can be affected by genetic and environmental factors. Modern diagnostic technologies such as molecular imaging and liquid biopsy accelerated cancer disease diagnosis. Early diagnosis is one of the essential treatment methods in cancer since cancer is not yet benign and non-metastatic. However, some tumors are diagnosed late due to late symptoms, which makes treatment difficult (Wong *et al.*, 2019). Tissue biopsy, molecular imaging, and liquid biopsy are some of the methods of a cancer diagnosis. The biopsy is an invasive method in which a small number of lumpy tissues or cells can be removed. However, it takes time for pathologists to identify the disease with this method. Therefore molecular imaging has become a more effective method by providing invasive imaging of disease regions even at the lowest level. This method is different from many imaging methods in which anatomical and biophysical features are revealed. The molecular imaging methods that

are single photon emission computed tomography (SPECT), sonography, positron emission tomography (PET), X-ray computed tomography (CT) scan, and magnetic resonance imaging (MRI) is commonly used for cancer detection. These methods may expose the patients to a high dose of ionizing radiation; therefore, risk and benefit assessments need to be made. On the other hand, the liquid biopsy method has become necessary in cancer diagnosis as it can be easily sampled (e.g., blood, urine). For example, protein markers such as CA125 and prostate-specific antigen (PSA) are used in fluid biopsies of some cancers. Detection of recently developed nucleic acids in biological fluids and next-generation sequencing (NGS) is vital for cancer diagnosis from liquid biopsy (Wu *et al.*, 2019).

1.2. BREAST CANCER

Breast tissue consists of lobules and some ducts. These are the channels that connect the adipose tissue and the lymph tissue. In addition, lobules are milk-producing glands. Breast cancer is most common in lobules and occurs as uncontrolled cell growth in any part of the breast (Winters *et al.*, 2017). Moreover, this cancer type is one of the most common cancers seen worldwide, which causes the most deaths in women. Breast cancer cases were expected to be approximately 276,480 invasive and 48,530 non-invasive in 2019, and 270,000 invasive breast cancer cases and 42,000 non-invasive in 2020 in the United States (Najminejad *et al.*, 2019; Kaur *et al.*, 2021). This type of cancer continues to be a significant health problem, and treatment methods continue to be investigated. However, aggressive breast cancer is increasing rapidly every year (Anastasiadi *et al.*, 2017). One in ten women has the risk of developing breast cancer. In many countries, breast cancer ranks second after lung cancer as the cause of death. However, it is one of the most common causes of death in underdeveloped countries. In South America, Asia, and Africa, the number of women with breast cancer increases due to environmental variability and screening programs, and mortality rates are increasing accordingly. The reason for the increase in mortality is the lack of diagnosis and treatment methods developed recently (Libson and Lippman, 2014; Harbeck *et al.*, 2019).

The development of breast cancer is affected by genetic or non-genetic factors. Genetic factors include mutations in BRCA1, BRCA2, and checkpoint kinase 2 (CHEK2) and non-genetic single nucleotide polymorphisms related to breast cancer (Gandhi and Das, 2019). Mutations in genes are the best genetic explanation for families with a history of breast

cancer. Mutations in the BRCA1 and BRCA2 genes are among the most common explanations to show the genetic changes in breast cancer. P53 mutations are also among the mutations that cause breast cancer. The P53 gene is an important tumor suppressor gene and protects the genome. Tumor suppressor P53 antigen is followed by a DNA damage checkpoint that occurs in the cell. It provides DNA damage by activating genes or initiating apoptosis. However, some studies have shown that it plays an essential role in hormone-induced breast cancer diseases (Schon and Tischkowitz, 2018).

Historically, breast cancer classification systems have been based on histopathological assessment, including histological type and grade of tissue. Breast cancer is divided into invasive diseases and ductal/lobular. Besides, it has been revealed by the WHO Classification of Tumours of the breast that there are more than 21 subtypes of invasive breast cancer type. The most common type is a non-specific ductal carcinoma (NST), accounting for 40%-75% percent of cases. The remaining tumor types are morphologically specific, such as lobular, tubular, mucinous, and metaplastic carcinoma. Furthermore, this includes medullary, neuroendocrine, or apocrine carcinomas (Vuong *et al.*, 2014). Invasive lobular carcinoma (ILC) is also one of the most common types of breast cancer (Figure 1.5) (Patil *et al.*, 2020)

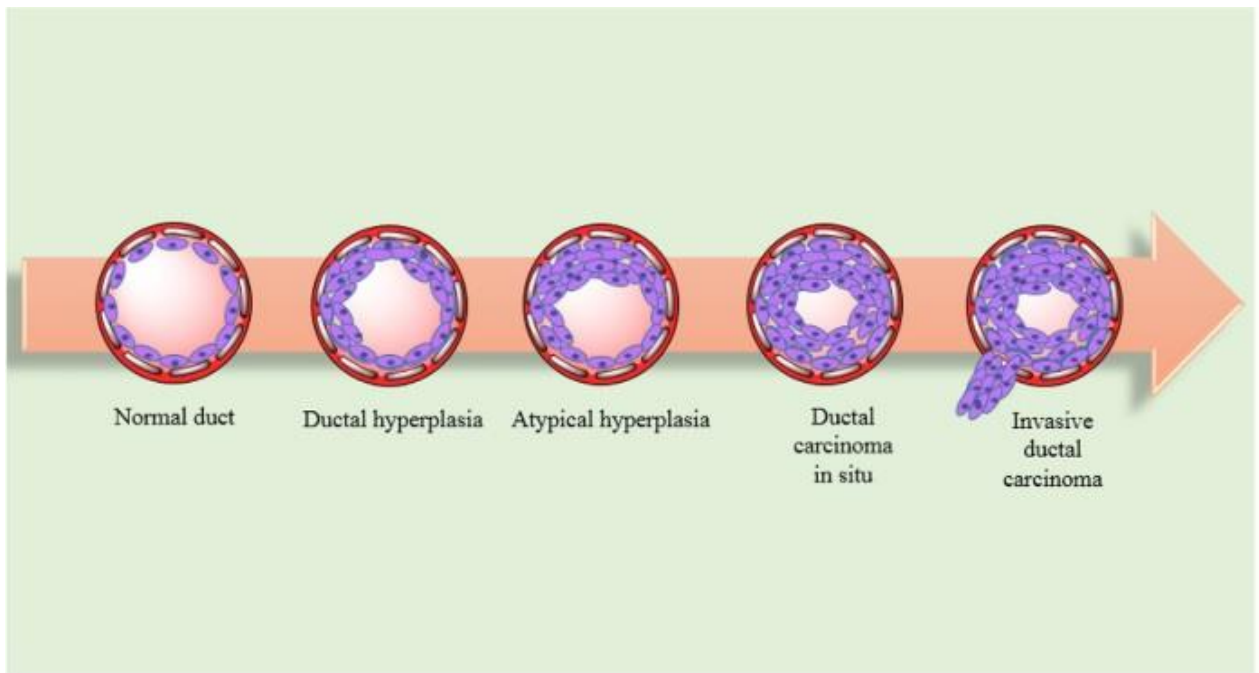


Figure 1.5: Ductal and Invasive Carcinoma (Patil *et al.*, 2020)

The best-known genetic feature of ILC is the loss of E-cadherin and specific mutations targeting genes such as PTEN, TBX3, and FOXA1. The association of PTEN reduction with increased AKT phosphorylation occurred in all breast cancer groups, with the highest occurrence in ILC. However, at the DNA, ILC cases have CDH1, TBX3, and FOXA1 mutations. After CDH1 mutations, GATA3 mutations and PTEN mutations are the most common conditions in ILC according to the ductal NST carcinoma and the others. Some genes regulate the metabolism of breast cancer. These are phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), MYC, ER, breast cancer sensitivity gene 1 (BRCA1), and P53 species (Rakha and Green, 2017; Gandhi and Das, 2019)

1.2.1. The Classification of Breast Cancer

Breast cancer used to be classified according to pathological clinic features, including stage and grade of the tumor. Specific subtypes of breast cancer are characterized by morphological characteristics and some biomarkers, such as hormone estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). These biomarkers express different levels in the cell and increase heterogeneity. For example, ER-positive tumors become less defined than PR-positive tumors. HER-2 positive tumors are the most aggressive according to the other subtypes. In addition, breast cancer cell lines are

characterized as triple-negative breast cancer when they do not express ER, PR, and HER2 (Rakha and Green, 2017; Januškevičienė and Petrikaitė, 2019).

1.2.1.1. Luminal breast cancer cell lines

The estrogen receptor (ER) and the progesterone receptor (PR) positive tumors are characterized as luminal carcinomas. Luminal cells have a high expression level of transcription factors (GATA3 and FOXA1) and genes/proteins such as ER α /ER, KRT8/18/19 (luminal keratins). However, cell lines with luminal features migrate less due to cell-to-cell connections (Wan *et al.*, 2002; Dai *et al.*, 2017). The most common type of breast cancer is usually ER-positive. In addition to the widespread use of endocrine therapy, it is also common for the disease to progressively cause death due to resistance to endocrine therapy. In response to this resistance, the PI3K signaling pathway takes an essential place in anti-estrogen treatments (Jézéquel *et al.*, 2019; Britt *et al.*, 2020).

- **Luminal A Type**

Luminal A breast carcinoma is the most common subgroup, 50–60 % of all the breast cancer cases. It is defined by genes expressed by the ER transcription factor located in the lumen epithelium of the mammary ducts. Also represented by luminal A expressing ER, PR; BCL-2, CK8 / 18 genes and not HER-2. GATA3 gene is the most expressed in luminal A-type breast cancer (Vidal *et.al*, 2016; Rakha and Green, 2017).

- **Luminal B Type**

Luminal B carcinomas include that influence mutation of TP53 and PIK3CA. Luminal B provides the high expression of genes that affect cell proliferation and cell cycles, such as MKI67 and AURKA, and low expression of genes and proteins such as PR and FOXA1. Besides, luminal B tumors have more somatic mutations and more P53 mutations according to the luminal A type of tumors. However, it has similar mutations for GATA3 and fewer mutations for PIK3CA and MAP3K1 than the luminal A type of tumors (Vidal *et al.*, 2016; Rakha and Green, 2017) .

1.2.1.2. HER2 Positive Breast Cancer

It is stated that necessary treatments have been developed for HER2-positive breast cancer, which constitutes 20% of breast cancer (Anastasiadi *et al.*, 2017). HER2, one of the four subtypes (HER1, HER2, HER3, and HER4) of epidermal growth factor receptors, is

overexpressed in 15% -20% of breast cancer. HER2 has tyrosine kinase activity. This receptor is involved in cell regulation, such as growth, survival, and differentiation. Overexpression of HER2 leads to unrestrained cell replication, causing cancer. However, HER2 dimerization leads to the phosphorylation of tyrosine residues. These receptor activations adhere to homodimerization or heterodimerization with HER2/HER3 dimer. Overexpression of the HER2 gene has led to an aggressive tumor for breast cancer and poor patient outcomes (Dai *et al.*, 2017; Jafari *et al.*, 2018). In HER2 positive breast cancer, the expression of ERBB2 / HER2 and GRB7 genes and proteins that provide proliferation in the cell is high, while the lumen, basal genes, and proteins such as ESR, PGR, FOXC1 are low (Vidal *et.al.*, 2016). PI3K is an essential pathway for decreasing the expression of HER2. PIK3CA mutations are seen in most HER2 receptor-positive cancers, which creates resistance to treating HER2 positive cases. Breaking the resistance in this signal pathway will be effective in HER2 targeted therapies (Zhang *et al.*, 2020).

1.2.1.3. Triple Negative Breast Cancer (TNBC)

Triple-negative cancer (TNBC) type that constitutes 20% of cancer cases is ER, PR, and HER2 negative type. TNBC type breast cancers are prevalent, a type of cancer associated with BRCA-1. TNBC with basal-like breast cancer is the most aggressive and deadliest type of breast cancer. Failure to perform targeted therapy in TNBC type cancer causes problems for patients. Therefore, therapeutic studies need to be defined to treat these types of cancer patients. The basal type of breast cancer forms the subgroup of TNBC, and these are expressed a high level of cytokeratins (CK5/6 and CK14) and expressed epidermal growth factor receptor (EGFR) (Vuong *et al.*, 2014; Jézéquel *et al.*, 2019). The PI3K / AKT / mTOR signaling pathway with TNBC is associated with decreasing PTEN and INPP4B phosphatases. Furthermore, this is associated with increased AKT phosphorylation. PIK3CA mutations are less common in TNBCs than hormone-positive and HER2-positive cancers. Activation of the PI3K signaling pathway is associated with chemotherapy resistance. Therefore PI3K inhibitors are used for the early stage of TNBC patients (Kennedy *et al.*, 2019).

1.2.2. The Metastatic Mechanism of Breast Cancer

Breast cancer can metastasize to different organs such as the brain, liver, bone, and lungs. This metastatic trend is called metastatic heterogeneity. It has been demonstrated that most

breast cancer patients can form metastases after treatment, and this metastasis can cause 90% death. However, bone metastasis is the first organ in which breast cancer metastasizes most. Bone metastases are found in 75 percent of breast cancer cases (Brook *et al.*, 2018). In addition, lung and liver metastases take the second and third-order (Liang *et al.*, 2020). Breast cancer uses many signaling pathways used by normal cells for tumor growth and metastasis. The PI3K/AKT cell signaling pathway is essential for breast cancer proliferation, differentiation, invasion, and metastasis. Decreasing the PTEN level inhibits the activation of the PI3K/AKT pathway. This pathway is related to the HER2 resistance of tumor cells (Joly *et al.*, 2016; Zhang *et al.*, 2019). The PI3K signaling pathway has been hyperactivated in hormone-positive and HER-2 negative breast cancers. In addition, genomic changes occurring in PIK3CA are seen in ER positive, HER2 negative metastasized breast cancer (Zhang *et al.*, 2020). Furthermore, some proteins and microRNAs (miRNAs) enable the development and progression of breast cancer. A great number of miRNAs, signaling pathways components, and growth factors cause breast cancer metastasis, which leads to death. Microenvironmental factors of the tumor cause cancer cells to move away from the immune system, and metastasis occurs. Various research is ongoing regarding exosomes causing breast cancer metastasis and invasion. Moreover, some studies have shown that the hypoxic environment increases the exosome release of cells into the tumor sites for tumor metastasis, and consequently, released exosomes contribute to breast cancer invasion by growth factor signaling pathways (Najminejad *et al.*, 2019).

1.2.3. Breast Cancer Diagnosis and Therapy

Early diagnosis and imaging are a vital part of breast cancer treatment. Surgery, chemotherapy, radiation, and combination therapies are among the most used methods in breast cancer treatment. In addition, endocrine and antibody therapies are among the techniques used (Jafari *et al.*, 2018; X. xi Li *et al.*, 2020). However, early diagnosis will not prevent breast cancer, but it helps to find the best time for effective treatment. There are different breast cancer detection methods such as ultrasound, mammography, molecular imaging, biopsy, and breast MRI in the clinic. Understanding the molecular mechanism of the tumor is essential for an effective diagnosis and treatment. In addition, molecular imaging can perform the biological and biochemical non-invasive evaluation of cancer cells in patients (Anastasiadi *et al.*, 2017).

1.3. CHEMOTHERAPEUTIC DRUGS

Chemotherapy is one of the cancer treatment methods applied with cancer drugs. Moreover, chemotherapeutics are pre-operative and postoperative adjuvant and neoadjuvant therapies (Shamsi and Islamian, 2017). Neoadjuvant therapy allows reduction of the tumor area. In addition, inoperable cancers bring a situation that can be operated on. Neoadjuvant therapy is used to diagnose early breast cancer and facilitates the treatments to be applied to the patient later by reducing the disease. However, neoadjuvant therapy can also be applied by combining chemotherapeutic drugs. Adjuvant therapy consists of radiation therapy, cytotoxic and molecularly targeted drugs, or a combination (Figure 1.6) (Fisusi and Akala, 2019). This therapy method has been shown to reduce the risk of cancer recurrence and increase survival in patients (Blondeaux *et al.* , 2019).

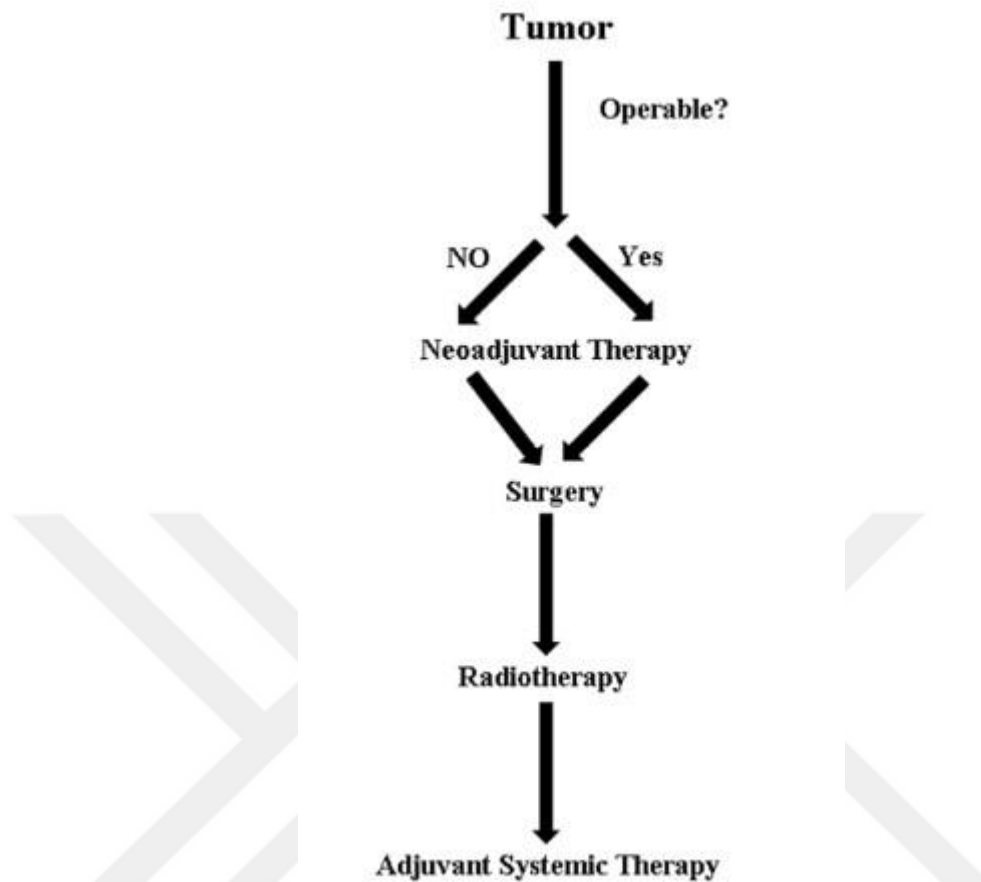


Figure 1.6: Breast Cancer Treatment (Fisusi and Akala, 2019)

Chemotherapy is an effective method used to treat tumors, despite its toxic effect on the healthy tissues (Bauersfeld *et al.*, 2018). Chemotherapy drugs inhibit cell cycle and DNA replication. Most drugs used in chemotherapy benefit from a lack of cell control mechanisms and genetic vacillation. Genetic instability occurs as a result of the failure of DNA repair mechanisms. Moreover, chemotherapeutic drugs are used in anticancer to protect the chromosomal structure by using the properties of cancer cells (Korgaonkar and Yadav, 2019).

Nevertheless, the studies have shown that chemotherapy increases the immune system's response by crossing the barriers to immunotherapy. In addition, chemotherapeutic drugs facilitate the apoptosis of cancer cells and create an immune response with an antitumor effect on the tumor (Lan *et al.*, 2020). Responses to chemotherapeutic drugs in breast cancer differ according to the class of breast cancer. This condition is called heterogeneity of breast cancer. In addition, breast cancer types create resistance to drugs according to their genetic, epigenetic, and metabolic characteristics (Varghese *et al.*, 2020).

1.4. TYROSIN KINASE INHIBITORS

Receptor tyrosine kinases (RTKs) are a subclass of tyrosine kinases involved in intercellular communication and controlling various biological processes such as cell growth and differentiation. RTKs are protein structures consisting of an extracellular binding domain, transmembrane regulator, and tyrosine kinase domain. Irregularity of RTKs by mutation can cause many diseases such as cancer (Du and Lovly, 2018). However, growth factors bind to receptors such as tyrosine kinases and activate various signaling pathways to induce cancer progression. In addition, tyrosine kinases are expressed different cell types. Vascular endothelial growth factor receptors (VEGFRs), insulin-like growth factor receptors (IGFRs), platelet-derived growth factor receptors (PDGFRs), epidermal growth factor receptors (EGFRs), fibroblast growth factor receptors (FGFRs) being breast cancer are tyrosine kinase receptors, which are found in many types of cancer. As the increase in the level of tyrosine kinase receptors accelerates the spread of breast cancer in the cell, the reduction of tyrosine kinase receptors by binding ligands causes a decrease in the activation of signal molecules. Moreover, tyrosine kinases are signal transducer and activators of transcription (STAT), Janus kinase (JAK)/signal transducer and mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/Akt. Since tyrosine kinase receptors has an important role in breast cancer progression, treatments targeting tyrosine kinase receptors are being developed (Butti *et al.*, 2018). The combination of tyrosine kinase receptors and CDK4/6 inhibitors has been promising in treating breast cancer (Jabbarzadeh *et al.*, 2020). Inhibition of tyrosine kinases, which are epidermal growth factor receptors, is a commonly used research in cancer therapy. However, several drugs have been and continue to be developed for the inhibition of tyrosine kinases (Basuli *et al.*, 2011). ERBB targeted chemotherapeutic agents are used in cancer treatment as tyrosine kinase inhibitors. Particular antibody drugs directed against the receptors inhibit ERBB receptors with tyrosine kinase activity at different levels (Hyochol Ahn *et al.*, 2017).

Tyrosine kinase inhibitors targeting HER2 are two specific monoclonal antibodies and tyrosine kinase inhibitors. Four drugs are approved by the Food and Drug Administration (FDA). Lapatinib, neratinib, trastuzumab, and pertuzumab are used to treat HER2-positive breast cancer. Tyrosine kinase inhibitors are small molecular drugs that induce apoptosis and inhibit cell proliferation. These drugs inhibit tyrosine kinase phosphorylation by binding the

ATP sites of growth factor receptors and stopping signaling pathways (Figure 1.7) (Xuhong *et al.*, 2019).

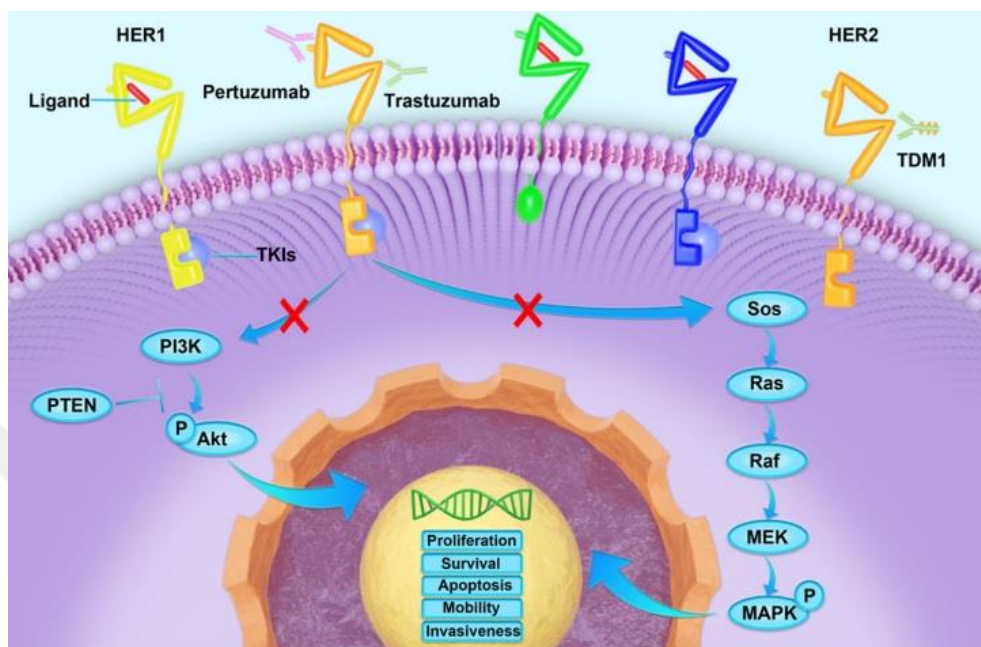


Figure 1.7: Mechanism of tyrosine kinase inhibitors targeting HER2 and EGFR receptors. (Xuhong *et al.*, 2019)

1.5. LAPATINIB

HER2 (ERBB2), the protein tyrosine kinase receptor, is one of the epidermal growth factors. Heterodimerization of the HER2 receptor causes the phosphorylation of tyrosine residues in the cytoplasmic domain with the overexpression of HER2. HER2 induces signaling pathways that lead to cell proliferation and tumorigenesis (Oh and Bang, 2020). Tyrosine kinase inhibitors are small molecules and attach to ATP-linked parts of the tyrosine kinase sites of the EGFR family. Different tyrosine kinase inhibitors differ according to the binding site. For instance, some bind either to the EGFR receptor alone or to the HER2 and EGFR receptors (Abo-Zeid *et.al.*, 2019).

Lapatinib is a tyrosine kinase inhibitor that can bind to both the EGFR receptor and the HER2 receptor, which involves normal and abnormal cell proliferation (Andriamanana *et al.*, 2013). However, it is a drug used in HER2-positive breast cancer (Jerusalem *et.al.*, 2019; Zajdel *et al.*, 2019). The FDA approved the usage of lapatinib with other chemotherapeutic agents for overexpression EGFR and HER2 of metastatic breast cancer treatments (Abo-Zeid *et.al.*, 2019). However, lapatinib becomes the first tyrosine kinase inhibitor effective in HER2-

positive breast cancer (Bredin *et.al.*, 2020). This drug inhibits the phosphorylation of HER1 and HER2 receptors and prevents phosphorylation of the EGF receptor and HER2 by inhibiting the ATP regions for the cell and in this case, the Raf, mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) signaling pathways are downregulated (Liao *et al.*, 2020). Cell proliferation is restricted, and apoptosis is induced (Huynh *et al.*, 2019; Xuhong *et al.*, 2019). However, lapatinib is better at inducing apoptosis of tumor cells than other monoclonal antibodies (Gao *et al.*, 2013, 2014).

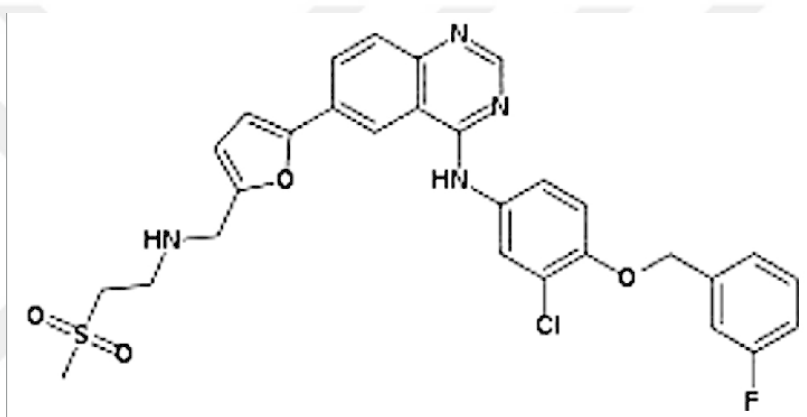


Figure 1.8: The structure of lapatinib (Kumar *et.al.*, 2012)

Clinical studies have shown that in the treatment of breast cancer, lapatinib can be used alone and with other drugs and is well tolerated. Another study showed that lapatinib, which inhibits EGFR and HER2 receptors, overexpress the STAT3 and pSTAT3 genes, which inhibit the proliferation of cancer cells (Guan *et al.*, 2018). Lapatinib is a drug that has proven its effectiveness in clinical and clinical models. In pre-clinical studies have shown that lapatinib reduces genes involved in nucleotide formation and exerts synergistic effects with other chemotherapeutic agents (Perry *et al.*, 2010). In addition, it has been clinically proven that lapatinib in combination with other chemotherapeutic drugs increases the efficacy of lapatinib in breast cancer metastasis (Basuli *et al.*, 2011). The FDA approved the combination of lapatinib with capecitabine in 2007 instead of the treatment method previously failed with trastuzumab in treating metastatic breast cancer. Approval was also obtained for the combination of lapatinib and letrozole for use in treating HER2 and ER-positive breast cancer by the FDA. Therefore lapatinib is an inhibitor drug that is also ideal for multiple anticancer

mechanisms (Wang *et al.*, 2014; McDermott *et al.*, 2019). However, the combination of lapatinib with other chemotherapeutic drugs makes the treatment of breast cancer patients effective and prolongs their survival. Lapatinib penetrates cells more quickly and crosses the blood-brain barrier more easily in treating brain metastasis in breast cancer patients than trastuzumab (Chen *et al.*, 2016). In addition, the combination of lapatinib with capecitabine is used effectively in patients with HER2 positive breast cancer who are resistant to trastuzumab and have had brain metastases (Chintalaramulu *et al.*, 2020). Studies have shown that chemotherapeutic drugs with lapatinib and endocrine therapy prevent brain metastasis in HER2 positive metastasized breast cancer patients (Gui *et al.*, 2020; Khan *et al.*, 2020). Although lapatinib is an effective drug, it has undesirable side effects. For example, it is poorly soluble in water, which reduces its absorption in the intestine, lowers bioavailability, and damages the gastrointestinal structure. Therefore, it does not allow lapatinib to be used as an injectable drug. Its low solubility in water has highlighted the application of lapatinib with nanoparticles (Gao *et al.*, 2014).

1.6. DRUG DELIVERY SYSTEM AND NANOPARTICLES

Nanotechnologies have been applied as new and effective intermediaries in many technological fields. Furthermore, these applied areas have led to the production and development of different types of nanomaterials. Nanotechnology, which aims to examine materials' properties and usage areas, is also used in various fields. In the biomedical field, it is widely used to develop nanotechnological drug delivery systems (Vangijzegem *et al.*, 2019).

Nanotechnology-based drug delivery systems are designed to affect ultrasound, physical signals such as heat, ultrasound, light, and the physical structure of the tumor, and it increases the therapeutic effect on cancer cells (Zheng *et al.*, 2020). Cancer nanotechnology is an interdisciplinary field of application that provides early diagnosis and prevention of cancer by covering various fields of science such as biology, chemistry, and engineering. Nanoscale drugs have begun to be produced, and these target-specific drugs only damage cancer cells and ignore normal cells. This situation has recently become an important treatment method for cancer treatments (Zhang, 2019). Encapsulated drug delivery systems reduce cytotoxicity in healthy cells in cancer treatments and increase a drug's effect on tumor cells (Iturrioz-

rodríguez *et al.*, 2019). Drug delivery systems have played an essential role in medicine as an excellent alternative to traditional drugs in cancer treatment. Moreover, drug delivery systems effectively increase the strength of biomolecules and protect them from degradation (Garbayo *et al.*, 2020). Many chemotherapeutic drugs are hydrophobic; therefore, their solubility in the systemic circulation is low, and they may have some problems in drug transport and side effects. Some nanocarriers have been developed to overcome these obstacles (Xue *et al.*, 2019). Nanomaterials are intended to be used for imaging purposes and for transporting drugs in targeting and distribution areas. Moreover, carbon nanotubes, polymers, metal oxides, metals are some of the nanomaterial types (Sábio *et al.*, 2019). However, nanocarriers consist of lipid-based, polymeric, and inorganic nanocarriers and subgroups (Figure 1.9). Nanocarriers and nanoparticle drug delivery systems increase the effectiveness of the drug users due to their permeability and retention properties to tumor tissues. With target-specific ligands such as antibodies, peptides, folic acid, and aptamers, nanoparticles become more functional and allow drugs to go directly to the target. In this way, drug delivery systems with reduced side effects can be developed according to the size of the surface physicochemical properties of the target (Jin *et al.*, 2020).

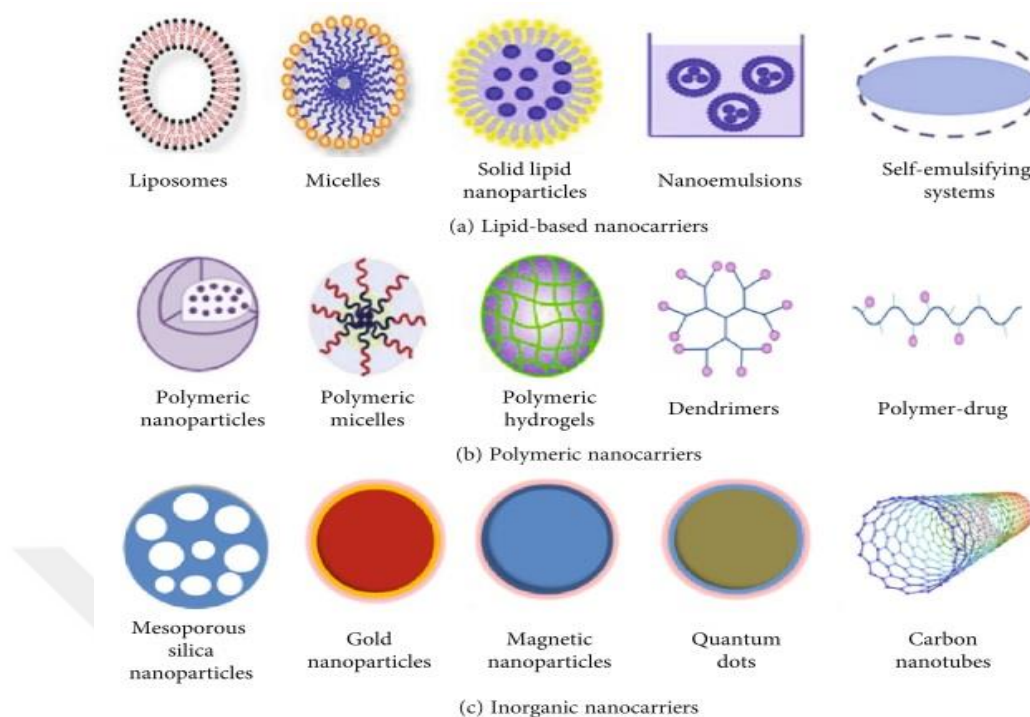


Figure 1.9: Various nanocarriers used in cancer treatment (Jin *et al.*, 2020)

Nanoparticles improve the stability and solubility of drugs, increase the efficiency of transport between membranes and extend circulation times (Mitchell *et al.*, 2020). In addition, drug delivery systems can control drug release based on signals and physical stimuli in the tumor microenvironment. These transport systems are classified as systems that respond to endogenous, exogenous, and multiple stimuli according to the signals' physical and chemical structures and sources (Li *et.al.*, 2019). In breast cancer treatments, chemotherapeutic drug-loaded nanoparticles administer active or passive drug therapy to tumors. The advantage of using nanoparticles is that they are designed in small size and make nanoparticles advantageous in the structure of the materials from which they are produced (Tran *et al.*, 2020). Mesoporous silica, viral nanoparticles, carbon, metal, and polymer-based nanoparticles are used for breast cancer treatment. As a result, there are many advantages of using nanoparticles in cancer treatments: 1) Overcomes the problem of poor solubility by increasing the bioavailability of the drug, 2) It provides a slow release by facilitating the permeability of the drug to cancer cells, 3) Nanoparticles are small in size (100 nm) can be loaded with cancer drugs and biodegradable particles (Liyanage *et al.*, 2020).

1.7. EXOSOMES

Cells communicate with neighboring cells and distant organs through extracellular vesicles (EVs) that carry various organic compounds (such as protein, genomic DNA, RNA). These vesicles are classified according to both their size and biogenesis. However EVs are divided into 3 subgroups (Yamashita *et al.*, 2018; Chung *et al.*, 2019; Fang and Vangsness, 2020). These are apoptotic bodies, microvesicles/ectosomes, and exosomes (Figure 1.10) (Sciences, 2020).

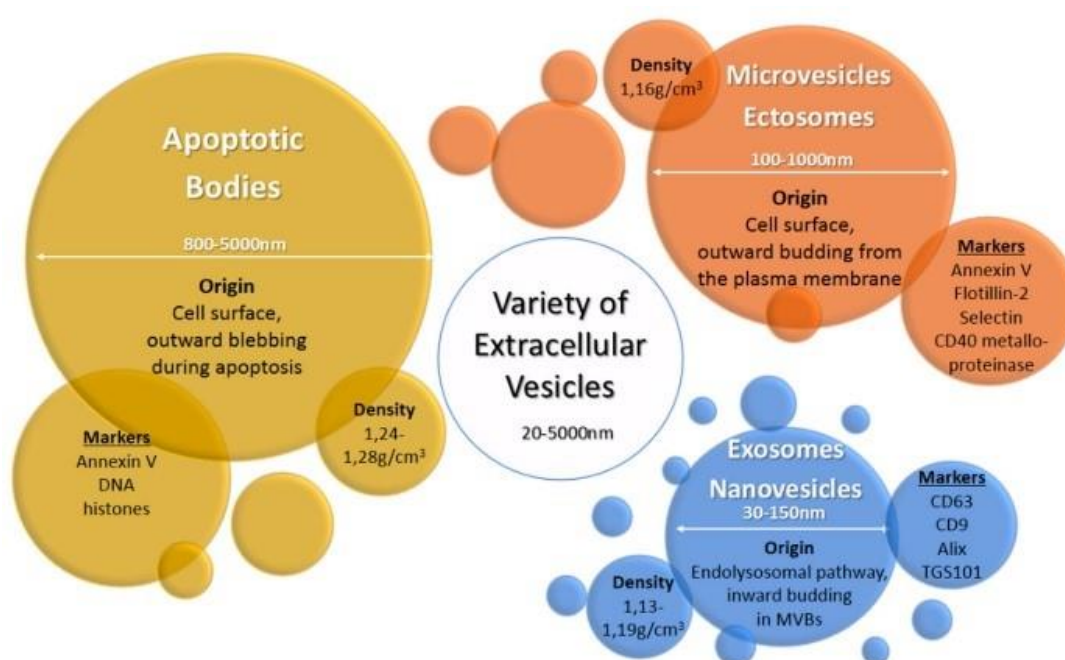


Figure 1.10: Subgroups of Extracellular vesicles (EVs)(Sciences, 2020)

Apoptotic bodies are vesicles that occur due to apoptosis in the cell. Exosomes and microvesicles are released from healthy cells, while apoptotic bodies are released from dying cells that undergo apoptosis. Moreover, apoptotic bodies carry nuclear DNA and protein histones (Zhang and Yu, 2019). Their diameters are usually around 500 to 5000 nm. It is also the largest type of EV (Yi *et al.*, 2020). Microvesicles/ectosomes produced by direct efflux from the plasma membrane range in size from 100 to 1000 nm. In addition, microvesicles play an important role in cell differentiation, cell communication, development, and remodeling of tissues and organs (Raghu Kalluri, 2014; Ratajczak, 2020). Exosomes are nano-sized and also called biological vesicles, are approximately 50-200 nm in size (Yu *et al.*, 2015; Agrawal *et al.*, 2017). Exosomes are intraluminal vesicles formed by the budding separation of multivesicular structures from the cell membrane (Fang and Vangsness, 2020).

Exosomes are known as heterogeneous collections of vesicles, and it also carries the phenotypic state of the cell from which it was produced. Besides, they are produced by cells such as immune cells, dendritic cells, macrophages, mast cells, neurons, tumor cells, oligodendrocytes, B cells, and T stem cells. They can circulate freely in the body's circulation (Faruqu *et al.*, 2018; Sharma *et al.*, 2020). Moreover, exosomes can be produced by non-hematopoietic cells like tumor cells, adipocytes, epithelial cells, neurons, mesothelioma cells, astrocytes, melanocytes, and fibroblasts (Baig *et al.*, 2020).

Previously, exosomes were thought to be a mechanism that expels wastes formed in the cell. In the 2000s, it was proved that exosomes play a role in intercellular communication. In the following years, it has been demonstrated that exosomes can load various molecules and transfer them between cells by acting as Cargo (Zhao *et al.*, 2020). They provide communication between close or far-range cells with the effect of signals such as growth factors, proteases, and cytokines (Harris *et al.*, 2015). Exosomes are excreted from the cell and move into the body fluid to change the activity of neighboring cells. They can be found in many body fluids such as lymph, amniotic fluid, mammary gland, blood, breast milk, tears, cerebrospinal fluid, saliva, nasal mucus, and urine (Sciences, 2020; Y. Wang *et al.*, 2020).

Exosomes consist of a lipid bilayer and also, like derivated cells, carry molecular structures such as DNA, protein, and RNA. The lipid bilayer structure of exosomes is similar to the cell from which they originated (Kalluri, 2016; Yin *et al.*, 2019). The plasma membranes of exosomes are composed of various lipids such as ceramides, phosphatidylserine, saturated fatty acids, cholesterol, and sphingomyelin (Logozzi *et al.*, 2018; Xunian and Kalluri, 2020). The lipid bilayer around the exosome protects the exosome content against RNase and proteases, making it a suitable carrier in therapy (Zheng *et al.*, 2019). Exosomes contain various RNAs like (mRNA, rRNA, tRNA, case RNA, Y-RNA, long-noncoding RNAs (lncRNAs), microRNA (miRNA)), and proteins (Kim *et al.*, 2018; McAndrews and Kalluri, 2019). The proteins in the exosome have various functions such as exosome biogenesis (ESCRT complex, ALIX, TSG101, and syntenin), membrane transport and fusion (annexins, flotillins, and Rab GTPases), tetraspanins (e.g., CD9, CD63, CD81, and CD82), integrins, major histocompatibility complex (MHC) class II proteins, and heat shock proteins (HSP70, HSP90) (Olejarz *et al.*, 2020). Rab family proteins provide the secretion of exosomes. Primarily, Rab 35 interacts with GTPase to regulate exosome secretion. Proteins involved in cytoskeleton and metabolism include podophyllin, tubulins, myosin, glyceraldehyde 3-

phosphate dehydrogenase, β -actin, and major histocompatibility complex (MHC) class I and II molecules, are included in the exosome-forming proteins (Figure 1.11). In addition, exosomes have proteins involved in cell signaling pathways such as interleukins, Wnt- β -catenin, and Notch ligand Delta (Azmi *et al.*, 2013).

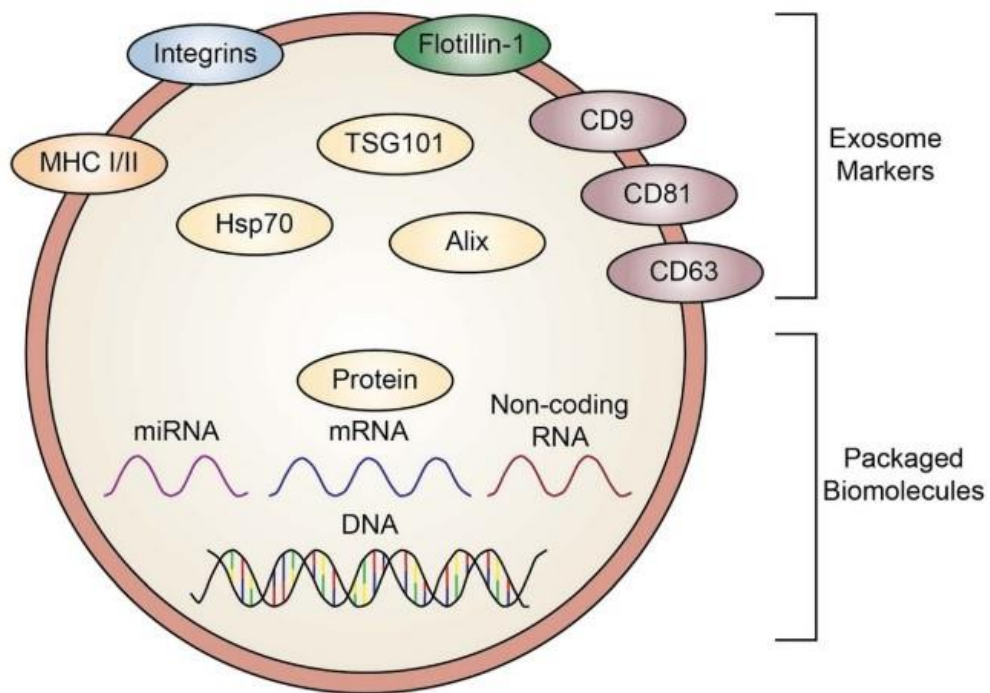


Figure 1.11: The structure of Exosomes (Azmi *et al.*, 2013)

Exosome biogenesis is regulated by the endosomal sorting complex required for transport (ESCRT). ESCRT consists of 4 complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. These complexes contribute to recognizing exosome-directed targeting proteins such as ALIX, VPS4, and VTA1 and the formation of MVB vesicles. Some studies have shown that ESCRT independence is significant for tetraspanin proteins (CD81, CD9, etc.) responsible for cargo loading and sorting into the exosome. Tetraspanins are exosome surface markers and transmembrane proteins that can interact with various receptors. ESCRT-dependent and ESCRT-independent pathways work synergistically by forming different pathways in different exosome biogenesis (Mashouri *et al.*, 2019; Xunian and Kalluri, 2020).

Exosomes induce many biological events through the biomolecules they carry. Studies have demonstrated that exosomes play important roles in intercellular information transfer in many diseases like cancer, Alzheimer's, and diabetes. Therefore exosomes can be used as

therapeutic agents in these diseases. They may even be effective as diagnostic markers (Yamashita *et al.*, 2018). Exosomes have been used as natural nanocarriers to transport various pharmaceutical molecules and drugs due to their stability in circulation, cell targeting abilities, and adaptation to the immune system by showing less toxicity than nanocarriers (Naseri *et al.*, 2020).

1.8. EXOSOMES ISOLATION AND CHARACTERIZATION METHODS

There are exosomes in various body biofluids, and there are multiple proteins in these currents that make the analysis of exosomes difficult. For this reason, isolation of exosomes from body fluids is among the most important requirements (Bu *et al.*, 2019). The methods developed and used to isolate exosomes are very important in isolating exosomes in high quality and large numbers. Exosomes can be isolated using ultracentrifugation, buoyant and differential intensity centrifugation, polymeric precipitation, size exclusion, filtration, immunoaffinity separation and column chromatography. The most commonly used method when isolating exosomes from body fluids or cell culture is buoyant and differential intensity centrifugation. After the exosomes are isolated, they can be analyzed by immunoblotting, proteomic techniques, or protein staining (Kesimer and Gupta, 2015; Gurunathan *et al.*, 2019).

Characterization of isolated exosomes can be done based on their morphology, surface marker proteins, and size. The most common method used to characterize exosomes is electron microscopy. However, flow cytometry and dynamic light scattering (DLS) are standard methods used to identify exosomes (Zhang *et al.*, 2016; Naseri *et al.*, 2020). Western blot and immunosorbent studies cannot determine the number of exosomes. However, they can not separate exosomes from microvesicles. Recently, atomic force microscopy has been used to determine exosomes' morphology and size distribution. DLS measures exosomes according to the size of light scattered from particles under Brownian motion. But it tends to measure larger particles. One of the most advanced methods used to analyze exosomes is nanoparticle tracking analysis (NTA), and this method performs real-time measurement. NTA visualizes exosomes by scattering light with a light microscope under Brownian motion. With the NTA program, the movement of the vesicles is monitored, and their size and concentration are determined (Dragovic *et al.*, 2011).

1.9. EXOSOMES AS NANOCARRIER

Recently, the most preferred drug delivery systems are polymeric and liposome nanoparticles. These synthetic nanoparticles provide both advantages and disadvantages. Although liposomes' stability and circulatory capacity are useful, their toxicity is not yet very developed. However, despite good stability, polymeric nanoparticles are considered unsafe for long-term use (Ha *et.al.*, 2016). The potential to transmit molecules such as RNA and protein, which exosomes carry in their natural structure, to the recipient cells has revealed the idea that exosomes can carry nano-sized agents (Faruqu, Xu and Al-Jamal, 2018). In this case, exosomes, which have the characteristics of an ideal drug transport system, as a vehicle in the transport system, come to the fore (Ha *et.al.*, 2016). Exosomes, which are structurally and functionally similar to nanoparticles, are an alternative used in cancer treatments. Therefore they can be used as nanocarriers in anticancer treatments (Vázquez-Ríos *et al.*, 2019).

In recent studies, the benefits of exosomes, which are used as natural transport vehicles, are investigated and applied (Kalluri, 2016). Moreover, exosomes isolated from epithelial cells, lymphocytes macrophages, cancer cells, and mesenchymal stem cells have been applied in the drug delivery system in studies carried out so far. Some anticancer agents such as doxorubicin, paclitaxel, and curcumin were used in these studies. These exosomes deliver drugs to the target cell and change its function (Johnsen *et al.*, 2014; Liu *et al.*, 2019). Biocompatibility, natural and high stability, and low toxicity are important features that distinguish the exosome from synthetic nanoparticles. Unmodified exosomes have a low cytotoxic effect, and also exosomes have low immunogenicity compared to other nanoparticles (Jiang and Gao, 2017; Pullan *et al.*, 2019). The exosome is in small structures allows it to be used efficiently by overcoming difficult obstacles such as the blood-brain barrier (BBB). In addition, since the exosome membrane is covered with lipid and protein, it is easy to reach the target cells (Zheng *et al.*, 2019). These nano-sized exosomes also have a phospholipid bilayer structure. In this way, it can pass directly through the targeted cell membrane. This fusion feature bypasses the endosomal lysosomal pathway used by synthetic nanoparticles and provides a direct effect (Tian *et al.*, 2014). Exosomes can reach tumor tissues by escaping from monocytes with enhanced permeability and retention (EPR) in tumor tissues and transmit the agents they mediate (Bu *et al.*, 2019). Exosomes provide all the advantages of synthetic nanocarriers and cell-mediated transport systems. The use of

exosomes in the drug delivery system eliminates the toxicity, low solubility and short half-life of chemotherapeutic drugs. All these advantages show that the exosome can be an effective and ideal vehicle for delivering chemotherapeutic drugs and can be used in cancer treatments (Tian *et al.*, 2014; Kim *et al.*, 2016a).

1.10. DRUG LOADING TECHNIQUES INTO THE EXOSOMES

Exosomes have been used in the drug delivery system and are as effective as synthetic nanoparticles. However, they are referred to as biological drug transport vehicles. In addition, exosomes can be used for drug targeting by modifying the their membranes (Figure 1.12) (M. Li *et al.*, 2020; Kim *et al.*, 2021).

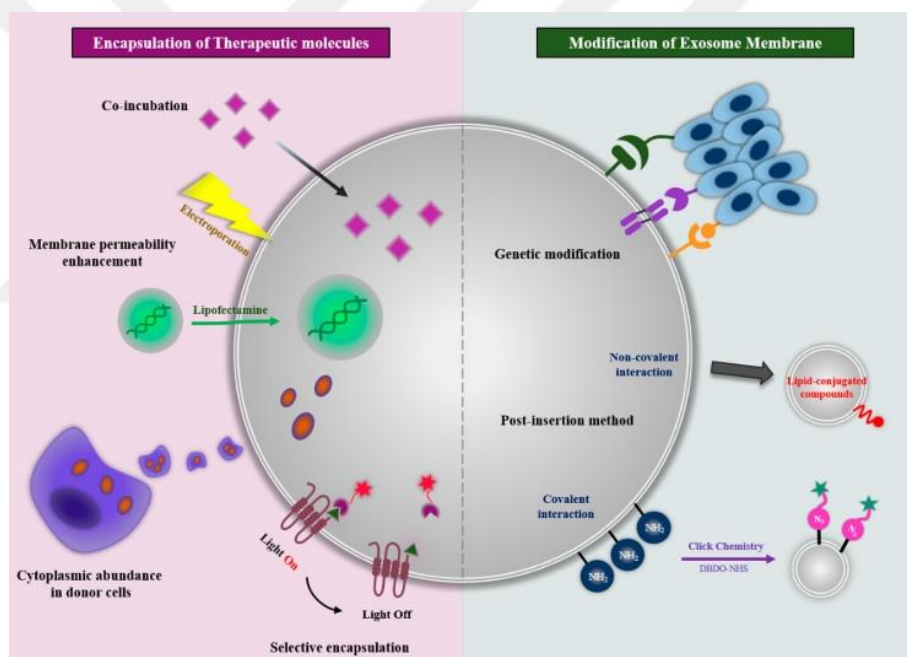


Figure 1.12: Encapsulation and Modification of Exosomes for Treatment (Kim *et al.*, 2021)

Efficient amounts of drug-loaded on exosomes are transported to the recipient cell, which depends on a successful and effective drug loading method. It has been shown in many studies that exosomes can be loaded with drugs using co-incubation, some chemicals such as saponin, electroporation, ultrasound, freeze-thaw flow, and ultracentrifugation methods (M. Li *et al.*, 2020). All methods used to load exosome drugs have their advantages and disadvantages. In the incubation loading method, less drug was loaded on the exosome. While loading the exosome through chemicals such as saponin, saponin may cause increased cytotoxicity in cells. On the other hand, mechanical methods such as ultrasonic or

ultracentrifuge can change the structure of exosomes. In this study, we preferred the electroporation method to load lapatinib into the exosome (Salarpour *et al.*, 2019).

In the electroporation method, the exosomes and the agents desired to be loaded mixed in a solution, and an electric field is applied. Pores form in the lipid bilayer membrane, and the therapeutic agent moves into the exosome. Some studies have shown that the exosomes are loaded effectively by applying electroporation in a voltage range of 150-700V. In addition, these optimal values differ according to the cells from which exosomes are obtained. The applicability and characterization of the electroporation method can be preferred because it is easy to perform; however, it has some disadvantages. Exosome particles can form aggregation by electroporation, but some optimized studies have shown that aggregation decreases at low voltage. As a result, electroporation is a method used in the literature for drug loading, and chemotherapeutic drugs were loaded with this method, and an effective delivery was achieved (Johnsen *et al.*, 2014).

1.11. THE AIM OF THIS THESIS

In this thesis, exosomes isolated from the healthy epithelial cell line MCF10 A were used as nanoparticles. Isolated exosomes were characterized with NTA, zeta sizer, AFM, and flow cytometry. To measure the amount of lapatinib loaded into exosomes was designed the HPLC method. Meanwhile, the cytotoxic effects of lapatinib on SKBR-3 and MCF10 A cells were evaluated. After IC_{50} values of lapatinib were determined, experimental groups were designed. We investigated and compared the cytotoxic and apoptotic effects of free lapatinib, exosome-encapsulated lapatinib, and empty exosomes on HER2 positive breast cancer cell line SKBR-3 and healthy epithelial cell line MCF10. We aim to increase the cytotoxic and apoptotic effects of exosome encapsulated lapatinib on the HER2 positive breast cancer cell line SKBR-3 at low concentration. We also aimed to investigate the effects of lapatinib and exosome-encapsulated lapatinib on specific cell signaling pathways and to examine how they affect the expression of particular genes and proteins in HER2 positive SKBR-3 and MCF10 A cells.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

MCF10 A human breast epithelial cell (ATCC CRL-10317) and SKBR-3 breast cancer cell (ATCC HTB-30) lines were obtained from ATCC, USA.

2.1.1. Cell Thawing

Cryovial tubes (TPP 89020, Switzerland) were dissolved gently after being taken from the nitrogen tank to revive each frozen cell line. Approximately 1.5 ml cells in the medium in the cryovial tubes were quickly passed to the 15 ml tube (Isolab, Germany) containing 2 ml of growth medium. Then the cells in the tube were centrifuged at 1300 rpm for 5 min, and the supernatant was discarded to remove dimethyl sulfoxide (DMSO, #D4540, Sigma-Aldrich, USA), and the pellet was resuspended with a fresh medium. After that, the cells were seeded into the tissue culture flask in a growth medium containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco 10500-056, Thermo Fisher Scientific, USA) and 1% Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK). All cells were incubated in a humidified incubator at 37°C, with 95% humidity and 5% CO₂ conditions (In-Vitro Cell ES NU-5800, NuAire, USA).

2.1.2. Cell Lines and Culture Conditions

In this study, healthy epithelial breast cell (MCF10 A) and HER2 breast cancer cell (SKBR-3) lines were used. MCF10A cells were grown in MEBM Bullet kit (2.00 mL BPE, 0.50 mL hEGF, 0.50 mL Insulin, 0.50 mL Hydrocortisone) (Lonza CC-3150, Switzerland) containing 10% FBS and 1% PSA. SKBR-3 cells were cultured in RPMI 1640 medium (Gibco BE12-702F, Thermo Fisher Scientific, USA) in supplemented with 10 % FBS and 1% PSA. All cells were cultured in the humidified incubator at 37°C, with 95% air and 5 % CO₂.

2.1.3. Cell Subculturing

Healthy breast cell (MCF10 A) in Figure 2.1 and breast cancer cell (SKBR-3) lines in Figure 2.2 were passaged at 70- 80% confluency. The cell monolayer was washed once with 1 x Phosphate Buffered Saline (PBS) (Lonza BE17-517Q, Switzerland) and incubated with 0.5%

trypsin-EDTA (Gibco 25200056, Thermo Fisher Scientific, USA) at 37°C for 5 minutes. A complete growth medium was added to the cell suspension to eliminate trypsin activity. After that, the cell suspension was centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in their unique, complete growth medium, and cells were seeded in cell culture flasks (SPL Life Sciences, Korea).

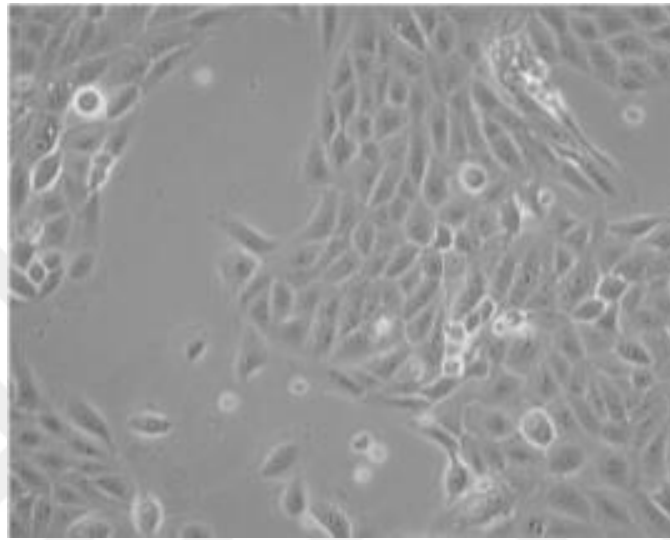


Figure 2.1: MCF10 A human breast epithelial cell (40X)

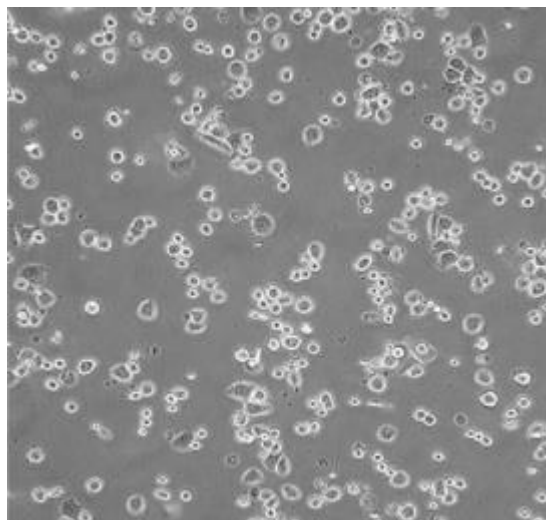


Figure 2.2: SKBR-3 breast cancer cell lines (40X)

2.1.4. Cryopreservation of Cells

The cell pellet was dissolved in 1 ml of freezing mix solution with a cell density of 1×10^6 cells/mL in each cryovial. The freezing solution was prepared by mixing 10% DMSO and 90% complete growth medium. Cells were immediately stored at -80°C freezers (New Brunswick, U410, Germany) overnight before transferring to a liquid nitrogen tank for long-term storage.

2.2. ISOLATION AND CHARACTERIZATION OF EXOSOMES FROM MCF10 A CELLS

2.2.1. Exosome Isolation from MCF10 A cells

Exosomes were isolated from a non-tumorigenic epithelial cell line (MCF-10A). Isolation of exosomes was performed using an aqueous two-phase system (ATPS) method introduced and reported by Kırbaş (Kırbaş *et al.*, 2019). First of all, MCF-10A cell lines were grown in T150 flasks with their recommended media supplemented with 10% FBS, 2.00 mL BPE, 0.50 mL VEGF, 0.50 ml Insulin, 0.50 ml Hydrocortisone, and 1% PSA, in a humidified 37°C incubator with 5% CO_2 . When reaching confluency of 60–70% in culture flasks, cells were cultured in exosome-depleted FBS media in 3 days. After harvesting, cell media were obtained for exosome isolation. The medium was centrifuged, the pellet was discarded, and the media was filtered. From each filtered, 20 ml of medium was added to 50 ml falcon tubes with 20 ml of PEG (Sigma Aldrich, 25322-68-3, Germany) / DEX (Sigma Aldrich, 9004-54-0, Germany) solution then was centrifuged. PEG/DEX solution was prepared, and 20 ml PEG/DEX along with 20 ml of distilled water was added to 50 ml tubes, then it was centrifuged at $3000\times g$ for the washing solution. 32 ml of supernatant was discarded from each tube and added 32 ml washing buffer (first washing). After the first centrifugation, discarded and added 32 ml washing solution was centrifuged again (second washing). The supernatant was discarded up to phase. As a result, the washings at the end of tubes were also formed in two phases. Formed dex exosomes sub-phase was collected in another tube. Then collected dex exosomes were stored at -80°C (Kırbaş *et al.*, 2019).

2.2.2. Characterization of Isolated Exosomes

Particle size, and density characterizations of exosomes that isolated from MCF10 A cells were performed by Nanoparticle Tracking Analysis (NTA), AFM (Atomic Force Microscopy (AFM)), and zeta sizer. Moreover, exosomes were confirmed by flow analysis according to the surface antigens.

2.2.2.1. Nanoparticle tracking analysis (NTA)

Using isolated exosomes, Nanoparticle Tracking Analysis (NTA) (Malvern Panalytical) measurements were performed with a NanoSight NS300 instrument. The exosomes were diluted 1:1000 with dH₂O. The particle size distribution and the isolated exosome data concentration were analyzed with the NTA 3.3 Analytical Software Suite. All procedures were performed at room temperature. The sample was evaluated in 5 replicates.

2.2.2.2. Atomic Force Microscopy (AFM)

Isolated exosomes derived from MCF10 A cells were characterized by the AFM method. Besides, AFM images of the exosome were obtained using the method reported in the literature (Skliar and Chernyshev, 2019). Briefly, the upper surface of the mica was treated with a 10 mM NiCl₂ (100 μ L) solution to change the surface charge from negative to positive at room temperature. The mica surface was washed 3 times with deionized water. The AFM sample disc was added to the surface-modified mica and placed in the petri dish. The exosomes we isolated were diluted 1:1 with PBS. Then 100 μ l of diluted exosomes were fixed on mica in a droplet form. The sample was covered with parafilm and incubated for 12 hours at 4 °C. After incubation, 80–90% of the sample was aspirated from the face without disturbing the surface, and exosomes were immobilized on the mica surface. Finally, the surface is washed 3 times with PBS, and the AFM image is taken.

2.2.2.3. Zeta Sizer

Dynamic Light Scattering (DLS) measurements were done at 25 °C using a Nano Zeta sizer (Malvern Panalytical). The isolated exosomes were determined using zeta sizer Nano instrument according to their size distribution and concentration. The exosomes were diluted in deionized water at 1:1000.

2.2.2.4. Flow Cytometry Analysis

5 μ l (5×10^6) latex beads and 2,5 μ l (25×10^6) exosomes were mixed and completed to 200 μ l with 2% BSA (Blocker BSA (10X) in PBS (Thermo Fisher Scientific, 37525, USA)) solution

and incubated for 1 hour at room temperature. 100 mmol glycine (Sigma Aldrich, 175838, Germany) was added into the mix and incubated for 30 minutes at room temperature. 800 μ l PBS was added and centrifuged at 5000 rpm for 5 minutes for washing. The supernatant was removed, and 800 μ l PBS was added and centrifuged at 5000 rpm for 5 minutes for washing again. The supernatant was discarded, and the pellet was dissolved with 500 μ l of PBS. 100 μ l from the sample was added into each of 5 micro tubes (Eppendorf Research, Germany). 1 μ l of FITCH anti-human CD9 Antibody (Biolegend, 312104, San Diego, CA), PE anti-human CD63 Antibody (Biolegend, 353004, San Diego, CA), PE anti-human CD81 (TAPA 1) Antibody (Biolegend, 349506, San Diego, CA) and Alexa Fluor 488 anti-HSP70 Antibody (Biolegend, 648004, San Diego, CA) antibodies respectively were added to each 4 micro tubes. No antibody was added to the 5th tube as it was a negative control. Each tube was incubated overnight. The tubes were centrifuged at 5000 rpm for 5 minutes. Then 500 μ l of PBS were added for washing and centrifuged at 5000 rpm for 5 minutes twice. All the samples were analyzed by flow cytometer (Merck Millipore, Germany).

2.3.DRUG LOADING WITH ELECTROPORATION AND CHARACTERIZATION

2.3.1. Encapsulation Lapatinib with Exosomes

Since the electroporation method was reported in several drug loading studies for exosomes (Tian *et al.*, 2014), we also performed our drug loading setting for isolated exosomes using electroporation. For the electroporation method, 50 μ M/L Lapatinib (Sigma Aldrich, CDS022971, Germany) was mixed in an equal volume of isolated exosomes (10^{10}) in 1 ml PBS at 350 V and 150 mF in 0.4 cm electroporation cuvettes using a Gene Pulser/MicroPulser Electroporation Cuvettes, (0.4 cm gap #1652081, Bio-Rad, USA), the mixture was incubated at 37°C for 30 min to restore the drug loaded exosomes. The unloadable lapatinib into the exosomes was discarded by the exosome spin column (MW 3000) kit (Thermo Fisher Scientific 4484449, USA).

According to this kit, the dry gel was collected at the bottom of the column with a spinner. The column of dry gel was hydrated with 650 μ L of double-distilled water. Then, the column was vortex to tap out air bubbles and hydrated at room temperature for 5–15 minutes. The spin column was placed in a 2 ml collection tube and centrifuged at 750 $\times g$ for 2 minutes at room temperature to remove the residual. After centrifuging, the collection tube was removed,

and added 500 μ L lapatinib-loaded exosomes were in the spin column. The spin column was placed in the 2 ml elution tube and centrifuged at $750 \times g$ for 2 minutes at room temperature. At the end of the centrifugation, drug-loaded exosomes were eluted into the elution tubes, and unloading drugs remained in the spin column.

2.3.2. Characterization of Lapatinib Loaded Exosomes by HPLC Analysis

First of all HPLC method was used to obtain the standard curve of lapatinib. For this, lapatinib were prepared at 5000 μ M in dimethylsulfoxide (DMSO). Then concentrations of lapatinib standards ranging from 4,1 to 333,3 μ M were arranged using acetonitrile. All analyses were performed using a C18 column (C18; 5 μ m; 4.6×250 mm) (XBridge, WT186003034, Germany) and measured absorbance of 268 nm. The mobile phase consisted of acetonitrile and double distilled water (80:20, v/v) at a 1 ml/min flow rate at 25°C. A standard curve was plotted using the area under the curve of the standards.

The amount of loaded drug in exosome studies was determined using the HPLC method. Accordingly, we optimized the HPLC method in our study and measured the amount of lapatinib loaded into the exosome (Kim *et al.*, 2016). Lapatinib-loaded exosomes were divided into the 2 ml microtubes as 1 ml of lapatinib-loaded exosomes, and 1 ml of methanol were added to these tubes. Afterward, centrifuged at 13000 rpm at 4°C for 20 min. Then, the methanol was evaporated from the collected supernatant, then dex was removed from the environment. Half of the lapatinib-loaded exosomes solvent was evaporated with a heating block (WiseTherm, HB-R, Switzerland) set to 75°C. Then, the sample was completed with an equal volume of acetonitrile, and the mixture was sonicated for 25 minutes. HPLC analyses of the samples were performed using a C18 column (C18; 5 μ m; 4.6×250 mm) and measuring the absorbance of 268 nm. The mobile phase consisted of acetonitrile and double distilled water (80:20, v/v) at a 1 mL/min flow rate at 25°C.

2.4. CELL VIABILITY ASSAY

2.4.1. Cytotoxic Effects of Lapatinib on SKBR-3 cells and MCF10 A cells

MCF10 A and SKB-3 cells were seeded onto 96-well plates (TPP, Switzerland) at a density of 5000 cells/well for MCF10 A and at a cell density of 7000 cells/well for SKBR-3. Lapatinib solution (10 mM) was prepared in DMSO. Different concentrations (0.1, 0.5, 1, 5, and 10 μ M) of the lapatinib were prepared in different media. After 24 hours, the cells were treated

with stated concentrations for 24, 48, and 72 hours. Cell viability was measured by the MTS reagent (3-(4,5-dimethyl-thiazol-2)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to the manufacturer's instructions. Briefly, after incubating cells with lapatinib for 24, 48, and 72 hours, 100 μ L of MTS solution was added to each well, and the plates were subsequently incubated for 2 hours at 37°C. Sample absorbance at 490 nm was detected with an ELISA plate reader (Biotek, Winooski, VT). This study was performed in triplicate.

2.4.2. Cytotoxic Effects of Lapatinib Loaded Exosomes on SKBR-3 Cells and MCF10 A Cells

MCF10 A and SKBR-3 cells were seeded onto 96-well plates (TPP, Switzerland) at a density of 5000 cells/well for MCF10 A and at a cell density of 7000 cells/well for SKBR-3. After 24 hours, the cells were treated with a determined concentration of lapatinib-loaded exosomes for 24, 48, and 72 hours. In this study, three different concentrations (0.1 μ M lapatinib (L)+ 10^8 exosomes (E), 1 μ M lapatinib (L)+ 10^9 exosomes (E) and 2.5 μ M lapatinib (L)+ 2.5×10^9 exosomes (E) particles/ml) of the lapatinib-loaded exosomes were prepared for RPMI 1640 medium and MEBM kit. The MTS assay was performed according to the manufacturer's instructions as mentioned section 2.4.1.

2.4.3. Cytotoxic Effects of Exosomes on SKBR-3 Cells and MCF10 A Cells

MCF10 A and SKBR-3 cells were seeded onto 96-well plates (TPP, Switzerland) at a density of 5000 cells/well for MCF10 A and at a cell density of 7000 cells/well for SKBR-3. Three different concentrations (10^8 exosomes (E), 10^9 exosomes (E), 2.5×10^9 exosomes (E) particles/mL) of the exosomes were prepared to RPMI 1640 medium and MEBM kit. After 24 hours of incubation, the cells were treated exosomes. The MTS assay was performed according to the manufacturer's instructions as mentioned section 2.4.1.

2.4.4. Cytotoxic Effects of Dex-phase on SKBR-3 Cells and MCF10 A Cells

In this section, MTS was done to measure the toxic effect of the dex phase remaining in the exosome isolation stage on SKBR-3 and MCF10 A cells. 10^8 dex phase (D), 10^9 dex phase (D) and 2.5×10^9 dex phase (D) particles/mL separate concentrations of the dex phase were prepared in different media. MCF10 A and SKBR-3 cells were seeded onto 96-well plates (TPP, Switzerland) at a density of 5000 cells/well for MCF10 A and at a cell density of 7000

cells/well for SKBR-3, after 24 hours incubation dex phase treated to the cells for 24, 48 and 72 hours. The MTS assay was performed according to the manufacturer's instructions as mentioned section 2.4.1.

2.5. EXPERIMENTAL GROUP DESIGN

According to the MTS results, we designed our experimental groups.

1. Control group: Non-treated MCF10 A and SKBR-3 cells.
2. Lapatinib group (Lap): 0.1 μ M lapatinib treated MCF10 A and SKBR-3 cells.
3. Lapatinib loaded exosomes group (LapExo): 0.1 μ M lapatinib and 10^8 exosomes particles/mL treated MCF10A and SKBR-3 cells.
4. Exosomes group (Exo): 10^8 exosome particles/mL treated MCF10 A and SKBR-3 cells.

2.6. CELL DEATH ANALYSIS BY ANNEXIN-V

SKBR-3 and MCF10 A cells were seeded onto 12-well plates (TPP, Switzerland) at a cell density of 1.5×10^6 cells/well for SKBR 3 and a density of 1×10^6 cells/well for MCF10 A and incubated for 24 hours in a humidified incubator at 37°C and 5 % CO₂. After 24 hours, while lapatinib (0.1 μ M lapatinib (L)), lapatinib loaded exosomes (0.1 μ M lapatinib (L)+ 10^8 exosomes (E)), and exosomes (10^8 (E) particles/mL) were applied to the experimental cell groups for 48h, no application was made to the control cell group. Following incubation, the cells monolayer was washed with 1 x PBS and trypsinized at 37°C for 5 minutes. Samples in each tube were centrifuged at $350 \times g$ for 5 minutes, then discarded the supernatant and added 1 mL of 1 x PBS. After that sample were centrifuged at $350 \times g$ for 5 minutes for washing. Dead Cell Apoptosis Kit with Annexin V Alexa Fluo 488 & Propidium Iodide (PI) kit (Thermo Fisher Scientific, V13245, USA) was used for apoptosis assay. Then the pellets were suspended in 100 μ L of Annexin binding buffer. 5 μ l of Annexin V and 1 μ l of PI solutions were added to each tube and incubated the cells under dark conditions at room temperature for 15 minutes. After incubation, 400 μ l of binding buffer was added to the samples and quickly analyzed by flow cytometry, (Merck Millipore, Germany). Gating parameters were set with untreated cells incubated with and without Annexin V or PI.

2.7. GENE EXPRESSION ANALYZES

2.7.1. RNA Isolation

SKBR-3 and MCF10 A cells were seeded onto 6-well plates (TPP, Switzerland) at a cell density of 2.5×10^5 cells/well for SKBR-3 and a density of 2×10^5 cells/well for MCF10 A and incubated for 24 hours in a humidified incubator at 37°C and 5% CO₂. After 24 hours, the control and experimental groups were created as mentioned section 2.5. After 48 hours, the cells were washed with 500 µl PBS, then incubated with 0.5 % trypsin-EDTA at 37°C for 5 minutes. When tyripsinization is completed growth medium added to the wells, and then the cells were centrifuged at 1500 rpm for 5 minutes. After removing the cell culture medium, the pellet was resuspended in 250 µl of NucleZOL reagent (MN, 740404.2, Netherlands) for homogenization and transferred into micro tubes, and added with 100 µL of nuclease-free water and vortexed for 15 seconds. Samples were incubated at room temperature for 5 minutes, then centrifuged at $12,000 \times g$ for 15 minutes. 250 µl of the supernatant for each tube was transferred to the new tubes, and 250 µl of 100 % isopropanol was added. Then the samples were incubated at room temperature for 10 minutes, centrifuged at $12,000 \times g$ for 10 minutes, and discarded the supernatant from each tube. 250 µl 75% ethanol was added to the pellet and centrifuged at $8000 \times g$ for 3 minutes for the washing twice. The supernatant was discarded after each washing. Furthermore, the last pellet was suspended in nuclease-free water (Thermo Fisher Scientific, 10-977-015, USA), and RNA concentration was quantified with Nano-Drop 2000 (Thermo Fisher Scientific, USA).

2.7.2. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA isolated from SKBR-3 and MCF10 A cells were transformed into cDNA products with Sensiscript Reverse Transcription (RT) kit (Qiagen, 205213, USA). The master mix was prepared according to Table 2.1. Template RNA and, master mix were added to the tube and then it was incubated at 37°C for 1 hour according to the manufacturer's protocol.

Table 2.1: Master mix of Sensiscript Reverse Transcription (RT) kit

Master Mix	Volume/ reaction
10 x Buffer RT	2 μ L
dNTP Mix (5 mM each dNTP)	2 μ L
Oligo dT primer (10 μ M)	2 μ L
Nuclease free water	Variable
SensiscriptReverse (RT)Transcriptase	1 μ L
Template RNA	Variable
Total reaction volume	20 μ L

2.7.3. Quantitative Polymerase Chain Reaction (QPCR)

The mRNA expression levels of target genes were determined with QuantiTect SYBR Green PCR Kit (Qiagen, 204145, USA). Reverse primer sequences and forward primer sequences used in gene analysis were given in Table 2.2. The reaction mixture was formed with 1000 ng (2 μ L) of cDNA, 1 μ L primer, 6 μ L RNase/DNase free water, and 10 μ L Syber Green PCR mix. Samples were studied in triplicate, and reaction conditions were listed in Table 2.3. The results of qPCR were evaluated by Bio-Rad CFX Manager software. The absolute quantification was determined using the obtained standard curve from the cycler, and relative quantification was normalized using the GAPDH reference gene.

Table 2.2: Forward and Reverse primer sequences

GENE	Forward Primer	Reverse Primer
ERBB1	AGGCACGAGTAACAAGCTCAC	ATGAGGACATAACCAGCCACC
ERBB2	TGCAGGGAAACCTGGAAGTC	ACAGGGGTGGTATTGTTTCAGC
AKT1	AGCGACGTGGCTATTGTGAAG	GCCATCATTCTTGAGGAGGAAGT
MAPK1	TACACCAACCTCTCGTACATCG	CATGTCTGAAGCGCAGTAAGATT
BCL-2	GGTGGGGTCATGTGTGTGG	CGGTTTCAGGTACTCAGTCATCC
TP53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
CASPASE-3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA
CASPASE-9	CTTCGTTTCTGCGAACTAACAGG	GCACCACTGGGGTAAGGTTT
GADPH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Table 2.3: RT qPCR reaction condition

STEP	TEMPERATURE	TIME
Initial Reaction	95°C	15 min
Denaturation	94°C	15 s
Annealing (40 repeat)	58°C	15s
Extension	72°C	15s
Final Extension	72°C	15 min

2.8. PROTEIN EXPRESSION ANALYSIS

2.8.1. Protein Isolation

SKBR-3 breast cancer and MCF10 A breast epithelial cells were seeded onto 6-well plates (TPP, Switzerland) at a cell density of 2.5×10^5 cells/well for SKBR-3 and 2×10^5 cells/well for MCF10 A. They were incubated for 24 hours in a humidified incubator at 37°C and 5% CO₂. After 24 hours, the cells were treated with lapatinib (0.1 µM lapatinib (L)), lapatinib

loaded exosomes (0.1 μ M lapatinib (L)+ 10^8 exosomes (E) particles/ML), and exosomes (10^8 (E) particles/mL) for 48 hours, the control group was formed from cells that did not receive any treatment. After 48 hours, the cells attached to the six-well plates were washed with 500 μ l PBS and incubated with 0.5% trypsin-EDTA at 37°C for 5 minutes. When tyripsinization was completed medium added to the cells, and then the cells were centrifuged at 300 $\times g$ for 5 minutes. After removing the cell culture medium, the pellet was resuspended RIPA buffer (Intron Biotechnology, IBS-BR002, Korean) and containing 0,1% protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78440, USA) on ice. The cell homogenized completely, lysates of the cell were centrifuged at 14000 $\times g$ at 4°C for 20 minutes. Then the supernatant was collected, and total protein concentrations were measured using a BCA Protein Assay kit (Thermo Fisher Scientific, 23225, USA). Then the protein was aliquoted and stored at -80°C until using for western blot analysis.

2.8.2. Western Blotting Analysis

All buffers used in this analysis, their ingredients and preperation methods were given in the Table 2.5. After the BCA assay, 50 μ g protein of each sample, 4X Laemmli buffer, and dH₂O were mixed at an equal volume, then heated each sample for denaturation at 95 °C for 5 min with a heat block. After that, each sample (20 μ l) were loaded in 12.5% SDS-PAGE gel (2% Mini-PROTEAN TGX Precast Protein Gels)(Biorad, 4561043, USA) for electrophoresis and run the gel with running buffer as first for 10 minutes at 85V after that for 45 minutes at 140V. At the end of the separation, proteins were transferred to the 0.45 μ m PVDF membranes (Thermo Fisher Scientific, LC2005, USA). The PVDF membranes were activated with methanol for 1-5 minutes. Proteins in the gel transferred to the membrane using 1X Transfer buffer for 2h at 200 mA. Then the membrane was incubated with blocking buffer with 5% nonfat dry milk to block for 1 hour at room temperature, and it was washed three times for 5 min each with 15 ml of TBST. Then the membrane was incubated with primary antibody (Table 2.4. 1:1000) in 10 ml primary antibody dilution buffer with soft shaking overnight at 4°C. After that, the membrane was washed three times for 5 min each with 15 ml of TBST, and incubated the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 hour. Then it rewashed three times for 5 min. each with 15 ml of TBST. Proteins were detected using ECL (WesternBright® ECL Luminol/Enhancer Solution and WesternBright® Peroxide

Chemiluminescent Detection Reagent) mixed Western Bright ECL components 1:1. Then the membrane was imaged with the BioRad Image System.

Table 2.4: Antibodies, their companies and dilutions.

Antibody	Company	Dilution
HER2	Cell Signaling Technology (#2242)	1:1000
EGFR	Cell Signaling Technology (#9922)	1:1000
ERK1/2 (P44/42 MAPK)	Cell Signaling Technology (#9102S)	1:1000
HSP90	Cell Signaling Technology (#4874)	1:1000
AKT	Cell Signaling Technology (#9272)	1:1000
pAKT	Cell Signaling Technology (#9271)	1:1000
P53	Cell Signaling Technology (#9282)	1:1000
Caspase 3	Cell Signaling Technology (#9915)	1:1000
GAPDH	Cell Signaling Technology (#5174)	1:5000

Table 2.5: Buffers used in Western Blot Analysis, their ingredients and preparation methods

Buffer	Ingredients	Preparation
1X Running Buffer	• 10X Tris Glycine SDS Running Buffer (BioRad, 1610732, USA) • dH₂O	100 mL TrisGlycine SDS Running Buffer and 900 ml dH ₂ O were mixed
1X Transfer Buffer	• 10X Tris-Glycine Transfer Buffer (Biorad, 1610771, USA) • Methanol • dH₂O	100 mL Tris-Glycine Transfer Buffer, and 200 ml Methanol, 700 ml dH ₂ O
1X TBST	• Tris base • NaCl • 12 N HCL • dH₂O • Tween 20	24 g Tris base and 88 g NaCl were dissolved in 900 mL dH ₂ O. Buffer pH was adjusted to 7.6 with 12 N HCl. It was completed 1 L by adding dH ₂ O to the buffer. Finally 0.1 % Tween 20 was added to the buffer.
Blocking Buffer	• 1X TBST • Non-fat dry milk (BioRad, #1706404, USA)	5 g non fat dry milk and 100 ml 1X TBST were mixed

2.9. STATISTICAL ANALYSIS

These are the standard deviation (SD) values of the results obtained from all experiments' three to six independent replicates. Statistical analyses were done with Graphpad prism 7 Software (USA). The statistical evaluation was performed with Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey post hoc test to multiple comparisons. The criterion for statistical significance was $P < 0,05$.



3. RESULTS

3.1. CHARACTERIZATION OF EXOSOMES FROM HUMAN BREAST EPITHELIAL CELL LINES (MCF10A)

Exosomes were isolated from a non-tumorigenic epithelial cell line (MCF-10A) by a polyethylene glycol/dextran aqueous two-phase system (ATPS) (see section 2.2). Different methods were used to characterize exosomes isolated from cells.

3.1.1. Nanoparticle tracking analysis (NTA) and Atomic Force Microscopy (AFM)

The average size distribution and intensity of exosomes in PBS were determined with NTA. As seen in NTA data in Figure 3.1 A, the size distribution of exosomes was determined as almost 50-200 nm, and it was observed that the size density of exosomes increased (Figure 3.1 B). In addition, the particle concentration of exosomes was measured by NTA. For MCF10 A, the concentration of exosomes was $3.03 \times 10^{11} \pm 3.80 \times 10^{10}$ particles/ml. The AFM method characterized the morphological and biophysical properties of isolated exosomes (Figure 3.1 C). Both NTA and the AFM analysis confirmed that exosomes were successfully isolated and harvested.

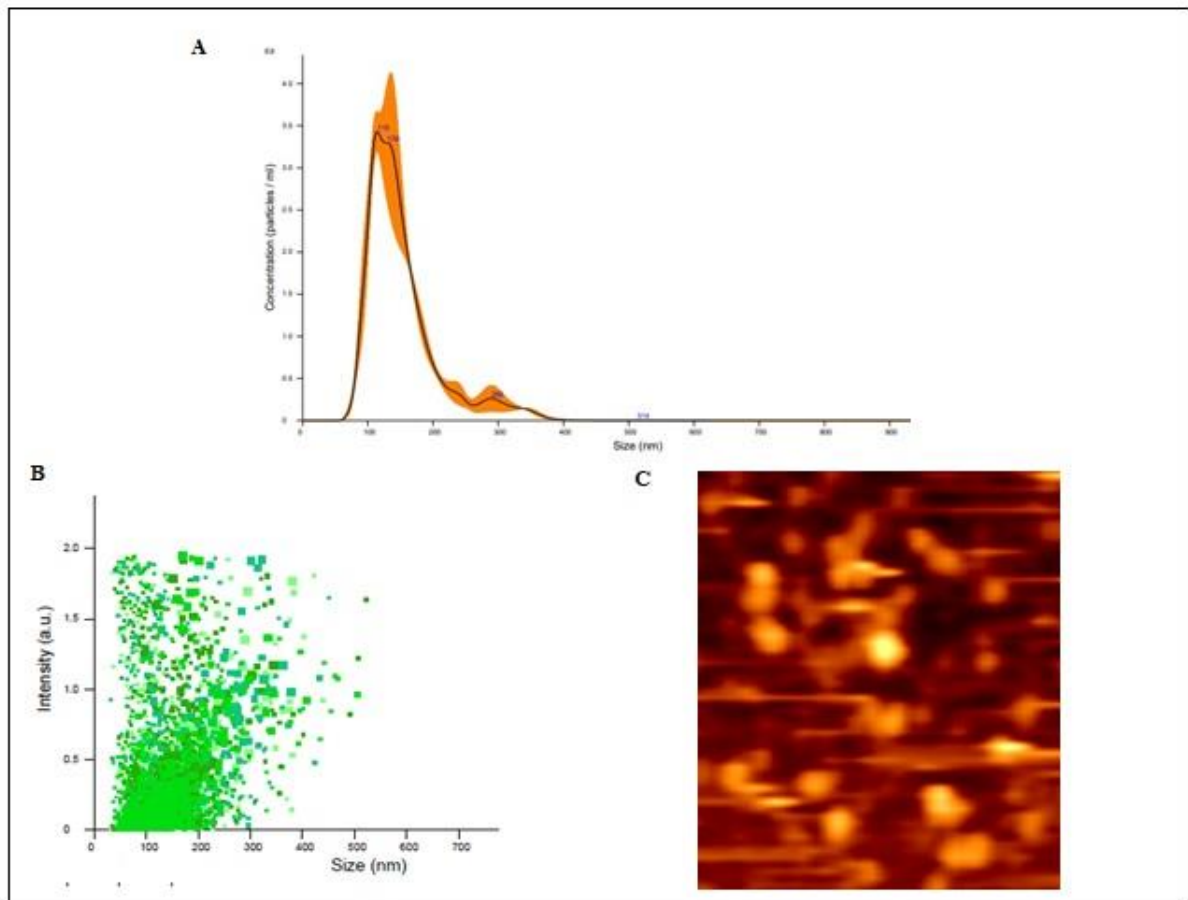


Figure 3.1: Characterization of exosomes released from MCF10 A epithelial cell lines. The average size (A) and intensity (B) of exosomes were analyzed by NTA. The AFM images of exosomes (C) were analyzed with AFM.

3.1.2. Dynamic Light Scattering (DLS or Zeta Sizer) Analysis

The isolated exosomes from MCF10 A cell lines were determined according to their size distribution (nm) and intensity (%) using a Zeta Sizer instrument. The isolated exosomes were diluted in 1:1000 with dH₂O. According to Figure 3.2, exosome particles' size was most likely around the 50 -200 nm size range.

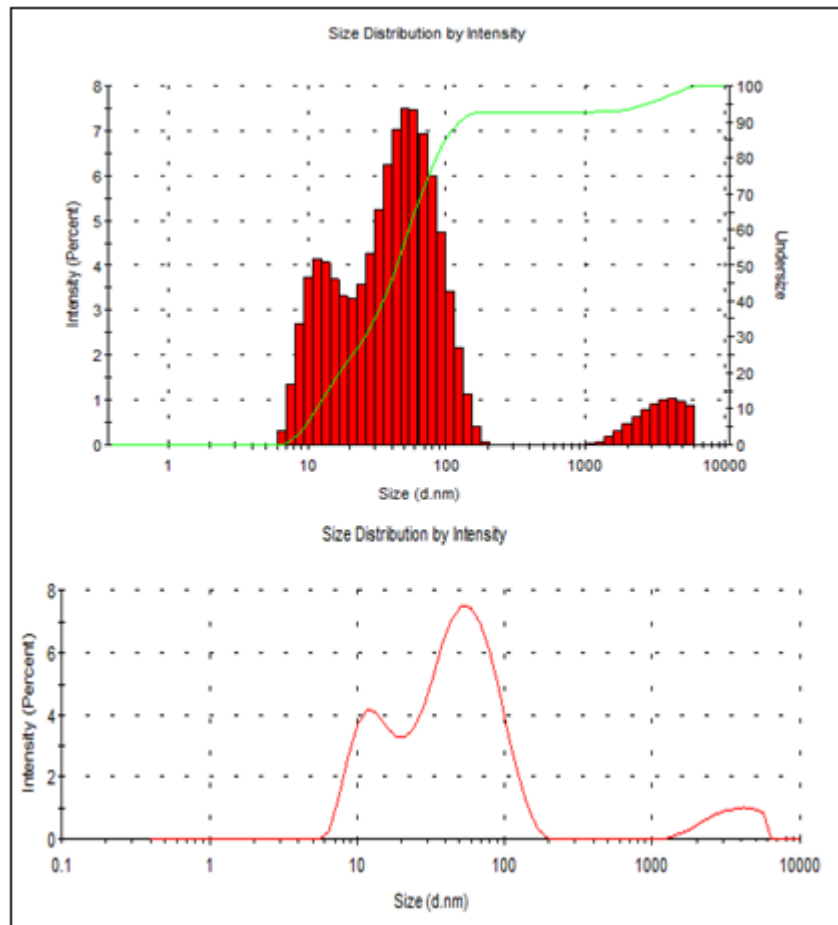


Figure 3.2: Particle size distributions of isolated exosome samples as measured by DLS.

3.1.3. Flow Cytometry Assay

Protein and lipid contents as well as the size of exosomes can be used to characterize exosomes. Exosomes can be definable with the existence of exosome surface markers. These exosome surface markers are known as tetraspanins (CD9, CD63, and CD81).

In this thesis, some surface molecules of exosomes derived MCF10 A cells were detected with flow cytometry. The exosomes were coated with latex beads and marked with monoclonal antibodies of the CD9, CD63, CD81, and heat shock protein (HSP70). In addition as a negative control (NC) unstained Latex beads was used. According to results, all antibodies were positive compared to unstained NC. As shown in Figure 3.3, the isolated exosomes expressed the tetraspanin proteins CD9, CD63, and CD81, which are known surface molecules.

As evidenced in Figure 3.3 E, HSP70 (9,23%), which was heat shock protein on the surface of exosomes, was less expressed according to the tetraspanins. However, it was demonstrated that CD81 (82,54%) was more expressed on the surface of the exosomes, CD9 (18,01%) was also detected well in the exosomes, while CD63 (10,10%) molecular marker was slightly positive.

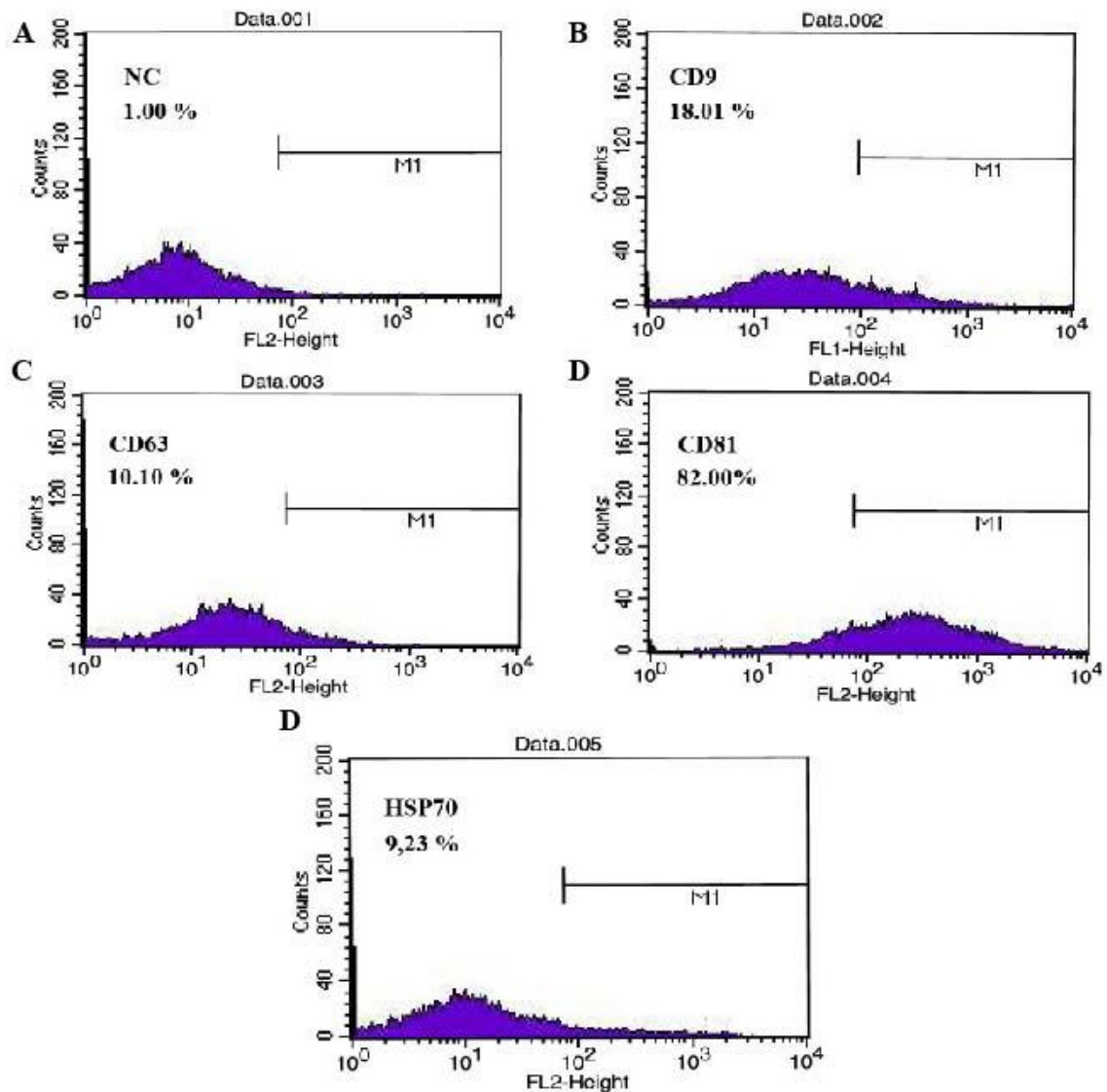


Figure 3.3: Flow cytometry was used to detect surface membrane molecules of MCF10 A exosomes. The exosomes were bound with latex beads and stained with FITCH anti-human CD9 (B), PE anti-human CD63 (C), PE anti-human CD81 (TAPA I) (D), and Alexa Flour anti-Hsp70 (E) and NC were unstained (A). All samples were incubated overnight at 4°C.

3.2.DRUG LOADING WITH ELECTROPORATION AND CHARACTERIZATION

3.2.1. Determination of the Standart Curve of Lapatinib with High Performance Liquid Chromatography (HPLC) Analysis

This study detected the amount of lapatinib loaded into the exosomes using HPLC. First of all HPLC method was used to obtain the standard curve of lapatinib.

The standard curve graph of lapatinib is shown in Figure 3.4. This standard curve analysis was made according to the range of 4.16-333.3 μM lapatinib concentrations by HPLC. The drug loading experiments were analyzed according to this standard curve graph.

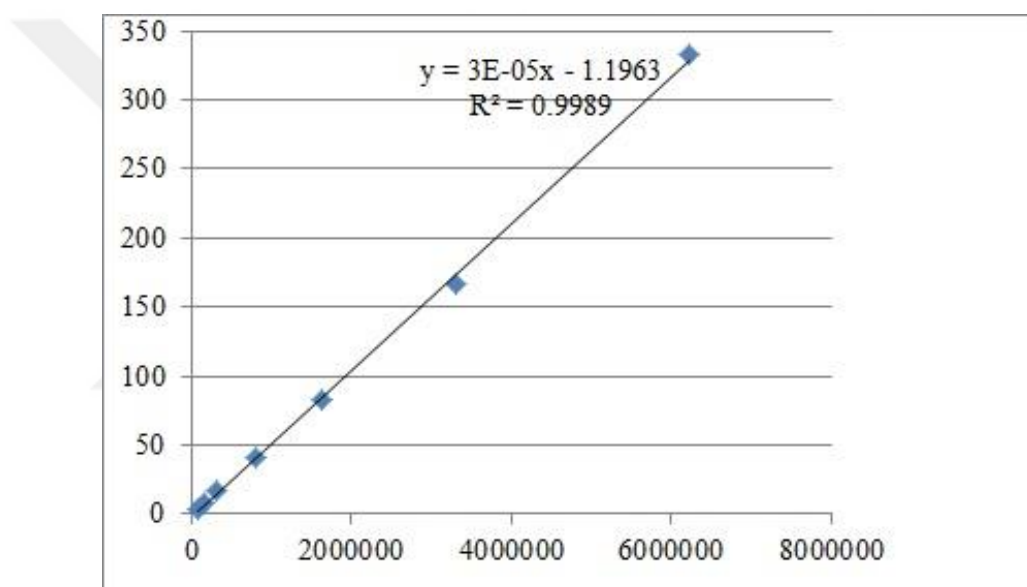


Figure 3.4: HPLC analysis of lapatinib (4.1, 8.3, 16.6, 41.6, 83.3, 166.6 and 333.3 μM) concentrations. The concentrations of lapatinib standards ranging from 2.5 to 200 μg were prepared with acetonitrile. All analyses were performed using a C18 column (XBridge C18; 5 μm ; 4.6 $\times$ 250mm) and measuring the absorbance of 268nm. The mobile phase consisted of acetonitrile and double dH₂O (80:20, v/v) at a 1 mL/min flow rate at 25 °C. A standard curve was plotted using the area under the curve of the standards.

3.2.2. Determination of the Amount of Lapatinib Loaded into the Exosomes with HPLC Analysis and Characterization of Lapatinib Loaded Exosomes with NTA

Lapatinib loaded into the exosomes as mentioned Section 2.3.1 and its amount was measured by HPLC analysis. Firstly, lapatinib loaded exosomes were disrupted as mentioned Section The released lapatinib from exosomes was calculated according to the lapatinib standard curve graph and its equation (Figure 3.4). HPLC analysis results were given in Figure 3.5. The area of released lapatinib was shown in Figure 3.5 A and between this area and areas of

lapatinib standards were compared in Figure 3.5 B. Our result showed that approximately 25 μM of lapatinib was loaded into the exosome.

After lapatinib loading the exosomes, the characterization of them was performed with NTA (Figure 3.6). According to the analysis, it was observed that exosomes sizes did not change despite electroporation, and the slight decrease a number of exosomes was regarded as a result of the aggregation of exosomes (Table 3.1).

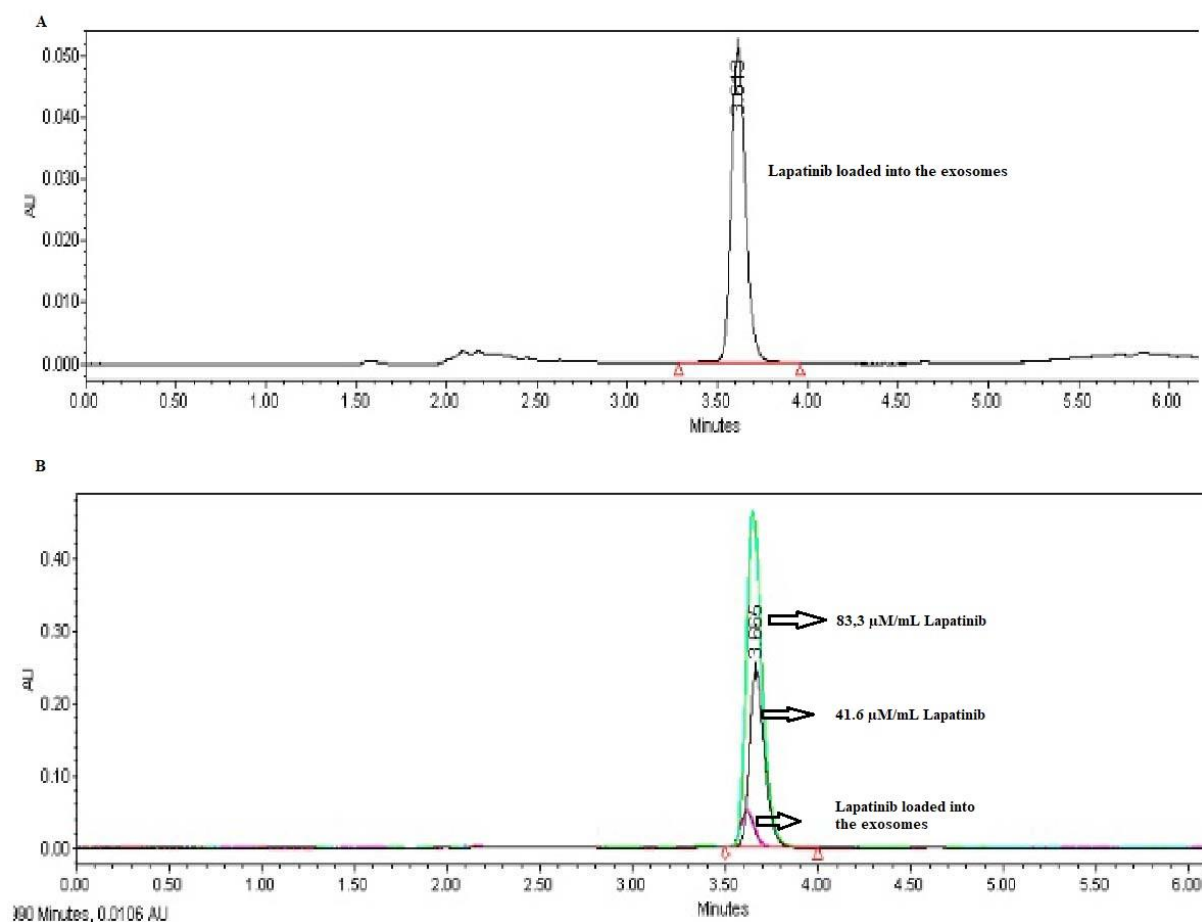


Figure 3.5: HPLC analysis of lapatinib loading into the exosomes (A) and HPLC analysis of 41.6 μM of lapatinib, 83.3 μM of lapatinib and lapatinib loaded into exosomes (B).

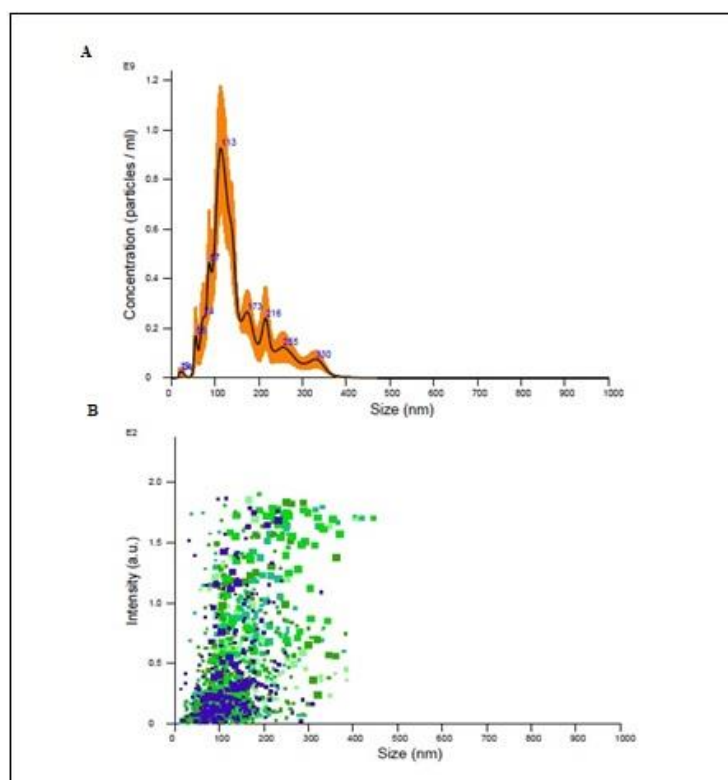


Figure 3.6: Characterization of lapatinib loaded exosomes. (A) The average size and (B) intensity of lapatinib loaded exosomes were analyzed by NTA. The sample was measured in 5 replicates. Mean +/- Standard Error: 64.0 +/- 7.0 nm

Table 3.1: The number of free exosomes and the number of exosomes after drug loading.

	Particles/ml
Free Exosomes	3.03e+11 +/- 3.80e+10
Lapatinib Loaded Exosomes	7.74e+10 +/- 8.07e+09

3.3. CYTOTOXIC ANALYSES

3.3.1. Cytotoxic Effects of Lapatinib on SKBR-3 and MCF10 A Cell Lines

SKBR 3 and MCF10 A cells were treated with different concentrations of lapatinib ranging from 0.1 to 10 μ M for the 24, 48, and 72 hours in order to determine the cytotoxic effects of lapatinib. The MTS cell proliferation assay was used to measure the cytotoxic effects of the drug. The percentage of cell viability was calculated according to the control cells (non-treated for lapatinib).

The viability results of SKBR-3 cells after treatment with lapatinib are shown in Figure 3.7. For the 10 μ M lapatinib concentration, there was a 70% reduction in cell proliferation in SKBR-3 cells at 24 hours and a further 80% reduction at 48 hours and to 90% at 72 hours. However, the cell viability of SKBR-3 was increased at 24 hours compared to the control cells at the lower concentrations (0.1 μ M, 0.5 μ M, and 1 μ M) lapatinib. Nonetheless, significant toxicity of lapatinib was observed at all concentrations (0.1 μ M, 0.5 μ M, 1 μ M, 5 μ M and 10 μ M) in SKBR-3 cells in 72 hour-treated groups. As shown in Figure 3.7 B, at 48 hours, it showed that 0.1 μ M concentration of lapatinib provided nearly 50 percent proliferation of SKBR-3 cells compared to the control.

As shown in Figure 3.8, different lapatinib concentrations were also applied to MCF10 A cells at 24, 48 and 72 hours. Lapatinib treatments were demonstrated statistically significant changes on MCF10 A cell viability at 24 (A) and 48 (B) hours, except 10 μ M lapatinib treatment for 48 hours. In Figure 3.8 C, it was shown that lapatinib treatments for 72 hours significantly decreased in MCF10 A cell proliferation. Consequently, although some lapatinib treatments were statistically significant changes in MCF10 A cell proliferation, IC_{50} values for 24, 48 and 72 hours could not determine.

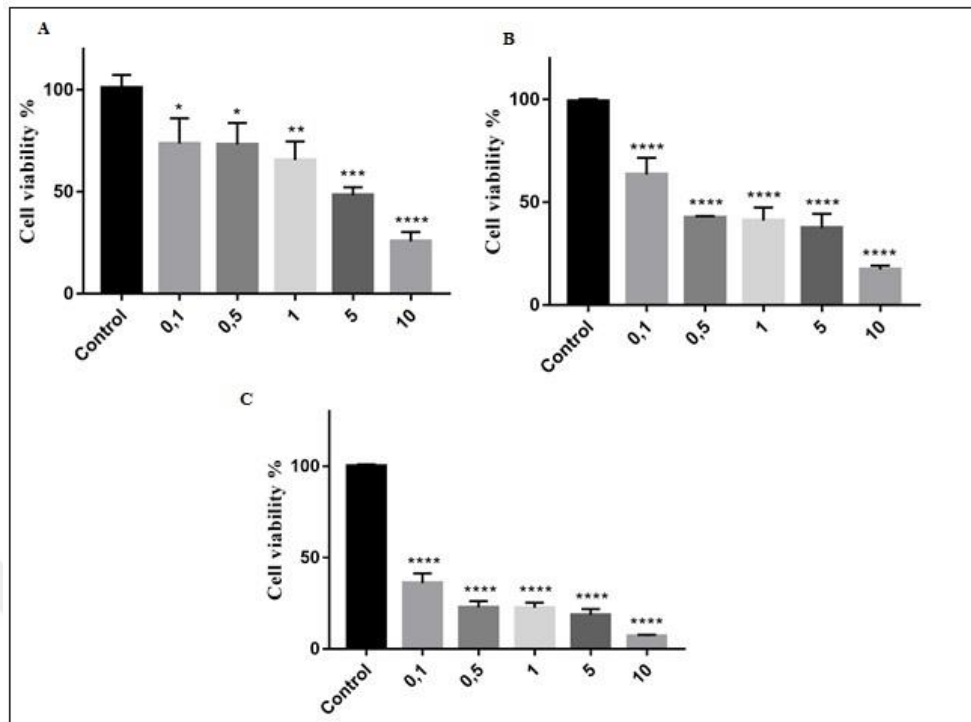


Figure 3.7: Effect of lapatinib on SKBR-3 cell line viability. 0.1-10 μ M of lapatinib was treated to SKBR-3 cells for 24 (A), 48 (B), and 72(C) hours. MTS assay was performed at the end of each time point. Cell viability was measured with an ELISA at 490 nm. Data shows average of experiments repeat three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$).

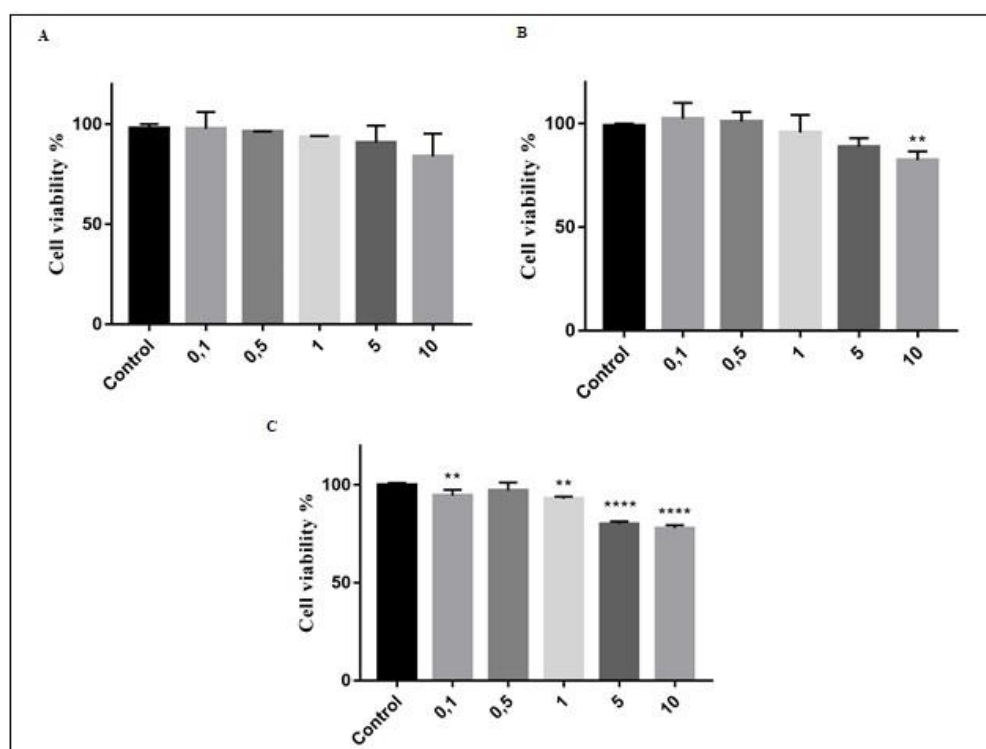


Figure 3.8: Effect of lapatinib on MCF10 A cell line viability. 0.1-10 μM of lapatinib was treated to MCF10 A cells for 24 (A), 48 (B), and 72(C) hours. MTS assay was performed at the end of each time point. Cell viability was measured with an ELISA reader at 490 nm. Data shows average of experiments repeat three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$).

3.3.2. Cytotoxic Effects of Free Lapatinib (Lap), Lapatinib Loaded Exosomes (LapExo), Free Exosomes (Exo) and Dextran Phase on SKBR-3 cells

In this experiments, the cytotoxic effects of lapatinib, lapatinib loaded exosomes (LapExo), exosomes (Exo) and dextran (used during exosome isolation from healthy MCF10 A cells) phase were determined and compared with each other. SKBR-3 cells were treated with different concentrations of lapatinib (0.1 μM , 1 μM , 2.5 μM) and lapatinib loaded exosomes (0.1 $\mu\text{M}/\text{ml}$ lapatinib (L)+ 10^8 exosomes (E), 1 $\mu\text{M}/\text{ml}$ lapatinib (L)+ 10^9 exosomes (E) and 2.5 μM lapatinib (L)+ 2.5×10^9 exosomes (E) particles/ml) for 24, 48, and 72 hours. The MTS cell proliferation assay was used to evaluate cytotoxic effects of the treatments. The percentage of cell viability was calculated according to the control cells (non-treated), whose cell viability value accepted as 100 percent.

The cell viability results of SKBR-3 cells treated with lapatinib is shown in Figure 3.9. As shown in Figure 3.9 A, all concentrations of lapatinib at 24 hours had more than 50% cell viability. As shown in Figures 3.9 B and 3.9 C, cell proliferation was decreased in a dose-

and-time dependent manner compared to the control cell viability (non-treated). While 0,1 μM concentration of lapatinib resulted in approximately 70% cell viability on SKBR-3 cells at 48 hours, the other lapatinib concentrations caused cell viability below 50% (Figure 3.9 B). Besides at the 72 hours, all treatments decreased cell viability and had statistically significant toxicity on SKBR-3 cells.

As mentioned before, approximately 25 μM of lapatinib could be loaded into the exosomes (see section 2.3.2). As shown in Figure 3.10, different concentrations of lapatinib-loaded exosomes (LapExo) were applied to SKBR-3 cells. According to the results, at 24 hours the highest lapatinib loaded exosomes 2.5 μM were caused nearly 60% cell viability (Figure 3.9 A). Although the 1 $\mu\text{M}/\text{ml}$ and 2.5 μM concentrations of LapExo decreased SKBR-3 cell viability, 0,1 $\mu\text{M}/\text{ml}$ of lapatinib-loaded exosomes treatment showed the best effectiveness as IC_{50} concentration at 48 hours (Figure 3.10 B). However, in Figure 3.10 C, all the treatments of LapExo for 72 hours reduced SKBR 3 cell proliferation below the half- maximal response (50%). Therefore, the treatments of cells were performed for the 48th hour for further studies. A viability comparison graph of Lap and LapExo treated SKBR-3 cells at 48 hours is shown in Figure 3.11. It was observed that the lowest concentration of 0.1 μM in LapExo treatment significantly reduced the cell viability at 48 hours compared to free lapatinib treated group.

Apart from these, SKBR-3 cells were treated at different concentrations of free exosomes (10^8 particles/ml, 10^9 particles/ml, and 2.5×10^9 particles/ml) and dextran (dex) phase 10^8 particles/ml, 10^9 particles/ml, and 2.5×10^9 particles/ml) (Figure 3.12 and Figure 3.13). The cells were treated with free exosomes containing no lapatinib in order to determine the loading effect of exosomes alone on SKBR-3 breast cancer cells. Free exosomes treatment had no significantly effects on the SKBR-3 breast cancer cells at 24 hours, 48 hours, or 72 hour. All particles/ml of free exosomes affected cell proliferation similar to the control for all hours (Figure 3.12). Since the isolation of exosomes was carried out with dextran (dex) using ATPS method (see section 2.2.1), the effect of dex phase was also determined whether remaining dex within exosome could effect the on SKBR-3 cell viability. The dex phase treatment without exosomes showed no cytotoxic effects on the SKBR-3 cells (Figure 3.13).

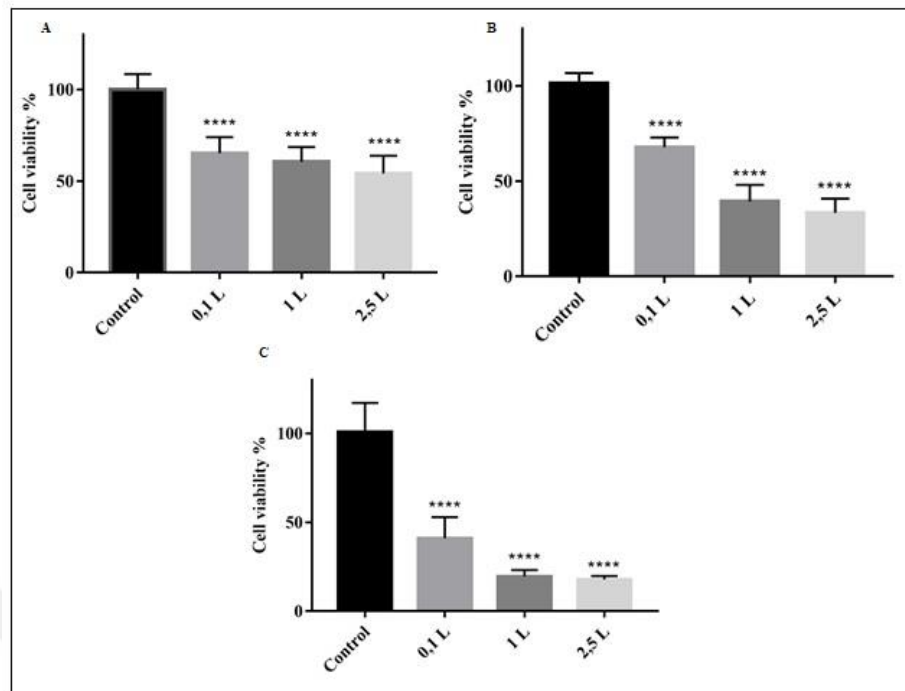


Figure 3.9: Cell proliferation assay on SKBR-3 breast cancer cell line. Different concentrations of lapatinib were treated SKBR-3 cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$). [0.1 L:0,1 μ M lapatinib; 1 L:1 μ M lapatinib; 2.5 L:2.5 μ M lapatinib]

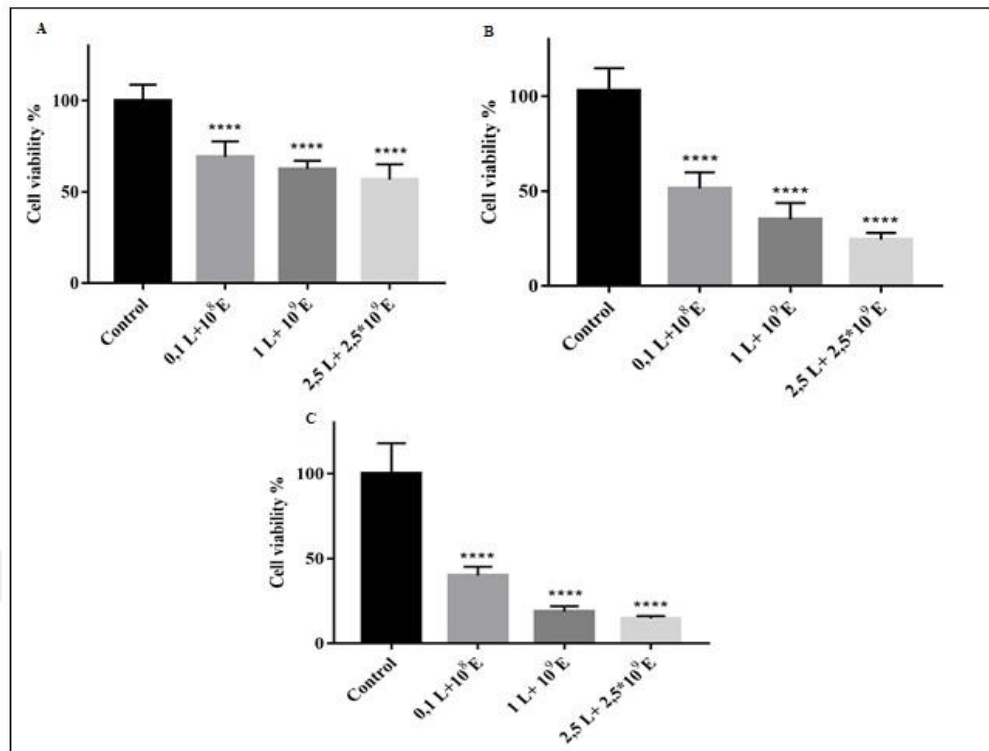


Figure 3.10: Cell proliferation assay on SKBR-3 breast cancer cell line. Different concentrations of lapatinib loaded exosomes were treated SKBR-3 cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$). [0.1 L+ 10^8 E: 0.1 μ M lapatinib+ 10^8 exosomes (particles/ml); 1 L+ 10^9 E: 1 μ M lapatinib+ 10^9 exosomes (particles/ml); and 2,5 L+ $2,5 \cdot 10^9$ E: 2,5 μ M/ml lapatinib+ $2,5 \cdot 10^9$ exosomes (particles/ml)]

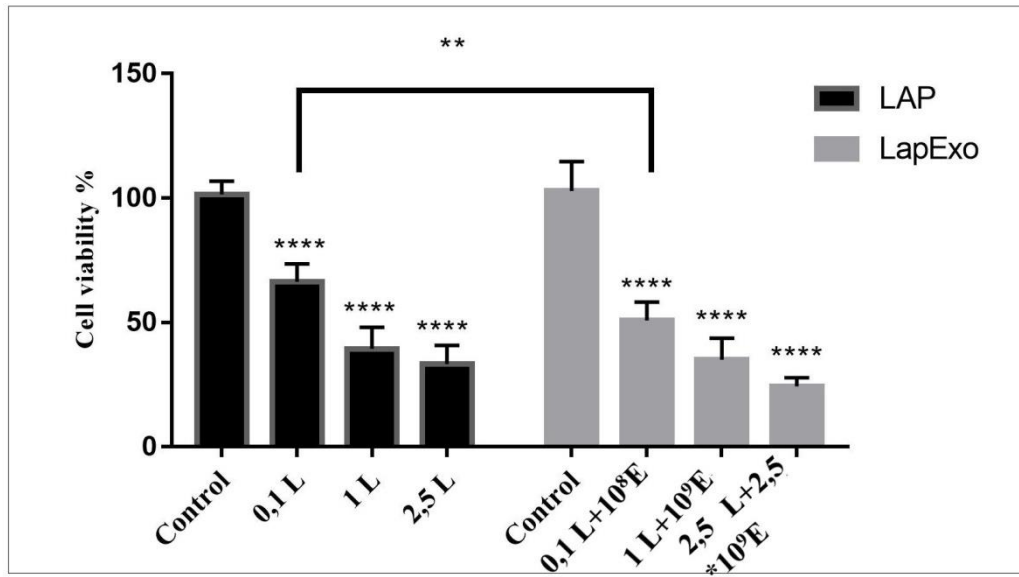


Figure 3.11: Comparison of lapatinib and lapatinib-loaded exosomes on the viability of SKBR-3 cells at 48 hours. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$). [0,1 L:0,1 μ M lapatinib; 1 L:1 μ M/ml lapatinib; 2,5 L:2,5 μ M lapatinib; 0,1 L+ 10^8 E: 0.1 μ M lapatinib+ 10^8 exosomes (particles/ml); 1 L+ 10^9 E: 1 μ M/ml lapatinib+ 10^9 exosomes (particles/ml); and 2,5 L+ 2,5* 10^9 E: 2.5 μ M lapatinib+2,5* 10^9 exosomes (particles/ml)]

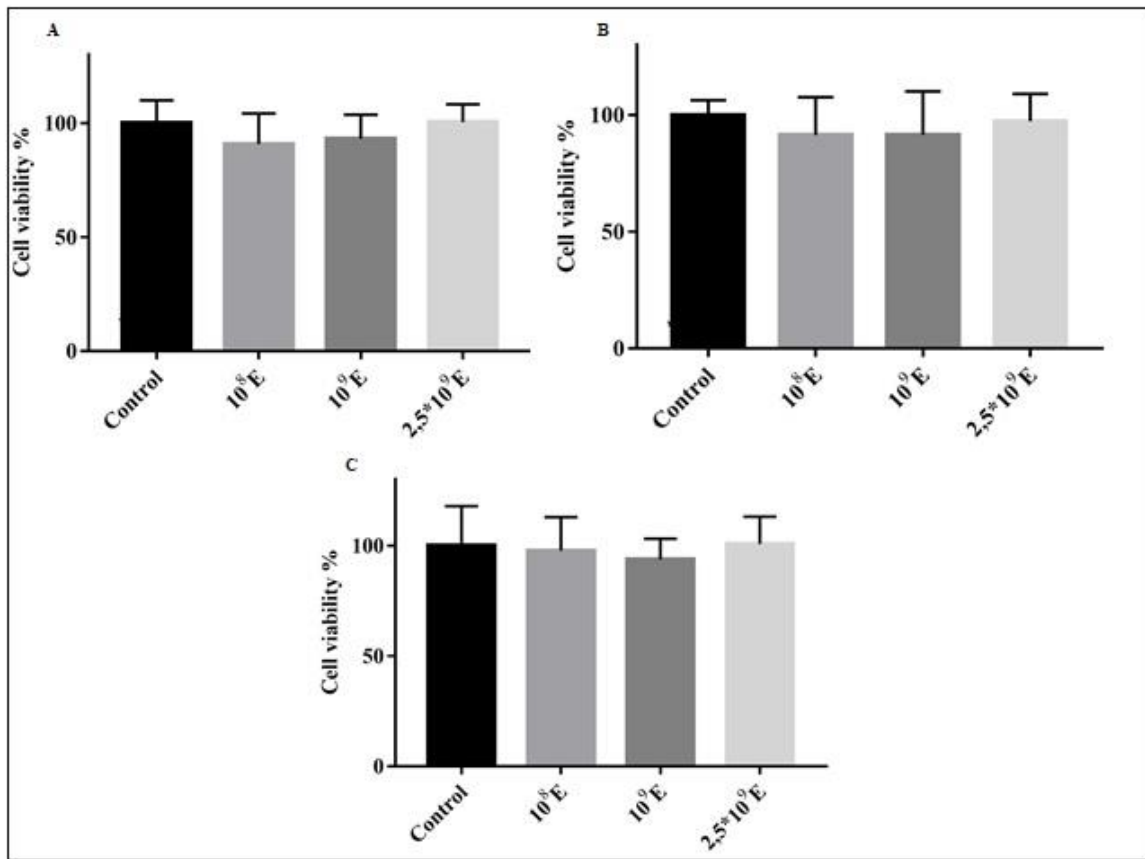


Figure 3.12: Cell proliferation assay on SKBR-3 breast cancer cell line. Different concentrations of exosomes (E) were treated SKBR-3 cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$). [10^8 E: 10^8 exosomes (particles/ml); 10^9 E: 10^9 exosomes (particles/ml); and 2.5×10^9 E: 2.5×10^9 exosomes (particles/ml)]

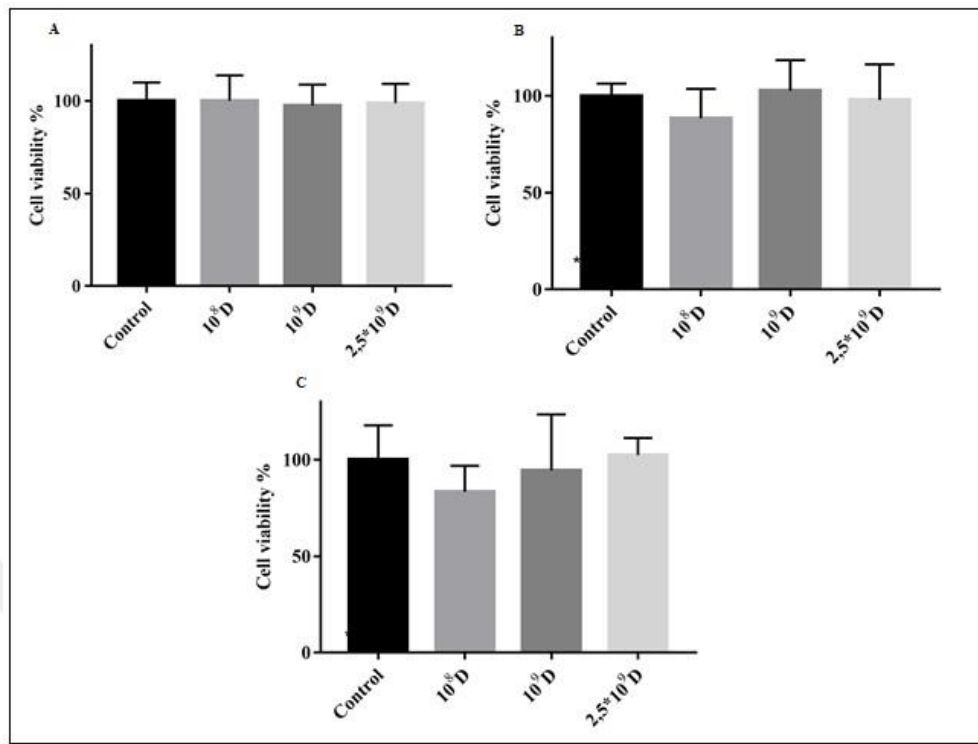


Figure 3.13: Cell proliferation assay on SKBR-3 breast cancer cell line. Different concentrations of dex phase (D) were treated SKBR-3 cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$). [10^8 D: 10^8 dex phase (particles/ml); 10^9 D: 10^9 dex phase (particles/ml); and 2.5×10^9 D: 2.5×10^9 dex phase (particles/ml)]

3.3.3 Cytotoxic Effects of Free Lapatinib (Lap), Lapatinib Loaded Exosomes (LapExo), Free Exosomes (Exo) and Dextran Phase on MCF10 A Cell Lines

Healthy MCF10 A cells were treated with different concentrations (0.1 μ M, 1 μ M/ml and 2.5 μ M) of Lap, lapatinib loaded exosomes (0.1 μ M lapatinib (L)+ 10^8 exosomes (E), 1 μ M lapatinib (L)+ 10^9 exosomes (E) and 2.5 μ M lapatinib+ 2.5×10^9 exosomes (E) particles/ml), free exosomes (10^8 particles/ml, 10^9 particles/ml, and 2.5×10^9 particles/ml) and dex phase (10^8 particles/ml, 10^9 particles/ml, and 2.5×10^9 particles/ml) for 24, 48, and 72 hours in order to determine the cytotoxic effects of lapatinib, lapatinib loaded exosomes, exosomes and dex phase. The MTS cell proliferation assay was performed to determine the cytotoxic effects of treatments. In this assay the percentage of cell viability was calculated with according to the control cells (non-treated) whose cell viability value is determined as 100%.

The cell viability of MCF10 A cells lines treated with lapatinib was shown in Figure 3.13. As shown in Figure 3.14, all concentrations have over 50 percent cell viability at 24, 48, and 72 hours. Eventually, lapatinib did not affect MCF10 A cell viability according to all hour trials.

The cells were exposed to lapatinib-loaded exosomes (LapExo) (Figure 3.15), LapExo treatments has not decreased cell viability by 50% or under 50% according to the all concentrations at 24, 48, and 72 hours similar to the lapatinib results. As shown in Figure 3.16 and 3.17, the empty exosomes and dex phase were applied to the cells. But these treatments were non-significant for healthy cells for all treated concentrations. According to these results, Lap, ExoLap, and empty Exo treatments did not affect cell proliferation and are non-toxic on MCF10 A cells.

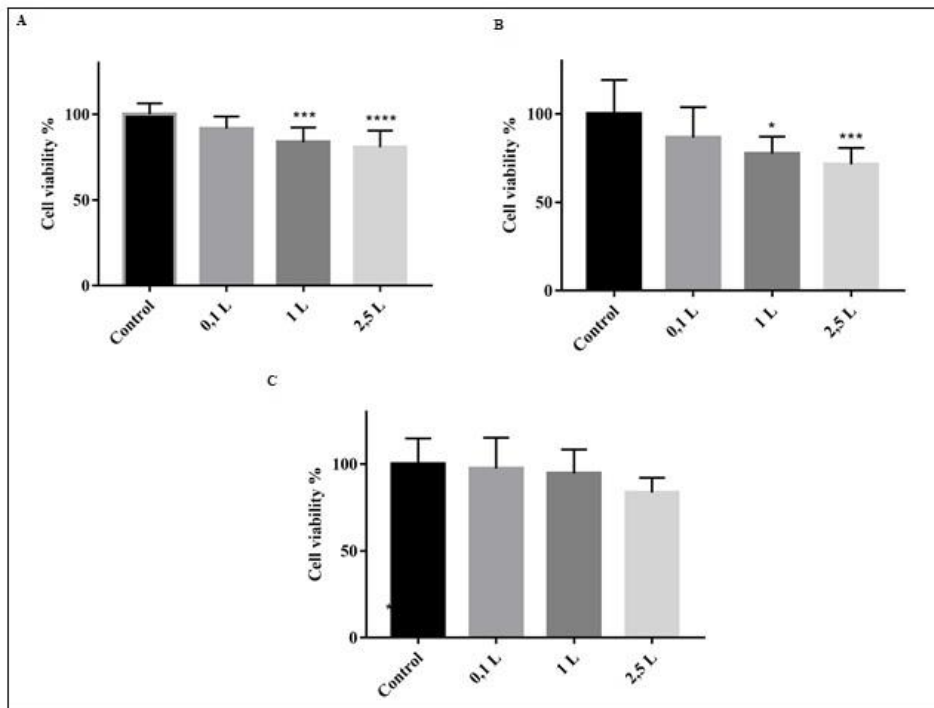


Figure 3.14: Cell proliferation assay on MCF10 A healthy breast cell line. Different concentrations of lapatinib were treated MCF10 A cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm by with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$). [0.1 L:0,1 μ M lapatinib; 1 L:1 μ M lapatinib; 2.5 L:2.5 μ M lapatinib]

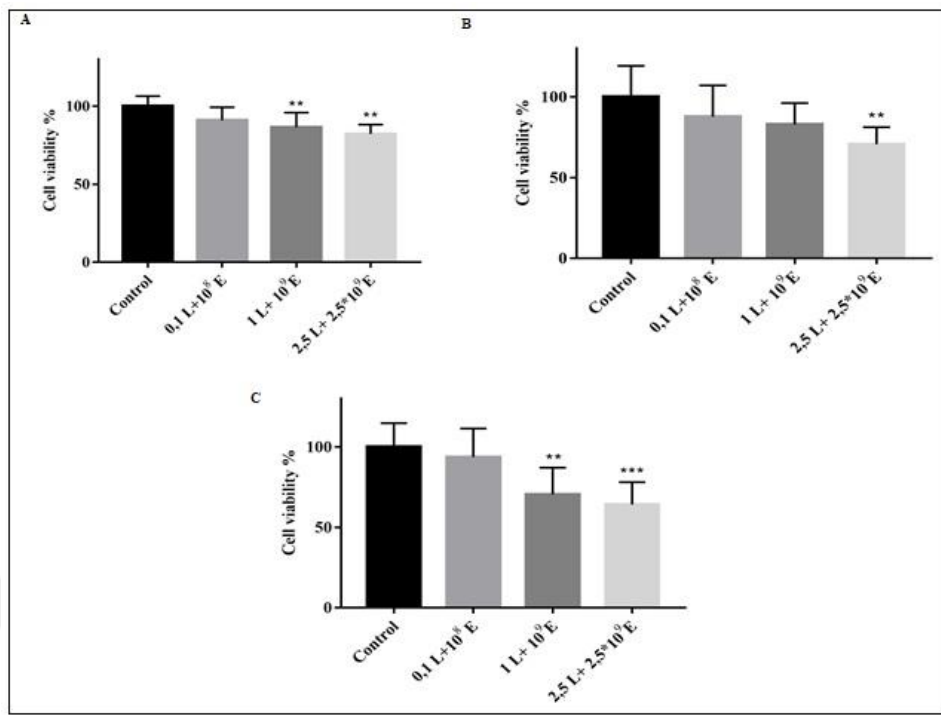


Figure 3.15: Cell proliferation assay on MCF10 A healthy breast cell line. Different concentrations of lapatinib loaded exosomes were treated MCF10 A cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$). [0,1 L+ 10⁸ E: 0.1 μ M lapatinib+10⁸ exosomes (particles/ml); 1 L+10⁹ E: 1 μ M lapatinib+10⁹ exosomes (particles/ml); and 2.5 L+ 2.5*10⁹ E: 2,5 μ M lapatinib+2,5*10⁹ exosomes (particles/ml)]

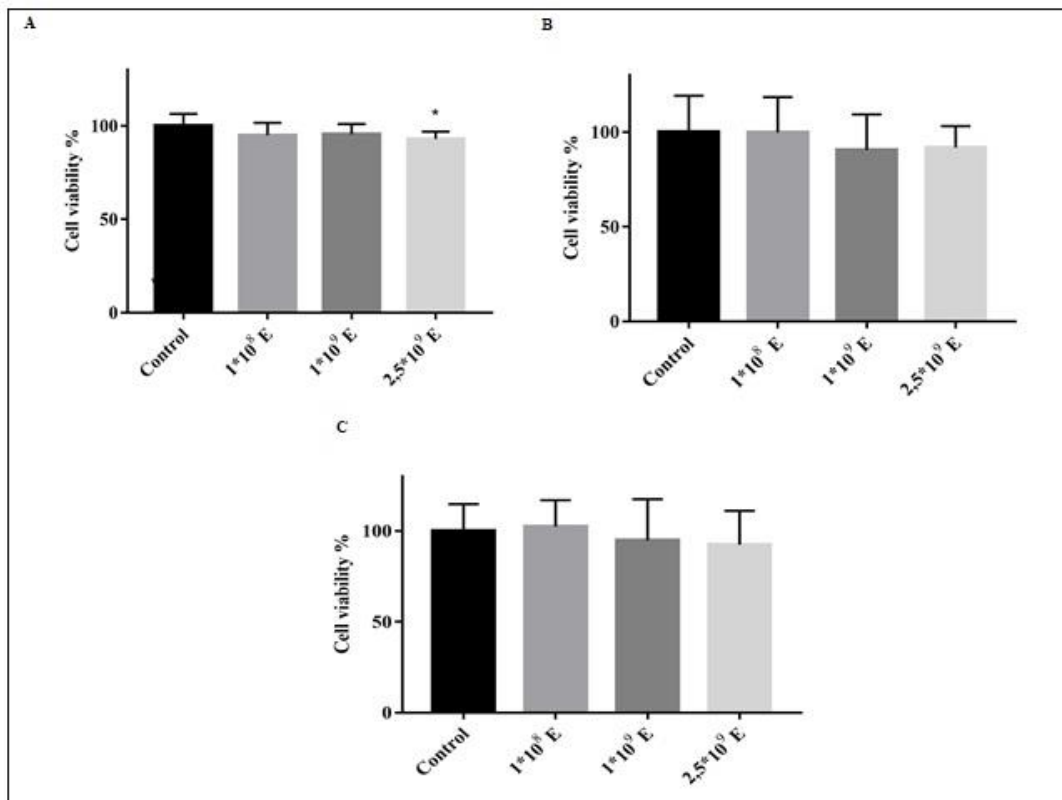


Figure 3.16: Cell proliferation assay on MCF10 A healthy breast cell line. Different concentrations of exosomes (E) were treated MCF10 A cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$). [10^8 E: 10^8 exosomes (particles/ml); 10^9 E: 10^9 exosomes (particles/ml); and 2.5×10^9 E: 2.5×10^9 exosomes (particles/ml)]

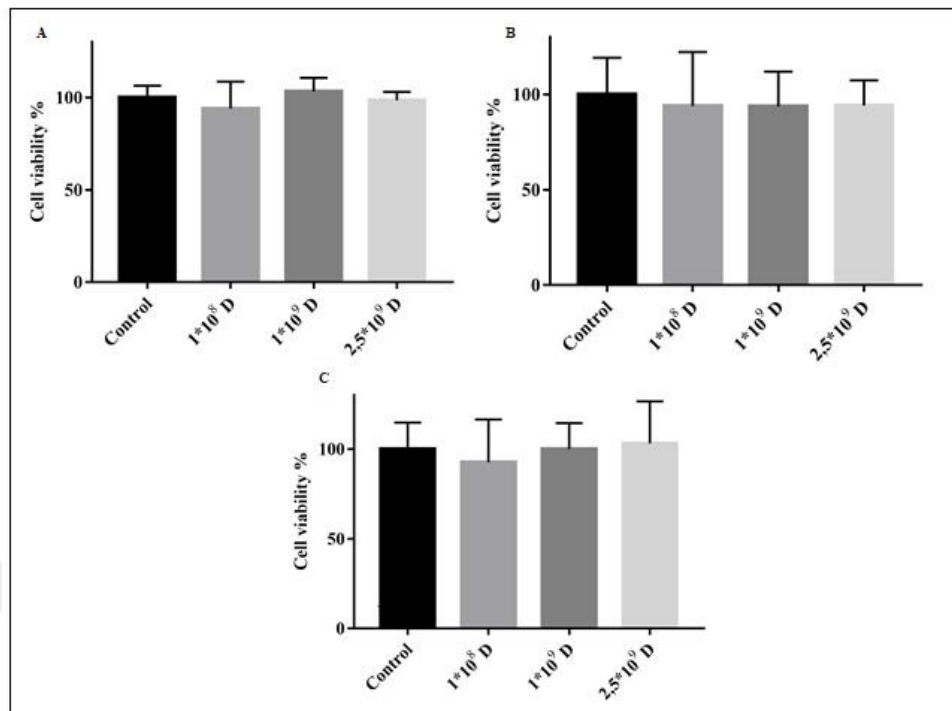


Figure 3.17: Cell proliferation assay on MCF10 A healthy breast cell line. Different concentrations of dex phase (D) were treated MCF10 A cells at 24 (A), 48 (B) and 72 (C) hours and cell viabilities were determined by using MTS assay. According to this assay, absorbance was measured at 490 nm with an ELISA. Cell viability values were calculated as mentioned section 3.3.1. Data shows an average of experiments repeated three times $\pm$ SD (**** $P \leq 0.0001$, ** $P \leq 0.01$, *** $P \leq 0.001$). [10^8 D: 10^8 dex phase (particles/ml); 10^9 D: 10^9 dex phase (particles/ml); and 2.5×10^9 D: 2.5×10^9 dex phase (particles/ml)]

3.4. CELL DEATH ANALYSES

Annexin V assay was applied to detect the apoptotic cells in the control and experimental groups on the SKBR-3 and MCF10 A cell lines. The cells were treated with the 0.1 μM concentration of Lap, 0.1 μM + 10^8 particles/ml concentration of LapExo, and 10^8 particles/ml Exo for this assay at 48h. All assays were performed as three biological replicates.

As shown in Figure 3.18 pre-apoptotic stage of lapatinib has 10,86% of annexin V positive cells, and 2,54% apoptotic cells were detected in post apoptotic. Average of 14% apoptotic cells were found after treatment with lapatinib for 48 hours (Figure 3.18 B). Moreover 18% pre-apoptotic cells and 3% of post-apoptotic cells were observed when the cells were treated with LapExo. In the results totally, 21 percent of cells were detected as apoptotic cells after being treated with LapExo for 48 hours (Figure 3.18 C). There was no significant cell death in the SKBR-3 cells treated with Exo. 1,31% pre-apoptotic, 1.87% post-apoptotic, and 7,31% of necrotic death were detected after treatment with empty Exo for 48 hours (Figure 3.18 D). Necrotic death was slightly increased compared to apoptotic death for lapatinib and LapExo treatment groups.

Our results indicated that the LapExo treatment was significantly more apoptotic than the Lap treatment on SKBR-3 cells.

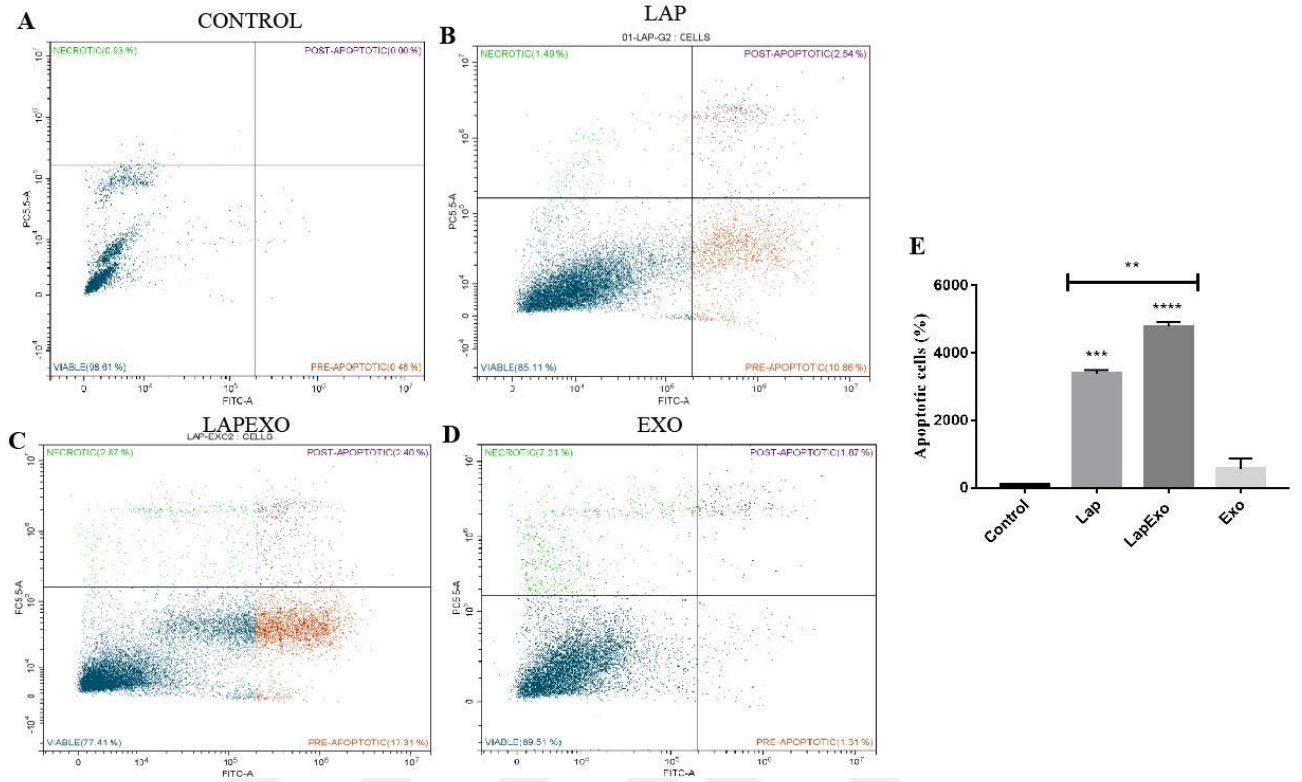


Figure 3.18: Apoptotic analysis of Lap, LapExo and Exo treatment on SKBR-3 cells. Flow plot representation of apoptosis analysis. Annexin V/PI staining was used to analyze the cell death of SKBR-3 cells. Treatment of 0.1 μ M Lap (B), 0.1 μ M+ 10^8 E particles/ml LapExo (C) and, 10^8 particles/ml Exo (D) on SKBR-3 at 48 hours. (E) Quantification of apoptosis analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$, *** $P < 0.001$, **** $P \leq 0.0001$)

In Figure 3.19 for MCF10 A cells, there was slightly death in the apoptotic and necrotic staining. Average 4.26% apoptotic death and 1.56% necrotic death were detected for Lap treatment for 48 hours (Figure 3.19 B). Total apoptosis was 3.76%, and necrosis was 4.52% in LapExo treated for 48 hours (Figure 3.19 C). Moreover, 4.24 percent of cells were apoptotic, and 4.87 percent were necrotic for exos treatment (Figure 3.19 D). A comparative graph of the apoptotic effects observed in SKBR-3 and MCF10 A cells treated with Lap, LapExo, and Exo is shown in Figure 3.20.

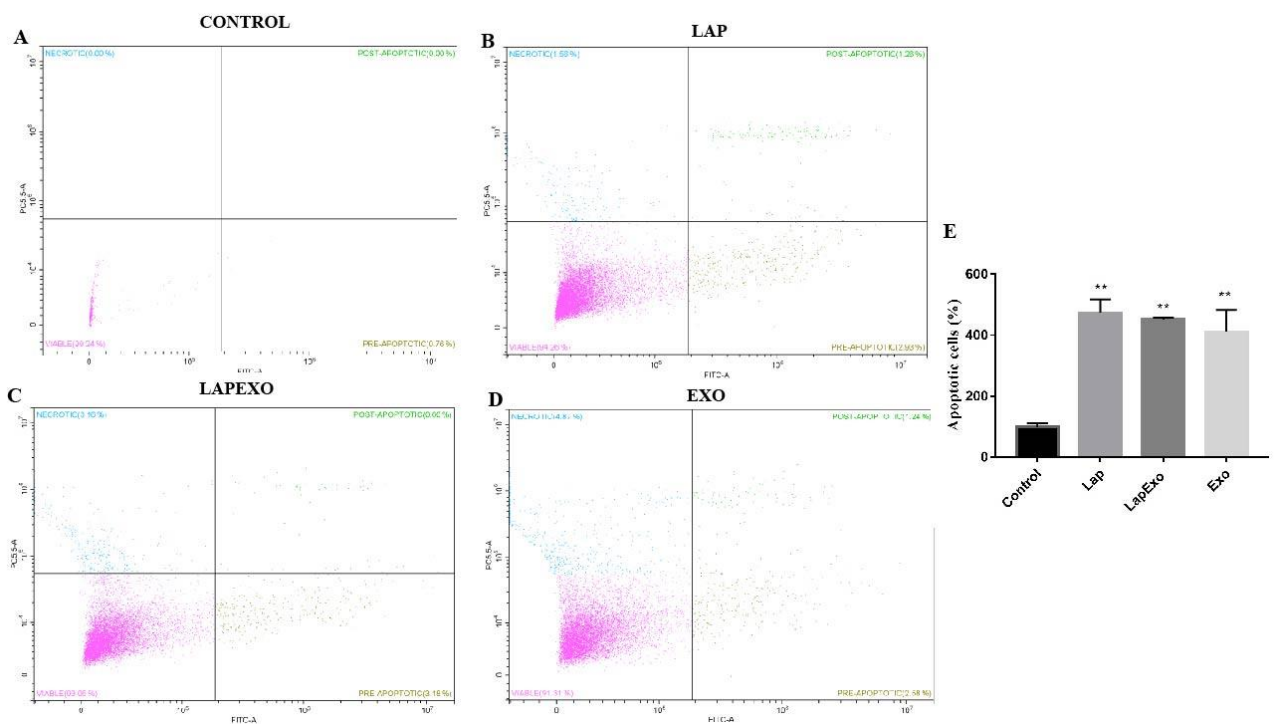


Figure 3.19: Apoptotic analysis of Lap, LapExo and Exo treatment on MCF10 A cells. Flow plot representation of apoptosis analysis. Annexin V/PI staining was used to analyze the cell death of MCF10 A cells. Treatment of 0.1 μ M Lap (B), 0.1 L μ M + 10^8 E particles/ml LapExo (C) and, 10^8 particles/ml Exo (D) on MCF10 A at 48 hours. (E) Quantification of apoptosis analysis. All groups were analyzed compared with control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$)

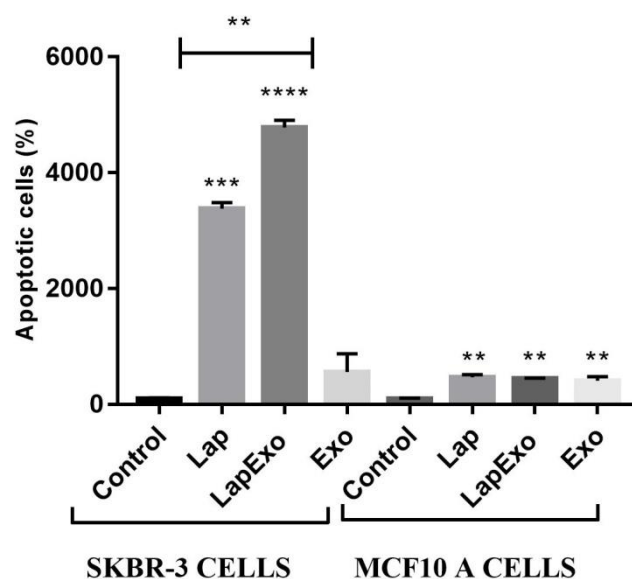


Figure 3.20: The comparison graph of SKBR-3 and MCF10 A apoptotic cells.

3.5. GENE EXPRESSION ANALYSES

3.5.1. RT-qPCR Analysis Results of SKBR-3 Cell Genes

To evaluate the effect of Lap, LapExo and Exo treatment on regarding ERBB1/ERBB2 and AKT/MAPK signaling pathways, gene expression analysis was performed in Lap, LapExo, and Exo treated cells. SKBR 3 and MCF10 A cells treated with 0.1 μ M Lap, 0.1 μ M lapatinib (L)+ 10^8 exosomes (E) LapExo, and 10^8 particles/ ml Exo for 48h. Then, EGFR (ERBB1), HER2 (ERBB2), MAPK, AKT, BCL2, TP53, caspase 9, and caspase 3 gene expression levels were measured with RT-qPCR assay. In Figure 3.21 A, EGFR mRNA level significantly increased in LapExo (≈ 9.3 -fold) treated groups. Besides, there was no statistically significant change in Lap (≈ 5.1 - fold) and Exo treated groups for 48 hour. While HER2 (ERBB2) gene expression levels significantly increased in LapExo treated group compared with the control group, Lap and Exo treatments did not significantly affect the mRNA level of the HER2 (ERBB2) according to LapExo treatment. In Figure 3.22 A, MAPK mRNA levels significantly decreased in Lap and LapExo treated (≈ 0.5 - fold) groups, but in Exo (≈ 1.6 - fold) treated group its gene expression increased. Furthermore AKT gene expression level did not show a statistically change in Lap, LapExo, and Exos treated groups compared to the control group according to the Figure 3.22. B. As shown in Figure 3.23. A, Caspase 3 gene expression significantly increased in Lap (≈ 6 -fold) and LapExo (≈ 3 - fold) treated groups, but no change

was detected in Exo treated group, and in Figure 3.23. B, Caspase 9 gene expression level significantly increased in Lap and LapExo (≈ 8 - fold) treated groups, but did not change in Exo treated groups. According to Figure 3.24 A, TP53 gene expression level increased significantly in Lap (≈ 7 -fold) and LapExo (≈ 8 -fold) treated groups. Moreover, the TP53 gene expression level did not significantly affect the cells treated with exosomes compared to the control group. Furthermore, in Figure 3.24 B, BCL-2 mRNA level did not significantly change in Lap, LapExo, and Exos treated compared to the control group.

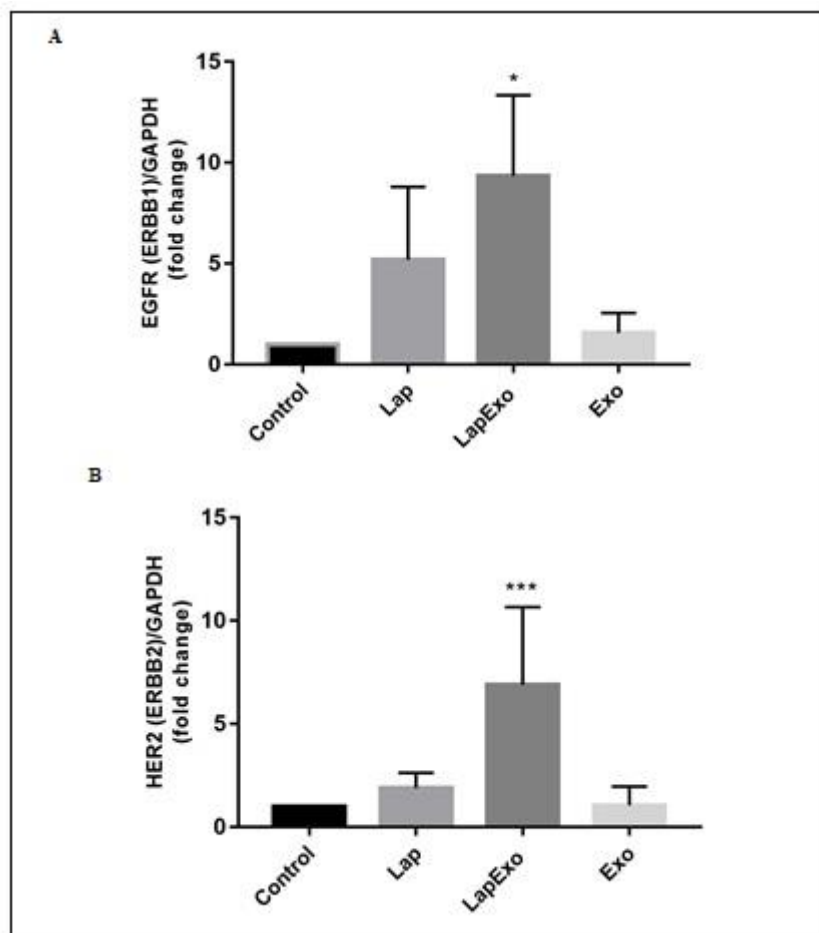


Figure 3.21: EGFR (ERBB1) (A) and HER2 (ERBB2) (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L + 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on SKBR-3 breast cancer cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (***) $P < 0.001$, * $P \leq 0.05$)

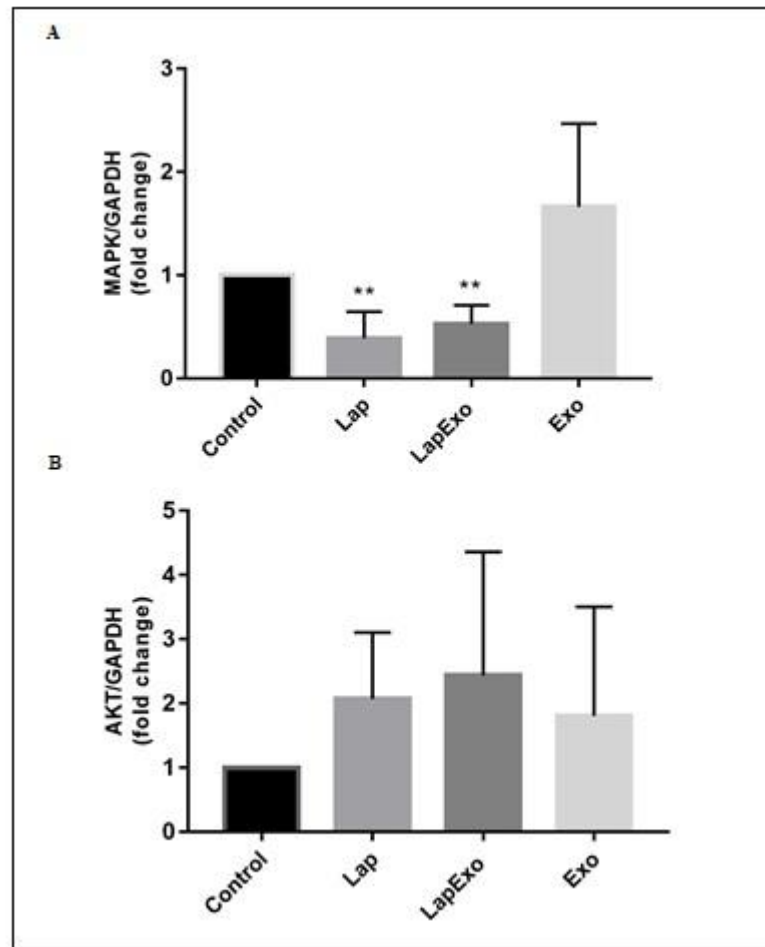


Figure 3.22: MAPK (A) and AKT (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L+ 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on SKBR-3 breast cancer cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$)

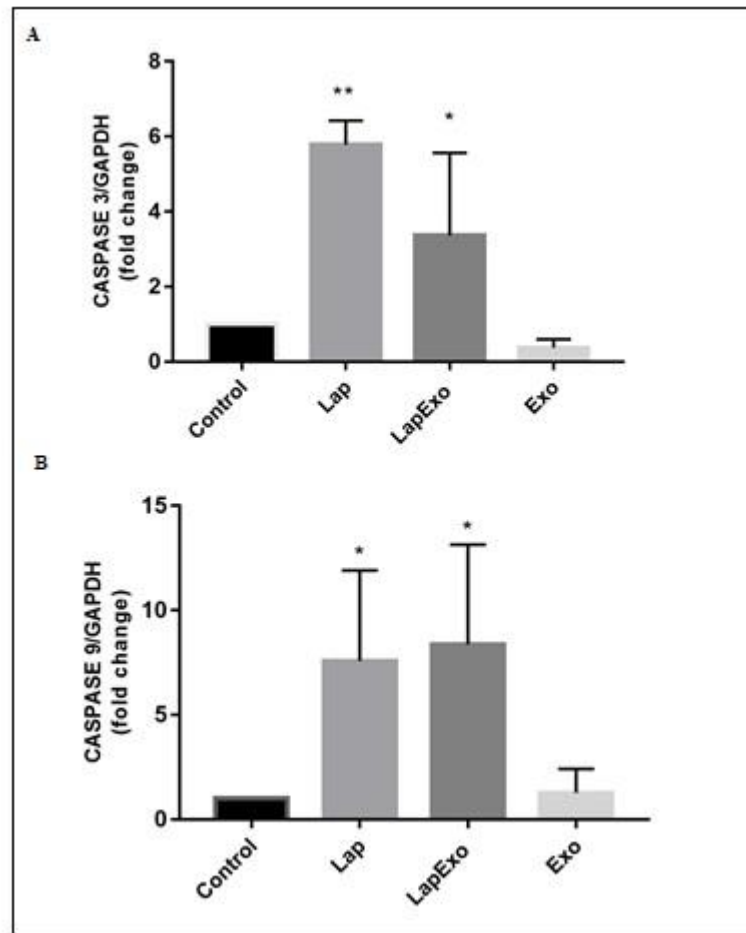


Figure 3.23: Caspase 3 (A) and Caspase 9 (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L+ 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on SKBR-3 breast cancer cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$, * $P < 0.05$)

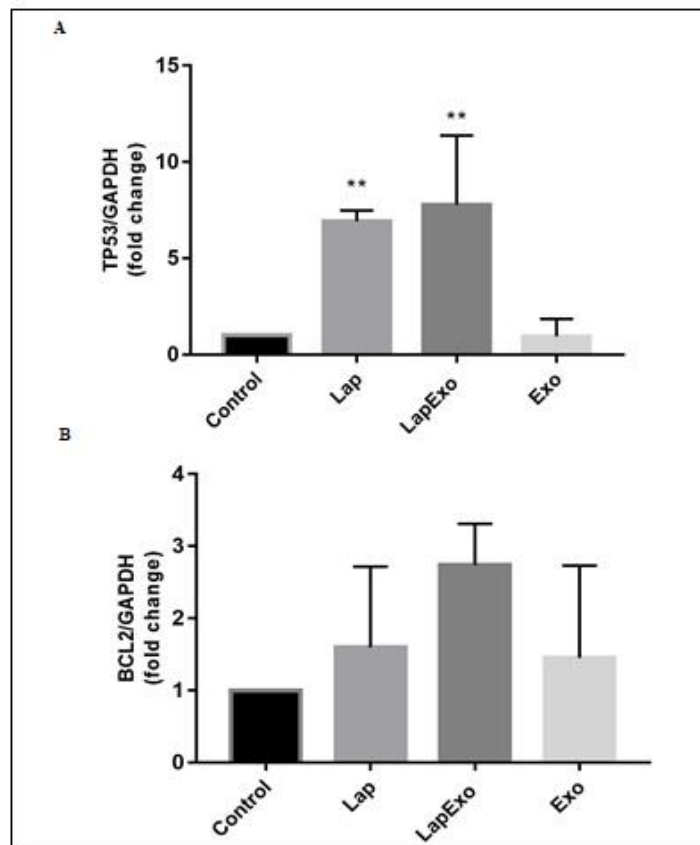


Figure 3.24: TP53 (A) and BCL-2 (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L + 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on SKBR-3 breast cancer cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$)

3.5.2. RT-qPCR Analysis Results of MCF10 A Cell Genes

Like SKBR-3 cells, MCF10 A cells also treated with 0.1 L μ M Lap, 0.1 L μ ML + 10^8 E particles/ml LapExo and 10^8 E particles/ml Exo. When all the treatments for 48 hours were completed, EGFR (ERBB1), HER2 (ERBB2), MAPK, AKT, BCL2, TP53, caspase 9, and caspase 3 expression levels were measured with RT-qPCR assay. ERBB1 Figure 3.25 A and ERBB2 Figure 3.25 B mRNA levels did not significantly change in all treated groups. In Figure 3.26 A, MAPK gene expression significantly increased (≈ 2 - fold) in LapExo, and Exo treated groups compared with the control, and in Figure 3.26. B, AKT gene expression levels did not change substantially in Lap, LapExo, and exosomes treated groups compared with the control group. Similar results can be seen in Figure 3.27, caspase 3 and caspase 9 mRNA levels did not change in all treated groups compared to the control. As shown in Figure 3.28, TP53 (A) and BCL-2 (B) gene expressions level did not change significantly in all treated groups compared to the control.

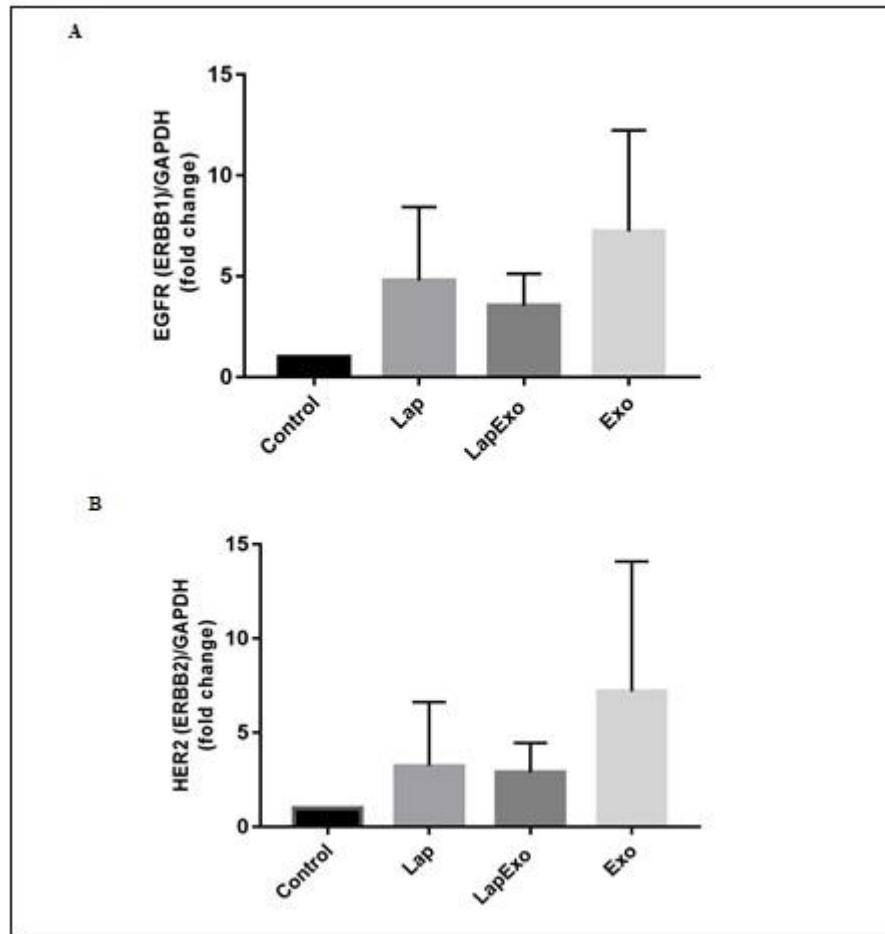


Figure 3.25: ERBB1 (A) and ERBB2 (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L + 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on MCF10 A healthy breast cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD.

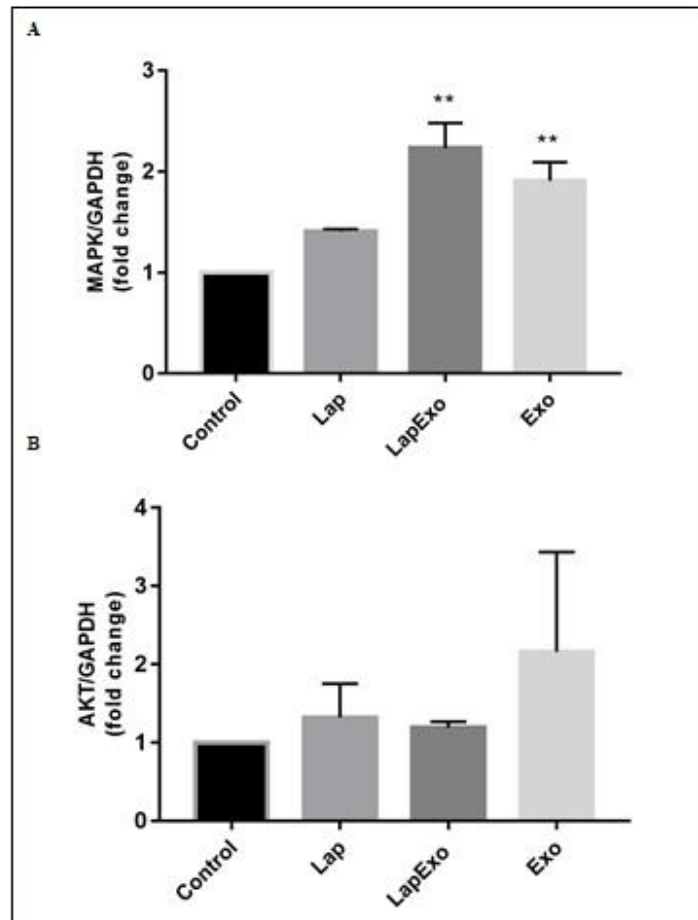


Figure 3.26: MAPK (A) and AKT (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L + 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on MCF10 A healthy breast cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$)

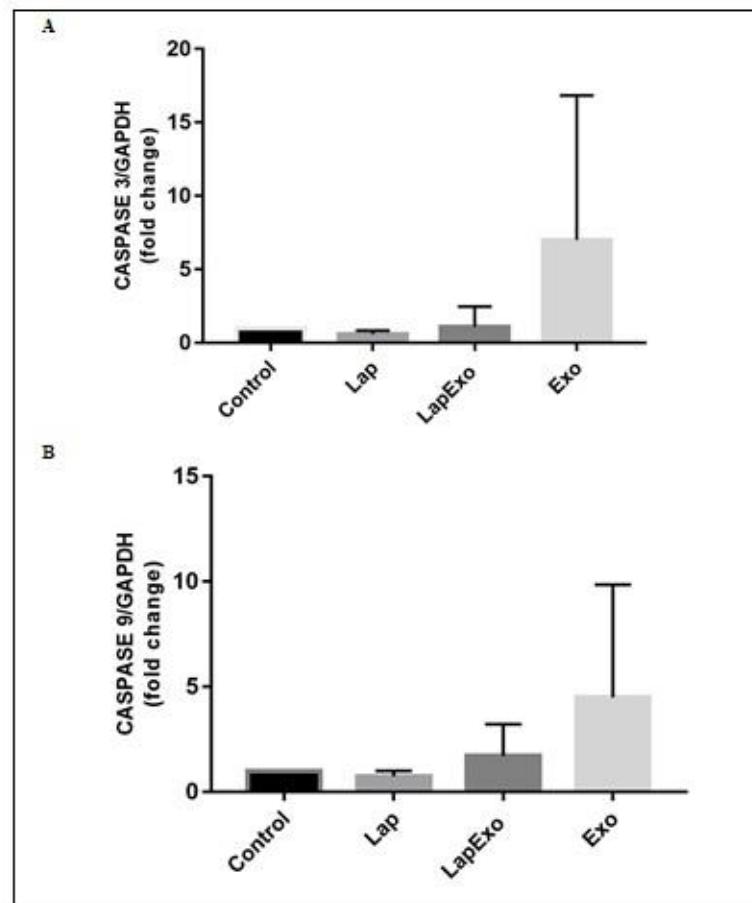


Figure 3.27: Caspase 3 (A) and Caspase 9 (B) gene expression analysis of Lap (0.1 μ M L), LapExo (0.1 μ M L + 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on MCF10 A healthy breast cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD.

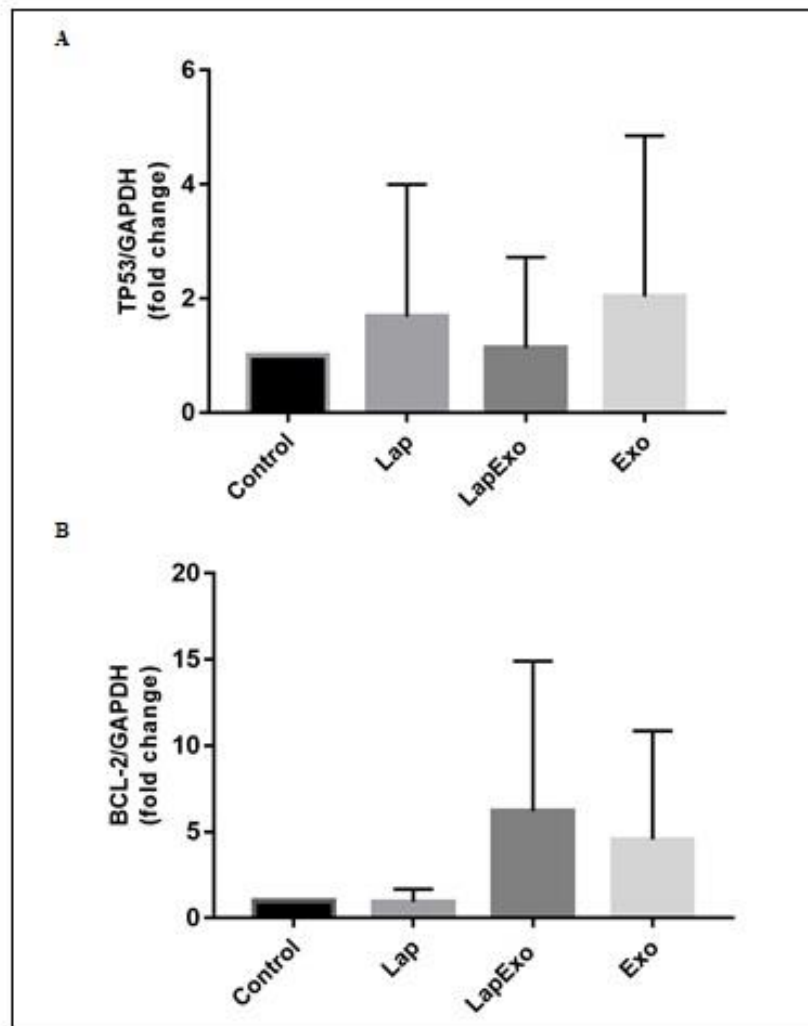


Figure 3.28: TP53 (A) and BCL2 (B) gene expression analysis of Lap (0,1 μ M L), LapExo (0.1 μ M L + 10^8 E particles/ml) and Exo (10^8 particles/ml) treatments on MCF10 A healthy breast cells at 48 hour. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD.

3.6. PROTEIN ANALYSES

3.6.1. Western Blot Analysis Results of SKBR-3 Cells

HER2, EGFR, 44/42 MAPK (ERK1/2), HSP 90, AKT, pAKT, p53 and caspase 3 protein expression levels were analysed in SKBR-3 and MCF10 A cell lines with Western blotting. The SKBR-3 cells were treated with the 0.1 μ M concentration of Lap, 0.1 μ ML+ 10^8 E particles/ml concentration of LapExo and 10^8 E particles/ml of exosomes for this assay at 48 hour.

As shown in Figure 3.29 A, HER2 protein expression level significantly increased in SKBR 3 cells treated with Lap ($\approx$ 1.87- fold) and LapExo ($\approx$ 2.08- fold) treated groups compared with control group. EGFR protein expression levels significantly increased in the LapExo treated group ($\approx$ 2.45- fold). Lap and Exo treatments were increased EGFR protein expression levels in the cells, but this increase was not significant compared with the control (Figure 3.29 B). In Figure 3.30 A , the ERK 1/2 (p44/42 MAPK) protein level was significantly decreased by treatment of Lap (73%), LapExo (61%) and Exos (74%). Moreover, HSP 90 protein expression level significantly decreased in Lap (74%) and LapExo (66%) treated SKBR-3 cells, but Exo treatment did not show any significant change compared with the control group (Figure 3.30 B). While AKT level did not show a statistically significant change in Lap, LapExo, and Exo treated groups (Figure 3.31 A), pAKT level was significantly reduced by treatments of Lap (31%) and LapExo (37%) for 48 hour (Figure 3.31 B). In addition, the pAKT protein level did not change in empty Exo treatment for 48 hours. The treatment of LapExo significantly increased the protein level of the P53 ($\approx$ 6- fold) (Figure 3.32 A). Although P53 level increased in Lap treated (257%) and Exo treated groups (368%), there was no significantly change in the SKBR-3 cell line. As shown in Figure 3.32 B Caspase 3 levels in SKBR 3 cells significantly increased by treatments of Lap (166%) and LapExo (206%) and Exo (200%) when compared to control groups.

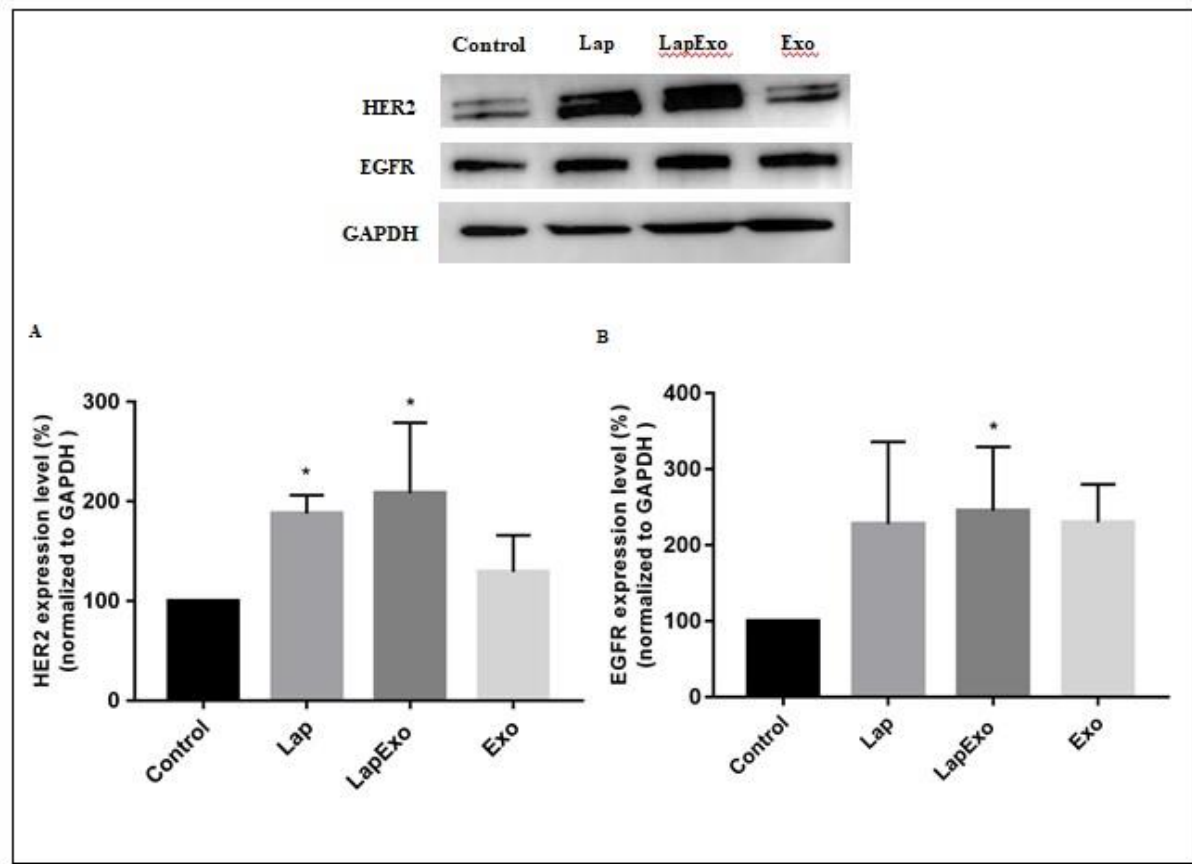


Figure 3.29: Effects of 0.1 μ M Lap, 0.1 μ M+ 10^8 particles/ml LapExo and 10^8 particles/ml Exo treatments on HER2 (A) and EGFR (B) protein expression in SKBR-3 cells at 48 hour. Protein expression levels were determined by western blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (*P<0.05)

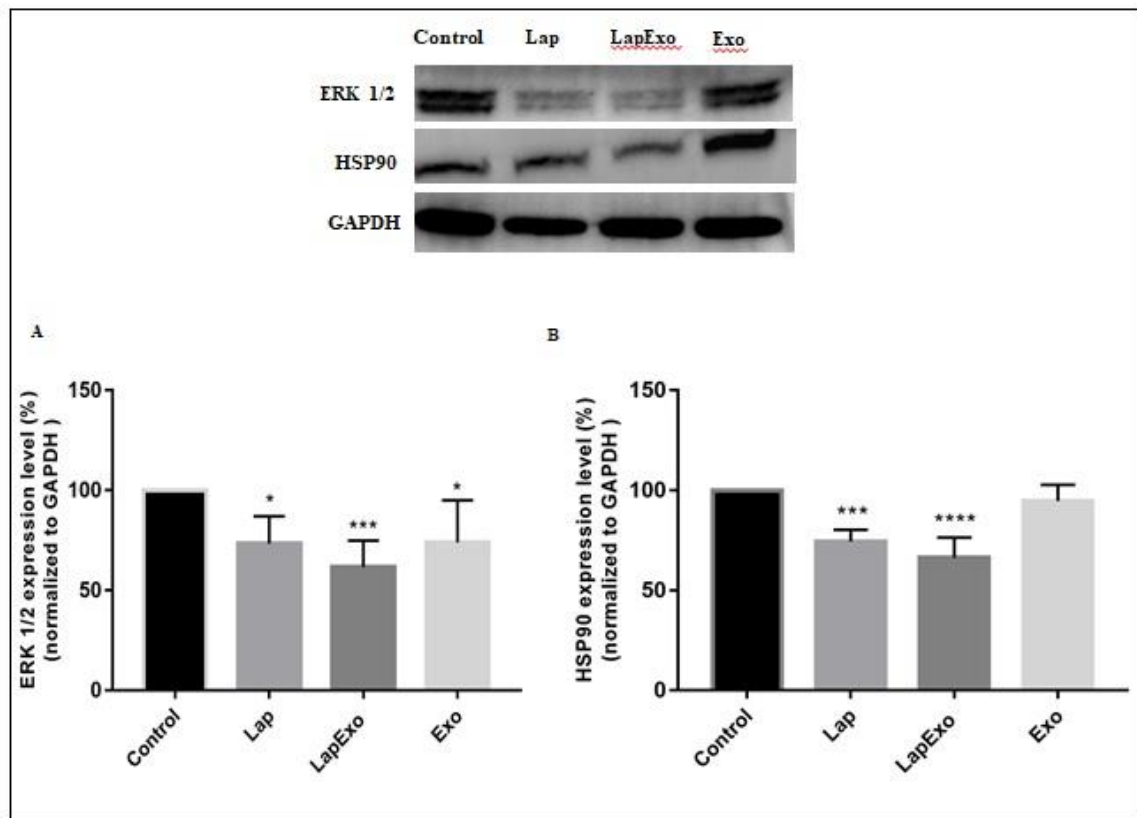


Figure 3.30 Effects of 0.1 μ M Lap, 0.1 μ M+ 10^8 particles/ml LapExo and 10^8 particles/ml Exo treatments on the ERK 1/2 (p44/42 MAPK) (A) and HSP90 (B) protein expression in SKBR-3 cells at 48 hour. Protein expression levels were determined by western blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (**** $P \leq 0.0001$, *** $p < 0.001$, * $P < 0.05$)

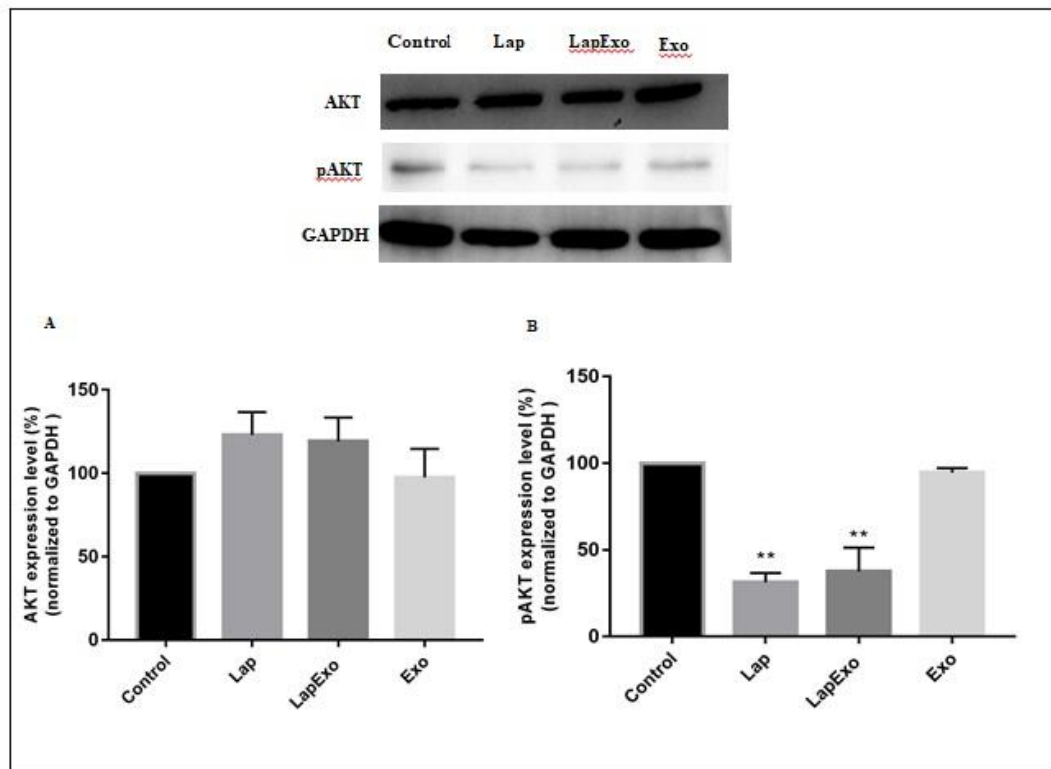


Figure 3.31 Effects of 0.1 μM Lap, 0.1 μM + 10^8 particles/ml LapExo and 10^8 particles/ml Exo treatments on AKT (A) and pAKT (B) protein expression in SKBR-3 cells at 48 hour. Protein expression levels were determined by western blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$)

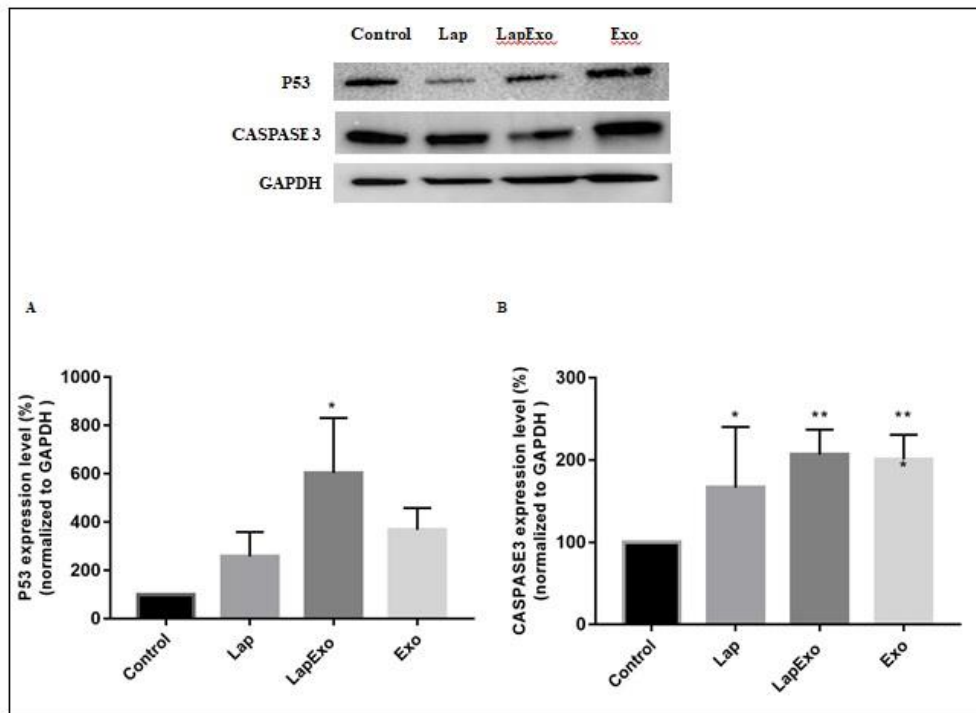


Figure 3.32: Effects of 0.1 μ M Lap, 0.1 μ M+ 10^8 particles/ml LapExo and 10^8 particles/ml Exo treatments on P53 (A) and caspase 3 (B) protein expression in SKBR-3 cells at 48 hour. Protein expression levels were determined by western blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (** $P \leq 0.01$, * $P < 0.05$)

3.6.2. Western Blot Analysis Results of MCF10 A Cells

MCF10 A cells were treated with the 0.1 L μ M concentration of Lap, 0.1 μ ML+ 10^8 E particles/ml concentration of LapExo and 10^8 E particles/ml of exosomes for this assay at 48 hour. In the cells, as shown in Figure 3.33 A, the protein expression level of EGFR did not change in the treatment of Lap, LapExo, and Exo compared with control groups. In Lap, LapExo, and exosomes treated MCF10 A cells, the ERK 1/2 (p44/42 MAPK) protein level did not change significantly (Figure 3.33 B). As indicated in Figure 3.34 the AKT protein expression level was increased during the treatments of LapExo (126%), and decreased during Exos treatment (71%). But the pAKT protein levels did not change in Lap, LapExo, and Exo treated groups. Similar to these results, HSP90 and caspase 3 protein levels in MCF10 A cells did not significantly change with the treatment of Lap, LapExo, and Exo (Figure 3.35).

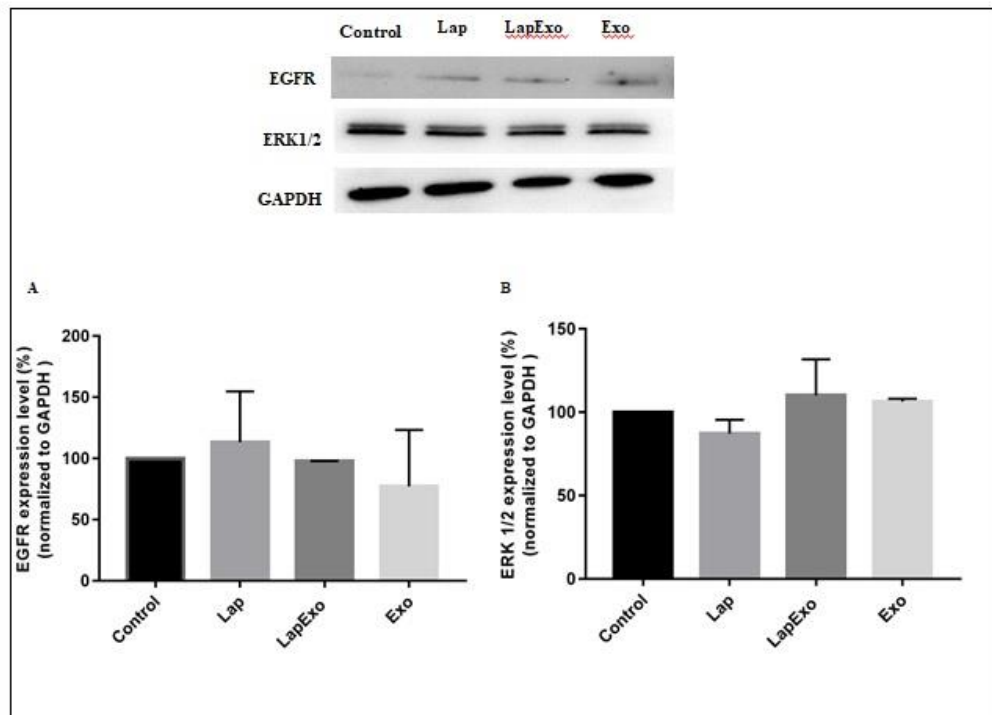


Figure 3.33: Effects of 0,1 μ M Lap, 0,1 μ M+ 10^8 particles/ml LapExo and 10^8 particles/ml Exo treatments on EGFR (A) and ERK 1/2 (p44/42 MAPK) (B) protein expressions in MCF10 A cells at 48 hours. Protein expression levels were detected by Western Blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD. (***) $p < 0.001$, (*) $p < 0.05$)

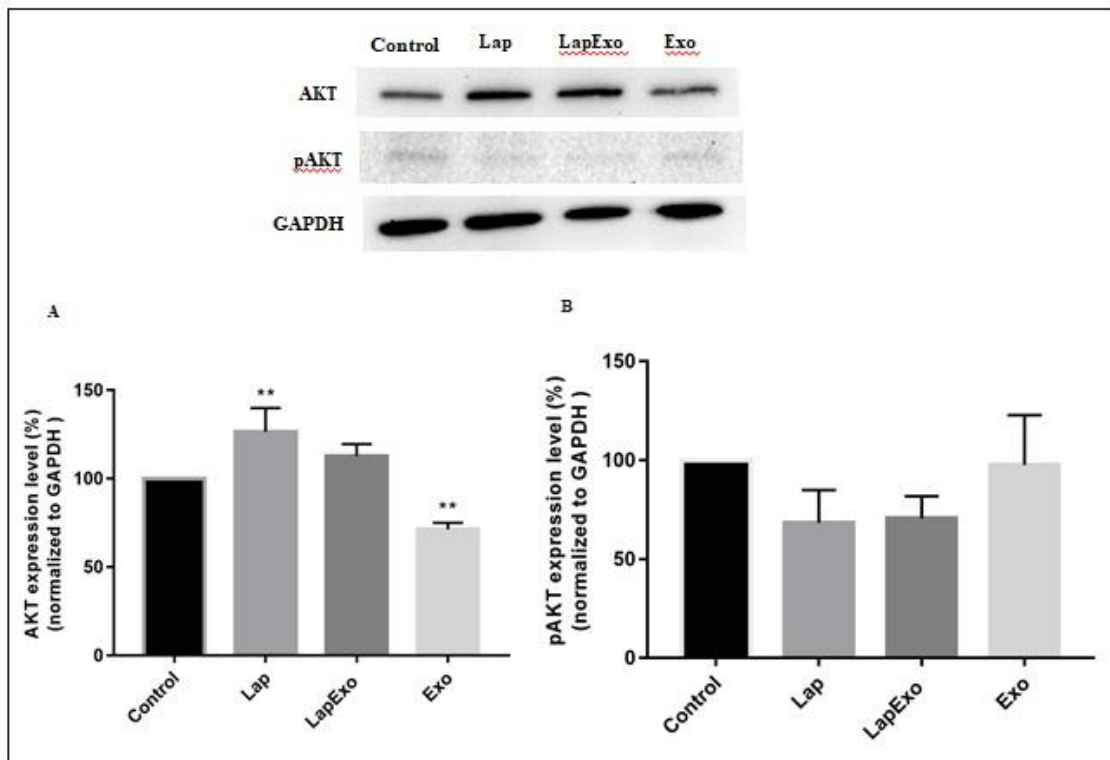


Figure 3.34: Effects of 0.1 μ M Lap, 0.1 μ M+108 particles/ml LapExo and 108 particles/ml Exo treatments on AKT (A) and pAKT (B) protein expressions in MCF10 A cells at 48 hours. Protein expression levels were detected by Western Blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD.

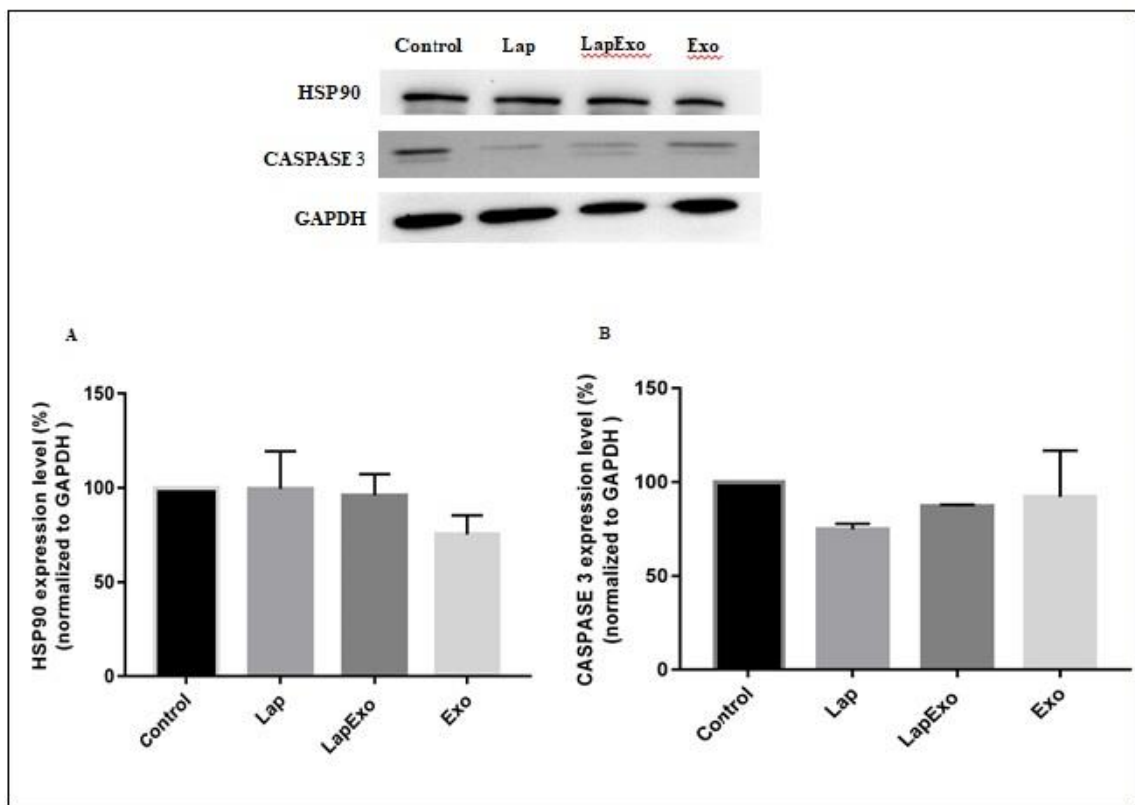


Figure 3.35: Effects of 0.1 μM Lap, 0.1 μM + 10^8 particles/ml LapExo and 10^8 particles/ml Exo treatments on HSP90 (A) and Caspase 3 (B) protein expressions in MCF10 A cells at 48 hours. Protein expression levels were detected by Western Blot analysis. All groups were analyzed compared with the control group. Data shows an average of experiments repeated three times $\pm$ SD.

4. DISCUSSION

From a molecular perspective, the pathology of breast cancer, which affects most women, is a heterogeneous disease (Harbeck *et al.*, 2019). Breast cancer is classified according to the stage and grade of the tumor. In breast cancer, the behavior of the tumor and its response to treatment are determined by its biological features. In addition, morphological features are crucial in classifying breast cancer types. Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression are the main determinants in breast cancer diagnosis (Rakha and Green, 2017). HER2 positive breast cancer, which is known as a more aggressive disease than estrogen receptor positive (ER positive) and HER2 negative breast cancers, is between 15% and 20%. That being the case, anti-HER2 agents have been developed recently and used to diagnosis of early and late HER2-positive breast cancer (Gonzalez-Angulo *et al.*, 2006; Cesca *et al.*, 2020). HER2 is a transmembrane receptor that promotes cell growth and differentiation. Moreover, ERBB2/HER2 gene is located in the 17q12 chromosomal region, and the amplification of this gene causes HER2 positive breast cancer. Overexpression of the HER2 protein is a event that occurs in most breast cancers (Ferrari *et al.*, 2016). However, the types of treatment were designed according to the heterogeneity of breast cancer and therapies were developed to reduce the side effects of treatment (Harbeck *et al.*, 2019). Before monoclonal antibodies against HER2 receptors were found, it was a form of breast cancer that was difficult to treat. However, in the last 30 years, the treatment options for this type of breast cancer have increased tremendously in the sense that entry of both monoclonal antibodies and tyrosine kinase inhibitors to target the HER2 receptor into clinical use have changed fate of the patients whose cancer overexpresses the HER2 receptor (B. Chen *et al.*, 2020; Kunte *et al.*, 2020).

Trastuzumab is the first drug used clinically as an anti-HER2 agent in both the early and metastatic stages of the disease. Recently, trastuzumab and pertuzumab as monoclonal antibodies against HER2 receptors. At the same time, lapatinib and neratinib have been used as small molecule tyrosine kinase inhibitors to block HER2 receptor kinase activity in treating of HER2 positive breast cancer patients (Yao and Fu, 2018). These drugs are small molecule tyrosine kinase inhibitors (TKI) that bind and block the intracellular tyrosine kinase domains of HER2 receptors. TKI play an active role in inhibiting HER2 functions. In addition, TKIs

are successful at crossing blood-brain barriers. *Lapatinib* (Lap) was the first tyrosine kinase inhibitor to be used and approved for metastatic HER2 positive breast cancer. Lap is an oral drug that is an anti-HER2 and anti-HER1 tyrosine kinase inhibitor and inhibits the intracellular domain of HER2 and HER1 (EGFR). Therefore, tyrosine phosphorylation is suppressed, and the downstream pathways of the MAPK/ERK1/2 and PI3K/Akt are also inhibited by lapatinib (Asif *et al.*, 2016; Piccart-Gebhart *et al.*, 2016).

Our aim in this thesis was to use exosomes to load lapatinib (Lap) as a tyrosine kinase inhibitor in the drug delivery system and thereby increase the cytotoxic effects of Lap treatment at a low dosage on HER2 positive breast cancer cell line SKBR-3. We demonstrated that the chemotherapeutic drug lapatinib was safely and successfully loaded into exosomes isolated from healthy breast epithelial cells (MCF10 A) by the electroporation method. The effects of free Lap and exosome-encapsulated lapatinib (LapExo) treatments on SKBR 3 and MCF10 A cells were evaluated comparatively. It has been shown that our LapExo treatment showed better cytotoxic effects on SKBR 3 cancer cells than the free Lap treatment. Similarly, the LapExo treatment had more apoptotic effects on cancer cells than the free Lap. Besides, we observed that the produced LapExo altered the mRNA and protein expression relating to the lapatinib targeting pathways, including HER2, EGFR, AKT, pAKT, MAPK, and ERK 1/2 on SKBR 3 cells.

In this study Lapatinib, a tyrosine kinase inhibitor was used on a HER2 positive breast cancer cell line (SKBR-3) and a model of normal human breast epithelial cell line (MCF10 A). The cytotoxicity assay was performed to determine the dose to be used in lapatinib to load into exosomes isolated from MCF10A cells. The highest Lap concentration (10 μ M) had a toxic effect on SKBR 3 cells (Figure 3.7), while it did not effect on MCF10 A cells (Figure 3.8) a time and dose dependent manners. All other selected concentrations of lapatinib at 72 hours are cytotoxic to SKBR 3 cells, while lapatinib different concentrations had no cytotoxic effect on MCF10 A cells at 24, 48, and 72 hours of treatment.

Chemotherapy has been the most commonly used treatment modality in cancer patients. When chemotherapeutic drugs are administered to patients, they destroy cancer cells and healthy cells simultaneously. However, the resistance of cancer cells to chemotherapeutic drugs may cause the tumor to spread and the disease to progress rapidly. This means that the treatment has failed (Iturrioz-Rodríguez *et al.*, 2019; J. Wang *et al.*, 2020). Nanotechnology-

based drug delivery systems have been developed to alter the pharmacokinetics and cytotoxic effects by targeting chemotherapeutic drugs selectively to the tumor site (Irvine and Dane, 2020). The aim of incorporating nanoparticles (NP) in cancer treatment is to increase the drug uptake by the targeted cancer cells, thereby reducing the drug's toxicity to the neighboring untargeted normal tissue (REF). When NPs are specifically targeted to a specific cell-type, they bind to the cell surface receptors, then become taken into the cell through endocytosis (Cho *et al.*, 2008). However, tumors consist of weak blood vessels that leak. Nanocarriers used as drug delivery systems can easily pass from leaky vessels to tumor tissues by using the enhanced permeability and retention (ERP) effect (Peer *et al.*, 2007). Nanoparticles can encapsulate drugs, genes, imaging agents, and proteins. This system increases the solubility and stability of encapsulated cargoes, allowing them to cross biological barriers such as blood-brain barriers (Kou *et al.*, 2018). Function, targeting, biocompatibility, and biodistribution of NPs are properties that require them to be a suitable carrier for cargo in drug delivery systems. At the same time they can increase circulation times in the body to increase safety and effectiveness (Jong, 2008; Mitchell *et al.*, 2020). Since NPs are small in size (100-200nm), they are easier to enter the cell than large molecules. They are used as drug delivery systems, and they have many different materials such as gold nanoparticles, solid lipid nanoparticles, liposomes, micelles, silica, and dendrimers that are used widely (M. Chen *et al.*, 2020; Vuja, *et al.*, 2020). On the other hand, nanoparticles' physicochemical properties, such as size, charge, and hydrophobicity, affect their metabolism, absorption, and distribution (Severino *et al.*, 2019).

Some studies have proven that HER1 and HER2 expression is too high in HER2-positive breast cancers. Therefore, FDA-approved lapatinib drug is used in the treatment of HER2 positive metastatic cancer. However, lapatinib is a hydrophobic drug. Because of this, lapatinib has low solubility and bioavailability in circulation. Peyvand *et al.* indicate that most free lapatinib binds to plasma proteins such as albumin and alpha-1 acid glycoprotein to protect the drug by encapsulating it in the bloodstream until it reaches its tumor target site. Therefore, encapsulation of lapatinib is essential to increase its solubility and effect on cancerous cells. Buss *et al.* demonstrated that encapsulated lapatinib was influential in the controlled release and targeting of the drug. Their study evaluated the cytotoxicity of lapatinib-loaded nanocapsules on HER2 positive bladder cancer (Buss *et al.*, 2019; Peyvand *et al.*, 2020). Although lapatinib was encapsulated with different nanoparticles in previous

studies in the literature (Gao *et al.*, 2013; Lee and Cho, 2019), it was not encapsulated with exosomes. In this thesis, lapatinib was encapsulated with a non-synthetic nanoparticle called exosome. Exosome encapsulated lapatinib, and free lapatinib were studied and compared on SKBR-3 and MCF10 A cell lines.

Exosomes (Exos) are vesicular structures originating from multivesicular bodies, and their sizes range from 40-150 nm. They with a lipid bilayer structure are released from the cell by the fusing multivesicular bodies with the plasma membrane. It has been revealed that exos released from almost all cells play a role in intercellular traffic (Article, 2008; Kanchanapally *et al.*, 2019). Exos carry some biological molecules that will guide their biological activity. These are proteins, lipids, carbonhydrates, miRNAs, and mRNAs. The contents of exos can indicate the cell's biological state from which they are separated (Walker *et al.*, 2019; Garbayo *et al.*, 2020).

Exosomes are found in several body fluids such as, blood, sweat, and urine. These biofluids contain many different macro-molecules and protein aggregates. For this reason, isolation and detection of the exosomes are challenging. Exosome isolation techniques need to be appropriate and effective for all samples. Some isolation methods have been developed based on the literature's surface proteins, density, and size of exosomes. In addition, the isolation methods developed are based on ultracentrifugation, precipitation, ultrafiltration, and immunoassay (Bu *et al.*, 2019; Y. Wang *et al.*, 2020).

Exosomes differ in their size, surface molecules, and the type of cargo they carry. However, the bioactive surface molecules on the exosomes are associated with the cell from which they originate. Strikingly, it has been shown that the same type of exosome donor cells is more effective for drug delivery systems than the use of different types of donor and recipient cells. For example, exosomes derived from tumor cells contain tumor antigens on their surface, such as FasL, TRAIL, or TGF- β . Therefore, it has been argued that exosome-related therapeutic studies should be obtained from normal cells and carefully purified (Kibria *et al.*, 2018; Chinnappan *et al.*, 2020). In this thesis, the HER2 positive SKBR-3 cell line belonging to the breast cancer type was used. Therefore, the exosomes used to load lapatinib were obtained from the healthy breast cell line MCF10 A. For drug delivery, we isolated exosomes from the human epithelial cell line (MCF10 A). The isolation method developed by Kırbaş *et al.* (2019) was used in this thesis, and this method is an aqueous two-phase system (ATPS).

Compared to other isolation methods, exosomes were obtained more effectively and efficiently (Kırbaş *et al.*, 2019). The characterization and identification of the exosome are based on the surface proteins, their number, size, and morphological structure. In this study, the physical characterization of the obtained exosomes was done with NTA and Zeta sizer, while the functional characterization was done with flow cytometry analysis. In addition morphological and physiological analyses were performed with AFM. NTA is a technique that can count and size exosomes released from cell culture. In addition, NTA is a laser light scattering microscope that can visualize and size individual exosomes in the liquid state (Oosthuyzen *et al.*, 2013; Y. Wang *et al.*, 2020). Our results showed that the significant peak detected that nanoparticles sizing occurred between 20 and 200 nm (Figure 3.1 and Figure 3.2). Further characterization of these nanoparticles by flow cytometry confirmed the presence of hallmark exosome-associated surface markers such as CD9, CD63, CD81 and HSP70 that were absent in the negative control (Figure 3.3). All results regarding the characterization of the exosome are consistent with the literature. In summary, exosomes obtained from healthy MCF10 A breast cells were used in the drug delivery system in this thesis.

The lack of a practice therapeutic application against different tumor types is due mainly to the lack of an ideal delivery system. The key characteristics of an ideal delivery system include having few side effects and basing on an effective and safe targeting system. Recently, nanomaterials such as viruses and liposomes have been used in cancer treatments. Over the last few years, studies have shown that naturally produced exosomes can be used as nanocarriers in anti-cancer treatments. Several studies in the literature report that when exosomes are used as a drug delivery system, they provide several advantages over other delivery systems (Kalluri, 2016; Bu *et al.*, 2019). First of all, since exosomes have a lipid bilayer membrane structure, they can tether to the membrane of their target cell, handing in the drugs to the recipient cells more efficiently and potently—this way, they increase the bioavailability of the drugs. Furthermore, compared to the conventional drug delivery systems, exosomes offer a more stable drug-transport system by protecting their cargo from enzymatic degradation and low immunogenic and toxic characteristics. Moreover, their nano-size enables their spread to the tumor tissue. Finally, it has been suggested that their cell surface receptors may play important roles in providing easier access through biological such as the blood-brain vessel barrier (Y. Wang *et al.*, 2020).

Drug loading into the exosome is accomplished using various techniques. Typically, exosomes consist of some of biomolecules in their structure. The loading of the drugs or molecules into the exosome can be achieved by two primary methods, either active or passive encapsulation processes. Passive encapsulation method is simple, and it does not require the introduction of different active substances into the loading process. The passive drug loading methods involve incubating the desired cargo molecules with exosomes. Meanwhile, active encapsulation methods include sonication, extrusion, freeze and thaw cycles, electroporation, incubation with saponin, and antibody binding. Each method differs in terms of drug loading stability and efficiency into the exosomes. At the same time, each method has its advantages and disadvantages (Luan *et al.*, 2017). Some studies in the literature regarding the use of exosomes in the drug delivery system. Munagala *et al.* used the exosomes obtained from bovine milk for drug delivery and proved that it has an anti-tumor effect on lung cancer (Munagala *et al.*, 2016). Hadla *et al.* (2016) proved that it could be used to treat tumors by loading the chemotherapeutic drug doxorubicin (DOX) into the exosomes with the electroporation method (Hadla *et al.*, 2016). In another study, Kim *et al.* used ultrasonic technology to encapsulate paclitaxel (PTX) chemotherapeutic drug with exosome. They showed that the anti-tumor effect of the exoPTX they produced increased on drug-resistant cells compared to the naked PTX (Kim *et al.*, 2016). Aqil and colleagues loaded curcumin into the exosome. 18-24% of curcumin was loaded into the exosome. In this study, they showed that the synthesized ExoCUR drug reduces the proliferation of tumor cells (Aqil *et al.*, 2017).

Since encapsulation of the chemotherapeutic agent lapatinib has not been reported so far in the literature, we chose to study the therapeutic potential of this drug on breast cancer in this study. First, 50 μ M of lapatinib was prepared for loading into exosomes obtained from MCF10 A cells. One of the loading methods found in the literature is the electroporation method. In this method, the drug and the exosome are suspended in a solution, and small pores are created in the exosome membrane by applying an electric field to the exosomes in the solution. Thereby, the penetrance of lapatinib into the exosome through the pores opened in the membrane was increased. After drug loading through electroporation, the pores opened in the exosome membrane are closed, and the membrane resumes its original state (Kim *et al.*, 2016; Luan *et al.*, 2017).

In the literature, it has been stated that the electroporation method may cause RNA aggregation and exosome instability. In this study, after loading lapatinib onto the exosomes by electroporation, characterization was performed with NTA to observe the status of exosomes. After electroporation at 350 V and 150 mF, free Lap drugs in the medium were removed, and the characterization of Lap-loaded exosome was done by the HPLC method. In order to characterize and calculate the amount of lapatinib loaded into the exosome, the standard graph of lapatinib (Figure 3.4) was first created by the HPLC method. The amount of lapatinib loaded was calculated using this graph. As shown in Figure 3.5, approximately 25 μ M Lap was loaded into the exosomes. Consequently the exosome loading efficiency of lapatinib in our studies was around 40-45% by the electroporation method.

After Lap was loaded into exosomes, cytotoxicity assay of free Lap, exosome-encapsulated Lap (LapExo), and empty exosome (Exo) were performed in the SKBR 3 and MCF10 A cells at different concentrations. It should be pointed out that Dextran remains in the final solution of the exosome preparations when the ATPS method is the exosome isolation method of choice. Dextran can affect the viability of cells. For this reason, in this thesis study, the effects of an equivalent concentration of dex phase found in the final exosome isolates were examined on cell growth. Cell viability assay was performed depending on dose and time, and the most effective concentration and time were selected according to the IC_{50} value obtained. When the results were examined, the cytotoxic effect of exosome-encapsulated lapatinib was greater than that of free lapatinib on SKBR-3 cells. Exosome-encapsulated lapatinib (51% cell viability) showed a significantly more cytotoxic effect than free lapatinib (68% cell viability) on SKBR-3 cells. The LapExo treatment statistically significantly decreased cell viability compared to free Laps in a dose- and time-dependent manner (Figure 3.11). These results showed that lapatinib was successfully loaded into exosomes, and the chemotherapeutic effect of exosome-encapsulated Lap was enhanced compared to free Lap. At the same time, no cytotoxic effects of either Lap and LapExo on healthy cells were observed as determined in a dose and time manner in this study. Furthermore, empty exosomes (10^8 particles/ml) had no cytotoxic effects on SKBR-3 and MCF10 A cells in a dose and time- dependent manner. It has been shown that exosomes are naturally existing nanoparticles used in drug delivery systems. A dose of 10^8 particles/ml, consistent with 0.1 μ M lapatinib, was chosen at 48 hours. Similarly, the dex phase remained in the medium after the isolation and did not affect the

proliferation of SKBR-3 and MCF10 A cells. The appropriate dose and time for Lap and LapExo are consistent with studies in the literature on lapatinib (Guo *et al.*, 2012).

Moreover, gene expression, protein expression, and apoptotic analyses and validation studies were performed by applying 0.1 μM concentrations of Lap, 0.1 $\mu\text{M/L}$ + 10^8 particles/ml concentration LapExo, and 10^8 particles/ml concentration of empty Exo to the cells at the 48th hour. Sholwer *et al.* (2019) applied 2 μM of lapatinib on SKBR-3 cells for 72 hours and investigated its apoptotic effects. Their results proved that up to 30% of the cells were subjected to apoptosis (Showalter *et al.*, 2019). As demonstrated by Guo *et al.* (2012), 11% apoptosis was observed in 48 hours of administration of 10 μM of lapatinib on a different HER2 positive cell line (Guo *et al.*, 2012). However, Ma *et al.* also examined the apoptotic effect of lapatinib on SKBR-3 cells and showed that 0.1 μM of lapatinib had a 17% apoptotic effect (Ma *et al.*, 2010). Our study evaluated cell apoptosis after applying the drugs by Annexin V/PI staining. Similar to the findings in the literature, free Lap has an apoptotic effect of 18% in SKBR3 cells. In comparison the apoptotic effect of LapExo (21%) is higher than that of free Lap. 7% necrotic death was observed in the empty exosome, which is used as a vehicle in drug transport. In MCF10 A, which we used as a control, apoptotic and necrotic death in all experimental groups was considerably lower than in SKBR-3 cells.

Apoptosis can occur in two ways: intrinsic and extrinsic pathways. The B-cell lymphoma-2 (BCL-2) protein family members are cell death regulators for the intrinsic pathway. It is also a control mechanism in the intrinsic pathway of apoptosis that governs membrane permeability of mitochondria and apoptogenic factors (Danial, 2008). Members of the BCL-2 protein family fall into two major groups based on their structure and function. Pro-apoptotic BCL-2 family proteins include: BAK, BAX, BIM, PUMA (p53 upregulated modulator of apoptosis), BH3 interacting-domain death agonist (BID), BAD (BCL-2 associated death promoter), NOXA, BIK (BCL-2 interacting killer), BCL-2 modifying factor (BMF) and HRK proteins (Raducka-Jaszul *et al.*, 2020). These pro-apoptotics induce cytochrome c release from the mitochondrial membrane (Shantanam, 2018). The other group includes anti-apoptotic BCL-2 proteins such as BCL-2, BCL-XL, BCL-w, BCLB, MCL1, and BFL1. These proteins bind to proteins belonging to the BCL-2 family and inhibit the apoptotic pathway (Raducka-Jaszul *et al.*, 2020). Another group, the extrinsic apoptotic pathway, begins when ligands bind cell surface receptors, stimulate death-inducing signaling complexes (DISC), and activate

caspases. Apoptosis can occur in an extrinsic or intrinsic pathway, and caspases are central to these pathways. They are initiators of caspase 8 extrinsic and caspase 9 intrinsic apoptotic pathways (Tian *et al.*, 2015). In the extrinsic apoptotic pathway, ligands initiate apoptosis by binding to the cell surface death receptors such as TNF- α ; this binding enables caspase 8 activations. Then, with the activation of caspase 3, the cell undergoes apoptosis. However, the intrinsic apoptotic pathway becomes induced by damage to the mitochondria upon which cytochrome C is released from mitochondria and complexes with APAF-1 resulting in activation of caspase 9. Cleavage and activation of caspase 3/7 upon caspase 9 activation allows execution of apoptosis. Caspase 3 can be activated by intrinsic and extrinsic apoptotic pathways and is located at the endpoint of caspase cascades. At the end of apoptosis that occurs with the activation of caspase 3 the plasma membrane is disrupted, the DNA is fragmented and the phosphatidyl serine on the extracellular side of the plasma membrane is inhibited (Jiang *et al.*, 2020). In our study, in addition to apoptotic analysis with annexin V/PI, the apoptotic mechanisms were also determined with gene expression analysis of caspase 9, caspase 3, BCL-2, and protein expression of caspase 3 to confirm the apoptotic effects of Lap, LapExo, and Exo treatments on SKBR-3 and MCF10 A cells. SKBR-3 cells treated with Lap and LapExo significantly increased caspase 9 gene expression level. However, caspase 3 gene expression significantly increased with Lap and LapExo treatments. For the BCL-2 gene expression, there was no significant change in SKBR 3 cells. The results show that Lap and LapExo can activate the intrinsic apoptotic pathway, making caspase 9 effective and enhancing caspase 3, causing SKBR-3 cells to apoptosis. Similar results were obtained from protein analysis. Caspase 3 protein expression showed a statistically significant changes in the Lap, LapExo and Exo treated groups. Beside Lap, LapExo and Exo treatments in MCF10 A cells did not demonstrate any significant change in neither caspase 3, caspase 9 or BCL-2 gene expression levels nor caspase 3 protein expression level. Treatment of Lap, LapExo, and Exo did not show any significant change in the mRNA levels of caspase 9, caspase 3, and BCL-2 for MCF10 A cells. Consequently annexin V/PI analysis, mRNA, and protein expression levels showed similar results for Lap, LapExo, and Exo treatments on SKBR 3 breast cancer cells and MCF10 A healthy breast cells.

BAX, a proapoptotic member of the BCL-2 family, is regulated by the P53 tumor suppressor gene (Nakano and Vousden, 2001). About 50% of HER2 positive breast cancers carry various TP53 mutations (Bertheau *et al.*, 2013). In this study, we analyzed both gene expression and

protein expression levels of P53 in the SKBR-3 and MCF10 A cells treated with Lap, Lapexo, and Exo. While the expression of the TP53 gene was significantly increased in SKBR-3 cells treated with Lap and LapExo, only LapExo treatment increased the protein expression of P53. LapExo treatment showed more effectiveness on the tumor suppressor gene P53 expression and its expression increased for cell death. Besides, P53 gene and protein expression level is no effect in MCF10 A cells confirmed with in RT-PCR and western blot studies.

When the HER2 gene is overexpressed in cells, it activates the RAS/RAF/MEK/MAPK pathway, allowing cells to grow, and differentiate (Asif *et al.*, 2016). However, previous studies have shown that the ERBB signaling pathway stimulates the MAPK pathway and the PI3 kinase pathway. MAP kinases stimulate cell growth, while PI3 kinases activate anti-apoptotic molecules, inducing cell immortality (Wang and Greene, 2005). Tumor inhibitor drugs such as lapatinib remove the barriers and activate cell death mechanisms in tumor cells. Inhibition of kinase signaling pathways such as MEK, AKT, ERK, RAS, RAF, and MTOR and ERBB families member growth factors such as EGFR and HER2 also lead to apoptosis (Shantanam, 2018).

Lapatinib binds to adenosine triphosphate (ATP)-binding pocket found in the intracellular tyrosine kinase domains of cell ERBB molecules EGFR and HER2. In addition, it inhibits the phosphorylation of HER2 and EGFR tyrosine kinase by binding ATPs. As a result of this inhibition, MAPK and PI3 kinase signaling pathways are blocked by down-regulation and, thereby, cell death takes place (Konecny *et al.*, 2006; Wang, 2014). In their study, Konecny *et al.* demonstrated that lapatinib increased the expression of HER2, EGFR, AKT, and ERK1/2 proteins while inhibiting the expression of pHER2, pEGFR pAKT, and pERK1/2 proteins in the concentration range of 2-0.2 μM (Konecny *et al.*, 2006). Our data indicate that, the gene expression levels of HER2 and EGFR increased and MAPK decreased at the SKBR-3 cells treated with 0.1 μM L + 10^8 E particles/ml concentration of LapExo. There is no change in gene and protein expression levels of AKT. The protein expression levels of HER2 and EGFR increased while protein expression levels of pAKT and ERK 1/2 (p44/42 MAPK) decreased in SKBR-3 cells treated with Lap and LapExo. Therefore, our findings are consistent with the literature findings. This study demonstrated that EGFR did not show any significant gene and protein expressions in MCF10 A cells treated with Lap, LapExo and Exo. LapExo did not

inhibit the tyrosine kinase receptors in these cells and no changes observed in the expression of proteins and genes of AKT and pAKT.

HSP90 is a chaperone molecule commonly found in mammals. It is more expressed in cancer cells compared to normal cells. The HSP90 chaperone is responsible for the ERBB1 and ERBB2 proteins, which belong to the ERBB reseptor family. HER2 and EGF receptors are susceptible to inhibition of HSP90 (Citri, 2004; Ye *et al.*, 2021). Previous studies in the literature stated that lapatinib with the combination of an HSP90 inhibitor showed a high anti-tumor effect against cancer (Leow *et al.*, 2009). Our study indicated that the HSP90 protein expression level was decreased significantly for Lap and LapExo treatment on SKBR-3 cells. When MCF10 A cells were treated with Lap and LapExo, there was no change in HSP90 protein level compared to the control. These findings also implied that the combinatory use of exosome-encapsulated lapatinib with an HSP90 inhibitor might provide a more effective approach to breast cancer cell therapy by inhibiting of both HER2 receptor and HSP90.

The metabolic pathways examined in this thesis, the proteins involved in these pathways, and their relationships with each other are shown in Figure 4.1. The results show that lapatinib and LapExo significantly affect these pathways.

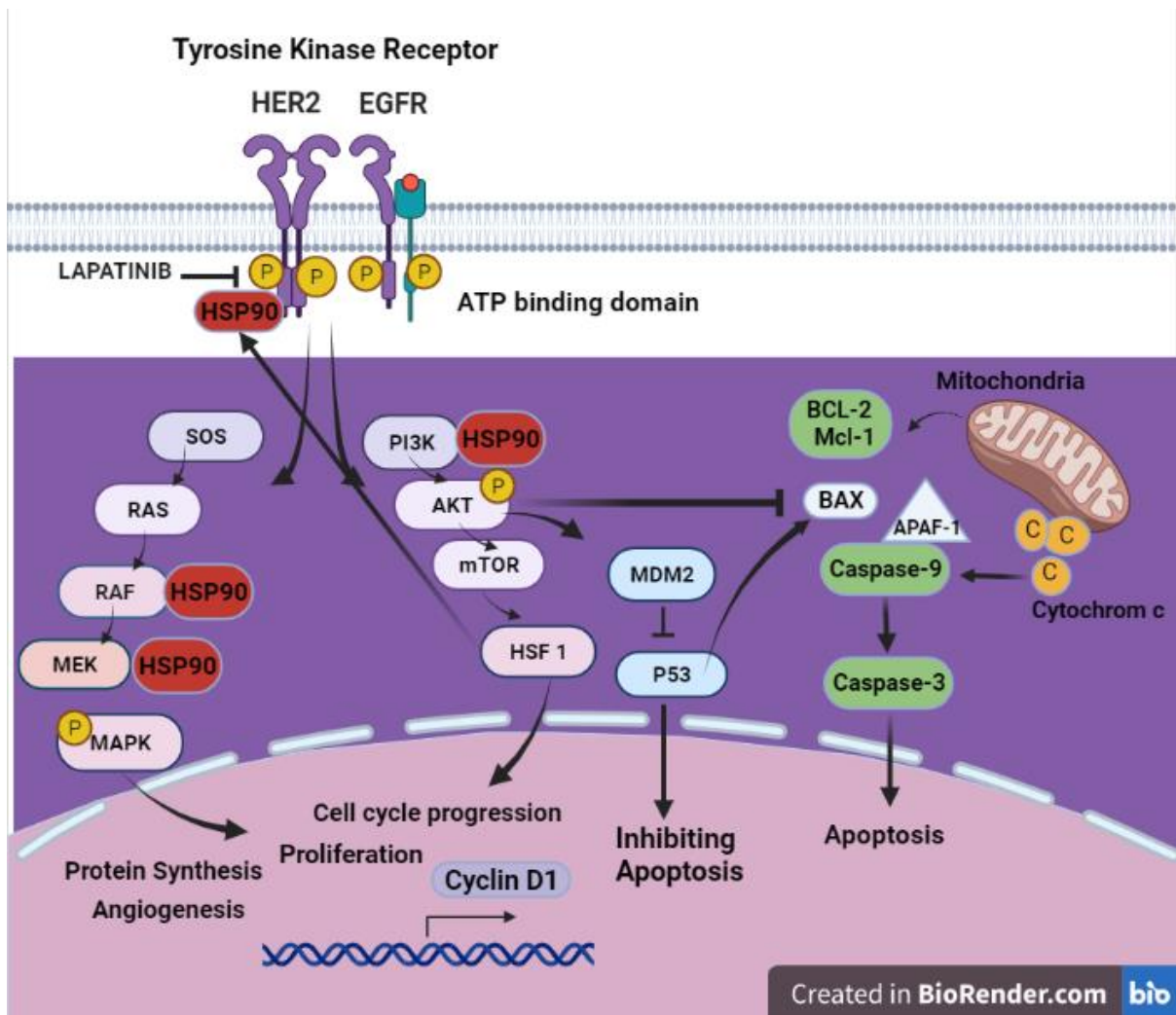


Figure 4.1: The metabolic pathway of Lap and LapExo on SKBR-3 and MCF10 A cells

5. CONCLUSION AND RECOMMENDATIONS

HER2 positive breast cancer is one of the subtypes of breast cancer. More specific treatment strategies are in place for this cancer type. As a tyrosine kinase inhibitor, lapatinib reduces the proliferation of cancer cells by binding to the intracellular kinase regions of HER2 and EGFR growth factor receptors, and inhibits their kinase activity. However, lapatinib is a hydrophobic drug and, it has low solubility in the bloodstream. In previous studies, lapatinib was combined with various nanoparticles to increase its utilization capacity. Recently, exosomes have been used for therapeutic purposes, considering their structural features and functions.

In our study, exosomes were used to improve the transport of hydrophobic lapatinib across the plasma membrane. In this context, first of all, was encapsulated with exosomes isolated from normal mammary epithelial cells (MCF10 A) using the electroporation method. Approximately 40-45% effective loading was achieved at the end of this. Then HER2 positive SKBR-3 and healthy MCF10 A cells were treated with free lapatinib, lapatinib loaded exosomes and free exosomes. Our results demonstrated that exosome-encapsulated lapatinib has more potent therapeutic effects on cancer cells at low doses than free lapatinib. Furthermore, this allows reducing the toxic effect of lapatinib on normal cells. Overall, Lapexo treatment showed an anti-tumor effect by inducing apoptosis and reducing cell proliferation and reducing tumor formation by inhibiting tumor angiogenesis and metastasis. In addition that exosomes, which we used as vehicles, did not have cytotoxic and apoptotic effects on the cells.

Exosomes acted as natural nanoparticles in this study. Our studies show that the exosome can also be used as a nanoparticle drug delivery system for breast cancer therapy. Future studies may also carry out one or more drugs to target cells and tissues by targeting motifs to exosomes. The clinical use of the exosome can be improved by developing different non-toxic isolation and drug loading methods to produce more exosomes without damaging them exosome.

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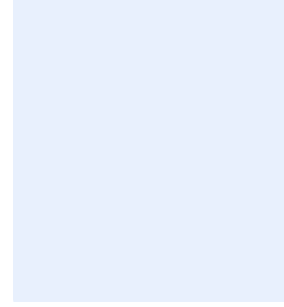
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