Liposomal Formulation of Lapatinib for the Treatment of Breast Cancer

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Dedication

I would like to dedicate this thesis to my dearest father Muhammad Idrees, as no one deserves this more than him. I am proud of the person that I have become and owe to his love. I'm highly obliged to him for his selfless love, care, and blessings. Long live Baba!

ABSTRACT

Breast cancer is the most common type of cancer among global women, even though numerous treatment options for breast cancer have been documented. Lapatinib, a tyrosine kinase inhibitor, modifies the cellular pathways and halts cell proliferation. The focus of this study includes the distribution of the drug to the tumor site in high amount and boost the efficiency of Lapatinib-loaded liposomes as a controlled drug delivery system against breast cancer. The synthesis of liposomes was followed by drug loading. The cargo was characterized under XRD, FTIR, SEM, EDX, and zeta analysis techniques. The overall findings demonstrated that the Liposomal formulation of Lapatinib has a high potential for sustained release of the drug for cancer therapy. As the successfully synthesized Lapatinib-liposomes were stable and exhibited increased in vitro anticancer activity as compared to free Lapatinib. Our study demonstrates that the combination of Lapatinib with liposomes improves the drug's long-term viability, effectiveness, and active targeting as a potential targeted therapeutic molecule for breast cancer treatment. To strengthen the utilization of this combination therapy in cancer therapy trials, further research is warranted.

Keywords: Drug delivery, Lapatinib, liposomes, Breast cancer, nanotechnology, anticancerous activity, cell viability.

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LIST OF ACRONYMS

BC	Breast Cancer
WHO	World Health Organization
BL	Bare Liposomes
LL	Liposomal-loaded Drug
L	Lapatinib
DNA	Deoxyribonucleic acid
EPR effect	Enhanced Permeability and Retention effect
XRD	X-ray Diffraction
FTIR	Fourier Transform Infrared
EDX	Energy Dispersive X-Ray Analysis
SEM	Scanning Electron Microscopy
MTT assay	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay
NPs	Nanoparticles

CHAPTER # 1

1. INTRODUCTION

Breast cancer, cancer that is receptor-positive, is one of the most occurrence types of cancer and is known as one of the most prevalent diseases in the world. The death rate is 14% and the risk of this disease in women is 30% [1]. According to the reported statistics, incidence rates differ almost 4-fold across different regions of the world's least developed countries [2]. Every year, metastatic breast cancer causes the death of almost 40,000 women. In developed nations, the prevalence of this disease ranges from 1 to 2 percent, whereas rates in Africa and Eastern Asia range from 27 per 100,000 to 96 per 100,000 people [3]. Age, geographic location, hormones, sleep patterns, radiation exposure, as well as certain genetic and environmental factors, are the key risk factors for breast cancer development. Breast cancer can be treated using a variety of approaches, the most common of which are chemotherapy, radiation, surgery, and targeted therapies based on nanodrugs.

Lapatinib was introduced in this study as a therapeutic drug. Lapatinib is a dual Tyrosine kinase inhibitor. It works by interacting with the transmembrane HER-2 receptor and preventing receptors from dimerizing normally. Cell migration and proliferation are impeded as a response. As lapatinib is hydrophobic and less soluble in water, liposomes are applied to enhance its effectiveness. In terms of their composition, liposomes resemble cell membranes and are amphipathic vesicles.

Lapatinib has a low water solubility, which causes poor and inefficient absorption from the gastrointestinal tract and less reliable results are being obtained. To address this problem, a stable and highly loaded liposomal formulation containing lapatinib was developed, and its therapeutic efficacy on the MCF-7 and MDA-MB-231 cell lines was tested in vitro. The controlled release of Lapatinib, compared to its commercial tablet formulation, had maximum clinical performance, reduced dosage and toxicity to the other organs, increased bioavailability, decreased plasma protein binding and showed higher water solubility.

Our research has the potential to assess a formulation's anticancer efficacy that contains lapatinib and has been encapsulated in liposomes to extend the shelf life of the drug.

The liposome synthesis process described by Bangham was followed. XRD, SEM, FTIR, and zeta analyses were used to characterize liposomes. According to our findings, liposomal Lapatinib significantly increases autophagy and death in cancer cells while limiting their growth in contrast to control groups.

CHAPTER # 2

2. LITERATURE REVIEW

After cardiovascular disorder, cancer is regarded as one of the most prevalent diseases in the world, and the likelihood of becoming infected is rising every day. One of the most prevalent cancers is breast cancer. The death rate is 14% while the risk of this disease in women is 30%. The highest mortality rate is seen in BC cases that have moved to other body areas [1]. The second most frequent cancer in women is breast cancer and the one with the highest incidence of new cases [2]. For women between the ages of 45 and 55, this illness is the leading cause of death. Metastatic breast cancer causes the death of about 40,000 people annually. According to the data, the incidence rate differed by nearly a factor of four between the less developed regions. While the prevalence of this disease ranges between 1 and 2 percent in developed nations, with rates in Western Europe reaching 96 per 100,000 people while those in Africa and Eastern Asia range from 27 per 100,000 [3]. In those nations with well-established screening programs, incidence rates are higher.

Older age groups (those over 50) have substantially lower incidence rates, whereas younger age groups have similar BC rates that are comparable to western nations. Lower recognized incidence rates might result from a lack of screening in older age groups. The proportion of BC at a young age is thus greater and does not necessarily reflect unique tumor biology in Africa due to lower reported prevalence in older women. Additionally, because of the declining frequency of BC, postmenopausal women had higher percentages of inflammatory BC.

Cancer has become a prominent issue of the century due to its recent rise in prevalence and its effects on various physical, mental, and social aspects of life [4].

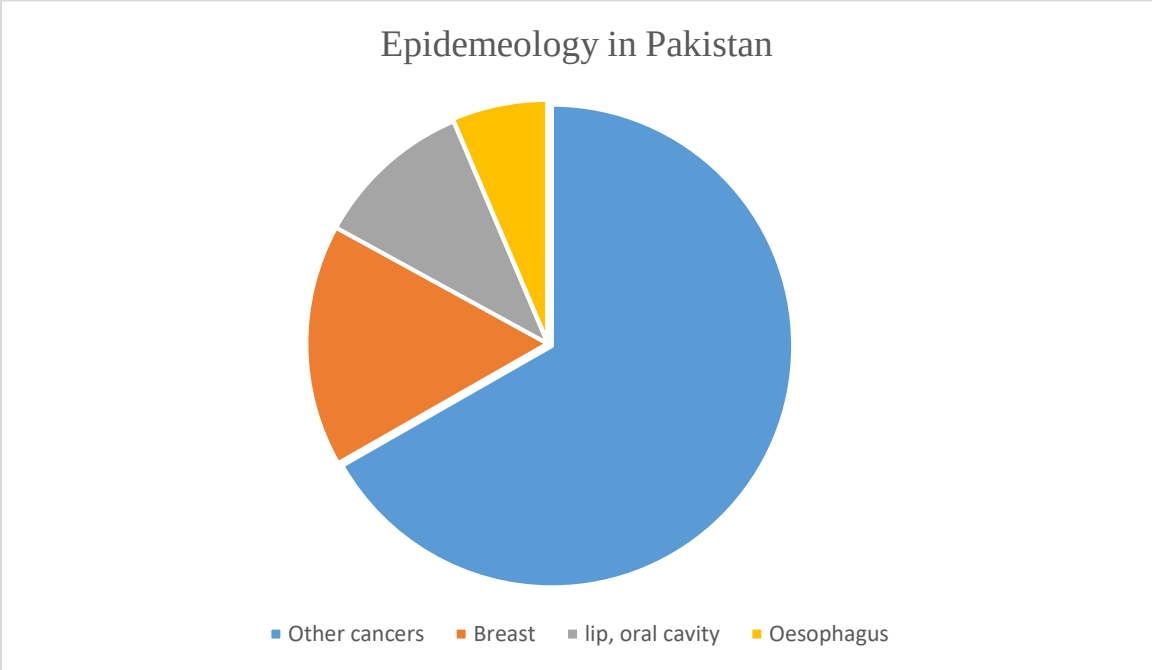
The prevalence of risk factors changes over time, by area, and by age group. Due to the large proportion of young patients and the late manifestation of breast cancer, the clinical picture of the disease is different from that of western nations (due to the high percentage of young people in the African population). International cooperation is needed to meet Africa's increasing need for improved breast cancer care.

With an expected 2.3 million cases of all cancers worldwide in 2020, breast cancer will represent 11.7% of cases [5]. Epidemiological studies predict that there will be approximately 2 million cases of BC worldwide by the year 2030. 526000 prevalent cases and an estimated 118000 incident cases involving 98.1% females occurred in India in 2016.

According to Globocan records, BC had a cumulative risk of 2.81 in 2020 and was responsible for 10.6% (90408) of all fatalities and 13.5% (178361) of all cancer cases in India. Compared to Western countries, India has a worse survival rate for breast cancer patients, because of early age at onset, advanced disease stage at diagnosis, a delay in the start of decisive management, and insufficient or fragmented care. The World Cancer Report 2020 [6] states that quick diagnosis and treatment are the best therapeutic methods for BC. A 2018 systematic review of 20 research found that a worse cancer stage at diagnosis was associated with higher BC treatment costs. Thus, BC should be discovered early to save lives and money [7].

2.1. Epidemiology of Pakistan

In 2020, the number of new cases for both sexes is 25 928 (14.5%) for breast cancer. Oral cavity cancer contributes 9.5% and esophagus cancer is having 5.7% of total cases. In Pakistani women, there were 34,066 new instances of breast cancer, according to a 2018 study from the International Agency for Research on Cancer. Due to regional delays in diagnosis and referral to appropriate institutions, the fatality rate for patients with breast cancer is unfortunately high (World Health Organization WHO).



2.2 Risk factors

Age [8], high hormone levels [9], race, economic position, and iodine deficit in diet [10-11] are the main risk factors for cancer. Viruses play a part in one stage of the pathogenic process that leads to breast cancer [12]. Typically, viruses play a role in various cancer forms [13]. One of the most significant risks to women's physical, emotional, and social

health is breast cancer. The patient's sense of acceptance, self-worth, and self-awareness may be impacted by certain treatment problems. The mental health of breast cancer patients is compromised by several variables, including the disease itself, worries about the future of the family, a dread of dying, difficulties with treatment, decreased performance, and mental imagery problems [14].

Losing breasts for a woman refers to losing her femininity. Additionally, though chemotherapy is a crucial component of cancer treatment, it adversely affects patients' physical, emotional, social, and spiritual welfare, as well as their quality of life [15].

2.4.3. Breast Cancer and Religion

Religion offers a helpful foundation for understanding the deeper significance of illness. Faith was viewed as a potent tool that reduces anxiety and tension and provides genuine solace, which can be useful in adjusting to and returning to life [16].

2.4.3. Genetic and environmental risk factors

Breast cancer is multifactorial [17]. It is caused by the interaction of environmental and genetic risk factors. It causes breast cancer cells to gradually amass genetic and epigenetic alterations. The best epidemiological evidence for breast cancer is familial history even when there are additional risk factors (such as age, obesity, alcohol consumption, and lifetime estrogen exposure). A specific susceptibility gene is etiologically connected to the family origin of about 20% of all cases of breast cancer [18, 19].

2.4.3. Smoking and breast cancer

Recent decades have shown a parallel rise in the occurrence of lung and breast cancer in women, which has drawn researchers to the increasing number of female smokers in an attempt to discover a common cause for this rising trend. The relationship between smoking and breast cancer has been under-research for about 20 years, but just 22 studies were published by the late 1980s. A weak relationship, a lack of relationship, or a supportive influence have all been suggested by various research. These articles have placed a strong emphasis on breast cancer and current cigarette smoking. There has been little research on the indirect link between smoking and breast cancer, but it has produced consistent findings. Women who were exposed to tobacco smoke as children or who were married to smokers are more likely to get breast cancer [20, 21]. A meta-analysis by Kuder et al. revealed a tenuous link between indirect cigarette smoke exposure and the prevalence of breast cancer; hence, more studies are necessary to establish the veracity of this association [22].

Results from research by Reynold et al. on 116,544 women revealed a higher risk of brea st cancer in smokers, supporting the idea that smoking contributes to the development of the disease [23]. By growing and differentiating breast tissue, Rousseau et al. were able to determine the susceptibility of the tissue. Breast cancer risk increases with earlier smok ing initiation. Breast cancer risk increased for women who began smoking between the ages of 10 and 14. The disease is more likely to affect women who have a family history of breast cancer, ovarian cancer, or both [24].

2.4.3. Diet and breast cancer:

High-calorie consumption and an increase in Weight are two nutritional variables that contribute to the development of breast cancer [25]. According to Kopans and Greenwald,

breast cancer risk increases with obesity and high BMI in post-menopausal women, but not in pre-menopausal women. According to research, higher fat consumption causes breast tumors in mice [26]. High fat intake and the occurrence chances of breast cancer are positively correlated, according to Howe and Goodwin [27].

Consumption of animal protein is linked with lower chances of breast cancer, according to research works [28]. In general, it's uncertain if eating animal protein contributes to breast cancer development [29]. On the one hand, calorie intake causes increased height in youth, weight gain, obesity, and preterm menopause. Both elements may set the conditions for cancer development in the future [30].

2.4.3. Hormones and breast cancer

The primary non-genetic risk factors for breast cancer are hormonal factors. Worthy of mention factors include gender, menarche, menopause ages, past reproductive experiences, nursing, and use of exogenous estrogens (estrogen with an external source). Non-genetic breast cancer is more often found in menopausal women with high estrogen receptor expression. In the development of breast cancer, estrogen has at least two key roles: (1) its metabolites can modify DNA or create DNA-damaging free radicals [31]; and (2) its hormonal action can stimulate cell proliferation in precancerous and malignant tumors.

Moreover, additional mechanisms are also connected to the development of breast cancer because of an important portion of estrogen-receptor negative (or ER-) breast carcinoma. A BRCA1 gene mutation increases the chances of breast cancer from 51% to 85%, respectively, at the ages of 50 and 70years, with this the chances of ovarian cancer also increase to 23% and 63% [32, 33].

2.4.3. Immune System and Breast Cancer

The immune system is entirely capable of combating cancers (a positive parameter). Numerous immunological factors utilizing cytokines like IL-12 and IFN- γ play important roles in this regard. In addition, IL-12 is a key cytokine in the differentiation of TH1 cells, which are powerful IFN-γ producing cells. IFN- γ plays a crucial function in cellular immunity as well as substantially boosting phagocytes' capacity to produce IL-12 [34, 35]. Following the discontinuation of hormonal contraceptives, an elevated risk of breast cancer declines five to ten years later [36].

2.4.3. Breast Cancer and Sleep

The amount of sleep you get and breast cancer are co-related. Women with longer sleep durations are more likely to get breast cancer than those with normal sleep durations [37, 38]. In this study, women who slept for fewer hours did not exhibit this connection. According to published research, inadequate sleep has been associated with an elevated risk of hormonal positive receptors breast cancer [39].

A retrospective cohort study's findings revealed that sleeplessness is linked to a higher risk of getting breast cancer [40]. Another study revealed that different parameters like sleep duration and quality can be correlated with an elevated risk of aggressive breast cancers. In those who have been rescued from cancer, the amount of sleep does not affect their prognosis in them [41, 42]. A prospective cohort study's findings revealed that there is no connection between sleep and breast cancer.

According to a study, working overnight shifts is moderately related to an elevated chance of breast cancer, and this association is higher in women who have worked for 20 years or more years [43].

2.4.3. Light pollution and breast cancer

Exposure to artificial light at night causes a significant drop in melatonin levels and is known to increase breast cancer risks. This decrease in melatonin production causes a rise in the amount of estrogen and other reproductive hormones, which is helpful in the growth of hormone-sensitive breast cancers. Shift work raises breast cancer risk by up to 48%, as indicated by the findings of a meta-analysis [44, 45].

2.4.3. Radiations and breast cancer

Those who have had treatment after overcoming a sarcoma or leukemia are generally more prone to get breast cancer at a younger age, alongside chest radiations, which may be brought on by high-dose anthracycline or alkylate chemotherapy [46].

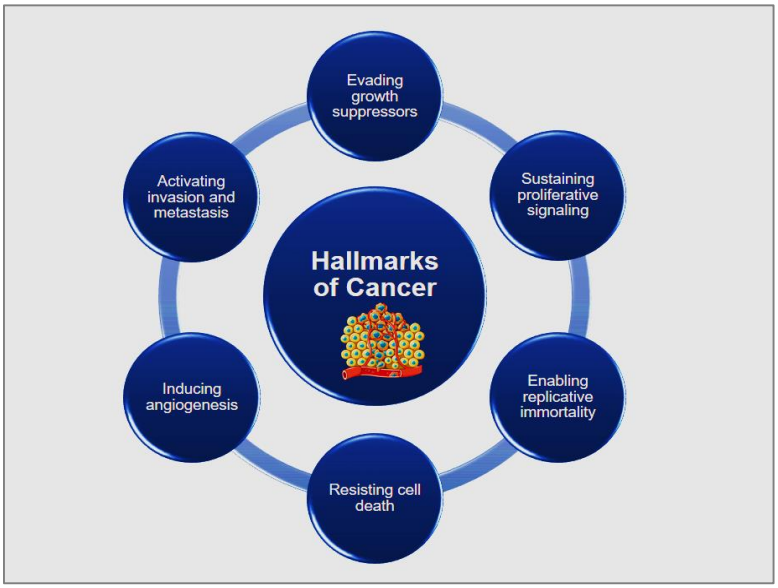


Figure 2.1: Hallmarks of cancer

2.3 Treatment Strategies for Breast Cancer Treatment:

The most common breast cancer therapies vary depending on several factors,

- Patient age
- The patients' physical and medical conditions
- Typical breast cancer therapy

2.3.1 Systemic Treatments

2.3.2 Chemotherapy

The method of treating cancer that is most frequently used is chemotherapy. Anti-cancer medications are delivered intravenously, orally, or directly into the spinal fluid during chemotherapy. For the treatment of cancer, both natural and synthetic medications have been utilized. The right chemo-drug selection is essential for treating specific cancer cells. On the market, there are more than 50 approved medications for chemotherapy [47,]. Combination chemotherapy is one of the most widely used methods of treating breast cancer since it employs both an alkylating agent and antimetabolites [48]. Chemotherapy has several safety concerns such as loss of hair, changing nails, mouth lesions, loss of weight, nausea, early menopause, infertility, tiredness, anemic condition, low blood levels especially hemoglobin level is reduced, and iron deficiency, among others [49]. The adverse effects vary depending on the cancer type, chemo-drug type, and patient. Cancer cells are stressed out by chemotherapeutic medicines, which also slow down their division (mitosis) and cause damage that results in apoptosis and cell death.

2.3.3 Hormone Therapy

Endocrine treatment, sometimes referred to as hormone therapy, slows or halts the development of breast cancer cells by preventing the body from producing hormones or by interfering with them [50]. Hormone treatment may be beneficial for tumors that express hormone receptors. Cancer that has metastasized to other body areas may rely on this treatment or it can begin even before surgery. It can be used after surgery to overcome the risk of cancer recurrence. The majority of treatments that involve the use of hormones work by down-regulation of estrogen production or lower estrogen levels, which encourage the growth of breast cancer cells. Along with discharge and dryness of the vagina, blood clotting, nausea, headaches, and bone soreness can also result from hormone therapy prescription side effects.

2.3.4 Targeted Therapy

Drugs, particularly designed to target the cell-level alterations that otherwise lead to cancer, are used in targeted therapy [51, 52]. Targeted medications can reach every part of the body where cancer may have spread, despite the fact that they function differently from chemotherapy medications. Targeted treatment medications, like chemotherapy medications, can cause certain unfavorable conditions including loss of hair, vomiting, diarrhea, lack of appetite, and constipation [53].

2.3.5 Immunotherapy

The use of immunotherapy halts the growth and metastasis of cancer cells [54]. Immunotherapy relies on employing medications or natural or synthetic chemicals to activate the immune system of the patient to fight and destroy cancer cells [55, 56].

Constipation, diarrhea, nausea, coughing, exhaustion, skin rashes, and loss of appetite are a few of the side effects of immunotherapy medications [57].

2.3.6 Local Treatments

Poor prognoses are associated with triple-negative breast cancer [58], and the only accessible treatment for triple-negative breast cancer is systemic chemotherapy. Immunotherapy, polyadenosine diphosphate-ribose polymerase inhibitors and antibody-drug conjugates may change the course of breast cancer treatment as it is now practiced. While there is a lot of discussion and concerns around newer medications that are flooding the market, the survival benefit has not changed significantly, which is a crucial factor to consider when formulating the therapy. Making a sensible decision is therefore essential. The discipline of breast surgery has advanced alongside the development of complete mastectomy, breast conservation treatment, and oncoplastic breast surgery.

Oncoplastic breast surgery, which is a fast-growing area, suggests a useful alternative to full mastectomy and breast conservation treatment. Due to its economic feasibility, low cost, and appropriateness for less developed countries, oncoplastic breast surgery is still in its development but is likely to become more commonly employed in the near future [59]. As these treatments pose a financial burden to families due to high costs, which in India are three times more for private cancer therapy centers, are a factor in the high attrition rates, and therefore, a very limited number of people go for follow-up for BC therapy. More than half of low-income patients receive BC treatment, which has disastrous results, at a cost of more than 20% of their yearly household income.

2.3.7 Surgery

BCS and mastectomy are the two primary surgical techniques used to remove breast cancer. BCS (a lumpectomy/ quadrantectomy/ partial mastectomy/ segmental mastectomy) involves simply the surgical removal of the cancer-containing portion of the breast. A mastectomy involves removing the entire breast. In cases when it is possible, BCS is chosen over mastectomy for aesthetic reasons.

2.3.8 Radiation therapy

To destroy cancer cells, radiation therapy treatments employ high-energy beams. It may be given as a supportive dosage together with other treatments, such as after breast cancer surgery or partial removal of the breast, to lower the risk of a cancer recurrence or the metastasis in body. The two primary categories of this therapy include radiation beam with an external source (EBRT) and brachytherapy, often known as internal radiation therapy. Potential side effects of EBRT shows swollen breasts or changes in the skin of the breast as well as changes in its size.

Brachytherapy involves temporarily introducing radioactive seeds or pellets into the tumor bed in the breast tissues. Some of its possible unfavorable effects include discomfort, redness, infection, or fluid retention in the breast [60].

2.4 Nanotechnology in the treatment of breast cancer

The tumor microenvironment (TME), an intricate environment made up of cells, different particles, and chemical compounds act as a usual barrier for tumor cells to grow, metabolize, infiltrate, and move. Extracellular matrix, vascular system, and cells work together to create a tumor microenvironment, or TME is recognized as a substantial

hindrance to the distribution of anticancer medications [61]. The TME is acidic, reductive, and hypoxic. Solid tumors are characterized by high cellular density, abnormalities in vesicles, acidosis, hypoxia (anaerobic), inadequate lymphatic drainage, an aberrant manifestation of extracellular matrix-related proteins and enzymes, and elevated pressure of the interstitial fluid.

A high number of cells and high pressure of fluid are two of these traits that avert drug absorption and hamper medicinal circulation. To boost the effectiveness of anticancer medication delivery, certain properties like acidosis, hypoxia, and overexpression of specific enzymes, can be subjugated to project stimuli-sensitive drug delivery systems.

Many different methods and approaches have been looked at over the years for medicine targeting to tumors. Systems including liposomes, polymers, micelles, nanoparticles, and antibodies are commonly used, and approaches like triggered drug delivery, active cancer cell targeting, active endothelial cell targeting, and passive drug targeting are a few examples [62]. This field's preclinical and clinical research has advanced significantly, and some nanomedicine formulations, mostly those with the passive mechanism of action against tumors, have been recognized for use in clinical settings. Noteworthy advancement has also been employed to study the pathophysiological principles underlying the therapeutic targeting of tumors. As a result, various substantial risks in the delivery of tumor-targeted medicines have been found.

(I) An excessive focus on the EPR impact.

(II) Poor penetration of nanomedicines into tumors and tissue.

(III) Misinterpretation of the possible effectiveness of active medication targeting.

(IV) Irrational drug production employs too complicated and narrowly applicable components.

(V) Inadequate use of nano-drugs in therapeutically applicable co-therapies.

(VI) Treatment of metastases rather than solid tumors is the most urgent medical requirement.

(VII) Insufficient use of theragnostic and non-invasive imaging methods, which might be utilized to modify treatments based on nanomedicine.

(VIII) There is a lack of efficacy studies using appropriate animal models being physiologically more significant and pertinent to the human scenario.

The literature suggest that future research should concentrate on some of the reason-based issues with tumor targeting ability of drugs and discover answers, in addition to developing novel nanomedicine formulations.

Additionally, the clinical trials of tumor-targeted nanomedicines may have been impeded by the use of appropriate animal models in many preclinical investigations and the absence of graphical data (lack of images) to modify nanomedicine-based treatments. To thoroughly investigate the drawbacks of tumor-targeted medication delivery and devise approaches to overcome them, it is imperative to continue developing nanomedicine materials while also working to understand more about the biological and pathophysiological principles of drug development [63].

2.4.1 Fake cell lines

The idea of "fake" cell lines is an additional essential factor that has, for the most part, been disregarded over the years. This was initially brought to light more than 20 years ago when

it was shown that numerous cell lines contained HeLa cells. Many journal editors and referees may not be aware of the severity of this cross-contamination issue, according to some reports. Cell lines must be precisely characterized to be utilized as models in a useful manner. One way to deal with this problem is to mandate that all publications use only fully identified and characterized before the publication of results.

The HER2 receptor stands to reason as a target for the improvement of targeted cancer therapeutics. In the development of breast and other malignancies, the HER2 proto-oncogene and its p185 HER2 (ErbB-2) receptor tyrosine kinase shows a significant role. In a significant portion of malignancies, there is a persistent overexpression of HER2.

Since HER2 is a cell surface receptor, antibody-based therapies can easily target it. By disrupting HER2 signal transduction, monoclonal antibodies against HER2 can slow the growth of tumors. For instance, muMAb4D5 and its improved version, rhuMAbHER2, suppress the development of breast cancer cells that overexpress the HER2 gene and improve the effectiveness of specific chemotherapy medicines. Sterically stabilized or "stealth" liposomes or conventional" liposomes are highly resistant to clearance by the reticuloendothelial system (RES). Therefore, they are most widely used in liposomal drug delivery [64].

Nanoparticles are very small in size with 1 to 100 nm (American Society for Testing and Materials, ASTM) [65]. Pharmaceutical preparations have several options due to the extensive number of materials available and the forms that nanoparticles can adopt. The therapeutic impact of medications on particular diseases may largely depend on the use of nano-carrier materials. Some nanomaterials may have distinct physiological functions under certain circumstances. For instance, manganese dioxide can promote the oxidation

of hydrogen peroxide and the production of oxygen in reductive BCME, which change TME to synergistically overcome drug resistance [66].

Drug resistance to various kinds of medication may be overcome by certain nanomaterials. Bypassing drug efflux pumps, and cellular absorption may be facilitated by the particular physical morphology and chemical makeup of nanocarriers [67].

2.4.1.1 Passive targeting NDDs

Traditional "passive" targeted NDDS rely heavily on EPR effects that might passively accumulate in tumor cells. Because of the particle size, or passive targeting, NDDS are kept in tumor tissue. doxorubicin (DOX) liposomal (Doxil®) and paclitaxel albumin nanoparticles (Abraxane®), are two industrially available nanoparticles that use the EPR effect. Based on the EPR effect, common forms of nanoparticles include solid lipid nanoparticles, polymer lipid nanoparticles, polymer nanoparticles, polymer micelles, nanoemulsions, and liposomes are examples of nanoparticles [68].

2.4.1.2 Receptor-targeted NDDs

To encourage active absorption by tumor cells, also known as "active" targeting, the proper ligands for the receptors that are preferentially or robustly expressed in tumor cells can be employed in NDDS. The nutrition that tumors cells receive is insufficient to provide them with the energy they need to continue growing. Thus, starving tumor cells develop extra receptors, such as the folic acid receptor, in order to absorb enough folic acid to finish the portion of DNA synthesis [69].

Hypoxia promotes tumor cells to produce VEGFR (vascular endothelial growth factor receptor) and angiogenesis in order to get the proper nutrition [70]. This receptor-mediated

NDDS allows tumors cells to accurately recognize and internalize nanocarriers modified with folic acid, VEGF, and other breast cancer-specific ligands. However, there are certain challenges with NDDS receptor-mediated targeting as well. A notable difficulty is the inconsistent expression of membrane receptors, which precludes active targeting from having the intended effect [71]. The NDDS particle size has been found to have an impact on how cells internalize, and nanoparticles with a diameter of around 150 nm tend to concentrate in tumors while eluding macrophage phagocytic uptake [72]. Contrarily, smaller nanoparticles with hydrodynamic diameters less than about 30–50 nm can more easily penetrate solid tumors [73, 74].

2.5 Diagnosis and pathophysiology

Depending on the presence of the estrogen or progesterone receptor and ERBB2 gene amplification, breast cancer is categorized into three primary tumor groupings. The risk profiles and therapeutic modalities of the three subgroups vary. The anatomic cancer stage, tumor subtype, and patient preferences all play a role in determining the optimal course of therapy for each patient.

The development of breast cancer has been linked to two main molecular targets. A portion of invasive breast tumors expresses the estrogen receptor alpha (ER alpha), which is one of them. Neoplastic growth pathways are sparked in breast cancer cells when estrogen stimulates the steroid hormone receptor and transcription factor ER.

The second main target is epidermal growth factor 2 (ERBB2, previously HER2 or HER2/neu). About 20% of breast tumors have this transmembrane receptor tyrosine kinase amplified or overexpressed, which is associated with a poor prognosis in the absence of

systemic treatment. It belongs to the family of epidermal growth factor receptors. Tumors that are ERBB2+ have an amplified or overexpressed form of the gene. For patients with breast cancer that have been amplified or overexpressed by ERBB2, anti-ERBB2 antibodies (such as trastuzumab and pertuzumab) and small-molecule tyrosine kinase inhibitors (such as lapatinib and neratinib) are effective treatment options [75].

15% of all breast cancers are triple-negative, meaning that neither the ER, PR, nor ERBB2 molecular targets are expressed in the tumor. In the first 3 to 5 years after diagnosis, there is a substantial chance of distant relapse in triple-negative cancers. There is still a lack of knowledge on the precise molecular pathophysiology of triple-negative breast cancer [76].

HER2/neu receptors are overexpressed in about 20–30% of all breast tumors. Lapatinib is a tyrosine kinase inhibitor that also inhibits HER2 and EGFR. In breast cancer, it manifests its anticancer activity by inhibiting the intracellular domain of the HER2 receptor [77].

The tyrosine kinase inhibitor lapatinib, which is used to treat breast cancer that expresses Her2/neu and inhibits both EGFR and HER2 receptors, has received FDA approval [78]. Lapatinib's ability to induce apoptosis and autophagy as well as lengthen survival time is being supported by more evidence [79]. Lapatinib has been studied in numerous clinical studies for both metastatic and aggressive Her2 overexpressing breast tumors. Capecitabine [80] or letrozole [81] have been approved for usage in conjunction with it in clinical trials.

For the treatment of aggressive malignancies, lapatinib is also being investigated in a number of studies. Consider the encouraging results of a recent study on metastatic colorectal cancer [82] and metastatic bladder cancer. Similar to malignant gliomas, which

are extremely difficult to treat and have a poor prognosis, palatinib's mechanism of action has been investigated against them. Breast cancer cells that are HER2-positive are prevented from migrating by lapatinib [83].

2.6 Mechanism of action of Lapatinib

Liposomes are employed to ensure the efficacy of lapatinib because it is hydrophobic and less soluble in water. Liposomes are amphipathic vesicles in the phospholipid structure s that, in terms of their contents, mimic cell membranes. Drug loading occurs either in the hydrophobic core or in the lipid membrane, depending on how soluble the active medicinal ingredients are. Liposomes have emerged as a viable drug delivery system for anticancer medications because of their pharmacological properties.

These characteristics include liposomes' capacity to release medications gradually and co ntinuously, to change the biological distribution of drugs, alter the toxicity of chemothera peutic agents to cells, and to promote drug accumulation in the target area [84]. Liposome s have been exploited as drug delivery methods in a number of academic studies.

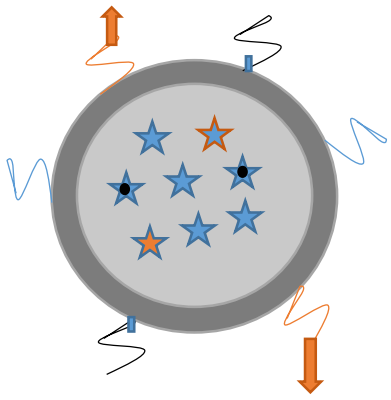


Figure 2.2: depicts the drug loading in liposomes.

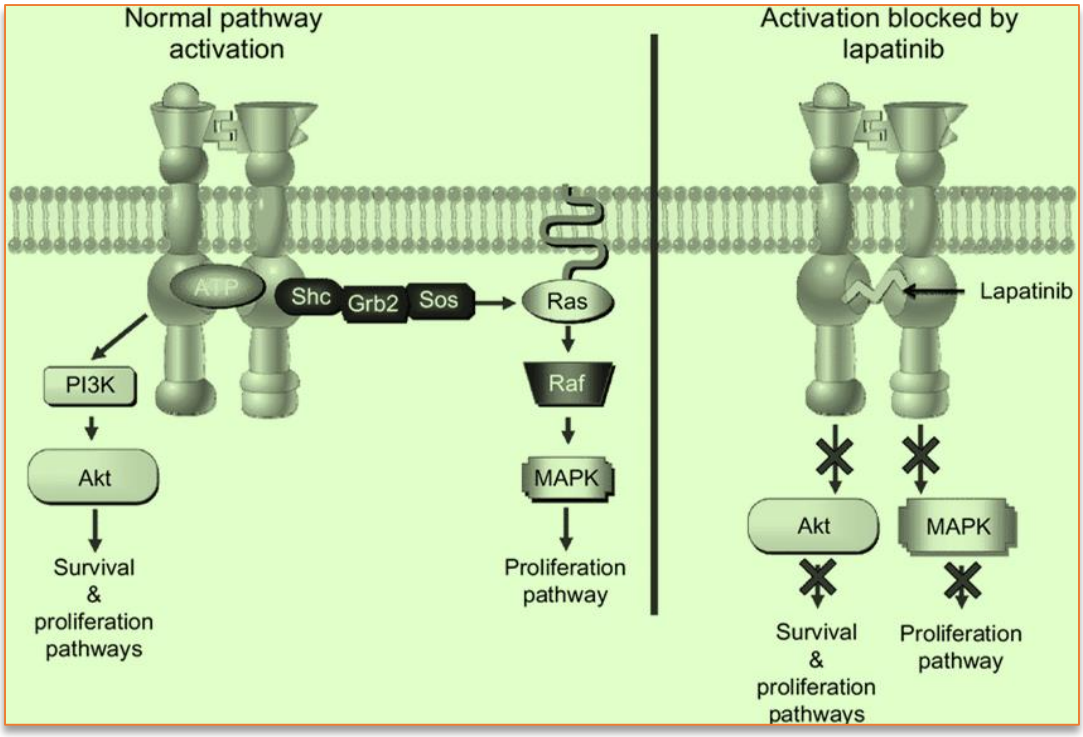


Figure 2.3: depicts the Palatinib metabolic route. According to the mechanism, high concentrations of ERBB2 result in homodimerization or heterodimerization with other ERBB family members, followed by autophosphorylation and the activation of downstream signaling pathways even in the absence of an activating ligand. Lapatinib inhibits the processes that lead to cell survival and proliferation to treat cancer [85].

CHAPTER # 3

3 METHODOLOGY

The cryovials preserved cell lines of MCF-7 and MDA in liquid nitrogen were collected from the cell culture lab founded at The University of Lahore. For the experimental work, these cryovials were revived. And the nanoparticles were synthesized in the Biochemistry Lab SMME NUST.

3.2 Materials and methods

3.2.1 Chemicals and Instruments

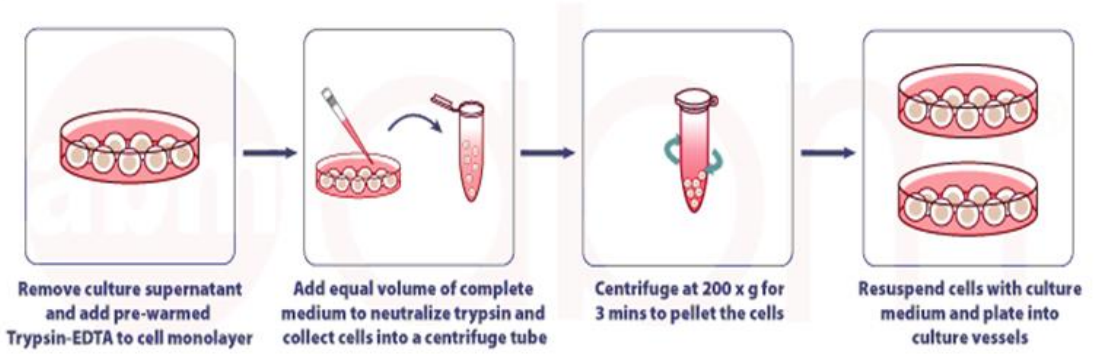
Chemicals used in this study include Soy lecithin (≥99% TLC) source (Wood Dale. IL 60191 USA), organic solvent (methanol), cholesterol powder (Sigma Grade, ≥99%), Lapatinib drug powder, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin (Pen Strap), Trypan Blue dye, Trypsin, Chloroform (99%) and MTT dye. Materials that were being used include cell culture flasks (15ml) and 50ml falcons, pipettes, filtered pipette tip, cell culture Petri dishes, 96-well plates, magnetic stirrer, rotary evaporator, and bath sonicator.

3.1 Synthesis of liposomal nanoparticles

The procedure developed by Alec Bangham is used to create liposomes. In addition to being utilized as a phospholipid, soy lecithin is a good stabilizer and emulsifier. To create a homogenized mixture, the soy lecithin (0.2g) and cholesterol (0.04g) powders required to improve the membrane's stability and fluidity were dissolved in 10ml chloroform. Then, for passive drug loading, 1 ml of methanol and 4 ml of chloroform (1:4 ratio) were added to the homogenized phospholipid mixture, along with dissolved 46.3ul of lapatinib drug solution (1 ml lapatinib/2 ml DMSO). For 15 to 20 minutes, stir this formed mixture. Rotary evaporation has been used for the distillation of particles. Add deionized water to the thin film of particles to rehydrate it. Afterward, the hydrated particles are applied to the magnetic stirrer for 3-4 hours for the encapsulation process. Bath sonication was done to get the small vesicle size of liposomes at 0.5sec on/off cycle for 15 minutes. The results solution was saved at 4°C for further procedures.

3.2 **Cell lines culturing**

The preserved cell lines cryovials were taken from the liquid nitrogen cylinder, thawed, and revived. Then, DMEM along with 10% fetal bovine serum (FBS), 100 mg/mL penicillin G (Sigma), and 100 U/mL streptomycins (Sigma) were provided to the culturing flask in which MDA and MCF-7 cell lines were cultivated. Cultures were sustained at 37°C in a humidified incubator with 5% CO2 supply. Three replicated experiments were conducted on MDA, and MCF-7 cells were subculture once they had reached 70–80% confluence in culture. To split the cells, they were first rinsed with 1X phosphate buffer saline (PBS) and then treated with 0.05% trypsin-EDTA until the cells became detached from the surface of culturing flask. The confirmation of detached cells was done through the inverted microscope. The detached cells were mixed with a few drops of FBS and stirred well. To get the cells the mixture was centrifuged at 2000rpm for 5 minutes, the supernatant was discarded and the pellet was re-suspended.



3.3 Cell counting

For cell counting, trypan blue dye was mixed with media containing cells in a ratio of 1:1 and 10µl of the trypan-blue cell mixture was added to the hemocytometer. Cells were counted using the grid lines observed under an inverted microscope and were calculated according to the given formula:

Cells per 1ml of media = dilution factor x number of cells counted / total area counted × 10,000

5000 cells per were added to a 96-well plate which was incubated for 24 hours for monolayer formation.

3.4 Cell lines treatment with nanoparticles

The 96-well plate was cultured with MDA and MCF-7 cell lines to check the effects of the drug and nanoparticles. The nanoparticles were prepared in two forms, bare liposomes (simple liposomes), and drug-loaded (lapatinib-loaded) liposomes. Along with the liposomes, there was the pure drug (Lapatinib) was also applied to the cell lines. The concentration of the liposomes, drug-loaded liposomes, and the drug was applied in triplets' form of serial dilutions. In the serial dilution, the concentration was achieved at 100% in serial diluted tube 1 and further diluted to 50% in serial tube 2 and 25% in serial tube 3. Then picked up the 1 µL media from each of the concentrations from serially diluted tubes and applied it to the 96-well plate, same procedure was repeated for the drug-loaded liposomes and bare liposomes. DMSO was applied as a control. Cell lines were kept in the incubator for the activity of the applied samples. Cell viability was checked after 24 and 48 h through microscopic observation and reading was taken through a plate reader.

3.5 Cell viability assays

A colorimetric procedure to be followed in which various concentrations of the previously stated samples were tested on cultured cells in 96 well plates using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen Inc., USA), assay to determine the viability of cancer (MDA & MCF-7) cell lines.

MTT dye is a water-insoluble yellow dye. When it is applied to cells, the mitochondria of living cells convert the MTT dye to purple color Formazen crystals with the activity of dehydrogenase enzymes.

3.5.1 MTT IC_{50} calculation

The time-dependent treated cell lines of MDA and MCF-7 with different concentrations were washed with PBS, and for 3-4 hours the washed cell lines were incubated with DMEM and MTT solutions. Formazan crystals were formed. To dissolve those crystals, isopropanol was used. After 4 hours, the absorbance value at 570 nm was measured. The formula is used to determine the percentage viability of cells.

CHAPTER # 4

4 RESULTS

Liposomes prepared by Bangham's method were studied under different characterization techniques. In this study, liposomes are characterized by the most popular approaches and cutting-edge techniques. The prepared samples were tested to confirm the size and stability of particles under **XRD** (X-ray Diffraction) (model: Bruker, D2 phase), **FTIR** (Fourier transform infrared spectroscopy), **SEM** (Scanning Electron microscopy) (model: JSM-6490LA, Jeol, Japan), and **Zeta analysis**. The size of the particles was measured through FESEM and zeta analysis whereas the elemental confirmation was done through the EDX analysis and the crystallinity of the particles was studied under X-Ray diffraction. FTIR was done for the bond presence.

4.1 X-Ray diffraction (XRD)

Critical characteristics including crystal structure, crystallite size, and strain have been extensively described by XRD in the characterization of nanoparticles. The XRD (model: Bruker, D2 phase) pattern was observed from 2q = 0 to 80 as shown in figure 4.1.1 The broadening diffraction peaks at 23˚ describe the amorphous and disordered crystalline structure of the Bare liposomes, drug-loaded liposomes, and the drug.

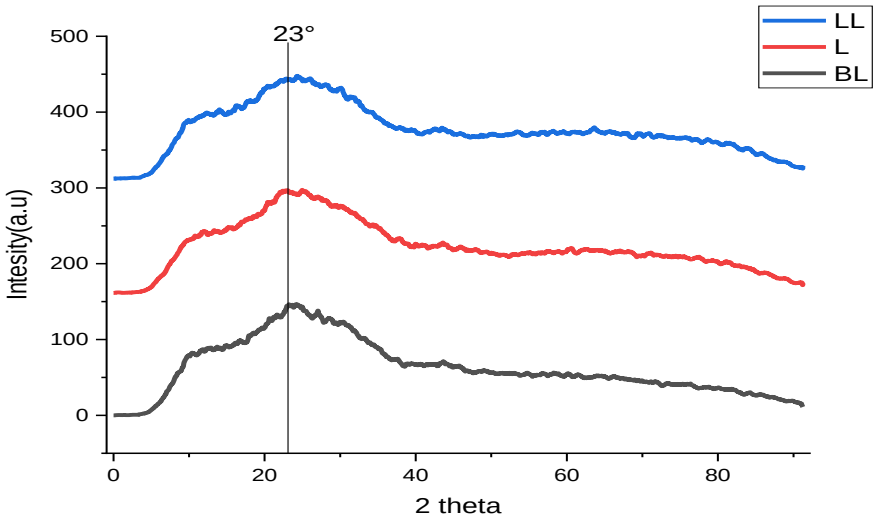


Figure 4.1.1: LL is the lapatinib-loaded liposomes, **L** is the lapatinib drug, and **BL** is the bare liposomes (simple liposomes)**.**

4.2 Fourier transform infrared spectroscopy (FTIR) analysis

Liposomes and drug were studied in order to check the association between them. Principle bands in bare liposomes are 680, 1640, and 3427 cm^{-1} whereas the drug contains bands at 685, 1044, 1417, 1640, 3003, and 3427. But the drug-loaded liposomes show peaks at 685, 1645, and 3427 (figure 4.2.1). These absorption peaks show the relation between the compounds and indicate the presence of the different bonds and functional groups. Table 1 shows the bond present in the compound which ensures the phospholipid nature of the samples. The stretching and bending motion of the bonds in the compound explain the nature of the presence of the functional group in the samples. In drug-loaded liposomes, the bare liposomes and drug shared the common peaks and don't show any new linkages between the liposomes and drug, ensuring the encapsulation of the drug in liposomes and helping in its unloading at the target site.

Table 1: FTIR Analysis

Wavelength (cm-1)	Bond	Motion
3427	O-H	Stretched vibration
3003	C-H	Stretched vibration
1640	O-H, C=N	BENDING vibration
1417	-CH2	BENDING vibration
1044	C-O	BENDING vibration
685	C-CL, C-H	AROMATIC BENDING

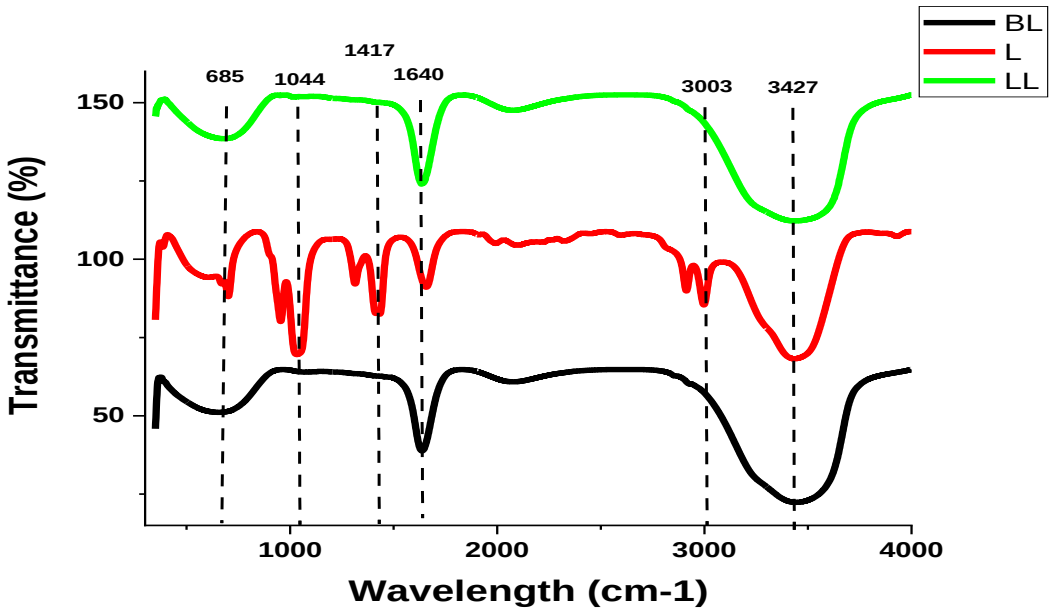


Figure 4.2.1: LL is the lapatinib-loaded liposomes, **L** is the lapatinib drug, and **BL** is the bare liposomes (simple liposomes).

4.3 Scanning electron microscopy (SEM) analysis

With the use of scanning electron microscopy, the liposomes' topography and surface morphology were studied (model: JSM-6490LA, Jeol, Japan). After being dried BL, LL, and drug particles on glass slides on SEM stubs, samples were analyzed for liposome particle sizes and shapes. The bare liposomes (BL) particles' size ranges from 20nm to 500nm shown in figure 4.3.1. The average size of the bare liposome is 350nm shown in figure 4.3.4. Whereas the LL particle's size ranges from 50nm to 700nm shown in figure 4.3.2. The average size of drug-loaded liposomal particles is 379nm shown in figure 4.3.5 and the lapatinib drug particle sizes range from 100nm to 250nm shown in figure 4.3.3, average size of the drug particles is 181nm shown in figure 4.3.6.

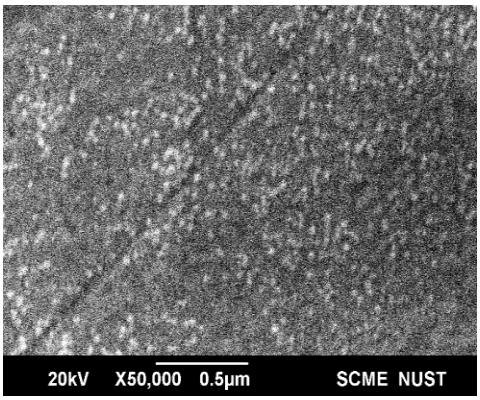


Figure 4.3.1: BL SEM size analysis

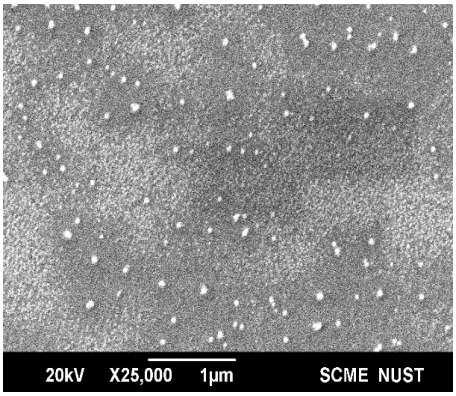


Figure 4.3.2: LL SEM size analysis

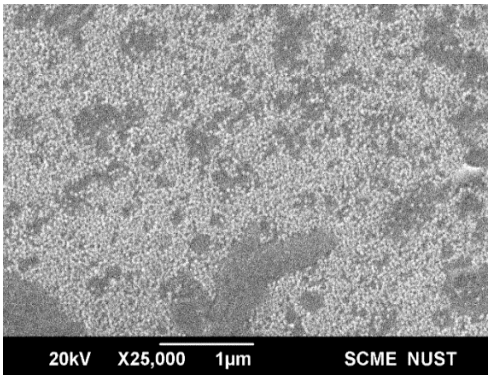


Figure 4.3.3: Lapatinib drug SEM size analysis of BL

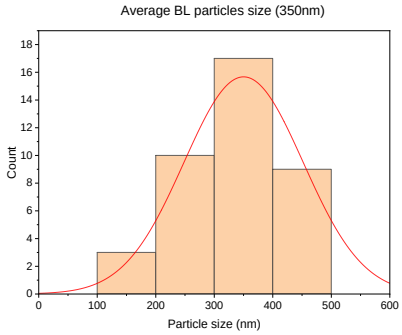


Figure 4.3.4: the average size

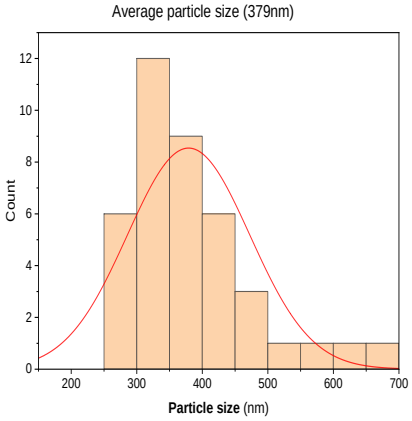


Figure 4.3.5: the average size of LL drug (L)

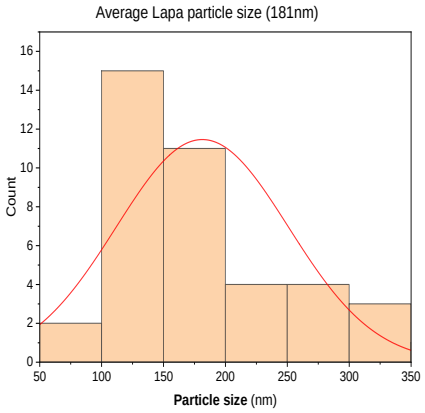


Figure 4.3.6: the average size of the

4.4 Zeta analysis

A method for estimating the surface charge of nanocrystals is the assessment of their zeta potential. This method may be used to comprehend the physical stability of nanosuspensions indicated by Jiang et al., 2009. Due to electrostatic attraction between individual particles, nanocrystals' zeta potentials with large positive or negative values suggest strong physical stability of nanosuspensions. According to basic consensus, a zeta

potential value outside the range of -30 mV to +30 mV possesses sufficient repulsive forces to enhance the physical colloidal stability. On the contrary side, because of the presence of attraction forces of van der Waals acting on the particles, a lower zeta potential value than the mentioned range might cause particle aggregation, flocculation, and physical instability of the compound. The size and charge analysis of the understudy compounds was done by the zeta sizer (model: **Zeta-sizer ZS 90 Malvern, UK**) from the pharmacy department of Quaid-e-Azam university Islamabad, Pakistan. Figure 4.4.1 shows the size distribution of the bare liposome (BL) with the mean value of 651.3nm. Two peaks are shown in the BL size distribution graph (figure 4.4.1) indicating the different sizes of the particles, which is due to the instability of the particles. Drug-loaded liposomes (LL) also possess two peaks with the mean value of 691.3nm shown in figure 4.4.2. Lapatinib drug (L) shows a stable peak in zeta size analysis shown in figure 4.4.3. Zeta size analysis of the lapatinib drug has also been done to check the stability of the compound. The surface charge of the bare liposomes (BL) is -13.3mV, drug-loaded liposomes (LL) is -153mV, and lapatinib drug is 16.5mV are shown in zeta analysis figures 4.4.4, 4.4.5 and 4.4.6 respectively.

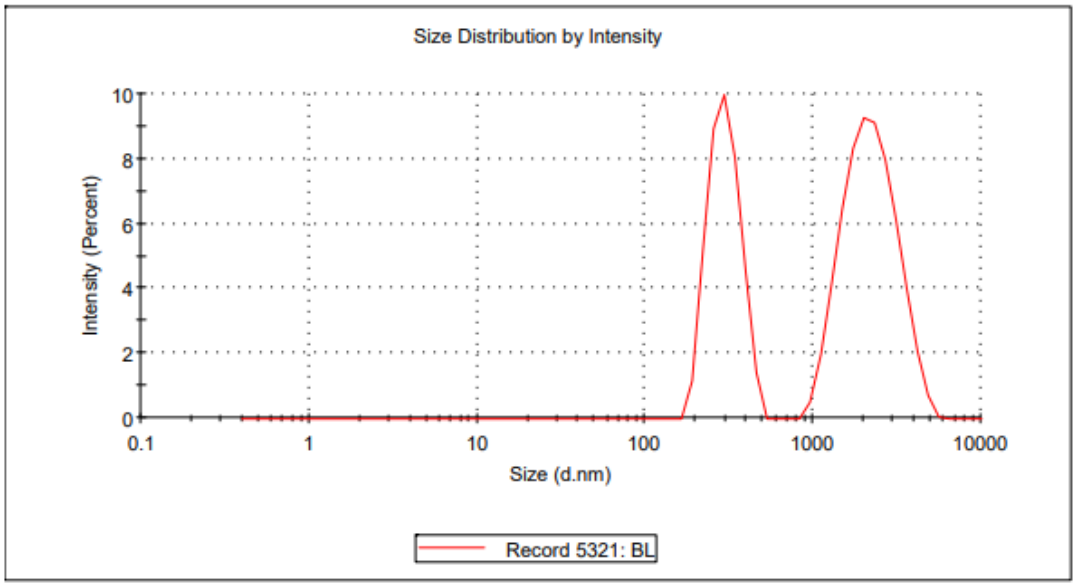


Figure 4.4.1: BL size distribution (zeta size analysis)

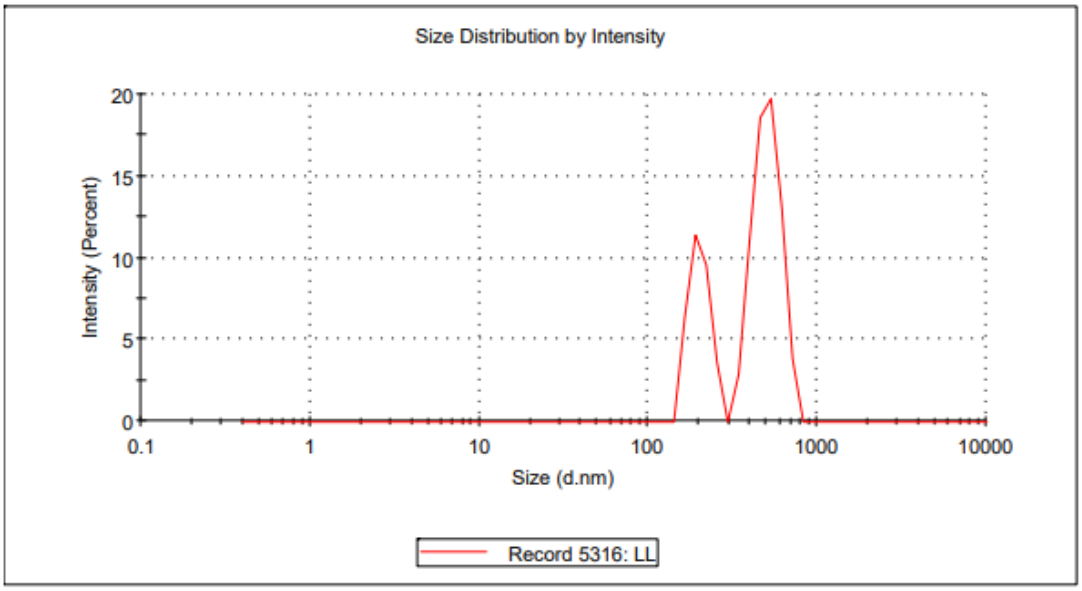


Figure 4.4.2: LL size distribution (zeta size analysis)

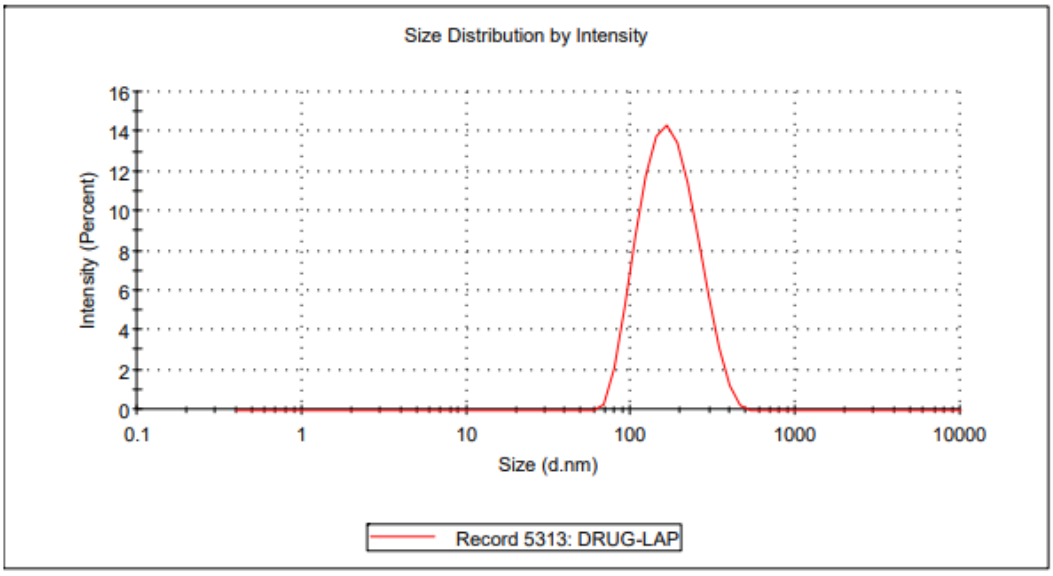


Figure 4.4.3: Lapatinib drug size distribution (zeta size analysis)

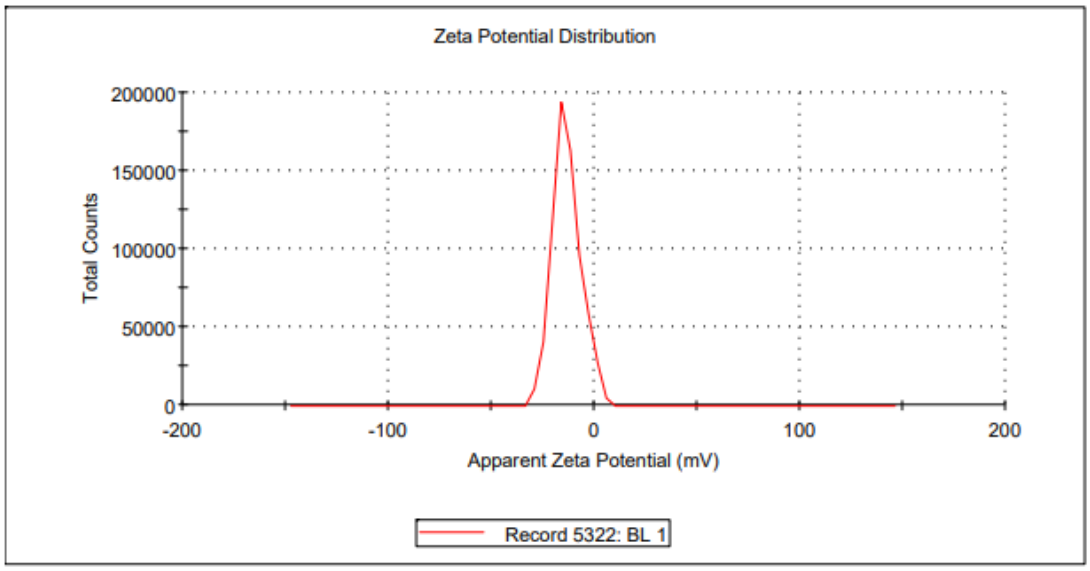


Figure 4.4.4: BL zeta potential

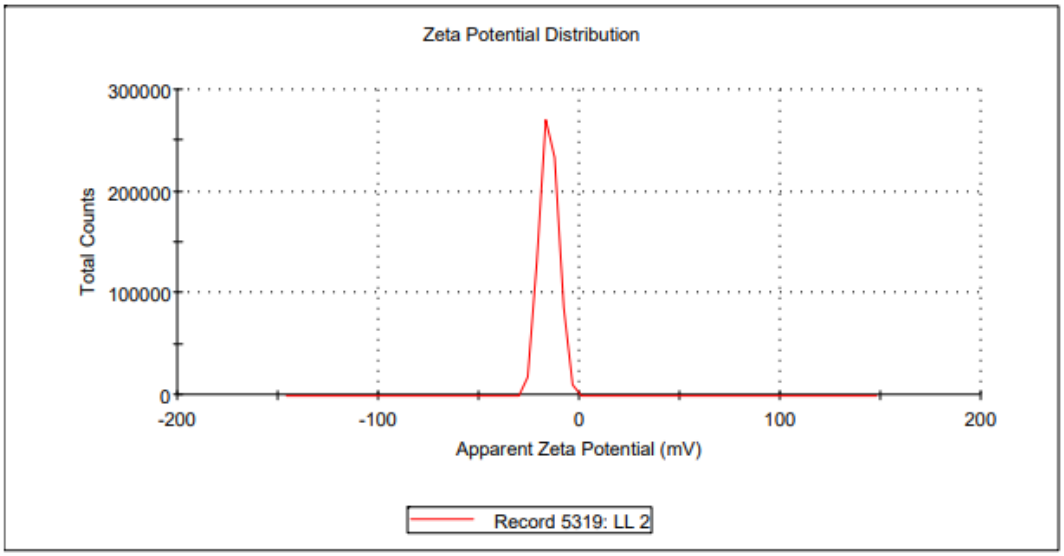


Figure 4.4.5: LL zeta potential

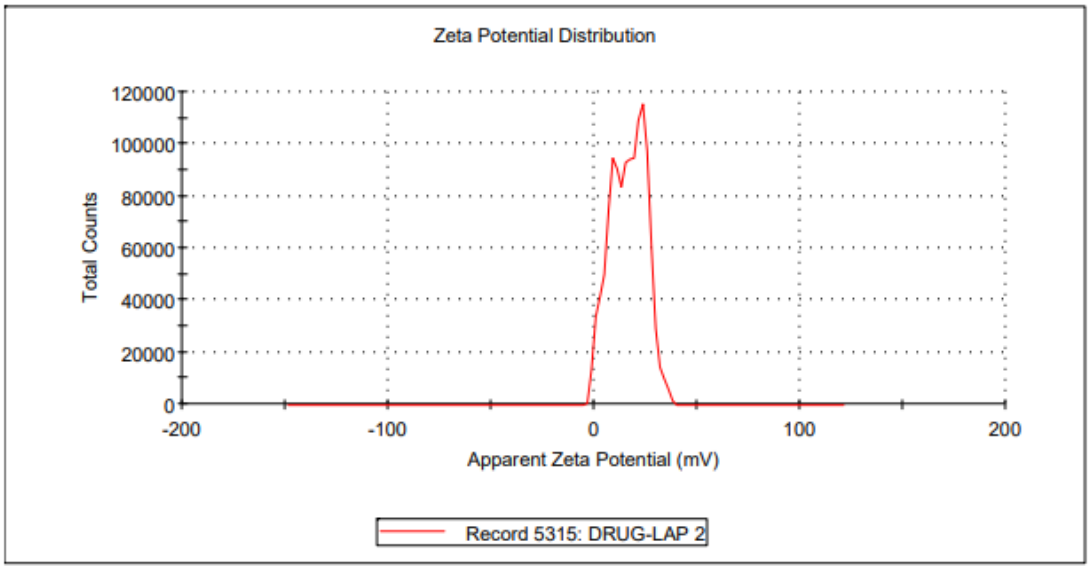


Figure 4.4.6: Lapatinib drug zeta potential

4.5 MTT assay analysis

Invitro study of the prepared samples was done through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) Assay analysis [86]. The BL, LL, and L samples

were diluted by serial dilution methods, in which the (100, 75, and 50 percent) concentrations of samples were achieved. These different concentrations were applied to the MCF-7 and MDA-MB-231 cell lines. Graph 12 and 13 show the efficacy of drug-loaded liposomes (LL), the unloaded drug lapatinib (L), and bare liposomes at 24 hrs and 48 hrs on the MCF-7 cell line. Whereas Graph 14 and 15 show the effects of BL, LL, and L on the MDA-MB-231 cell line at 24 and 48 hrs with different concentrations.

In the MCF-7 cell line with the increased concentration of drug and drug-loaded liposomes the cell viability become decreases. But in the case of the MDA-MB-231 cell line, the 75% concentration of drug-loaded liposomes at 24 and 48 hrs shows high cytotoxicity as compared to 100 and 50%. Bare liposomes also show somehow cytotoxic effects on both cell lines. In combination of drug and liposome, it enhances the cytotoxic effects of drug-loaded liposomes.

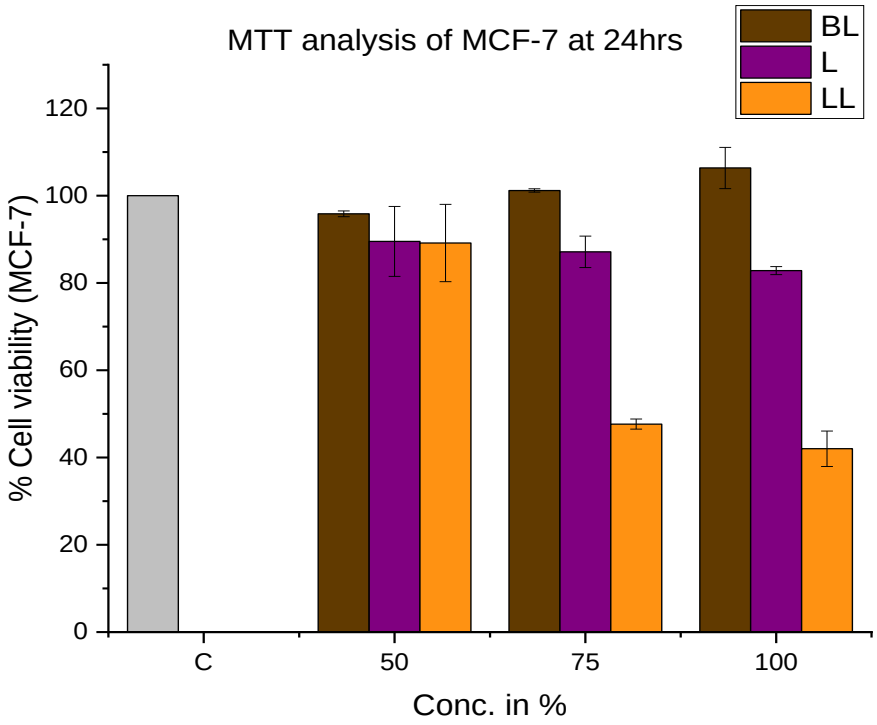


Figure 4.5.1: showing the % cell viability of cells when treated with the liposomal formulation of Lapatinib at 24h. BL (bare liposomes), L (lapatinib), LL (liposomal lapatinib).

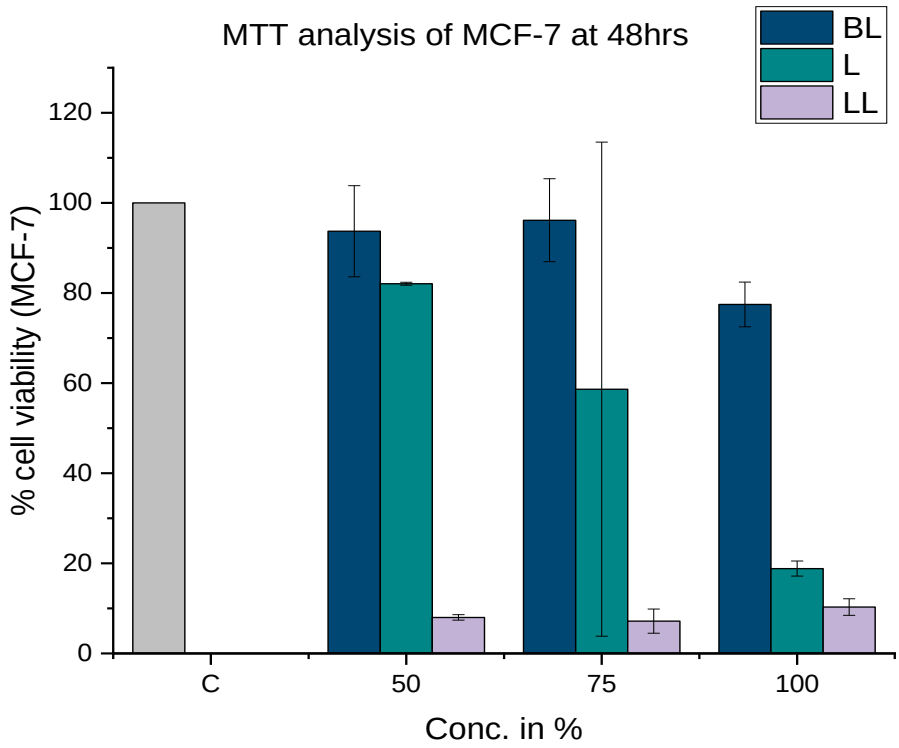


Figure 4.5.2: showing the % cell viability of cells when treated with the liposomal formulation of Lapatinib at 48h. BL (bare liposomes), L (lapatinib), LL (liposomal lapatinib).

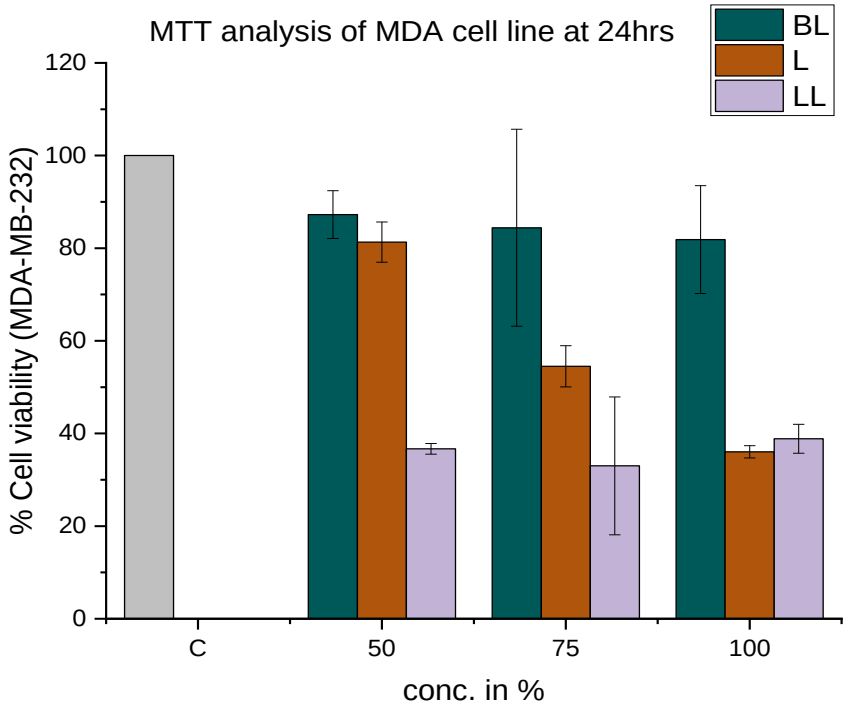


Figure 4.5.3: showing the % cell viability of MDA-MB-232 cells when treated with the liposomal formulation of Lapatinib at 24h. BL (bare liposomes), L (lapatinib), LL (liposomal lapatinib). The activity was assessed in a concentration-dependent manner.

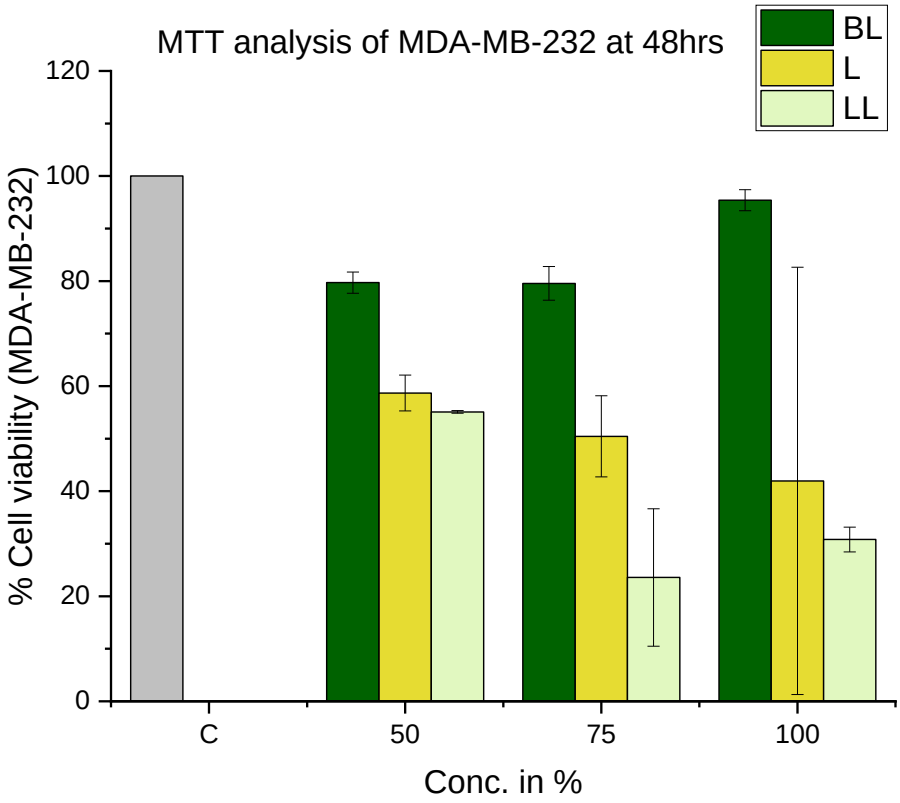


Figure 4.5.4: showing the % cell viability of MDA-MB-232 cells when treated with the liposomal formulation of Lapatinib at 48h. BL (bare liposomes), L (lapatinib), LL (liposomal lapatinib).

CHAPTER # 5

5 DISCUSSION

In this study, we used drug-loaded liposomes to check the anticancer activity on breast cell lines of humans, MDA, and MCF-7. To ensure the activity of drug-loaded liposomes, it is important to study the characterization of the liposomes. Liposomes were prepared by the conventional method (Bangham's method) and the drug is loaded by the passive loading technique. Bare liposomes are also prepared to compare the results of drug-loaded liposomes. To check the characteristic of liposomes, different characterizing techniques have been used including XRD, FTIR, SEM, and zeta analysis. XRD results describe the amorphous nature of the liposomes and drug at 2theta where the angle ranges from 0 to 80˚angles. No crystalline structure is observed in the lapatinib drug as well. There are no new linkages found in the drug-loaded liposomes compared to bare liposomes and lapatinib drugs. It helps the unloading of drug particles from the liposomes at the target site as indicated in literature [87]. The principal peaks in FTIR analysis of BL, LL, and L are 3427, 3003, 1640, 1417, 1044, and 685 comes from the stretched, bending and aromatic vibrating motion of the molecules showing the presence of the functional group, O-H, C-H, C=N, -CH2, C-O, and C-Cl respectively. The encapsulation effectiveness of liposomes, which comprise an oligolamellar phospholipid bilayer, solely for hydrophilic compounds, increases with liposome size and decreases with the number of bilayers. The circulation half-life of liposomes is significantly influenced by the size of the vesicles. The amount of the medicine enclosed is influenced by the size and quantity of bilayers. The prepared liposomes have a good size. Numerous ideas explain how liposomes interact with cell

membranes, including selective or nonspecific endocytosis (modified by receptor-mediated absorption), phagocytosis, and absorption into the membrane. A few of the factors influencing liposome-cell interactions include composition, liposome diameter, surface charge, targeting ligand on the liposome surface, and biological environment.

Two speculative theories about the beginning and spread of breast cancer. (A) The stem cells or progenitor cells that give rise to all tumor subtypes are the same. Then, subtype-specific transforming events choose different tumor morphologies. (B) A single cell type initiates each tumor subtype (stem cell, progenitor cell, or differentiated cell). Any breast cell can gradually develop random mutations, which can eventually cause them to become tumor cells when a sufficient number of mutations have accumulated.

Lapatinib, A tyrosine kinase inhibitor, was used in this study. Liposomes are being employed to ensure their efficacy because it is hydrophobic and less soluble in water, as the properties are clearly stated in the literature [88]. Because cycloheximide prevents the production of lap-induced Stress-Granules (SG), they also have similar dynamic properties to classic SG. Cycloheximide is an antibiotic that is thought to suppress the development of SG by preventing the release of mRNPs from ribosomes that are translating. SG development carried on by Lap therapy is not just limited to T47D; it also occurs in the breast cancer MCF-7 cell line but less effectively However, neither the MDA-MB-231 nor the Hs578T breast cancer cell lines displayed SG development, demonstrating that SG formation generated by Lap in breast cancer is cell-type specific. After formulation of simple and drug-loaded liposomes, they are characterized and anticancerous activity was evaluated. The MTT assay has shown that the MCF-7 cell line has shown a concentration-dependent response. When cells were introduced with liposomal Lapatinib formulation and

gave a contact time of 24 hours, the cell viability was decreased with the high concentration of drug formulation to about 45%. After 48hours, a 20 percent viability value was calculated. It showed that the liposomal formulation has a high value of anticancerous activity against cancerous cells as compared to liposomes and drugs alone. Similarly, the anticancerous activity of this formulation was evaluated on the MDA cell line. The calculated values have shown that liposomal formulation didn't show better activity against the MDA cell line. Our study showed that when Lapatinib was used in combination with liposomes, it showed better results due to an increase in exposure to the environment.

Conclusion & Future Prospects

Lapatinib, a tyrosine kinase inhibitor, modifies the cellular pathways and halts cell proliferation but faces the working issue because of its water-insoluble nature. The objective of our study was the distribution of Lapatinib drug to the tumor site in high amounts and boost the efficiency of Lapatinib against breast cancer by using liposomes as cargo. Our study demonstrated that Lapatinib has a high level of cytotoxicity against cancerous cells when use in combination with liposomes. To strengthen the utilization of this combination therapy in cancer therapy trials, further research is warranted. To further support the findings, pharmacological screening on primary cell lines and animal models is required. Although dissected tumor biopsies can offer more reliable data in the context of ethical concerns, the primary culture clinical model of this disease is more likely to be useful for additional validation. Additionally, it offers a more direct path to cytogenetic analysis. There is a need to work on the increase in the amount of medicine that can be

loaded. The increased payload may lead to longer release and prevent the recurrence of breast cancer.

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