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An Impact Of Liposomal Drug Delivery System Of Docetaxel On Prostate Cancer Cell line

A Thesis Submitted To The Council Of College Of Medicine, University Of Babylon In a partial Fulfillment Of The Requirements For The Degree Of Master Science (M.Sc) In Pharmacology &Toxicology.

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ فَأَمَّا الزَّبَدُ فَيَذْهَبُ جُفَاءً وَأَمَّا مَا يَنْفَعُ النَّاسَ فَيَمْكُثُ فِي الْأَرْضِ كَذَلِكَ يَضْرِبُ اللَّهُ الْأَمْثَالَ ﴾

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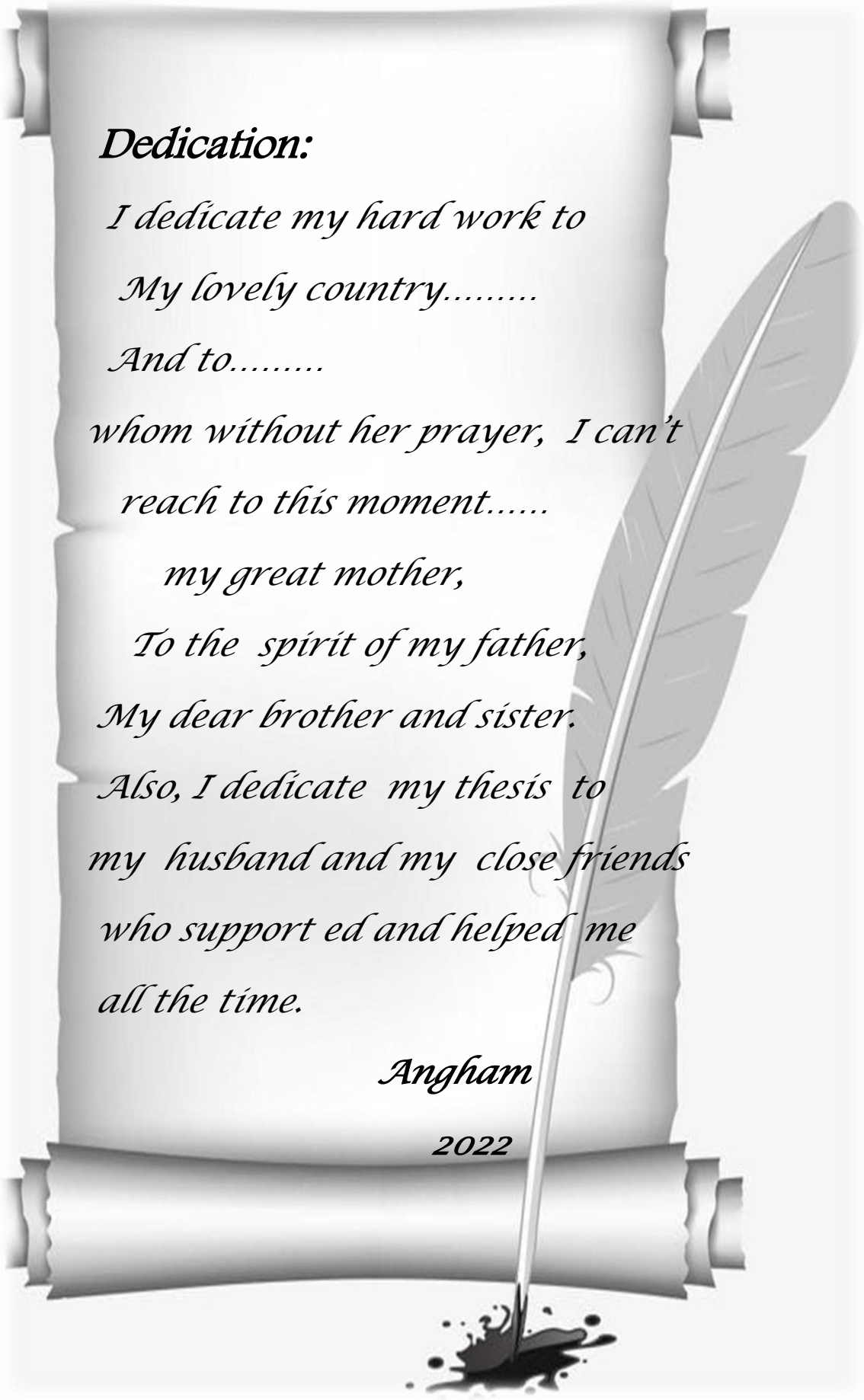

Dedication:

I dedicate my hard work to

My lovely country.....

And to.....

whom without her prayer, I can't

reach to this moment.....

my great mother,

To the spirit of my father,

My dear brother and sister.

Also, I dedicate my thesis to

my husband and my close friends

who support ed and helped me

all the time.

Angham

2022

Certification :

We certify that this thesis entitled (**An Impact Of Liposomal Drug Delivery System Of Docetaxel On Prostate Cancer Cell line**) was prepared by (Angham Mohsen Abed) under our supervision at the Department of Pharmacology and toxicology, College of Medicine, University of Babylon (Iraq) under partial fulfillment of the requirements for the Master degree of Sciences in Pharmacology and Toxicology.

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The Recommendation of the Head of Department

In view of the available recommendation, I forward this thesis for debate by the examining committee.

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Date:

Examining Committee Certificate

We certify that we have read this thesis entitled "**An Impact Of Liposomal Drug Delivery System Of Docetaxel On Prostate Cancer Cell line**" and as an examining committee examined the student "**Angham Mohsen Abed**" in its contents and that in our opinion it meets the standard of a thesis for the degree of Master, in Pharmacology and toxicology.

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Summary:

Cancer starts when cells in the body begin to grow out of control. Cells in nearly any part of the body can become cancer cells, and can then spread to other areas of the body. Prostate cancer begins when cells in the prostate gland start to grow out of control. The prostate is a gland found only in males. It makes some of the fluid that is part of semen. Prostate cancer is the most common cancer among men, except for skin cancer.

Nanomedicine is a branch of science that uses nanotechnology in conjunction with medications or diagnostic molecules to improve the ability to target specific cells or tissues. These materials are made at the nanoscale and are completely safe to consume. Nanotechnology's applications in medicine include imaging, diagnostics, and drug administration, all of which will aid medical practitioners in treating a variety of illnesses.

Docetaxel is a taxoid antineoplastic drug that is used to treat diseases such as locally advanced or metastatic breast cancer, metastatic prostate cancer, gastric adenocarcinoma, and head and neck cancer. The toxicity of the traditional docetaxel formulation is largely attributable to the formulation vehicles polysorbate 80 and ethanol. To overcome the toxicity concerns, a nanoliposomal docetaxel formulation was created without the use of the formulation carriers polysorbate 80 and ethanol. The lipids were produced using patented Nanotechnology and are considered safe by the US FDA. Due to compromised tumor vasculature, nanosomal lipid-based particles (<100 nm) may increase docetaxel delivery to tumor tissues, resulting in improved treatment outcomes.

The primary aim of this study is to improve the drug delivery system of docetaxel by manufacturing it as liposomal docetaxel.

Firstly, The docetaxel was formulated as liposomal nanoformulation and the different characteristics on the nanoscale were studied.

Secondly, an attempt has been made to examine the effect of liposomal docetaxel on the viability of prostate cancer cell lines. The present work was performed in the Postgraduate Laboratory /Department of Pharmacology at the College of the Medicine\ University of Babylon from October 2020 – to August 2021.

The prostate cancer cell lines LNCaP were seeded in 96 well plates and treated with different concentrations (500, 250, 125, 62.5, 31.25,15.62) µg/ml of Liposomal docetaxel, pure docetaxel powder, formulated docetaxel (Taxoter), and control group (not treated).

All of this well was incubated for 24,48,72 hours at $37C^0$. Then the MTT cytotoxicity assay (**3-(4,5- dimethylthiazol)-2,5 diphenyl tetrazolium bromide)** was used to assess the effect of this drug on the viability of the cell in the mentioned groups.

Results showed docetaxel in liposomal form caused a significant ($p\leq 0.001$) decrease in cell viability of the LNCaP cell line, at all tested concentrations except the lower concentrations (31.25 and 15.6)µg/ml only at 24 hr, incubation period. The pure docetaxel effect on LNcaP cell line caused significant decrease in cell viability (P value<0.001) at concentration (500,250,125) µg/ml. The effect of formulated Docetaxel vial on the LNCaP cell line caused a significant decrease in cell viability (P<0.005) at all concentrations of the drug at different incubation periods. The IC50 of

liposomal docetaxel was (112.71,213.11 and 356.70) µg/ml, Pure docetaxel (385.33,310.32 and 126.82), and Formulated docetaxel was (362.75,345.65 and 530.7) µg/ml at 24m48 and 72 hr of incubation respectively.

The conclusions from this study were this modification in the drug delivery system of docetaxel lead to improvement in the characterization and physicochemical properties and antitumor efficacy of this drug.

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List Of Abbreviations

Abbreviation	**Meaning**
AFM	Atomic force microscopy
°C	Centigrade
Conc.	Concentration

DDW	deionized distilled water
DNA	Deoxyribonucleic acid
DHT	dihydrotestosterone
DMSO	Dimethyl sulfoxide
DXL	Docetaxel
DDS	Drug delivery system
DL	Drug Loading
EPR	Enhanced permeability and retention
EE	Entrapment Efficiency
FBS	Fetal bovine serum
Fig	Figure
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
Hr	Hour
LUV	Large unilamellar vesicles
L.DXL	Liposomal docetaxel
RPMI-1640	Liquid Roswell Park Memorial Institute
MTT	3,-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide

µg	Microgram
µL	Microliter
mL	Milliliter
IC50	Minimum Inhibitory Concentration
MLV	Multilamellar vesicles
MLVs	Multivesicular vesicles
Nm	Nanometer
NPs	Nanoparticles
P-gp	P-glycoprotein pump
PBS	Phosphate-buffered saline
pH	power of hydrogen
PCa	Prostate cancer
PSA	Prostate-specific antigen
RPMI	Roswell Park Memorial Institute
SEM	Scanning electron microscopy
SUV	Small unilamellar vesicles
SLNs	solid lipid nanoparticles
SLP	Soy Lecithin-Derived Liposome
TEM	Transmission Electron Microscopy

EDTA	Trypsin- Ethylenediaminetetraacetic acid

Chapter One

Introduction

and

Literature Review

1.1 Introduction :

Prostate cancer (PCa) is one of the major causes of morbidity and mortality in developing and under-developed countries. The most frequent non-skin cancer causes the second largest number of deaths in men as compared to other cancers. Prostate cancer can be localized and advanced depending upon its severity. Prostate cancer can metastasize via the lymphatic system and invade bones. Various factors like age, genetics, environmental toxins, chemical hazards, and radiation seem to be involved in the pathogenesis of prostate cancer but the exact mechanism is still unknown. Androgens are involved in the normal developmental phase of the prostate and their functions, but, in that phase, they can still steep towards carcinogenesis(Barani *et al.*, 2020).

Over the past few years, taxanes have emerged as a new class of anticancer drugs. Docetaxel (DXL) the prototype of this class has been approved for the treatment of a broad range of cancers including breast, prostate, non-small cell lung, and head and neck cancers (Naguib *et al.*, 2014). However, to date, the commercial preparation of DXL is accompanying side effects, intolerance, and poor solubility, which can be overcome by encapsulating them using different types of nanoparticles (Sumera *et al.*, 2017).

1.2 Aim of the study:

Improvement of the drug delivery system of docetaxel by manufacturing it as liposomal docetaxel.

1.3 Literature Review:

1.3.1 Mechanisms of Carcinogenesis:

The multistage model of carcinogenesis (Figure 1.1) adequately explains the age-incidence patterns of non-hormone-dependent carcinomas, as well as the effects of the timing and dose level of various agents alone and in combination (particularly smoking, alcohol, ionizing radiation, and some occupational carcinogens(Wark and Peto, 2016). Carcinogenesis occurs in three stages: initiation, promotion, and progression. The genotoxic carcinogen generates initiated cells with a mutant genotype; during promotion, tumor-promoting carcinogens can stimulate these cells to proliferate and form clusters of initiated cells; the resulting lesion is predisposed to progress to cancer; however, additional exposure to genotoxic and tumor-promoting substances accelerates the progression stage by increasing genomic instability and cell proliferation rate, thereby converting a preneoplastic lesion to cancer (Carreón and Zajgla, 2012).

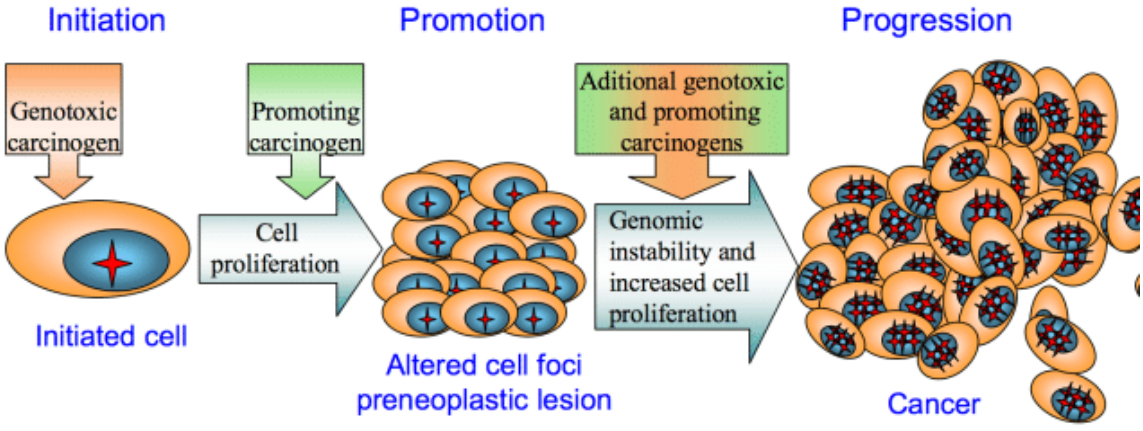


Figure 1.1 Mechanism Of Carcinogenesis (Carreón and Zajgla, 2012)**.**

1.4 Prostate gland and prostate cancer:

The prostate is a glandular organ located beneath the bladder. It is made up of epithelial cells that are arranged in a fibromuscular stromal network. Although definitive etiological indications relating to prostate cancer development and incidence have been difficult to establish, multiple studies have consistently connected the disease to common risk factors, including age, race, diet, and physical activity. PCa incidence is heavily influenced by age, as the likelihood of being diagnosed increases with age. The prostate glands are anterior to the rectum and produce a thick, white liquid that combines with sperm from the gonads to form semen. This white prostatic liquid, along with the overwhelming majority of spermatozoa, is discharged into the urethra via the major discharge divisions. It produces a protein called prostate-specific antigen (PSA) (Siddiqui *et al.*, 2015), which converts the sperm to a fluid. The prostate gland and a cancerous prostate are depicted in Fig. 1.2. Typically, the prostate gland is located between the urethra (bladder) and the rectum. (Garg *et al.*, 2021).

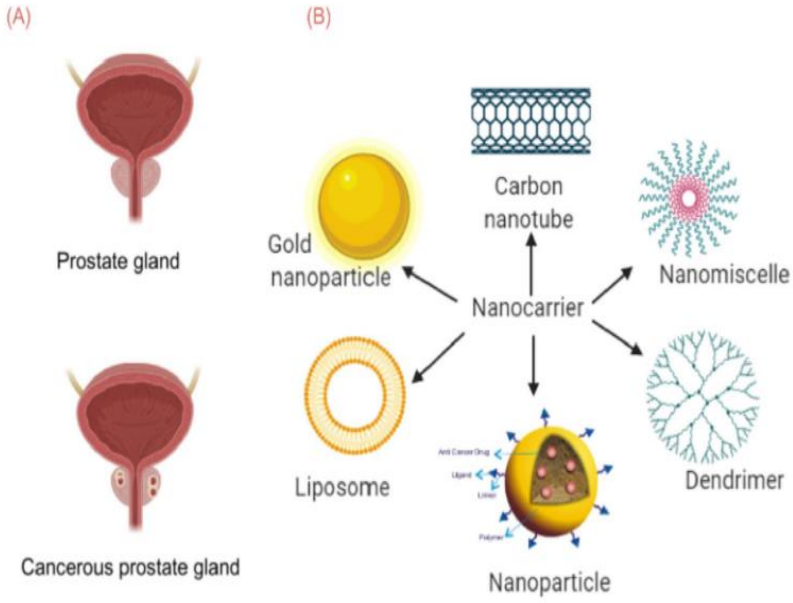


Figure 1.2 (A) Prostate Gland Structure And Tumor Within The Prostate Gland; (B) Schematic Representation Of Various Nanocarriers Used For The Treatment Of Prostate Cancer (Garg *et al.*, 2021)**.**

PCa was the second most often diagnosed cancer and the fifth top cause of death from cancer in men worldwide in 2012, according to the WHO. PCa incidence varies by more than 25-fold throughout countries; it is often higher in Western countries and lowers in Asian countries. However, mortality rates vary less globally than incidence rates. (Kimura and Egawa, 2018).

While PCa may be asymptomatic in its early stages, it may manifest with urine retention and back discomfort in its advanced stages, as the axial skeleton is the most prevalent site of bone metastatic illness. Numerous prostate malignancies are identified when plasmatic levels of prostate-specific antigen (PSA > 4 ng/mL), a glycoprotein usually produced in prostate tissue, are increased. However, because men without cancer have been reported to

have increased PSA levels, a tissue biopsy is the gold standard for confirming the existence of malignancy (Pernar *et al.*, 2018).

1.4.1Epidemiology:

Prostate cancer is the second most common cancer in men, affecting elder men: over 80% of cases are diagnosed after the age of 65. Despite this, only 10% of men who develop prostate cancer die from it. prostate cancer is found in 39 % of men aged 70–79 years old and in 43 % of men aged 80 years old (Daniyal *et al.*, 2014).

1.4.2 Risk Factors:

The etiology of prostate cancer is not fully understood, but the incidence increases with age from 10 percent in men in their fifties to 90 percent in men in their nineties. Male hormones (androgens) such as testosterone also need to be present because the disease does not occur in men castrated before puberty. Family history is one of the few established risk factors. Men who have a first-degree relative with prostate cancer have a twofold risk of developing the disease. The risk also increases as the number of family members affected increases; men with two or three first-degree relatives affected have a five to tenfolds increased risk of developing the disease. These findings suggest that exogenous factors affect the risk of progression from latent prostate cancer to clinical prostate cancer and may include factors such as a diet high in fat, sexual behavior, alcohol consumption, and occupational exposure but these risk factors are still under investigation. Higher body mass index and adult weight gain increase the risk of dying from prostate cancer (Drudge-coates, 2015).

1.4.3 Symptoms :

Patients with prostate cancer may be asymptomatic or they may have some of the following :

- Urinary symptoms, such as frequency, urgency, poor flow, difficulty in starting to void, or incomplete emptying.
- Haematuria
- Haematospermia.
- Erectile dysfunction due to local spread.
- Unexplained anemia or pain in the hip, back, or pelvis due to metastases(Drudge-coates, 2015).

1.4.4 Treatment:

There are many treatment options for prostate cancer which include surgery, radiation, hormonal, and chemotherapy. Surgery is not considered a monotherapy in men with prostate cancer; rather it is a part of the multimodality approaches. Surgery is highly suggested for high-risk locally advanced prostate carcinoma. Radical prostatectomy and pelvic lymphadenectomy are mostly used types used in prostate cancer. The second major therapeutic modality in radiotherapy for localized high-risk prostate cancer. Prostate tumors are hungry for androgens, thus androgens are considered fuel for this type of tumor. The systemic androgen function includes testosterone which accounts for 90% and dihydrotestosterone DHT which is a cytosolic variant. The treatment with androgen deprivation therapy along with a medical or surgical approach is considered the initial therapy for metastatic prostate cancer. The beneficial clinical effect of this therapy is rapid. Fortunately, bilateral surgical removal of the testicle is associated with

fewer side effects than medical deprivation therapy. The common chemotherapeutic drugs used in the treatments of advanced prostate cancer include mitoxantrone, doxorubicin, vinblastine, paclitaxel, docetaxel, and some others. Mitoxantrone is an anthracene Dione antineoplastic agent (Chen and Zhao, 2013).

1.5 Nanotechnology:

The term 'nanotechnology' first gained widespread public recognition in 1986 with K. Eric Drexler's book 'Engines of creation: the future era of nanotechnology.' Richard Feynman, the 1959 Nobel laureate, first proposed the concept in 1959 during a presentation titled 'There's Plenty of Room at the Bottom.' Initially, nanotechnology was envisioned to be used for chemical synthesis via the use of nanoscale devices and atomic-level information storage. Since then, nanotechnology has been applied to a variety of industries, including wastewater treatment, textile manufacturing, advanced batteries, biology, and medicine. Nanotechnology has made significant advancements in cancer therapy, disease diagnostic imaging, tissue engineering, and, most significantly, drug and gene delivery methods for medical applications. (Bayda *et al.*, 2020).

In recent years, nanotechnology has become a big deal in medicine because of how easy it is for nanostructures to work together on a molecular level. Nanomedicine is a branch of nanotechnology that is used in medicine. People use molecular tools and the body's molecular information to identify, evaluate, and prevent disease. They also use the body's molecular information to protect and improve human well-being. Material's properties may be different when it's smaller than it is in its bulk form. This happens because a certain surface area grows to the same size as the molecule. In addition, the arrangement of

atoms changes when the surface area changes. This could change the optical, magnetic, electrical, and thermal properties of biological communication. Changing the surface or size of something can make it more or less likely to get into cells. Nanoparticles (NPS) can be made to fit a specific need. Even though NPS are small, they can be layered with atoms or DNA to make curative and investigational agents. (Garg *et al.*, 2021).

DDS based on NPs Recent advancements in the field of delivery systems has resulted in huge advancements in the delivery of therapeutic agents or natural-based active chemicals to their target region for the treatment of a variety of diseases. While numerous drug delivery methods have been effectively deployed in recent years, certain problems remain and sophisticated technology must be created to ensure the successful delivery of medications to their target sites. (Patra *et al.*, 2018).

1.6 Drug delivery system (DDS):

DDS has a vital node of biotechnology, intending to deliver a specific substance to its site of action to achieve the desired pharmacological effect. Along with the target component, other variables such as the carrier's type and the sequence of execution should be considered when developing the DDS strategy. The most difficult aspect of manufacturing DDS products is ensuring the biocompatibility system. This word refers to the ability to get past the body's defenses without hurting the organism's immune response. Stability, dispersibility, penetration, and better interactions with the cell membrane are also important parts of a good DDS system design. Advances in the understanding of how cells and DDS systems interact with each other have made it easier to improve these systems (Garg *et al.*, 2021).

Liposomal drugs following administration can circulate through the bloodstream and accumulate in diseased lesions in the target region. It acts on tumors by accumulating at the site of action, being absorbed by tumor cells, and being released as free drugs. The entrance of Liposomal medications into the human body through several different mechanisms and then communicate with molecules in a variety of different ways. Techniques for tumor targeting are typically categorized as active or passive(Rommasi and Esfandiari, 2021).

1.6.1 Active and passive targeting:

Nanoparticles can accumulate in cancerous tissue through the use of both passive and active targeting methods. Choosing a ligand-receptor fusion that is upregulated in tumor cells is the first step in tumor targeting. Using nanocarriers, ligands can be attached to the nanocarriers, which could then attach to the overexpressed position on cancerous cells for specific targeting medication orders. Examples of ligands for malignancies include folate, transferrin, and galactosamine. The drug-loaded nanocarrier can penetrate the tumor's leaking vasculature to achieve passive targeting (Barani *et al.*, 2020).

Diffusion and convection are used to achieve a passive target. Large molecules are primarily transported through the endothelium's aperture by convection, while smaller molecules and lipophilic compounds are transported by diffusion following the gradient established by blood flow. Nanocarrier aggregation in malignancies is accelerated by the enhanced penetration and retention (EPR) effect(Danhier, Feron, and Préat, 2010) This was observed in prostate malignancy by S.andanaraj et al. utilizing a fluorescent nanoprobe and microscopy(Sandanaraj *et al.*, 2010). Receptor-specific associations between the carrier and target are achieved by altering NPS surface to

facilitate point-specific interactions. Nanocarriers must first arrive at the tumor before they can bind to the target cells, requiring the EPR effect. Although active targeting by itself does not increase medication accumulation in tumors, it does enhance cell identification and uptake. Examples of ligands used to attack tumor cells include galactosamine. (Akberzadaeh *et al.*, 2013). As shown in figure 1.3 The passive targeting nanoparticle is the mechanism by which the drugs leak from blood vessels supplying cancer cells and accumulate in the cells by enhanced permeability and retention (EPR) effect. while The active targeting nanoparticles, target ligands conjugated on the surface of nanoparticles, resulting in increased cellular uptake by receptor-mediated endocytosis and therefore increased drug accumulation in cancer cells(Muhamad, Nadda, *et al.*, 2018)

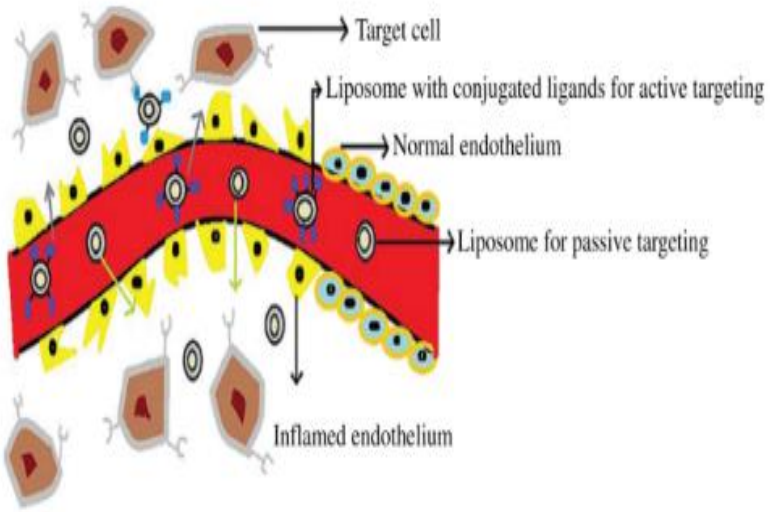


Figure:1.3 Passive And Active Targeting Of Cancer Cells For Drug Targeting By Liposome (Alavi and Hamidi, 2019).

1.7 Routes of drug delivery to the prostate :

- Anticancer medicines delivered systemically exhibit nonspecific biodistribution and severe side effects in patients. These constraints have been partially overcome by nanomedicine.
- Localized drug delivery has an advantage over systemic drug delivery in that it minimizes the medication's systemic exposure and is more focused on a specific region during the initial phase of nanomedicine administration.
- Nanomedicine enhances a drug's therapeutic load while minimizing nonspecific dispersion and adverse effects. Additionally, the prostate is a candidate for locoregional cancer therapy, which has emerged as a viable alternative to systemic administration in solid tumors.
- Intraprostatic, vas deferens, and transrectal are three locoregional delivery routes for nanomedicine to the prostate that gives an advantage over systemic delivery for prostate cancer therapy(Gupta *et al.*, 2017). The different routes of drug delivery in prostate cancer cases are demonstrated in figure 1.4.

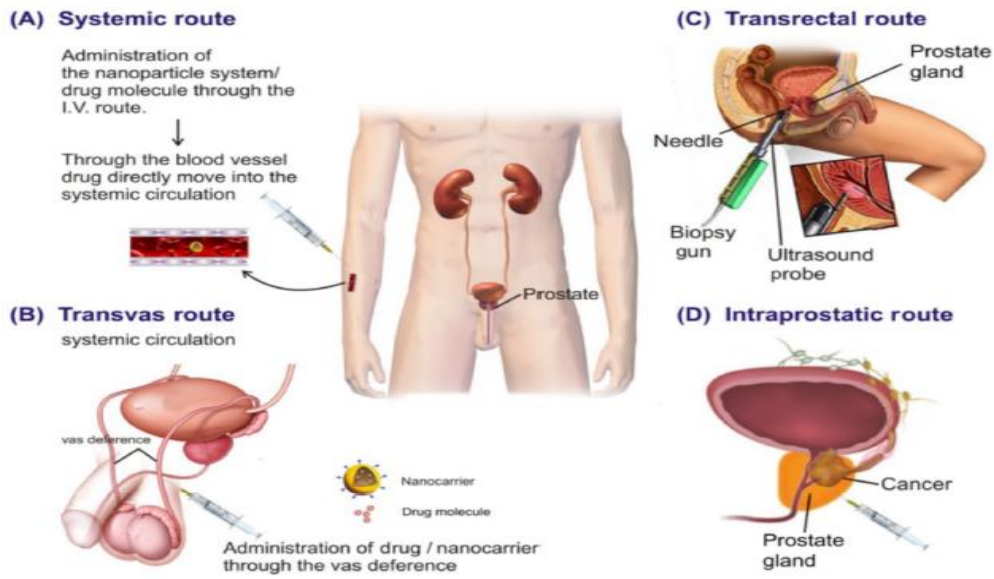


Figure 1.4: Routes of drug delivery and prostate cancer therapeutics. (A) via the systemic route; (B) via the transverse route, The transrectal route, and (D)the intraprostatic route(Garg *et al.*, 2021)**.**

1.8 Treatment of prostate cancer with nanotechnology:

Recent breakthroughs in cancer nanotechnology have made it possible to improve the diagnosis and treatment of prostate cancer. There is a need to enhance the availability and efficacy of traditional chemotherapeutic drugs for prostate cancer. Numerous therapeutic nanoparticles have been designed utilizing nanotechnology that can precisely target and distribute a variety of chemicals to destroy prostate cancer cells while causing no harm to healthy cells. Theranostic NPS have been created to specifically target prostate cancer cells with targeting ligands and to deliver anticancer medicines in a controlled and time-dependent manner for cancer therapy, in conjunction with aided imaging to evaluate the therapy's effectiveness in real-time. Natural products, surface-modified polymers, and metallic NPS have all developed into

promising nanomaterial for prostate cancer therapeutic targeting (Aleena Mary Cherian and Lakshmanan, 2014).

1.9Nanomedicine:

Nowadays, the nanotechnologies of biomedicine and healthcare, in general, are referred to as 'nanomedicine' and this is considered a high-growth area of nanotechnology. Throughout the previous few decades, the US Food and drug administration approved the marketing of over 100 nanoparticle-based applications and devices. This highlights the critical role of nanotechnology in contemporary biomedical science. Nanotechnology, according to a new Forbes assessment, is the sixth most significant growth technology to monitor over the next decades(Farjadian *et al.*, 2019).

Nanomedicine has transformed the world of medicine and diagnosis by bypassing traditional treatment protocols in the treatment of infamous malignancies and numerous intracellular disorders. The application of nanotechnology to medicine can also result in improved transmembrane penetration, permeability retention, higher solubility, and controlled drug delivery(Barani *et al.*, 2020).

1.9.1 Advantage of Nano formulation :

- Nano drug formulation is one method for delivering medicinal compounds more precisely to the target organ while minimizing the total dose and potential for side effects.
- The EPR effect which enables passive targeting and accumulating of nanoparticle-sized medications in malignant tumors and other sick areas.

- Nano-sized formulations outperform conventional microsized formulations in terms of active concentration and bioavailability.
- NPS outperforms in terms of safety and efficacy.
- Nanomedicines have the potential to be significantly less expensive than traditional therapy.
- Over the specified timescale, drug release can arise at a persistent rate (Patra *et al.*, 2018).

- The development of intelligent or response delivery vehicles that can respond to specific internally or externally, physical or chemical signals was recently identified as a new area in nanocarrier technology. Physical stimuli like light and temperature have been employed in the construction of smart DDS, whilst chemical stimuli such as ph changes, reducing agent concentration, or ionic strength have been used. Furthermore, advanced active or passive targeted strategies can be employed to deliver a variety of drugs directly to specified targets. Diverse ligands or targeted agents can be put to the nanoparticle surface to direct that towards specific cells or tissues, depending on the molecular recognition processes. Active targeting has been widely employed in cancer therapy, where the targeting moieties enable the drug carriers to attach to specific biomarkers highly expressed in tumors. The critical parameter in passive targeting, on the other hand, is the nanocarrier size, which results in accumulation at tumor sites due to the tumor's characteristic leaky vasculature and absence of lymphatic drainage (EPR effect)(Rivankar, 2014).
- The other advantages of Nanomedicine include increased non-polar drug solubility, prolonged biodistribution, EPR effect in tumor tissue's

leaky vasculature, increased intracellular infiltration, controlled drug delivery, and trying to minimize nonspecific uptake and avoiding the unwanted off-target and side effects (Loukanov *et al.*, 2019).

1. 9.2 Classification of Nanoparticles:

Nanoparticles can be classified into three categories based on the materials that they are made from (**A**) polymeric nanoparticles (**B**) inorganic nanoparticles, and (**C**) lipid-based nanoparticles. Figure 1.5 illustrated the different types of nanoparticles

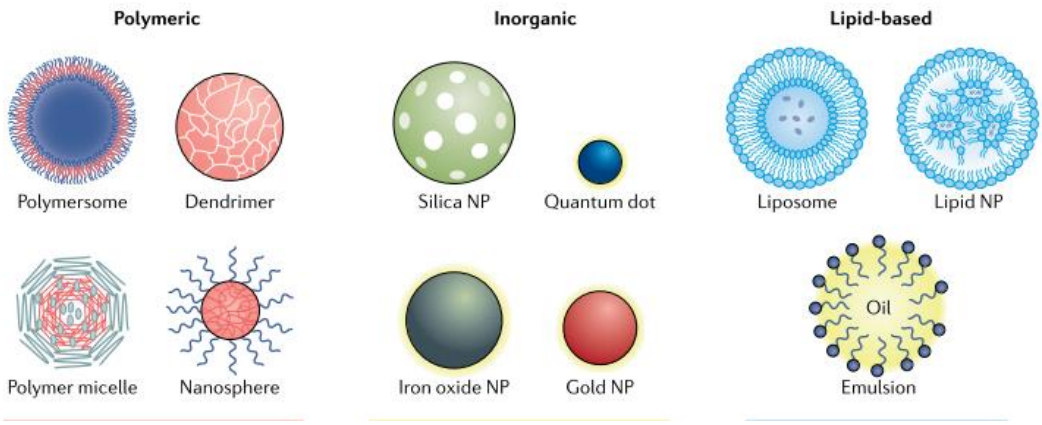


Figure 1.5: Different Types Of Nanoparticles (Mitchell *et al.*, 2021)

1.10 Liposome:

Dr. Alec D. Bangham, a British hematologist, described liposomes for the first time in 1961 (published in 1964) at the Babraham Institute in Cambridge. Dr. Alec Bangham and R. W. Horne noticed these while putting the institution's new electron microscope through its paces by staining dry phospholipids with negative stains. The liposome and plasma lemma is similar, and the

microscope image offered the first real confirmation that now the cell membrane is a bilayer lipid architecture. A liposome is derived from the two Greek words, Lipo, meaning fat, and Soma, which means body; it is so named due to its compositional similarities to phospholipids. This liposome is a vesicle that was created artificially and is mostly composed of a lipid bilayer. The liposome acts as a vehicle for the transfer of nutrients and therapeutic drugs. These are composed of natural phospholipids and may have mixed lipid chains incorporating surfactants (e.g. egg phosphatidylethanolamine). Surface ligands on liposomes may allow them to link to sick tissue. Multilamellar vesicles (MLV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV) are the three forms of liposomes (Pawar, Bhosale, and Derle, 2012). The forms of the liposome are demonstrated in figure 1.6.

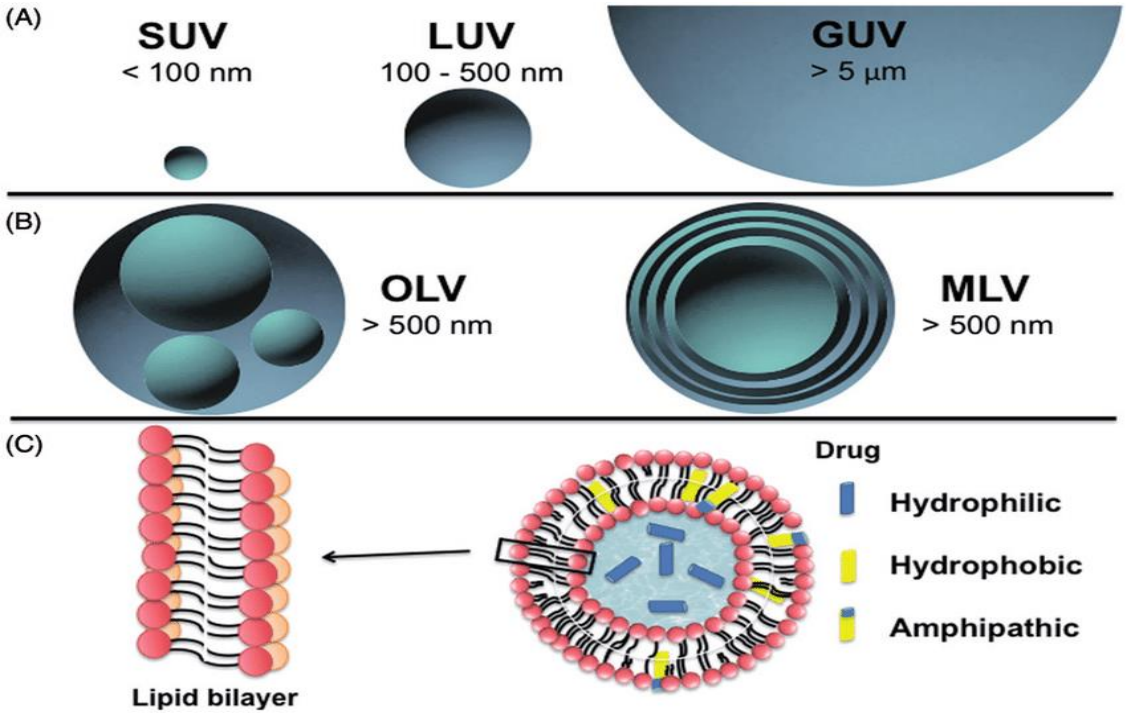


Figure 1.6: liposomes in drug delivery. (a) unilamellar vesicles of increasing size: suv, luv, and guv. (b) oligo (olv) and multivesicular (mlv) vesicles. (c) representation of the lipid bilayer and incorporation of hydrophilic, hydrophobic, and amphipathic compounds inside the liposomes(Ortega, Giorgio, and de Paula, 2017)**.**

1.10.1 Liposome Application:

Over the last century, liposomes have been used in a wide number of applications. In addition to their use as model biological membranes, liposomes are frequently used in drug delivery, gene delivery, vaccine distribution, molecular imaging, cosmetics, and the food industry. Recently, liposomes have been used in genome editing. (Zhang, 2017).

Anticancer medication liposome formulations have been created to extend drug circulating lifespan, improve anti-tumor activity by boosting tumor drug deposition, and reduce drug toxicity by bypassing key normal tissues (Ait-Oudhia, Mager, and Straubinger, 2014).

1.10.2 Structural Components Of Liposomes:

Liposome membranes are made up of natural and/or synthetic lipids that are biocompatible, biodegradable, and non-immunogenic. Liposomes are utilized as carriers for both lipophilic and water-soluble compounds due to their unique bilayer structure. In the interior aqueous compartments, hydrophilic compounds are enclosed. Lipophilic medicines are mostly entrapped in lipid bilayers. (Laouini *et al.*, 2012).

1.10.3 The Main Components Of Liposomes Are:

• Phospholipids.

• Cholesterol (Maheswaran *et al.*, 2013).

1.10.3.1 Soy Lecithin-Derived Liposome (SLP):

Lecithin is found in triglycerides, phospholipids (phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine), and glycolipids. The Natural phospholipids can be obtained from animal resources including egg yolk, seaweed, as well as milk. Lipids derived from cattle or eggs, for instance, have stability difficulties due to their high amount of polyunsaturated fatty acids. As a result, lecithin produced from such sources is less stable than lecithin derived from soybeans or another plant source, which contains fewer polyunsaturated fatty acids. Additionally, bovine or egg-derived lecithin (e.g., egg yolk and milk lecithin) may be contaminated with proteins and/or dangerous organisms such as viruses (Le *et al.*, 2019), But on the other side, lecithin derived from soybeans is believed to be more cost-effective, safer, and more stable in terms of production (Lan, Hiep, and Hai, 2017), Soybean lecithin has several advantages, including the fact that it is more stable

(contains less polyunsaturated fatty acids), safe, available in both pure and unpurified form, and less expensive for laboratory and pharmaceutical liposome synthesis. (Le *et al.*, 2019).

1.10.4 Liposome Preparation Protocols:

1.10.4.1 Thin-Film Hydration Method:

That's the most often used method for producing liposomes. It is based on the creation of lipid thin films inside a round flask inside a rotary evaporator under lower pressure, followed by hydration at a temperature greater than the major transition temperature of phospholipids. If the encapsulated bioactive molecule is lipophilic, it is injected during the creation of the lipid film; if it is hydrophilic, it is inserted in the aqueous medium. (Torres-Flores *et al.*, 2020).

Thin-film hydration results in the formation of multilamellar vesicles. These liposomes can be reduced to (SUV) or (LUV) with a more uniform size distribution. This can be performed by sonicating the probe or by extruding the solution via porous polycarbonate filters. Sonication and extrusion both provide the system with energy (thermal and mechanical) necessary to divide the liposome bilayers of MLVs into smaller lipid vesicles, hence reducing the hydrophobic part that is exposed to the aqueous environment. Typically, the liposomal MLVs solution is fusioned before utilizing polycarbonate filters to reduce their size. The freeze-thaw process entails a rapid change from the cold to the hot temperatures of dry ice or liquid nitrogen, and the fundamental phospholipid transition temperature used in liposome structural units. These extreme conditions cause the bilayers to break, resulting in the formation of microscopic multilayer lipidic vesicles. (Franzé *et al.*, 2018). The thin film hydration method is illustrated in figure 1.7.

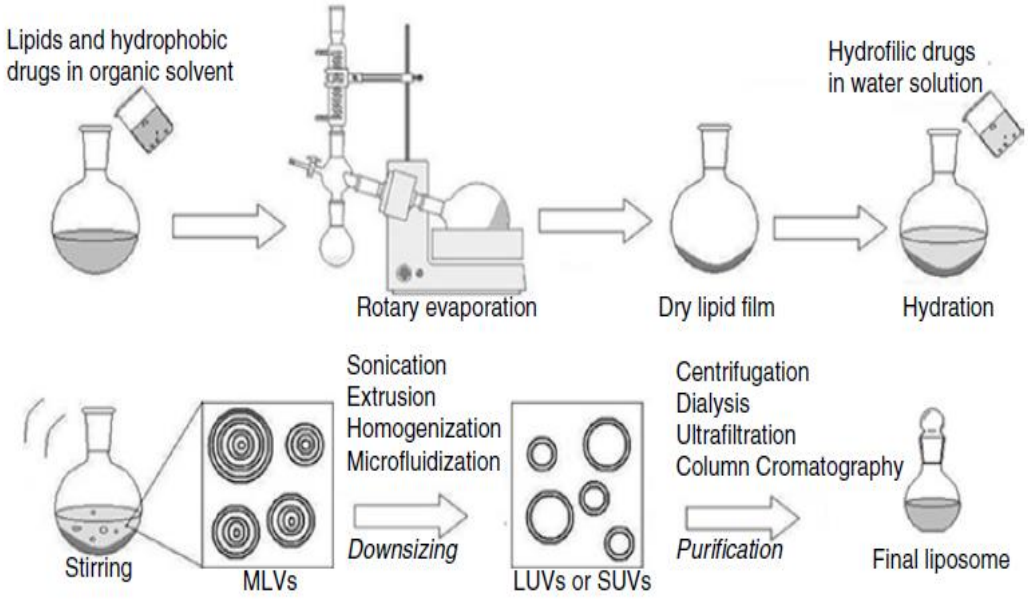


Figure 1.7 Thin Film Hydration Technique(Gallego-Yerga *et al.*, 2017).

1.11 Method of encapsulation of drugs into liposomes :

1.11.1 Passive Loading

Passive loading describes the procedure in which liposomes are formed concurrently with drug loading (Figure 1.11 A). In general, hydrophilic chemicals are distributed uniformly in the aqueous phase (both inside and outside the liposomes), whereas hydrophobic medicines are kept within the liposome's lipid bilayer. When working with poorly water-soluble pharmaceuticals, the drugs are first dissolved in an organic solvent with lipids, followed by solvent evaporation to form a drug-containing thin film, which is then hydrated with an aqueous phase to prepare liposomes. When loading water-soluble medicines, the lipid film is disseminated in an aqueous phase containing the drug(Pauli, Tang, and Li, 2019). The passive and active methods of encapsulation are demonstrated in figure 1.8.

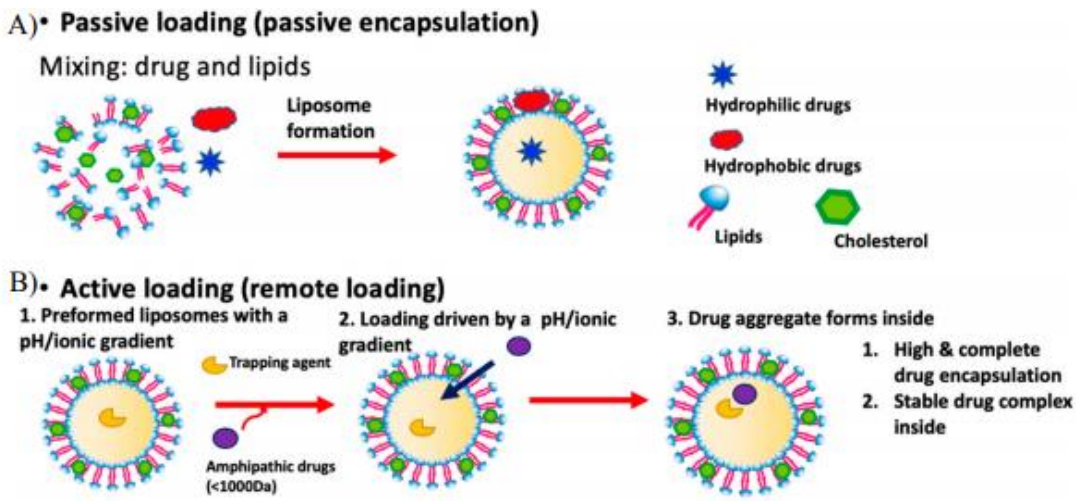


Figure 1.8 Method Of Drug Encapsulation(Pauli, Tang, and Li, 2019)**.**

1.11.2 Active Loading

Liposomes with a transmembrane gradient, i.e. different aqueous phases inside and outside the liposomes, are first formed during inactive loading. Following that, an amphipathic drug dissolved in the external aqueous phase can permeate across the phospholipid bilayer(s), followed by interactions with a trapping agent in the core to lock the drug in place(fig 1.11B).

Bioactive compounds with varying degrees of solubility They have, nonetheless, emerged as intriguing Nanosystems for delivering bioactive chemicals to specific organs. Their capacity to expand the active and passive targeting methods is critical for the discovery of new illness targets. Liposomes are the best prevalent and fascinating lipidic carriers (Demetzos, 2016).

Medication carriers have advantages over typical drug formulations. They prevent the entrapped medication from degradation and premature

metabolization during human body release. Liposomes are vesicular delivery systems that can transport either hydrophilic or hydrophobic molecules. They have a spherical shape, a tiny particle size, a high drug encapsulation efficiency, an inner aqueous core, and an exterior double lipidic layer, and are biocompatible with human cells (Maja, Željko, and Mateja, 2020).

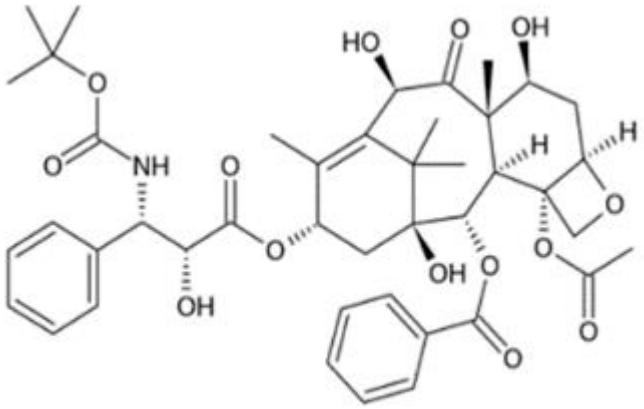
Lipidic nanocarriers are regarded as the most advantageous and promising technological platforms for encapsulating bioactive compounds. Because of their lipidic composition, biocompatibility and biodegradability are favorable qualities, as is the great ability to trapping of bioactive compounds using varied solubilities. They have, nonetheless, arisen as intriguing nanosystems for delivering bioactive chemicals to specific organs. Their capacity to improve active and passive targeting methods is critical for the discovery of different disease targets. Liposomes are the best prevalent and fascinating lipidic carriers(Demetzos, 2016).

Most conventional chemotherapeutics' clinical efficacy is restricted by their failure to deliver therapeutic drug concentrations to target tissues or by significant and detrimental adverse effects on normal organs and tissues. Liposomes are small, spherical, enclosed compartments that are separated from one aqueous medium by a phospholipid bilayer. Hundreds of drugs, including anticancer and antimicrobial agents, chelating agents, peptide hormones, enzymes, proteins, vaccines, and genetic materials, have been incorporated into the aqueous or lipid phases of liposomes of varying sizes, compositions, and other properties to provide selectively and delivery to the target site for in vivo application. Several procedures have been published for generating liposomes with high entrapment efficiency, narrow particle size distribution, and long-term stability, including the Bangham, detergent-

depletion, ether/ethanol injection, reverse phase evaporation, and emulsion methods. Recently, certain alternate methods for liposome preparation, such as dense gas and supercritical fluid procedures, have been established that do not require the use of an organic solvent. Liposomes are classed according to their lamellarity (uni- and multilamellar vesicles), size small [100 nm], intermediate [100–250 nm], or large [>250 nm]), and surface charge due to differences in manufacturing methods and lipid compositions (anionic, cationic, or neutral). Liposomes exhibit enhanced pharmacokinetics and biodistribution of medicinal drugs in clinical investigations, reducing toxicity by accumulating in the target tissue (Chang and Yeh, 2012).

1.12 Docetaxel (DXL):

Docetaxel (C43H53NO14) (DXL) is a semisynthetic medicine created by chemically modifying a compound derived from European yew needles. DXL's unique method of action determines a broad spectrum of anticancer activity. The drug promotes tubulin buildup in intracellular structures known as microtubules and delays their breakdown, resulting in aberrant mitotic and interphase processes in tumor cells (Krasnopolsky and Dudnichenko, 2017). The chemical structure of DXL is illustrated in figure 1.9.



Docetaxel

Figure 1.9 Chemical Structure Of Docetaxel(Alken and Kelly, 2013)

DXL's physicochemical features include white to off-white powder that is normally crystalline. It has an atomic weight of 807.89 Dalton and the chemical formula C43H53NO14. DXL has a melting point of 232° Celsius. The most significant physicochemical parameters to evaluate for every medicine, according to Lipinski's rule, are water solubility and membrane permeability. DXL has a log-P partition value of 4.1 and a pKa of 10.97, which results in low aqueous solubility (0.025 g/mL) and membrane permeability (1 cm/s 10^{-6}) (A. Razak *et al.*, 2021).

DXL is a taxoid antineoplastic medication discovered in the 1980s by Pierre Potier from the needles of the western yew tree, Taxus baccata. DXL is structurally similar to paclitaxel, except for the phenylpropionate and functional group on carbon 10. DXL is a powerful anti-mitotic chemotherapeutic medication that is used to treat a variety of human cancers. DXL's anticancer action is attributed to its high affinity for the tubulin protein binding site, which initiates tubulin assembly into microtubules. As a result, during the G2/M phase, microtubule depolymerization is inhibited, resulting

in cell cycle arrest and apoptosis. DXL is also anti-angiogenic and is regarded to be a more potent anti-microtubule agent than doxorubicin, paclitaxel, and fluorouracil , The mechanism of action of DXL is demonstrated in figure 1.10. Despite these advantages, DXL has some disadvantages, including limited water solubility, high systemic toxicity, and non-specific dispersion. Researchers have focused on designing nanocarriers for DXL delivery to address these difficulties. These methods appear to be a promising option to enhance water solubility and target anticancer drug delivery to the tumor site, hence reducing side effects. Several DXL nanocarriers have been developed using polymers, dendrimers, inorganic nanoparticles, solid lipid nanoparticles (SLNs), and liposomes(Sumera *et al.*, 2017).

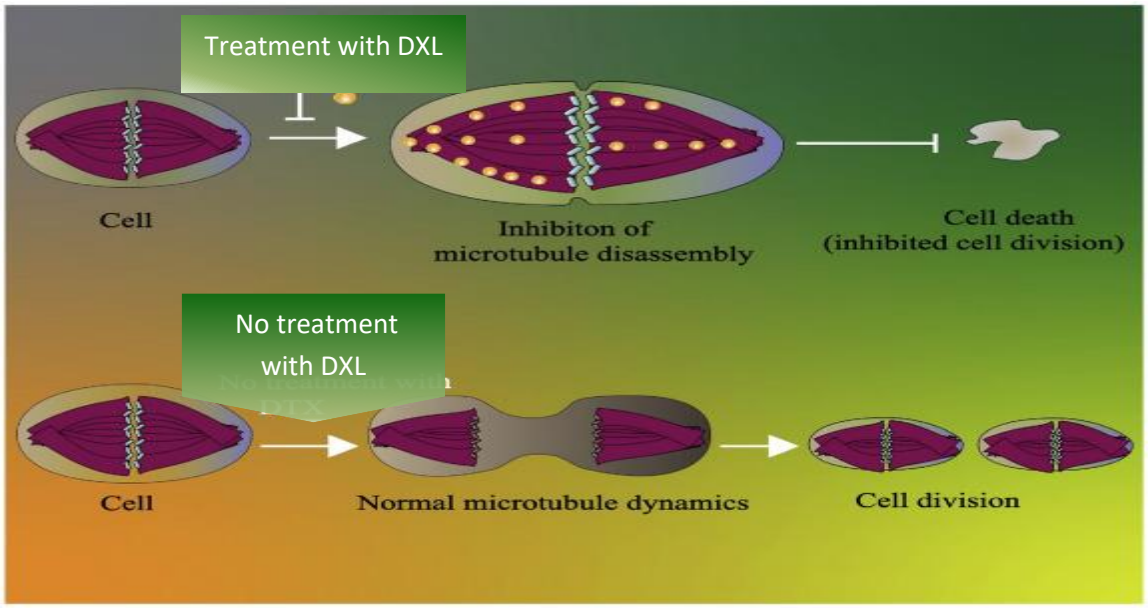


Figure 1.10. Mechanism of action of dxl in the treatment of cancer. DXL binds to the β-tubulins and promotes its assembly into microtubules, simultaneously inhibiting disassembly which leads to the stabilization of microtubules and results in inhibition of mitotic and interphase cellular functions, causing cell cycle arrest and ultimately leading to cell death(Imran *et al.*, 2020)**.**

DXL is a highly effective chemotherapy medication that is used to treat several types of cancer. Because of it is low water solubility, limited membrane permeability, and vulnerability to hepatic first-pass metabolism, the medication has a low oral bioavailability. DXL is provided intravenously to alleviate these issues. DXL is now supplied in a single vial containing polysorbate 80 and ethanol which solubilizes the poorly soluble medication. This preparation, on the other hand, causes short- and long-term side effects such as hypersensitivity, febrile neutropenia, tiredness, fluid retention, and peripheral neuropathy. DXL is also a substrate for the drug efflux pump P-glycoprotein (P-gp), which reduces it is concentration in the proximity of the cells and leads to drug resistance. As a result, the inclusion of DXL into various nanocarrier systems has received a lot of interest in recent years to overcome these shortcomings. The drug-delivery systems' surfaces can be functionalized by modifying them with different ligands for smart targeting of malignant cells(A. Razak *et al.*, 2021).

1.12.1 Docetaxel Resistance:

Known mechanisms of docetaxel resistance include limiting intracellular drug concentration, antagonizing the drug-stabilizing effect on microtubules, and antagonizing or circumventing the cytotoxic effect of taxanes through alternative growth pathways or apoptotic escape(Hwang, 2012).

1.12.2 DXL-Loaded NPs:

Recent research indicates an increase in the use of NPs, a branch of nanotechnology, for DXL delivery. This is mostly due to the ease with which NPS may be altered to improve medication delivery efficiency as well as DXL bioavailability. NPs are particles with diameters ranging from 1–1000 nm, and

the word is used to refer to a wide range of shapes and sizes of Nano vector structures. NPs can change the basic characteristics and the bioactivity of medications due to their high surface area to volume ratio. Because of their nanoscopic size, NPs can potentially aid in the intracellular uptake of a medicine. The ability y of NPs to encapsulate a drug can improve it is pharmacokinetic characteristics and biodistribution, reduce toxicity, and boost a drug's solubility and stability. It can also control the release of an anticancer medicine while delivering it to a specific spot. A few characteristics of NPs can be easily modified when building a drug delivery system: composition, size, shape, and surface qualities (A. Razak *et al.*, 2021).

1.12.3 Formulations For Taxotere:

To address the previously mentioned DXL solubility issue, the first commercialized DXL formulation, Taxotere, is created with 40 mg /mL of DXL polysorbate 80, and ethanol for intravenous delivery. Taxotere has been packed as a set of two vials since its debut on the market in 1996, with the first vial containing a concentrated formulation of DXL in polysorbate 80 and the second vial containing ethanol (95 percent v/v) as a diluent. Taxotere has launched in 2010 in a single vial with the same docetaxel to polysorbate 80 ratios as the two vials. Also, there were numerous generic DXL formulations approved by the FDA, including Docefrez® (SunPharma) and Docetaxel Accord (Accord Healthcare company), as well as many other generic DXL formulations, containing polysorbate 80.

Taxotere has been linked to several acute and chronic adverse effects, including hypersensitivity, febrile neutropenia, tiredness, fluid retention, and peripheral neuropathy. Polysorbate 80 has been linked to the incidence of

hypersensitivity reactions which can range from a simple skin rash to systemic anaphylaxis, necessitating corticosteroid premedication. (Dou *et al.*, 2014),and fluid retention, neutropenia, and baldness. Polysorbate 80 can also decrease taxanes binding to albumin, impairing albumin-based drug delivery. The formulation also results in decreased drug uptake by the tumor tissue and increased drug exposure in other bodily compartment which may lead to systemic toxicity and subsequent discontinuation of therapy (Naguib *et al.*, 2014). To address these concerns, an alternate drug delivery system (DDS) that does not contain polysorbate 80 was considered. A nanoparticle-based drug delivery method is one of the most preferred solutions(A. Razak *et al.*, 2021).

Chapter Two

Materials

and

Methods

2. Materials And Methods:

This chapter includes a detailed description of all materials and equipment used in the experimental work. The present work was performed in the Postgraduate lab /Department of Pharmacology at the College of the Medicine\ University of Babylon from October 2020 – to August 2021. A control viability study was investigated to determine the suitable enhancement concentration of liposomal docetaxel, pure docetaxel powder, and formulated docetaxel vials.

2.1 Materials:

2.1.1.Chemicals:

This study uses the following chemicals which are listed in(table 2-1) with their suppliers:

Table 2-1 Chemical used in the study:

Chemical	Company	Country
Acetonitrile	Biosolve	France
Alcohol spray (ethanol 70%)	AMEYA FZE	UAE
Chloroform	THOMAS BAKER	India
Cholesterol	Dyc	Korea
Dimethyl sulfoxide (DMSO)	Roth	Germany
Docetaxel powder	Sigma Aldrich	USA
Docetaxel vial	Sanofi Aventis	france
Fetal bovine serum (FBS)	Gibco	UK

Gentamycin (80 mg ampule)	Menarini	Italy
Lecithin	Sigma Aldrich	USA
Liquid nitrogen	Cleaver	USA
Methanol	THOMAS BAKER	India
MTT dye powder	Roth	Germany
Penicillin- streptomycin solution	Capricorn	Germany
Phosphate-buffered saline (PBS) tablet	Himedia	India
Roswell Park Memorial Institute-1640 (RPMI-1640) powder medium	Gibco	UK
Trypsin- Ethylenediaminetetraacetic acid (EDTA) powder	US Biological	USA

2.1.2.Instruments And Tools:

Table 2.2 Include The Instruments And Tools Used In The Study:

instrument or tool	**Company**	**Country**
Autoclave	Jeiotech	Korea
Automatic micropipettes (different sizes)	Human	Germany
Cell culture flask (25ml)	SPL	Korea
Cell culture flask (25ml)	SPL	Korea
Cell culture plate (96- wells)	SPL	Korea

Cell culture plate (96- wells)	SPL	Korea
Centrifuge	Rotanta	Germany
Deionized distilled water	Promega	USA
Distiller	ROWA	Germany
Electric oven	Memmert	Germany
Incubator	Memmert	Germany
Inverted microscope	T.C Meiji techno	Japan
Laminar airflow cabinet	Labtech	Korea
Lyophilizer	LTE SCIENTIFIC LTD	Great Britain
Magnetic stirrer	Labinco	Netherland
Magnetic stirrer	Scotech	Germany
Microcentrifuge	Memmert	Germany
Millipore filter (0.45, 0.22µm)	Biofil	Australia
pH Meter	WTW	Germany
Probe sonicator	Qsonica	U.S.A
Refrigerator	Arcelik	Turkey
Rotary evaporator	Heidolph	Germany
Sensitive Balance	Labtech	Korea
UV-Spectrophotometer	Optima	Japan
Vortex	Kottermann	Germany
Water bath	Memmert	Germany

2.2 Methods:

Part 1: Preparation And Characterization Of Liposomal Docetaxel:

2.2.1Calibration Curve Of Docetaxel:

A-Preparation of docetaxel Stock Solution

The Primary stock solution of docetaxel in acetonitrile (250µg/ml) was prepared by dissolving 2500 µg of docetaxel in 10ml acetonitrile. The primary stock solution was stored at 2-8ºC (Sheetal, 2013).

B-Determination λmax :

The absorption maximum (λmax) of docetaxel was determined by scanning 10 µg/ml solution against Acetonitrile as a reagent blank in spectrum mode between 200 to 400nm. Docetaxel in acetonitrile exhibits a sharp peak at 229 nm when scanned in the UV region between 200-400 nm and hence it was selected as the analytical wavelength (Sheetal, 2013).

C. Preparation of Calibration Curve of docetaxel in Acetonitrile:

The standard curve was created from DXL standards with known concentrations in the range of 5-30µg/mL by using a spectrophotometer at wavelength 229 nm (Sheetal, 2013). The calibration standard curve showed linearity in the range of 5-25µg/mL, with an R2=0.9969 (Figure 2.1). The amount of DXL in the liposomal formulations was subsequently quantified based on the standard curve.

Table 2.3 Data Of Drug Concentrations With Their Absorbance At 229 Wavelength.

CONCENTRATION µg/ml	ABSORBANCE
0	0
5	0.219
10	0.527
15	0.766
20	0.986
25	1.312

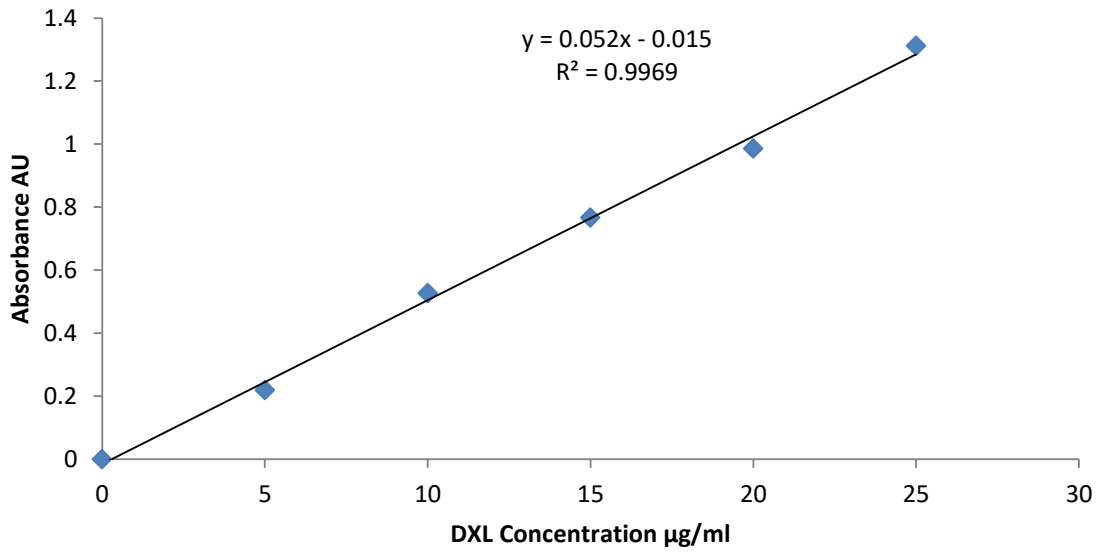


Figure 2.1: The Docetaxel Standard Curve Obtained Using Spectrophotometer At 229 Wavelength.

2.2.2 Cholesterol Stock Solution Preparation:

To prepare a stock solution of cholesterol weighed 300 mg of cholesterol and dissolved it in a mixture containing 20 ml chloroform and 10ml of methanol to obtain 10 mg of cholesterol in every 1 ml.

2.2.3 Lecithin Stock Solution Preparation :

The same method above was used to prepare a stock solution of lecithin also weighed 300 mg of lecithin and dissolved in a mixture containing 20 ml chloroform and 10ml of methanol to obtain 10 mg of lecithin in every 1 ml.

2.2.4 Preparation Of Docetaxel-Loaded Liposomes:

A-preparation of liposomes:

1. Rinsing a glass round-bottom flask with 5 mL of chloroform and using it right away (fig.2.2 A).

2. To make 2 mL of 10 mg/mL Lecithin/cholesterol liposomes (1:1, molar ratio), mix 670 µL of 10 mg/mL lecithin stock solution and 1330 µL of 10 mg/mL cholesterol stock solution,1mg of pure docetaxel powder and mix briefly. To speed up the evaporation process, place the flasks in a warm water bath (typically 40–45 °C) and the organic solvent was removed by using a rotary evaporator at 40 Cº for 15 minutes (fig 2.2 B).

3. Place the flask in a vacuum desiccator overnight to eliminate any remaining organic solvent.

4. To prevent dust or pollutants from entering the flask, cover the hole with a piece of stretched parafilm and punch a few small holes in it with a needle(Zhang, 2017).

B-Hydration of the Thin Film and drug encapsulation:

1. To the dry lipid film (fig.3.2 C), add 2 mL of phosphate buffer saline (fig.2.2 D).

2. Vortex the solution three times for 10 seconds at the maximum speed to suspend the lipid materials in the solution.

3. To help the lipids suspend in the solution and not stick to the flask's edge, sonicate them for 15 seconds in a water bath sonicator. Allow the suspension to stand at 4 °C overnight to efficiently hydrate all the lipid materials.

C-Extrusion:

1. To eliminate all visible precipitates, sonicate the liposome preparation in a water bath for 10–20 minutes. The obtained milky crude liposome sample contains mostly MLVs. With more sonication time, the particle size will decrease.

2. Then use a microfilter with sizes 0.45 and 0.22 nm.

3. Pass the liposome suspension through the polycarbonate membranes five times to extrude the liposome preparation. The sample will have a semi-translucent appearance.

4. The sample will appear more translucent after extrusion through 100 nm polycarbonate membranes. Transfer the sample from the syringe to a storage vial. Store the liposome sample at 4 degrees Celsius (Zhang, 2017).

5. To separate suspended liposomal particles which contain the drug, then used a centrifuge at 5Cº for 20 minutes and 12000 rpm to get rid of the untrapped DXL prodrug before any assay (fig. 2.2 E) (Ren *et al.*, 2016)

6. Then use a lyophilizer for tonight to remove any remaining liquid (fig. 2.2 F).

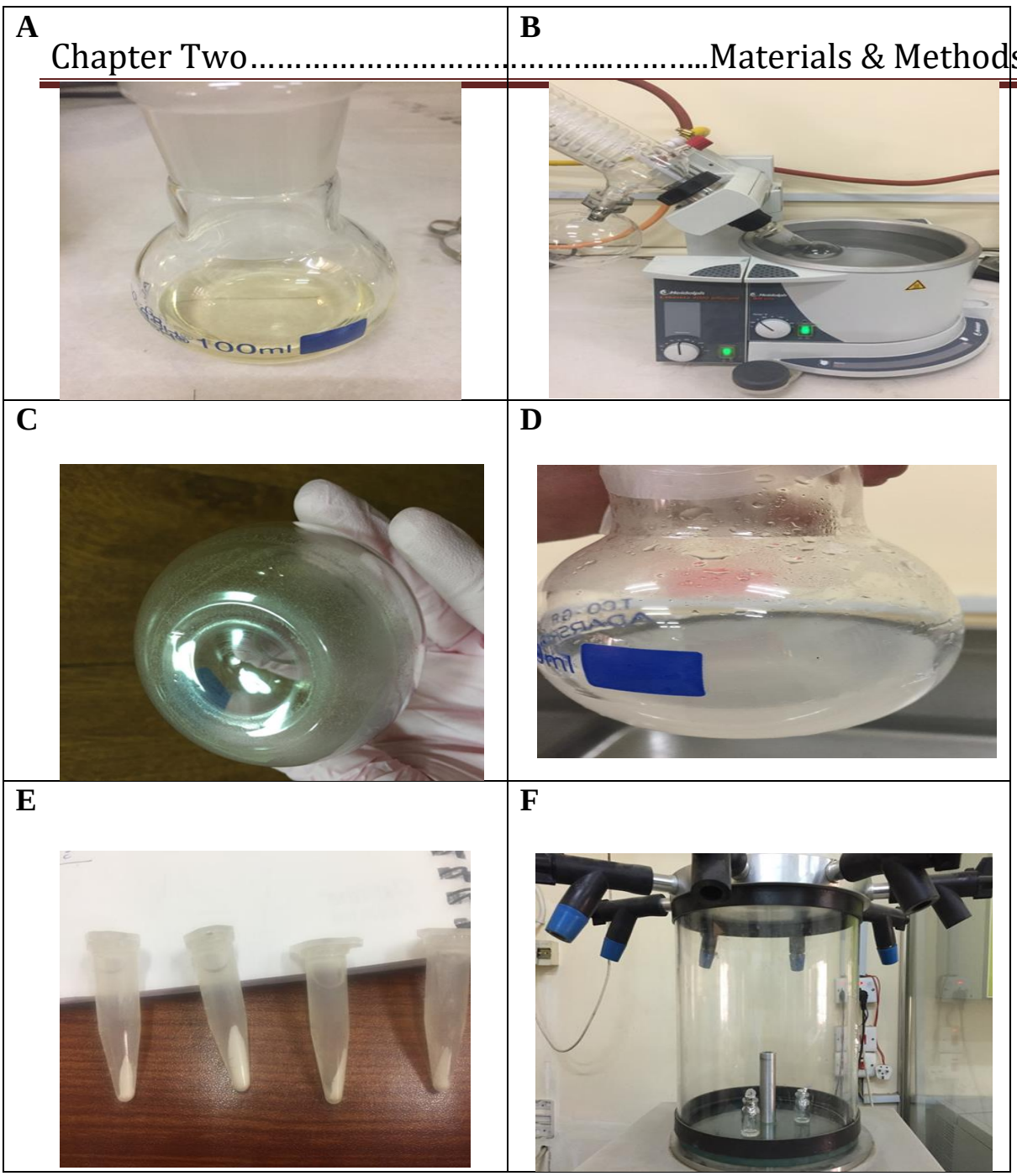


Figure 2.2: Steps Of Preparation Of Liposomal Docetaxel

2.2.5 Physicochemical Characterization Of DXL-Loaded Nanoliposomes:

2.2.5.1Encapsulation Efficiency Of Docetaxel Nanoformulations :

The percentage of drug encapsulated in liposome nanoformulations was determined by separating the unentrapped drug from nanoformulations by centrifugation at 10,000 rpm for 30 min using a cooling centrifuge (Rotana, Germany).

2.2.5.2 Size Characterizations And Morphology:

The Size of The particle is one of the most basic and important measurements for nanoparticle characterization. It determines the size and distribution of the particle and whether it falls under the nano or micro scale. The particle size and distribution are most commonly measured using the electron microscope. The images of the Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) are used for the measurement of particles and clusters.

Scanning electron microscopy was used to verify the uniformity of particle shape and size. The lyophilized nanoparticles were dropped onto double-sided carbon tape. They were then vacuum-coated for 45 seconds with a platinum mixture and examined for morphology with an SEM (JEOL JSM7500, Thermo Scientific) at 5 kV. (Keum *et al.*, 2011).

Atomic force microscopy(AFM)analysis was performed by placing a drop of NPs on the silicon mica and then allowed to dry for ~30 min which was also used for detection of morphology(Kushwah *et al.*, 2018).

2.2.5.3 Fourier Transform Infrared Spectroscopy (FTIR) :

FTIR Spectroscopy was used to determine any possible interactions and the physical mixtures of the drug and the liposome. Briefly, the samples were dried in a hot air oven at50°C for 2 h. The samples were compressed under a pressure of 10 t/nm2. The samples were scanned in the range of 400 to 4000 cm^{-1}, and the procedure was done according to(Rarokar *et al.*, 2016).

2.2.5.4 Zeta Potential :

The Zeta potential of the prepared DXL nanosuspensions was measured by a zeta sizer (Nano ZS, Malvern Instruments, UK). All measurements were carried out at room temperature(25C^{o}).

Part 2:

2.3 Cytotoxicity assay:

2.3.1 Preparation of Reagents and Solutions :

2.3.1.1 Phosphate Buffer Saline (PBS):

According to the Gibco manufacturer manual, the PBS was prepared by dissolving only one tablet(PBS pH: 7.4) which contain (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.47 mM KH2 PO4) in 500 ml of deionized distilled water (DDW) with continuous stirring by a magnetic stirrer at room temperature resulting in a pH value of 7.45 without need for adjustment. Autoclaving is required for complete sterilization and stored in a closed bottle until used to keep sterile.

2.3.1.2Gentamycin Stock Solution:

A gentamycin ampoule of 40 mg/ ml solution was considered as a stock solution and stored at a temperature of 4 C˚ until use. The final concentration of gentamycin in the medium was 50 µg/ml (Freshney, 2010).

2.3.1.3 DXL Stock Solution For Cell Line :

From this different form of docetaxel (liposomal docetaxel, pure docetaxel powder ,formulated docetaxel vial) we take amount equal to 1mg of docetaxel concentration and diluted with serum-free media ,then make serial dilution with different concentration (500,250,125,62.5,31.25,15.61)µg/ml.

2.3.1.4 Trypsin-(EDTA) Solution:

As indicated by US Biological headings, a weight of 10.1 gm of trypsin-EDTA powder dissolves in 0.9 Liter of double distilled water (DDW) with continuous mixing at room temperature. 7.2 of PH value should be reached and complete the volume to 1 Liter by DDW, the solution was sterilized by using Millipore filters of 0.45 and 0.22 µm respectively, after that, the solution was kept at (- 20C˚) temperature.

2.4 Preparation of Tissue Culture Medium:

2.4.1 Preparation of Serum-Free Medium:

Liquid Roswell Park Memorial Institute (RPMI-1640) medium:

Liquid RPMI-1640 medium was prepared from powdered RPMI-1640 medium according to the Gibco product manual as the following:

From the RPMI-1640 powdered medium, 10.43 g was dissolved in approximately 900 ml of DDW in a volumetric flask. The other components

include: 2 g sodium bicarbonate powder or according to need and 1.25 ml from gentamycin stock solution had been added with continuous stirring. The volume was completed by DDW to one liter and the pH of the medium was adjusted to 7.4. Sterilization was done by 0.4 and 0.2 μm millipore filters subsequently. After the end of the procedure, 5 ml of the medium was incubated at 37 ºC in a sterile flask for 4 days with daily examination for signs of bacterial and fungal contamination. It was considered sterile only in case of no signs of contamination during the four days of incubation. Then the medium was stored at 4ºC until use.

2.4.2Preparation of Serum-Medium :

Medium with serum was prepared as described in the preparation of serum-free medium in (2.3.1) in addition to 10 percent of fetal bovine serum.

2.4.3Preparation of Freezing Medium:

The freezing medium was prepared from the following compositions: 6 ml serum-free medium, 3 ml FBS, and 1 ml DMSO. The solution was stored at (-20) C˚ temperature between uses (Meleady and O'Connor, 2006).

2.5 MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)Assay:

2.5.1 MTT Principle:

The general use of the MTT assay is to quantify viable cells in moderately high throughput (96-well plates) without the requirement for elaborate cell counting. Therefore the most widely recognized use is to evaluate the cytotoxicity of several drugs at various concentrations. The principle of the

MTT assay is that the mitochondrial activity of viable cells is consistent and thereby any change in the number of viable cells is directly proportional to the mitochondrial activity. The mitochondrial activity of the cells is reflected by the transformation of the pale yellow colored tetrazolium salt (MTT dye) into other dark purple formazan crystals by NADH (Figure 2.3) which can be solubilized for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected by optical density (absorbance) using a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells (Meerloo, Kaspers, and Cloos, 2011)

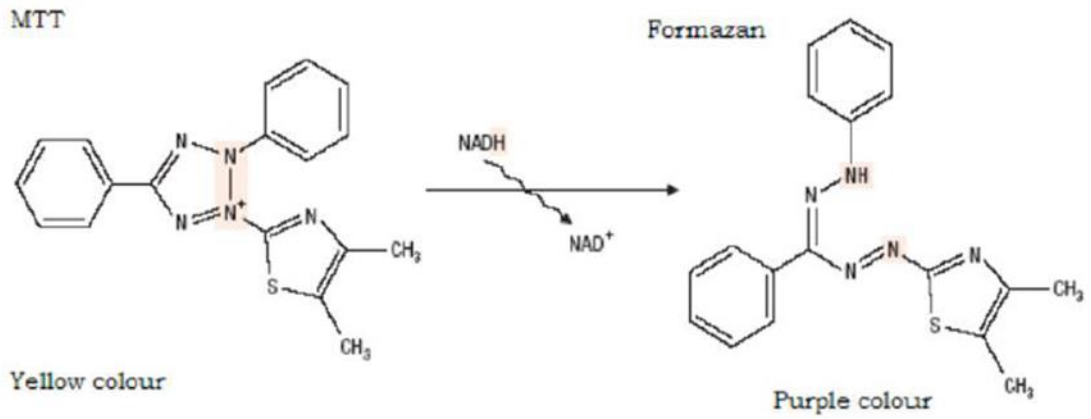


Figure 2. 3 Principle Of MTT Assay (Sukhramani et al., 2016).

2.5.2 MTT Solution:

MTT powder (0.5 gm) was dissolved in PBS (100 ml) to achieve 5 mg/ml concentration. A 0.2 µm millipore filter was utilized to sterilize the MTT solution and stored in a sterile and light-protected bottle. The solution was stored at 4°C of temperature for multiple uses or -20°C of temperature for long storage (Meerloo, Kaspers, and Cloos, 2011).

2.5.3 Procedure (Meerloo, Kaspers and Cloos, 2011)**:**

1. The media was withdrawn from the wells after the extract's exposure period, and the cells were rinsed with PBS. To assess unspecific formazan transformation, a blank control was performed.

2. To produce the final concentration of 0.5 mg/ml, 1.2 ml of MTT solution (5 mg/ml) was added to a 10.8 ml medium. The final solution is added to every well in the amount of 200 ml.

3. The 96-well plate was incubated at 37°C for 3 hours until intracellular purple formazan crystals could be seen under an inverted microscope.

4. The supernatant was collected, and 100 ml DMSO was added to each well to dissolve the formazan crystals that had formed.

5. The 96-well plates were then incubated at room temperature for 30 minutes, or until the cells lysed and the purple crystals disintegrated.

6. The absorbance was measured using a microplate reader set to 570 nm.

The blank and control readings of absorbance must be subtracted from each sample. Absorbance readings from samples should then be divided by those of the control and multiplied by 100 to get the percentage of cell proliferation or viability. Greater absorbance values compared to the control demonstrate cell proliferation, while lower values propose cell death or inhibition of proliferation. The percentage of cell viability or percentage of inhibition was calculated by the following formula:

$$\% \text{ viability} = (AT - AB) / (AC - AB) \times 100\%$$

Where, AT = Absorbance of treated cells (drug).

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.6Preparation Of Trypan Blue Solution :

It is the test used to determination of the number of viable cells present in the suspension. This test is based on the principle in which the live cells are composed of intact cell membranes that normally exclude certain dyes, such as trypan blue dye, whereas dead cells cannot exclude the dye. In this test, a cell suspension is mixed with trypan blue dye and then visually examined to determine whether cells take up or exclude dye. a viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm (Strober, 2015).

Cells mixed with trypan blue solution in a 1:1 ratio. Inspect and count dead and alive cells under a light microscope in a hemocytometer. Dead cells will stain blue due to trypan blue uptake, while live cells appear transparent. Cells should not be exposed to a trypan blue solution for more than 20 min. Prolonged incubation will increase cell death and reduce viability(Verhoeckx *et al.*, 2015).

Calculation of viability percentage can be done using the formula:

V = N / T x 100%, where V is the percentage viability, N is the number of viable cells counted per 10 sub-grids, and T is the number of total cells counted per10 sub grids (1 mm^3).

2.7 Cell lines:

Frozen vials of human prostate cancer LNCaP cell lines which are exhibiting epithelial morphology that was isolated from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old, White, male (blood type B+) with a confirmed diagnosis of metastatic prostate carcinoma. These cells were received from the cell culture laboratory in the College of medicine//university of Babylon.

2.7.1 Thawing of LNCaP Prostate Cancer Cell Lines:

With caution, the frozen cell line vial was withdrawn from the liquid nitrogen box and placed straight into a glass beaker pre-warmed (37°C) sterile DDW. The vial was taken from the water before the ice floccule completely disintegrated, and it was then cleaned with 70% ethanol. Without delay, the cell suspension substance of the vial was pipetted into a 15 ml sterilized plastic centrifuge tube containing 10 ml of pre-warmed serum-free media using a laminar flow cabinet. After 5 minutes of centrifugation at 1000 rpm, the supernatant was drained and eluted. The cells pellet were resuspended in 5ml of warm (37C) serum medium and transferred to a 25 ml culture plate, incubated at 37C, and the serum medium was changed the next day (Vanella *et al.*, 2013).

2.7.2harvesting And Sub-Culturing Of Prostate LNCaP Cell Lines:

Harvesting is a procedure that uses proteolytic enzymes such as trypsin to detach and disaggregate the monolayer of adherent cells from the bottom of the flask of culturing. It was performed at whatever point the cells should be collected to be harvested for cell counting and sub-culturing of the cell line. This methodology was done according to the following:

1. The inverted microscope was utilized to analyze whether the cells are healthy and sub-confluent without contamination.

2. The spent medium had been emptied by a pipette and added sufficient amount of pre-warmed trypsin-i(EDTA) solution was washed in the monolayer to guarantee the removal of all medium from the cell culture flask. This step could be repeated if the cells were still adherent strongly.

3. The convenient volume of trypsin-i (EDTA) solution was included in the washed cell monolayer utilizing (1-2 ml) per i25-cm2 flask. Flask was rotated to completely cover the monolayer with trypsin.

4. To detach the cells from the surface of the flask, the flask was returned to the incubator at 37°C. Usually, the cell's detaching period depends on a cell line which could take 2 to 10 minutes.

5. An inverted microscope could be used to evaluate the cells and whether they are detached and floating or not. The flask might be tapped gently on its side to detach any remaining cells.

6. An equal volume of serum-containing medium was added to deactivate trypsin in the flask.

7. If there are enough cells in the flask, an aliquot of cells could be transferred to another flask labeled with the cell line name with a pre-warmed serum-containing medium (5–7ml for a 25-cm2 flask).

8. The flask was incubated at 37C° temperature.

9. This process has been repeated according to the characteristics of the growth for each cell line.

2.7.3 Freezing of Prostate LNCaP cell lines:

The cell lines source were kept frozen at (-196) ºC in a nitrogen tank according to the following protocol:

1. Cultures were checked to utilize an inverted microscope to evaluate the degree of cellular development and to guarantee that the cells are free of contaminations. Adherent cells are gathered (as mentioned above) for cryopreservation in the exponential phase of growth.

2. one ml of the freezing medium was included and then the content of the flask was moved into a 1.5 ml sterile freezing vial. All freezing vials were marked with the cell line name, date of freezing, and passage number.

3. These vials were put in the vapor phase of liquid nitrogen, which is equivalent to a temperature of -80°C for at least three hours (or overnight).

4. The vials were expelled from the vapor phase of the liquid nitrogen and moved to the liquid phase for storage (-196°C).

2.7.4 Cell lines groups:

LNCaP cell line was treated with a different form of docetaxel for different incubation periods, which include:

1- Liposomal docetaxel.

2- Formulated Docetaxel vial

3- Pure Docetaxel powder.

2.8Experimental Design:

According to (Freshney, 2010), cytotoxicity assays have been applied for the determination of the effect of different docetaxel forms on Prostate LNCaP cell lines. Different concentrations of docetaxel were tested for defined time durations. The cell monolayers were harvested and re-suspended in a serum-free media at a concentration of $5*10^5$ cells/ml and plated in a 96-well cell culture plate after the growth in the flask reached the exponential phase. The calculation of the cell concentration, taking the average number of viable cells in the four sets of 16 squares and multiply by 10,000 to get the number of cells per milliliter. Then, multiply this by five to correct for the one in five dilution from the trypan blue addition. When cell development reached 80%, the well was treated with repeated dilutions of each variety of docetaxel, as shown in the following trials.

2.8.1Experiment No.1:

The effect of liposomal docetaxel on LNCaP cell lines at 24,48 and 72 hours incubation time.

LNCaP cell lines were planted in four columns of six repetitions of 96-well plates at the concentration of $5*10^5$ cells. Column No. 1 was used as a control group, with six replicates, and the three consecutive columns are exposed to L.DXL in serial dilutions of (500, 250, 125, 62.5, 31.25, 15.62 g/ml), with three duplicates for each concentration. The plate was then sealed with a self-plastic lid and incubated for 24 hours, 48 hours, and 72 hours. The wells were rinsed with 200 µl of phosphate Buffer saline (PBS after the exposure. The MTT test was used to determine the effect of L.DXL on the growth of the LNCaP cell line.

2.8.2Experiment No.2:

The effect of pure docetaxel powder on LNCap cell lines at 24,48 and 72hours incubation time:

Four columns of six replicates of 96-well plates were seeded with **LNCap** cell lines in a concentration of $5*10^5$ cells. Column number .1 with six replicates was considered as a control group, and the three subsequent columns were exposed to docetaxel powder in serial dilutions of (500, 250, 125, 62.5, 31.25, and 15.62 µg/ ml), with three replicates for each concentration. Then the plate was covered with a self-plastic lid and incubated once for 24 hours and the other for 48 96hours. Afterward the end of the exposure, the wells were washed with 200 µl of sterile PBS. The effect of pure DXL on the growth of the LNCap cell line was assessed by MTT assay.

2.8.3Experiment No.3:

The effect of formulated docetaxel on LNCaP cell lines at 24,48 and 72 hours incubation time:

LNCaP cell lines were planted in four columns of six repetitions of 96-well plates at a concentration of $5*10^5$ cells. Column No. 1 was used as a control group, with six replicates, and the three consecutive columns were subjected to formulated docetaxel in serial dilutions of (500, 250, 125, 62.5, 31.25, 15.62 g/ml), with three replicates for each concentration. The plate was then sealed with a self-plastic cover and incubated for 24 hours,48 hours, and 96 hours. The wells were rinsed with 200 µl of sterile PBS after the exposure. The MTT assay was used to evaluate the effect of formulated docetaxel on LNCaP cell line proliferation.

2.9 The Half Maximal Inhibitory Concentration (IC50) Calculation:

The half maximal inhibitory concentration (IC50) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function. The simplest estimate of IC50 is to plot x-y and fit the data with a straight line (linear regression). The IC50 value is then estimated using the fitted line, according to the following equation:

$Y = a * X + b,$

$IC50 = (50 - b)/a.$

2.10 Statistical analysis

All data were collected and analyzed by Microsoft Office Excel 2010 and Sigmaplot version 13 software. The differences between the means of the different group concentrations were analyzed using a T-test. And one-way ANOVA. P-values ≤ 0.001 and <0.050 were considered to be statistically significant.

Chapter Three

Results

3.1 Characterization Techniques:

3.1.1 Scanning Electron Microscopy (SEM):

Morphology, shape, and particle size have been characterized by using a Scanning electron microscope (SEM). The size of liposomes in the current study was approximately 28nm(fig 3.1) and liposomal docetaxel was approximately 20 nm(fig.3.2).

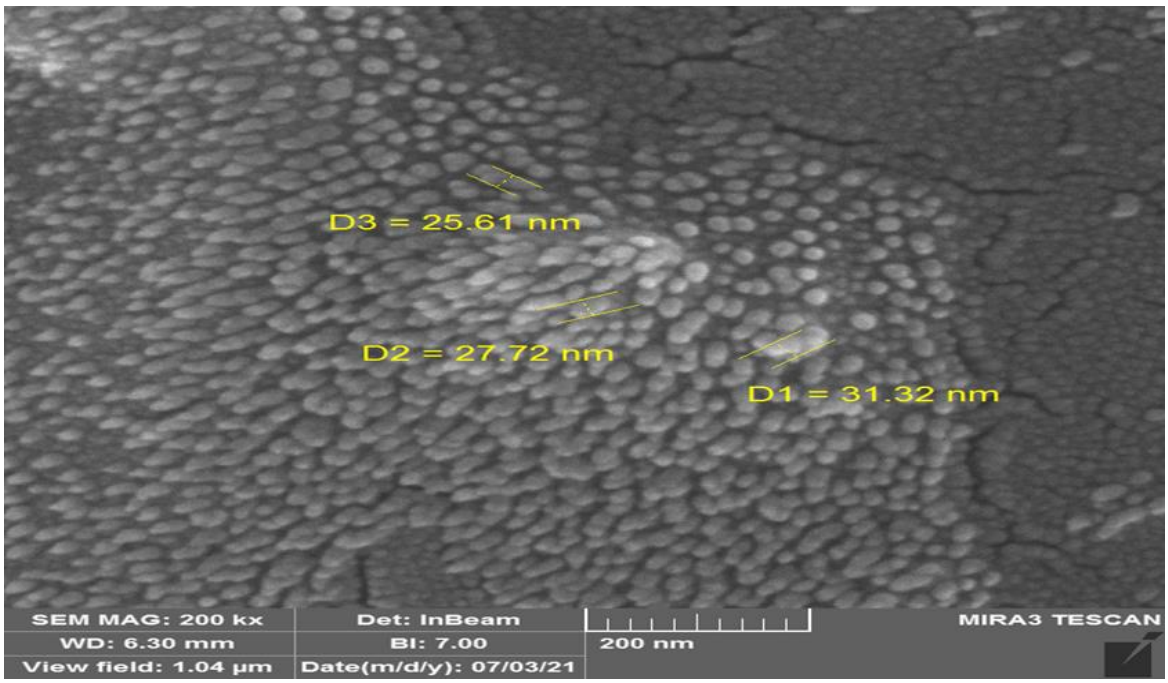


Figure3.1: Microphotograph SEM Of Liposome

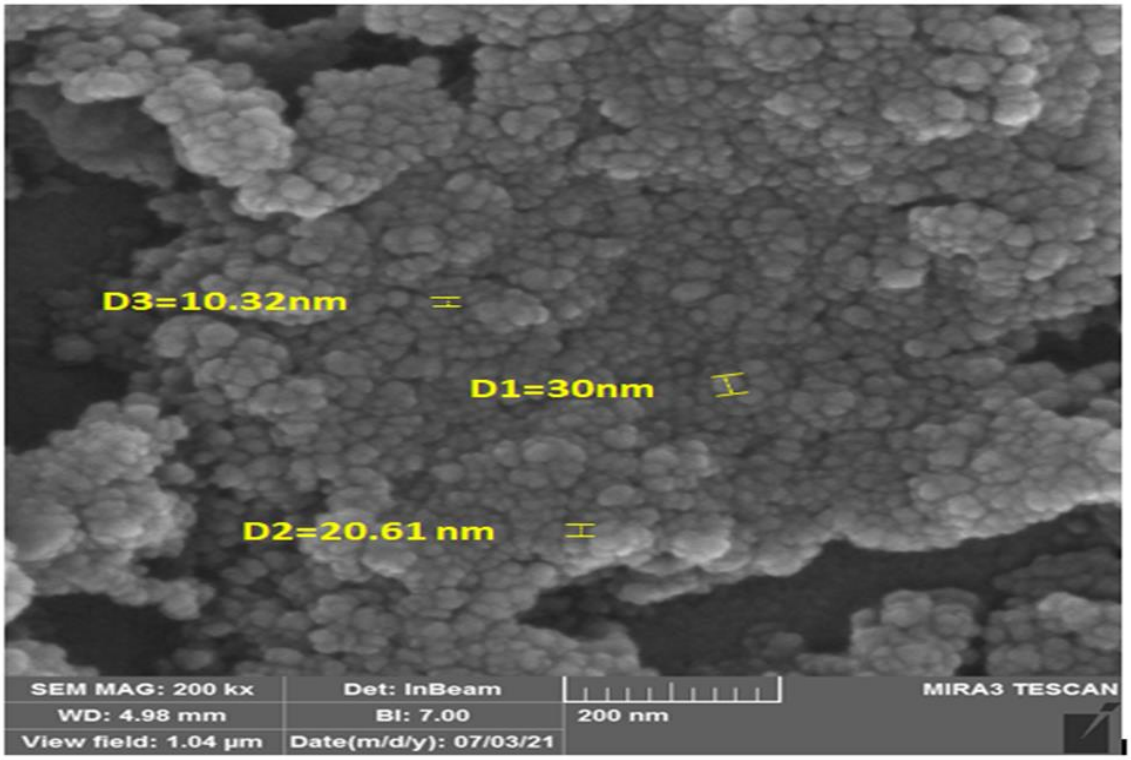
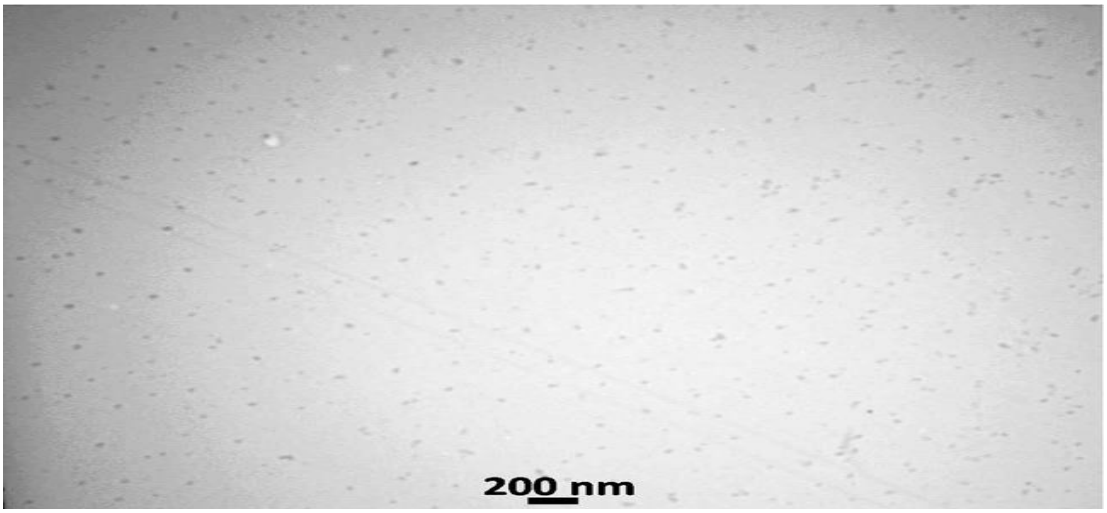


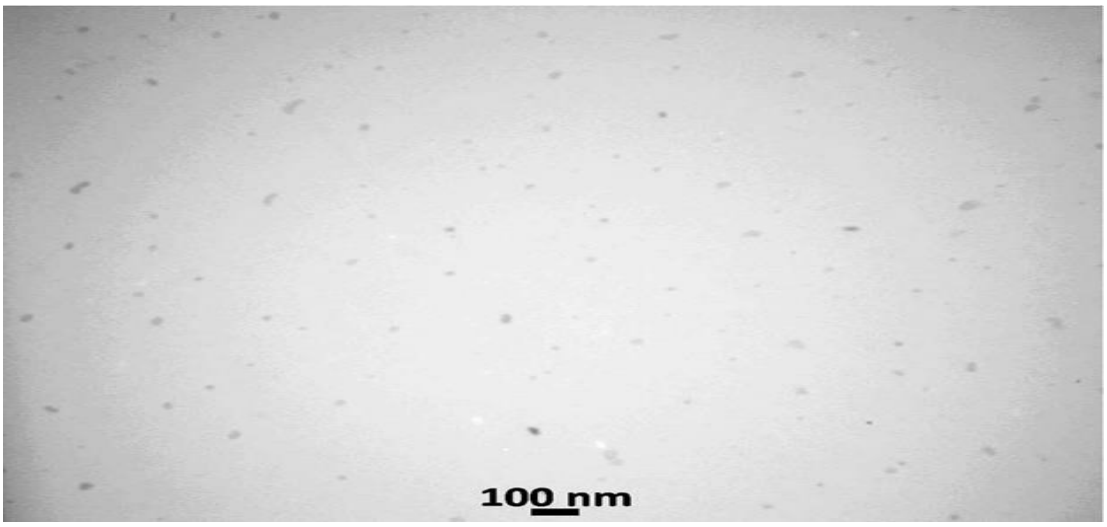
Figure 3.2: Microphotograph SEM Of Liposomal Docetaxel

3.1.2 Transmission Electron Microscopy (TEM):

The TEM image shows a monodisperse and spherical shape, for the nanoliposomes prepared by a Thin-Film Hydration (TFH) method. Figure3-3(A and B) represent the TEM of liposomes at 200 and 100 nm. Figure 3.4 for liposomes which represent the relative frequency, the size obtained by this method is equal to 20 nm. For liposomal docetaxel figure, 3.5(A and B)illustrate TEM images at 200 and 100nm, figure 3.6 act the relative frequency and the size which obtain by this method equal to 14 nm.



(A)



(B)

Figure 3.3: TEM Image Of Liposome, (A) At 200nm,(B) At 100nm.

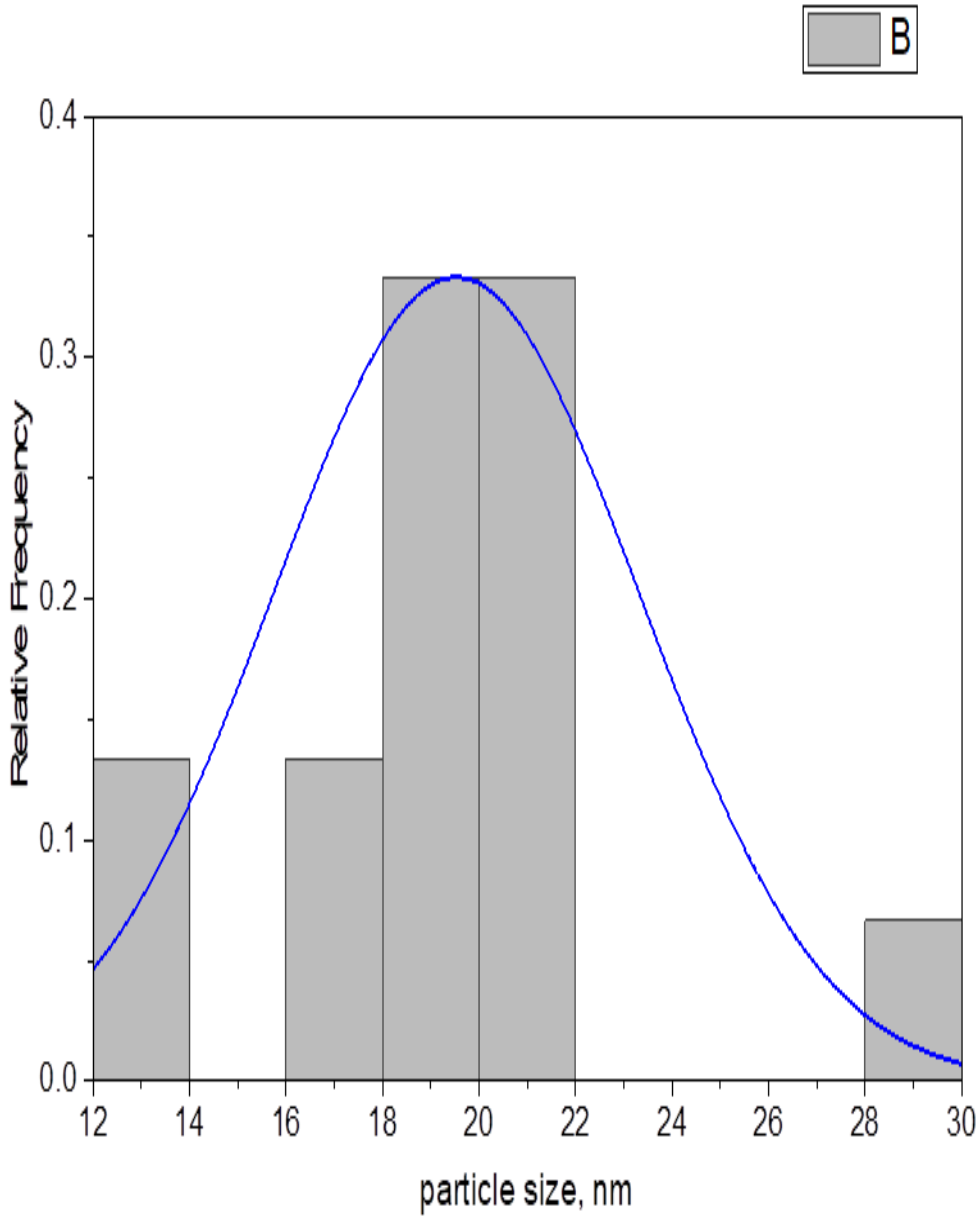
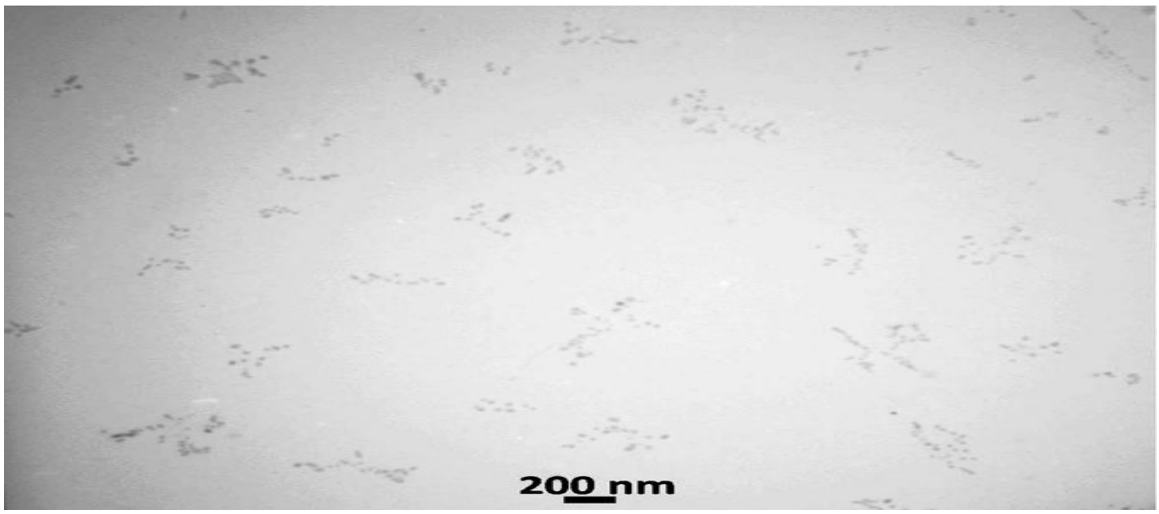
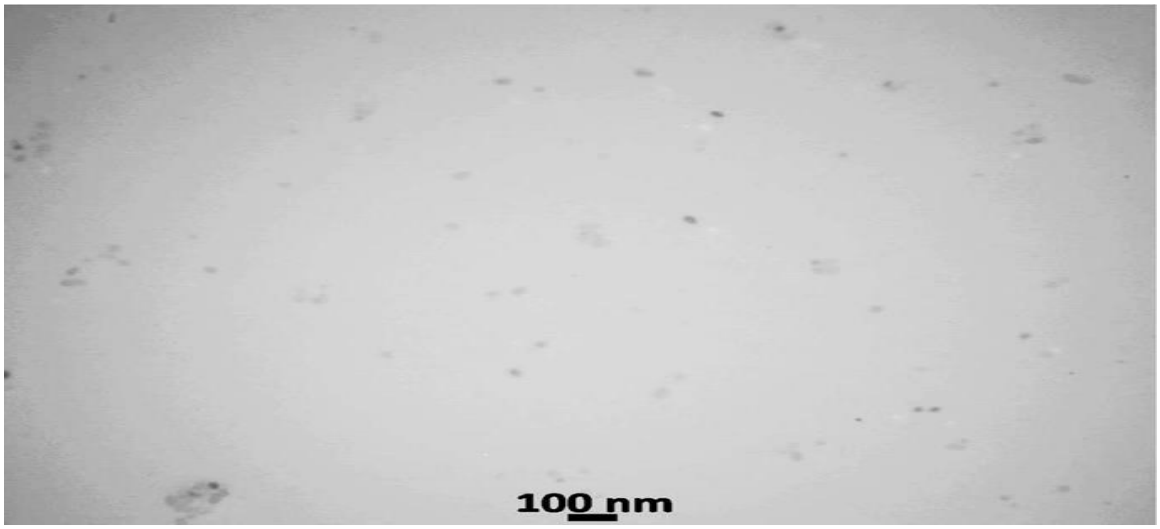


Figure 3.4: Relative Frequency With The Size Distribution Of Liposome



(A)



(B)

Figure 3.5: TEM Image Of Liposomal DXL, (A) At 200nm,(B) At 100nm.

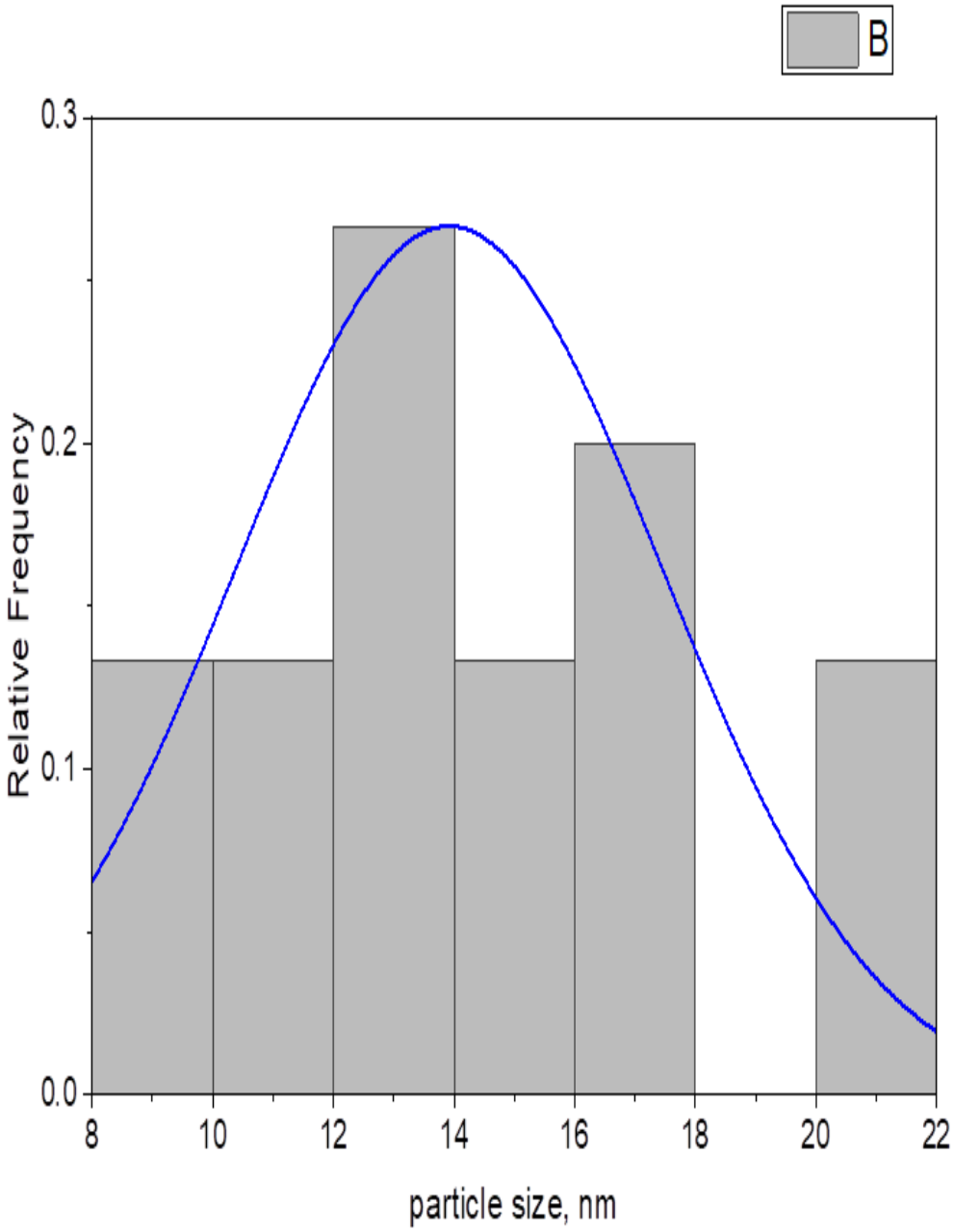
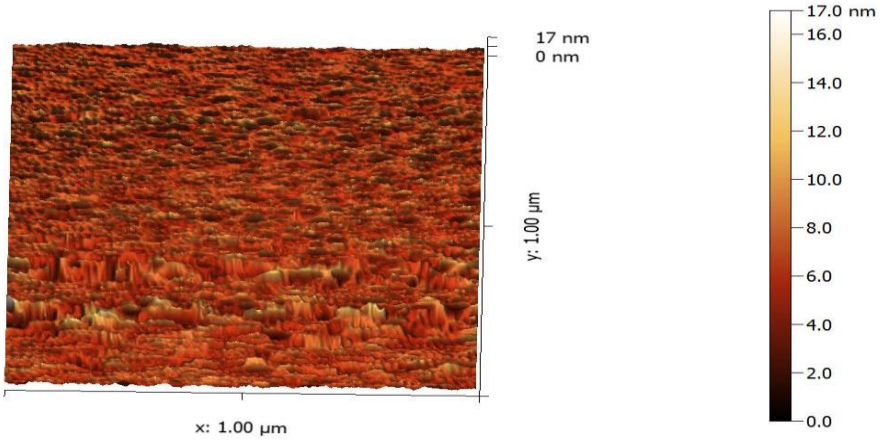


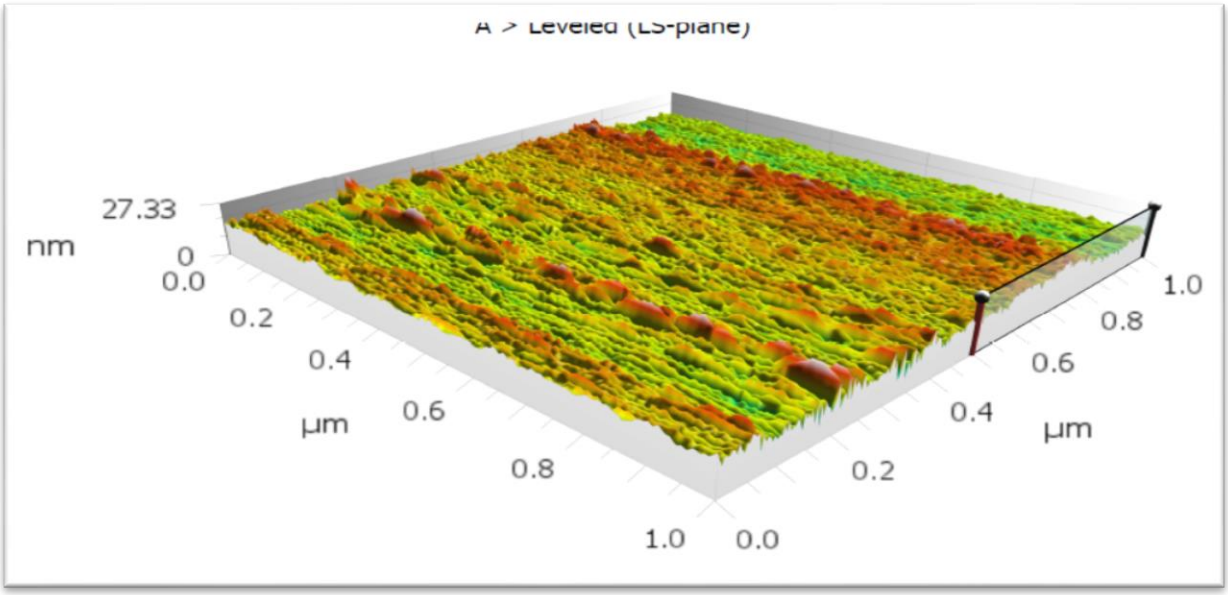
Figure 3.6: Relative Frequency With The Size Distribution Of Liposomal DXL.

3.1.3 Atomic Force Microscopy :

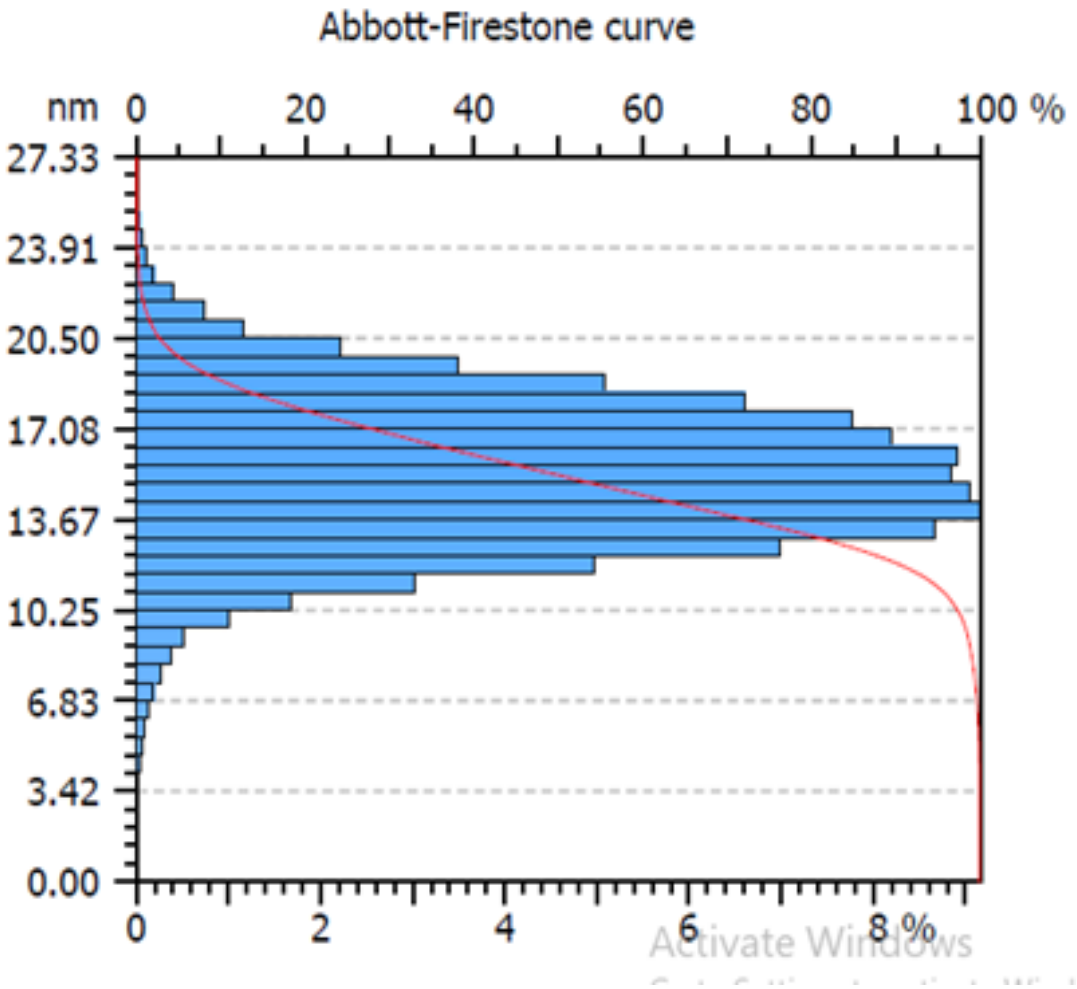
According to AFM images, the NPs were almost spherical (Figures 3.7 A and B) with a diameter of about 17 nm (Figure 3.7C). The measured size of NPs by the probe-based method.



(A)



(B)



(C)

Figure 3.7 : (A) AFM image of L.DXL . (B) Three-dimensional view of a DXL-loaded NP. (C) Particle size distribution of NPs according to AFM image.

3.1.4 Zeta Potential:

The Zeta potential of the liposome and encapsulated docetaxel was shown in (table 3.2 and figure 3.8)for liposome and (table 3.3 and figure 3.9) for liposomal docetaxel:

Table 3.2:Calculation Result Of Zeta Measuring Of Liposome

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-23.0 mV	-0.000179 cm^2/Vs
2	……..mV	……cm^2/Vs
3	…….mV	……cm^2/Vs

Zeta Potential (Mean) : -23.0 mV

Electrophoretic Mobility Mean : -0.000179 cm^2/Vs

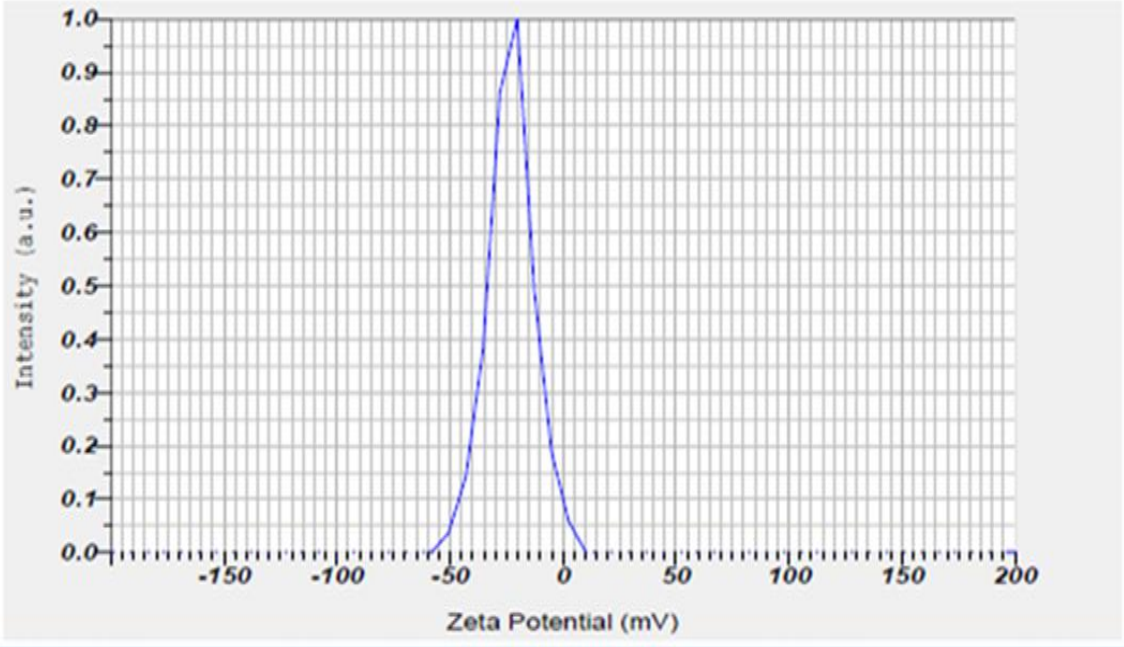


Figure3.8:Zeta Potential Of Liposome

Table 3.3: Calculation Result Of Zeta Measuring Of Liposomal .DXL

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-28.3mV	-0.000220 cm^2/Vs
2	……..mV	……cm^2/Vs
3	…….mV	……cm^2/Vs

Zeta Potential (Mean) : -28.3mV

Electrophoretic Mobility Mean : -0.000220 cm^2/Vs

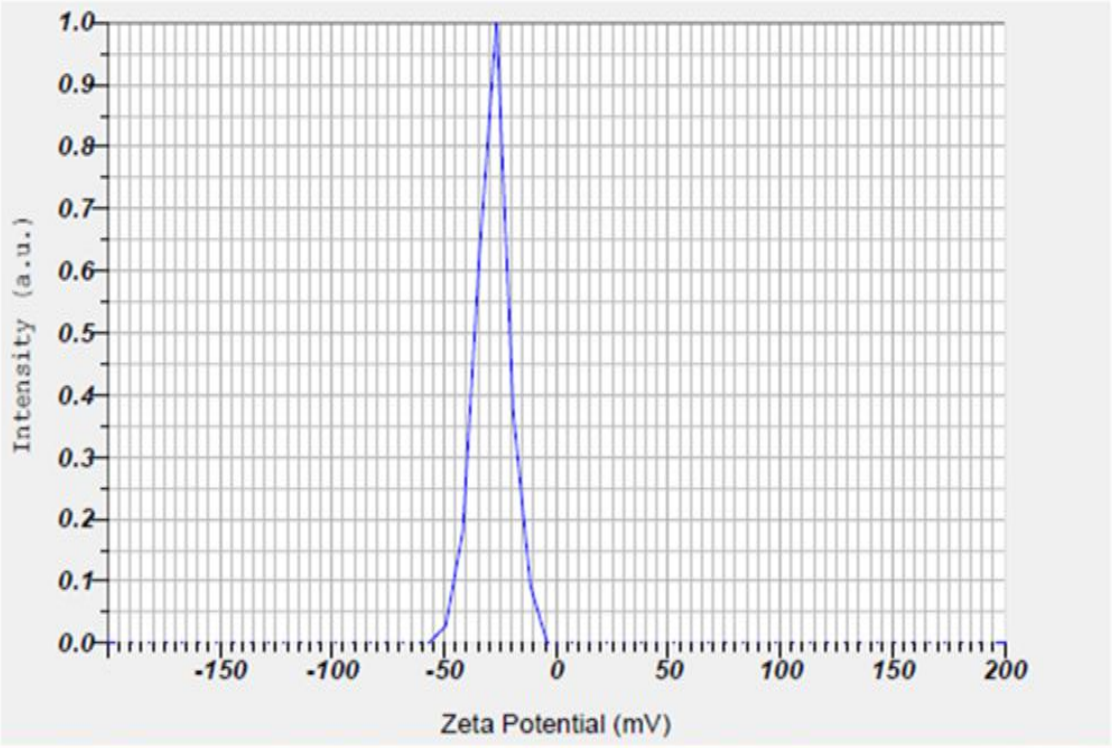


Figure 3.9:Zeta Potential Of Liposomal Docetaxel

3.1.5 Stability :

To examine the stability of the liposomal drug, the measuring of zeta potential for liposomal docetaxel was done after three months, the result showed in table 3.4and figure 3.10:

Table 3.4: Calculation Result Of Zeta Measuring Of L.DXL After Three Months

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-36.5mV	-0.000284 cm^2/Vs
2	……..mV	……cm^2/Vs
3	…….mV	……cm^2/Vs

Zeta Potential (Mean) : -36.5mV

Electrophoretic Mobility Mean : -0.000284 cm^2/Vs

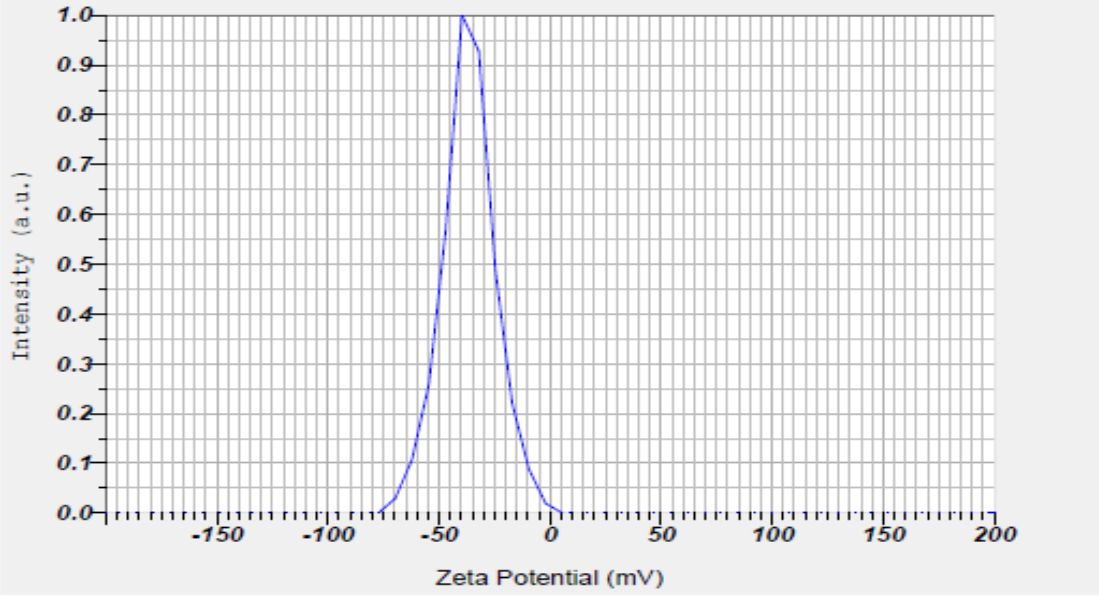


Figure 3.10: Zeta Potential Of Liposomal Docetaxel

3.1.6 Fourier Transform Infrared Spectroscopy (FTIR):

FTIR analysis measures a sample's absorbance of infrared light at various wavelengths to determine the material's molecular composition and structure, the result of this measuring was shown in figure 3.11 for liposome and figure 3.12 for liposomal docetaxel .

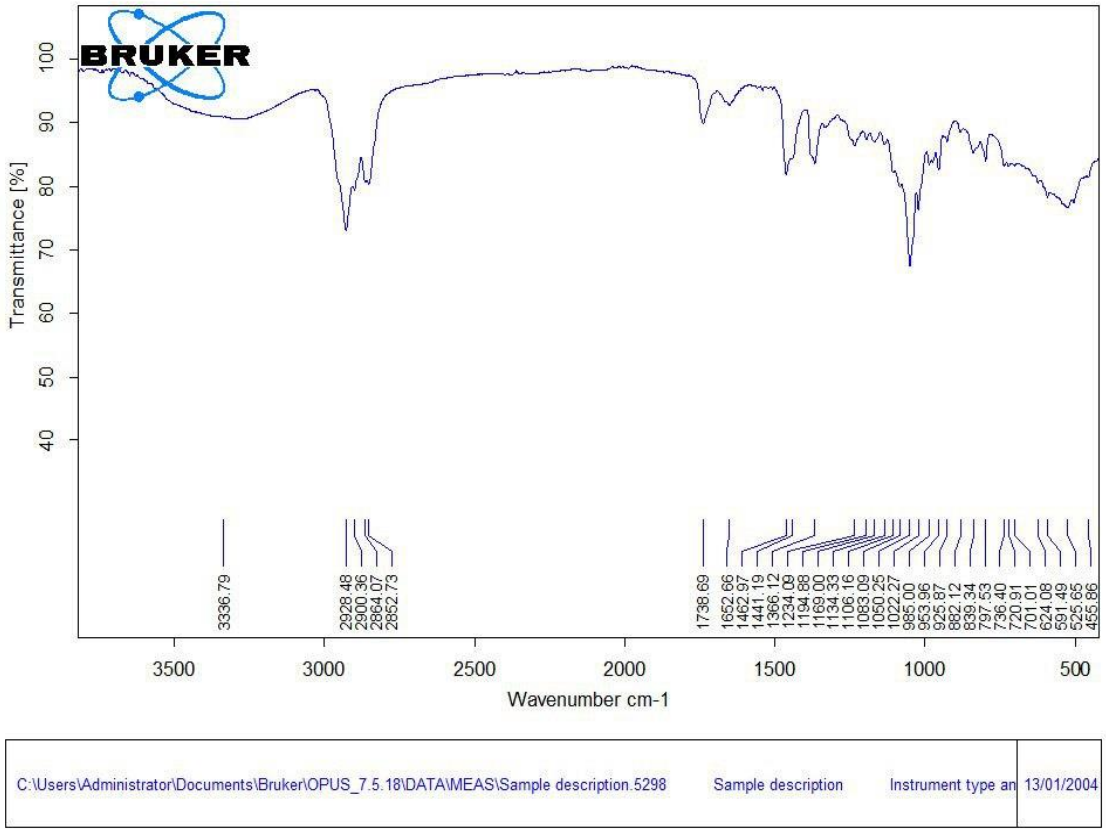


Figure 3.11: FTIR Of Liposome

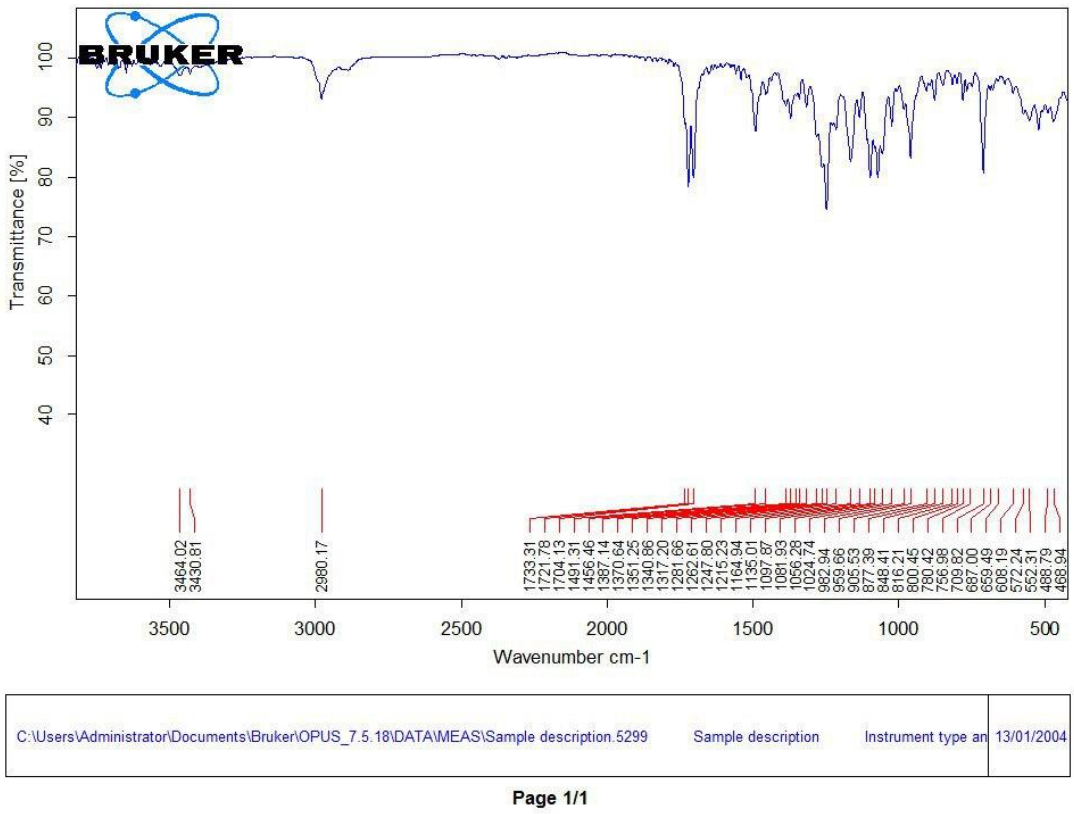


Figure 3.12:FTIR Of Encapsulated Docetaxel

3.1.7 The Entrapment Efficiency (EE)%:

The Entrapment Efficiency (EE) was calculated according to the following equations:

$$EE(\%) = \frac{amount\ of\ Doc\ \ entrapped\ in\ liposomes}{amount\ of\ total\ Doc\ \ applied\ in\ the\ preparation} \times 100\%$$

Table 3.5: Calculation Of Optimal EE% With Change In Amount Of Cholesterol And Phospholipids.

Molar ratio	Cholesterol amount(µl)	Phospholipid amount (µl)	Con.of free docetaxel (mg)	EE%
1:1	1330	670	23.49	81.2
0.25:1	670	1330	23.97	80.8
0.5:1	1000	1000	30	76
1:14	250	1750	23.97	80.8
0:1	0	2000	30.1	75.9
2:1	1600	400	28.96	76.8

Amount of DXL entrapped in a liposome

= total conc. Of drug- Conc. of free docetaxel (mg) in solution

=1000-(23.49*8) where 8 represents the dilution factor.

= 1000-187.92=812.08 mg

EE(%)= $\frac{812.08}{1000} \times 100\%$

EE(%)=81.82% (molar ratio 1:1 provided optimal encapsulation efficacy)(Zhang, 2017).

3.1.8 Drug Loading (DL)% :

The entrapment efficiency of the liposome was evaluated after the removal of the free drug, The following equations were used for calculation:

Firstly we calculate the amount of encapsulated drug by using a spectrophotometer to measure the absorbance of free drug in the solution and compare it with the calibration curve to find the recent amount which is equal to the amount of encapsulated drug.

$$\text{Drug loading (\% DL)} = \frac{amount\ of\ drug\ released\ from\ nanoparticle}{amount\ of\ liposome} \times 100$$

%DL=(795.4/1000)*100=79.54%

3.1.9 In Vitro Drug Release:

In vitro drug release profile using dialysis bag and measure the absorbance by spectrophotometer at 227 for encapsulated docetaxel at pH 7 and pH 5.5, the result has been observed after 24, 48, 72, and 96 hours which is illustrated in figure 3.13.

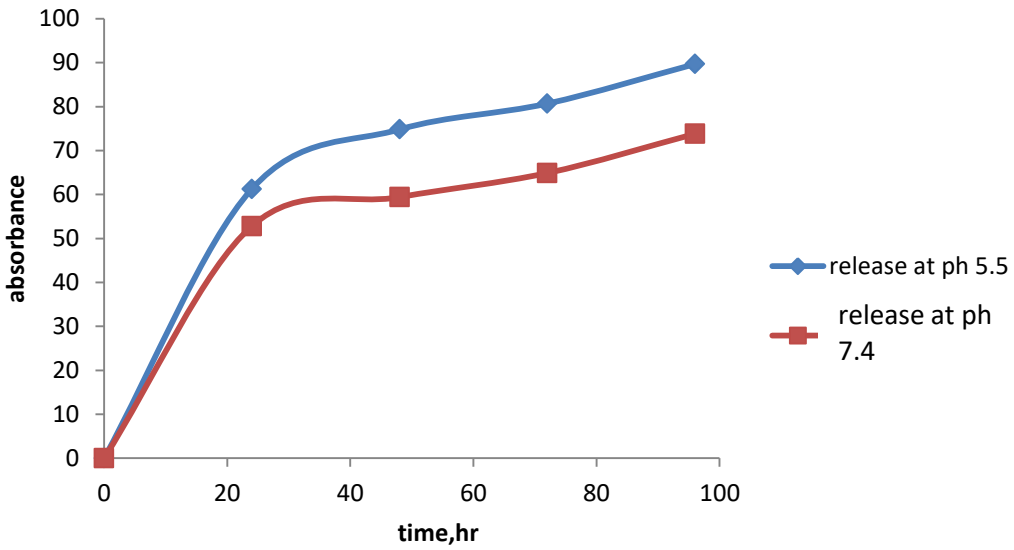


Figure 3.13: Liposomal Docetaxel Release Profile At Ph 7 And 5.5 After 24, 48, 72, And 96 Hours.

3.1.10 The Kinetics models Study of drug release:

At pH 5.5 (figure 3.14), liposomal docetaxel was analyzed to assess the in vitro release patterns of L.DXL

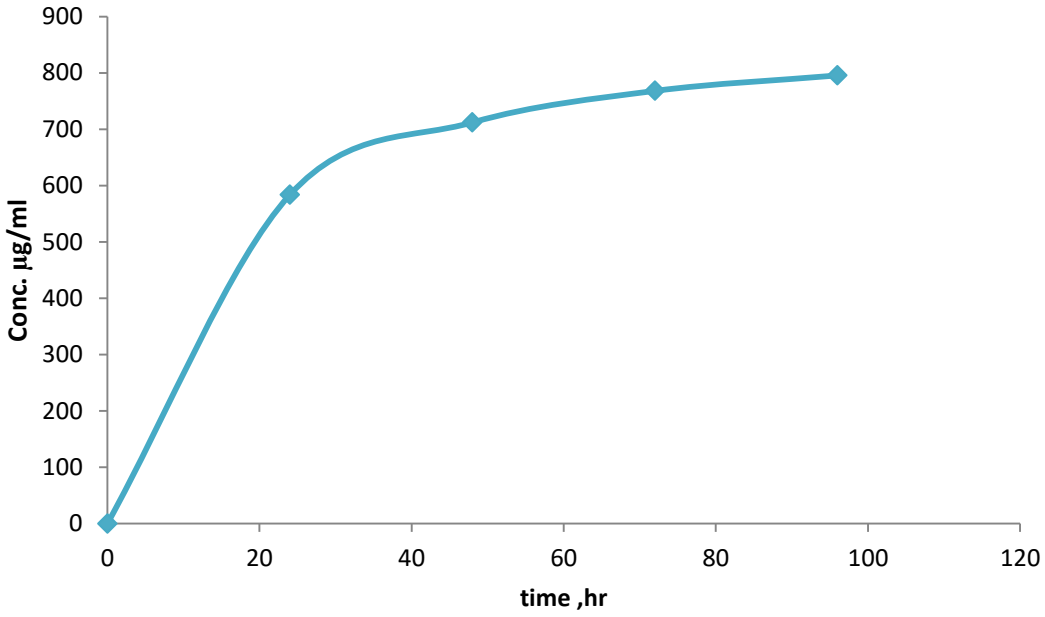


Figure 3.14: Liposomal Docetaxel Release Profile At Ph 5.5 After 24, 48, 72, And 96 Hours.

Four drug release kinetic models (figure 3.16 A, B, C, and D), including the zero-order kinetic model, first-order kinetic order, Higuchi model, and Korsmeyer-Peppas model, were employed in this study. It was possible to describe the steady-state release of a drug from such a drug delivery system using a zero-order kinetic model, which relates time and cumulative percent drug release. For now, the link between time and log cumulative percent of drug left was used in the first-order kinetic model. This model was used to examine the concentration-dependent manner in which the drug release was

evaluated. Using the Higuchi model, researchers were able to determine whether or not the drug release mechanism was diffusion-controlled or not based on the link between time and cumulative percent drug release. Finally, the relationship between time and log cumulative percent drug release in the Korsmeyer-Peppas model aided in deciphering the mechanisms by which pharmaceuticals dissolve in the matrix. Equations (2), (3), (4), and (5) were used to generate the graphs of the zero-order, first-order, Higuchi, and Korsmeyer-Peppas models in Microsoft Excel 2010, and then a linear regression fit was used to determine the rate constant and correlation values. (Vu *et al.*, 2020).

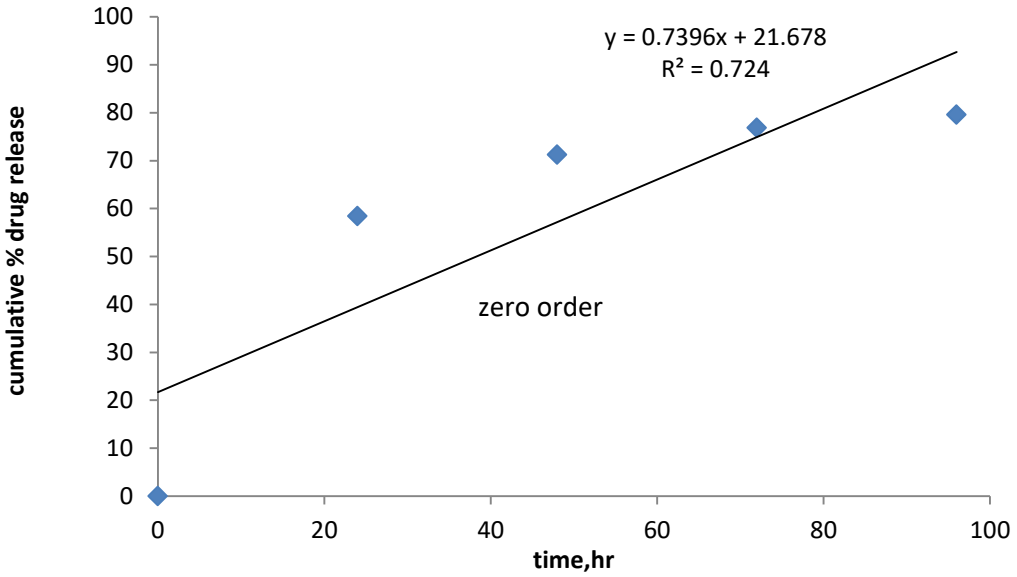
$$C = k_0 t, \quad (2)$$

$$\log(100 - C) = -\frac{k_f t}{2.303}, \quad (3)$$

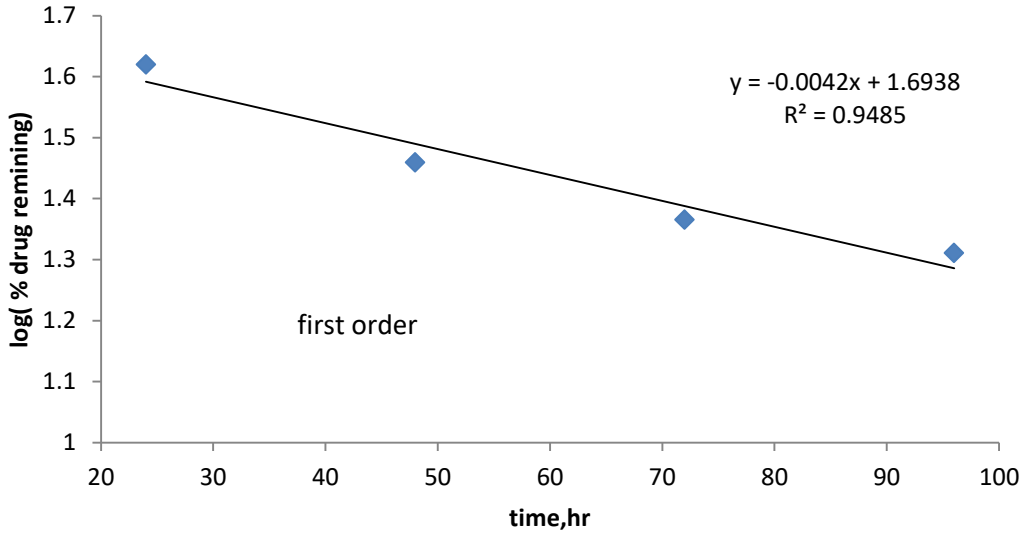
$$C = k_H \sqrt{t}, \quad (4)$$

$$C = k_K t^n, \quad (5)$$

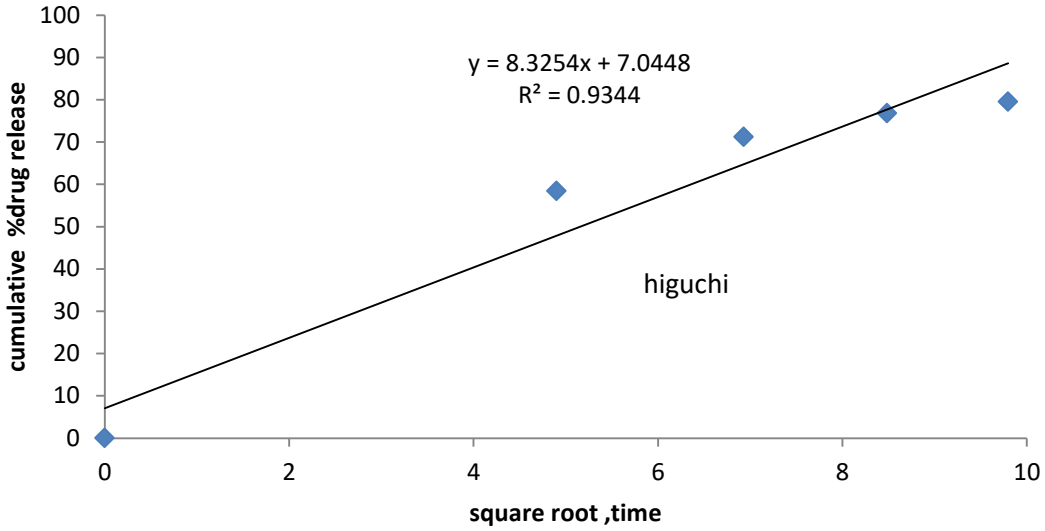
where is C the cumulative % drug released at the time, K0 is the zero-order rate constant, Kf is the first-order rate constant, KH is the Higuchi dissolution constant, Kk is the Korsmeyer-Peppas constant, and n is the exponent that describes a particular diffusion mechanism, each of this constant was displayed in table 3.6.


y = 0.7396x + 21.678
R² = 0.724
zero order
cumulative % drug release
time,hr
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100

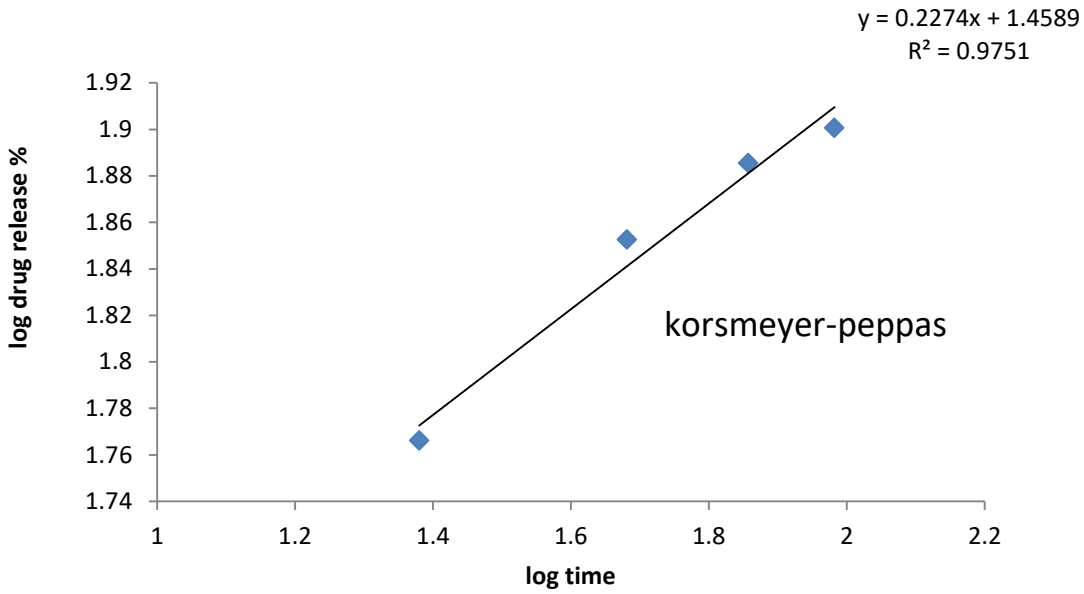
(A)


y = -0.0042x + 1.6938
R² = 0.9485
first order
log(% drug remining)
time,hr
1
1.1
1.2
1.3
1.4
1.5
1.6
1.7
20
30
40
50
60
70
80
90
100

(B)



(C)



(D)

Figure 3.15: Release Kinetics Of Liposomal Docetaxel Fitted To Four Kinetic Models: (A) Zero-Order Kinetic Model, (B) First-Order Kinetic Model, (C) Higuchi Model, And (D) Korsmeyer-Peppas Model.

Table 3.6: Rate Constants And Correlation Coefficients Of Liposomal Dxl, Obtained Through The Zero-Order Kinetic Model, First-Order Kinetic Model, Higuchi Model, And Korsmeyer-Peppas Model.

Models	L.DXL
zero	K0= 0.7396 R^2 = 0.724
first	Kf= -0.0096 R^2 = 0.9485
Higuchi	KH= 8.3254 R^2 = 0.9344
Korsmeyer-pepas	K= $-0.641*10^{1.5}$ R^2 = 0.9741

3.2.In Vitro Cytotoxicity:

3.2.1 Effect of Liposomal docetaxel on cell proliferation after 24,48hr and 72 hrs of incubation.

The results showed that there was a significant decrease in the viability percent ($P<0.001$) for all concentrations at the different time intervals except with the lowest concentration (31.25,15.6 µg/ml)at 24hr only which has no significant decrease ($p > 0.001$) in the viability percent in comparison with the control group as shown in figure 3.16. Also, the result of the IC50 calculation showed in figure 3.17.

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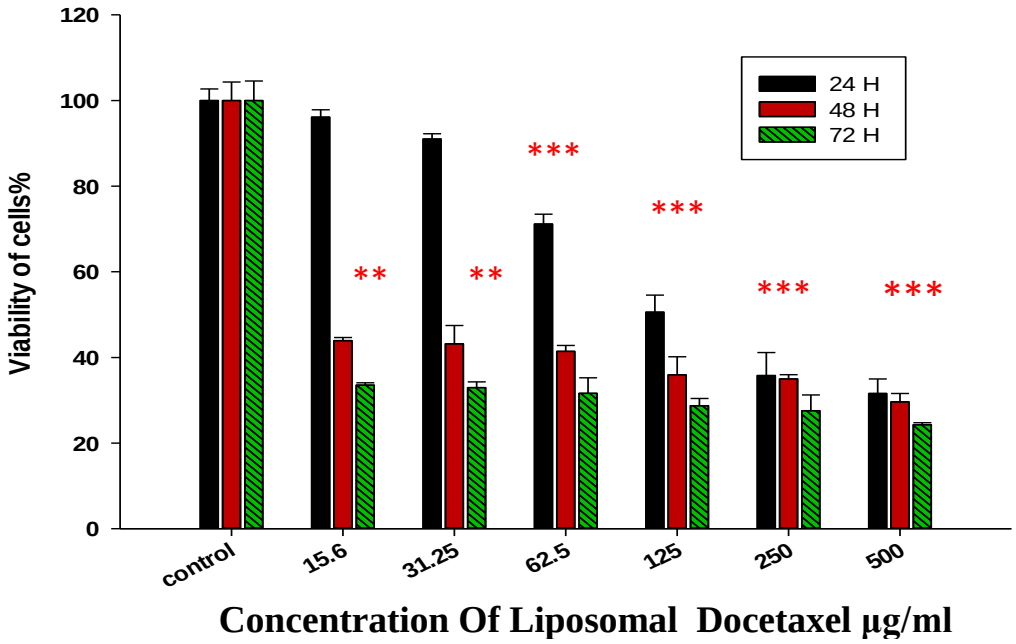

Figure 3.16: The Effect of Different Concentrations of L.DXL on prostate cancer LNCaP Cell Line After Incubation for 24,48 and 72 hr. *mean significant

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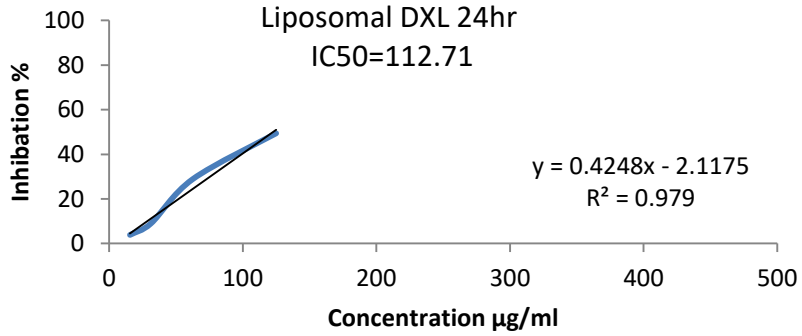
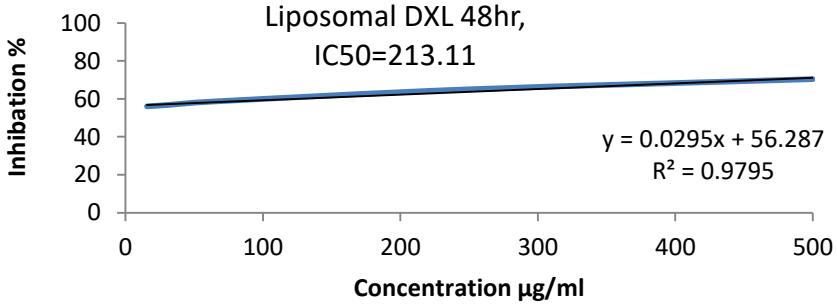


(A)



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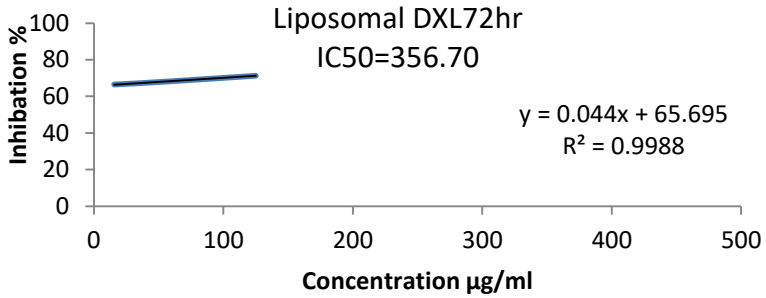


(C)

Figure 3.17:IC50 of Liposomal docetaxel on LNCaP cell line after incubation periods (A) 24hr,(B) 48hr, and (C) 72hr.

3.2.2 Effect of pure docetaxel powder on the prostate cancer cell line after 24,48hr and 72 hrs of incubation.

The results showed that there was a significant decrease in the viability percent (P≤0.001) for all concentrations, at the different time intervals except with the lowest concentration (62.5,31.25&15.6 µg/ml)at 24hr only which has no significant decrease (p > 0.001) in the viability percent in comparison with the control group as shown in Figure 3.18. And the result of the IC50 calculation showed in figure 3.19.

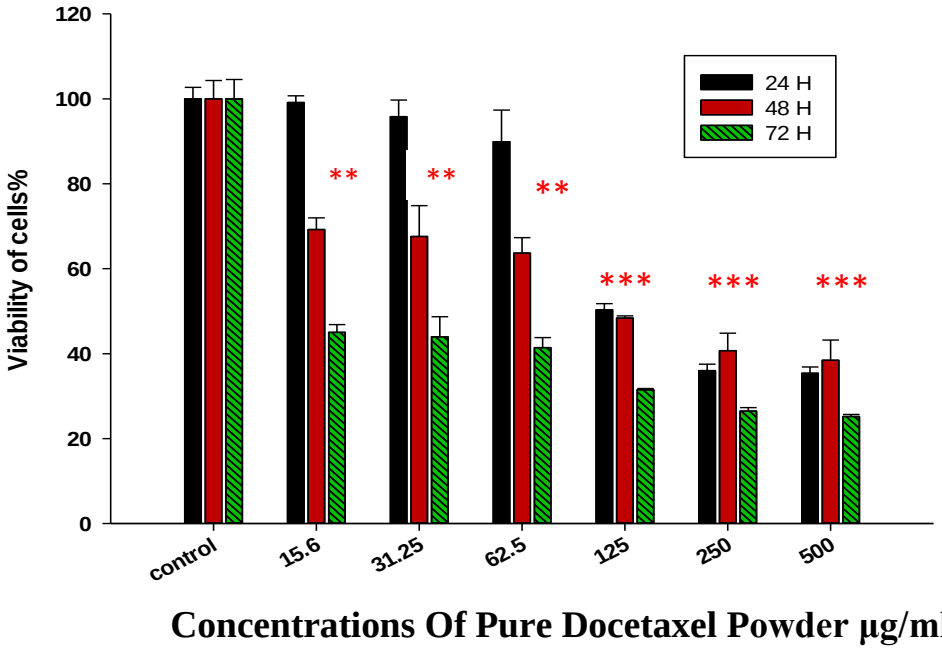
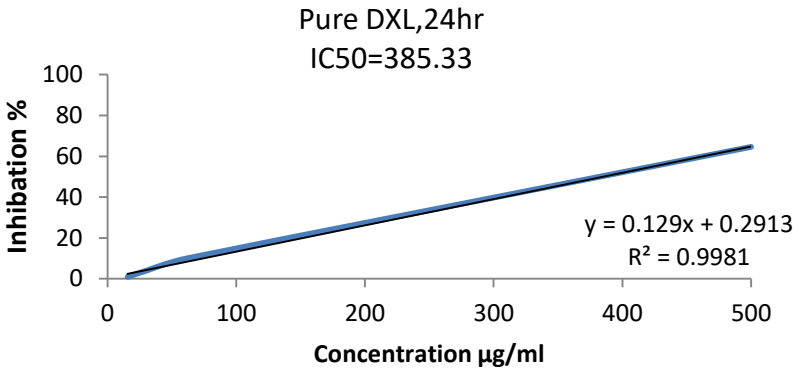


Figure 3.18: The Effect of Different Concentrations of pure. DXL on prostate LNCaP Cell Line After Incubation for 24,48 and 72 hr.



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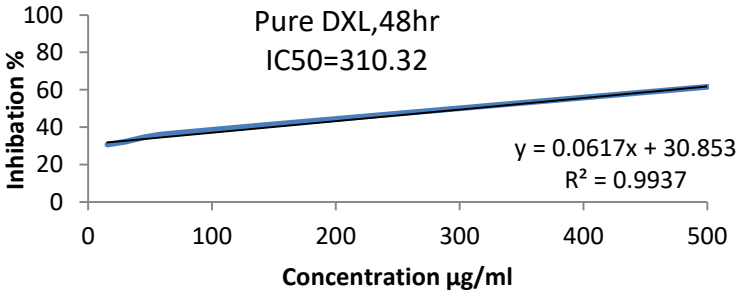
(A)



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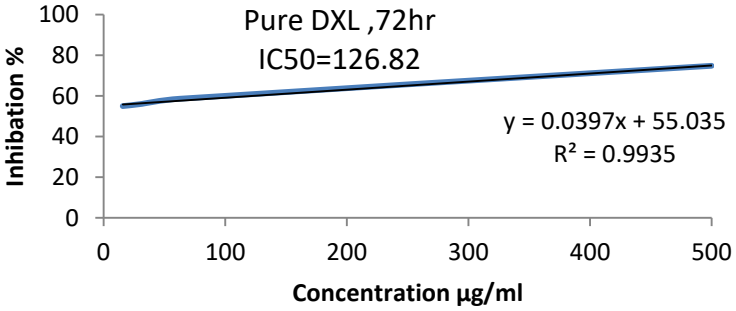

(C)

Figure 3.19:IC50 of pure docetaxel powder on LNCaP cell line after incubation periods (A) 24hr,(B) 48hr, and (C) 72hr.

3.2.3. Effect of formulated docetaxel vial on the prostate cancer cell line after 24,48hr and 72 hrs of incubation.

The results showed that there was a significant decrease in the viability percent ($P<0.005$) for all concentrations, at the different time intervals in comparison with the control group as shown in Figure 3.20. And the result of the IC50 Calculation showed in figure 3.21.

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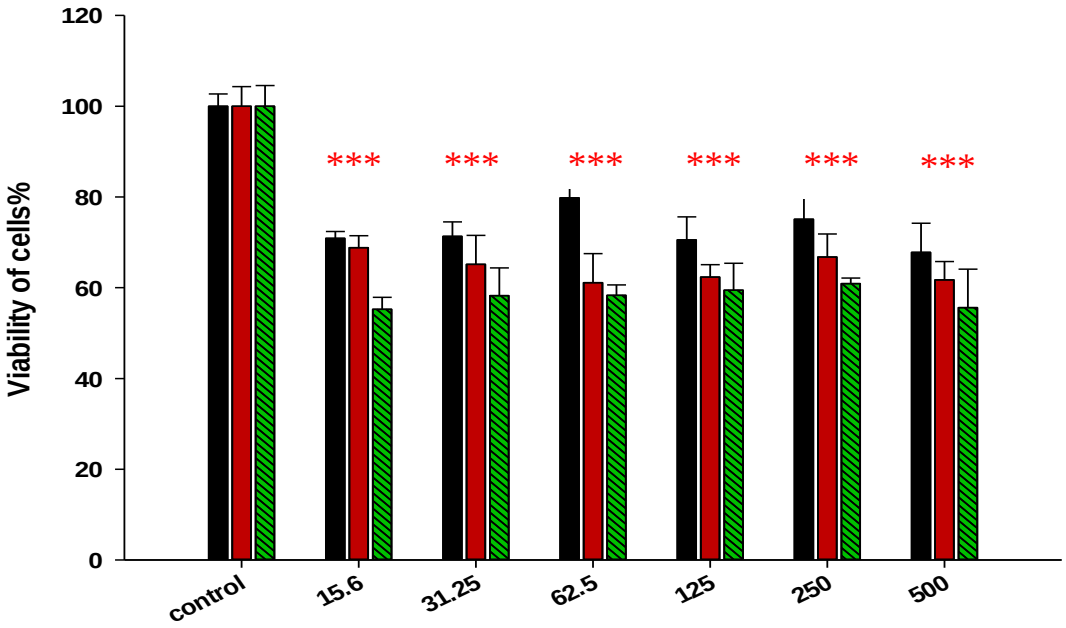
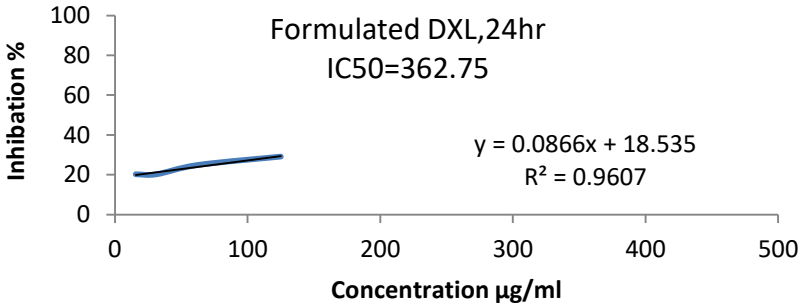
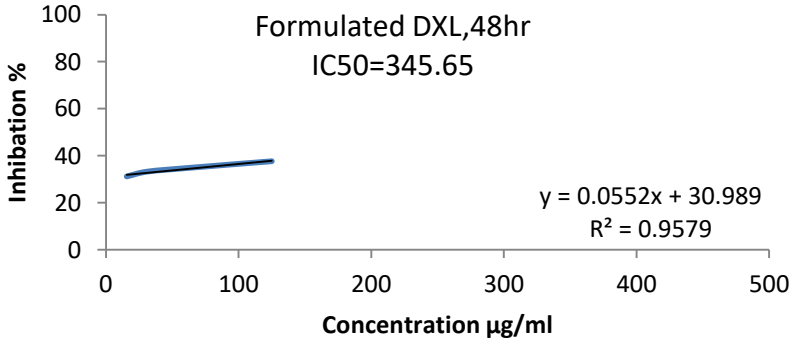


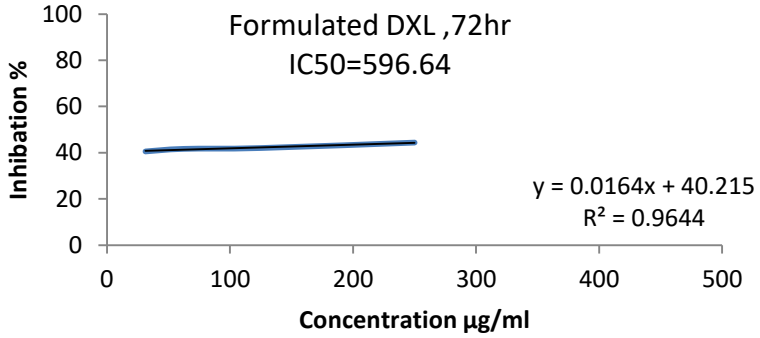
Figure 3.20: The Effect of Different Concentrations of formulated DXL vial on prostate LNCaP Cell Line After Incubation for 24,48 and 72 hr.



(A)



(B)



(C)

Figure 3.21:IC50 of formulated docetaxel vial on LNCaP cell line after incubation periods (A) 24hr,(B) 48hr, and (C) 72hr.

3.2.4 Comparison In Cell Viability Between Liposomal Docetaxel, Formulated Docetaxel Vial, And Pure Docetaxel Powder :

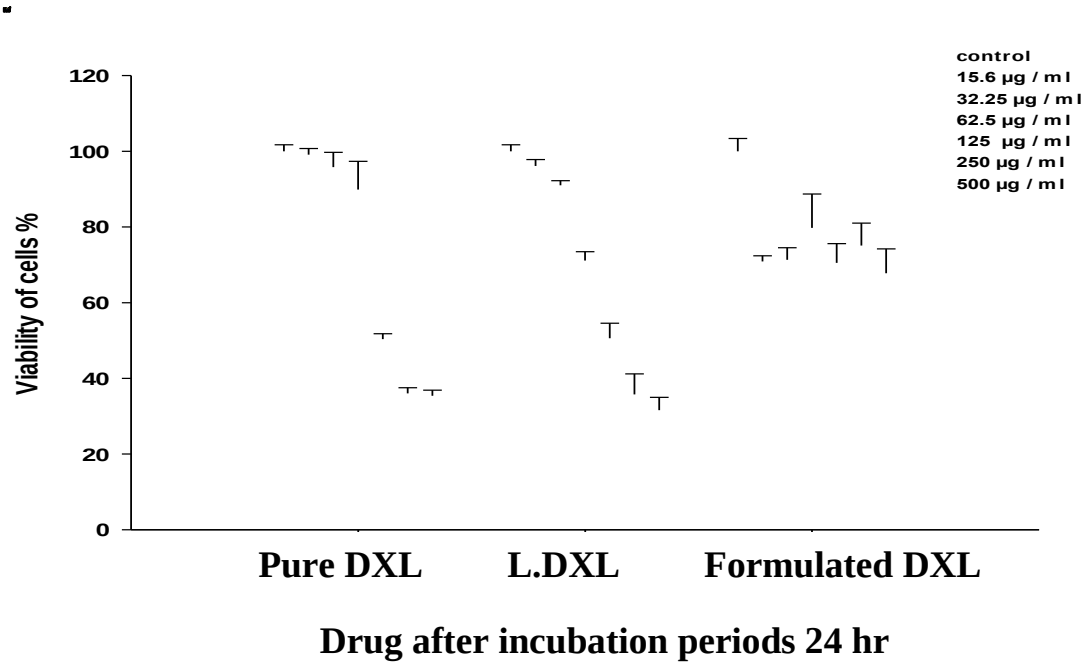


Figure 3.22 A: Difference between drugs in cell viability percent at 24 hr

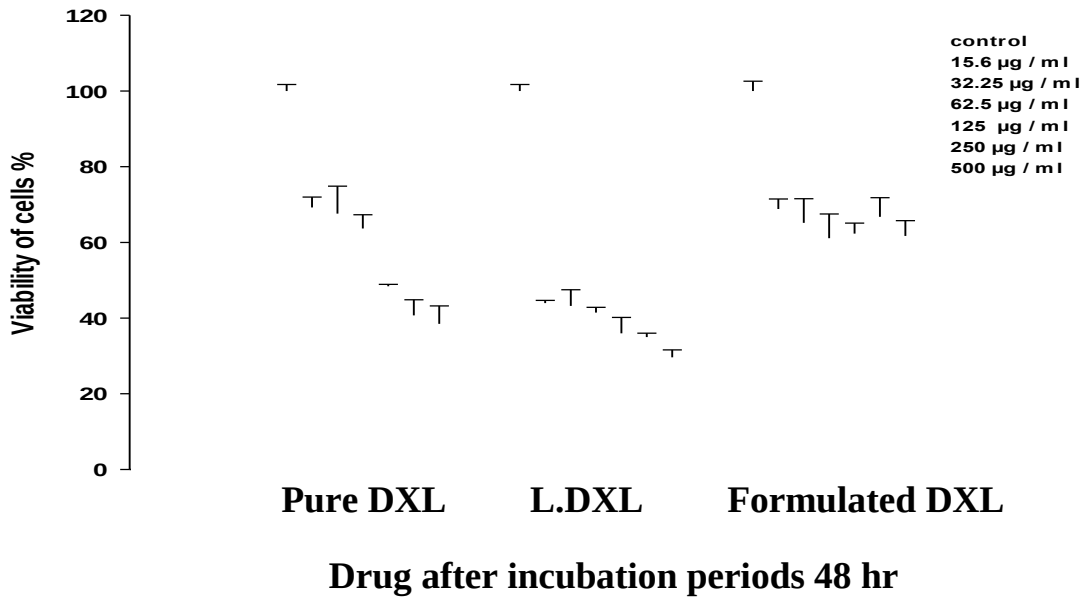


Figure 3.22 B: Difference between drugs in cell viability percent at 48 hr

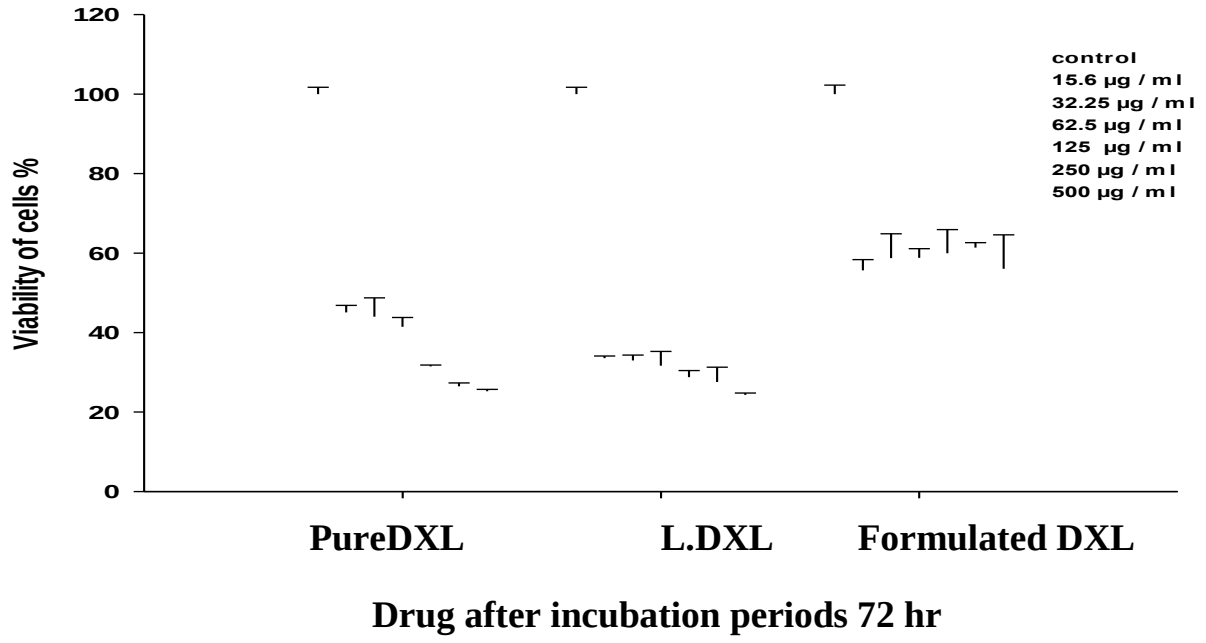


Figure 3.22 C: Difference between drugs in cell viability percent at 72 hr

3.2.5 Comparison In Cells Growth Inhibition Between Liposomal Docetaxel Formulated Docetaxel Vial And Pure Docetaxel Powder :

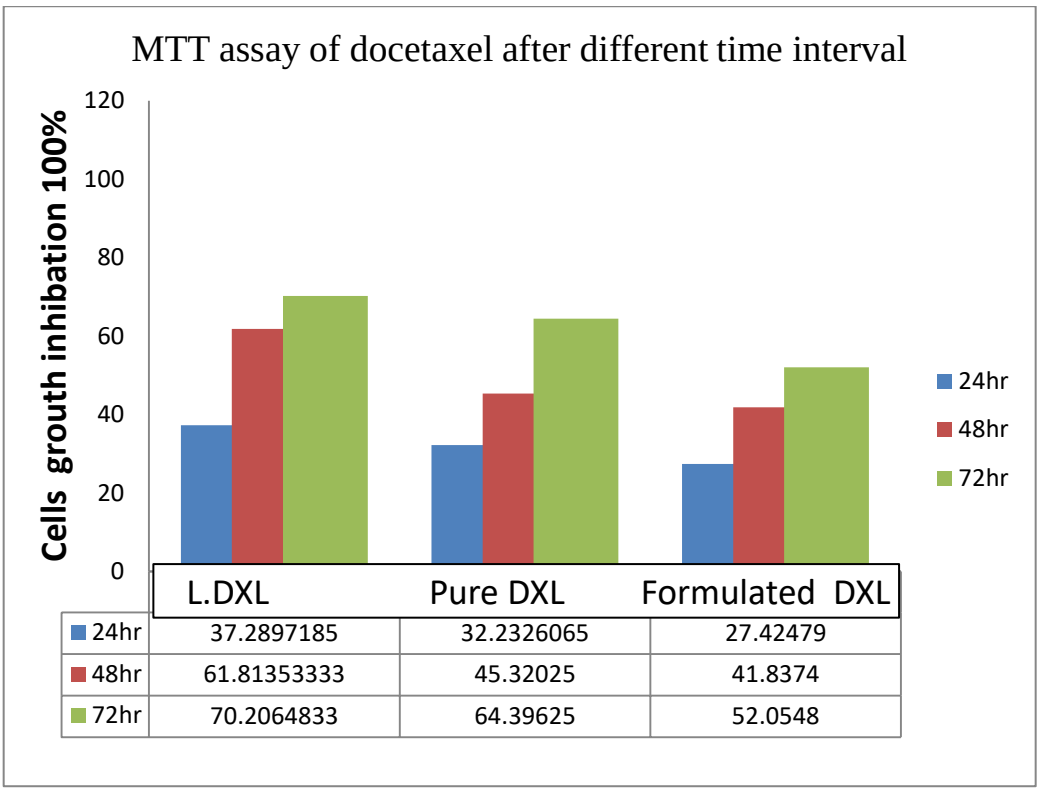


Figure 3.23: Rate of cell growth inhibition of different forms of Docetaxel at different periods for 24,48,72 hr of incubation.

Chapter Four

Discussion

4.1 Characterization Techniques:

4.1.1 Scanning Electron Microscopy (SEM):

According to the SEM photographs, the liposomes all had a fine spherical shape with a relatively monodispersed size distribution confirming the size distribution measurement studies (Fig. 3.1&Fig.3.2)(Yousefi *et al.*, 2009).

The size of conventional liposomes determines the fraction cleared by the MPS and it has been shown that small liposomes (e.g. size below 100 nm) are opsonized less rapidly and to a lower extent compared bigger liposomes (e.g. bigger than 100 nm) (Fanciullino and Ciccolini, 2009). On the other hand, liposomes with a diameter < 10 nm were rapidly cleared by the kidney (Kumari et al., 2016), the size of liposomes in the current study was approximately 28nm(fig 3.1) and liposomal docetaxel was approximately 20 nm(fig.3.2), this difference in size is due to increase the time of sonication in case of liposomal docetaxel which leads to downsized particles(Kushwah *et al.*, 2018).

4.1.2 Transmission Electron Microscopy (TEM):

The surface morphology of the drug-loaded liposome was found by TEM to be spherical. The particle size obtained from the DLS can also be confirmed from the TEM image which was found equal to 20 nm for liposomes (fig.3.3,3.4), and 14 nm for L.DXL (Fig. 3.5m3.6), this result is in agreement with (Sarfraz *et al.*, 2017) who said the hydrophobic substance has a higher percentage of incorporation, which is related to its tendency to position between the bilayer shell of liposomes rather than an aqueous phase, also this smaller size Of liposomal drug may be attributed to sonication time which as

it increases causes the size of the nanoparticles to become much smaller(Silva *et al.*, 2010).

4.1.3 Atomic Force Microscopy (AFM):

According to AFM images, the NPs were almost spherical (Figures 3.7A and B) with a diameter of about 17 nm (Figure 3.7C). which is closely related to the size of nanoparticles obtained by SEM. The result agreed with(Ju *et al.*, 2018) who find AFM images of docetaxel indicated that functional docetaxel nanoparticles were round and uniform with smooth surfaces.

4.1.4 Zeta Potential:

Zeta potential determination is a significant characterization technique of nanoparticles to estimate the surface charge, which can be employed for understanding the physical stability of nanosuspensions (Jiang et al., 2009).

Zeta potential values are typically in the range of +100 to -100 mV according to (Shnoudeh *et al.*, 2019) study. A large positive or negative value of zeta potential of nanocrystals indicates good physical stability of nanosuspensions due to electrostatic repulsion of individual particles.In this present procedure, the value of ZP is -23.0 mV for liposome alone(table 3.2&fig.3.8) ,and for liposomal docetaxel equal to -28.3mV (table 3.3 & fig. 3.9) which are considered optimum for good stabilization of a nanodispersion (Samimi *et al.*, 2018). To avoid liposome aggregation in buffer solution, a high absolute value of the zeta potential implies that the drug-loaded liposomes have a high electrical charge on their surface. Liposomes had a negative Zeta potential because of the lipids' terminal carboxylic groups (Yousefi *et al.*, 2009). Also, the zeta potential is a good indicator to quantify the stability of nanoparticles in physiological conditions. It was reported that negative charges resulted in

preventing fusion and aggregation and also reduced phagocytosis (Vu *et al.*, 2020).

4.1.5 Stability :

The formulations were stored at refrigerating (4 °C) for 3 months and the zeta potential of the formulations was measured(-36.5 mv), as shown in (Table 3.4 &fig.3.10) and compared with those at the time of preparation, this value means the nanoformulation is still physical stable according to (Mahbubul, 2019), This matter could be resulted by the presence of cholesterol that has stabilizing effect against aggregation and fusion of the liposomes(Sarfraz *et al.*, 2017).

4.1.6 Fourier Transform Infrared Spectroscopy (FTIR):

Drug-excipients interaction (if any) was investigated using FTIR spectroscopy to assess the type of interaction among the various functional groups of the drug and the excipients of a formulation (Figures 3.11 and 3.12). When the FTIR spectra of the excipients (cholesterol and lecithin) and the pure drug (DXL) were compared with their physical mixtures, it was found that the characteristic bands of all the excipients and the drug were present in their physical mixtures. The N-H and O-H group vibration band was found at 3464.02 cm-1 (Invernizzi *et al.*, 2018). The C =C asymmetric stretch of medium intensity bending vibration and C=O variable weak intensity out of plane bending vibration (at 709.82 cm-1), strong intensity C=O stretching vibration (at 1733.31cm-1) and medium intensity bending vibration of –CH3 deformation (at 1370.64cm-1) of lecithin were present in the physical mixture. Similarly, the characteristic bands of Cholesterol were observed in the spectra of the physical mixture with the drug and the spectra of liposome alone. For

example, strong intensity stretching vibration of the C–OH group (at 1056.28 cm1) and medium intensity bending vibration of –CH3, –CH2, –CH deformations (at 1456.46 cm-1) indicate the presence of Cholesterol in the physical mixture L.DXL. Further, in the case of DXL, medium intensity out of plane bending vibration C=C (at 800.45 cm-1) and C–O stretching bands were observed in the spectrum of liposome alone and L.DXL. This suggests that there is no chemical interaction seen between the drug and the excipients and suggests that the drug was well encapsulated in the bilayer (Shaw *et al.*, 2017)**.**

4.1.7 The Entrapment Efficiency (EE%):

The most important finding was the results of measurement of liposomal encapsulation efficiency EE(%)=81.82%(Table 3.5) for docetaxel nanoliposomes, which consider the higher percent of encapsulation according to (Eloy *et al.*, 2020), who ranging the high efficiency of encapsulation of docetaxel from 68.53% to 99.45%, being the main contribution given by the lipid to drug ratio.

4.1.8 Drug loading (LD%):

DL% depends on the composition of the lipid matrix and its crystalline state. It has been reported that in lipid-based drug delivery systems, Among the different types of lipid nanostructures, solid lipid nanoparticles (SLNs) are an attractive DDS due to their high structural stability and biocompatibility in comparison to nanoemulsions and are considered a less toxic alternative to polymer-based nanoparticles. Liposomes are made from physiologically tolerable lipid components, which remain in the solid state at room and body temperature. Some advantages of lipid nanoparticles include low toxicity, controlled drug release, physical stability, large-scale production, protection of

incorporated drug, high drug loading, low cost, avoidance of organic solvents in preparation, biodegradability, biocompatibility, and capability of incorporating hydrophilic and hydrophobic compounds(Da Rocha *et al.*, 2020).

As calculated previously DL% was equal to 79.54% which consider a high amount in drug loading and a sign of successful remote loading and good encapsulation of DXL into the liposomes (Vakili-Ghartavol *et al.*, 2020).

4.1.9 In Vitro Drug Release And Drug Release Kinetic Study:

At pH 5.5 the *in vitro* drug release profile of L.DXL has been observed after 24, 48, 72, and 96 hours(figure 3.13). pH 5.5 represents the favorable pH of prostate cancer (Maloney *et al.*, 2020). At pH 5.5 higher absorption has been obtained compared with pH 7 the biodegradation of the liposome in the acidic environment. Also, sustainable slow-release has been obtained where after 24 hours the absorbance was 61.25, after 48 hours the absorbance was 74.81, after 72 hours the absorbance was 80.68 and after 96 hours the absorbance was 89.67. The well-encapsulating core of liposomes may be responsible for the sustained and delayed release of docetaxel. This allows for continual combat against cancer cells, resulting in a reduction in cancer cell viability (Drugs and Olusanya, 2018). generally, the utilization of liposomes for the administration of anticancer medicines for docetaxel encapsulation can prolong and regulate the release profile (Zheng *et al.*, 2020).

The pattern of drug release from the formulation was best fitted with the Korsmeyer–Peppas kinetics(R^2 = 0.9741) shown in figure 3.15 (D), which demonstrates the involvement of anomalous diffusion which is controlled by more than one parameter(Shaw *et al.*, 2017).

Docetaxel's release from liposomes takes longer than expected, according to the findings, since cholesterol stabilizes lipid bilayers. Liposomes could thus be used to create a depot effect. The liposomal formulation appears to meet the criteria for a successful drug delivery system based on these results. (Yousefi *et al.*, 2009). The current study result of the release profile of liposomal docetaxel shows a typical sustained and prolonged drug release(as seen in figure 3.14).

4.2 In Vitro Cell Viability Assay:

The antineoplastic effects of the prepared agent Liposomal docetaxel(L.DXL), as well as formulated agent(Taxoter) and the pure drug powder docetaxel on LNCaP cells, were performed by MTT assay because this procedure has been reliably applied in proliferation and cytotoxicity studies to screen the new drug products potential on cell growth (Van Tonder, Joubert, and Cromarty, 2015).

Docetaxel whether as pure agents, formulated, or loaded in the nanoliposome shows a conc- time-dependent cytotoxicity effect(Liu *et al.*, 2013) on cancer cell lines. Docetaxel is believed to have a twofold mechanism of antineoplastic activity: (1) inhibition of microtubular depolymerization, and (2) attenuation of the effects of BCL-2 and Bcl-xL gene expression (Pienta, 2001).

This action of DXL mostly results in cytotoxicity in cancerous cells. In this study, the action was increased when these toxic agents were encapsulated in nanoliposome which agrees with (Sánchez-Moreno *et al.*, 2012) study.

In vitro cell viability or anti-proliferative effect of pure DXL, L-DXL, and formulated DXL was evaluated in prostate cancer cells (LNCaP cell line).

The plot of cell viability percent against concentration shows that as the concentration of DXL increased, the death rate of cells increased. L-DXL mediated cellular inhibition was found to be more compared to the cells treated with the pure-drug solution and Taxotere (figure 3.23,3.24 and 3.25) at an all-time interval, this result was also confirmed by (Shaw *et al.*, 2017) study.

The cytotoxicity of DXL-Sol displayed time and concentration-dependent cytotoxic patterns. After 24,48 72hours of incubation, pure DXL-Sol and Liposomal DXL and Taxotere, the inhibition rate of LNCaP cell proliferation was (37.2897185% at 24hr, 61.81353333% at 48hr, and 70.2064833% at 72hr), for the cell treated with liposomal docetaxel, the percent of inhibition are much higher than for the cell treated with pure DXL and with Taxotere (figure 3.24), this result agreement with (Ren *et al.*, 2016) study.

Liposomal docetaxel was used to treat the LNCaP cell line in different concentrations as shown in figure 3.16, after incubation periods 24m48 and 72 hr at 37c. The results of incubation showed that there was a significant decrease in the viability percent ($P<0.001$) for all concentrations, at the different time intervals except with the smallest concentration (31.25,15.6 μg/ml)at 24hr only which has no significant decrease ($p > 0.001$) in the viability percent if we compared with the control group. The result agrees with (Ju *et al.*, 2018) who suggested that cell viability decreased with increasing the concentration of L.DXL the cell viability of L.DXL treated cells was reduced with an increase in the DXL concentration at different.

While The results of pure docetaxel powder showed that there was a significant decrease in the viability percent ($P\leq0.001$) for all concentrations, at

the different time intervals except with the lowest concentration (62.5,31.25&15.6 μg/ml) at 24hr only which has no significant decrease ($p > 0.001$) in the viability percent in comparison with the control group as shown in Figure 3.18. The result was also confirmed by (Saqr *et al.*, 2021) study, who illustrate the low concentrations of DXL did not affect cell death in all cell lines. Compared with the control group and with the cells treated with a low concentration of DXL, treatment with a high dose of DXL significantly increased the proportion of apoptotic cells.

The results of cells treated with DXL vial showed that there was a significant decrease in the viability percent ($P<0.005$) for all concentrations, at the different time intervals in comparison with the control group as shown in Figure 3.20.

The IC50 of DXL in the formulated vial (Taxotere), liposomal docetaxel, and pure docetaxel powder on LNCaP cells after 24 hours, 48 hours, and 72 hours of cell culture at the same DXL dose could lead to conclusions, Each of these treatments has a different effect on different cells at different time intervals, which may be attributed to the controlled drug release manner of the nanoliposome formulations, the cellular internalization rate of DXL into the cells, and resistance to the DXL due to causes such as cancer cells up-regulating protein transporter pumps. This conclusion is consistent with (Vakili-Ghartavol *et al.*, 2020) study. The IC50of DXL loaded liposomes(Figure 3.17) showed lowerIC50 compared to a formulated DXl (Figure 3.21)which means the liposomal DXL is more potent than formulated drug. The reason for the reduction in IC50 values as compared with Taxotere could be the enhanced cellular uptake of DXL using nanoparticles, this result was also confirmed by (Sumera *et al.*, 2017), who also compared between the

cytotoxicity of taxotere and DXL loaded in nanoparticle and notice increased cellular uptake of DXL loaded in nanoparticles and enhanced accumulation of DXL in tumour could be the reasons for enhanced antitumour activity .

Conclusions

and

Recommendations

From Our Research Study, We Can Conclude That:

1. We can overcome the problems associated with the current conventional formulations of DXL, and efforts have been made to develop the DXL-loaded nanosystems. These systems have improved the characterization of drug and bioavailability as well as anti-tumor efficacy toward tumor site.

2. Liposomal docetaxel exerts its effect in a dose and time-dependent manner, as the concentration of the drug increase, the cytotoxic effect was also increased.

3. Liposomal docetaxel extract has a better anti-proliferative effect on LNCaP prostate cancer cells in comparison to the pure docetaxel and formulated one (Taxoter).

Recommendation :

We Recommended That:

1. Further studies are required to assess the Liposomal docetaxel efficacy, safety profile, and pharmacokinetic properties in laboratory animals.
2. Investigation of the L.DXL cytotoxicity on other cell lines such as breast cancer, lung cancer, gastric cancer, and other types of solid tumors.
3. Molecular study of the cells to determine the mechanism of the anti-proliferative effect of the L.DXL.

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الخلاصة:

مرض السرطان يبدأ عندما تبدأ خلايا الجسم في النمو خارج نطاق السيطرة. و يمكن أن تتحول الخلايا الموجودة في أي جزء من الجسم تقريبًا إلى خلايا سرطانية ، ويمكن أن تنتشر بعد ذلك إلى مناطق أخرى من الجسم .اما سرطان البروستات فيبدأ عندما تبدأ خلايا غدة البروستات في النمو خارج نطاق السيطرة. البروستاتاهي غدة موجودة فقط في الذكور. وظيفتها المساهمة في تصنيع السائل المنوي. يعد سرطان البروستات أكثر أنواع السرطانات شيوعًا بين الرجال ، بعد سرطان الجلد.

الطب النانوي هو فرع من فروع العلم يستخدم تقنية النانو جنبًا إلى جنب مع الأدوية أو الجزيئات التشخيصية لتحسين القدرة على استهداف خلايا أو أنسجة معينة. هذه المواد مصنوعة بالمقياس النانوي وهي آمنة تمامًا للاستهلاك. تشمل تطبيقات تقنية النانو في الطب التصويري والتشخيصي وإعطاء الأدوية ، وكلها ستساعد الاطباء في علاج مجموعة متنوعة من الأمراض.

الدوسيتاكسيل هو دواء مضاد للأورام من مجموعة التاكسويد يستخدم لعلاج أمراض مختلفة مثل سرطان الثدي ، وسرطان البروستات، وسرطان المعدة الغدي ، وسرطان الرأس والعنق. تُعزى سمية تركيبة الدوسيتاكسيل التقليدية إلى حد كبير لوجود مركبات البولي سوربات 80 والإيثانول.و للتغلب على مخاوف السمية ، تم إنشاء تركيبة دوسيتاكسيل نانوية بدون استخدام البولي سوربات 80 اوالإيثانول. تم إنتاج الدهون المغلفة للدواء باستخدام تقنية النانو الحاصلة على براءة اختراع وتعتبر آمنة من قبل إدارة الغذاء والدواء الأمريكية. ونظرًا لضعف

الأوعية الدموية للورم ، فقد تزيد الجزيئات الدهنية النانوية (<100 نانومتر) من توصيل الدوسيتاكسيل إلى أنسجة الورم ، مما يؤدي إلى تحسين نتائج العلاج.

الهدف الأساسي من هذه الدراسة هو تحسين نظام توصيل دواء الدوسيتاكسيل عن طريق تصنيعه على شكل دوستاكسيل شحمي.

أولاً ، تمت صناعة الدوسيتاكسيل كمكون نانوي شحمي ودُرست الخصائص المختلفة له على المقياس النانوي.

ثانياً ، جرت محاولة لفحص تأثير الدوسيتاكسيل الشحمي على خط خلايا سرطان البروستات. تم تنفيذ العمل الحالي في مختبر الدراسات العليا / فرع الادوية في كلية الطب / جامعة بابل من تشرين الاول 2020 الى آب 2021.

تم تعريض خلايا سرطان البروستات LNCaP الى تراكيز مختلفة (500 ، 250 ، 125 ، 62.5 ، 31.25 ، 15.62) ميكروغرام / مل من اشكال الدوسيتاكسيل المختلفة (دوسيتاكسيل الشحمي ، دوكيتاكسيل نقي ، والدوسيتاكسيل مركب (تاكسوتر) ، ومجموعة السيطرة السالبة (لم تتم معالجتها)ز

تم حضن جميع الخلايا المعالجة لمدة 24،48،72 ساعة وبدرجة حرارة 37 درجة مئوية ثم تم استخدام مقايسة السمية الخلوية MTT، لتقييم تأثير هذا الدواء على قابلية تثبيط الخلايا للمجموعات المذكورة.

أظهرت النتائج أن الدوسيتاكسيل في شكل شحمي تسبب في انخفاض معنوي ($p\leq 0.001$) في حيوية الخلية لخط خلية LNCaP ، في جميع التركيزات المختبرة باستثناء التراكيز المنخفضة (31.25 و 15.6) ميكروغرام / مل فقط عند 24 ساعة من فترة الحضانة. تسبب تأثير الدوسيتاكسيل النقي على خط الخلايا LNcaP في انخفاض معنوي في قابلية بقاء الخلية (قيمة P <0.001) عند التركيز (125،250،500) ميكروغرام / مل. تسبب تأثير Docetaxel المركب على خط الخلايا LNCaP في انخفاض معنوي في حيوية الخلية (P <0.005) في كل تركيز الدواء في فترات حضانة مختلفة ، وكان IC50 للدوسيتاكسيل الشحمي (112.71،213.11 و 356.70) ميكروغرام / مل ، الدوسيتاكسيل النقي385.33،310.32 و 126.82) ، وكان الدوسيتاكسيل المركب (345.65،362.75 و 530.7) ميكروغرام / مل عند 24 م 48 و 72 ساعة على التوالي.

استنتاجات هذه الدراسة هي أن هذا التعديل في نظام توصيل دواء الدوسيتاكسيل أدى إلى تحسين خصائص هذا الدواء وخصائصه الفيزيائية والكيميائية و كذلك الفعالية المضادة للأورام لهذا الدواء

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامـــعة بابـل

كليـة الطب

تأثير نظام توصيل دواء الدوسيتاكسيل الشحمي على خط خلايا سرطان البروستات

رســـالـــــــة

مقدمـــــة إلـــى مجلس كليـــة الطـــب / جامعـــة بابـــل

كجزء من متطلبـــات نيـل درجـــة الماجستيـر في علم الأدوية والسموم

من قبل

انغام محسن عبد

بكالوريوس صيدلة

٢٠١٣-٢٠١٤

اشراف

أ.م. ماجد كاظم عباس **أ.م. امجد مرزة عودة**

١٤٤٤هجري **٢٠٢٢ميلادي**