Synthesis and Characterization of Etoposide Loaded Nanostructured Lipid Carriers and Their Targeted Delivery to Cancerous Cells

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A thesis submitted in partial fulfilment of the requirements for the degree of

MS Biomedical Sciences

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2021

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Dedicated to my exceptional parents whose tremendous support and cooperation led me to this wonderful accomplishment!

Abstract

Etoposide (ETP) is one of the most widely used drugs for the treatment of many forms of cancer. The main target of Etoposide is Topoisomerase-II, hence the name Topoisomerase-II Inhibitor. But its low solubility causes slow and less effective therapeutic response in patients, so to overcome these drawbacks, the liposomal nanoparticles are being used owing to their large surface area and ability to deliver drugs through cell membrane. ETP loaded Liposomal, prepared by “Thin Film Hydration” method, were characterized with the help of different characterization techniques. The Release Kinetics of particles was evaluated by Drug Diffusion method, the slow release of drug from nanoparticles. ETP-LIP showed significantly improved properties as compared to Blank Liposomes and Etoposide.

Improvement of ETP-LIP with attachment of proteins which have their receptor on cell surface, would make them target specific with minimal exposure to normal cells and with enhanced activity so a small amount of drug results in more toxicity in cancerous cells.

Key Words: *Etoposide, Liposome Nanoparticles, Topoisomerase-II, Cytotoxicity, Anticancer.*

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Chapter 1 INTRODUCTION

1.1 Objective

The research has been planned and performed in two parts. The first part of the research emphases on the formulation and characterization of an anti-cancer drug delivery vehicle, for targeted delivery to the specific cancer sites. The drug selected for the study is a widely used anticancer drug Etoposide, and its liposomes are synthesized according to Thin Film Hydration method, which has already been reported in our established work. Then different aspects of the nanoparticle formulation are characterized using standard tests, ultimately making them equipped for the in-vitro & in-vivo analysis.

The second part of this project is more elaborate, which focuses on the in-vitro analysis of anticancer activity of our liposomes. The in-vitro treatment response of the etoposide loaded liposomes was determined through cytotoxicity assay and biocompatibility assay, to observe their efficacy and biocompatibility in cultured cells. The results of these in-vitro assays allow us to calculate the precise dosage and amount of liposomal formulation employed for in-vivo administration for future purpose.

1.2 Nanotechnology

Nanotechnology is a field regarding the manipulation of matter on Nano level, with at least one dimension sized from 1 to 100 nanometers. The nanotechnology was first proposed in 1959 by renowned Noble laureate Richard Feynman in a lecture 'There's Plenty of Room at the Bottom', in which he explained the possibility of synthesis of particles via direct manipulation of atoms. Japanese researcher Norio Taniguchi, in 1970, defined nanotechnology for the first time(L. Zhang et al., 2008).

1.2.1 Advancement of Nanotechnology

The application of Nanotechnology for synthesis of novel products has been going all over the world. The ancient Romans used to color glass with shades of mauve and yellow by using different concentrations of gold and silver (Daniel & Astruc, 2004). Gold and silver nanoparticles were also used for aesthetic purpose in the famous Lycurgus cup from the 4th century, which is now placed in the British museum (Barber & Freestone, 1990). Similarly, in

Middle Ages, colloidal silver and gold nanoparticles were used to produce bright colored stained windows, mostly red and purple in European cathedrals. For example, in Notre Dame, the red and purple color of the rose window of cathedrals is due to presence of gold nanoparticles (Dreaden et al., 2012). The technique of nanoparticle synthesis was studied in the 9th century by Muslim scientists, who carried out the reduction of metal oxides upon heating at high temperature which were already deposited on ceramic surfaces(Daniel & Astruc, 2004; Padeletti & Fermo, 2003). This technique of glass coloring was further refined in 15th and 17th century by using precipitates of different colloids added to the glass (Daniel & Astruc, 2004). The first ever documented chemical synthesis of metal nanoparticles was performed in 1857 by Michael Faraday(Turkevich et al., 1951) who reduced solution of chloroauric acid with carbon disulfide to obtain deep red colored gold nanoparticle solution, and then by Zsigmondy in 1906 , who reduced chloroauric acid in the presence of formaldehyde to obtain monodisperse gold solutions. Zsigamody's method was then modified in 1951 through Turkuvish method (Alp et al., 2017)(Turkevich, Stevenson et al. 1951) that involves chloroauric acid reduction in the presence of sodium citrate to synthesize gold nanoparticles. This method has also been employed for the synthesis of silver nanoparticles.

1.2.2 Role of Nanotechnology in Clinical Therapeutics

The nanotechnology has a prominent impact on clinical therapies and therapeutics in the last decades(Hu et al., 2010) and enormous advancements have been done in developing the field of nanomedicine in cancer studies to detect, diagnose and effectively treat cancerous tissues (Babu et al., 2013).

Nanomedicine as per national institute of health is a formulation of drug whose end product's size is less than a micron(Babu et al., 2013). Nanomedicine has gained much advantage due to its ability to overcome biological barriers, enhances the bioavailability of drug(Lavan et al., 2003), effective and targeted hydrophobic therapies. (Kumari et al., 2016).

1.3 Cancer and Chemotherapeutics

Cancer is one of the leading causes of death worldwide. It is a group of diseases that affects millions of people all over the world irrespective of age group and sex. The chance of getting cancer in one's lifetime is one out of every two men and one out of every three women. (Popova et al., 2019)

Its treatment is always dependent on the type of tumor, place and stage at the time of diagnosis. It ranges from surgery, chemotherapy, radiation therapy and sometimes combination of afore mentioned therapies. (Kumari et al., 2016) Chemotherapy, always the first-line treatment for cancer, is intravenous administration of drugs. The drug circulates throughout the body ultimately destroying cancerous tissue. (Saroj & Rajput, 2018)

1.3.1 Adverse Effects of Cancer Chemotherapeutics

The hydrophobic nature of most of the cancer chemotherapeutics limits their administration at high dose (Lu et al., 2007). Most of the chemotherapeutics have low molecular weight and so are easily excreted from the body soon after administration and so a high concentration dose is required, thus a high toxicity it causes (Singh et al., 2011). Furthermore, chemotherapeutics is non-targeted and cause damage to healthy tissues as well. The side effects it causes include suppression of bone marrow, sloughing of the epithelial cells of alimentary canal and the most common and most unwanted side effect in all patient subjected to chemotherapy is increased hair loss also known as alopecia (Picart et al., 2002).

1.4 Applications of Nanoparticles in Medicine

Drug delivery systems (DDS) such as nanoparticles of lipids or polymers can be synthesized to enhance the pharmacological and therapeutic properties of drugs administered parenterally. (Shi and Wan et al. 2014)

The nanoparticles are the small unit whose dimensions almost resembles to the building blocks of biological macromolecules such as proteins and DNA, this feature give a benefit to nanoparticles of being used for therapeutic purpose. Surface functionalization of nanoparticles can be done by various functional groups, signaling molecules, targeted molecules to make it target specific.(Amendola & Meneghetti, 2009) It can also be made biocompatible by binding with various functional groups and it is conjugated with drug as drug delivery vehicle. The surfaces of nanoparticles can be modified in such a manner so as it can bind to various functional groups that defines the fate of the nanoparticles that where should it be targeted (Kuo & Lee, 2015).

One of the biggest applications of using nanomaterials as biomedicine is that it has an internal core or void where the drug or the material to be targeted is encapsulated. So not only the toxicity caused by the drug is minimized but also sustained release of the drug is achieved (Kuo

& Chen, 2015; Yavuz et al., 2018). Nanoparticles encapsulate radio labelled molecules and other small molecules in its internal core or void to be used in imaging techniques. Such molecule encapsulated by nanoparticles do not cause harmful effects in the rest of the body due to its target specificity and is also biocompatible (Ghasemiyeh & Mohammadi-Samani, 2018).

The most important property of using nanoparticles in medicine and diagnostics is its biocompatible nature. The outer surface of nanoparticles is modified by binding small functional group molecule or encapsulating it with polyethylene glycol (PEG) and other such chemicals to make it biocompatible and so it does not provoke immune reactions and so other inflammatory processes as well and is considered as self-molecule (Bhatt et al., 2018).

Surface functionalization of nanoparticle with small functional group molecules or with other ligands make nanoparticle highly targeted, also the controlled and sustained release of drug is because of the surface group attached. (Lian & Ho, 2001a) Furthermore, the functionalization has a lot to do with the bio-distribution of drug and plays an important role in its pharmacokinetic behavior. Nanoparticle surface modified by small functional group enhance their role in the mode of excretion of nanoparticle from the body and its bio-distribution gives an idea of the type of clearance the nanoparticle follows (Bharali et al., 2009).

1.4.1 Nanoparticle Based Drug Delivery Vehicle for Cancer Treatment

Drug delivery system based on Nanoparticle increases therapeutic value and decreases side effects of the drug payloads by improving their pharmacokinetics (Peer et al., 2007). It also results in better cell penetration and preservation effect caused by permeable tumor vasculatures for better drug increase at the tumor sites. (R. X. Zhang et al., 2018) All of the above mentioned benefits have made nanoparticles an efficient and promising tool instead of traditional chemotherapy, where side–effects of drug's toxic agents cause damage to healthy tissues surrounding the tumor eventually resulting in dose-limiting side effects. (De Nicola et al., 2017)

Nanoparticles positively modify the bio-distribution of anticancer drug by increasing its therapeutic efficacy, pharmacological properties and decrease nonspecific toxicity of potent anticancer drugs, a major problem with traditional mode of treatment, due to its higher biocompatibility, protection of nucleic acids from degradation in cellular environment, and

delivery of therapeutic agents to cancer cells in vivo mark nanoparticles, “the ideal delivery vehicle” (Lu et al., 2007).

1.4.2 Anti-cancer Nano-medicines in Practice

Two well-known nano-formulations that are approved for cancer treatment by FDA-US are Doxil and Abraxane (Bharali et al., 2009). Doxil® has been derived from doxorubicin and has much higher therapeutic value than doxorubicin (Sakai-Kato et al., 2012; Yoo et al., 2002). Abraxane® is a nano-formulation of paclitaxel and is used for the metastatic breast cancer’s treatment (Moreno-Aspitia & Perez, 2005). Other nano-formulations used for cancer treatment are DaunoXome® a liposomal formulation daunorubicin, DepoCyt® (Jaeckle et al., 2002), a nanoformulation for cytarabine and ONCO-TCS(L. Zhang et al., 2008), a nanoformulation of vincristine(Hu et al., 2010).

1.5 Liposomal Nanoparticles

The word, “liposome” is a combination of two Greek words, ‘lipos’ and ‘soma’ meaning fat and body respectively. Liposomes consist of a central aqueous compartment encapsulated by lipid bilayers. These lipid bilayers are composed of natural or synthetic lipids. (Lian & Ho, 2001a)

Liposomes are of multiple types, either unilamellar/multilamellar, with varying degree of sizes from smaller to larger in diameters ranging from nanometers to micrometers. Liposomes are formed when naturally occurring or synthetic phospholipids or other hydrophilic and hydrophobic molecules having specific properties are hydrated. (Chen, Zhu et al. 2013) The acyl chains of phospholipids have hydrophobic properties results in formation of shells. There are many forces at molecular levels present in liposomes which are hydrogen bonds, van der Waals forces and other electrostatic attractions (Pentak, 2014).

Liposomes are being used as Nano-carriers, capable of transporting and delivering both hydrophilic and hydrophobic drugs with integration of Hydrophilic compounds in the central aqueous compartment of the liposome, whereas hydrophobic compounds/molecules are trapped in the lipid bilayers as shown in Figure 1 . The targeted/site specific delivery of drugs by Liposomes makes them beneficial in the treatment of malignancy or other syndromes (Pimple et al., 2012).

There are also countless other gains in using liposomes as drug transporters. Firstly, the ease in changing of properties, which have effect in different sizes, targeted delivery of drugs/ toxic agents into specific tissues or cells and release of drug in an orderly manner (Duan et al., 2017; Kuo & Chen, 2015). Liposomes are produced from biocompatible components making them biodegradable and less toxic (Tang et al., 2010). Secondly, the enhanced drug solubility and improved opposition to biological and chemical degradation during storage and in patient's body are also the perks of liposomal drug delivery method (Athawale et al., 2014). Further advantages include a better therapeutic index, higher efficacy, along with a decrease in direct contact of sensitive/normal cell and tissues to the toxic drugs for cancerous cells and tissues. Liposomes possess extraordinary encapsulation efficiency and enhanced drug loading capacity with further changes in surface ligands present on the liposomes can result in better usefulness and higher active site targeting of the drug.(Kanásová & Nesměrák, 2017)

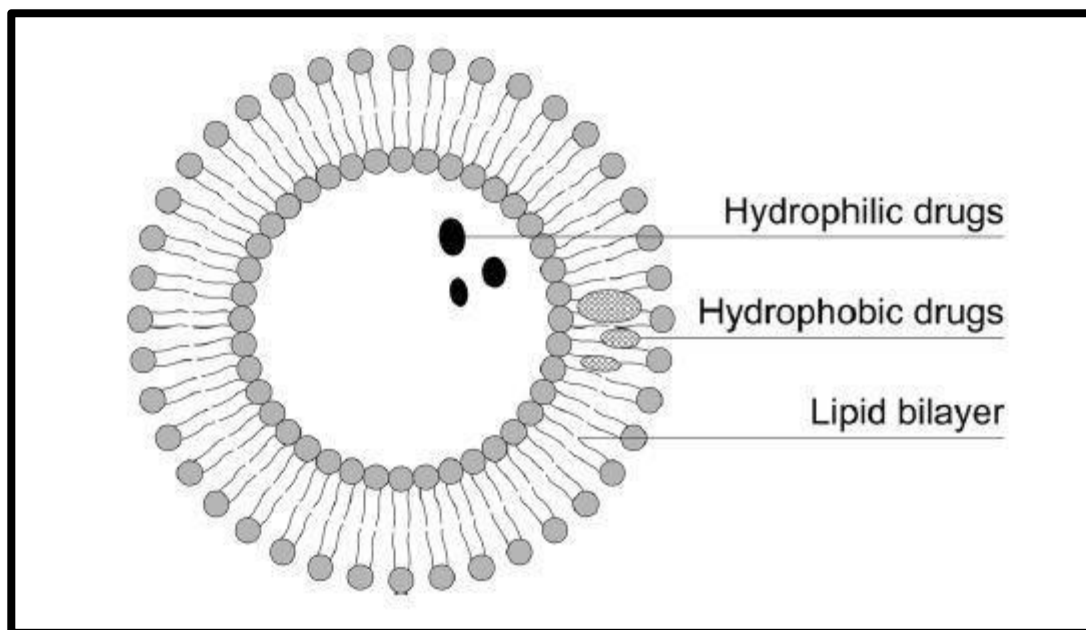

Figure 1:Liposome carrying hydrophilic & Hydrophobic drugs

Chapter 2 Literature Review

2.1 Etoposide: A Widely Used Anticancer Drug

Etoposide is a derivative of podophyllotoxin, is a chemotherapy agent and an immune system suppressant with antimitotic and antineoplastic properties. Etoposide (a semi-synthetic podophyllotoxin) derived from Podophyllum peltatum roots. It has therapeutic activity against a variety of cancers; Lung Cancer, Lymphoma, Testicular Cancer, Liver Cancer and Leukemia etc. It is among few of those anticancer drugs that can be used against Metastatic cancers.

The bioavailability of this drug varies in range of 24-74% in oral mode of drug administration and 1.5 hours of terminal half-life by intravenous route of administration. The general dosage, for the patients in last stages of lung cancer, is 600mg/m2 after 24 hours for three weeks.

Etoposide was originally developed and continues to be used for chemotherapy, either alone or in combination with other agents. Because of the antineoplastic property of drug, the pharmaceutical grade is reportedly effective in the treatment of various types of malignant neoplasms such as those of stomach, testis, brain and lungs etc.

The conventional parenteral therapy results in discomfort and pain to the patients because of its continuous and lengthy administration time of over 24–34 h. The lipophilicity and solubilizers available in the conventional drug found in market are major hurdles in the formulation of Etoposide. These solubilizers are associated with adverse side effects such as bronchospasm, hypotension and anaphylaxis, etc. Due to its poor bioavailability, low solubility and drug resistance, it is imperative to develop a drug delivery system to overcome these disadvantages and advance the clinical therapy effectiveness (Wang et al., 2014).

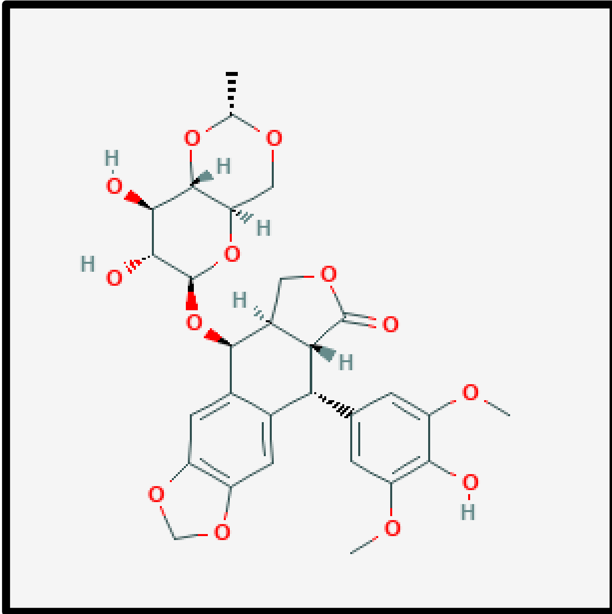

Figure 2: Chemical Structure of Etoposide

2.1.1 Mechanism of Action

Etoposide (ETP), deoxyribonucleic acid (DNA) topoisomerase 2 Inhibitor, works by stopping the cell replication in the dividing cells. The main stopping point in the DNA replication due to Etoposide is in the G2 stage of the cell cycle in mammalian cells. These topoisomerase 2 is an enzyme present in the DNA replication responsible for the deviations in DNA structure by cleaving and bonding/rejoining of the phosphodiester bond which is the backbone of DNA strands in normal cell as shown in Figure 3 (Montecucco et al., 2015).

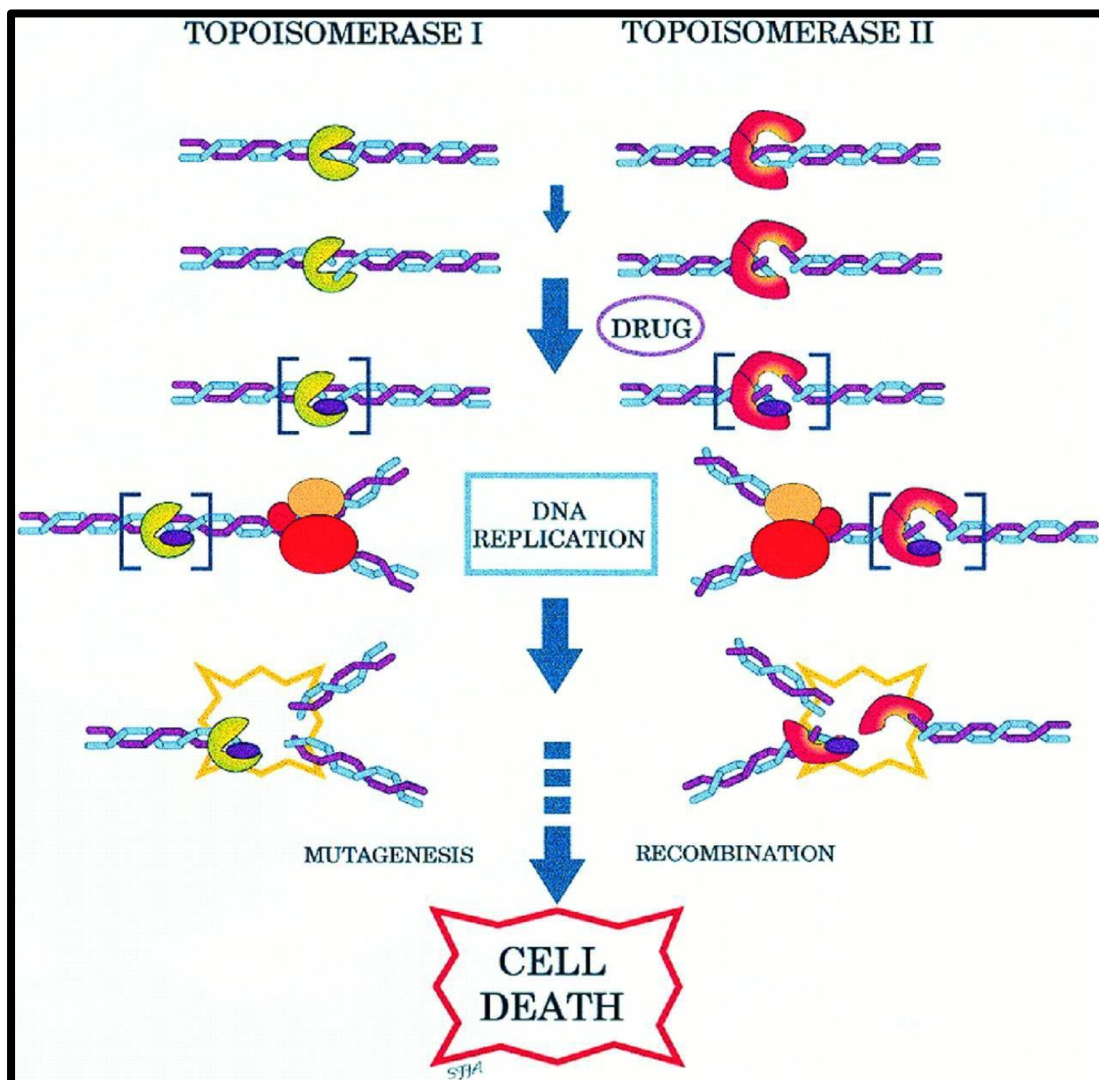

Figure 3: Representation of Molecular Mechanism of Etoposide.

2.1.2 Limitations of Etoposide Chemotherapy

The conventional/usual parenteral therapy results in discomfort and ache to the patients due to its continuous Intra-Venous administration of over 24-34 hours. The Lipophilicity and Solubilizers used in the most of the conventional formulations of Etoposide are the major drawbacks in the conventional therapy. These solubilizers are the reason for many severe side effects such as bronchospasm, anaphylaxis and hypertension etc. (Narvekar et al., 2014)

Further, the conventional process of etoposide administration also deals with short half-life in-vivo and toxicity. These problems have commended the need for development of the new/specialized systems having the ability of selective delivery of one or more

chemotherapeutic/toxic agents to cancer cells, without posing any danger to the normal cells (Akbarzadeh, Rezaei-Sadabady, et al., 2013).

The work on these specialized systems have been in progress over the last two decades, during this time a number of strategies have been developed. There are numerous possible techniques which have been investigated to find a comprehensive and active means of payload delivery of anti-cancer drugs to the target sites, a few of these techniques;

1. microcapsules,
2. microspheres,
3. polymeric nanoparticles,
4. liposomes,
5. cationic liposomes and
6. emulsions etc. (Akbarzadeh, Rezaei-sadabady, et al., 2013)

2.2 Need for an Efficient Drug Carrier

In all of the Novel drug delivery methods, the most attention is received by Nanoparticles. For the cancer treatment. In a tumor, the malfunctioned lymphatic system and in blood vessels the Angiogenic factor and nutrients are not supplied in an adequate amount. The smaller size particles such as sub-micron sized particles accumulate at tumor site and cause their effect "Enhanced Permeability & Retention (EPR)". This kind of targeting and accumulation is known as Passive Targeting which is more beneficial in achieving the desired results.

Polymeric nanoparticles & solid lipid nanoparticles etc. are designed for successful Passive Targeting of tumors. The polymeric drug delivery system has a number of problems associated with it. a few of them are mention below, scale-up, less physical stability, and polymeric cytotoxicity are major drawbacks of the polymeric drug delivery systems. Whereas, Drug delivery using Lipids have benefits, which most of the other systems lack, the advantages are stability, protection from early drug degradation & metabolism, controlled drug release and exceptional permissibility and less severe side-effects.

Solid lipid nanoparticles (SLN) are known as excellent carriers of hydrophobic/poorly water-soluble drugs. Here, Etoposide which is hydrophobic anticancer drug/agent, has been choosen for lipid-based delivery systems. Due to the high cholesterol needs of tumor cells they have high uptake of lipoproteins (low density) and this uptake is the base of lipid drug delivery system.

The Etoposide entrapment in the liposomes results in higher drug specificity and reduction in the off –site targeting of cytotoxic drug. The liposomal nanoparticles have benefits over other methods of drug delivery such as,

i. Extremely Small size
ii. High surface area to volume ratio
iii. high stability,
iv. permeability

2.3 Significance of Liposomal Nanoparticles

Liposomes are frequently used to simulate biological membranes and for the targeted drug delivery systems to specific sites. They are basically spherical/round structures and are classified into three types,

1. Small unilamellar vesicles (SUV)
2. Large unilamellar vesicles (LUV)
3. Multi lamellar vesicles (MLV)

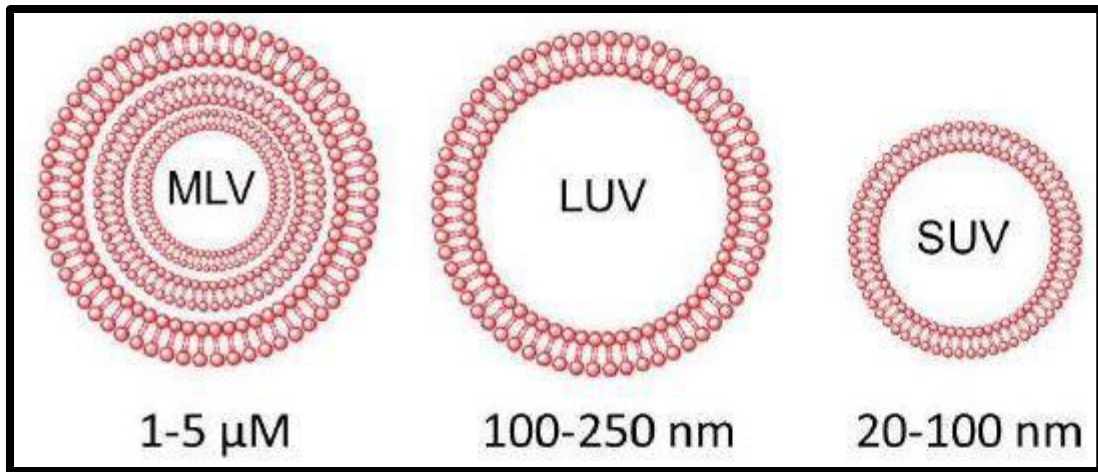

Figure 4: Types of Liposomes by size

The Cholesterol molecules used in the preparation of liposomes is present in phospholipid's bilayer thus helping to improve properties by causing an improvement in distance between the choline head groups, increases the hydrogen bonding and other electrostatic interaction. This eventually stables the membrane and depresses its water permeability and permeability to other molecules (Lian & Ho, 2001a). The mechanism of action is shown below in the Figure 4: Mechanism of action of Liposomes.

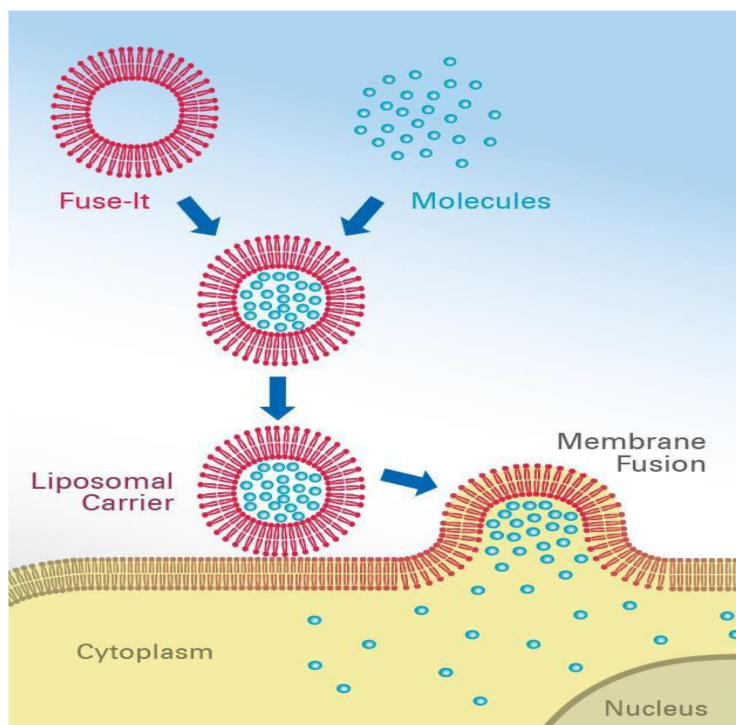

Figure 4: Mechanism of action of Liposomes

Nanoparticles have been of great importance from the last few decades. Because of their extremely small size, they form a bridge between the large bulk molecules and the small entities at molecular and atomic level and it differs in physical properties from its bulk form. Among all nanoparticles used for pharmaceutical and for biomedical purposes, liposomal and this unique property is because of high surface area to volume ratio. This is the reason that its different chemical, physical and electrical properties changes due to change in surface area, charge distribution and composition of nanoparticle.

2.4 Synthesis of Liposomal Nanoparticles

Lipid nanoparticles are synthesized by following different methods such as hot and cold high pressure homogenization, solvent evaporation, micro-emulsion formation technique, and ultrasonic solvent emulsification etc. (Ghasemiyeh & Mohammadi-Samani, 2018) as shown in following **Error! Reference source not found.** (Ong et al., 2015) . (Ghasemiyeh & M ohammadi-Samani, 2018). Here, Lipid Film Hydration method is preferred for the liposomes synthesis.

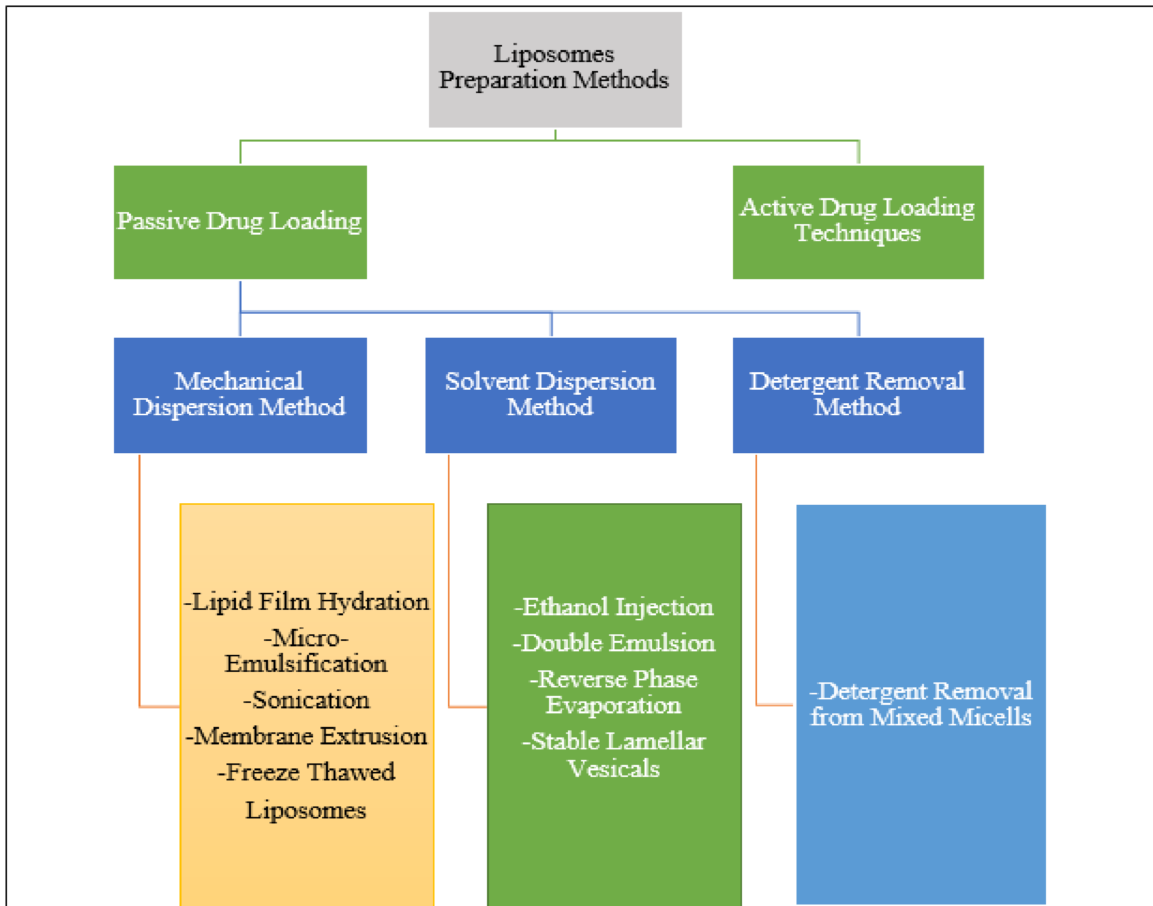

Figure 5: Methods for Liposomal Synthesis

2.4.1 High pressure homogenization technique

2.4.1.1 Hot (High Temperature) High Pressure Homogenization

- The heating of lipid phase at 90 °C,
- Dispersion of this lipid phase in aqueous phase which has surfactants with same temperature.
- Homogenization at 90 °C of pre-emulsion under 3 cycles
- The resulted emulsion is cooled down to room temperature to prepare SLNs or NLCs.

2.4.1.2 Cold High-Pressure Homogenization

- In this method, the solidification is achieved by cooling the melted lipid phase and then ground to produce lipid particles.

- The lipid micro particles are then dispersed in cool aqueous phase including surfactants to form pre-suspension.

- Homogenization of pre-suspension is achieved in 5 cycles of homogenizer at room temperature. The pressure here is 1.5×108 Pa.

2.4.2 Solvent Evaporation Technique

In this method,

- lipid is dissolved in an organic solvent such as acetone/ chloroform (organic phase).

- Then the organic phase is added to the aqueous phase (surfactant solution in water) under continuous stirring in Rotary Evaporator at 50-60 °C depending upon the lipid used.

- The stirring will be continued until the organic phase is completely evaporated.

- Then resulted emulsion is sonicated to obtain smaller size and cooled to solidify lipid nanoparticles.

2.4.3 Micro-Emulsion Formation Technique

In this method,

- lipids are melted at appropriate temperature and aqueous phase containing surfactants are heated up to same temperature.

- Then the hot aqueous phase will be added to the melted lipids under stirring at the same temperature.

- The hot oil in water micro emulsion is dispersed in cold water at 1:50 ratios to solidify lipid nanoparticles.

2.4.4 Ultrasonic Solvent Emulsification Technique

In this method,

- lipid phase is dissolved in an organic solvent such as dichloromethane and heated up to 50 °C.

- After partial evaporation of dichloromethane, the aqueous phase is added to the organic phase under stirring at 50 °C.

- Obtained emulsion is sonicated for appropriate time and finally cooled in an ice bath to solidify lipid nanoparticles.

Chapter 3 MATERIALS AND METHODS

3.1 Experiment Design

3.1.1 Materials

All used chemicals were purchased from Sigma-Aldrich (USA), unless stated otherwise. These chemicals were 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Cholesterol, Etoposide, Chloroform, Phosphate Buffer Saline (PBS) Tablets and distilled water.

3.1.2 Synthesis of Blank Liposomal Nanoparticles

Blank Liposomal nanoparticles were synthesized by Thin Film Hydration. In this method, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and Cholesterol were mixed together in ratio of 4:1 in 10 ml of chloroform and the solvent was evaporated at least 10 °C above the phase transition temperature of DPPC which is 41 °C with the help of Rotary Evaporator. This process resulted in the formation of Thin lipid film in flask. The hydration of lipid film with 10 ml of Phosphate Buffer saline solution was performed. Then, the obtained sample was sonicated for 40 min to decrease the size of synthesized liposomes. (K & P, 2015)

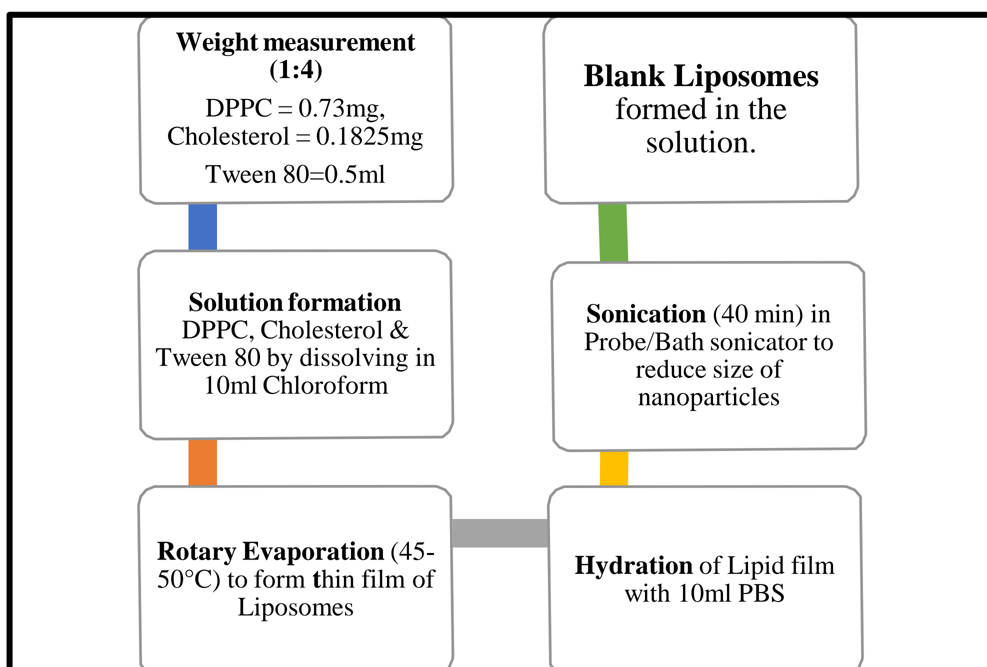

Figure 6: Method for production of Blank Liposomes

3.1.3 Synthesis of Etoposide loaded Liposomal Nanoparticles

For the synthesis of Etoposide loaded Liposomal Nanoparticles, the Film Hydration Method was employed as described in the above paragraph except the addition of drug. 1 mg of Etoposide drug was dissolved in 10ml of chloroform and 500 µL of etoposide solution was dissolved in DPPC and Cholesterol solution of chloroform. Then, Rotary evaporation, Hydration and Sonication was performed in the conditions same as for blank liposomes. (K & P, 2015)

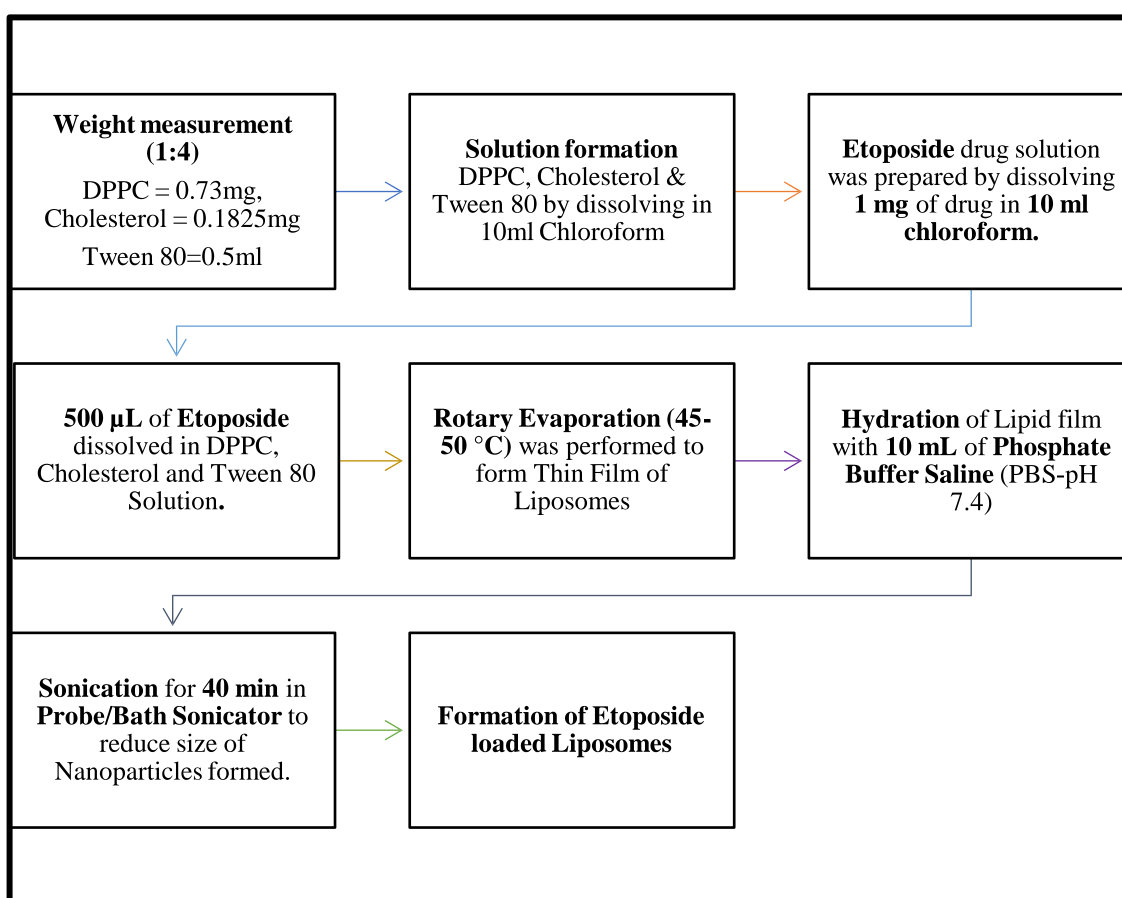

Figure 7: Protocol for the production of Etoposide Loaded Liposomes

3.2 Characterization of Etoposide loaded Liposomal Nanoparticles

The characterization of Etoposide Liposomal nanoparticles was performed to assess and investigate their particle size and surface charge, drug encapsulation and release efficiency, then it was proceeded to further characterization tests, explained as follows.

- Spectral Characterization

- Structural Characterization

3.2.1 Optical Characterization

Etoposide, Blank Liposomes (without drug) and Etoposide loaded liposomes were tested for their optical properties through following techniques.

a) UV-Vis Absorption spectroscopy

b) Fourier Transform Infrared Spectroscopy

3.2.1.1 UV-Vis absorption spectroscopy (UV-Vis)

The most used technique in laboratories is UV-Vis absorption spectroscopy. A beam of light is passed through a sample placed inside a cuvette. A specific wavelength of light is absorbed by the specific compounds in sample. While some wavelengths are transmitted through the sample, hence, the extent of absorption is measured. First, a cuvette filled with only solvent (Reference) is placed inside hood and absorption is recorded. After this, a second cuvette filled with sample is placed in hood and absorption spectrum is generated. The beam of light splits into two halves, one directed towards the Reference solution while the second one directed towards the second cuvette containing sample. An absorption spectrum is generated against whole range of wavelength. lambda max is the maximum absorption value at any given wavelength. It follows Beer Lambert Law, which is absorbance of sample is in direct relation with the concentration (molar) in the sample cuvette, this value of absorption is called molar absorptivity and is used for the comparison of spectra of different compounds (Lian & Ho, 2001b)(Kanásová & Nesměrák, 2017).

Beer-Lambert Law says

Figure 8: UV-VIS spectroscopy

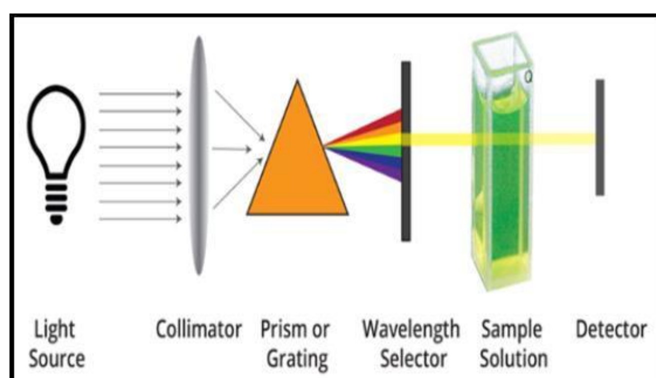

Figure 9: Mechanism of UV-Vis Spectroscopy

$$A = EcL$$

$$\text{Molar absorptivity } E = A / cl$$

A= absorbance,

c= sample concentration (moles/Litre)

L= length of light path through the cuvette (cm)

This Beer-Lambert law makes this technique of spectroscopy a useful tool for quantitative analysis.

3.2.1.2 Fourier Transform Infrared Spectroscopy

3.2.2 Structural Characterization

The following characterization techniques were carried out to determine the shape, size, morphology and structure of Liposomes.

a) Scanning electron Microscopy b) Surface Charge and Zeta Potential

3.2.2.1 Scanning Electron Microscopy

Particle structure and size was studied and analyzed by scanning electron microscope (SEM). A drop of liposomal nanoparticles was dropped on a glass slide which was further sputtered with a layer of gold as coating material to make the sample conductive for SEM analysis. (Zare et al, 2019)

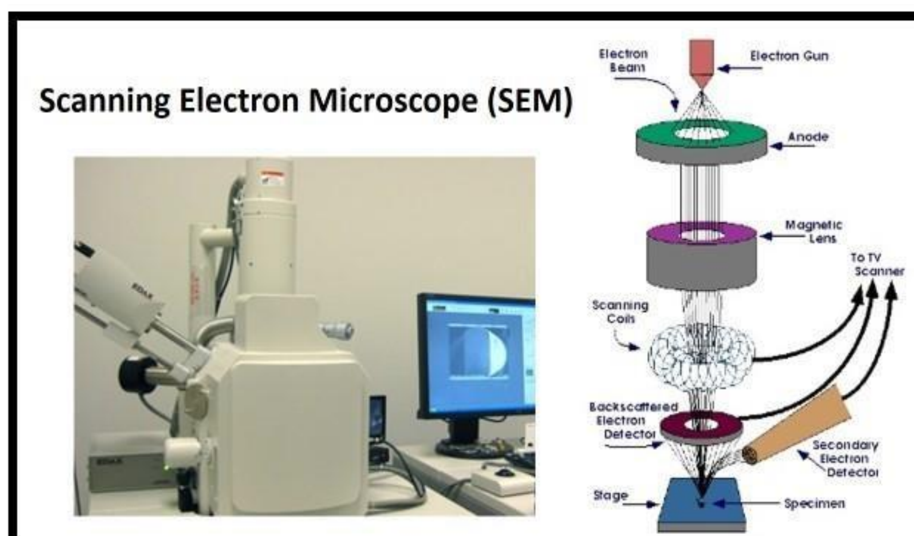

Figure 10: SEM and its mechanism

3.2.2.2 Surface Charge and Zeta Potential

Zeta potential (surface charge) of Etoposide loaded Liposomal nanoparticles was evaluated by Dynamic Light Scattering (DLS) using Zetasizer Nano (Malvern® Instruments Ltd.). The sample in liquid state was further dissolved in a solvent to suspend the particles- i.e., water. The Set Zero measurement was taken of sample in a cell. Sample dilutions of 1:100 were made with double-deionized water, followed by the particle size measurement at 25 °C. For mean particle size and zeta potential three batches of sample were analysed through zetasizer. (Pinto et al, 2014).

Figure 11: Zetasizer Nano zs (Malvern Instruments Ltd. UK)

3.2.3 Drug Encapsulation Efficiency

Drug encapsulation efficiency was calculated by comparing the values of UV analysis of Etoposide loaded liposomes with the standard curve obtained from the dilutions of Etoposide solution. An appropriate volume of sample was loaded in a mini-column centrifuge tube and then centrifuged on 4500 rpm at room temperature for the separation of Non-Entrapped drug from the entrapped drug. Non-entrapped drug was filtered through the centrifuge tube in supernatant. The UV spectral analysis of supernatant at 285 nm absorbance showed the absorbance value of Non-entrapped drug. The concentration of unknown drug in supernatant was calculated by using R equation from Standard Curve Graph.

Drug Encapsulation Efficiency was calculated by the following formula.

% Encapsulation efficiency = (Entrapped drug / total drug added) * 100

3.2.4 Drug Loading Capacity

After the determination of Encapsulation efficiency, the sample present in centrifuge tube was then filled with PBS buffer to make up the total volume up to 5ml in tube. This process was performed to wash the Liposomal Nanoparticles. Centrifugation of sample was carried out until 500 microliter sample was left in column tube. The supernatant was separated.

This process was repeated this time with Distilled Water instead of PBS. The remaining 500 microliter solution in mini-column centrifuge tube was dried overnight to weigh the liposomal nanoparticles.

Loading Capacity was calculated by using the following formula.

%Loading Capacity = (Amount of Entrapped Drug/ Weight of Nanoparticles) * 100

3.2.5 Cytotoxicity Assay (MTT Assay)

The MTT analysis is the most common type of Viability Assay performed involving cell lines. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a dye used for the in-vitro cell proliferation measurement. Tetrazolium salts are widely used in cell biology for determining the metabolic activity of cells ranging from microbial origin to mammalian cells. For this purpose, HuH-7 cells were cultured, using DMEM Supplemented with 10% FBS

and 1% Penicillin-Streptomycin solution, at 37°C for 24 hours. When the cultures achieved desired confluence, 10,000 cells were plated in each well of a 96-well culture plate. The plate was incubated in CO_2 incubator for 24 hours. The concentrations of the compounds were prepared by dissolving them in deionized water and making subsequent dilutions. The drug dilutions along with the solvent control were loaded in the 96-well culture plate with the final volume of 200ul per well and the plate was incubated for another 24 hrs. MTT was prepared in the medium to a final concentration of 5 mg/ml. The plate was then inoculated with 15ul of prepared MTT in each well and incubated for 3 hours at 37°C, until intracellular purple formazan crystals became visible under microscope. Then MTT was removed and solubilizing solution i.e., 150 µl DMSO was added in every well. The Incubation at room temperature was done for few minutes while pipetting up and down the materials of each well, so that the cells had been lysed and purple crystals had been dissolved. The plate was covered in foil to protect from light and immediate measurement of the absorbance in Spectrophotometer was carried out at 550 nm wavelength. The experiment was performed in triplicates and the results are the average of the three experiments.

Chapter 4 RESULTS AND DISCUSSION

4.1 Visual Confirmation of Liposomal Nanoparticles

When liposomes were synthesized, the solution was turned into turbid one depicting the synthesis of Liposomes. (Figure 4.1)

4.2 Characterization of Liposomes

The characterization of liposomal nanoparticles was done with following mentioned techniques to evaluate and analyze their different properties.

4.2.1 UV-Vis absorption spectroscopy

UV-Vis absorption spectroscopy of ETP-LIP showed liposomal peak at 210 nm, while that of ETP is at 283 nm.

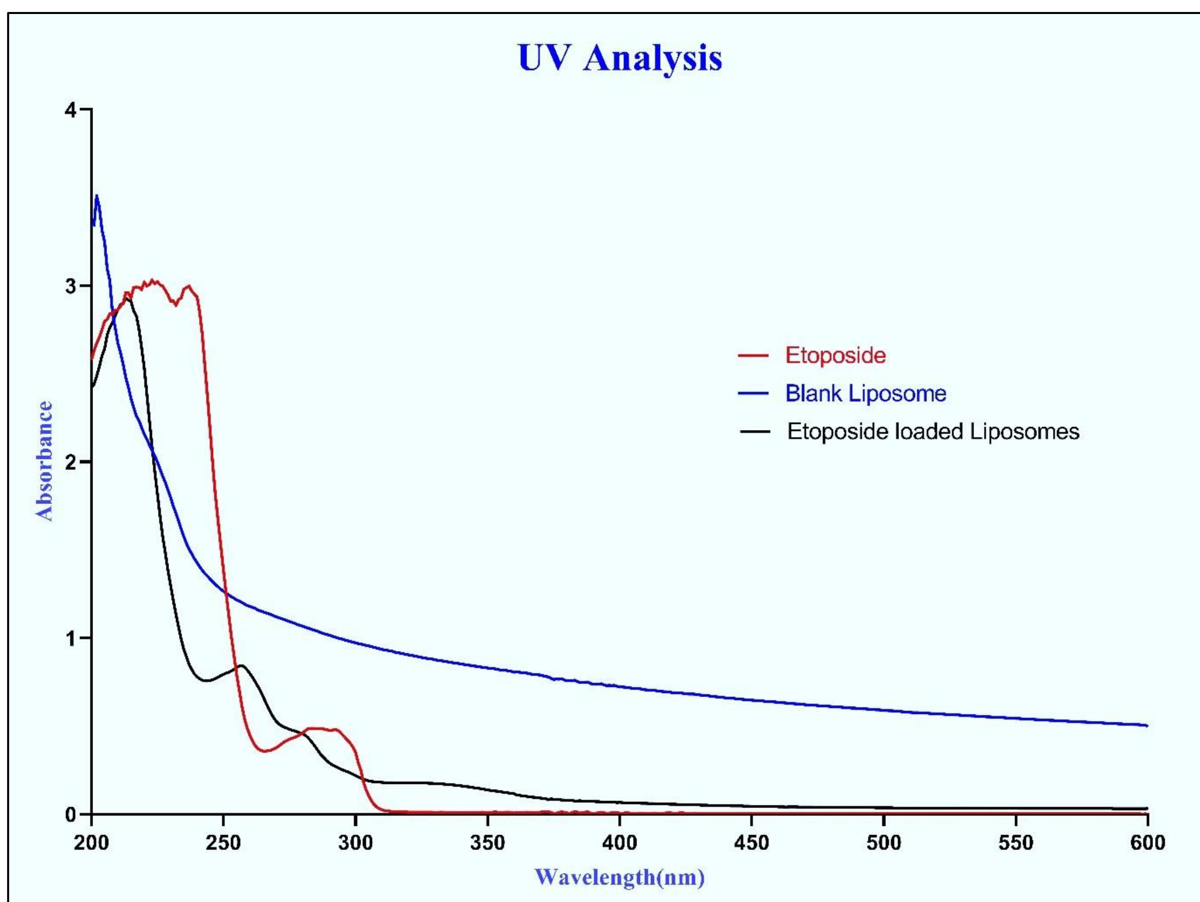

Figure 12: Comparative UV-Vis spectra of ETP, Liposomes and Etoposide liposome

4.2.2 Fourier Transform Infrared Spectroscopy

The FTIR spectrum of Cholesterol indicated peaks or bands at 2850/cm (CH stretch, Alkanes), 873/cm (Tri-substituted Aromatics). DPPC spectrum indicated peaks at 2919/cm (CH stretch, Alkanes), 1632/cm (R-NH2, Amines), 1115/cm (C-O stretch, Ether), 720/cm (RCH2CH3, Bending mode). PEG-6000 spectrum delineated peaks at 3429/cm (O-H stretch, Alcohol), 2923/cm (CH stretch, Alkanes) and 1638/cm (C=C stretch, Alkenes). In distinction, the spectrum of Blank liposomes and ETP-liposomes depicted the disappearance of cholesterol's 2850/cm (CH stretch, Alkanes) and 1736/cm (C=O) of DPPC. ETP-liposomes spectrum illustrated the appearance of 2917/cm (CH stretch), 2856 (CH stretch) and a new peak at 1257/cm (P=O) of phenol group of Etoposide drug. These observed differences in the spectrum of Blank liposomes and ETP-Liposomes indicate the incorporation of Etoposide in the liposomes.

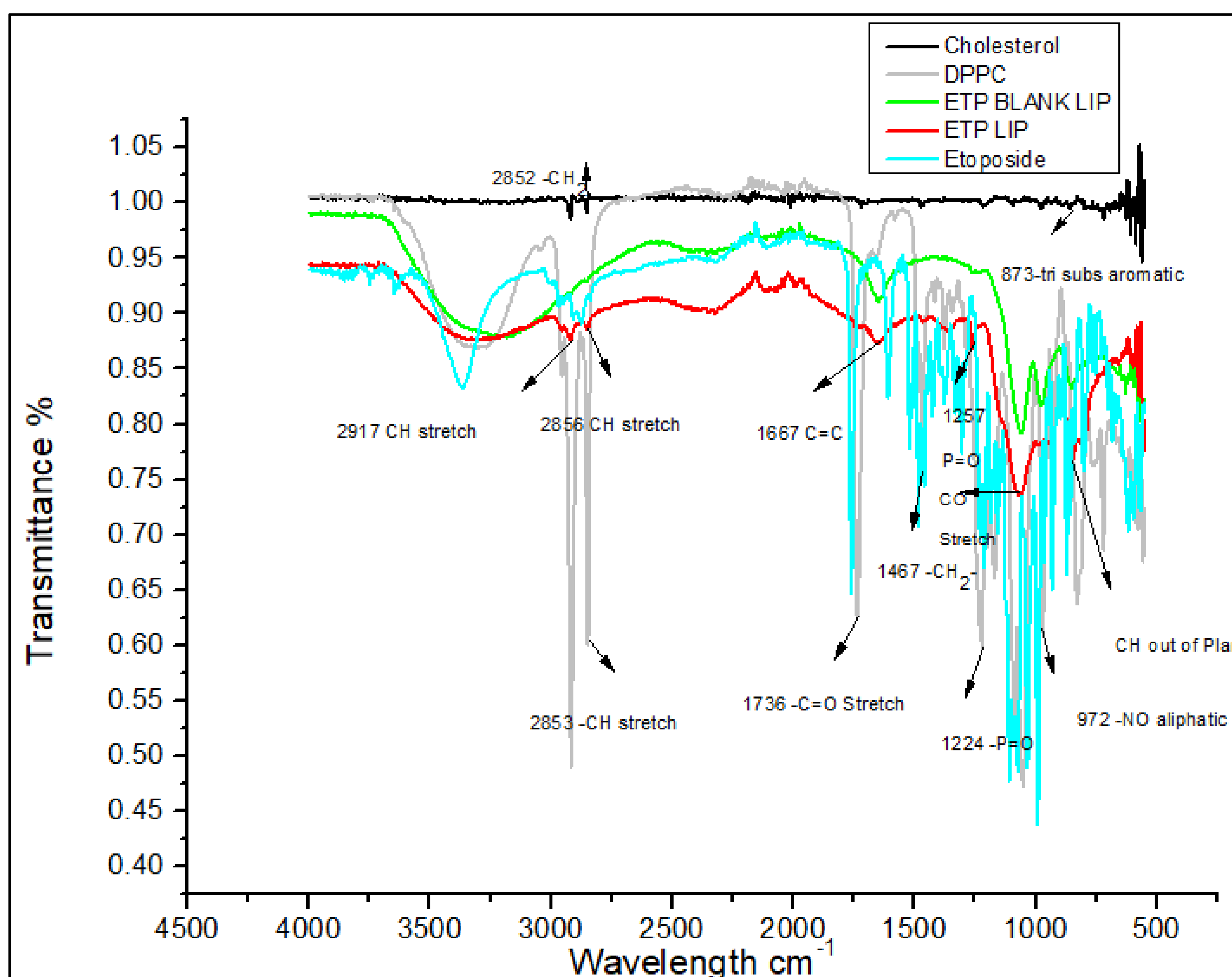

Figure 13: Comparative FTIR spectra of DPPC, Cholesterol, Blank LN Ps, ETP- LNPs

4.2.3 Particle Size and Area Distribution

The particle size was determined by the Scanning electron microscopy. (Figure 4.4) The particles ranged from 180 nm to 230 nm which is a considerable size for nanoparticles as it can cross cell membrane barriers.

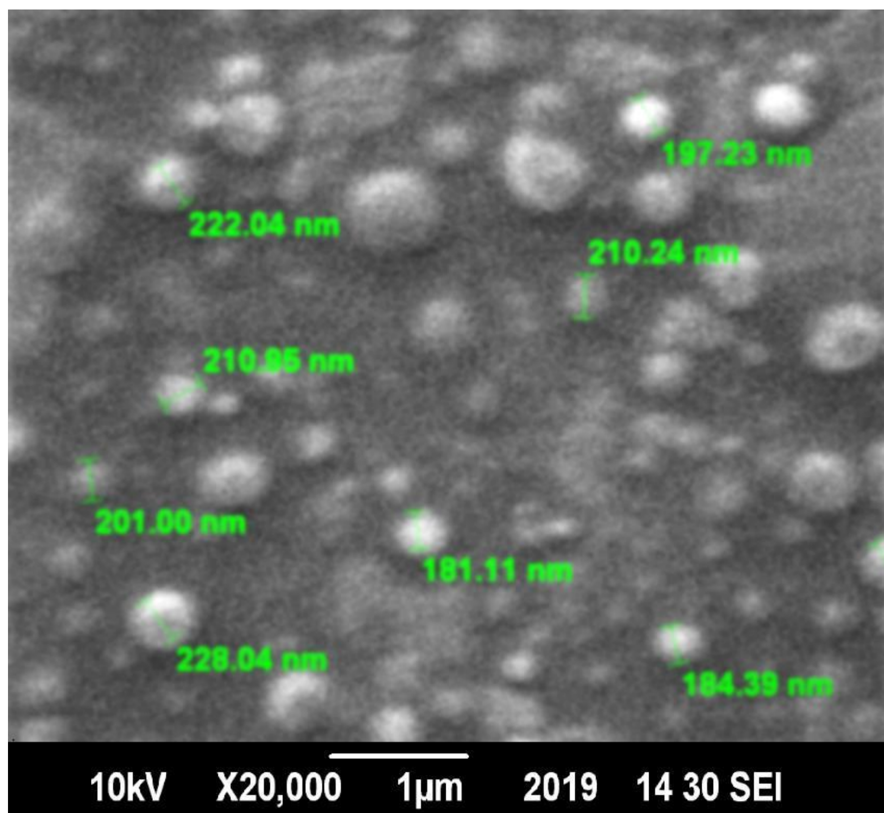

Figure 14: SEM Image of ETP Liposomes

4.2.4 Surface Charge or Zeta Potential

Zeta potential tells us about the stability of formulated liposomal nanoparticles. The value of zeta potential from +30 mV to -30 mV are considered as stable particles, whereas the potential of isoelectric point indicates less stability. Zeta potential or surface charge of ETP-LIP nanoparticles was evaluated by Dynamic Light Scattering (DLS) using Zetasizer Nano (Malvern® Instruments Ltd.). Zeta potential is the measurements of charge difference between the surface of nanoparticles and the medium around the particles. The stability of particles is larger when they are more positive or negatively charged. The zeta potential of Liposomal nanoparticles was found to be -14.5mV, which is very stable.

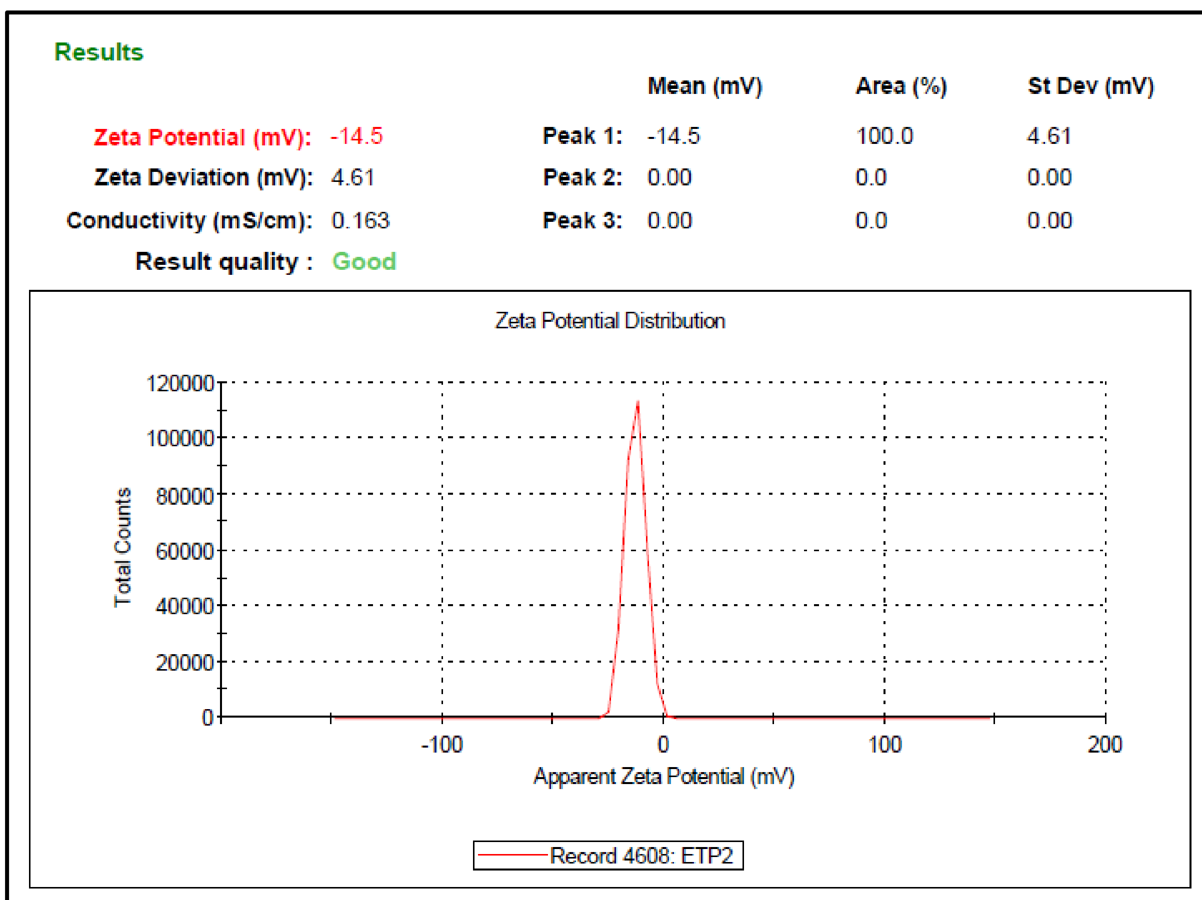

Figure 15: Zeta Potential of Etoposide Liposomal nanoparticles

4.2.5 Drug Release Efficiency

The release pattern of ETP-LIP nanoparticles at the desired site is of a great importance for formulating an ideal cancer-targeted drug delivery system, so in-vitro release studies were performed. The drug release analysis of nanoparticles was carried out using a UV spectrophotometer with empty nanoparticle solution used as control, over a time duration of 48h. The results were tabulated, and a cumulative drug release percentage graph was obtained using graphpad prism 6, as shown in figure 4.6. This graph shows little burst effect, with a prolonged sustained release of methotrexate from nanoparticles for 48 h. This is very helpful to eliminate the side effects of etoposide that are associated with its high doses and low retention time in the body (Cooper and Harirforoosh, 2014).

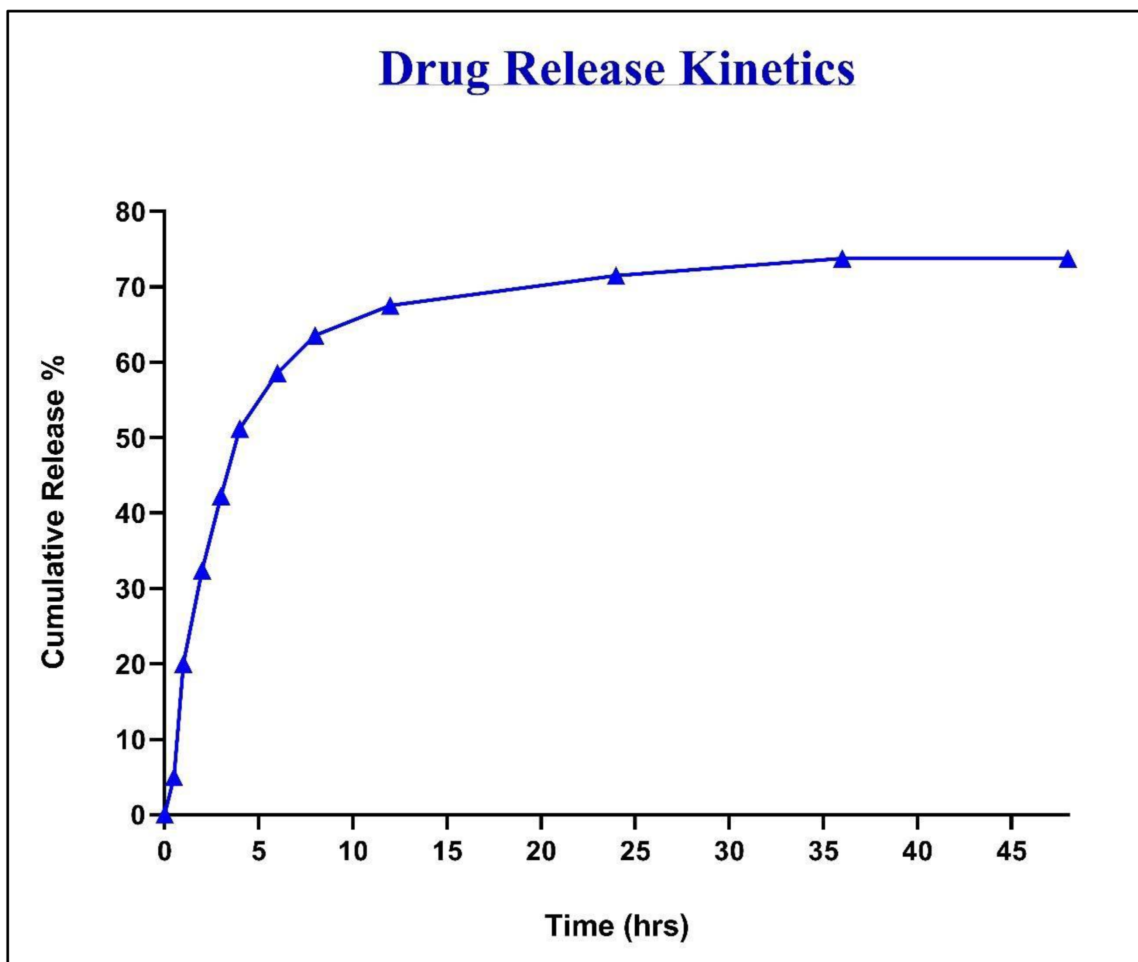

Figure 16: Cumulative Release % of Etoposide Drug over Time period of 48 Hours

4.2.6 Cytotoxicity Assay

The MTT analysis is the most common type of Viability Assay performed involving cell lines. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a dye used for the in-vitro cell proliferation measurement. Tetrazolium salts are widely used in cell biology for determining the metabolic activity of cells ranging from microbial origin to mammalian cells. For this purpose, HuH-7 cells were cultured, using DMEM Supplemented with 10% FBS and 1% Penicillin-Streptomycin solution, at 37°C for 24 hours. When the cultures achieved desired confluence, 10,000 cells were plated in each well of a 96-well culture plate. The plate was incubated in CO_2 incubator for 24 hours. The concentrations of the compounds were prepared by dissolving them in deionized water and making subsequent dilutions. The drug dilutions along with the solvent control were loaded in the 96-well culture plate with the final volume of 200ul per well and the plate was incubated for another 24 hrs. MTT was prepared in the medium to a final concentration of 5 mg/ml. The plate was then inoculated with 15ul of prepared MTT in each well and incubated for 3 hours at 37°C, until intracellular purple

formazan crystals became visible under microscope. Then MTT was removed and solubilizing solution i.e., 150 µl DMSO was added in every well. The Incubation at room temperature was done for few minutes while pipetting up and down the materials of each well, so that the cells had been lysed and purple crystals had been dissolved. The plate was covered in foil to protect from light and immediate measurement of the absorbance in Spectrophotometer was carried out at 550 nm wavelength. The experiment was performed in triplicates and the results are the average of the three experiments.

% Cell Viability in MTT Assay			
Concentration	**Etoposide Loaded Liposomes**	**Blank Liposomes**	**Etoposide Drug**
Control	100.00	100.00	100.00
50 µg/µl	65	88	4.11
100 µg/µl	47	59	4.11
150 µg/µl	41	59	0.03

Table 1:Values of Percent (%) Cell Viability

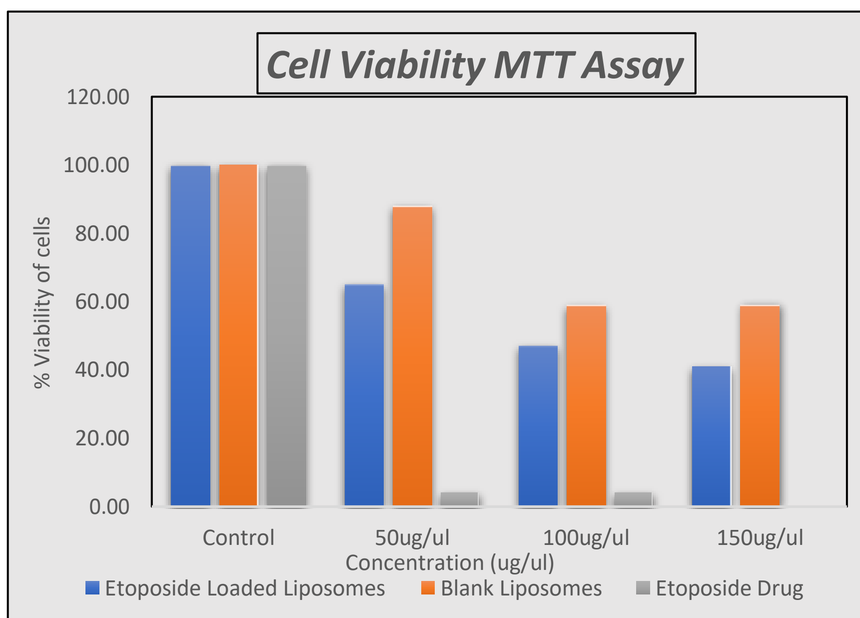

Figure 17: In-vitro Cytotoxicity on HuH7 Cells of ETP-Liposomes, Blank Liposomes and Etoposide

The afore-mentioned results were obtained from Spectrophotometric analysis after normalized interpretations. The normalization was done by the following mentioned formula. Absorbance values greater than the control indicates cell proliferation, while lower values suggest cell death or inhibition of proliferation.

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

*Abs= absorption

Chapter 5 Conclusion

After the synthesis of nanoparticles, successful synthesis is proved by different characterization techniques. Liposomes enhanced the Encapsulation efficiency, stability that resulted in the long-term Drug Release proved in Drug Release Study. This would result in high bioavailability and better therapeutic effects as compared to the simple drug. The % Cell Viability in Etoposide Loaded Liposomal Nanoparticles is considerably good but in comparison to Free drug's Viability, the Cytotoxicity of Nanoparticles is less. Which is in contrast to the proposed Hypothesis. It was assessed that this problem happened due to the lipid nature of MTT and Formazan used in this MTT assay, they get accumulated in the cell membrane giving the appearance of small granules and causes disturbance in the Cell count. These granules are later replaced by needle-like filaments produced by the continuous aggregation of MTT-formazan that later extrude through the cell plasma membrane by physical/chemical molecular aggregation mechanisms.

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