Effect of Etoposide loaded Nanoparticles in the Treatment of Advanced Liver Diseases

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Copyright Statement

Dedication

Dedicated to my beloved Parents

Acknowledgments

First and foremost, I am thankful to Allah Subhana-Watala, the Most Gracious and Most Merciful for His blessings given to me and to have guided me throughout this work. I could have nothing without Your priceless help and guidance. Whosever helped me throughout the course of my thesis, whether my parents, siblings, and friends were Your will, so indeed none be worthy of praise but you.

I am profusely thankful to my beloved parents for their love, prayers, caring and sacrifices for educating, preparing me for future, and raised me when I was not capable of walking and continued to support me throughout in every step of my life.

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بسم الله الرحمن الرحيم

Abstract

Advanced liver diseases (ALD) continue to pose significant health challenges due to their high global prevalence and limited currently available curative options, besides liver transplantation. Liver disease therapeutics include many substances that may have insufficient effectiveness and severe adverse effects, such as Etoposide, having toxic nature with low compatibility and solubility in an aqueous solution. Nanoparticle-based therapeutics emerged as an efficient and safe treatment option to minimize side effects. Etoposide used for ALD is more toxic with low biocompatibility and solubility in an aqueous solution which makes it less efficient. The purpose of the study is to use nanoparticle-based etoposide for the treatment of ALD by improving its biocompatibility and solubility which makes this target-based therapy less toxic and more effective. Rat models used in this study were introduced with CCL4 and Urethane co-administration to develop liver cirrhosis. The thin film hydration method was used to synthesize etoposide-encapsulated liposomal nanoparticles. The stability and stealth effect of liposomes were enhanced by polyethylene glycol (PEG) coating. Etoposides and PEG-coated etoposide liposomes were administered intravenously, into diseased rats. Results showed that etoposide and PEG-coated liposomal encapsulation are highly effective as compared to etoposide alone and blank nanoparticles and hence, be a substantial strategy for the treatment of ALD through intravenous drug delivery system.

Keywords: *Advance liver diseases (ALD), Etoposides, PEGylate etoposide loaded liposomal nanoparticles, Liver treatment*

TABLE OF CONTENT

List of Figures

List of Tables

Abbreviations

HC	Hepatocellular Carcinoma
DPPC	Dipalmitoylphosphatidycholine
DMPC	Dimyristoylphosphatidylcholine
CH	Cholesterol
IV	Intra-Venous
IVP	Intra-Peritoneal
NASH	Non-Alcoholic steatohepatitis
NAFLD	Non-Alcoholic Fatty Liver Disease
ECM	Extra-Cellular matrix
TNF	Tumor Necrosis Factor
ELNP	Etoposide loaded Nanoparticles
BLN	Blank Nanoparticles
SEM	Scanning Electron Microscope
RES	Reticulo endothelial system
PEG	Polyethylene glycol
CCL4	Carbon tetrachloride
UV-VIS	Ultraviolet-Visible Spectroscopy
AST	Aspartate Transaminase
ALT	Alanine transaminase
ALP	Alkaline Phosphatase
NIH	National Institute of Health

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Aim of Study

The study in this dissertation introduced into two parts. The first part of this study elaborates the liposomal nano formulation of Etoposide drug and its characterization. By using the different types of characterization procedures nanoparticles formulation performed to permit them in vivo analysis in hepatic cirrhosis.

The second part of this study is based on the development of liver cirrhosis in animal model of rats to improve the pharmacokinetic behavior of encapsulated Etoposide drug. Treatment strategies were explored by intravenous (IV) route in living systems. However, our chief focus of study was to demonstrate the anti-cirrhotic and anti-fibrotic activity of Etoposide loaded liposomal nanoparticles in cirrhosis model and will be considered a notable step to boost these Etoposide liposomal nanoparticles to the level of preclinical trials.

1.2 Overview of Nanotechnology

The word nanotechnology has derived from the word nano, meaning small. The nanotechnology can provide the revolutionary changes in medicine, robotics and in communication. Nanotechnology described as the ability to reconstruct and hatch the matter approximately to 1-100nm in range at atomic and molecular level and utilize the various properties and abnormalities (Roco, 2011). Generally, the definition of nanotechnology is imputed to Feynman (1960) and his well-known speech ‘‘there is plenty of room at the bottom’’ Taniguhi (1974) after 15 years slightly mint the word itself (Zingg & Fischer 2018). The basic concept of nanotechnology was confirmed after discussion of more than 20 countries experts from 1998-1999 (Siegel et al 1999).

1.2.1 Nanotechnology Development

Nanotechnology is the growing field that has immediate effect on drug delivery system. Nanotechnology can be used to enhance the effect of drugs which have poor bioavailability (Emerich et al,. 2003).

Nanotechnology has proved greater involvement in variety of study areas such as chemistry, physics, engineering, nanomedicine, and health medicine (Sainz et al, 2015).

Now the main target of nanotechnology are medicine, green energy, environmental protection, and electronics. While some nanotechnology's bi products have been launched in markets like cosmetics, articles, textiles, sports, and housing (Forloni, 2012).

1.2.2 Nanotechnology as Clinical Equipment

To improve the health care system in case of Cost effective and efficiency, nanotechnology has been used as a nanomedicine, a critical factor for the availability of affordable drugs and treatments (Sainz et al,. 2015).

In comparison with some other drugs having low molecular weight, nanomedicine potentiated the several advantages. In instance, 1) they caused longer circulation by reducing the renal excretion and hepatic deterioration, 2) possessed avoidance for non-targeted site by reducing the volume distribution, 3) enhanced the capacity of drug to assemble at pathological site. Moreover, the formulation of nanomedicine help chemotherapeutic agents having low molecular weight to cross the barrier and enter into pathological sites (Rizzo et al,. 2013).

1.3 Liposomal Nanoparticulation

The liposomes are sphere shaped vesicles, first introduced in mid of 60S containing the one or more lipid by layer (Akbarzadeh et al,. 2013). The liposomes have size ranges from 30nm to several micrometers (Wagner & Vorauer-Uhl, 2011). One approach is to enhance the chemoradiotherapies to exploit the nanoparticles (Zhang et al 2021). Nanoparticles have been shown an effect in the field of drug delivery system which improves the efficacy of treatment and decrease the toxicity of drug (Peer et al,. 2020). Many types of nanoparticles have been developed for decreasing the side effects of drugs and increasing the therapeutic effects. Liposomes are defined as closed vesicles (Zare Kazembadi et al 2019). Due to amphipathic properties, liposomes facilitate the drug delivery of both lipophilic and hydrophilic drugs. Hence etoposide have low water solubility and liposomal nanoparticles are acceptable for novel drug delivery system (Sercombe et al,. 2015).

Nanoparticles have been extensively used in imaging, diagnosis, and treatment of many diseases (Skourtis et al,. 2020). Among others, currently lipid-based nanoparticles are emerging nano constructs for drug delivery. They have biodegradable and biocompatible

composition, therefore, have a unique quality to encapsulate hydrophobic and hydrophilic drugs, making liposomes best nanocarriers.

To prolong the circulation time and increase stability in aqueous medium, they are often coated with biocompatible polymers like PEG (Nekkanti et al,. 2015). The coating of liposomes with PEG decreases the uptake by macrophages and facilitate its prolonged presence in bloodstream (Singh et al,. 2019). These liposomes are prepared by dispersing the liquid in aqueous medium and that dispersion performed by several methods including solvent dispersion and mechanical (sonication and micro emulsification Akbarzadeh et al ,. 2013).

1.2.3 Liposome as Bearer

Many medical researchers are performing their research on liposomal encapsulating procedure, which is new and emerging field, to introduce the drug delivery system to targeted organs. It resembles to submicroscopic generation, namely liposomes which encapsulates several contents (Akbarzadeh et al 2013). In pharmaceutical industries and cosmetics, liposomes are greatly used as a carrier for various molecules (Atrooz, 2011).

Liposomes could encapsulate both hydrophobic and hydrophilic contents by preventing the disintegration of trapped stuff and release at their target sites (Shehata, Ogawara, Higaki, & Kimura, 2008). Liposomes are FDA approved and exhibit good clinical efficacy, proved as a mature technology (Meng et al., 2016).

1.4 Cirrhosis of Liver

It is essential to recognize the pathogenesis of advance liver diseases, such as cirrhosis, NASH, Cancer, Fibrosis, Viral Hepatitis, and alcohol induced liver disease. Lack of this reorganization facilitate the development of biomarkers of diagnostic and prognostic (Palma et al,. 2019). Cirrhosis represents the occurrence of histological nodule that surrounded by fibrous band due to chronic liver injury which causes portal hypertension and end-stage liver disease. Previous studies in understanding the pathophysiology and natural history of cirrhosis, and effective treatment for its complications have improved its management, life expectancy, and quality of life of patients (Schuppan et al,. 2008).

Liver cirrhosis is generally considered as advance phase of scarring (when hepatocytes damaged scar formation occurs), and damaged of hepatic tissues. Scarring is also defined as fibrosis, after scarring liver becomes hard and contract, develops cirrhosis. When hepatocytes damaged, then it progresses to an advance stage, as a result fluid accumulates in the legs, defined as edema and the fluid which accumulates in abdomen, defined as ascites. Ascites may develop into bacterial peritonitis (Vas et al,. 1981) which is serious infection.

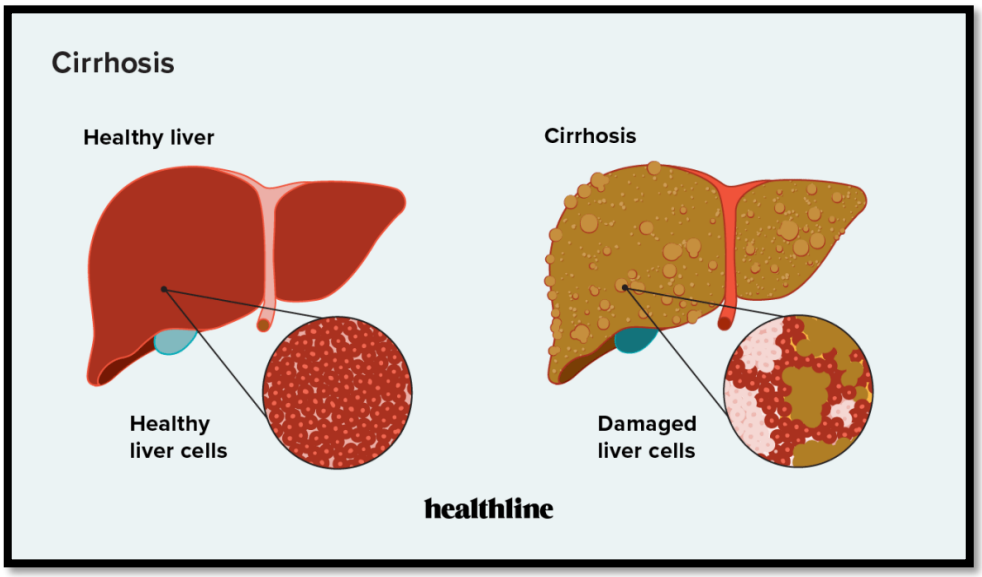


Figure 1Healthy and Cirrhotic liver

The crucial clinical outcome of cirrhosis are an increased hepatic resistance, impairment of hepatocyte function, and the occurrence of hepatocellular carcinoma. There are other systems abnormalities in cirrhosis (hypoperfusion of kidneys, vasoconstriction, salt, and water retention leads to increased cardiac output) are closely linked with hepatic vascular modification and that leads to portal hypertension. Cirrhosis and its vascular deformation are known as irreversible but recent studies shown that its regression and reversal is possible (Desmet et al,. 2004 & Wanless et al 2000).

One of the Worldwide commonest solid tumors is Hepatocellular Carcinoma that caused by progression of cirrhosis. It develops from the regenerative nodule through small cell dysplasia to invasive hepatocellular carcinoma.in developed countries, the mortality of hepatocellular carcinoma associated with cirrhosis is arising (Fattovich et al ,2004). In worldwide, Cirrhosis due to Hepatitis B, is the major risk factor for hepatocellular

carcinoma. The 5-year progressive incidence of Hepatocellular carcinoma in USA and Europe is 10% while 15% in endemic areas (El-Serag et al,. 2002). In cirrhosis, the scar tissue is composed of network gathering of Extracellular matrix molecules (ECM), compromising of basement membrane of collagen type IV, elastic fibers, non-collagenous glycoprotein such as laminin and glycoprotein, and proteoglycans (Schuppan et al,. 2001).

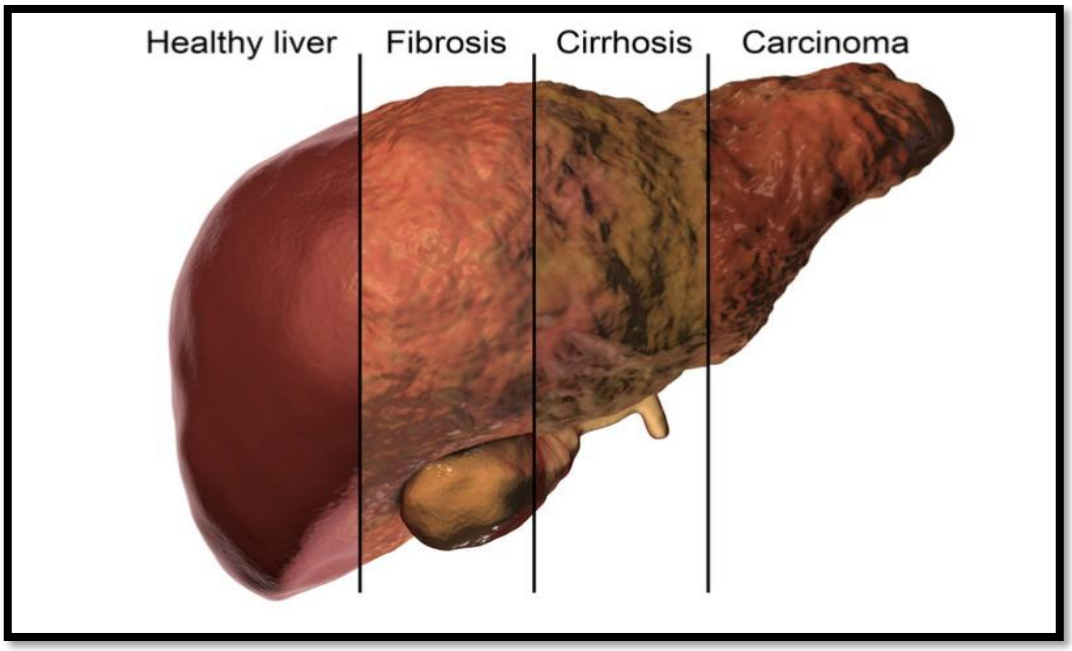


Figure 2Stages of liver Diseases

Liver fibrosis is the process of wound healing that causes liver injury by the reason of various factors such as hepatitis B &C, alcohol consumption, non-alcoholic steatohepatitis (NASH), non-alcoholic fatty acid liver disease (NAFLD), autoimmune hepatitis, and cholestatic liver diseases. The chronic inflammation causes abnormal wound healing process which effect the liver (Sun et al,. 2015 & Bataller et al,. 2005). The destruction of liver is caused by the existence of fibrous scar, leads to the loss of hepatocytes and the disruption of normal liver functioning as a result causes liver failure (Kisseleva et al,. 2008). If fibrosis is not progress into cirrhosis, then it is reversible process (Seki et al,. 2015 & Brenner et al 2013). Angiogenesis has an essential role in liver regeneration, and it promotes hepatic carcinogenesis. The vascular disorganization in some areas due to progressive hepatic injury causes hypoxia and induces angiogenesis (Rosmorduc et al,. 2010).

1.5 Treatment Strategies for Advanced Liver Diseases (ALD)

Generally, among other hepatic disorders, cirrhosis is known as last stage of disease which could develop into cirrhosis or cause death if not treated in early stages (D'Amico et al 2006). The hepatocyte transplantation is the alternative treatment of liver transplantation for hepatic failure. This procedure has been proven an effective against cirrhotic liver in animal model, improved hepatic functions and prolong survival rate (Nagata, et al 2003).

The process of self-regeneration of liver was established in the model of hepatic injury and carcinoma. The proliferation of mature hepatocytes could replace the liver after partial hepatectomy (Malhi et al 2002). The hemopoietic cells could regenerate hepatocytes in the liver. The capabilities of system cells and progenitors is comparatively less, and this implant would cause risk of hepatic failure (Thorgeirsson & Grisham 2006). Hepatocytes regeneration can also boost by genetic replacement via telomerase (Rudolph, Chang, Millard, Schreiber-Agus, & DePinho, 2000) but increase in telomerase activity cause hepatocarcinogenesis (Martin & Dufour, 2008).

In previous studies, animal cirrhotic model demonstrated the reversibility of cirrhosis by using anti-fibrotic substances and cessation of substances which responsible for liver damage (van Leerdam, 2008). The anti-fibrotic agents available in market have focused on decrease of hepatic inflammation instead of decreasing the fibrosis. However, it is essential to introduce the therapeutic agents, to diminish the fibrosis and reversal of cirrhosis.

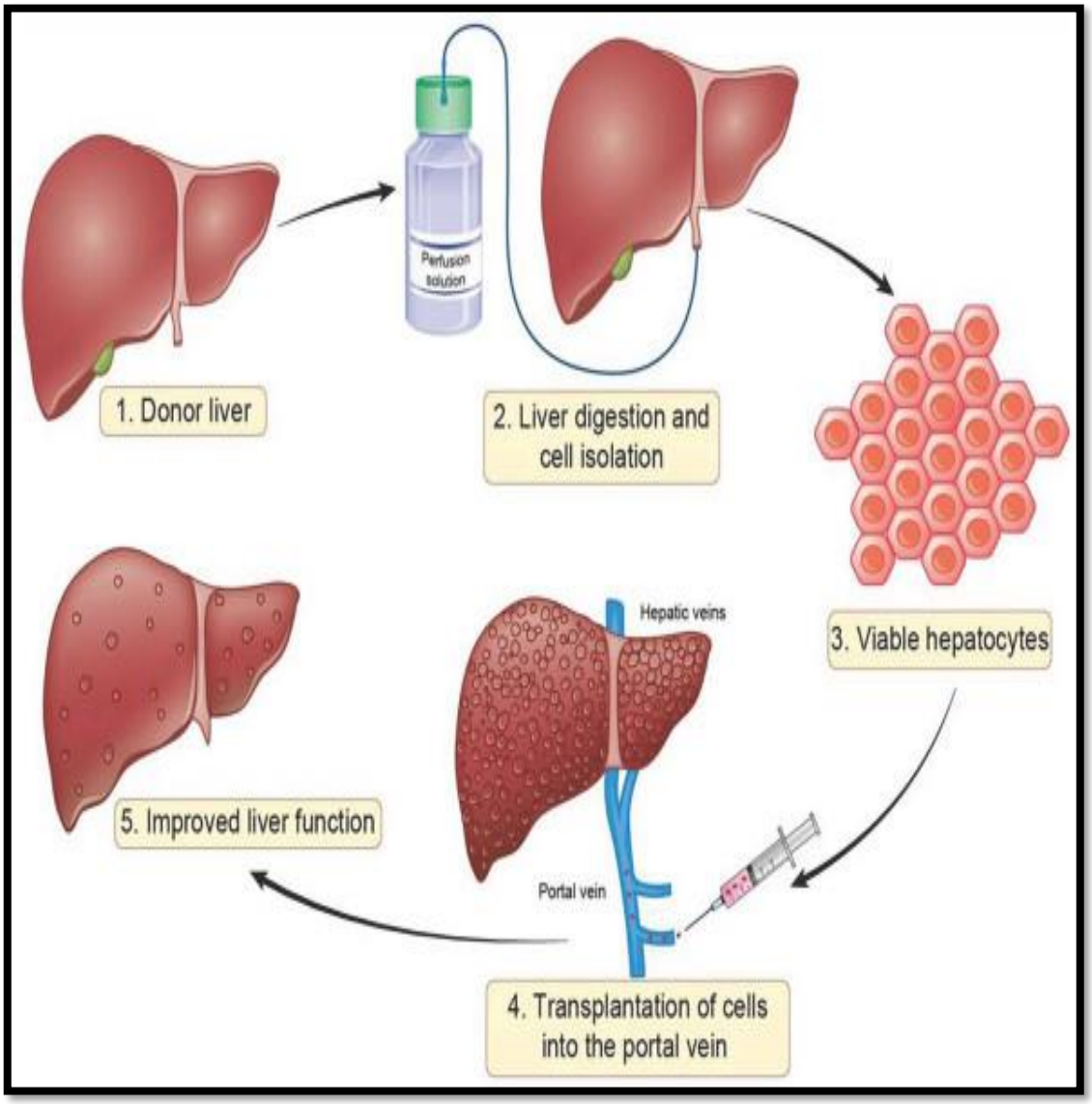


Figure 3Hepatocyte Transplantation https://doi.org/10.3727/096368916X691286

CHAPTER 2

LITERATURE REVIEW

RELATED WORK

2.1 Etoposide: A Universal Drug

Etoposide was first discovered in 1966 and approved by food and drug administration in 1983. It is semisynthetic and derived from rhizome of a plant namely wild mandrake (podophyllum peltatum). Basically, it is a glycoside of podophyllotoxin having D glucose derivative. Chemically it is like anti-cancer drug teniposide, distinguished only by methyl group and teniposide have thienyl. It has white to yellow brown colored crystalline powder and soluble in organic solvent. It is used in its salt etoposide form.

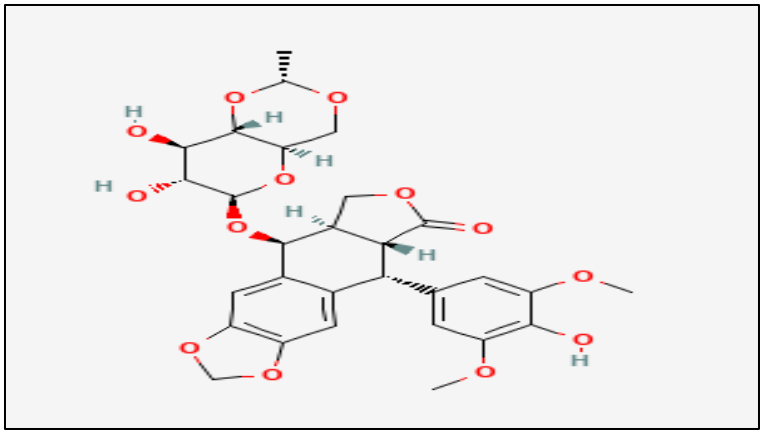


Figure 4structure of Etoposide

Etoposide is a topoisomerase II inhibitor and is used for the treatment of various disease including Hodgkin's disease and leukemia. Normally, topoisomerase II maintains the covalent bond complexes between topoisomerase 5 and II hewed end of DNA molecules and impede with DNA delegation. Moreover, etoposide persuade the apoptosis of malignant cell lines, like in human leukemia cell line and results in DNA fragmentation. Although the mechanism of etoposide is unclear, but it induces the apoptosis of macrophages. In hemophagocytic syndrome, the level of serum inflammatory cytokines and TNF-α are extremely high, and these patients died with intravascular coagulopathy. These features are like fulminant liver failure in which multiorgan failure occur. (Nakama et al,. 2001)

Etoposide is a derivative of podophyllotoxins, a toxin found in American Mayapple which delays the process of cell cycle by acting on S phase and G2 phase. The clinical side of

etoposide has been well established in the treatment of lung cancer and testicular cancer. In combination with cisplatin, shown antineoplastic activity in testicular cancer, small cell lung cancer, and myelogenous leukemia. This combination has shown beneficial effects as salvage chemotherapy in different types of tumours compared to other chemotherapeutic reignms. In combination with other cytotoxic drugs etoposide shown its effect in various types of cancers and as well as in Hodgkin's and non-Hodgkin's lymphomas and some other trophoblastic diseases. The route of administration of etoposide alone is intravenously, and the dose dependency was shown in vitro regardless of human malignant cell lines and in animal models of leukemia (Henwood et al,. 1990).

2.2 Etoposide as Topoisomerase-II Poison

There are some important enzymes, like DNA Topoisomerase which regulates the configuration state of genetic material by inducing the DNA molecule breaks. They are involved in various elementary biological processes like transcription, translation, replication, chromatin remodelling and DNA repair (Montecucco et al,. 2015). The first transesterification is initiated by tyrosine which forms covalent bond with the backbone of DNA. After second transesterification, DNA breaks occur and will regenerate tyrosine (Chen et al,. 2013).

Etoposide poison diminishes the second step of reaction. It's difficult for etoposide to enter in topoll DNA complex to make the interaction with specific amino acid of enzymes (Wu et al,. 2011). Somehow, previous studies shown that etoposide has great aversion for histones and chromatin, particularly in H1, indicates that instead of topoll, chromatin could be target for drug (Chamani et al,. 2014).

There are two different topoll present in mammals, namely Topolα and β which regulated during cell growth (Nitiss et al,. 2009). Topoll α is known as proliferation marker and it adheres in tumour cells nevertheless, beta isoenzyme act as proliferating and present in post-mitotic cell.

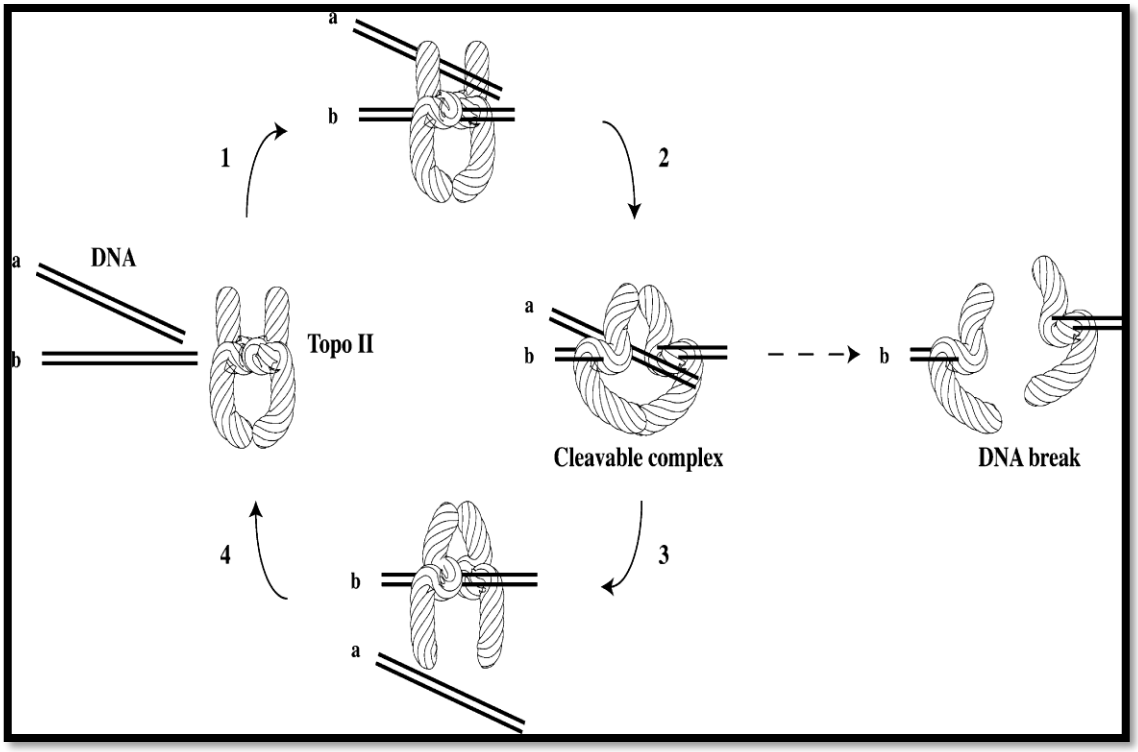


Figure 6Catalytic cycle of Topoisomerase-II

According to previous studies Etoposide can increase DNA breakage of Topo II by inhibiting the activity of enzyme to constrain adhere nucleic acid molecules (Burden et al., 1996).

2.3 Etoposide for Liver Inflammatory Diseases

Hepatic diseases ranging from Hepatitis, fibrosis, and cirrhosis to advance stage Hepatocellular carcinoma, are one of the global socioeconomic burden due to their high morbidity and mortality rate (Li et al,. 2015). There are some organic foods and plants that contains several numbers of phytochemicals, which shown their efficacy against treatment of hepatitis from many years ago. One of them an important group of phytochemicals which have polyphenols that has been used for the treatment of liver diseases. The polyphenols shown their efficacy in insulin resistance, oxidative stress, inflammation, and lipid metabolism, motivated the attention for liver disease therapy (Tang et al,. 2018).

A natural product namely GA, were extracted from the Garcinia of hanburryi tree, exhibits its potential effects in anticancer activity for several types of cancers in studies both in vitro and in vivo (Wu et al,. 2014; Li et al,. 2013 & Ishaq et al,.). GA diminishes cancer cells via multiple mechanisms like angiogenesis, anti-inflammation, and anti-metastasis (Yu et al,. 2007; Prasad et al,. 2011; Cascao et al,. 2014 & Park et al,. 2015). GA has shown its efficacy against other chemotherapeutic agents (Xia et al,. 2017).

There are some experiments in previous studies shown that Etoposide has been effective against liver diseases. Treated animal models with etoposide shown reduced apoptosis against hepatocytes, reduction in lethality, hence other topoisomerase II was not effective. The treatment with Etoposide shown reduction in CPP32/Casepase3 activity of hepatocytes and not altered the serum TNF- α levels. However, Etoposide increased the protein and mRNA expression of Bcl-xL, which is antiapoptotic molecule present in liver. That study suggests that Etoposide prohibits the endotoxin via up-regulation Bcl-xL, caused by lethal liver injury and it could be beneficial for the treatment of TNF-α-mediated liver diseases (Nakama,. Et al 2001).

Etoposide is a topoisomerase II inhibitor which has been used for the treatment of various neoplasms, as well as Hodgkin's and non- Hodgkin's disease, and leukemia (Ganser, A et al,. 1993 & Joel, S, 1996). Moreover, topoisomerase II inhibitor formed covalently bound complexes among topoisomerase II and 5 in the Hew ending of DNA molecules, and impede DNA relegation (Yang, et al 1985). Additionally, Etoposide enhance the apoptosis of malignant cell lines, like human leukemia cell line caused DNA fragmentation (Kaufmann et al,. 1989).

Etoposide improved the efficacy in hemophagocytic syndrome (Brown et al,. 1987). Although the mechanism is unclear, but Etoposide enhanced the apoptosis in activated macrophages. In hemophagocytic syndrome, some inflammatory cytokines from activated macrophages were high, and patients died because of intravascular coagulopathy (Wong et al,. 1992 & Ishii et al 1991). The characteristics aspects of hemophagocytic syndrome are like liver failure which could cause the serum inflammatory cytokines and multiorgan failure (Bernal et al,. 1998).

2.4 Carrier Essentials for Etoposide Drug

The etoposide possessed some drawback along its beneficial clinical effects because of poor solubility in aqueous medium and deficient bioavailability. The rate of administration of Etoposide is limited due to its less solubility in aqueous solution. Some variabilities of Etoposide exist in pharmacokinetics parameters of intravenous (IV) and oral dosage. Almost 30% to 40% drug excretion occur via urine, whereas biliary excretion is less. The oral bioavailability of Etoposide ranges from 24% to 72% and 1.5hr is the half-life of IV route and 0.44hr via oral route (Hande et al,. 1992).

Although the dose dependency of Etoposide for myelosuppression activity is clear but it's hard to evaluate the dosage for anti-tumor activity. Recent studies shown some hematological toxicities due to influences of pharmacodynamics effects of Etoposide (Joel et al,. 1994). However poor solubility and bioavailability causes unfruitful drug delivery approaches. Hence, to improve the efficacy and reducing the toxicity of drug, some previous studies shown an approach of nano ionization and nanoparticulation of drug (Kalepu et al,. 2015; Tanaka et,. 2009).

2.5 Consequences of Liposomal Nanoparticles in case of Liver Fibrosis

Liposomes are small sizes biodegradable which has been used for the drug delivery system to decrease the toxicity and improve the efficacy of drug. This liposomal encapsulation has been used a chemotherapeutic drug namely, doxorubicin (Goram et al,. 2001). This liposomal drug delivery system has proven its efficacy in the treatment of various inflammatory diseases (Crielaard et al,. 2012) by a reason that administrative induction of anti- inflammatory drugs causes immuno-suppression, resulting in severe infection called sepsis (Kawarabayashi et al,. 2010).

Previous research shown that the therapeutic use of liposomal loaded Dexamethasone is effective in liver fibrosis and hepatic inflammation. These nanoparticles reduced the T cell by showing its activity on Kupffer cell through immune rection in liver, caused liver fibrosis. Dexamethasone can cause toxic effects especially in liver compartment in which GC and nanocarrier are cleared during circulation (Bartneck et al,. 2015).

Cationic liposomal encapsulation has been reported as beneficial for the delivery of artificial microRNA to by targeting the connective tissue, and beneficial for liver fibrosis reduction. One recent study designed fibrotic mouse model for the delivery of microRNA by the induction of dimethyl nitrosamine, caused inhibition of fibrotic collagen marker through targeting the growth factor of connective tissue (Yang et al,. 2013).

Another study used a nanoparticle having size of 130nm to target the M6P receptor by inducing CCL4 fibrosis mouse model, results in decrease of hepatic fibrosis symptoms both in vivo and in vitro (Zhang et al,. 2013). The hydrophilic and as well as hydrophobic drug can encapsulate by liposome in its aqueous medium and provide greater stability and increase circulation when PEGylated for drug delivery of specific site (McCormack & Gregoriadis 1994b; Bernsdorff et al,. 1999; Sampedro et al,. 1994; Sharma et al,. 1994).

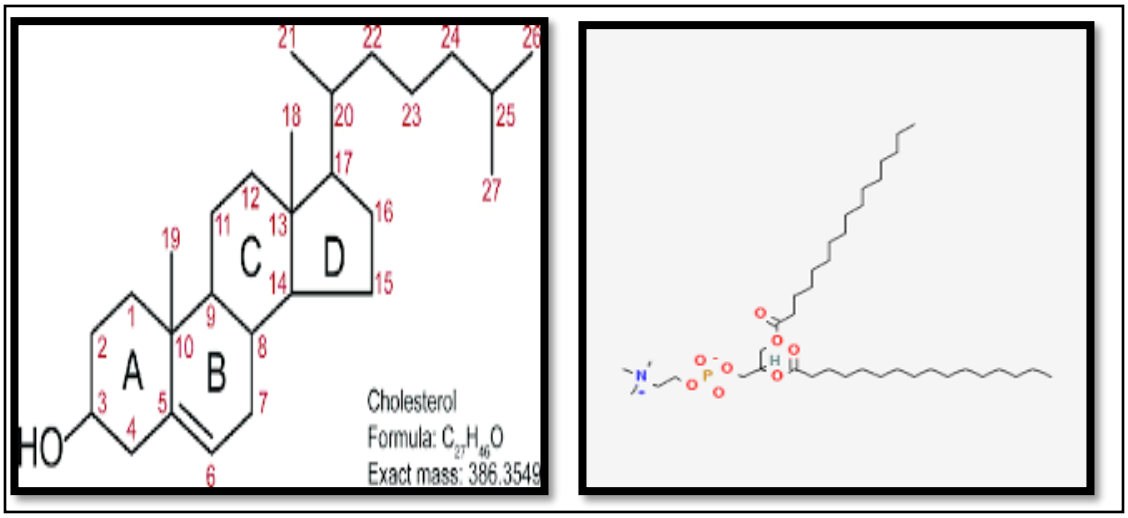


Figure 7Structure of DPPC (left) and Cholesterol (right)

2.6 Composition of Liposomal Nanoparticles

To synthesize the liposome nanoparticles, various methods were applied including scattering of lipid in an aqueous solution, drying of lipid from organic solvent, purification of resultant liposomes and then final analyzation. To formulate the different properties of liposomes like size, biocompatibility, surface charge, drug release kinetics, and cell targeting are used (Anderson et al,. 2014; Tang et al,. 2018).

Liposomes can synthesize by using several methods. These procedures were used to affect the size, to enhance the solubility of encapsulated drug, to decrease the toxic effect of drug,

to prevent the biological degradation, and lamellarity (Dimov et al,. 2017; Maeki et al,. 2018; Pattni et al,. 2105).

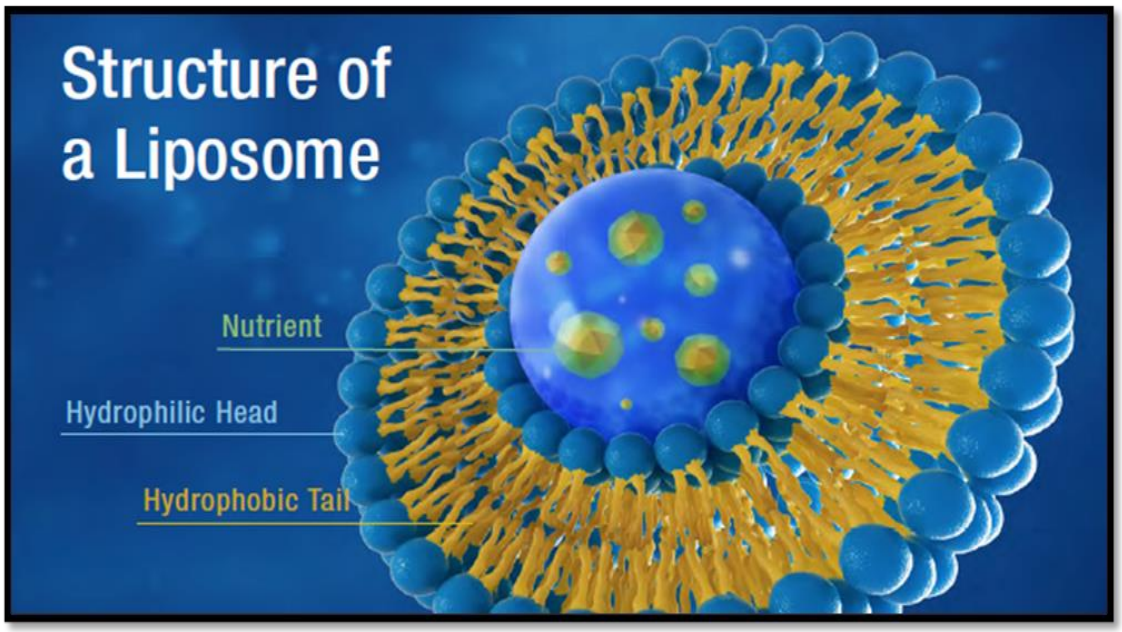
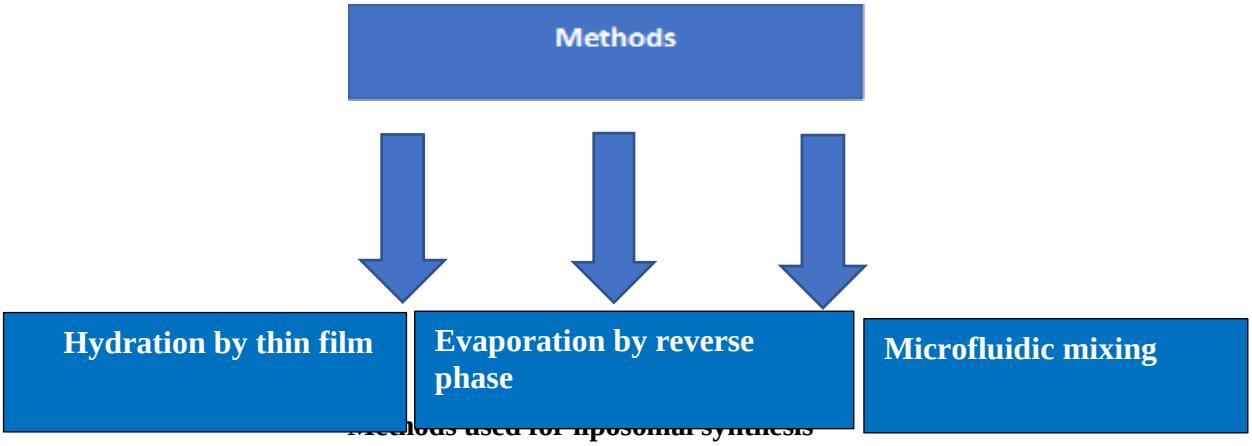


Figure 89Structure of Liposome



Methods used for liposomal synthesis

1.2.4 Drug Loading

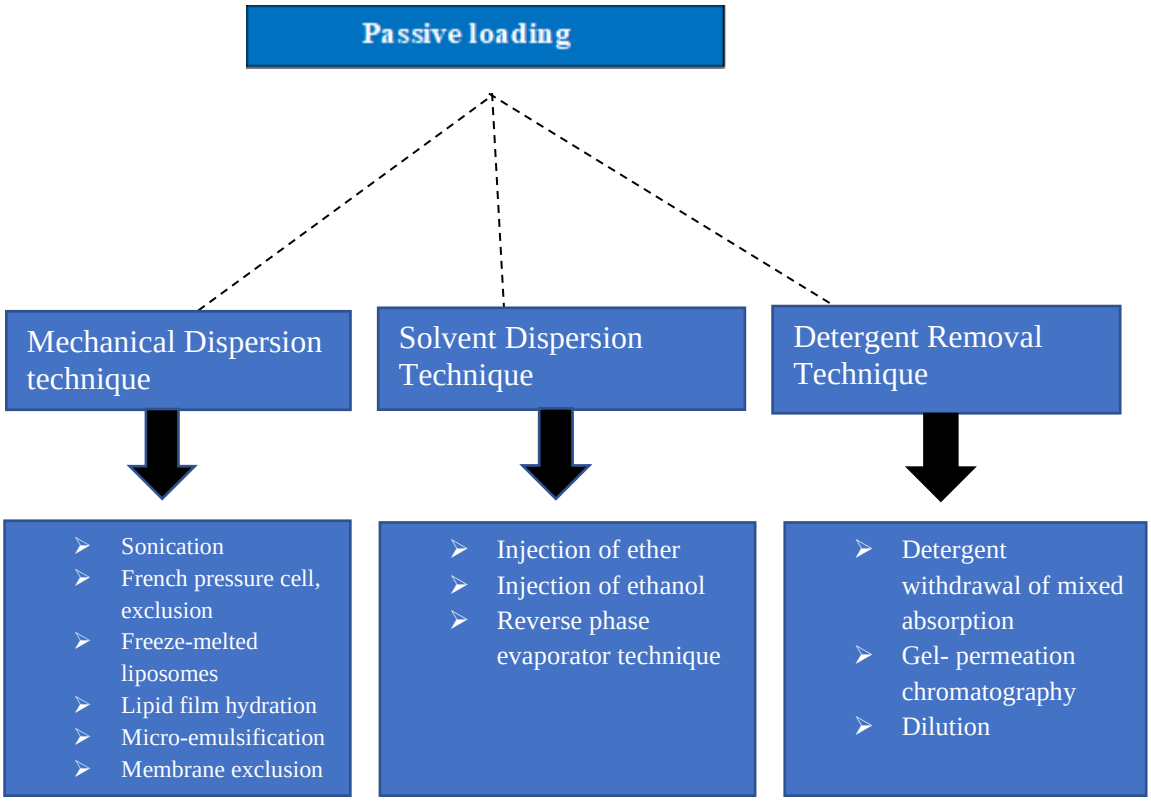
There are two procedures that has been used for the loading of drug.

1.2.4.1 Active loading

The aqueous phase of liposome in inside and outside are different. The liposomes containing transmembrane gradient were generated in active loading. Consequently, an amphiphilic drug was dissolved in external aqueous media which penetrated via phospholipid bilayer, supervene the interlinkage among trapping agent for trapping the drug. However, active loading proven an effective method to trap the drug effectively in liposomal core (Bhatt et al,. 2018).

1.2.4.2 Passive Loading

The passive loading is the process in which both drug loading and drug formation occur concurrently. Hydrophilic substances were consistently diffused in aqueous media while hydrophobic drug was adhered within the lipid liposomal bilayer. However, the lipid and drug were dispersed in liposomal bilayer, interacted them with water, followed by solvent evaporation, obtained thin film, and hydrated that to acquire liposome (Gubernator, 2011) Following water soluble drug loading, the lipid bilayer was dispersed in drug containing aqueous medium. Due to various factors such as, drug solubility, the concentration of lipid, the vesical size, the preparatory method, and the trapped drug efficiency varied. According to passive loading procedure, the average lipid to drug (D/L) ratio <0.05 W/W were obtained in mostly cases (Zhao et al,. 2015).



1.2.5 Stealth Liposomal Nanoparticles

Somehow, liposomes are identical to bio membranes but still they behave as foreign bodies in body. However, they are identified by human system namely reticulo endothelial system, following communication with plasma proteins. The liposomes can merge with our cell membrane, then deliver liposome contents in body. Afterwards, they are removed via blood stream (Akbarzadeh et al., 2013).

The mechanism of liposomal removal of body from circulation is much fast, so its important to overcome this process for longer circulation. To overcome this effect, an important finding was ruled out, that was liposomal coating with PEG, reduction of stealth effect, resulting in prolonged liposomal survival in circulation from the leakage of endothelium (Shaheen et al,. 2006).

Stealth liposomes are circle shaped sacs with membrane having the composition of lipid bilayer which has been used as a drug delivery system and the delivery of genetic

components into cell. These were achieved in new drug delivery system for controlled release. This stealth concept has been used in doxorubicin loaded liposome which are still used in market by the name of Doxil (Janssen Biotech, Inc., Horsham, USA) or Caelyx (Schering-Plough Corporation, Kenilworth, USA) for the treatment of solid tumors. In previous study, an arthritic model was demonstrated with corticosteroid loaded liposome to enhance the therapeutic strategies. Hence, liposomes with longer circulation behave container for the prolonged drug release (Akbarzadeh et al,. 2013).

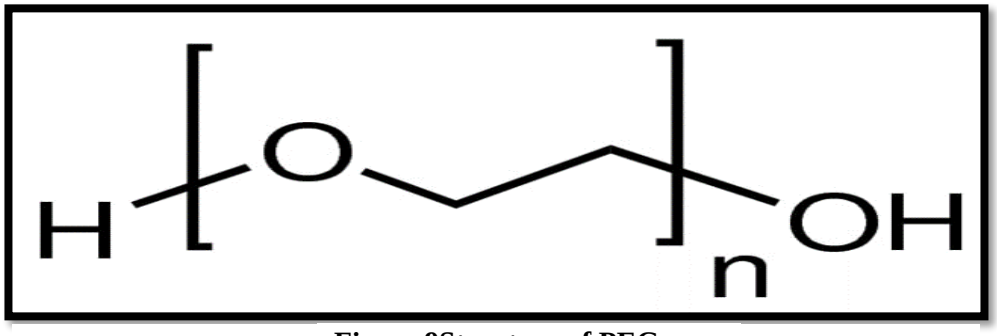


Figure 9Structure of PEG

2.7 Hepatic Cirrhosis Induction with CCl4

The administrating of CCl4 and bile duct ligation are the principal techniques for introducing the Liver Cirrhosis in rat's model. Although the CCl4 is being used but it is harmful process. It damages the liver tissues by forming the linkage with free radical metabolites, resulting in activation of metabolites, hemostasis of calcium, and covalent bonding. The administrating of CCl4 cause hepatic inflammation and ultimately results in hepatic necrosis and fibrosis then it spreads to neighbored vascular structure that drains into portal vein. However, it causes apoptosis of hepatocytes, necrosis, and fatty penetration by stimulating the hepatic stellate cells upon continuous administration (Marques et al,. 2012).

The process of CCl4 toxicity induction take place in two or three phases. Primarily, necrosis formed in two to three weeks followed by enhancing the hepatic enzyme activities and reducing the cholinesterase range. Moreover, Aspartate aminotransferase has increased during two to three weeks and liver fat deposition happen. There is a specific increase in AST around three weeks and reduction of other hepatic functions, also raised the level of

triglyceride and hydroxyproline. The Liver atrophy and reduction in pseudocholinesterase range were noted at the end (Paquet et al,. 1975). This might be caused by the reduction of serum albumin level and decrease in weight, caused loss of hepatic functions followed by constant fibrogenesis (Scholten et al,. 2015).

1.2.6 Process of Hepatic Damage

In liver, the CCl4 is converted to trichloromethyl radical CCl3 after metabolization through the activity of cytochrome P450 monooxygenases super family, namely FYP family. These radicals caused damage in cellular functionalities by making connections with lipids, proteins, and nucleic acid, resulting in lipid metabolism changes such as steatosis, fatty degradation, and decrease in protein level. Additionally, mutation occur because of adduct formation in DNA and CCl3. As a result, lipid peroxidation and polyunsaturated fatty acid degeneration occur due to evolution of trichloromethyl per oxy radicals which were initiated CCl3 oxygenation. At the End, membrane permeability of various cell organelles was decreased such as mitochondria, plasma membrane, Golgi apparatus and endoplasmic reticulum results in heavy destruction of hepatic tissues, characterized by inflammation, fibrosis, necrosis, and finally Hepatic Cellular Carcinoma (Weber et al,. 2003).

CHAPTER 3

MATERIALS & METHODS

MATERIALS and METHODS

3.1 Experiment Design

1.2.7 Materials

All chemicals were purchased from Sigma Aldrich, unless indicated. Female rats were purchased from National Institute of Health (NIH), Islamabad.

1.2.8 Synthesis of Etoposide loaded liposomal Nanoparticles

DPPC, DMPC, and Cholesterol were used for the synthesis of liposomes in 4:1 (percent molar ratio). To make a 100μM solution, solution has measured and disintegrated in ethanol. The 200μM of Etoposide drug solution has formulated in ethanol, and 500μl solution was obtained from that and blended in lipid solution. The sonication of the mixture has done for 40mins. Then added 10 ml of water to the lipid phase, mix and let it for a water bath till reaches 60°c. This dispersion mixture of water in the lipid phase allowed to be water bath sonication for 10 min at 90rpm. Then again probe sonication was performed for 40 min (at 50mhz) allowing the rotary evaporator to remove ethanol.

1.2.9 PEGylating of Etoposide loaded Liposomal Nanoparticles

For the synthesis of PEG-ELPS, the same method has been used as ELPS with some additional steps. Similarly, 200 μM solution of Etoposide and 100 μM solution of lipid were processed in ethanol, 500μl was taken from the drug solution to be mixed in the lipid solution. Then mixed the Water phase & lipid phase by following the sonication, then this mixture led to rotary evaporation. Following the pegylation of ELPS, the mixture has undergone dilution up to 50ml and is put on 0.25% peg drop by drop on stirring, after that again let for continuous rotary evaporation till the 10ml solution has left. The entrapped drug has released by centrifugation (at 4500 rpm) via mini-column filtration for 1hr.

3.2 Physical Characterization

200 μl sample was placed on cover slip to visualize the ELPS and PEG-ELPS. Then surface of slide was coated with gold for 50 s in a sputter coater at 20 mA. Imaging was conducted using VEGA3 LMU Scanning Electron Microscope (Tescan, Czech Republic).

1.2.10 Fourier Transform Infrared Spectroscopy (FTIR) and UV-VIS Analysis

The identification of organic and non-organic material was by using a technique called Fourier Transform Infrared Spectroscopy (FTIR). The material of sample was measured through absorbing radiations verses wavelength. The infrared absorption band defines the structure and molecular components. The absorbed infrared radiations cause excitation of molecules into state of vibration upon hitting of infrared radiation to substance. A single molecule absorbed the wavelength of light demonstrates the energy difference between vibrational excitation and resting state. The characteristics of molecular structure observed by absorption of wavelength (Khan et al,. 2019 & Mohammad et al,. 2017).

For the analysis of FTIR, first, samples were air dried and then processed by using KBr discs. FTIR spectra were obtained between 4000-350cm1 by using Bruker FTIR Spectrophotometer Alpha II (USA). FTIR was done for all components including DMPC, DPPC, Cholesterol, PEG (2000), Blank Liposomes, Etoposide drug, and Etoposide loaded liposomal nanoparticles.

1.2.11 UV-VISIBLE Spectroscopy

UV-Vis Spectroscopy is a non-invasive procedure used to identify the material by using reflectance of spectra in a short time. UV-Vis spectroscopy is the connection between matter and electromagnetic radiation in the UV-Visible region. The ultraviolet and visible radiations communicates the matter by hitting the surface in different methods including, absorption, emittance, reflection, and transmission (Picollo et al,. 2019). The absorption of sample measured from reflection of beam when light hits the beam. The Splitting of light beam occurs, one beam acts on Cuvette having sample while other focused on Cuvette having a solvent used as control. The absorption could measure at target range and measuring spectrum obtained absorption of sample verses wavelength. Maximum absorption named as lambda max at significant wavelength. It demonstrates the molecular electronic information and commands the Beer Lambert Law theory which said:

$$A=EcL$$

Molar absorption E = a/cl (A= absorbance, C= concentration of sample in moles/liter, and L= length of path light though cuvette in cm). this law makes beneficial to UV-VIS

Spectroscopy for quantitative analysis (Amendola et al,. 2009; Perkampus, 2013; Tomaszewska et al., 2013).

UV-Vis spectrum of Etoposide loaded LNPs, and PEG Etoposide loaded LNPs were analyzed by using Shimatzu 2800 BMS Scientific Technical Corporation (PVT) spectrophotometer, by using resolution of 1nm in range of 200-450nm. The reference for UV analysis de-ionized water was used. The UV spectrum of Etoposide drug alone and loaded with liposomal nanoparticles and PEGylated Etoposide loaded liposomal nanoparticles were recoded.

1.2.12 Zeta Potential, Surface Charge, and Dispersity Index

The potential difference between liquids and solids called zeta potential. The particle's electrical is measured by suspension of liquid. It measures the electrostatic potential at electric double layer enclosing the nanoparticle in liquid. The nanoparticles having a range between -10 to +10 mv consider as neutral, whereas the potential difference in the range of > +30mv or < -30mv are strongly suggested for cationic and anionic accordingly. The measurement of zeta potential in mv and called electro kinetic potential. The zeta potential demonstrates about nanoparticles' size, stability, and surface charge. The more stable colloid denotes high potential. The beginning of particles aggregation is denoted by the zeta potential < -15mv. The colloidal precipitation occurs when zeta potential becomes zero (Glawdel, et al,. 2008; Clogston et al,. 2011).

Size distribution, zeta potential and dispersion of PEG-ELPs and ELPs were assessed by Dynamic Light Scattering utilizing Malvern Zeta Sizer Ver.7.12 (Malvern, UK).

1.2.13 Area Distribution and Particle size

The Scanning Electron Microscope was used to demonstrate the particle size. The area distribution was calculated by 'image J software'. The SEM works through image scanning in range unless item edge defined. The values were given in range of 0 to infinity to demonstrate the particle size. The particles in circulatory area of outside range were ignored. The 8 bits image was analyzed containing best curve of measured particle. (gray levels: Ellipses: 0; background: 255) (Goldstein et al., 2017).

The Etoposide loaded liposomal nanoparticles were analyzed by placing a small portion of liquid on glass slide by using micropipette. Then, the surfaces of slide were coated with gold for 50second at mA in sputter coater. Finally, images were obtained using VEGA3 LMU Scanning electron microscope at National University of Sciences and Technology Islamabad.

1.2.14 Drug Release Efficiency

The release of drug from nanoparticles has a great importance for treatment in nanomedicine. The main concern of nano formation is the release of drug delivery to target site in time dependent manner, results in controlled release.

The solution of 3ml of ELPs and PEG-ELPs were separately centrifuged for 10 min at 4500 rpm at 25^{o}C by using 15ml centrifuge tube. 3ml buffer solution was mixed in ELPs and PEG-ELPs solutions. Then the mixture has been analyzed using UV-spectrometry interpretation by following centrifugation. The same process has repeated after 1,2,4,6,12,24 and 48hrs. With an empty nanoparticle solution analysis was executed and utilized as a control. This process was carried out by using empty nanoparticle as a reference.

3.3 Development of Model

1.2.15 Animals

Firstly, 10 female rats with ages of 4-6 weeks having weight 85-105 g were used. The rats with the accessibility of food and water were kept in a 12hr dark and light room in separate cages. The temperature in the room was set at 37^{o}C with the humidity 60-70%.

1.2.16 Chemical

We used different chemicals to induce liver cirrhosis in our study for example strong carcinogens namely Urethane, a hepatotoxic carbon tetrachloride (CCl4), a delivery agent called peanut oil, A de-contaminant namely Ethanol, and neutral formaldehyde 10% dissolved in phosphate buffer solution.

3.3.3. Experimental Design

The rats were divided into four groups, Control (n=5), Diseased (n=5), Etoposide treated (n=5), ELPs treated and PEG-ELPs treated.

1.2.17 Liver Cirrhosis Induction

The six-week protocol was conducted for the induction of liver cirrhosis (n=15). Rats were acclimatized for 1 week. Intraperitoneal injections of 2.5% urethane solution in Dimethyl Sulfoxide (DMSO were given to diseased groups (n=15) for first two weeks. The injections were subjected twice a week at 1ml/kg dose. For the next four weeks, CCl4 infused in peanut oil (50% v/v) was intraperitoneal injected to the rats twice a week at 1ml/kg dose (Fortea et al 2018); Gitiara et al 2017). For every group five rats were used.

1.2.18 Drug Dosage

The protocol designed for treatment strategies was four weeks. For negative control, three rats were used, and 5 rats were used for the remaining groups. Etoposide was given Via Intravenous route for four weeks with a dose of 0.5mg/kg to Etoposide treated group. The second group was treated with PEG-ELNP, Intravenous injections were given at the dose of 0.9mg/kg. However, the third group was treated with Blank Liposomal Nanoparticles with a dose of 0.9mg/kg via the IV route.

1.2.19 Physical Parameters of rats

Several conditions were observed such as body and liver weight, ascites, food, and water consumption.

1.2.20 Serological Indices

For serological studies like LFTS (AST, ALP, ALT, and Bilirubin) blood was extracted directly from heart of rats.

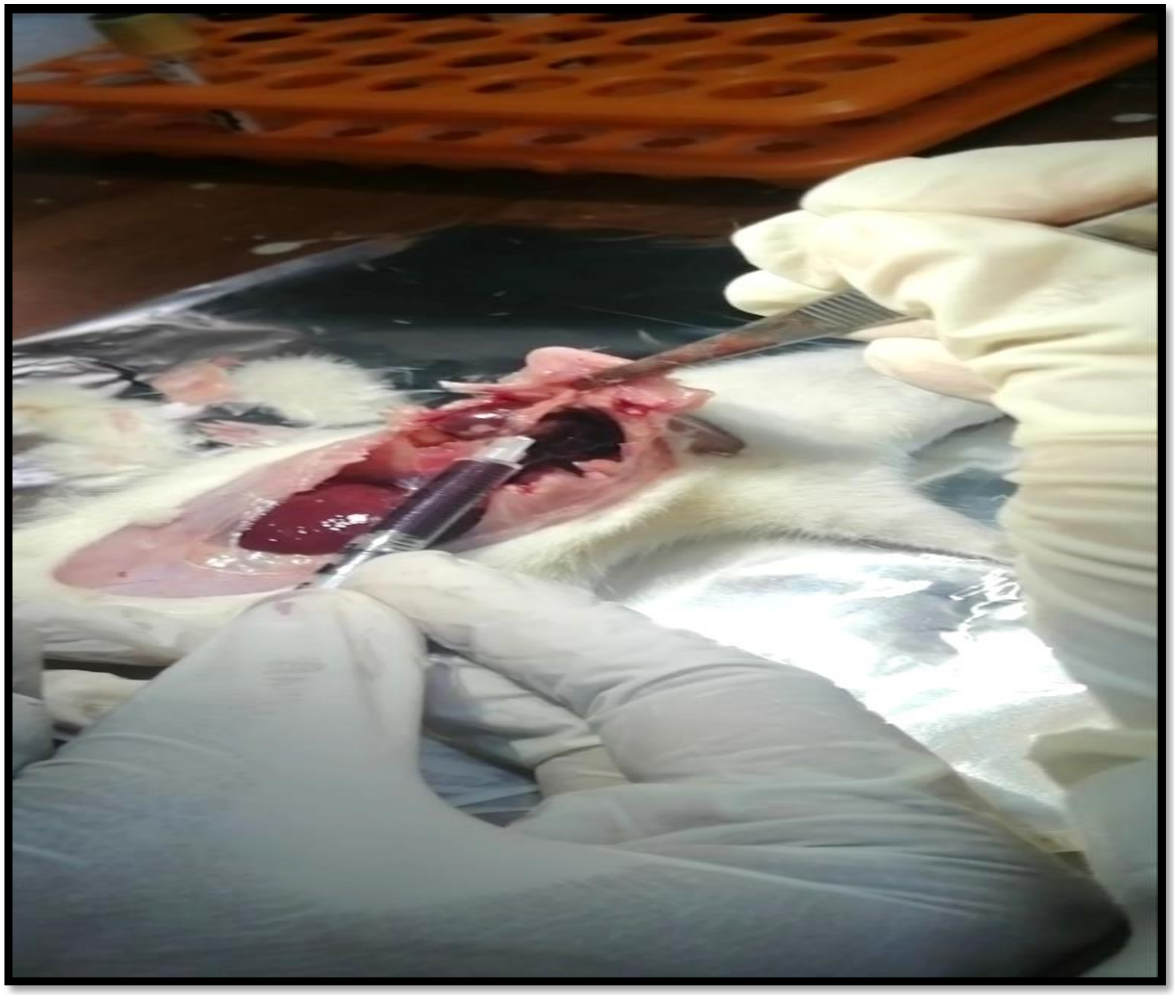


Figure 10Blood Collection for serological findings directly from heart

1.2.21 Histological Examination

To unconsciousness the rats for histopathological findings, chloroform was used. By taking into consideration the US FDA (Food and Drug Administration 1978) instructions, care and handling are carried out. Six rats were dissected, from which five rats were diseased, three were normal, five were Etoposide treated, and five PEG-ELPs treated, and their organs were collected such as kidney, liver, and spleen. At the same time, the dimension, form, and structure of diseased rats were picked by hepatic tissue, firm by 10% formaldehyde solution and following the paraffin embedding, 4um serial sections were secured. Afterward, to detect the structural modifications in liver tissues HE (Hematoxylin and Eosin) staining has performed, and hyperplasia of collagen fibers was detected in

histological slides. According to the NASH/NAFLD Clinical Research Scoring Network description and outcomes, the grading of pathological conditions is performed based on Histological categorization and the process of fibrosis (Tajima et al 2013).

Some modifications were performed due to changes in our histological observations. Modifications were associated with 'Piecemeal Necrosis' containing outcome = 1,2,3,4 for the stages of Necrotic cell inflammation. According to that lightly inflammation includes mild (few portal areas), moderate (<50% of tract or septa), and severe (>50% of tract or septa) (Knodell et al., 1981).

In 1993, in France, METAVIR developed a scoring system for the histological staging of hepatic disorders and chronic hepatic disorders (Bedossa, 1993; Bedossa Poynard, 1996).

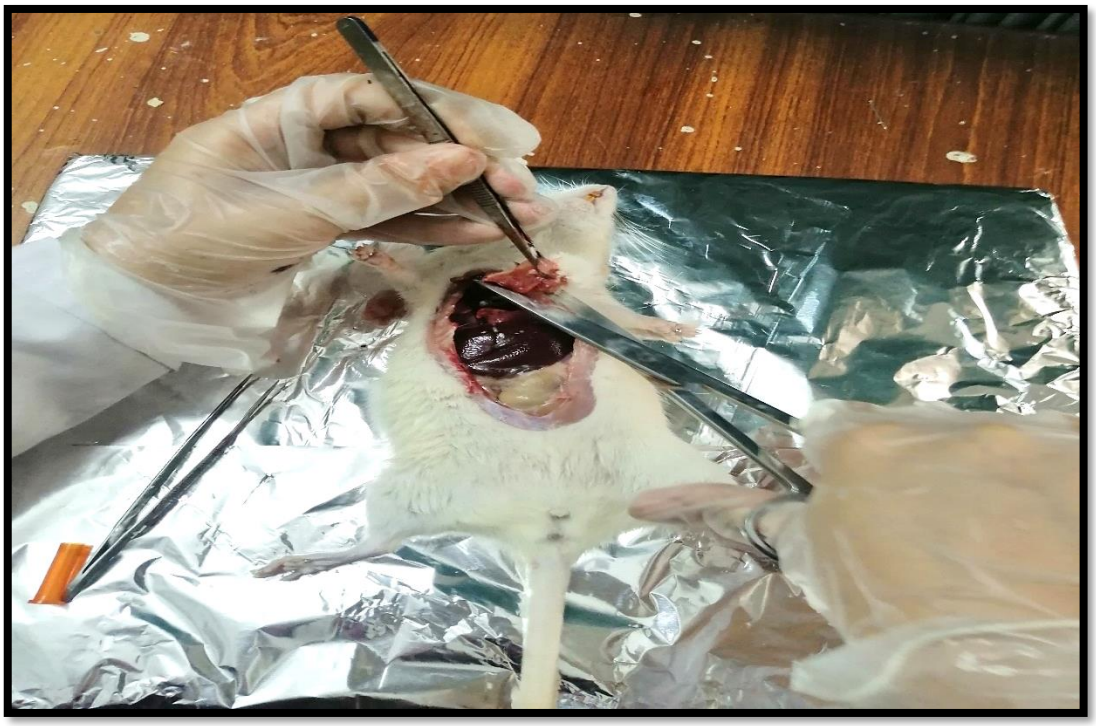


Figure 11Dissection for Histological Examination

Table 1 Clinical research and network scoring system NASH and NAFLD

Steatosis		
Grade	Parenchymal Involvement	
	<5 %	0
	5-33%	1
	33-66%	2
	>66%	3
Inflammation		
Lobular inflammation	Judgement of inflammatory foci	
	No foci	0
	< 2 foci x 200 field	1
	2-4 foci x 200 field	2
	>4 foci x 200 field	3
Portal inflammation	Judgement with low magnification	
	Minimal	0
	>Minimal	1
Microgranulomas	Little aggregation of macrophages	
	Absent	0
	present	1
Large Granulomas	Portal vein involvement or near to central vein	
	Absent	0
	present	1
Piecemeal necrosis	Absent	0
	Mild (Limited foci)	1
	Moderate (<50% of tract)	2
	Severe (>50% of tract)	3
Fibrosis stage	Method of brunt	

3.4 Treatment Design

The rats were divided into four groups to demonstrate the anti-cirrhotic and anti-fibrotic activity of Etoposide loaded liposomal Nanoparticles.

1.2.22 Negative Control Group

A set of two diseased rats were separated and labelled them as negative control group. These rats were kept as untreated during the whole procedure and at the end of experiments, these rats were sacrificed to carry out the serological and histological analysis. Ascites, liver and body weight was acknowledged.

1.2.23 Positive Control Group

A set of two normal rats were separated and labelled them as positive control group. These rats were kept normal throughout the experiment. During dissection, after experiments these rats were sacrificed and liver, body weight noted to compare with diseased and treated rats.

1.2.24 Etoposide treated Intravenous (IV) Group

The only one rat was placed in this group. The etoposide was given via IV route with the dosage of 10mg/kg for 15 days. During dissection, at the end of experiment, ascites, liver, and body weight were acknowledged.

Figure 12Drug Administration via IV Route

1.2.25 Etoposide loaded Liposomal Nanoparticles treated Group

The only one rat was placed in this group. The Liposomal loaded nanoparticles were given via IV route with the dosage of 500µ/kg for the 15 days. During dissection, at the end of experiment, ascites, liver, and body weight were acknowledged.

1.2.26 Blank Liposomal Treated Group

The only one rat was placed in this group. The Liposomal loaded nanoparticles were given via IV route with the dosage of 500µ/kg for the 15 days. During dissection, at the end of experiment, ascites, liver, and body weight were acknowledged.

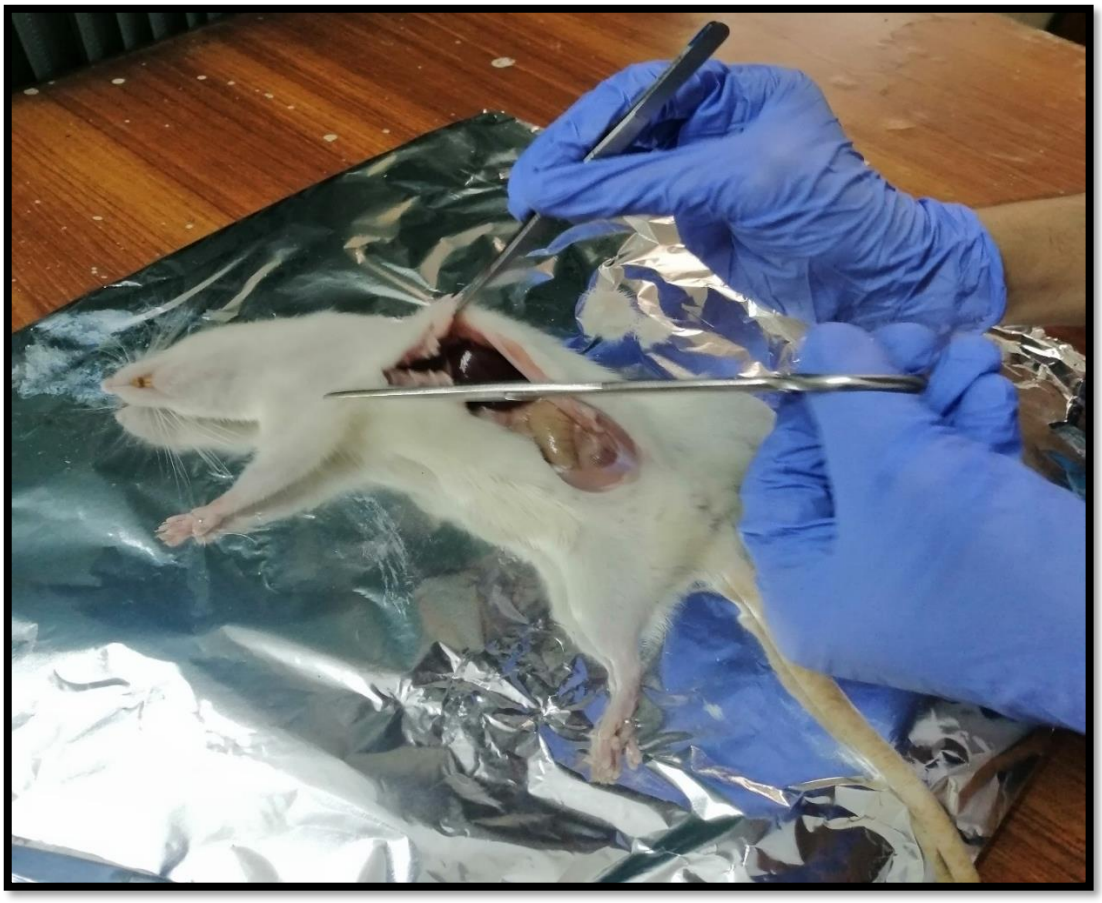


Figure 13Dissection for removal of Kidney, Liver, and Spleen

CHAPTER 4

RESULTS

RESULTS

4.1 Physical Characterization of Etoposide loaded pegylated liposomal Nanoparticles

The thin-film hydration was used for the preparation of ELNPs. For example, Dimyristoylphosphatidylcholine (DMPC), cholesterol, and Dipalmitoyl phosphatidylcholine (DPPC) were used to formulate phospholipid bilayer vesicle. The DMPC & DPPC are phospholipids used to prepare the liposomes. They contain C16 palmitic acid groups which attached at the head of phosphatidylcholine. The coating of PEG was performed for peaks after preparation.

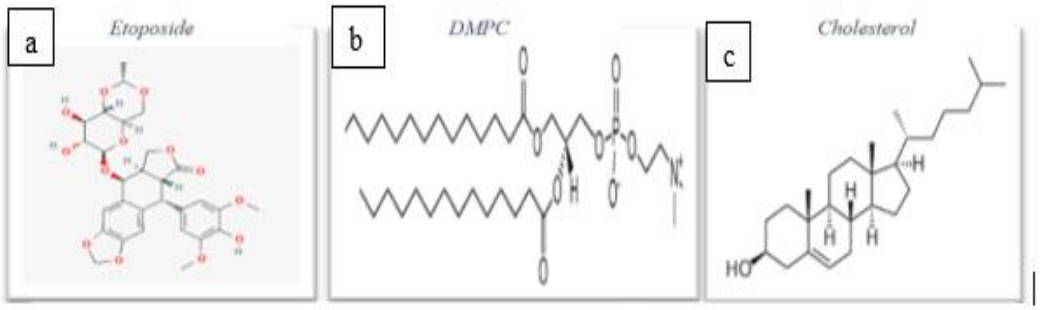


Figure 14Structure of Etoposide (a) DMPC (b) and Cholesterol (c)

1.2.27 UV-VIS Spectroscopy Analysis

UV-VIS absorption analysis of Etoposide shown the peak of absorbance at 280nm. The ELNP showed absorbance peak at 300nm. The absorption peak of blank liposomes shown at 230nm. The absorbance peak of cholesterol shown at 220nm. The absorbance peak of DMPC and DMPC was shown at 225nm.

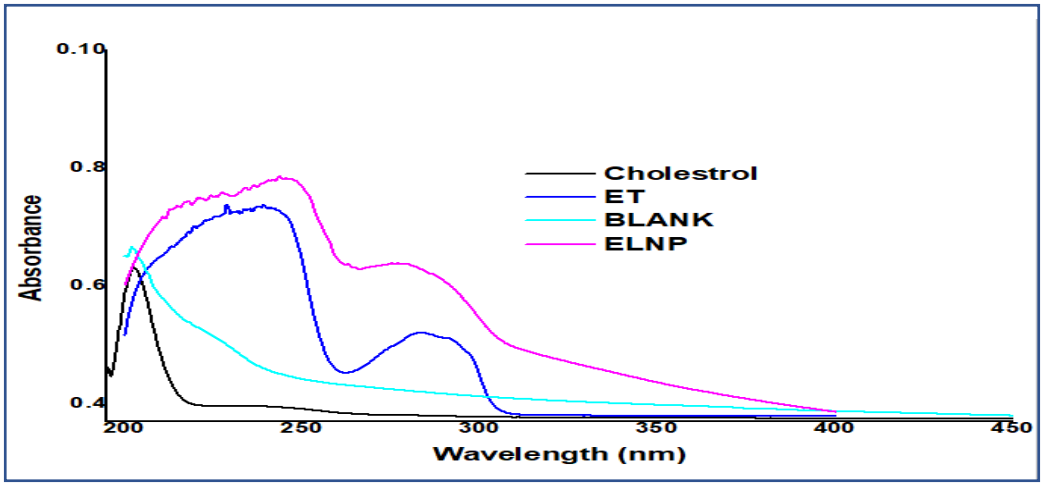


Figure 15UV VIS Analysis

1.2.28 Fourier Transform Infrared Spectroscopy

Cholesterol indicated peaks or bands at 2883/cm (C.H. stretch, Alkanes), 874/cm (Tri-substituted Aromatics). **DPPC** spectrum showed peaks at 2901/cm (C.H. stretch, Alkanes), 1649/cm (R-NH2, Amines), 1086/cm (C-O stretch, Ether), 876/cm (RCH2CH3, Bending mode), peaks at 3348/cm NH stretching vibration, 2977/cm for R-CH2-CH3,1632 R-NH2.dmpc shows 2971/cm (C.H. stretch, Alkanes), 1632/cm (C=0), 1068/cm (C-O stretch, Ether), 880/cm (RCH2CH3, Bending mode), peaks at 3336/cm NH stretching vibration, 1277/cm C-N, Phosphate group at 11-1200/cm. **DLNP** contains phosphate group at 962-1156/cm, 1638/cm for C=O, 1632 R-NH2, 1068/cm (C-O stretch, 1277/cm C-N, 2108/cm for C=N, OH stretching at 3348/cm. In **simple drug** OH band vibration observes at 3341/cm, CH stretching vibrations at 2973/cm, C=O stretching at 1661/cm, C-O-C stretching at 1056/cm, and C=C stretching was observed at 1438/cm.

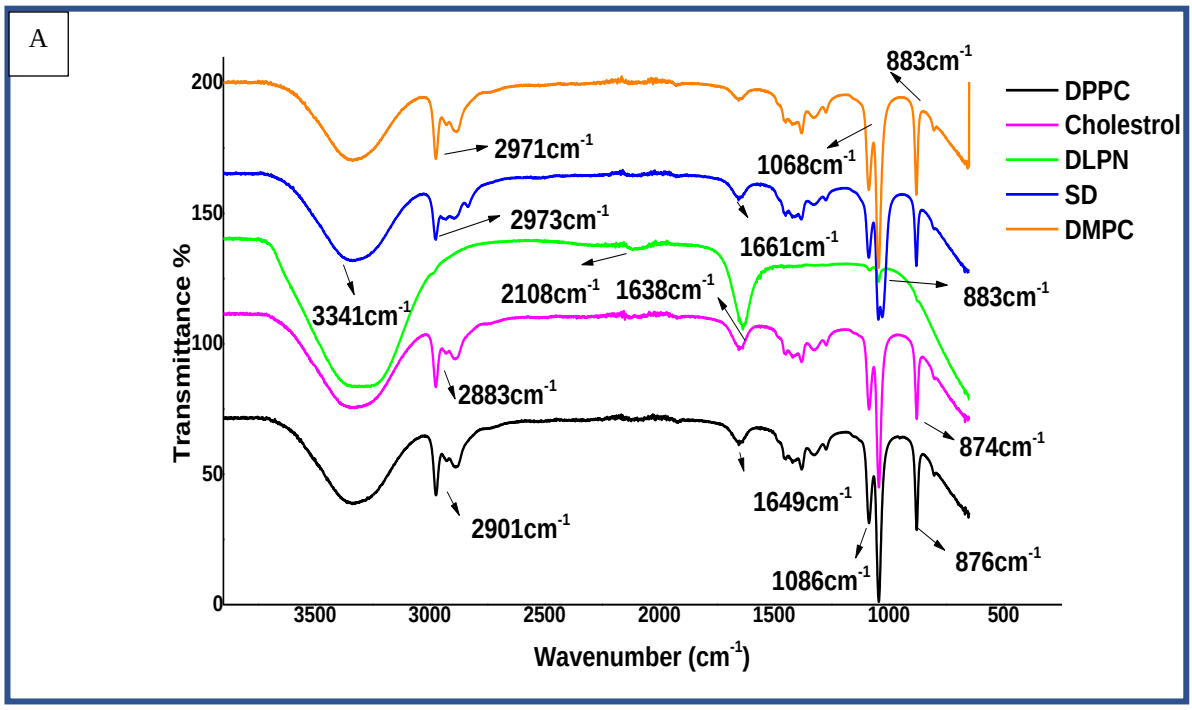


Figure 17FTIR Analysis

1.2.29 Area Distribution and Particle size

The Scanning Electron Microscope was used to demonstrate the size and morphology of Etoposide loaded nanoparticles. The image showed the sphere-shaped nanoparticles with average size of 73nm.

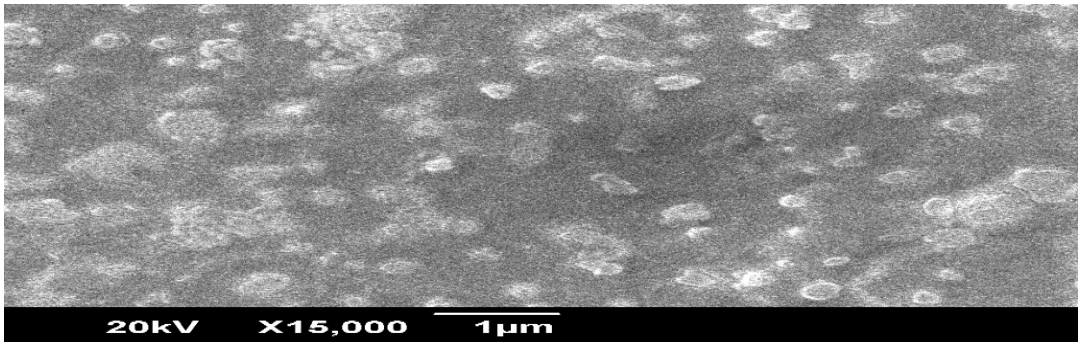


Figure 18SEM Analysis

The Zeta Sizer was used to evaluate the mean size of ELPs having 224 and 219 respectively. The average zeta potential for ELNP were -3.95 and -3.65 with the polydispersity index of 5.24 and 7.7. the increase in zeta potential of ELP occur due to the presence of PEG-6000, exposing the enhanced stability of ELP.

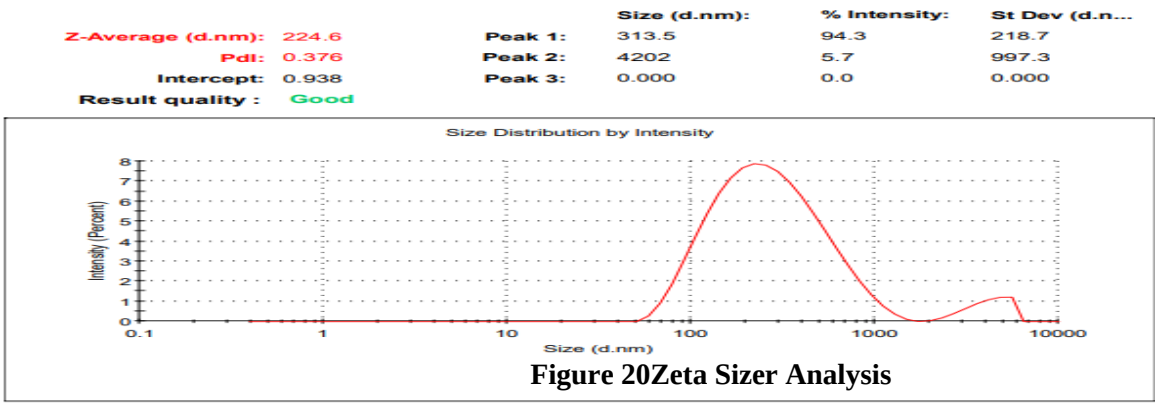


Figure 20Zeta Sizer Analysis

Figure 19Zeta Sizer Analysis

4.2 Drug Release Kinetics

The percentage cumulative drug release was measured for 48 hours by the time difference of two hours. The release of Etoposide drug from PEG-ELNP was noted for 48 hours.

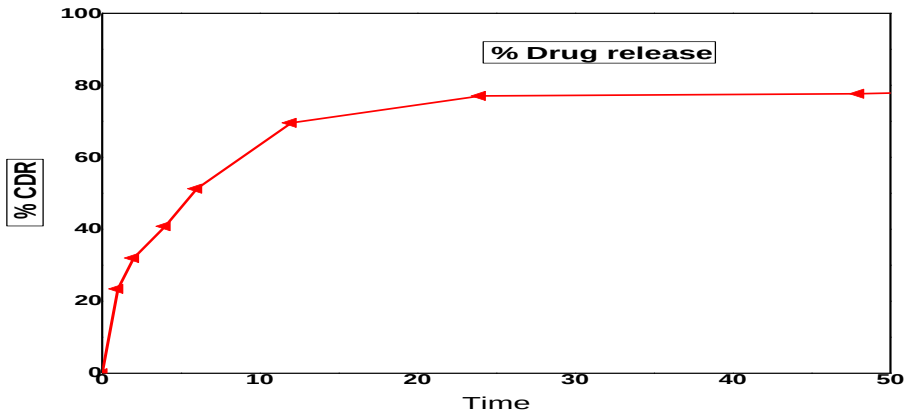


Figure 21Drug release kinetics

4.3 Induction of Cirrhosis in animal model

To induce the cirrhosis, CCl4, urethane, and peanut oil were used. However, some different parameters were performed including histopathology, serology, liver and body weight analysis, and ascites observation to rule out the outcoming findings.

4.4 Treatment of Cirrhosis induced animal model.

After the successful induction of cirrhosis in female Sprague- Dawley rats, subjected forward to the treatment by using Etoposide intravenously (IV), ELNP (IV), and Blank liposomal nanoparticles (IV). Moreover, some testing parameters were performed including histopathology, serology, liver & body weight, and ascites observation to find out the efficacy of Etoposide and ELNP in the treatment of Advanced Liver Diseases in comparison with normal rats.

1.2.30 Histopathological Analysis of Liver, Kidney, and Spleen

The histopathological analyses of the liver, kidney, and spleen are provided in Figure22. The negative group/normal denoted, normal liver cells and tissue, with no hepatic structural and morphological changings [Figure (a)]. The positive control group/diseased shows, portal extension, hepatocellular dysplasia, cirrhotic lesions, & bile duct aligned by

epithelial and columnar cells, & fibrosis. [Figure (b)]. In the Etoposide treated IV group, hepatic cellular dysplasia, and early cirrhosis were identified [Figure (c)]. While in the group of Etoposide loaded pegylated nanoparticles IV treatment, no presence of cirrhotic nodules and no fibrosis, hepatocytes were normal, only dilated veins were seen, had histopathology in normal limits [Figure (d)]. In the Blank nanoparticles treated IV groups, mild fibrosis, moderate cirrhosis, epithelial and columnar lining, and portal extension were observed [Figure (e)]. However, the results with PEG-ELNP treatment are more effective as compared to other groups.

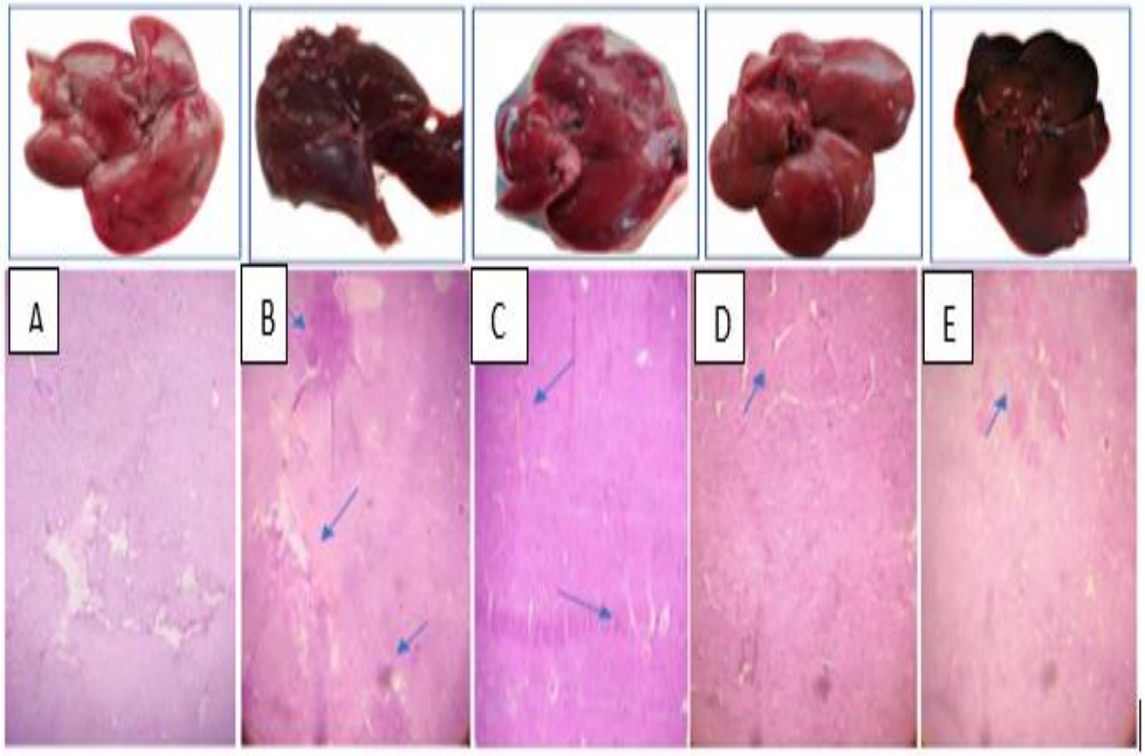


Figure 22HISTOPATHOLOGY OF LIVER

The histopathology of kidney in negative control group/normal, no histopathological and morphological changes were observed [Figure B, (a)]. In the renal tissues of diseased group, Tubular atrophic injury, cortical fatty changings, shrunken glomeruli, congestion, necrosis, >40% cortical tubular damage were identified [Figure B, (b)]. In the treatment of Etoposide treated IV group, renal tissues denoted, necrosis, congestion, and cortical fatty changings [Figure B, (c)]. The renal Histology of ELNP treated group 1 [Figure B, (d)] & 2 [Figure

B, (e) showed mild tubular injury, <20% tubular damage, and mild congestion. The renal histopathology of Blank treated IV group [Figure B, f)], >30% cortical tubular damage, necrosis, and congestion were noted.

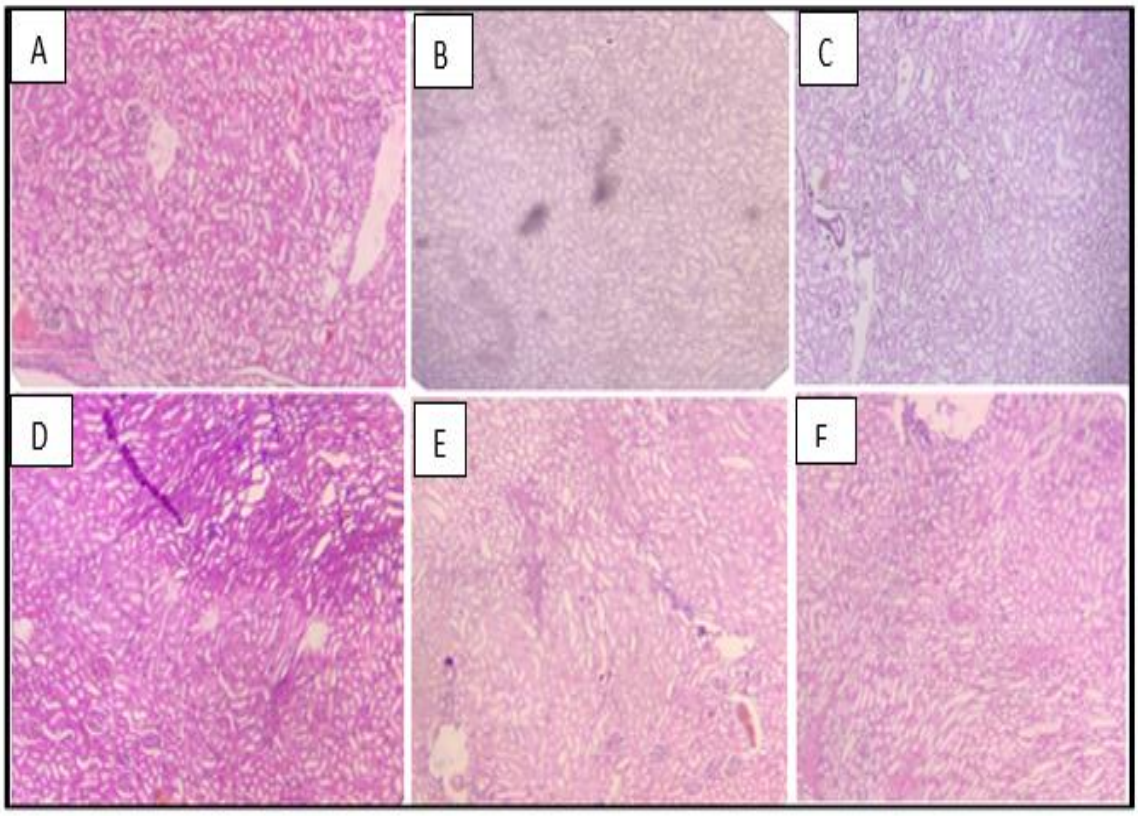


Figure 23Renal Histopathology for Different groups

The Histology of Etoposide IV treated spleen showed expanded white pulp, mild fibrosis [Figure C, (a)]. In the negative control group/normal [Figure C, (b)], no histopathological changings were observed. In the spleen of ELNP treated IV group, expanded red and white pulp were observed but no congestion present [Figure C,]. The spleen of positive control group/diseased demonstrated, hyperplasia of red and white pulp, congestion, mild fibrosis, follicular formation [Figure C, (d)].

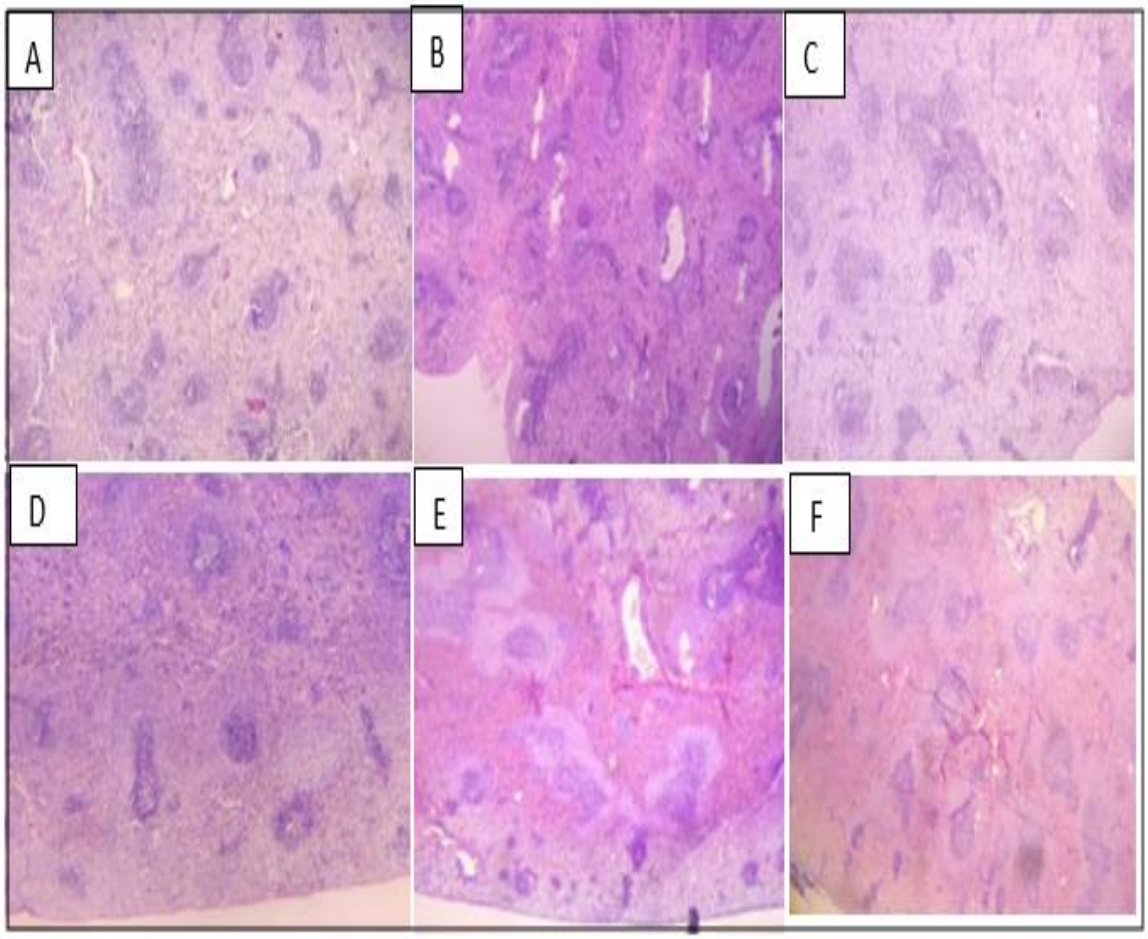


Figure 24Splenic Histopathology for Different groups

4.5.2. Serological Indices of Treated Rats

The serological analysis showed a difference between Diseased/ positive control, negative control/normal, Etoposide treated, and PEG-Etoposide loaded liposomal nanoparticles. The serological indications of LFTS shown in the diseased group as compared to the normal as (AST; p = 0.0001, T.B; p = 0.289 ALT; p = 0.001, ALP; P = 0.001). According to this consideration an eminent injury of the liver has been noted, causing cirrhosis, while the comparison of Etoposide with PEG-ELNPs shown as (AST; p = 0.224, T.B; p = 0.144 ALT; p = 0.0029, ALP; p = 0.009), according to this difference it is observed that significant decrease was only observed in the ALT levels. Whereas the contrast of Blank liposomal nanoparticles with ELNPs (AST; p = 0.10, T.B; p = 0.205 ALT; p = 0.0013, ALP; p =

0.0001) shown that it is a lesser remarkable difference as compared to other serological findings taken by the blood of rats via cardiac puncture. The ALT results showed mean and standard deviation for the diseased group as (91.667 ± 2.082), For Etoposide treated group as (115.3 ± 1.528), For ELNP treated group as (104.33 ± 2.517), For BLN treated group (124.333 ± 3.512). The ALP results show the Mean and standard deviation among different groups as diseased group (646.333 ± 4.041), ET treated group (249.0 ± 7.937), normal group (166.0 ± 1.000), ELNP treated group (223.3 ± 4.726), whereas in Blank treated group (533.333 ± 5.132). The total Bilirubin demonstration for Mean and Standard Deviation among distributed groups as, diseased group (0.500 ± 0,100), For normal group (0.400 ± 0.100), For Et treated group (0.767 ± 0.153), For ELNP treated group (0.567 ± 0.115), and For BLN treated group (0.700 ± 0.100). Whereas the AST demonstration of Mean and Standard Deviation in different groups is shown as, For the diseased group (131.000 ± 1.000), For the Normal group (153.000 ± 1.000), For Et treated group (148.667 ± 6.351), For ELNP treated (154.333 ± 2.517), and BLN treated as (151.000 ± 1.000).

1.2.31 Physique and Liver Weight

Body and liver weights reveal a significant reduction in diseased rats as compared to normal rats. According to notified calculations of different groups of rats, weight analysis of the body and liver was performed in a normal group, diseased group, Etoposide treated, ELNP treated, and BLN treated were observed in Figure 6. Body weight analyses were observed on a weekly basis whereas liver weight analysis was observed upon Dissection after treatment. The liver weight was higher in ELNPs treated group as compared to Et group. The weight of the ELNPs treated group decreased in second week and increased till 4th week of treatment. Whereas weight of the Et treated group kept on increasing after treatment. (Figure 7b).

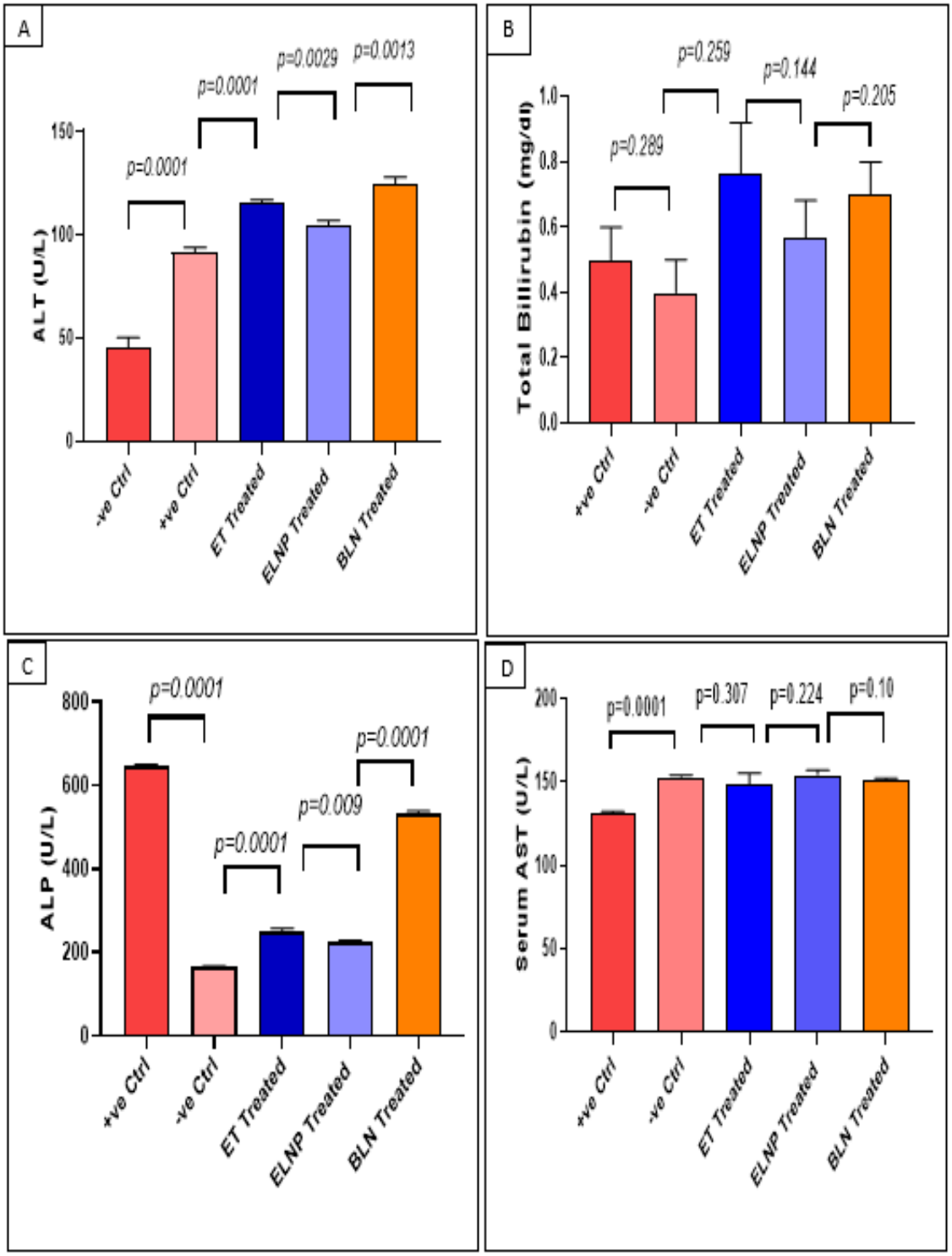


Figure 25Graphical representation of LFTS (A) ALT, (B) Total Bilirubin, (C) ALP, (D) AST

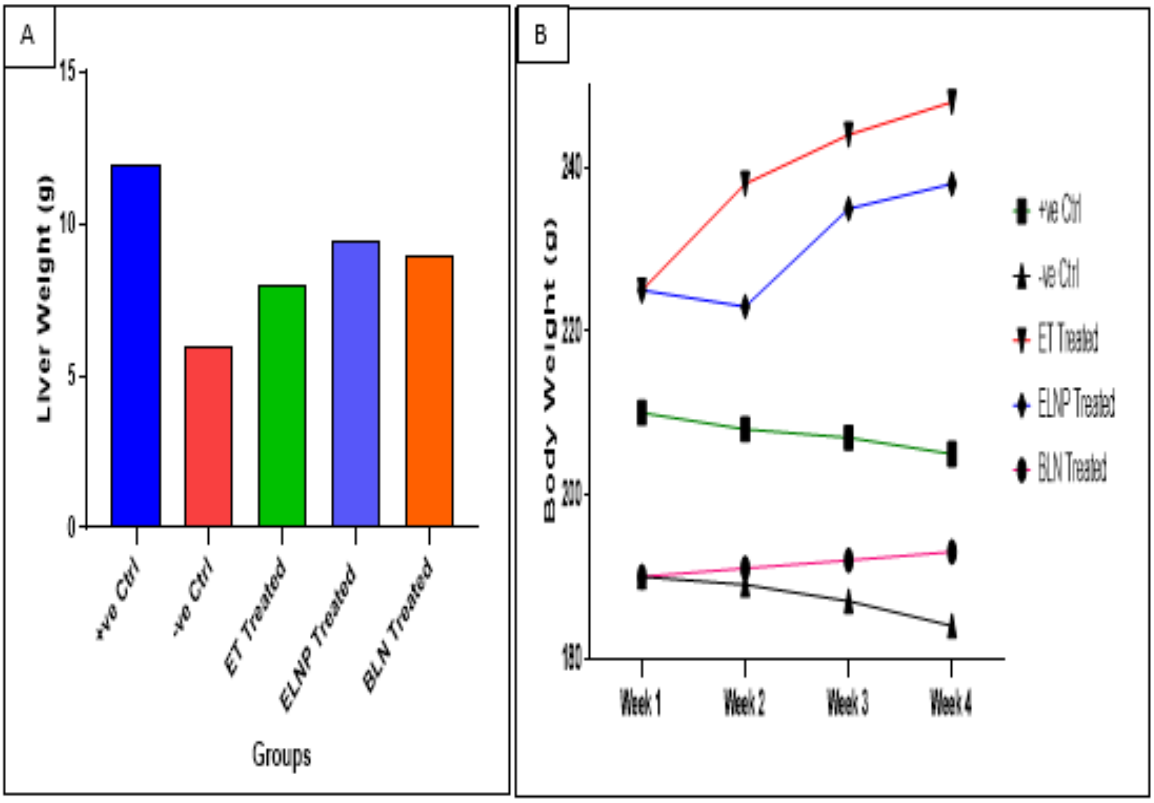


Figure 26A) Liver & (B)Body weight among Control and treated groups

CHAPTER 5

DISCUSSION & CONCLUSSION

DISCUSSION

In general, hepatic cirrhosis is investigated as an advanced phase of scarring/fibrosis, if left untreated or delayed diagnosis, more likely it develops into advanced stages of the liver such as bacterial peritonitis and even liver cancer. Therefore, it is necessary to focus on the management of liver cirrhosis. Various treatment strategies are available for that purpose, but limited effects have been shown. A therapeutic drug Etoposide has been used for the treatment of advanced liver diseases but due to its toxic effects and biocompatibility, does not show fruitful results [35]. Through the involvement of nanotechnology, several drugs with less solubility and biocompatibility are introduced for treatment strategies by the combination of nanoparticles [36]. Etoposide shows its action by activating the topoisomerase II and by enhancing the cell cycle at the end of the synthesis phase and gap phase [37]. In this study, we used etoposide with the combination of nanotechnology. For this purpose, PEG-coated Etoposide has been used for the treatment of liver cirrhosis in an animal model of rats and examined their results. Etoposide is a hydrophobic, encapsulated in the hydrophobic tail of DMPC and DPPC. Pegylation increases the steric aversion, therefore compensating the formulation [38]. The previous studies also demonstrated the use of CCl4 and Urethane for the induction of liver cirrhosis. CCl4 is a strong hepatotoxin. The biotransformation of CCL4 in the liver causes the release of free radicals and reactions of lipid peroxidation which leads to hepatocellular necrosis [39]. Moreover, prolonged administration of CCl4 caused portal duct fibrosis [40]. The current research scenario is one of that type, using CCl4 and Urethane for induction of liver diseases and monitoring results through histopathological study and Liver Function tests.

After the 5 weeks interval of induction of hepatic disorders, the antifibrotic activity of Etoposide, ELNPs, and BLNPs was carried out. By using the ELNPs and Etoposide, a successful reversible of collagenous scar to normal hepatocytes become possible. Hepatic necrosis and fatty changes were noticed which extended from mild to severe during five weeks of induction. The Lymphoid follicular involvement, hyaline accumulation, and hyperplasia of red and white pulp in the spleen have been observed following the 5 weeks of induction which confirm the liver cirrhosis and dysplasia of hepatocytes. Some characterization methods like UV-VIS Spectrometry, FTIR, Zeta potential, and SEM were

performed for Nanoparticles absorbance and size. Several testing methods, including histopathological studies, serology, and body and liver weight interpretations showed a notable improvement in the treatment through both Et and ELNPs. According to serological consideration an eminent injury of the liver has been noted, causing cirrhosis, while the comparison of Etoposide with PEG-ELNPs shown as (AST; $p = 0.224$, T.B; $p = 0.144$ ALT; $p = 0.0029$, ALP; $p = 0.009$), according to this difference it is observed that significant decrease was observed in the ALT levels, generally, ALT, AST, and ALP levels increased in liver disorders. The ALT results showed mean and standard deviation For Etoposide treated group as (115.3 ± 1.528), For ELNP treated group as (104.33 ± 2.517). However, suggested that encapsulated Etoposide is beneficial for the treatment of advanced liver diseases as compared to Blank Etoposide as it decreases the toxicity of the Et towards liver cells as observed in the histological analysis. According to histological interpretation of liver, diseased liver demonstrates the Cirrhotic lesions, fibrosis, epithelial columnar lining, and hyperplasia of hepatocyte, while in ELNP treated group no presence of cirrhotic nodules and no fibrosis, hepatocytes were normal, only dilated veins were seen, had histopathology in normal limits, in contrast to etoposide showed early cirrhosis and hepatic cellular dysplasia. The histopathological interpretations of diseased kidney demonstrated hyperplasia of red and white pulp, congestion, mild fibrosis, follicular formation, the Etoposide treated group showed, expanded white pulp, mild fibrosis, and in contrast to ELNP treated group no congestion, only expansion of red and white pulp were noted. By these considerations of results, its proven that the treatment in the group of ELNP treated is more effective than other groups. Hence, Etoposide-loaded liposomal encapsulation coated with PEG leads to sustained release which eventually enhances the bioavailability and pharmacokinetics of Etoposide. The LFTs were almost similar for both Et and ELNPs treated groups. This effect must be explored in future studies. PEG-ELPs are proven to be effective for hepatic cirrhosis treatment, and this is a positive indication for the management of NASH/NAFLD. Future studies are required to rule out the effect of PEG-coated ELPs in the treatment of Hepatocellular Carcinoma, gene & protein study, tumor necrosis factor-alpha, and growth factor beta to understand the mechanism of Etoposide in detail.

LIMITATION

The present study was short term study which included treatment for only 4 weeks. We couldn't see any effect on LFTs after this time period. Long term in vivo studies with different dosages of ELNPs must be explored in future.

CONCLUSION

Etoposide has been used in combination with PEG-liposomal Nanoparticles to reduce toxicity, improve bioavailability, pharmacodynamics and prolong circulation for the treatment of Advance Liver diseases. Histological analysis confirmed the limited toxicity of ELNPs towards liver cells hence treatment with PEG-ELNP was more significant than simple Et treatment. In future studies, long term effects of the ELNPs must be studied and compared with simple Et and their effects on the LFTs must be explored.

CHAPTER 6

REFERENCES

REFERENCES

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