LIPOSOMAL DOXORUBICIN FOR THE TREATMENT OF ADVANCED LIVER DISEASE

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ISLAMABAD

2022

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

MS Biomedical Sciences

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Acknowledgements

In the name of Allah, Beneficent the Merciful

First of all, I have no words to express my deepest and infinite sense of gratitude to ALLAH the Almighty, Who knows all the hidden and exposed things in the entire Universe, Who gives me the courage to complete the work.

I want to express sincere acknowledgment to my Supervisor, Dr. Nosheen Fatima, for her queen supervision and practical advice during the research work. It is a great honor for me to be a student of such an enlightened personality. I would like to give my supervisor special regards for her support and concern during my whole research. She always guided me in a time of need.

I am profusely thankful to my beloved parents who raised me when I was not capable of walking and continued to support me throughout every department of my life and thankful to my siblings for their endless support.

I would also like to thank Dr. Omer Gilani, Dr Faheem Amin, and Dr Aneeqa Noor for being a part of my thesis guidance and evaluation committee.

I would also like to pay special thanks to Sir Saeed, a best friend of mine, for his tremendous support and cooperation. Without his help I wouldn't have been able to complete my thesis. I truly appreciate his patience and guidance throughout my whole thesis.

I would also like to express my profound gratitude to my most amazing seniors Tehreem Tanveer, Ayesha Naeem and Afrah Nawaz and my class fellows Arfa Iqbal and Shabia Azhar for their support and cooperation. Their constant support and continuous guidance throughout the whole journey of this project enabled me to complete my thesis on time. They were my guide and inspiration during my research work. I would not have been able to complete my thesis without their assistance throughout my research work.

I am forever indebted to my educational institute, The National University of Sciences and Technology (NUST), Islamabad, for giving me an opportunity for the completion of an MS Degree in such an excellent educational environment by providing knowledgeable faculty during the whole course.

Dedicated to my exceptional mother and adored siblings whose tremendous support and cooperation and to my father (late) whose prayers led me to this wonderful accomplishment.

LIST OF ABBREVIATIONS

LNPs	**Liposomes Nanoparticles**
PEG-LNPs	**Pegylated Liposomes Nanoparticles**
DPPC	**Dipalmitoyl phosphatidylcholine**
NASH	**Non-Alcoholic Steatohepatitis**
NAFLD	**Non-Alcoholic Fatty liver Disease**
DMPC	**1,2-Dimyristoyl-sn-glycero-3-phosphocholine**
HCC	**Hepatocellular Carcinoma**
RES	**Reticulocyte Endothelial System**
AMPK	**AMP activated Protein kinase**
HSC	**Hepatic Stellate cells**
Ccl4	**Carbon tetrachloride**
Ccl3	**Trichloromethyl radical**
PEG	**Polyethylene glycol**
AST	**Aspartate transaminase**
ALT	**Alanine transaminase**
ALP	**Alkaline phosphatase**
T.B	**Total Bilirubin**
RPM	**Rotation per minute**
PDI	**Polydispersity Index**
FTIR	**Fourier Transform Infrared**
I.V	**Intravenous**

Abstract

Nanoscale materials are utilized as diagnostic instruments or to administer therapeutic substances to specific targeted regions in a controlled manner in the fields of nanomedicine and nano drug carriers, which are still relatively young but are quickly evolving. Nanoparticles can potentially deliver medications more accurately because they are currently made from biocompatible materials. The treatment of advanced liver disease has benefited greatly from nanomedicine in previous decades. Nano-based drug delivery systems improve the effectiveness of medications. Today, advanced liver disease is being treated with liposomal nanoparticles. Nano-based drug delivery systems increase the efficacy of both new and existing treatments through a detailed investigation of nanoparticle manufacturing and utilization.

One of the most often used anticancer medications is doxorubicin. Doxorubicin (DOX) is a medication that is frequently used to treat HCC. Doxorubicin is a medicine that belongs to the anthracyclines class and is commonly used to treat different types of cancers, including lymphomas, leukemias, breast, ovary, thyroid, and lungs.

Doxorubicin interacts with nitrogen - containing bases of DNA and prevents the production of macromolecules. This, in turn, prevents the action of the enzyme topoisomerase II (Top II), which inhibits the replication process. Consequently, malignant cells are prevented from proliferating. According to early research, doxorubicin's cardiotoxicity is reduced when it is encapsulated inside liposomes.

Due to its cardiotoxicity, the "thin film hydration approach" was utilized to create doxorubicin-encapsulated liposome nanoparticles, which were then used to treat advanced liver disease. The liposome nanoparticles were coated with polyethylene glycol (PEG) to boost their stability and provide a stealth effect. Pegylation improves steric repulsion and is therefore regarded as a superior stabilizer for various kinds of nanoparticles. PEG adopts the drug's erosion-controlled release mechanism, which led to continuous release. It is noted that a significant technique to treat NAFLD is to encapsulate the doxorubicin drug within liposomes and modify these liposome nanoparticles via PEG.

Key Words: *Doxorubicin, Liposome Nanoparticles, Pegylation, Advanced Liver Disease, NASH, Fibrosis, Animal Model, In vivo administration, Histological and Serological Examination.*

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CHAPTER 1: INTRODUCTION

1.1 Objective

Two sections make up the research work of the dissertation. The liposomal nano formulation of doxorubicin medication and its characterization were the primary focus of the first section of this study. The chosen medicine has been utilized to treat fibrosis and NAFLD because of its versatility. "Thin film hydration method" is the process used to create nanoparticles. With the use of several characterizing procedures, various characteristics of the formed nanoparticles are characterized, allowing for the eventual in vivo investigation of liver cirrhosis.

The construction of a liver cirrhosis model and in vivo testing for the encapsulated drug's better pharmacokinetic characteristics were highlighted in the second extended section. In the second portion, a model for liver copper toxicity was created and its effects were addressed. By contrasting the drug and nano-formulations administered via intravenously, the efficacy of the treatment was determined. When these liposome-encapsulated doxorubicin liposome nanoparticles are evaluated in the well-established animal model for liver copper toxicity, they will be seen as a significant step towards preclinical trials.

1.2 Nanotechnology; An Overview of the New Era

The ability to organize and manipulate matter at the molecular or atomic level, between 1 and 100 nm, and to make use of unique characteristics and anomalies is known as nanotechnology (Roco, 2011). Most people credit Feynman (1960) and his well-known lecture "There is plenty of room at the bottom" with defining nanotechnology, however Taniguchi (1974) may have originated the term itself roughly 15 years later (Zingg & Fischer, 2018). The notion of nanotechnology was agreed upon and acquired a certain level of worldwide recognition after consultation with specialists from more than 20 nations (Siegel et al. 1999). Conceptually, it differs from earlier ideas that focused on surfaces with atom and molecule patterns, ultra-precision engineering, ultra-dispersions, or minuscule features at a given scale (Roco, 2011).

1.2.1 Nanotechnology advancement

Nanotechnology is a young subject that is growing quickly and has the potential to bring about improvements, provide innovative goods, enable new and enhanced human capabilities, and generally redefine partnerships in a variety of industries through invention (Forloni, 2012). According to Roco, the first generation of nanoparticles were passive at first (polymers, ceramics, and nanostructured metals), turned active (targeted drugs and 3D transistors, etc.) with the second generation in 2005, reached third generation in 2010, and eventually will have the atomic layout of molecular nano structures (Roco 2006, 2007).

The discipline of nanotechnology today offers a wide range of opportunities in many traditional fields of research. including health sciences and biology, allowing integrated systems that rely on unmet issues, physics, chemistry, engineering, and biotechnology (Sainz et al., 2015). Currently, the application of nanotechnology is focused on the fields of medicine, green energy, electronics, and environmental protection. However, some nanotechnology-specific products have already hit the market, including those for clothing, cosmetics, sporting goods, and housing and construction (Forloni, 2012).

1.2.2 Therapeutic Uses of Nanotechnology

Functional systems are created at the cellular scale in nanotechnology. These systems' physical, visual, and electrical characteristics make them intrigue to a variety of disciplines, from biology to materials science. To prevent, diagnose, and treat disease, highly tailored medicinal treatments are made possible by nanotechnology (Bamrungsap et al., 2012; Wagner et al., 2006). The current surge in nanomedicine research has accelerated commercialization efforts globally, with a variety of treatments already available and others waiting in line (Lobatto et al., 2011).

Nanotechnology has received a lot of attention during the last 20 years and is now considered a "new technological revolution." Over the past few decades, significant molecular and macromolecular nanoscale-level nanomedicines and tailored nanodevices have been created (Sainz et al., 2015). An important step toward making pharmaceuticals and treatments more accessible and affordable is the utilization of nanotechnology in nanomedicine, which has the potential to lead to significant advancements in efficient and cost-effective treatment (Sainz et al., 2015). In comparison to conventional low molecular weight pharmaceuticals, nanomedicines have several

advantages. For instance, they (1) inhibit hepatic degradation and renal excretion, extend circulation, and (2) modify volume distribution reduction, causing non-targeted site avoidance. (3) increase the medications' ability to assemble in diseased conditions. Additionally, nanomedicine formulation aids in the passage of low molecular weight chemotherapeutic drugs across numerous obstacles to diseased areas (Rizzo, Theek, Storm, Kiessling, & Lammers, 2013).

1.3 Liposomes and its Nanoparticulation

First described in the middle of the 1960s, liposomes are sphere-shaped vesicles made up of one or more lipid bilayers (Akbarzadeh et al., 2013). Liposomes can range in size from 30 nm to several micrometres (Wagner & Vorauer-Uhl, 2011). With respect to lipid content, size, surface charge, and manufacturing method, liposome characteristics vary greatly. The surface charge and fluidity of a bilayer are also determined by the bilayer's content choice. Unsaturated phosphatidylcholine species found in naturally occurring sources, such as egg or soybean, call for less stiff and permeable bilayers, whereas saturated phospholipids with long acyl chains, like dipalmitoyl choline, do the opposite. Among the many effective current drug delivery methods, liposomes stand out as a cutting-edge method for getting active molecules to the action site (Sharma, Aara, Ali, & Trivedi, 2018). Thin-film hydration, which entails lipid component dissolution in an organic solvent, is the most used method for liposome formation. Using rotary evaporation, the solvent will be evaporated before the film is rehydrated in an aqueous solvent (H. Zhang, 2017). It is also possible to use techniques like ethanol infusion and freeze-drying. The size and distribution of the particles can be altered by using procedures including homogenization, sonication, membrane extrusion, freeze-thawing, or membrane extrusion. Many different morphological and functional liposomes can be created using liposomal preparations and processing (Bulbake et al., 2017).

1.3.1 Liposomes as carrier

Amphiphilic phospholipids are used to create artificial vesicles called liposomes. These vesicles are composed of an aqueous core domain surrounded by a spherical bilayer structure that can range

in size from 10 nm to several micrometers. Medical researchers employ the new delivery method known as liposome encapsulation technology to deliver drugs to the desired body organs. Liposomes are a type of submicroscopic foam production that encapsulates different components (Akbarzadeh et al., 2013). The pharmaceutical and cosmetic sectors frequently employ liposomes as molecular carriers (Atrooz, 2011). Both hydrophilic and hydrophobic compounds can be encapsulated by liposomes, limiting their decomposition, and allowing them to release at their intended locations (Shehata, Ogawara, Higaki, & Kimura, 2008). Recently, the FDA approved liposomes, which constitute an advanced technology with shown clinical effectiveness (Meng et al., 2016).

1.4 Advanced Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver disease in the West, and its prevalence is expected to rise with that of diabetes, obesity, and other metabolic syndrome symptoms. (Mengesha et al., 2021a). NAFLD can induce a wide variety of liver damage, from simple steatosis to cirrhosis, inflammation, non-alcoholic steatohepatitis (NASH), and fibrosis. (Lam & Younossi, n.d.). The metabolic syndrome's hepatic manifestation, NAFLD, is currently acknowledged as one of the most prevalent liver illnesses in the world. In most Westernized countries, 2-3% of people develop NASH, while 20-30% of the adult population as a whole has hepatic steatosis, according to estimates. (Longato, 2013; Qiu & Chen, 2015).

Nonalcoholic fatty liver disease (NAFLD) is the buildup of fat (steatosis) in the liver without consideration of the secondary causes of fatty liver, such as excessive alcohol consumption, viral hepatitis, or medicines. Some of the metabolic conditions that are frequently connected to NAFLD include diabetes, hypertension, obesity, hypertriglyceridemia, and hyperlipidemia. (Neuschwander-Tetri, 2017). Studies show that NAFLD-related liver diseases are becoming more common. Due to the increased incidence of type 2 diabetes and obesity worldwide, NAFLD is increasingly becoming a substantial risk factor for liver cancer and end-stage liver disease. NAFLD is anticipated to be among the most common reasons for liver transplantation within the next ten years. (Martins & Oliveira, 2018).

Liver cirrhosis is an advanced form of fibrosis that is characterized histologically by the development of regenerating nodules encircled by thick fibrotic septa. Cirrhosis is viewed

clinically as an advanced condition that, if left untreated, will eventually result in death. Liver cirrhosis is an advanced form of fibrosis that is defined histologically by the development of regenerating nodules surrounded by thick fibrotic septa, leading to end-stage liver disease. (Schuppan & Afdhal, 2008; Tsochatzis, Bosch, & Burroughs, 2014). These fibrotic septa disrupt the blood flow in the liver parenchyma and inhibit regular oxygen delivery. Due to these circumstances, portal hypertension and hepatocellular dysfunction are also present. (Berzigotti, 2017). (Garcia-Tsao, Abraldes, Berzigotti, & Bosch, 2017), ascites (Liver, 2010), and hepatic encephalopathy (Hytiroglou et al., 2012). The possibility of cirrhosis reversibility is based on results obtained in animal cirrhosis models, the use of a potential anti-fibrotic agent, or the withdrawal of the substance causing liver injury. (van Leerdam, 2008). Instead of fighting fibrosis, available anti-fibrotic medicines have concentrated on reducing hepatic inflammation. Therefore, a potent therapeutic drug that can reverse liver cirrhosis and eradicate fibrosis is required.

1.4.1 Available Treatment for Liver Cirrhosis

Cirrhosis is a condition that, if left untreated, is thought to be in its last stages and will eventually result in death. (D'Amico, Garcia-Tsao, & Pagliaro, 2006). Hepatocyte transplantation improves and reverses advanced stage fibrosis and the physiology of the liver. (Cai et al., 2002; Nagata et al., 2003). An optimistic transplantation paradigm may have resulted from in vitro growth after isolating progenitor or hepatocyte stem cells. (Malhi, Irani, Gagandeep, & Gupta, 2002; Nowak et al., 2005), however progenitor or stem cell competence is very low, and the necessary alterations for optimal engraftment in humans would carry extremely high risks of liver failure. (Thorgeirsson & Grisham, 2006). Telomerase can also enhance hepatocyte regeneration by restoring the genome (Rudolph, Chang, Millard, Schreiber-Agus, & DePinho, 2000) although enhanced telomerase activity may also be a sign of hepatocarcinogenesis. (Martin & Dufour, 2008).

Reversibility of cirrhosis is determined by results obtained in animal advanced liver disease models, by employing a potential anti-fibrotic agent, or by stopping the substance causing liver injury (van Leerdam, 2008). Instead of fighting fibrosis, available anti-fibrotic therapies have concentrated on reducing hepatic inflammation. Therefore, a potent therapeutic drug that can reverse the advanced liver disease and eradicate fibrosis is required.

CHAPTER:2 LITERATURE REVIEW

2.1 Doxorubicin: A Multipurpose Drug

The bacteria Streptomyces peucetius is the source of the antibiotic doxorubicin. As a chemotherapeutic agent, it has been widely used since the 1960s. Doxorubicin belongs to the anthracycline class of chemotherapy drugs, along with daunorubicin, idarubicin, and epirubicin. Doxorubicin is a medication frequently used to treat solid tumors in both adults and pediatric patients. Breast, ovarian, bladder, thyroid, and soft tissue and bone sarcomas can all be treated with doxorubicin. It is also used to treat Hodgkin lymphoma, acute myeloblastic leukemia, acute lymphoblastic leukemia, and small cell lung cancer. The FDA has approved the liposomal doxorubicin formulation for the treatment of Kaposi sarcoma associated with AIDS, multiple myeloma, and ovarian cancer in patients who failed platinum-based chemotherapy. (Kelly Johnson-Arbor; Ramin Dubey,2022).

A powerful antineoplastic drug having activity against a variety of human malignancies is doxorubicin. Doxorubicin's bioavailability, biodistribution, and hence biological activity are drastically changed by liposomal encapsulation. Drug persistence and pharmacokinetics are significantly influenced by the physical characteristics of the lipid nanoparticles (size, lipid dosage, and lipid components)

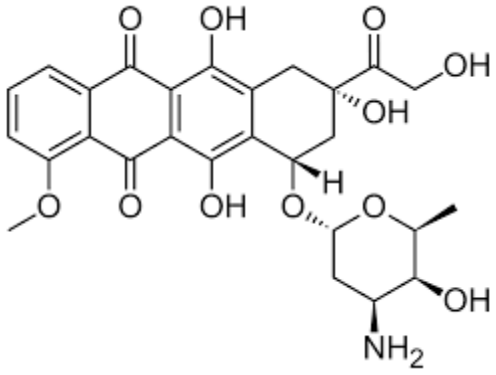


Figure 2.1: Structure of Doxorubicin

2.1.2 Mechanism of Action

Doxorubicin is thought to work in cancer cells through two different mechanisms: intercalation into DNA, interruption of topoisomerase-II-mediated DNA repair, and production of free radicals that cause damage to cellular membranes, DNA, and proteins. In a nutshell, doxorubicin is oxidized to semiquinone, an unstable metabolite, and then reconverted to doxorubicin in a procedure that causes reactive oxygen species to be released. Reactive oxygen molecules can cause DNA damage, increased lipid peroxidation, lipid peroxidation and mitochondrial dysfunction, as well as start the apoptotic pathways that cause cell death. (Thorn, C. F., Oshiro, (2011).

2.1.3 Doxorubicin against Advanced Liver Disease

Pegylated doxorubicin liposome may be a safe and efficient hepatocellular cancer treatment, even in the condition of weakened liver function. It merits additional research to determine how it can cure advanced hepatocellular cancer. (Hong, R.-L., Tseng, Y.-L., & Chang, F.-H. ,2000). The medication DOX is capable of selectively targeting HSCs and prevents hepatic steatosis in BDL rats. The use of antiproliferative medications for antifibrotic objectives may become more feasible because of this. (Greupink, R. 2006).

Doxorubicin is a popular antibiotic used in chemotherapy for cancer patients. According to theory, it inhibits topoisomerase II and DNA intercalation, which results in the generation of reactive oxygen species, DNA crosslinking, and apoptosis3.(Sergazy, S., Shulgau, 2020)

2.2.2 Carrier Necessity for Doxorubicin Delivery

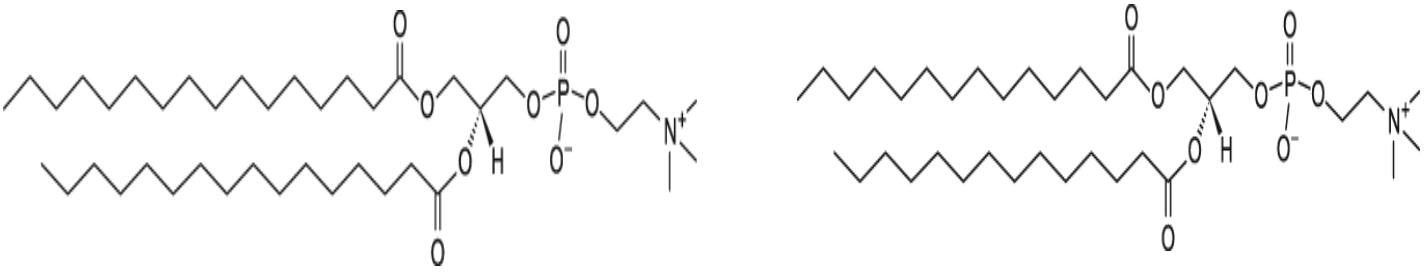
A powerful and often used cancer chemotherapy drug, doxorubicin has a chronic dose-dependent side effect known as cardiotoxicity, which significantly hinders its use. To get over this restriction, a medication delivery system based on nanoparticles has been developed. (Du, Y., Xia, L.2018).

The FDA has licensed the anticancer medication doxorubicin (DOX) for the treatment of tumors, but it has clear drawbacks in clinical use, including severe toxicity, poor absorption, and high dose

requirements. Numerous investigations have been concentrated on developing DOX-based drug delivery systems that concurrently demand high medication consumption and low adverse effects. (Wang D, Zhang X and Xu B 2021)

2.3 Significance of Liposome Nanoparticles Against Advanced Liver Disease

Dexamethasone-loaded liposomes have been shown to have therapeutic potential, and their use has also been shown to significantly reduce liver fibrosis and inflammation. According to research, these nanoparticles influence Kupffer cells by decreasing T cells through an immunological response in the liver, which reduces fibrosis (Bartneck et al., 2015). It has also been reported that cationic liposomes with microbubbles can deliver artificial microRNA to target the connective tissue growth factor (CTGF) and can be effective in inhibiting hepatic fibrosis. In their investigation, the introduction of synthetic microRNA reduced levels of the fibrotic marker collagen and alpha-smooth muscle actin (alpha-SMA) in a fibrotic animal model generated by dimethyl nitrosamine (Yang et al., 2013). Targeting hepatic stellate cells with peroxisome proliferator-activated receptor-gamma (PPAR-) ligand-loaded mannose-6-phosphate (M6P)-human serum albumin (HSA)-conjugated liposomes has begun a new trend in the treatment of hepatic fibrosis. In a Ccl4-induced fibrosis animal model, M6P receptor targeting nanoparticles as small as 130 nm reduced the signs and symptoms of liver fibrosis both in vitro and in vivo (Zhang, Kong, Lu, & Zheng, 2013).



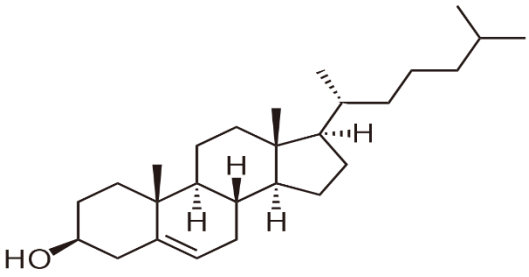


Figure 2.3: (a) Structure of DPPC (b) Structure of DMPC

(c) Structure of Cholesterol

2.4 Synthesis of Liposome Nanoparticles

All processes for making liposomes involve steps like drying down lipids from organic solvent, dispersing the lipid in aqueous media, purifying the resulting liposome, and evaluating the finished product. The formulation of liposomes involves the use of many lipid types, each of which has unique qualities such as size, zeta potential, biocompatibility, drug release kinetics, and cell targeting (Anderson & Omri, 2004; Tang et al., 2018).

There are numerous ways to make liposomes. The techniques employed to create the liposomes have an impact on their lamellarity and size (Dimov, Kastner, Hussain, Perrie, & Szita, 2017; Maeki, Kimura, Sato, Harashima, & Tokeshi, 2018; Pattni, Chupin, & Torchilin, 2015).

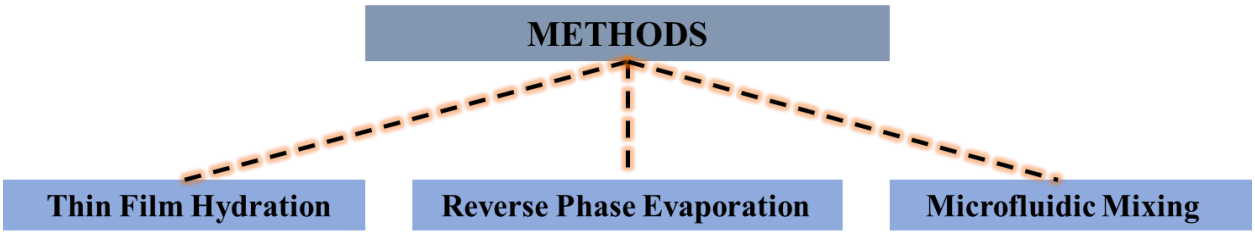


Figure 2.4: Different methods used for the synthesis of liposomes Nanoparticles

2.4.1 Drug loading

Two types of technique are used for drug loading

a) Passive loading

The term "passive loading" describes a mechanism in which drug loading and liposome production occur simultaneously. Hydrophobic medications are kept within the liposome bilayer, whereas hydrophilic compounds are uniformly diffused in the aqueous phase (both inside and outside the liposomes). In specifically, the medication and lipids are first dissolved in a suitable solvent, and then they interact with water as the solvent evaporates, resulting in a thin film that is then hydrated to produce liposomes. When loading water-soluble pharmaceuticals, the lipid layer is dispersed in a drug-contained aqueous environment. The trapping efficacy of passive loading varies depending on several variables, such as lipid concentration, vesical size, drug solubility, and technique of production. Most of the time, the passive loading strategy results in an average drug to lipid ratio (D/L) of 0.05 (w/w) (Gubernator, 2011; Zhao, May, Chen, Undzys, & Li, 2015).

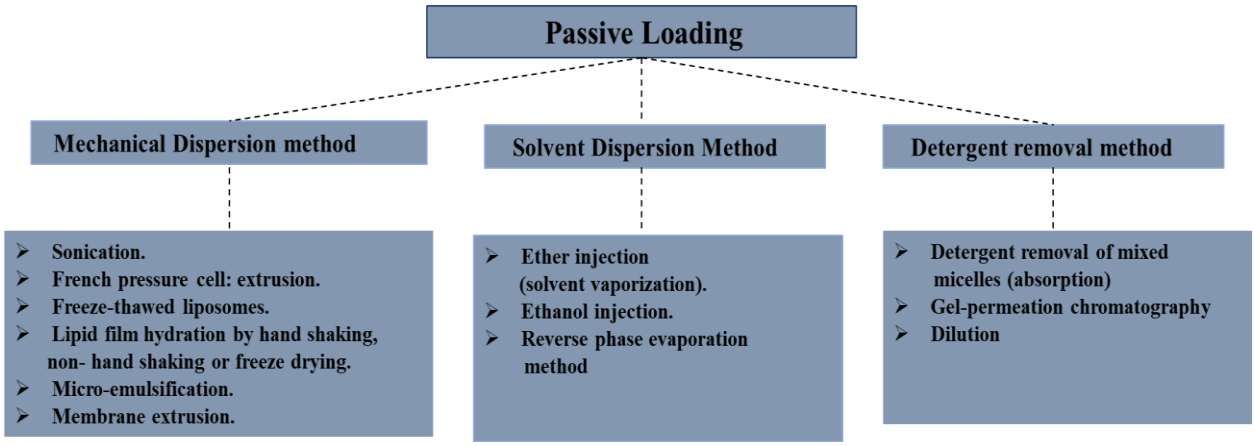


Figure 2.41: Different types of methods used in Passive loading

b) Active Loading

Active loading produces liposomes first that have a transmembrane gradient (the aqueous phase outside and inside the liposomes are different). The phospholipid bilayer(s) can then be penetrated by an amphipathic drug that has been dissolved in the exterior aqueous phase after interacting with an entrapment agent in the center to trap the drug in. Drugs can be held effectively and steadily in the core of liposomes by active loading, which is still a viable technique (Bhatt et al., 2018).

2.5 Stealth Liposomes Nanoparticles

Even while liposomes resemble biological membranes, they are nonetheless foreign substances to the body. Therefore, these are detected by the body's Reticulocyte Endothelial System after interacting with plasma proteins (RES). As a result, they are removed from the blood stream (Akbarzadeh et al., 2013). These stability-related restrictions were overcome by using synthetic phospholipids, polyethylene glycol (PEG), chitin derivatives, freeze drying, polymerization, and ganglioside micro-encapsulation to coat the liposome particle (Shaheen et al., 2006). Because PEG coating leads in long-term circulation and lower levels of liposomal phagocytosis, there is more opportunity for these liposomes to escape the bloodstream through the endothelium.

Stealth liposomes are sphere-shaped vesicles with bilayer membranes made of phospholipids with various lipid chains that are stabilised or coated with PEG or colloidal polymer to deliver medications or genetic material to certain cells. By making liposomes stealthy, new medication delivery systems for controlled release are created. This stealth approach has been applied to enhance the well-known doxorubicin-loaded liposomes, known as Doxil (Janson Biotech, Inc., Horsham, USA) or caelyx (Schering-Plough business, Kenilworth, USA) and now marketed for treating solid tumours (Akbarzadeh et al., 2013).

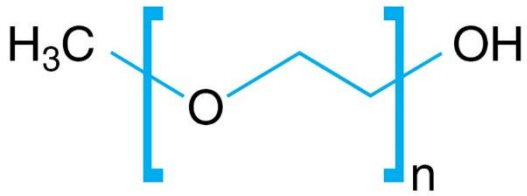


Figure 2.5: Structure of Polyethylene glycol (PEG)

2.6 Induction of Advanced Liver Disease by CCL4

The main techniques for causing liver cirrhosis in rats are bile duct ligation and CCL4 injection (BDL). CCL4 is a technique that is widely employed nowadays but is also very harmful. Hepatic tissues are harmed, and covalent bonding, metabolic activation, lipid peroxidation, altered calcium homeostasis, and reactive free radical metabolites are all associated with it. When CCL4 is administered, it causes fibrosis, necrosis, and inflammation that disseminate to join the vascular system that feeds into and drains the hepatic sinusoid (portal tract and central vein respectively). It produces liver cell death, zone 3 necrosis, and fatty infiltration upon repeated treatment by stimulating the hepatic stellate cells (HSC) (Marques et al., 2012).

Three or four separate steps are involved in CCL4-induced toxicity. The first two or three weeks are often characterized by necrosis, which is suggested by rising liver-specific enzyme activity and

falling pseudocholinesterase values. The following two to three weeks see considerable hepatic fat accumulation, and serum triglyceride and aspartate aminotransferase (AST) levels considerably rose. thereby decreasing hepatic activity. The third step's AST increase causes an increase in hydroxyproline and lipid levels while decreasing overall liver function. The final step shows a further decline in pseudocholinesterase levels and liver shrinkage (Paquet & Kamphausen, 1975). This might be connected to the significant drop in serum 13 albumin levels and weight loss, which would indicate a progressive decline in hepatic function while maintaining fibrogenesis (Scholten, Trebicka, Liedtke, & Weiskirchen, 2015).

2.6.1 Mechanism behind the liver damage

In the liver, the cytochrome P450 monooxygenases superfamily converts CCL4 to the trichloromethyl radical (CCl3) (CYP family). Following their interaction with lipids, proteins, and nucleic acids, these radicals change lipid metabolism, resulting in fatty degeneration and steatosis as well as a decrease in the quantity of proteins. Additionally, mutation results from ccl3-DNA adduct formation. By producing trichloromethyl per oxy radicals (CCl3OO*), the oxygenation of ccl3 further starts the lipid peroxidation and destruction of polyunsaturated fatty acids. As a result, cellular organelles such the mitochondria, endoplasmic reticulum, and plasma membrane lose some of their membrane permeability, which leads to significant hepatic damage that is eventually characterized by inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Weber, Boll, & Stampfl, 2003)

CHAPTER:3 MATERIAL AND METHODS

3.1 Experiment Design

3.1.1 Materials

The following items were bought from Sigma-Aldrich USA: Dipalmitoyl phosphatidyl choline (DPPC), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), cholesterol, doxorubicin medication, and polyethylene glycol (PEG. Molecular weight 2000). We purchased urethane and carbon tetrachloride (Ccl4) from Strem chemicals. The National University of Science and Technology (NUST), Islamabad's ASAB (Atta-ur-Rehman school of Applied Biosciences), provided the Wistar female rats that were used in this study. I carried out this work entirely using deionized water.

3.1.2 Synthesis of doxorubicin loaded-liposomes Nanoparticles

DPPC, DMPC, and cholesterol were utilized in a 3:1:1 (percent molar ratio) ratio to create the components for doxorubicin liposomal nanoparticle production. The initial step was to weigh each lipid—cholesterol, DPPC, and DMPC—and dissolve them in ethanol separately to create a 100Um solution. Following this, a 200Um solution of the drug doxorubicin was made in ethanol. 500UL of the 200Um doxorubicin drug solution was taken and added to the lipid solution. This lipid phase solution underwent a 40-minute bath sonication (at 80 MHz). Then, 10 ml of the lipid phase solution and deionized water were heated individually in a water bath until the temperature reached 60 OC**.** Lipid phase and water phase were combined, and the resulting dispersion mixture was continuously stirred at 90 RPM for 10 minutes. Following rotary evaporation (above the phase transition temperature, i.e., 50 OC) to remove ethanol, the fresh mixture was once more sonicated for 40 minutes at 50 MHz. (Chorachoo, Amnuaikit, & Voravuthikunchai, 2013; Meng et al., 2016).

3.1.3 Pegylation of doxorubicin loaded- LNPs

The Doxorubicin-loaded nanoparticle mixture was diluted to a volume of 50 ml, at which point 0.25% Peg 2000 was added while being stirred in, and the mixture was then subjected to rotary evaporation until 10 ml of solution remained. Using a dialysis tube, the drug that wasn't trapped was removed. (Stiufiuc et al., 2013).

3.2 Physical Characterization

Peg loaded nanoparticles characterization was determined to assess and examine to ensure that they are in proper size and to examine their charge, dispersity index, shape, drug loaded encapsulation and release efficiency.

3.2.1 U. V-Vis Absorption Spectroscopy

UV-Vis spectrometry is used to measure the extent of absorption in the sample. It is commonly used in analytical chemistry, chemical and clinical laboratories. A cuvette is placed, and a light beam is passed through a sample. When a sample is placed, then a beam of light is passed through the sample and certain wavelength is absorbed because different individual of samples absorbs different wavelength. First a sample set as a reference is put in cuvette inside the hood and then absorption of sample is measured. And then we can obtain the absorption spectrum by placing the second sample in cuvette inside the hood. A laser beam passing through two compartments, in which one of the half laser beams is focused on reference sample and other half laser beams is pointed on another second sample and now absorption spectrum is obtained, and we can measure the wavelength. At specific wavelength, maximum absorption is called lambda max. According to Beer Lambert Law theory, sample absorbance is proportional to the molar concentration of sample in cuvette, this value we measure is known as molar absorptivity and it is used to compare the different spectra of different chemicals.

The equation of Beer Lambert Law is

$$\mathbf{A = EcL}$$

Molar Absorptivity E = A/cL

(Where A= absorbance, c= sample concentrations in moles or liter and L = length of light in cm)

(Amendola & Meneghetti, 2009; Perkampus, 2013; Tomaszewska et al., 2013)

3.2.2 Fourier transform infrared spectroscopy (FTIR) analysis

Fourier transform infrared spectroscopy is often used in the labs to identify the organic and inorganic materials and for the characterization of nanoparticles. FTIR is used to obtain the transmission and absorption of specimens and identify unknown materials. When the sample undergoes the radiation of IR, several radiations are transmitted and some of them radiation are absorbed. In result, resultant spectrum shows molecular transmission and absorption making molecular fingerprint of the sample. (Khan, Saeed, & Khan, 2019; Mohamed, Jaafar, Ismail, Othman, & Rahman, 2017).

3.2.3 Particle size and Area Distribution

By using Scanning Electron Microscope, the morphology of Pegylated doxorubicin loaded liposomal nanoparticles and blank liposomal nanoparticles were studied. For particle size values are defined between 0 to infinity. (Gray levels: Ellipses: 0; background: 255) (Goldstein et al., 2017). A tiny amount of sample is placed on cover slip by micropipette. Both glass slides were coated with gold to make them conductive for SEM analysis. In SEM the size and distribution of both particles were evaluated.

3.2.4 Zeta Potential

Zeta potential measures the electrical charge of the nanoparticles. Zeta potential is also called electro kinetic potential and it is always expressed in millivolts (mV). It tells about the stability, surface charge and average size of nanoparticles.

3.2.5 Drug Release

Drug release has massive significance in treating with nanomedicine. Drug release from pegylated doxorubicin loaded liposomal nanoparticles were examined up to 48 hours. 5ml solution of Pegylated doxorubicin loaded liposomal nanoparticles and 5ml phosphate buffer were placed in 15 ml of centrifuge tubes. Solution was placed on an electronic shaker for 1 hr at 40 rpm. After this 3ml solution was separated and placed in centrifuge tubes and centrifuged for 10 minutes at 4500 rpm. In contrast, 3 ml of phosphate buffer was added to solutions of pegylated doxorubicin-

loaded liposomal nanoparticles. After this supernatant was permitted for analysis using a UV Spectrophotometer. The process was repeated 1, 2, 3, 5, 6, 7, 8, 12, 24, and 48 hours later. In UV spectrophotometer at 495nm wavelength was set as an absorbance value for drug release procedure and before these blank nanoparticles is set as a control or reference.

3.3 Development of Liver Cirrhosis Model:

3.3.1 Animals

Twenty Wistar female rats weighing 100-120g and aged 7-8 weeks were purchased from ASAB (Atta-ur-Rehman School of Applied Biosciences), National University of Islamabad (NUST), Islamabad, for the development of a liver cirrhosis model. Rats were housed in separate cages with food and water at a temperature of 27 ° c temperature for less than 12 hours during a light and dark cycle

3.3.2 Chemicals

For the induction of cirrhosis different chemicals were used i.e., Urethane, Carbon tetrachloride (CCl4), peanut oil, 10 % neutral formaldehyde and Ethanol.

3.3.3 Advanced Liver Disease Induction

Rats were allowed for two weeks in a regular environment. Chemicals were injected intraperitoneally to induce liver cirrhosis in groups 2, 3, and 4. In the beginning, 2.5% urethane was dissolved in DMSO (Dimethyl sulfoxide) and a dose of 1ml/kg of urethane was given intraperitoneally into rats twice a week for two weeks [23], [24]. After two weeks, CCl4 and (50%v/v) peanut oil was combined, and 1mg/ml of this solution was injected twice a week through intraperitoneally for 6 weeks. First 6 weeks 1mg/ml dose was injected but after 6 weeks amount of dose was reduced for next 7 weeks because of rat's condition. (Fortea et al., 2018; Gitiara et al., 2017).

3.3.4 Outcomes

Food, water intake and body weight were measured during this method. Body weight was measured once a week.

3.3.5 Serological Indices

Different serological liver function tests like AST (Aspartate transaminase or aspartate aminotransferase test), T.B (Total Bilirubin), ALT (Alanine transaminase) and ALP (Alkaline Phosphatase) were done by taking blood sample from heart of rats.

3.3.6 Histological Examination

Organs such as the liver, spleen, and kidney were removed after rats were sacrificed. We recorded the liver, spleen, and kidney's weight, size, and color. After dissection organs were washed with PBS solution and then placed immediately in 10% neutral formalin solution to avoid decomposition. Organs from the liver, spleen, and kidney were stained with hematoxylin and Eosin (HE) to examine the structural alterations. (Bedossa, 1993; Bedossa & Poynard, 1996).

Steatosis		
Grade	Parenchymal involvement	**Score**
	< 5%	0
	5-33%	1
	33-66%	2
	> 66%	3
Inflammation		
Lobular inflammation	Assessment of all inflammatory foci	
	No foci	
	< 2 foci per X 200 field	1
	2-4 foci per X 200 field	2
	> 4 foci per X 200 field	3
Portal inflammation	Assessed under low magnification	
	None to minimal	0
	Greater than minimal	1
Piecemeal Necrosis	Absent	0
	Mild (focal, few portal areas)	1
	Mild moderate (focal most portal areas)	2
	Moderate (continuous around <50% of tracts or septa)	3
	Severe (continuous around >50% of tracts or septa)	4
Microgranulomas	Small aggregates of macrophages	
	Absent	0
	Present	1
Large lipogranulomas	In portal areas or adjacent to central vein	
	Absent	0
	Present	1
Fibrosis Stage	Method of Brunt	
	None	0
	Perivenular Perisinusoidal Fibrosis	1
	Combined Pericellular portal fibrosis	2
	Septa bridging fibrosis	3
	Cirrhosis	4
		(__/17)

Table: 3.36 NASH/NAFLD Clinical Research Network Scoring System (Definition and score)

3.4 Treatment Design

To compare the anti-cirrhotic properties of Pegylated doxorubicin-loaded liposomal nanoparticles, pegylated blank liposomal nanoparticles and simple drug, rats were divided into various groups.

3.4.1 Negative Control Group

Six diseased rats were separated and assigned to the negative group. This group was left untreated throughout the experiment and at the end rats were sacrificed for histological examination and serological analysis. After dissection, ascites, liver weight, and body weight were observed.

3.4.2 Doxorubicin treated Intravenous (IV) group

Four rats were placed in this group. Doxorubicin drug at the dose of 10mg/kg were injected via intravenously for the duration of 3 weeks. Body weight, liver weight and ascites were noted after dissection for histological and serological analysis.

3.4.3 Pegylated blank liposomal nanoparticles treated intravenously (IV) group

Four rats were placed in this group. PBLNPs at the dose of 10mg/kg were injected via intravenously for the duration of 3 weeks. Body weight, liver weight and ascites were noted after dissection for histological and serological analysis.

3.4.4 Pegylated Doxorubicin loaded liposomal nanoparticles treated intravenously (IV) group

Four rats were placed in this group. PDLNPs at the dose of 10mg/kg were injected intravenously for the duration of 3 weeks. Body weight, liver weight and ascites were noted after dissection for histological and serological analysis.

CHAPTER 4: RESULTS

4.1 Characterization of Pegylated Doxorubicin loaded LNPs and PBLNPs

Both nanoparticles were characterized by UV spectrophotometry, FTIR, SEM and zeta analysis to find out their size, charge, dispersity index and absorbance.

4.1.1 UV-VIS absorption spectroscopy

UV-VIS absorption spectroscopy of doxorubicin drug showed absorption peak at 495nm, pegylated blank liposomal nanoparticles at 280nm and pegylated doxorubicin loaded liposomal nanoparticles showed absorption peak at 255nm and 290nm. Changes in the peaks describe the successful loading of doxorubicin drug in nanoparticles.

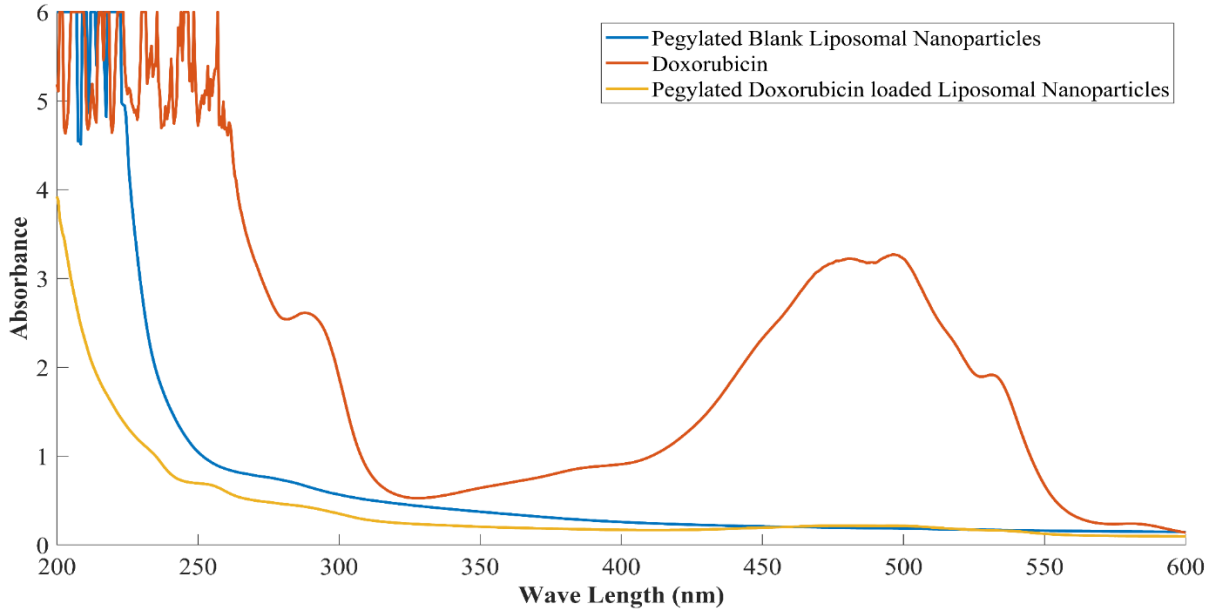


Figure 4.11: Comparative UV VIS spectra of DPPC, Cholesterol, PEG 2000, Doxorubicin, Pegylated Blank LNPs, Pegylated Doxorubicin loaded LNPS

4.1.2 Particle size and surface area distribution

The size of pegylated doxorubicin loaded liposomal nanoparticles was examined by Scanning electron microscopy and image j software measured area distribution of nanoparticles. They were irregular spherical shaped, and they were around 22-35nm in size.

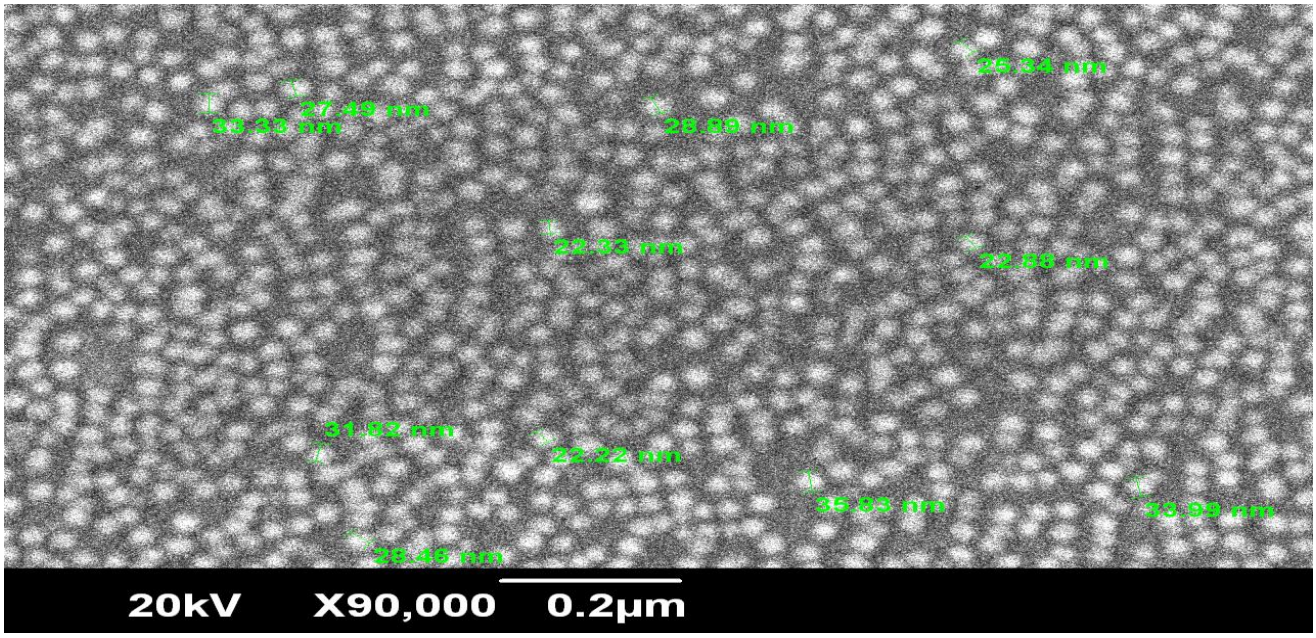


Figure 4.1.2: SEM Image of Pegylated Doxorubicin loaded LNPs

4.1.3 FTIR Analysis

The peak at 2850/cm indicated the CH of cholesterol. Whereas the bond at 2919/cm represents the CH group of DPPC. The peak at 1599 showed NH3 group. C-C group spectrum is seen at 1406/cm. The bond spectrum at 3430 represents the O-H group which is ideal and responsible for bonding to doxorubicin.

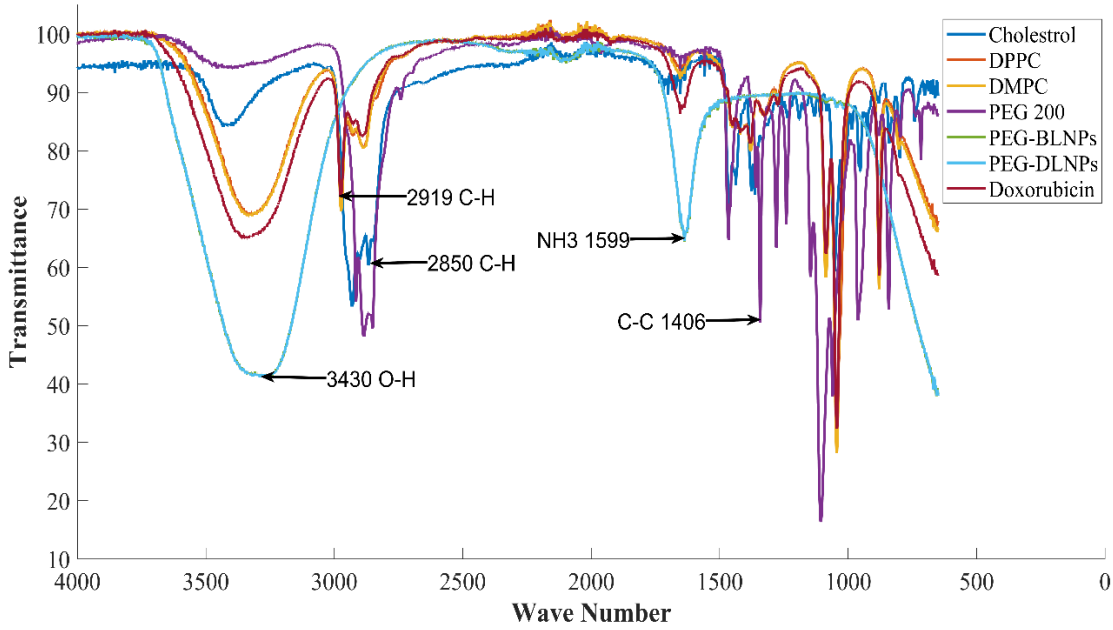


Figure 4.1.3: Comparative FTIR spectra of DPPC, Cholesterol, PEG 2000, Doxorubicin, Pegylated Blank LNPs, Pegylated Doxorubicin loaded

4.1.4 Zeta Potential

The average zeta potential of pegylated doxorubicin loaded liposomal nanoparticles were -7.57 Mv and -5.52. And their poly dispersity index of DPLNPs were 1.000 and 0.992. There was an increase in zeta potential by using Peg 2000 which enhanced its stability.

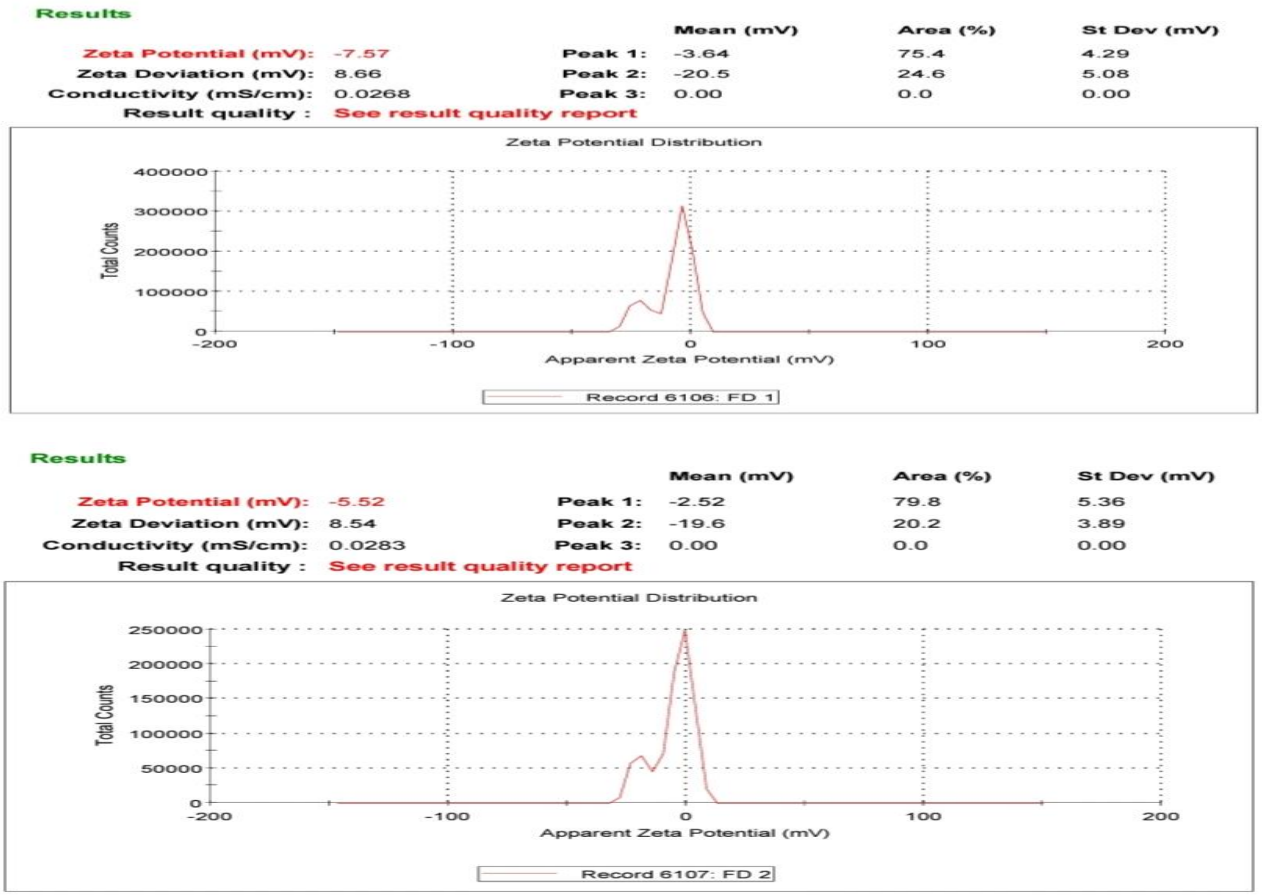


Figure 4.14: Zeta Potential of Pegylated Doxorubicin loaded LNPs

4.1.5 Drug Release Efficiency

Every one hour until 48 hours, the cumulative drug release (CDR) percentage was measured. PEG-DLNPs released doxorubicin at rate of 67% up to 48 hours. Increased bioavailability is achieved because of the drug's long stay, that ultimately results in high disease-treating efficacy.

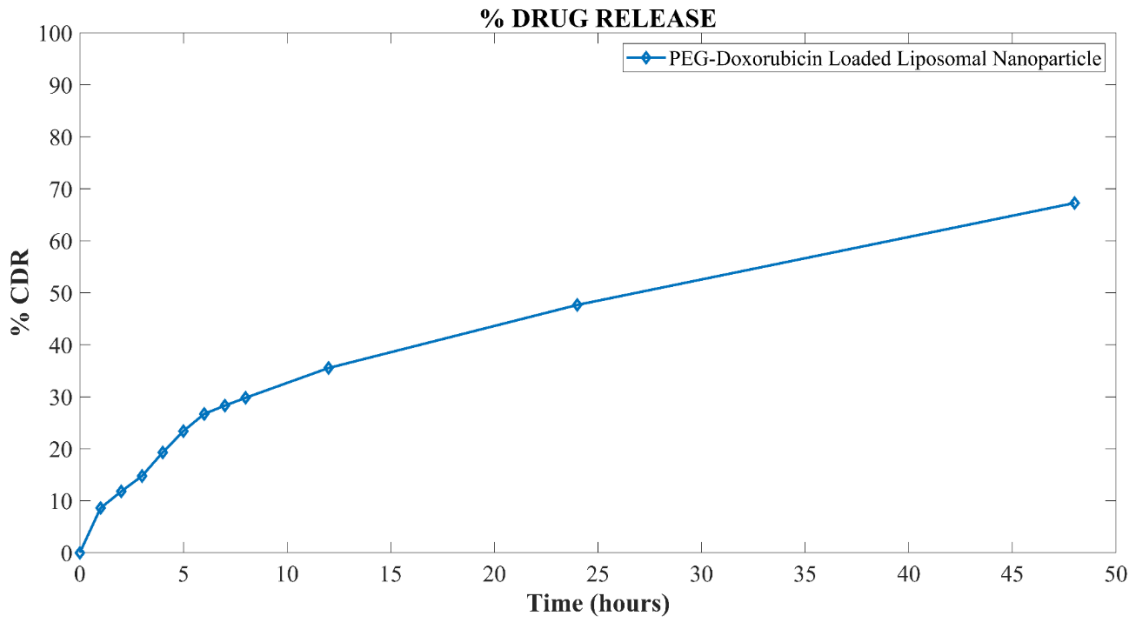


Figure 4.15: Drug release graph of. Pegylated Doxorubicin loaded LNPs

4.2 Hepatic, Renal and Spleen Histopathology

Using a combination of CCL4, urethane, and peanut oil, cirrhosis was induced. Rats' successful induction of cirrhosis was confirmed by histological slides. The histopathological examination showed that cirrhosis had been induced after 13 weeks. From the examinations of the normal liver histology, these alterations demonstrated a significant difference, in this cirrhosis develops in 13 weeks of induction. Fig 1 shows the normal histopathology of liver in which portal area and central vein were observed. Complete cirrhosis was seen in diseased slide with bridging fibrosis, balloon degeneration, cirrhotic nodules, and cholestasis. Diseased renal histology showed Tubular atrophy, congested blood vessels and vascular congestion. And diseased spleen histology showed Hyperplastic thick trabeculae, high line deposition, and fibrosis.

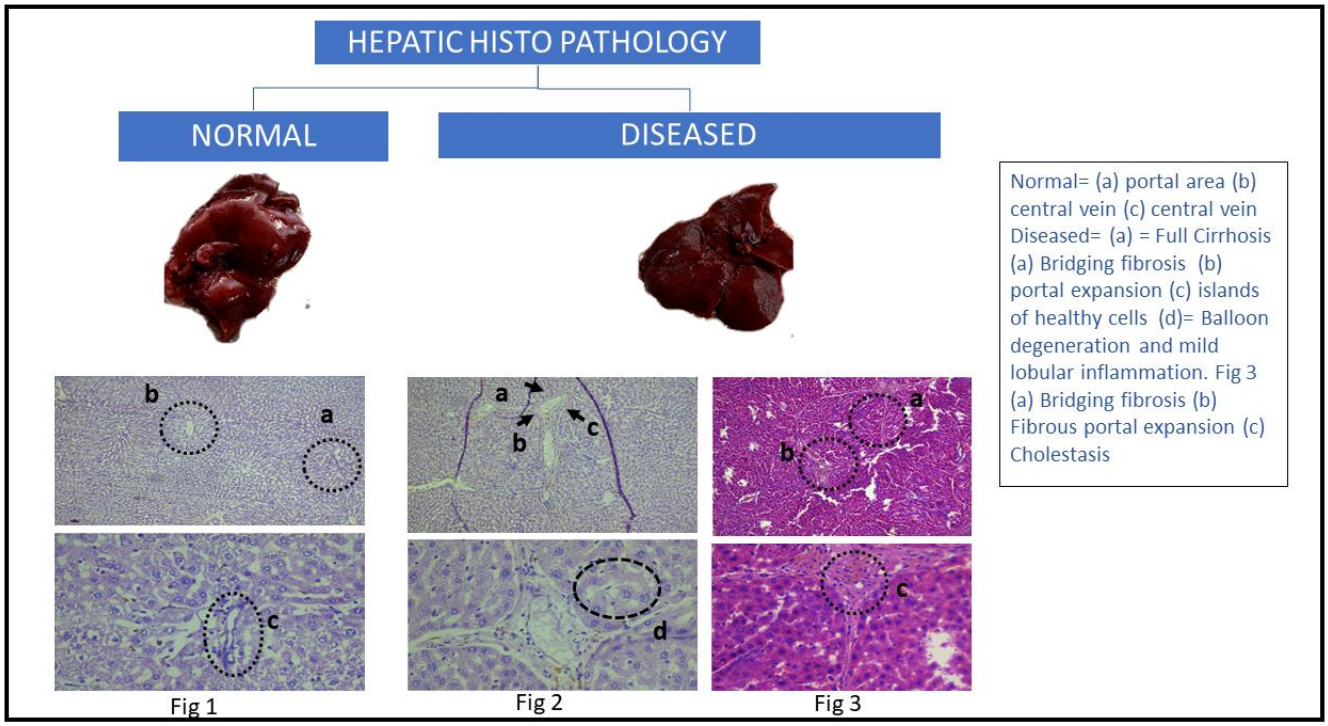


Figure 4.22: Hepatic Histopathology

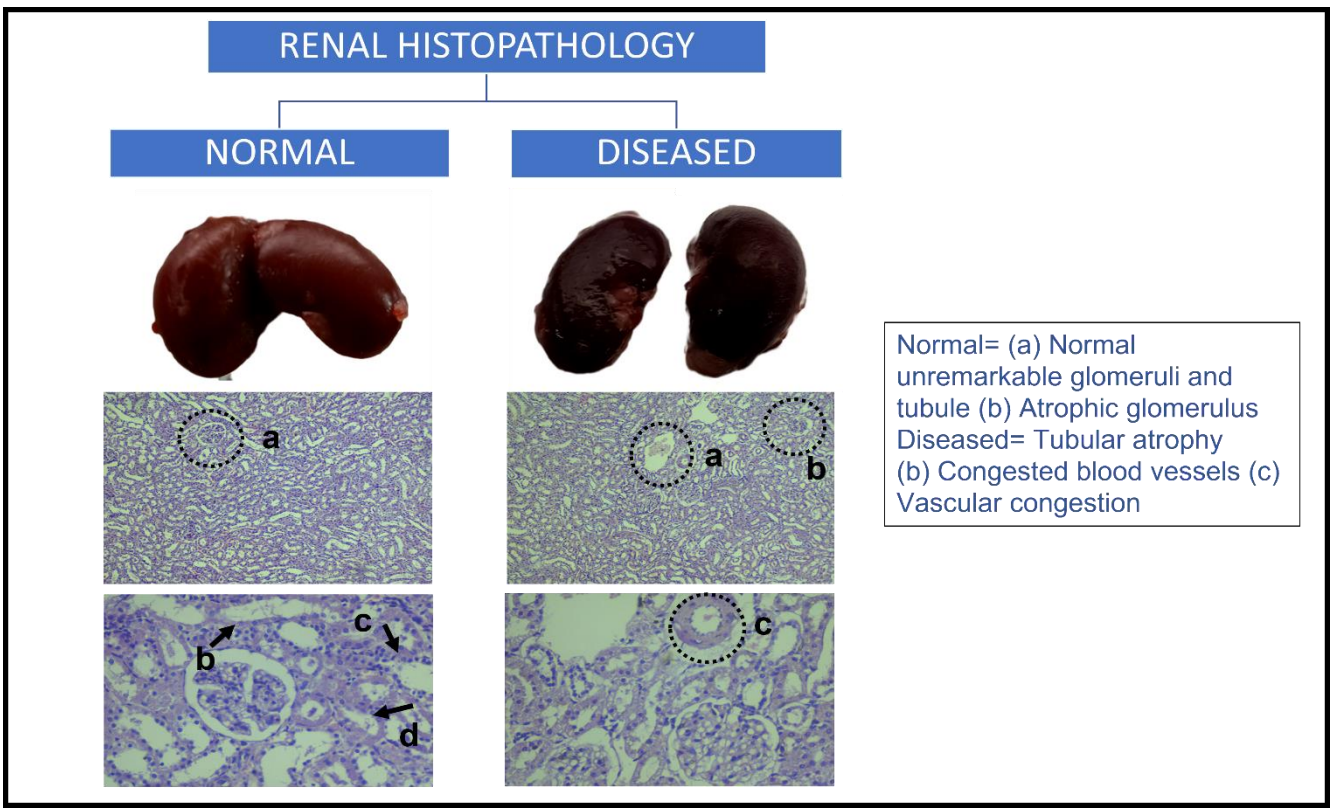


Figure 4.22: Renal Histopathology

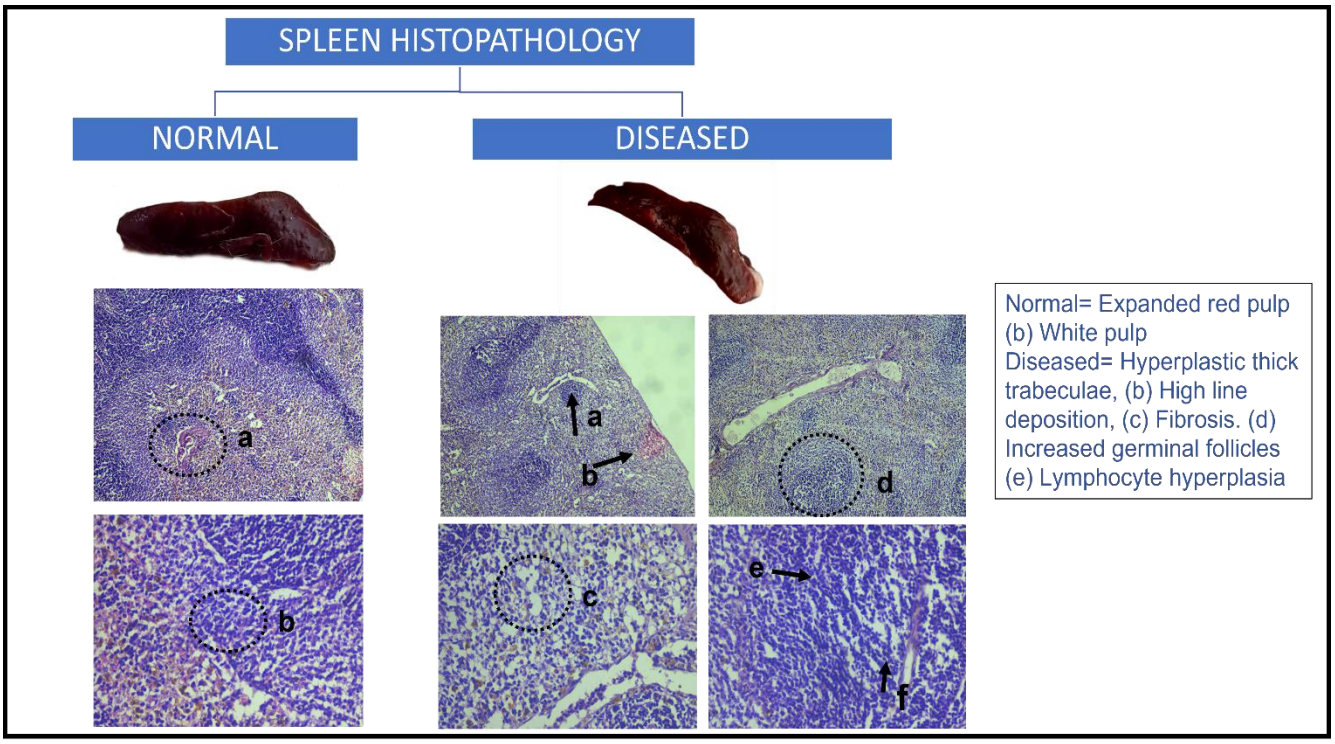


Figure 4.22: Spleen Histopathology

4.3 Treatment

4.3.1 Hepatic, Renal and spleen Histopathology

Complete cirrhosis was seen in diseased slide with bridging fibrosis, balloon degeneration, cirrhotic nodules, and cholestasis. In the group treated with pegylated blank liposomal nanoparticles showed Proliferating hepatocytes and liver cell dysplasia. In the group treated with doxorubicin drug, fatty change dilated vein degenerating hepatocyte were observed. And treated with Pegylated doxorubicin liposomal nanoparticles, prominent Kupffer cells and within normal limits except dilated central vein were observed. Diseased renal histology showed Tubular atrophy, congested blood vessels and vascular congestion. In the group treated with pegylated blank liposomal nanoparticles showed no cellular cast and no necrosis. In group treated with doxorubicin drug showed regenerating tubules, atrophic glomerulus and hypercellularity. And with treated with Pegylated doxorubicin liposomal nanoparticles, regenerating tubules, mild necrosis, and no cellular cast were observed. Diseased spleen histology showed hyperplastic thick trabeculae, high line deposition and fibrosis. In the group treated with pegylated blank liposomal nanoparticles showed congestion of red pulp and lymphocyte's hyperplasia. In a group treated with doxorubicin

drug, expanded white pulp was observed. And with the treatment of Pegylated doxorubicin liposomal nanoparticles, mild expansion of red pulp and white pulp were observed.

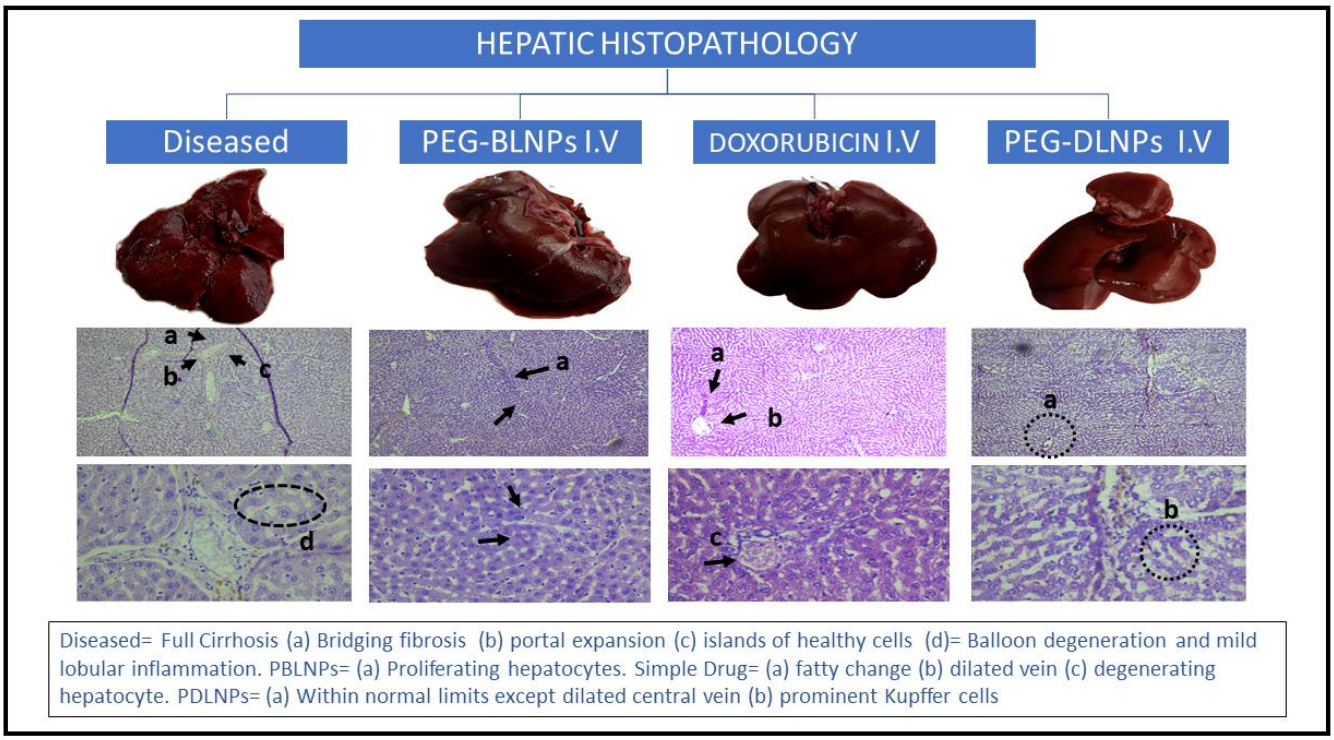


Figure 4.3: Treatment of Hepatic Histopathology

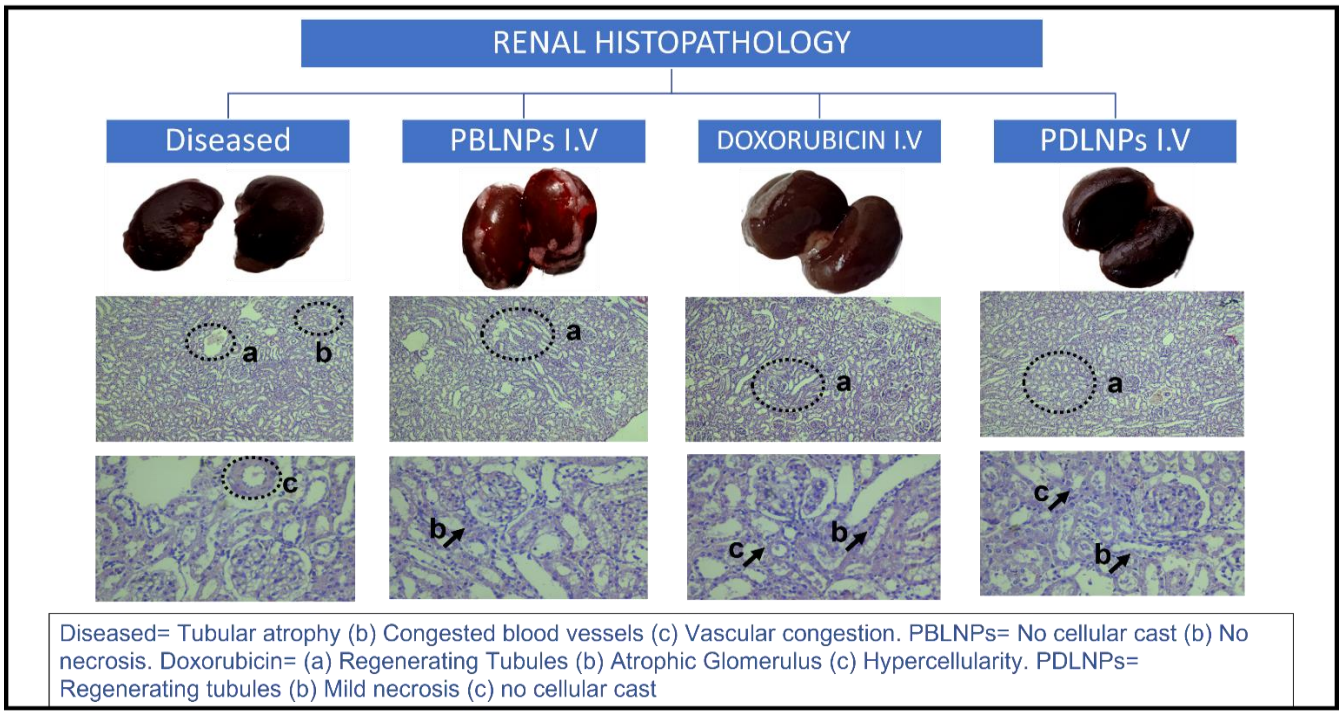


Figure 4.3: Treatment of Renal Histopathology

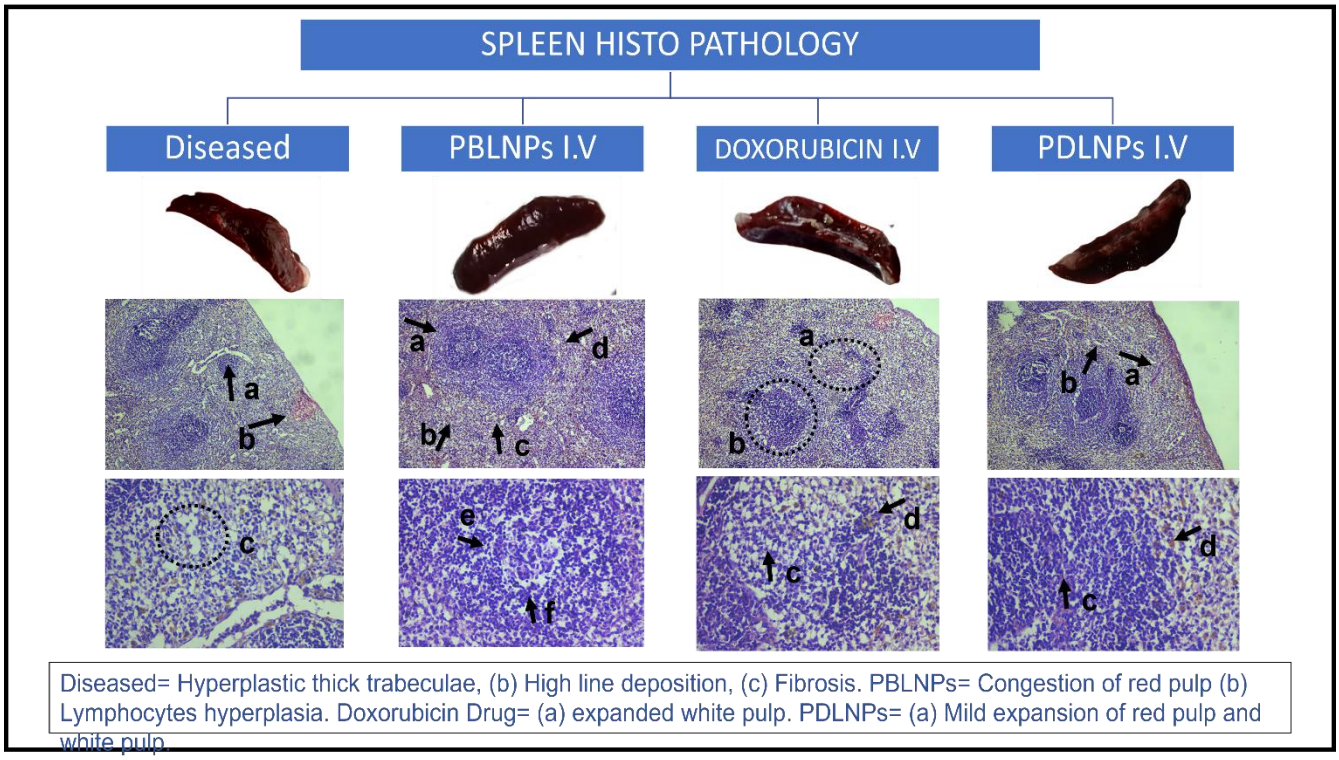


Figure 4.3: Treatment of Spleen Histopathology

4.3.2 Serological Analysis treated with CCL4 and urethane

To evaluate how well the liver is working, a serological investigation was performed. The results of the liver functional tests (LFTs) performed on the blood samples taken from the rats that were treated with CCl4 and urethane and the rats who were given a normal diet revealed a substantial difference. This shows a difference between normal and diseased rats as shown in graph, from Aspartate transaminase (AST) (P=0.0375) Alkaline Phosphatase (ALP) (p=0.0008), Alanine transaminase (ALT) (p=0.0021) and Total Bilirubin (TB) (p=0.0114) indicating the development of cirrhosis and ultimate liver damage.

4.3.3 Serological Analysis of treated rats

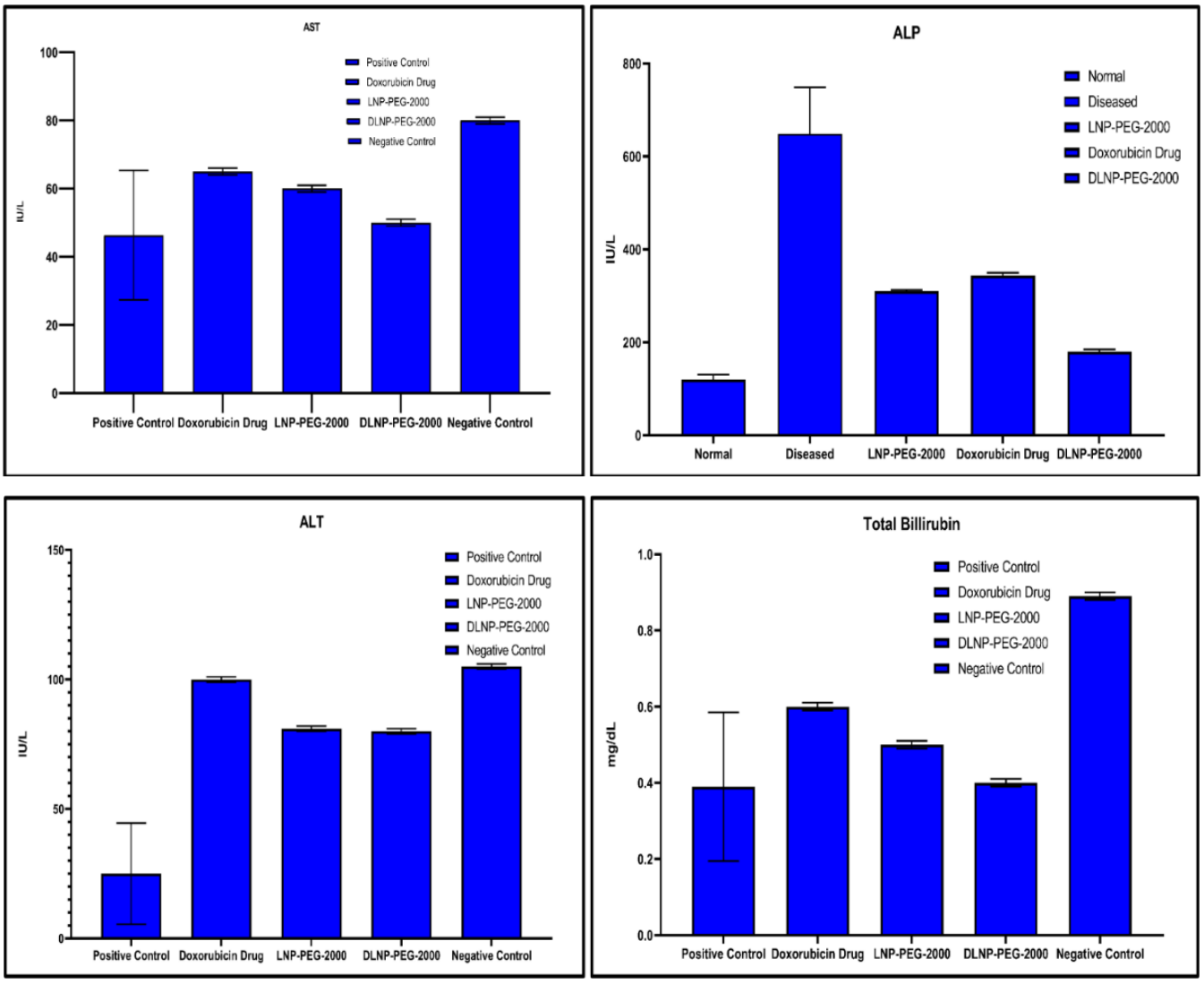


Figure 4.3.3: Serological Analysis

As a result of the substantial liver damage, cirrhosis was evident. When rats were given pegylated doxorubicin-loaded liposomal nanoparticles through IV, the serological indices taken from their blood revealed a significant difference (AST; p = <0.0001, T.B.; p = <0.0001, ALP; p =0.0013, and ALT; p = <0,0001). When rats were given pegylated blank liposomal nanoparticles intravenously, the serological indices taken from their blood showed a difference (AST; p = <0.0001, T.B.; p = <0.0001, ALP; p =0.0042, and ALT; p = <0,0001). And doxorubicin drug administered through IV showed (AST; p = <0.0001, T.B.; p = <0.0001, ALP; p =0.0062, and

ALT; p = <0,0036). This shows that pegylated doxorubicin loaded liposomal nanoparticles is more effective in the difference in ALP readings.

4.3.4 Body Weight treated with CCL4 and urethane

The body weight of rats given CCl4, and urethane administration significantly reduced over time in comparison to positive control rats. Weight of the body (p = 0.0025)

4.3.5 Body weight of treated rats

The weight differences between rats given Pegylated doxorubicin loaded liposomal nanoparticles intravenously varied significantly (p = <0.0001), for the body. Pegylated blank liposomal nanoparticles given through IV (p = 0.0018) and for doxorubicin drug (p = <0.0001). This finding suggests that Pegylated doxorubicin loaded liposomal nanoparticle and doxorubicin drug given by IV is more successful in treating liver cirrhosis. The group of rats given doxorubicin drug and PEG-DLNPs showed the least amount of weight loss, and their results were also noticeably better than those of the rats given PEG-BLNPs intravenously.

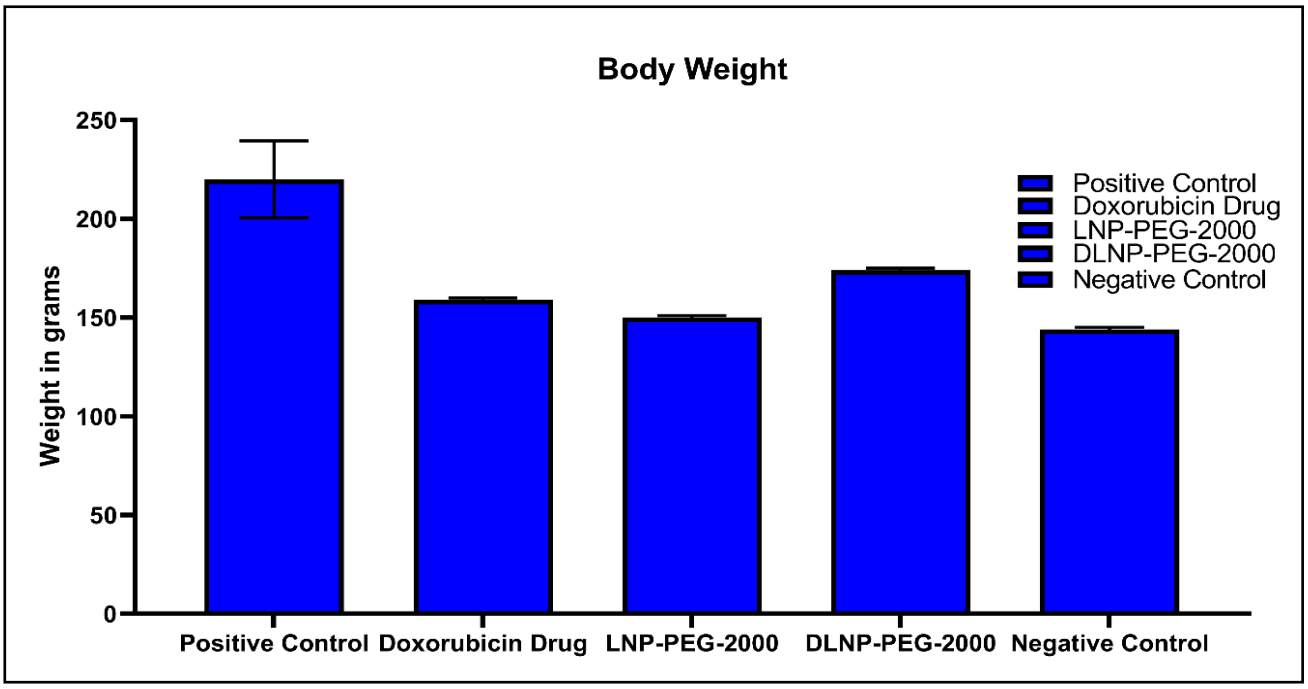


Figure 4.3.5: Body Weight

4.3.6 Liver Weight treated with CCL4 and Urethane

The liver weight of rats given CCl4, and urethane administration significantly reduced over time in comparison to positive control rats. Weight of the liver (p = 0.0582).

4.3.7 Liver weight of treated rats

The liver weight differences between rats given Pegylated doxorubicin loaded liposomal nanoparticles intravenously varied significantly (p = <0.0001). Pegylated blank liposomal nanoparticles given through IV (p = <0.0001) and for doxorubicin drug (p = <0.0001).

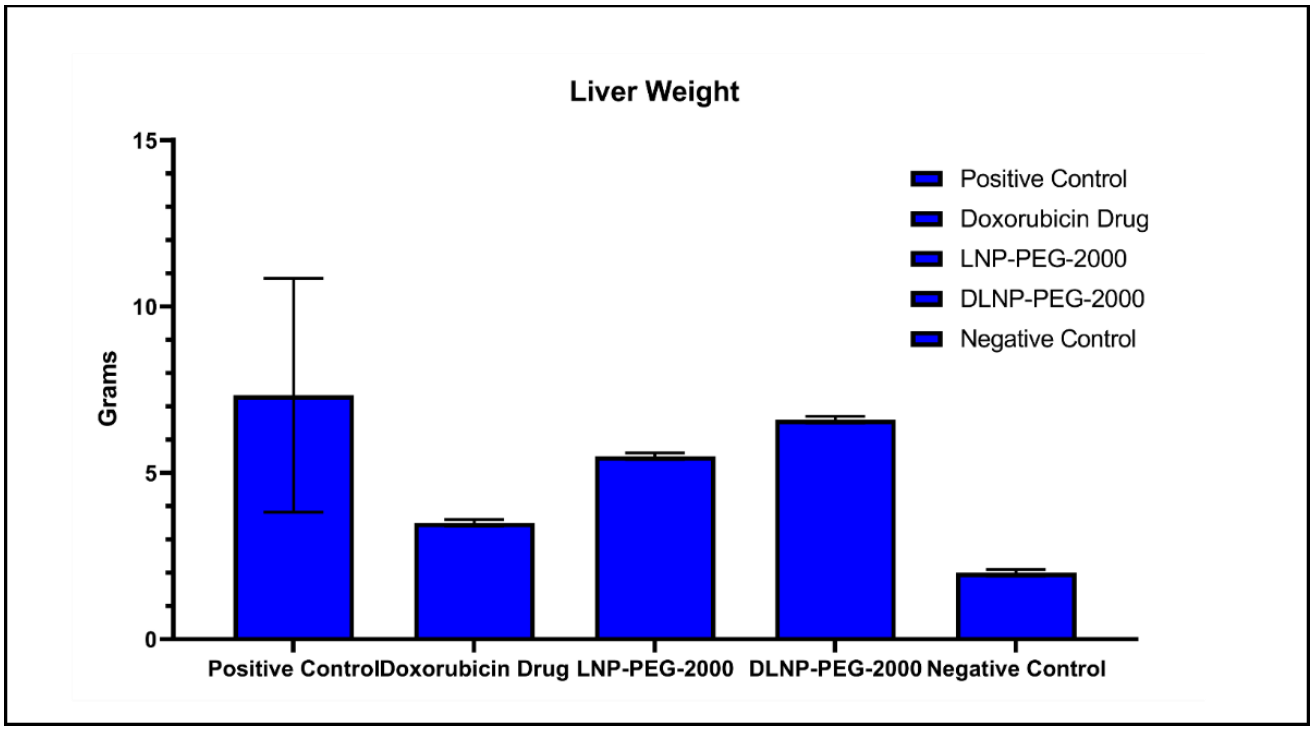


Figure 4.3.7: Liver Weight

4.3.8 Ascites

Diseased rats show severe ascites. But after being treated with doxorubicin drug and PEG-blank liposomal nanoparticles shows moderate ascites and in case of PEG-doxorubicin loaded liposomal nanoparticles, this treatment shows no ascites.

ASCITES				
Normal control	Diseased	PEG-BLNPs	Doxorubicin Drug	PEG-DLNPs
--	+++	+	++	--

No Ascites: --, Mild Ascites: +, Moderate Ascites: ++, Severe Ascites: +++

Table 4.3.8: Ascites observation after treatment

CHAPTER: 5 DISCUSSIONS

The second most prevalent reason for liver transplantation, NAFLD, is a major and growing issue globally (Lindenmeyer & McCullough, 2018). Because it can induce portal hypertension, which is mostly brought on by severe fibrosis and abnormal tissue remodeling, and because it can have adverse effects that include cirrhosis. However, when fibrosis is significantly less advanced, and cirrhosis is absent, higher portal venous pressure has also been noted in clinical NAFLD and animal liver fibrosis models. Increases in portal vein vascular resistance at an early stage may help liver disease develop (Iwakiri, 2014).

Cirrhosis of the liver is often regarded as a dynamic condition with an advancing and regressive disposition. A crucial step towards reducing mortality, according to this contemporary view of the continuum of changes that characterize ACLD (advanced chronic liver disease), is early detection before decompensation has taken place. To avoid decompensation, which is a concerning development in the course of this disease, there are several pharmacological and nonpharmacological techniques that may be applied (Berzigotti, 2017). For individuals with end-stage liver disease, effective artificial liver support continues to be a critical unmet need. The only effective treatment option currently is liver transplantation (in those without contraindications). The development of regenerative medicine is a much-anticipated 21st-century innovation that has immense potential (Sampaziotis et al., 2017). The necessity for liver transplantation in cirrhotic patients must be balanced with potential options in the twenty-first century. (2014) Tsochatzis et al.

Doxorubicin, an anthracycline-class chemotherapeutic antibiotic, kills not only cancer cells but also healthy cells, even in organs that are not specifically targeted, leading to toxicity. (P. L. Prasanna, K. Renu, 2020). The most efficient treatment option for advanced liver disease is doxorubicin, which has quantitative response rates that typically range from 15% to 20%. (H. Pokorny *et al.*, 2005).

Doxorubicin is hydrophilic and easily soluble. The PEG-DLNPs were effectively created in this research with the intention of treating liver cirrhosis. Doxorubicin is hydrophilic in nature, or it may encapsulate in aqueous cavity of lipid bilayer. The 3:1:1 DPPC, DMPC and cholesterol ratio produced the smaller size of liposomal nanoparticles. Pegylated doxorubicin loaded liposomal

nanoparticles were observed to have average sizes of around 22-35nm. PEG-DLNPs released doxorubicin at rate of 67% up to 48 hours. Stabilizing the formula-tion through Pegylation, which increased steric repulsion (A. A. D'souza and R. Shegokar, 2016). This study's comparative FTIR analysis of doxorubicin, PBLNPs, and PDLNPs showed that different functional groups participated as a distinctive quality of different ingredients participating in nanoparticle synthesis.

This study also formed an advanced liver disease model by using chemicals of CCL4 and urethane. CCL4 is the liver toxin that is most frequently used to cause cirrhosis in rats. The trichloromethyl radical that is produced by the liver bioconversion of carbon tetrachloride causes several free radical and lipid peroxidation reactions, which together generate a painful stage effect defined by the apoptosis of central vein of liver cells. (S. Crespo Yanguas *et al.* 2016.) Additionally, hepatic vein fibrosis caused by extended urethane treatment has also been documented (I. Brodsky, H. Johnson). The current study is unique in that it reported the coadministration of these substances and examined their effects on the hepatic, kidneys, and spleen through histopathological analysis in addition to liver function testing. Collagenous scarring indicated effective cirrhosis induction 13 weeks after urethane/CCL4 injection.

The antifibrotic effects of doxorubicin, PBLNPs, and PDLNPs were examined after successfully inducing cirrhosis to verify the model. In contrast to the doxorubicin, the histopathological, serological, body composition, and the liver's weight demonstrated the considerable conversion of extracellular matrix scarring into healthy liver tissue model. By administering the PBLNPs and PDLNPs in contrast to the doxorubicin, the histology, serological, body composition, and liver's weight demonstrated the considerable conversion of extracellular matrix scarring into healthy liver tissue. By the end of 13 weeks, signs of the successful de-velopment of the cirrhosis model included the establishment of hyperplastic thick trabeculae, high line for-mation, and fibrosis, proliferation of red pulp in the spleen.

The histology, serological, body, and liver's weight after treating diseased rats showed a considerable im-provement in the PDLNPs group. Additionally, the group that received PBLNPs treatment also showed good outcomes—up to a point. As a result, the most successful tactic for improving the bioavailability and phar-macokinetics behavior of doxorubicin medication is the liposome encapsulation combined with PEG coating. Additionally, significant treatments against

liver cirrhosis have been shown to have anti-fibrotic efficacy. The intravenous route has demonstrated superior results. As a result, it has been discovered that PDLNPs and PBLNPs are effective in treating advanced liver disease. To fully understand the mechanism of action of advanced liver disease by doxorubicin, future studies are needed to examine the coating of different types of PEG in synthesis of liposomal nanoparticles.

CHAPTER6: CONCLUSION

Following the synthesis of liposomal nanoparticles, several characterization procedures are used to demonstrate that the synthesis was successful. In comparison to traditional drugs and non-coated liposome nanoparticles, pegylation improved the stability, leading to long-term circulation in the body. The histology, serological, body, and liver weight data showed a considerable improvement in the Peg-LNPs treated group after treating the sick rats. Following that, the PDLNPs-treated group likewise showed improved outcomes to some extent. Therefore, using the encapsulated doxorubicin medicine is a better option than using a blank drug for the reversibility of fibrous tissue scars to normal liver tissues. The liver enzymes AST, ALP, ALT, and bilirubin were all considerably lowered by PDLNPs. Therefore, liposome encapsulation in conjunction with PEG coating is demonstrated to be the most successful strategy in boosting the bioavailability and pharmacological behavior of doxorubicin medication, and it has demonstrated significant therapy against advanced liver disease or anti-fibrotic activity. The intravenous route has been shown to be a superior choice. Additional research should be conducted on the proposed theory to treat liver cirrhosis and validate this approach for usage in clinical settings.

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