Effect of PEG coating on the Vitexin liposomes for Oral treatment of ALD

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Acknowledgements

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

List of Abbreviations

ALD	**Advanced Liver Disease**
PEG	**Polyethylene glycol**
NASH	**Non-alcoholic steatohepatitis**
NAFLD	**Non-alcoholic fatty liver disease**
VLP	**Vitexin-loaded liposomal particles**
SLN	**Solid lipid nanoparticles**
NLC	**Nano-structured lipid nanoparticles**
CC	**Compensated cirrhosis**
DC	**Decompensated cirrhosis**
HCC	**Hepatocellular carcinoma**
PVT	**Portal vein thrombosis**
TIPS	**Transjugular intrahepatic portosystemic shunt**
ROS	**Reactive oxygen specie**
EAH	**Essential arterial hypertension**
DSS	**Dextran sulfate sodium**
RES	**Reticulocyte endothelilal system**
BDL	**Bile duct ligation**
HSC	**Hepatic Stellate cells**
LNP	**Liposomal nanoparticles**
AFM	**Atomic force microscopy**
SFM	**Scanning force microscopy**
SPM	**Scanning probe microscopy**
LFT	**Liver functioning test**
AST	**Aspartate transaminase**
ALT	**Alanine transferase**

Abstract

Vitexin a natural flavonoid, occasionally used in pharmaceutics due to its variety of medicinal effects and its roles in fat metabolism, hepatoprotection, and anticancer therapies. It has been rendered important for diseases leading to chronic liver disease, such as NASH/NAFLD, diabetes, cardiovascular ailments, and Liver cirrhosis. Recently, it has been administered in pure drug formulation, in combination with other chemicals, and nanoparticulate form mostly intravenously for various types of liver diseases. In its pure form it shows lower bioavailability due to its insolubility in aqueous environments and a lower rate of intestinal absorption. Since vitexin is known for its role in reducing and reversing the complications arising from decompensated liver cirrhosis, liposomal nanoparticles encapsulating vitexin were prepared by the 'thin-film hydration' process along with passive drug loading. Polyethylene glycol (PEG) coating of vitexin-loaded nanoparticles was used in the oral treatment of advanced liver disease in this research. Wistar rat models of intense liver injury was prepared and treated with vitexin-loaded liposomal nanoparticles coated with PEG-1000 and PEG-2000. Histopathological, serological analysis and various other parameters observed and analyzed during and after the disease induction and completion of the treatment protocol presented better results and a possible reversal of liver cirrhosis when nanoparticles were coated with PEG-2000 in oral treatment. PEG-2000 also shows positive role in the oral administration to match the effectiveness of previously used vitexin-loaded nanoparticles for intravenous treatment of liver cirrhosis.

Key Words: *Vitexin, Liposome Nanoparticles, Polyethylene glycol, ALD, Liver Cirrhosis, Animal Model, Histological Examination*

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CHAPTER 1: INTRODUCTION

1.1   Objective

This research focused on preparing Vitexin-loaded liposomal Nanoparticles (VLPs) and then using different types of Polyethylene glycol (PEG) coatings to attain a favorable size for enhanced bioavailability in oral treatment of advanced liver disease (ALD). Vitexin being already studied for its effectiveness in liver cirrhosis model when administered intravenously using liposomal nanoparticles as an effective delivery system in recent researches, has been opted for the preparation of similar Nanoparticles by 'thin-film hydration method'. Addition of PEG coating for improving its stealth effect and stability has been studied using varying types of chain lengths of PEG, i.e., PEG-1000 and PEG-2000.

Animal model of liver cirrhosis were prepared and treatment effects of these varying liposomal formulations in the *in vivo* analysis by histology and serological analysis along with various other parameters when administered through oral route have been studied. Using different chain lengths of PEG coating over drug encapsulated nanoparticles for understanding the pharmacokinetic behavior and proficiency in Oral treatment may be considered as the next step after intravenous treatment.

1.2   Nanotechnology

Nanotechnology, or nanotech, is the potential to control, restructure, and manipulate the matter within the small range of approximately 1-100nm at molecular or atomic level and exploiting the distinct properties and anomalies (Jabeen et al., 2021). Lately, field of nanotechnology has offered a multitude of possibilities across many conventional areas of study. Such as physics, chemistry, engineering, biotechnology, and particularly in health sciences and biomedicine, enabling integrated systems based on unmet challenges solutions (Picciolini et al., 2021).

1.2.1   Evolution in Nanotechnology

Nanotechnology has largely been investigated over the past two decades and has emerged as a 'new technological revolution'. Many significant engineered nanodevices, molecular, and macromolecular nanoscale-level nanomedicines have been designed and developed (Bodunde et al., 2021). Through nanomedicine quite a lot of improvements in the healthcare sector have been

brought about that will eventually offer significant breakthroughs in terms of efficient and economical healthcare, an essential factor in making available the affordable drugs and treatments (Liu et al., 2020).

The prime objective of using nanotechnology for the purpose of engineering the delivery systems is using them for transporting the desired payload exactly at target site. Active and passive targeting approaches are used where drug encapsulation within the nanoparticles is an example of the passive targeting as the drugs are linked to the macromolecules and then the target tissues passively owing to their now improved permeability (Saxena et al., 2020).

1.2.2 Nanotechnology in Biomedicine

Particles of nanoscale size are now being used in various biomedical applications including drug carriers and as imaging contrast agents in the microscopic analysis. For example, anisotropic nanoparticles can be used in biomolecular detection. Owing to the tunability of nanoparticles along with their diverse physico-chemical properties, these particles tend to be readily functionalized with any type of biomolecule moieties and then used for selective targeting (Mendes et al., 2022). It is a preferred branch of novel medicine due to its characteristics such as target-orientation and site-specificity for delivering the precise medicines while treating chronic illnesses (Patra et al., 2018).

1.3 Liposomal Nanoparticles as drug carriers

Liposomes are a type of vesicles enclosed of a phospholipid bilayer structure that are intrinsically biodegradable and biocompatible making them an attractive choice of vehicle for the purpose of drug delivery. Owing to this structural confirmation, they can encapsulate both the hydrophobic and hydrophilic moieties and drugs when required. Additionally, their novel technological developments have included their benefits in diagnostics and therapeutics in anticancer treatments and other fatal diseases (Lila et al., 2017).

The size of liposomes ranging from 30nm to several micrometers is studied for drug delivery systems (Saraswat & Maher, 2020). The properties of the liposome considerably differ with their composition, size, surface charge and process of preparation. Components of the bilayer define the fluidity, rigidity, and surface charge (Dalmoro et al., 2018). Amid numerous capable modern drug delivery technologies, liposomes signify an advanced technology for the transportation of active molecule at the site of action (Sharma et al., 2018). The

pharmacokinetics of the encapsulated and non-encapsulated drugs distinctly varies when they are loaded in the liposomes (Lin & Qi, 2021). Currently, liposome technology has emerged into 'second generation liposomes' and are being used clinically in various formulations serving as an ideal carrier for the systemic delivery (Gao et al., 2019).

Liposomes, due to their biocompatible nature, are responsible for improving the bioavailability of drugs in oral administration. Such nano carriers adhere to the biological membranes improving the solubility of insoluble drugs including hydrophilic and the lipophilic drugs. Since orally induced liposomes require stability under the specific Gastro-Intestinal conditions approaches such as use of appropriate polymer coating, specified liposomal concentration, and addition of stabilizing lipids to liposomal structures have been beneficial (Daeihamed et al., 2017).

1.4 Advanced Liver Disease (ALD) & Liver Cirrhosis

Liver being the largest internal organ of a human body is involved in major function of detoxification and clearance of chemically harmful metabolic by products. Cirrhosis is the generic term used for the final stage of the advanced liver disease, characterized by the destruction, scarring, and necrosis of the normal hepatic architecture. It is chronically progressive with diffused damage to the hepatocytes which are then replaced by fibrotic tissue and nodular regeneration (Zuñiga-Aguilar & Ramírez-Fernández, 2022).

The scarring of the tissue is formed when the liver is injured again and again because of various forms of diseases, and it tries to repair itself owing to the regenerative capacity of the liver tissue. Building up of this scar tissue worsens the liver condition causing it to lose its functions slowly progressing into cirrhosis (Jangra et al., 2022).

1.4.1 Symptoms & Causes of ALD

Generally liver cirrhosis lacks any prominent signs or symptoms unless the liver damage has become extensive. The basic clinical symptoms include fatigue, weakness, itchy skin, bruising easily, nausea and lack of appetite, weight loss, bleeding easily, ascites, varices, swelling in lower legs, ascites, spider naevi, confusion, and mental disturbances (Kreitman et al., 2020). These early signs also resemble to various other diseases and thus if not recognized can later lead to major complications including peripheral edema, peritonitis, splenomegaly, portal hypertension, gastric and esophageal varices, hepatorenal syndrome, hepatic encephalopathy, and liver cancer (Peng et al., 2019).

Liver cirrhosis is most commonly caused due to other conditions effecting liver function such as excessive alcohol abuse, chronic hepatitis B&C, drug abuse e.g., heroin, and NASH/NAFLD (non-alcoholic steatohepatitis/ non-alcoholic fatty liver disease) (Asrani et al., 2019). Obesity, diabetes, drug abuse, and malnutrition are some of the underlying causes of advanced stage liver disease whereas, the less common causes include primary biliary and sclerosing cholangitis, hereditary hemochromatosis, autoimmune hepatitis, cardiac cirrhosis, cystic fibrosis, and Wilson's disease etc (Valamparampil & Gupte, 2021; Al-Khazraji et al., 2021). HCV and NASH are the leading causes of liver cirrhosis and have also been increasing globally (Enomoto et al., 2020).

1.4.2 Stages of ALD

Cirrhosis is generally classified into three different stages as follows:

- Steatosis (Stage I). Inflammation of the liver and bile duct causes damage to liver aggravating the illness.

- Scarring or Fibrosis (Stage II). Scarring and progressing inflammation causes obstruction of the normal blood flow in the liver.

- Cirrhosis (Stage III). Permanent liver scarring causes it to be hard and lumpy losing its normal function due to obstruction of blood flow through the portal vein.
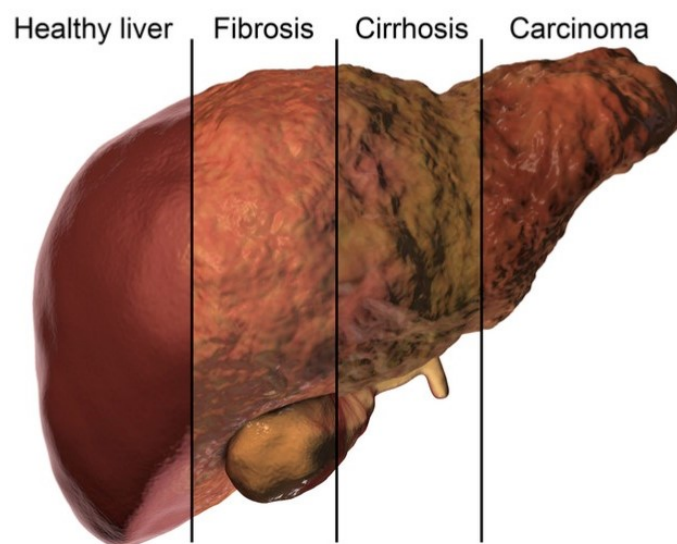
Figure: 1. *Progression of ALD*

Stage I and II can be controlled if detected in time and stop the liver from progressing into cirrhosis or hepatic failure. There is also a Stage IV classified as the ultimate liver failure and ALD that is fatal if immediate action is not taken (Khan et al., 2020; Bahirwani & Griffin, 2022).

1.4.3 Compensated and Decompensated cirrhosis

Compensated liver cirrhosis (CC) is an asymptomatic phase that can still function on the basis of its healthy cells that compensate the scar tissue. The final stage of the advanced liver disease initiates from the compensated phase which if not treated leads to decompensated phase reducing mortality time from 12 years to up to 2 years. Decompensated liver cirrhosis (DC) is a progressive stage that has high mortality due to various other complications such as hepatic encephalopathy, portal hypertension, cardiovascular dysfunction, and gastrointestinal bleeding etc (D'Amico et al., 2021).

1.4.4 Ascites

Bodily fluid accumulation in the abdominal cavity or peritoneum is known as ascites and is a predominant symptom of ALD. This fluid when collects has adverse effects on various other organs such as lungs and kidneys etc. (Yatsuhashi et al., 2021). Patients with ascites suffer from abdominal pain, nausea, and vomiting etc. It is the landmark of the liver cirrhosis entering the decompensated state. Poor prognosis is the cause of higher mortality rate in severe cases of liver cirrhosis (Labenz et al., 2019). Gastrointestinal dysfunction is associated with liver cirrhosis, and it furthers the complications of the liver disease. Food intake is affected by dysfunctionality of gut as well as the ascites developed due to the loss of proper function of liver and accumulation of sodium inside the body cavity (Kalaitzakis, E., 2014) progressing into the decompensation of the liver. All these are the causes due to which the liver cirrhosis eventually progresses into hepatocellular carcinoma (HCC) (Kanda et al., 2019).

1.4.5 Prevalence & Prevention

Liver cirrhosis causes an enormous amount of economic and health burden upon many countries. Due to a worldwide growth rate of obesity, age and population, NASH-related liver cirrhosis is surpassing the viral hepatitis associated cirrhosis (Zhai et al., 2021). Its prevalence and absence of any antifibrotic or regenerative medicine is causing it to be globally a leading cause of death and the ultimate way out to avoid death is liver transplant which has its limitations. Currently drug development for NASH is a largely researched field for as it contributes towards liver cirrhosis. Lack of proper diagnostics for successfully detecting liver

cirrhosis is also an obstacle for carrying out clinical research in a patient population hence prodding a need for non-invasive targeted treatment (Fallowfield et al., 2021).

Diabetes along with obesity is a prevalent cause of advanced liver disease leading to liver cancer which is subsequently becoming the major cause of mortality in Asian countries (Talawar et al., 2021). Cases of liver disease are often diagnosed with other related diseases such as NAFLD etc., due to overlapping symptoms and lack of specific diagnostic method for liver cancer or decompensated stage of liver cirrhosis (Pang et al., 2018). There is an understanding that an improvement in the management of diabetes and of obesity may be able to diminish the chances of NASH cirrhosis causing HCC (Tarao et al., 2019).

1.4.6 Treatment & Diagnostics:

ALD at the stage of cirrhosis limits the use of interventional approaches regarding surgery in cancer treatment by influencing the pharmacokinetics of the anticancer drugs being used and by increasing the chemotherapeutic side effects. Due to this the patients become susceptible to hepatotoxicity with an increased ultimate risk of morbidity (Pinter et al., 2016). The gold standard for the diagnosis of the liver cirrhosis is liver biopsy guided by ultrasound but is not a very applicable method due to the sampling bias and its invasive nature. Diagnostic imaging is also another favorable alternative including various techniques for estimating the stages of liver fibrosis and cirrhosis, such as ultrasound (US) and US Doppler (Soresi et al., 2014). Another less invasive and economically applicable alternative is the use of Serum markers which is theoretically devoid of any complications (Zirnis et al., 2020).

The decisions regarding the treatment liver cirrhosis is significantly influenced by the prognosis of the underlying degree of the disease. Clinical trials of the patients suffering from liver cirrhosis leading to liver cancer are not generally held to know about adequate treatment dosage (Pinter et al., 2016). Frequently bacterial infections cause insult to injury in case of severe cirrhotic patients and use of antibiotics becomes mandatory. But since liver is the main metabolic site for all these antibiotic drugs, it is essential to avoid any incidences that can elevate the liver disease. It is preferred to use lower doses of the drug with a lower frequency of dose administration (Zoratti et al., 2021). Portal vein thrombosis (PVT), a major complication in ALD has been treated with anticoagulation therapy, thrombolysis, and transjugular intrahepatic portosystemic shunt (TIPS) but there is no evidence to support the successful reversal of variceal

bleeding after these treatments in the patients suffering from acute liver disease (Hepatobiliary Disease Study Group, 2021).

Reversal of liver fibrosis was studied to find evidence and successful treatment for the reversal or avoidance of liver cirrhosis. Role of hepatic stellate cells was found to be contributing towards fibrogenesis (Jung & Yim, 2017). Progression of liver disease is aided by spleen acting as lymphoid reservoir. Portal hypertension is also affected by splenomegaly in case of liver cirrhosis. Splenic modification for the invention of novel nanomedicine is studied to be critical for the therapies of liver cirrhosis (Li et al., 2017).

CHAPTER 2: LITERATURE REVIEW

2.1 Vitexin

Vitexin (apigenin-8-C-β-D-glucopyranoside), a c-glycosylated flavone of apigenin is found in the medicinal plants including hawthorn leaf, buckwheat, Passiflora, pearl millet, and bamboo. Flavonoids exhibit many pharmacological activities being antioxidant, hypotensive, anti-inflammatory, anti-spasmodic, and anti-metastatic (Timalsina et al., 2021; Noor et al., 2022).
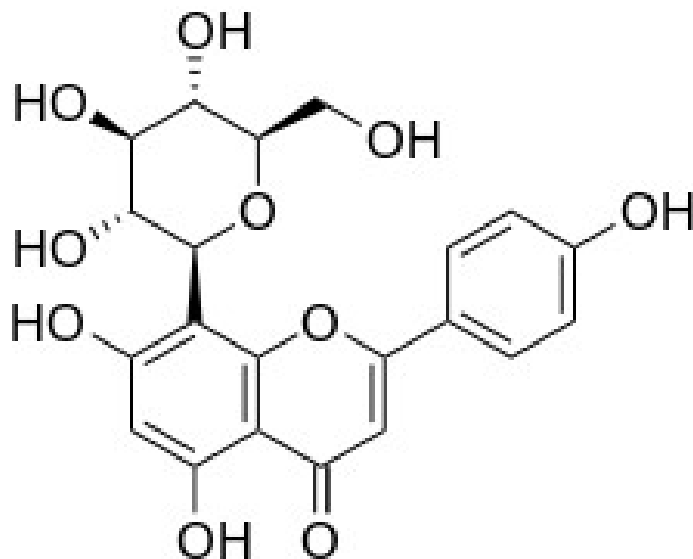
Figure: 2 *Structure of Vitexin ($C_{21}H_{20}O_{10}$)*

Vitexin has other benefits, such as anticonvulsant effects, anti-nociceptive effect, antiglycation and memory restoration stimulation (Aslam et al., 2015). Vitexin imposed antineoplastic activity in both in vitro and in vivo models as it promotes the process of apoptosis and autophagy as well as inhibits the proliferation and migration via multiple signaling pathways (Ganesan & Xu, 2017). It is an alternative dietary supplement that acts in combination with the prevention and also as a therapeutic strategy against complications associated with obesity (Peng et al., 2019).

2.1.1 Vitexin against Liver Inflammatory Diseases

Liver diseases such as hepatitits, steatosis, cirrhosis, and HCC, are globally the leading causes of mortality that result in immense socio-economic burden (Li et al., 2015). Number of food and herbs having polyphenols have been documented that showed therapeutic potential on

hepatic injuries through complex mechanisms (Tang et al., 2018). Hawthorn extracts have shown beneficial effects against various hepatic dysfunctionalities such as hepatic toxicity, inflammation, fat deposition, liver fibrosis, and hepatic cancer (Kim et al., 2022). The experimental results revealed the potential role of Alysicarpus monilifer's methanolic extract and its bioactive molecule i.e. vitexin in shielding liver function and improving the histological structures in the rat model of liver damage induced by CCl_4 (Ravan et al., 2017).

2.1.2 Mechanism of action of Vitexin

The hepatoprotective role of Folium Microcos fraction (FMF) in liver tissues delineated as a crucial junction in cascade reactions through the dual regulation of the apoptosis signaling via effects on the axis of ROS / MAPK and response of the antioxidant defense system mediated by Nrf2. Vitexin presence in FMF from 'Folium Microcos' might be the key bioactive compounds which corresponds to its antioxidant and hepatoprotective properties (Wu et al., 2017). Vitexin treatment significantly reduced the serum levels of AST and ALT and was seen to attenuate the hepatic injury caused by oxidative stress. Study showed that vitexin was responsible for ameliorating the hepatic injury in EAH mice by activating the AMPK/AKT/GSK-3β pathway and up regulation of the Nrf2 gene. It was also identified to inhibit the fat accumulation in 3T3-L1 adipocytes as well as it normalized the lipid contents in liver tissues via the AMPK-mediated pathway which also inhibited NAFLD (Zhang et al., 2022). Anethum graveolense capacity L. (AGME) containing vitexin inhibits TGF-β1 release and to reduces oxidative stress in the treatment and prevention of hepatic cirrhosis (Babaei et al., 2020).

Vitexin has shown hepatoprotective effects in the treatment of extensive liver injury induced by the ulcerative colitis. It has also been used to alleviate the dextran sulfate sodium (DSS)-induced injury of the liver through suppression of the inflammation caused by TLR4/NF-κB signaling. Vitexin has been reported to have multiple pharmacological activities (Duan et al., 2020).

2.2 Carrier Necessity for Vitexin Delivery

Vitexin along with its therapeutics versatility, possess poor solubility in water. Poor bioavailability and lower aqueous solubility limits its clinical applications (Gu et al., 2017). These issues lead to lower bioavailability of the drug which is then reformulated by nanoparticulation and nanonization for improving their affectivity and safety (Kalepu & Nekkanti, 2015). Vitexin also shows poor absorption in the gastrointestinal tract as it is

eliminated almost instantly from the bloodstream, essentially in the bile and then urine and thus has a poor absolute oral bioavailability (Chen et al., 2021).

2.3   Stealth Liposome Nanoparticles for Oral Delivery

Although liposomes are similar to bio membranes, but these are still foreign antigens for the body. Hence, after interaction with plasma proteins, these are recognized by body's Reticulocyte endothelial system (RES). Consequently, these are eliminated from blood stream (Akbarzadeh et al., 2013). Such limitations linked with stability, overcome with the use of synthetic phospholipids and coating the liposome particle by PEG, chitin derivatives, freeze drying, polymerization, ganglioside micro-encapsulation (Shaheen et al., 2006). PEG coating reduces the percentage of liposomal phagocytosis and results in a long-term circulation and thus provides frequent time to the liposomes for leak out of the circulation through endothelium.

Stealth liposome vesicles are sphere shaped with bilayer membrane, that consist of phospholipids with assorted lipid chains, stabilized or coated with PEG or colloidal polymers that are used for transporting drugs to targeted cells with a controlled release. This concept has been used for improvement of popular doxorubicin-loaded liposomes, which are currently marketed for treating solid tumors as Doxil (Janson Biotech, Inc, Horsham, USA) or caelyx (Schering-plough company, kenilworth, USA) (Akbarzadeh et al., 2013). Studies have also suggested that the oral administration of vitexin can be fabricated to significantly improve the symptoms of chronic colitis during clinical treatments, and for suppressing the incidence and burden of the tumor (Chen et al., 2021).
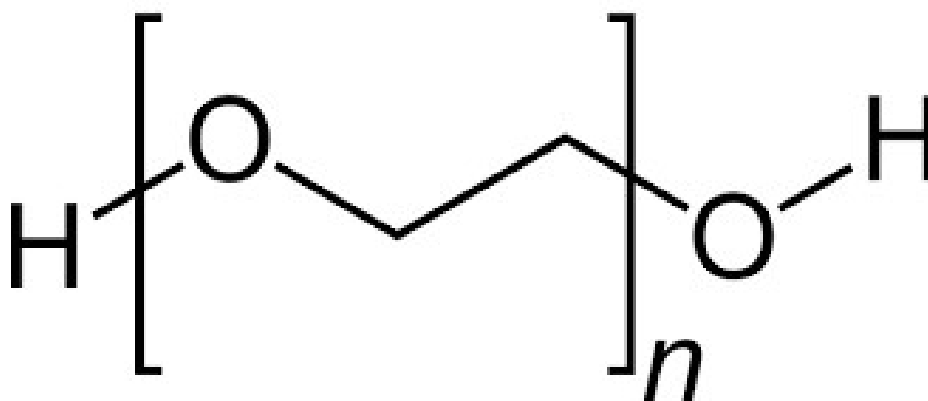
Figure: 3 *Structure of Polyethylene glycol (PEG)*

2.4 Induction of ALD by CCL_4

The key methods for the induction of ALD in rats include administration of CCL_4 and the bile duct ligation (BDL). Nowadays, CCL_4 is commonly used, but is known as extremely toxic process. It damages hepatic tissues and is linked with reactivity of free radical metabolites, metabolic activation, lipid peroxidation, disturbed calcium homeostasis, and covalent bonding. The CCL_4 administration results in inflammation, necrosis, and consequently fibrosis, which later spreads to connect the vascular structure that drains into the hepatic sinusoid (portal tract and central vein respectively). It stimulates the hepatic stellate cells (HSC) that induces liver cells apoptosis, zone 3 necrosis and fatty infiltration, upon incessant administration (Marques et al., 2012).

CCL_4 induces toxicity occurs in four distinct phases. Predominantly, first 2-3 weeks are described as necrosis, proposed by increasing liver-specific enzyme activities and declining pseudocholinesterase value. Significant hepatic fat deposition occurs during the next two to three weeks and the serum triglyceride and aspartate aminotransferase (AST) levels increased significantly. Although diminishing the hepatic activity. The increase in AST persists throughout the third step, and raises the hydroxyproline and triglyceride levels, while overall liver function decreases. Pseudocholinesterase values further decrease in the final step and liver atrophy is observed (Paquet &Kamphausen, 1975). This could be associated with serum albumin's substantial reduction and weight loss, signifying a gradual hepatic function loss while sustained fibrogenesis (Scholten et al., 2015).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1   Experiment Design

3.1.1 Materials

Vitexin drug, Dipalmitoyl phosphatidyl choline (DPPC), Cholesterol, Polyethylene glycol (PEG of MW-1000 & 2000) were purchased from Sigma-Aldrich USA. Dimethyl sulfoxide (DMSO), Formaldehyde, Urethane and carbon tetrachloride (CCl_4) were purchased from Strem chemicals. Wistar female rats were purchased from Atta-ur-Rahman school of Applied Biosciences (ASAB) National University of science & technology (NUST), Islamabad, Pakistan. Deionized water was used in all formulation preparations and experimental tasks.

3.1.2 Synthesis of Vitexin loaded-liposome Nanoparticles

For the synthesis, liposome constituents, i.e., DPPC and the Cholesterol were used in a percent molar ratio of 4:1. Firstly, the lipid components were weighed and dissolved in solvent (ethanol) to form 100 µMolar solutions. 200 µMolar solution was prepared of Vitexin drug in ethanol out of which a quantity of 500 µL drug solution was mixed in the above lipid solution. This mixture was now sonicated at 80 MHz for duration of 40 minutes. Next 10mL water and the lipid phase were separately warmed to reach a temperature of 60°C in a water bath. Both these, the lipid phase and water phase were then mixed to form a dispersion mixture which was constantly mixed at 90 RPM for 10 minutes duration. This new mixture formed was once again sonicated now at 50 MHz for 40 minutes and then allowed to rotary evaporation (above the phase transition temperature i.e. 50 °C) for getting rid of the ethanol (solvent) (Farooq et al., 2022). Finally, the unentrapped drug was removed using dialysis tube in PBS buffer solution for 40 minutes.

3.1.3 Pegylation of vitexin loaded liposomal nanoparticles

The mixture of Vitexin-loaded liposomal nanoparticles (VLPs) was divided into 2 parts and each part was diluted by adding 50 mL of Deionized water. 0.25% (w/v) of PEG-1000 was added drop wise in one part of solution and PEG-2000 in the other part of this solution was

added with continuous stirring for capping of the nanoparticles. Similarly, the blank liposomal nanoparticles (LNPs) were also divided into 2 parts and Pegylation was done using these 2 types of PEG. The final four solutions obtained were, a) blank liposomal nanoparticles with PEG-1000 coating (LNP-PEG 1000), b) blank liposomal nanoparticles with PEG-2000 coating (LNP-PEG 2000), c) Vitexin-loaded liposomal nanoparticles with PEG-1000 coating (VLP-PEG 1000), d) Vitexin-loaded liposomal nanoparticles with PEG-2000 coating (VLP-PEG 2000).

3.2 Physical Characterization

3.2.1 Particle Size, thickness and topography

For analyzing the size, thickness, and topography of the nanoparticles produced, atomic force microscopy (AFM) was used. AFM synonymously known as scanning force microscopy (SFM) is a very-high-resolution type of microscopy. It is also called as scanning probe microscopy (SPM) due the probe used in this microscopy for the scanning purpose. This has the ability to produce images of resolution on the order of fractions of a nanometer, making it 1000 times better than the usual optical diffraction limit. The topographical, thickness, and size related information of the specimen is obtained by "feeling" or "touching" the surface of the particles or subject matter with a mechanical probe. Precise scanning of the material is facilitated by the aid of piezoelectric elements that function with tiny but accurate and precise movements on an electronic command (Ganesh, 2022).

The components of AFM include a probe made up of a cantilever with a sharp tip at its end used for scanning the surface of the specimen. This cantilever is usually of silicon or silicon nitride material and it's a tip has a radius of curvature in nanometers. As the sample surface comes close to the tip of the cantilever the forces between them cause deflection in the cantilever according to the Hooke's law. Forces generally measured in AFM are; mechanical contact force, capillary forces, van der Waals forces, chemical bonding, magnetic forces, electrostatic forces, and solvation forces, etc. This bending of the cantilever changes the amount of the laser light that is reflected to photodiode (Kenkel et al., 2020).

The topographical image is formed by a plotting method that generates a color mapping by changing the position of the x–y plane of the tip during conducting a scan and records the variable that is measured, i.e. control signal intensity for each of the x–y coordinate. The

measured value of the variables is shown by the color mapping through its corresponding coordinate. The intensity value in the image is expressed as a hue whose correspondence with the intensity value is displayed in the explanatory notes of the color scale.
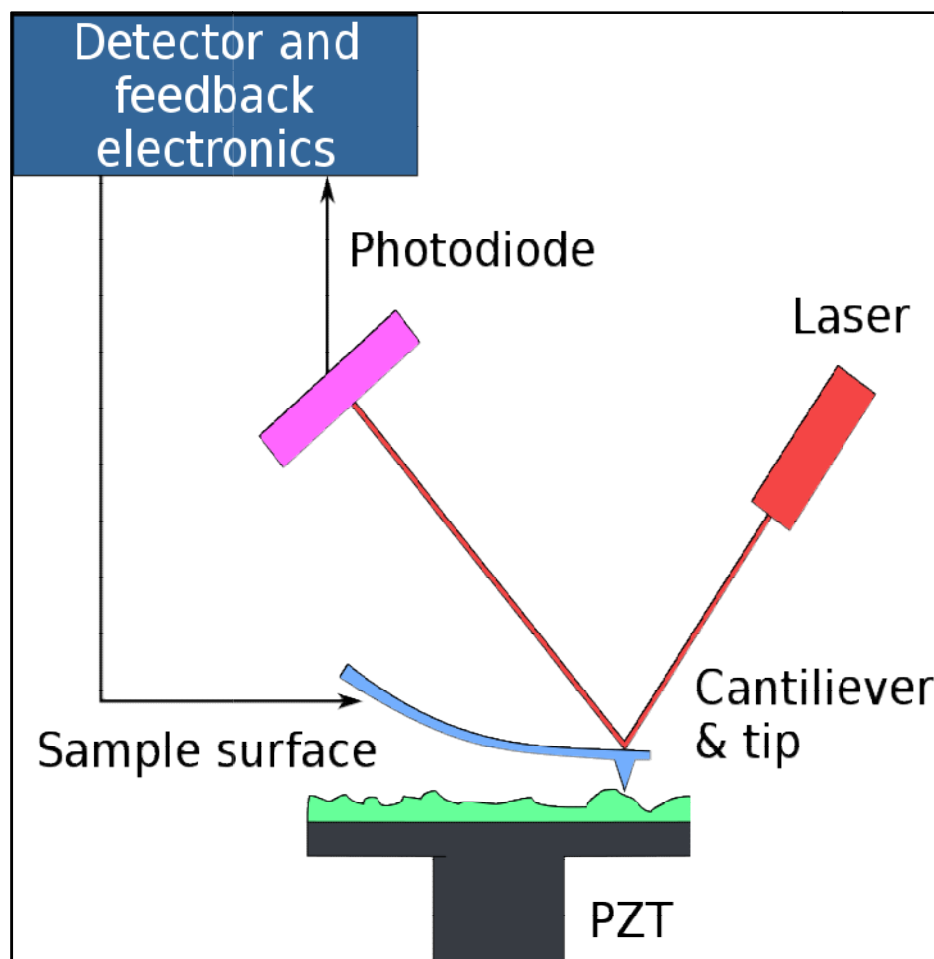
Figure: 4. *Working principal diagram of AFM*

3.2.2 Efficiency of Drug Release

The behavior of the release of drug from the nanoparticles has great importance in treating with nanomedicines. Release of drug cargo in time dependent manner at targeted site is the main concern of nano formulation that results in controlled or sustained release.

Drug release from PEG-LNPs was examined up to 48 hours along with the addition of specified volume of phosphate buffer saline. From 25ml solutions of each VLP, 3ml samples

were placed into separate 15ml of the centrifuge tubes and then were allowed to centrifuge for 10min at 4500rpm and 25ºC while on other hand 3ml of PBS was added in the original solutions of VLPs solutions. Following the process of centrifugation, the supernatant was taken for UV spectrophotometer analysis. Similar procedure was later followed after duration of 1hr, 2hr, 4hr, 6hr, 12hr, 24hr and 48hr. At 330nm of wavelength, absorbance values were taken and used as cumulative drug release. Entire analysis was executed using blank nanoparticles solution as standard control.

3.3 Development of Advanced Liver Disease Model

3.3.1 Animals

35 Wistar female rats having weight of 105-170g and age of 5-8 weeks were obtained and kept under 12-hour dark and light cycle in the separate cages given the access of food and water. Temperature of the room was set to approximately 27°C with a 60-70% of humidity. When conducting histological procedures, rats were anesthetized using chloroform. The caring and the handling of the rats were done according to the regulation of good laboratory practice as issued by US FDA (Food and Drug Administration) in 1978.

3.3.2 Chemicals

In our study different chemicals were used for induction of liver cirrhosis i.e. less potent carcinogen i.e. Urethane, a potent hepatotoxin - carbon tetrachloride (CCl_4), Ethanol (de-contaminant), peanut oil (as a delivery agent), and 10% neutral-balanced formaldehyde buffer (dissolved in PBS).

3.3.3 Induction of ALD

At the start of the study, the rats were set free to acclimate with their group and environmental conditions for one week. A total of 35 rats were divided within 2 groups. 5 rats considered as positive control, devoid of exposure to any adverse condition in the entire procedure whereas 30 rats were exposed to injurious chemicals via intra-peritoneal injections. Initially, 2.5% (w/v) Urethane was dissolved in DMSO and 1ml/kg dose of this Urethane

solution was injected in the intra-peritoneal twice a week for the first 2 weeks. The exposure with urethane is intended in order to cause great liver damage resulting in the reduction of induction time frame of cirrhosis. For the rest of 4 weeks CCl_4 mixed in pure Peanut oil in a 50% proportion (v/v) and was injected into intra-peritoneal cavity having the dose of 1ml/kg twice a week.

3.3.4 Physical parameters and other outcomes

Conditions such as liver weight, body weight, ascites, and consumption of water & food were taken in consideration.

3.3.5 Serological Indices

For serological liver function tests like, ALP (Alkaline Phosphatase), AST (Aspartate transaminase or aspartate aminotransferase test), ALT (Alanine transaminase) and T.B (Total Bilirubin) according to manufacturer's guidelines.

3.3.6 Histological Examination

After 6 weeks of disease induction, 2 rats were dissected and organs were harvested including liver, kidney and the spleen. The shape, color, and the size of each affected liver tissue were noted. Organs were obtained and placed immediately in 10% neutral-balanced formalin solution to avoid postmortem autolysis and decomposition. 5μm serial sections of organs were taken, followed by the paraffin imbedding. Organs were stained using Hematoxylin and Eosin (HE) to observe the structural variations as mentioned in histological slides. The pathological grading and scoring was done, centered upon the criteria of Histological grading and staging of Fibrosis by NASH/NAFLD Clinical Research network scoring system- definitions and scores (Davison et al., 2020).

3.4 Treatment Design

For evaluating the anti-fibrotic/anti-cirrhotic impacts of using PEG-1000 & PEG-2000 in LNPs and VLPs that were administered orally, diseased rats were taken within experiment. Rats were distributed into various groups according to different types of treatment formulations.

3.4.1   Negative Control Group

A set of five diseased rats were isolated and assigned the tag as negative control. This group of rats was left untreated throughout the experiment, and the survived rats were sacrificed upon completion of the treatment protocol to carry out the histological and serological analysis. Body and liver weight and ascites were noted.

3.4.2 Vitexin treated Group

Five rats were placed in this group. Vitexin drug at the dose of 10mg/kg were given intravenously for the duration of 20 days. Body and liver weight and ascites were noted upon completion of the treatment protocol prior to dissecting for the histology and serologic analysis.

3.4.3 PEG-1000 coated Blank LNPs treated Group

Five rats were placed in this group. PEG-1000 coated blank liposomal nanoparticles (devoid of Vitexin drug), at the dose of 500μg/kg were given via oral gavage for the duration of 20 days. Body and liver weight and ascites were noted upon completion of the treatment protocol prior to dissecting for the histology and serologic analysis.

3.4.4 PEG-2000 coated Blank LNPs treated Group

Five rats were placed in this group. PEG-2000 coated blank liposomal nanoparticles (devoid of Vitexin drug), at the dose of 500μg/kg were given via oral gavage for the duration of 20 days. Body and liver weight and ascites were noted upon completion of the treatment protocol prior to dissecting for the histology and serologic analysis.

3.4.5 PEG-1000 coated VLPs treated Group

Five rats were placed in this group. Vitexin-loaded liposomal nanoparticles coated with PEG-1000 at the dose of 500μg/kg were given via oral gavage for the duration of 20 days. Body and liver weight and ascites were noted upon completion of the treatment protocol prior to dissecting for the histology and serologic analysis.

3.4.6 PEG-2000 coated VLPs treated Group

Five rats were placed in this group. Vitexin-loaded liposomal nanoparticles coated with PEG-2000 at the dose of 500μg/kg were given via oral gavage for the duration of 20 days. Body and liver weight and ascites were noted upon completion of the treatment protocol prior to dissecting for the histology and serologic analysis.

CHAPTER 4: RESULTS

4.1 Characterization of Pegylated Liposomal Nanoparticles

4.1.1 Particle Size, thickness and topography

Atomic force microscopy was conducted to determine the size and surface topography of the nanoparticles. By considering the corresponding hue identified in the image obtained of the electrical intensity to the color map given (Fig.5) the thickness of the blank liposomes and size was noted. Whereas the 3D image of the slide used for specimen placement showed the topography of the blank liposomes coated with PEG (Fig.5b and d).
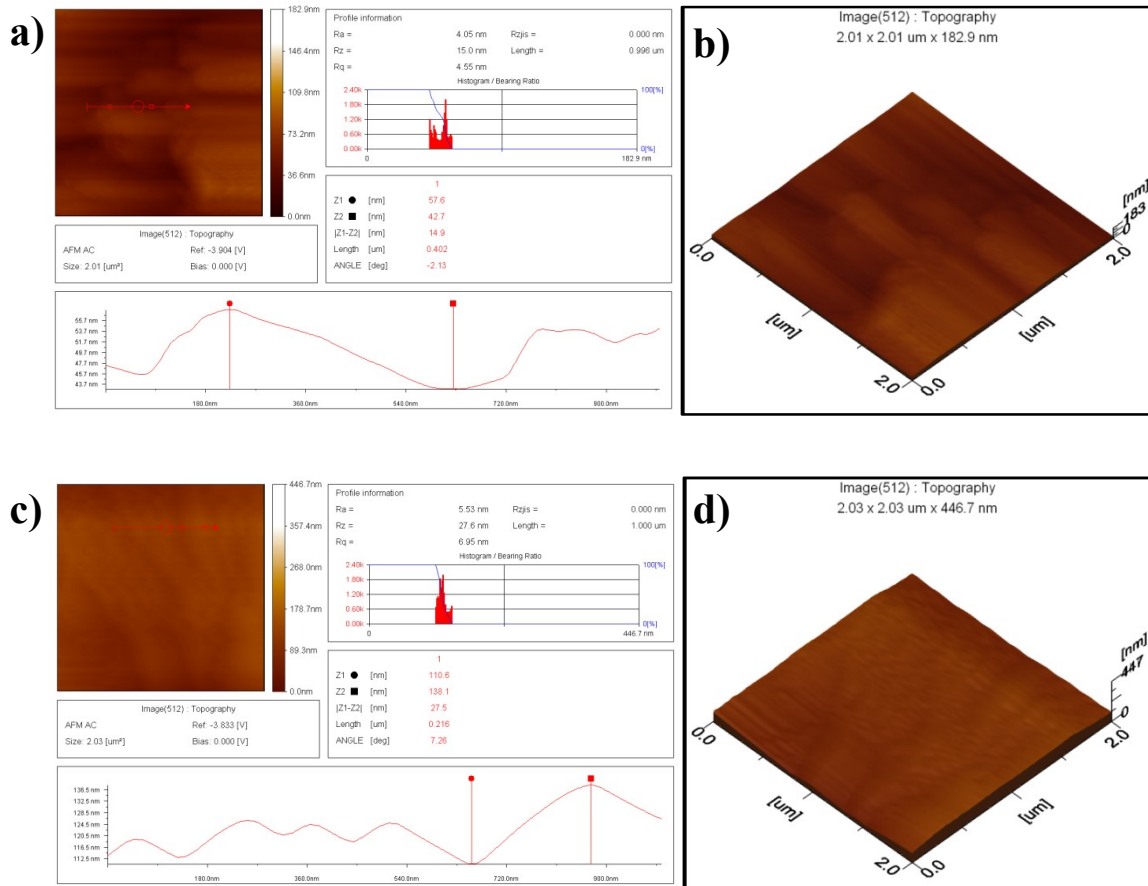
Figure: 5 *AFM image of a) Thickness of VLP-PEG 1000, b) 3D topographic view of the surface of VLP-PEG 1000, c) Thickness of VLP-PEG 6000, d) 3D topographic view of VLP-PEG 6000*

The sizes obtained for vitexin-loaded liposomal nanoparticles coated with PEG from AFM were 183nm of VLP-PEG 1000 (Fig.5a) and 447nm of VLP-PEG 6000 (Fig.5c).
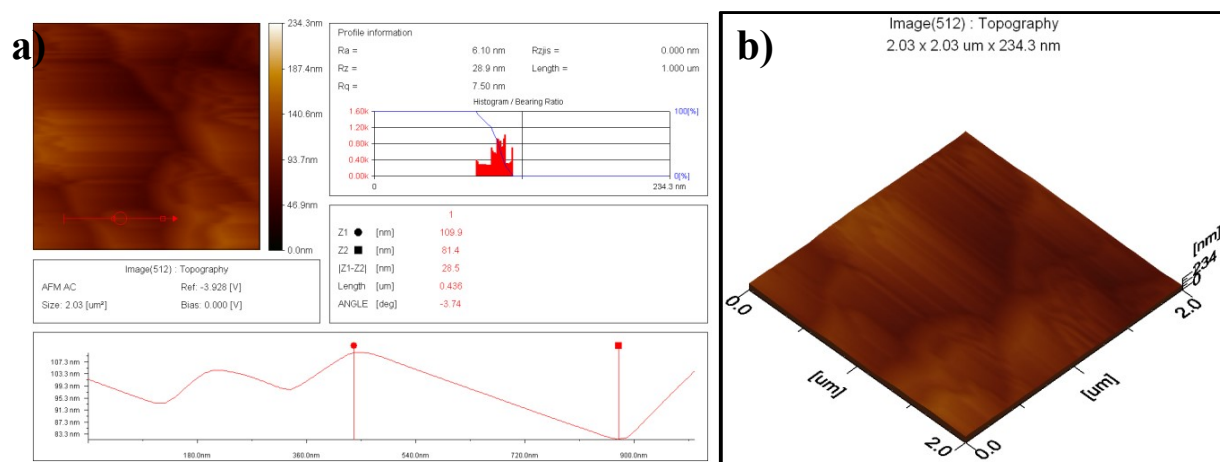
Figure: 6 *AFM image of a) Thickness of VLP-PEG 2000, d) 3D topographic view of VLP-PEG 2000*

The images obtained of Vitexin-loaded liposomes coated with PEG showed the sizes were 234nm of VLP-PEG 2000 (Fig. 6a). Whereas the 3D image showed the topographical view of the vitexin-loaded liposomes coated with PEG (Fig. 6b).

4.1.2 Drug Release Efficiency

The drug release efficiency was calculated to be 40% from VLP-PEG 1000 and 32% from VLP-PEG 2000 noted up to 48 hours suggesting the sustained release of drug from PEG-VLPs. Experiment demonstrated that the Vitexin drug releases faster from VLPs when coated with PEG-1000 as compared to VLPs coated with PEG-2000 which accordingly further improved the release profile of drug and increases its circulation time. This long stay of drug results in attaining the increased bioavailability and ultimately leads to high efficacy in treating the diseases (Fig. 7).
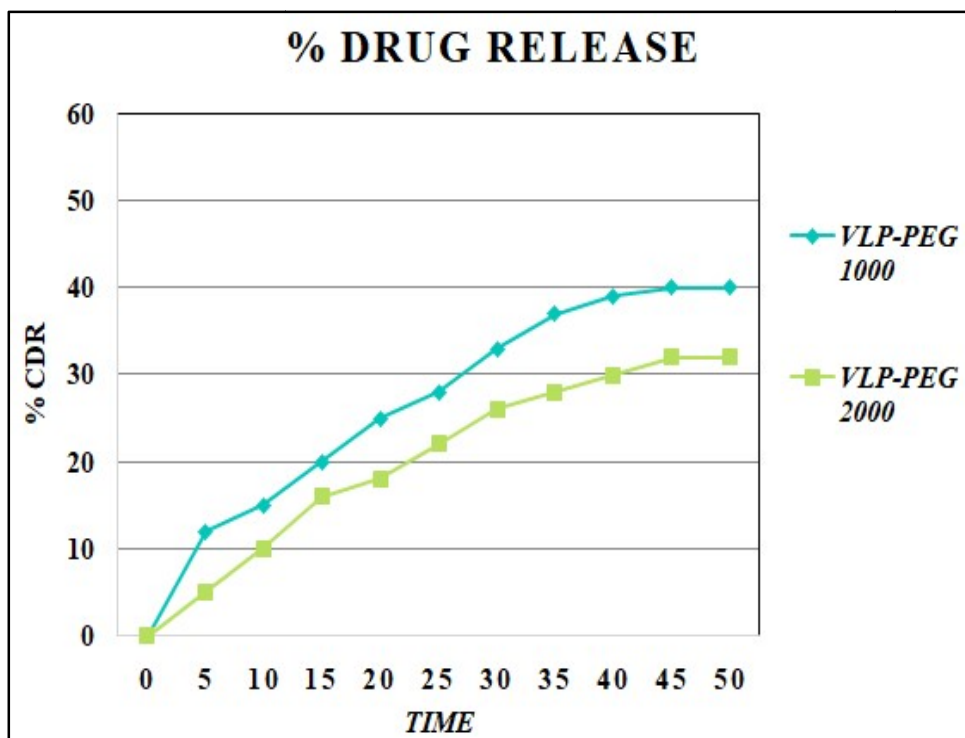
Figure: 7 *Comparative drug release graph of VLPs coated with PEG-1000 and PEG-2000*

4.2   Induction of ALD

4.2.1   Effects on Liver

Table: 1 Scoring of normal control and diseased control groups by the NASH/NAFLD Clinical Research Network Scoring System during disease induction.

GROUPS	SCORE
Normal Control	0/17
Disease Control	12/17

4.2.2   Hepatic Histopathology
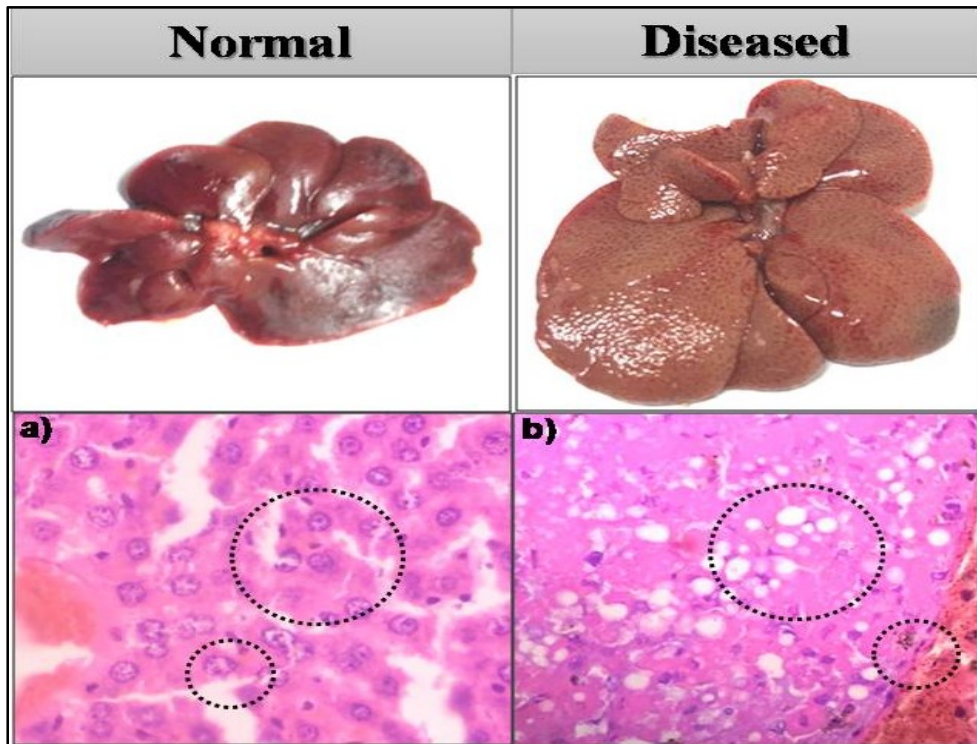
Figure: 8 *Morphology of complete liver and histology of a) normal and b) diseased liver tissue*

The section of normal control group revealed four cores of liver tissue, each core showing 4-5 portal tracts. Liver parenchyma showed sinusoidal congestion, unremarkable hepatocytes, and no dysplasia, fibrosis, or inflammation was seen. No portal tract abnormality was noticed and central vein was dilated and congested (Fig. 8a). Whereas, section of liver displayed two cores showing 5-6 portal tracts. Hepatocytes showed vacuolization and ground glass appearance with balloon degeneration. Portal vessel dilation, chronic inflammation of perivenule, and central vein dilation and congestion was observed. Periportal inflammation, dysplasia of bile duct, marked sinusoidal congestion, and irregular nuclear membrane with pleomorphism was seen (Fig. 8b).

4.2.3 Renal Histopathology
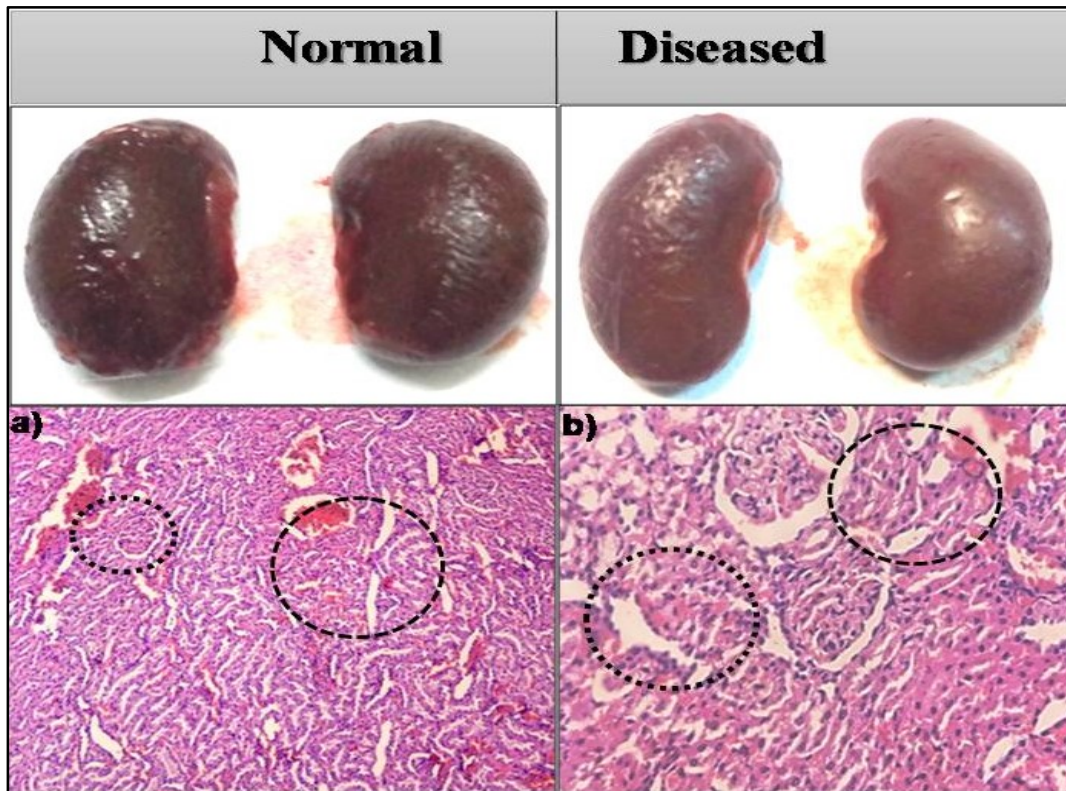
Figure: 9 *Morphology of kidneys and histology of a) normal and b) diseased renal tissue*

Kidney section showed a portion of renal cortex and medulla consisting of glomeruli, renal tubules and vasculature. Glomeruli showed normal capillary wall thickness and no increase in mesangium. There was no sclerosis, hypercellularity or atrophy of glomeruli. Tubules showed mild congestion, no necrosis or apoptosis of tubules was seen. No cellular cast of regeneration was identified (Fig. 9a). Whereas the diseased section of kidney tissue showed two fragments consisting of unremarkable glomeruli with capillary congestion and mesangial hypercellularity. Severe tubular apoptosis and congestion of vessels was seen. Tubules showed single cell necrosis spread at many places (Fig. 9b).

4.2.4 Spleen Histopathology
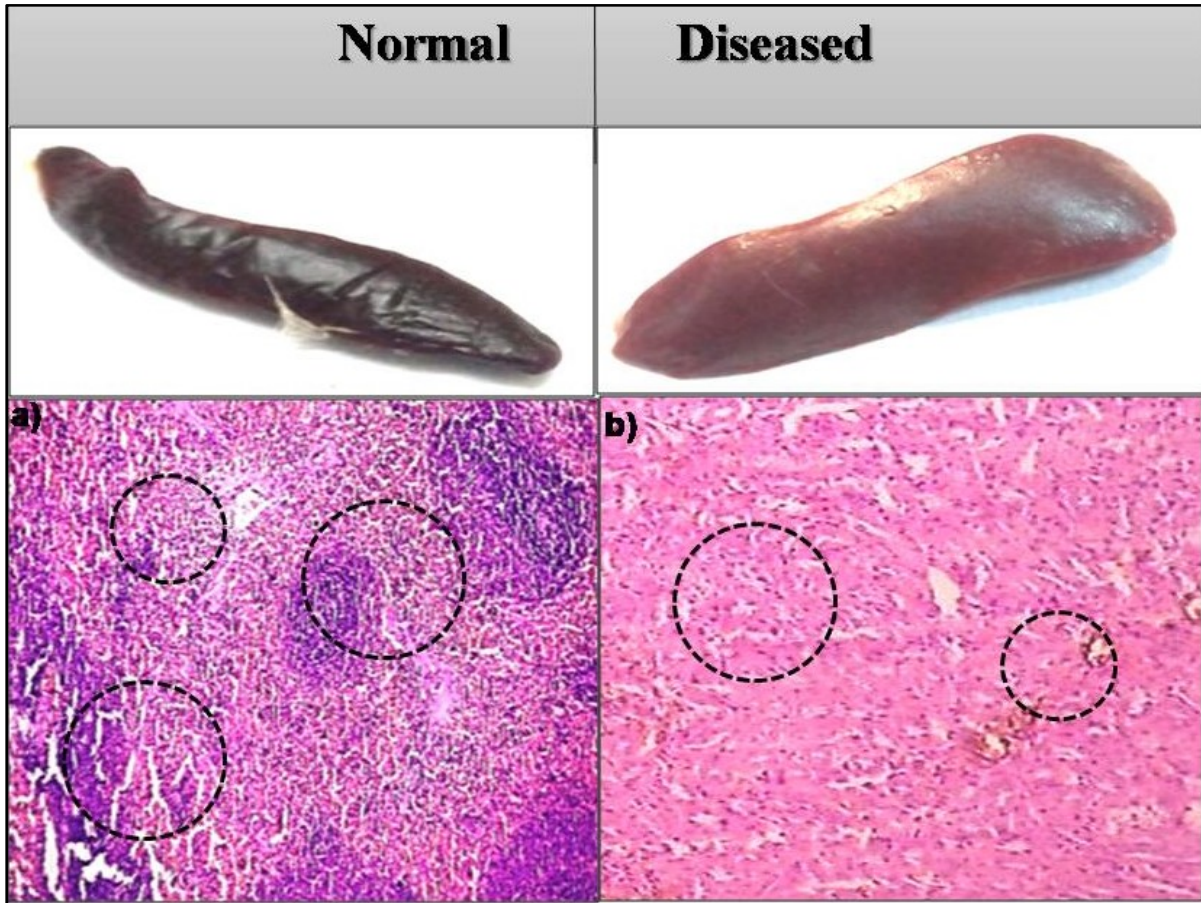
Figure: 10 *Morphology of spleen and histopathology of a) normal and b) diseased spleen tissue*

Sections of spleen showed a single core consisting of red and white pulp. Red pulp consisting of splenic sinusoid and cord showing congestion was noticed. White pulp showed lymphoid follicles with germinal centers around arterioles. Few trabeculae were seen transversing the red pulp, no fibrosis or hyperplasia of the parenchyma was noticed (Fig. 10a). Whereas in diseased splenic tissue showed two fragments consisting of red white pulp with severe congestion and hemosiderin laden cells. White pulp showed hyperplasia along with vesicular dysplasia and hyalinization of vessels was seen. Inflammatory cells were consisting of lymphocytes (Fig. 10b).

4.2.5 Ascites & Body Weight

Table: 2 Ascites after disease induction

Normal Control	Disease Control
--	+++
No Ascites: --, Mild Ascites: +, Moderate Ascites: ++, Severe Ascites: +++	
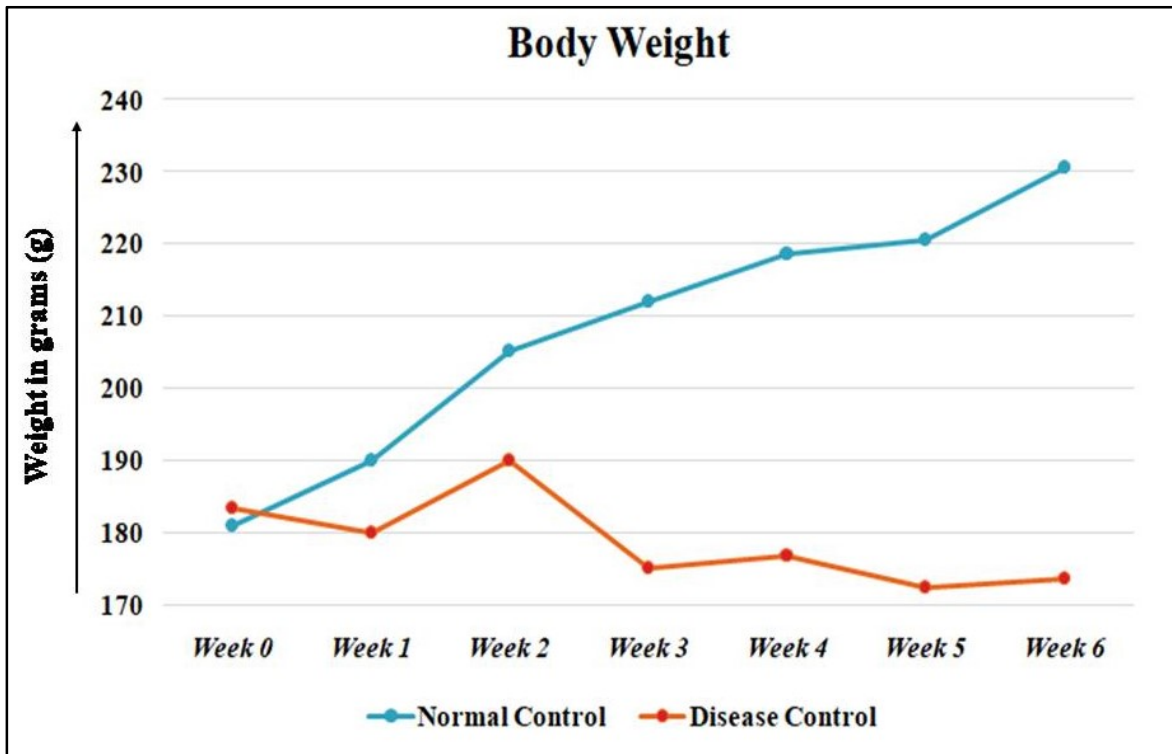
Figure: 11 *Weight of body during the development of ALD*

4.3   Treatment

4.3.1   Hepatic Histopathology
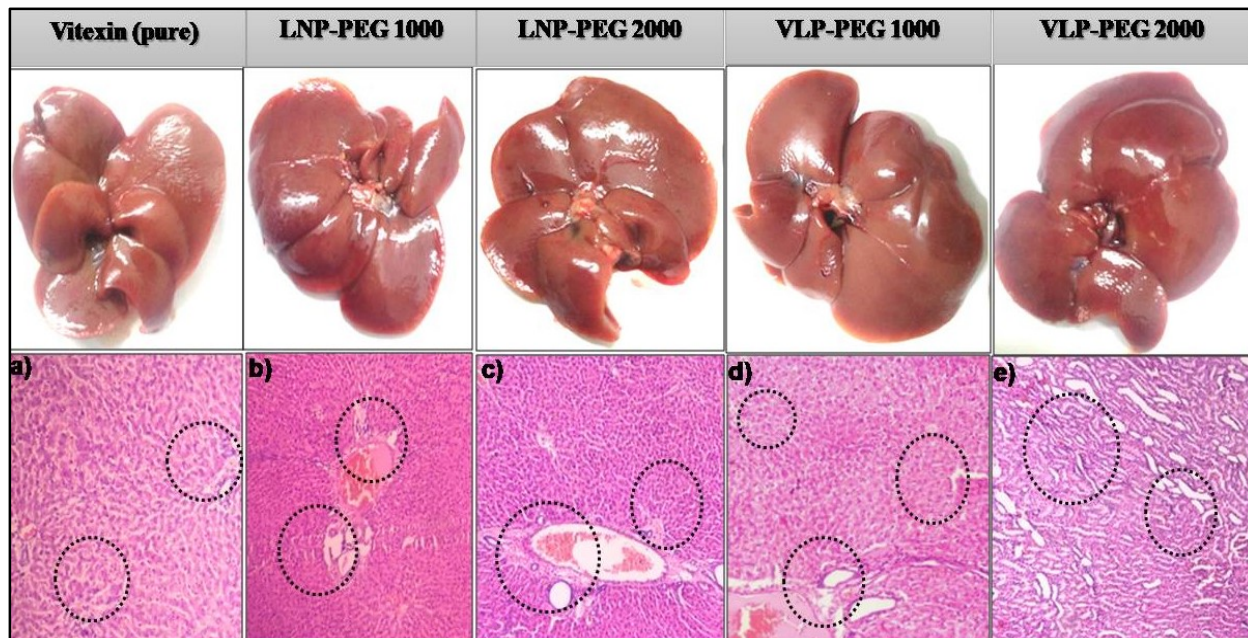
Figure: 12 *Liver morphology along with respective histopathology of hepatic tissue*

The liver section of the group treated with orally administered pure vitexin drug showed four portal tracts. Hepatocytes exhibited feathery degeneration and nuclei had conspicuous nucleoli. Few binucleated cells were seen. Central vein showed mild congestion. Mild portal inflammation was also noticed (Fig. 12a). Liver section of PEG-1000 coated blank liposomes treated group (LNP-PEG 1000) showed seven portal tracts in a fragment with atypical nucleus of hepatocytes along with feathery degeneration. There was an absence of ductular reaction in portal tracts. Some periportal inflammation remained as well as dilation and mild to moderate congestion of central vein (Fig. 12b). Two cores showing 5-6 portal tracts were seen in a section of liver treated with PEG-2000 coated blank liposomes (LNP-PEG 2000). Hepatocytes showed vacuolization and mild ground glass appearance. Mild periportal and chronic inflammatory filtrate was observed. Central vein showed dilation and congestion. No fibrosis and cirrhotic nodule was observed along with a lack of dysplasia or ductular reaction (Fig. 12c). A section of liver from the treatment group of vitexin-loaded liposomes coated with PEG-1000 (VLP-PEG 1000) showed one fragment consisting of seven portal tracts. Feathery degeneration and nuclear

atypia was seen in hepatocytes. Portal tracts showed no ductular reaction and periportal inflammation. Central vein showed dilation and mild congestion (Fig. 12d). In the rats treated with vitexin-loaded liposomes coated with PEG-2000 (VLP-PEG 2000), the liver section viewed contained a fragment consisting of 6 portal tracts, feathery and ground glass appearance of the hepatocytes, mild congestion of portal tract, and no ductular reaction. The central vein showed normal congestion (Fig. 12e).

Table: 3 NASH/NAFLD Clinical Research Network Scoring System at the end of treatment protocol.

| GROUPS	SCORE
Normal Control	**0/17**
Disease Control	**12/17**
Vitexin drug	**9/17**
VLP-PEG 1000	**6/17**
VLP-PEG 2000	**2/17**

4.3.2   Renal Histopathology
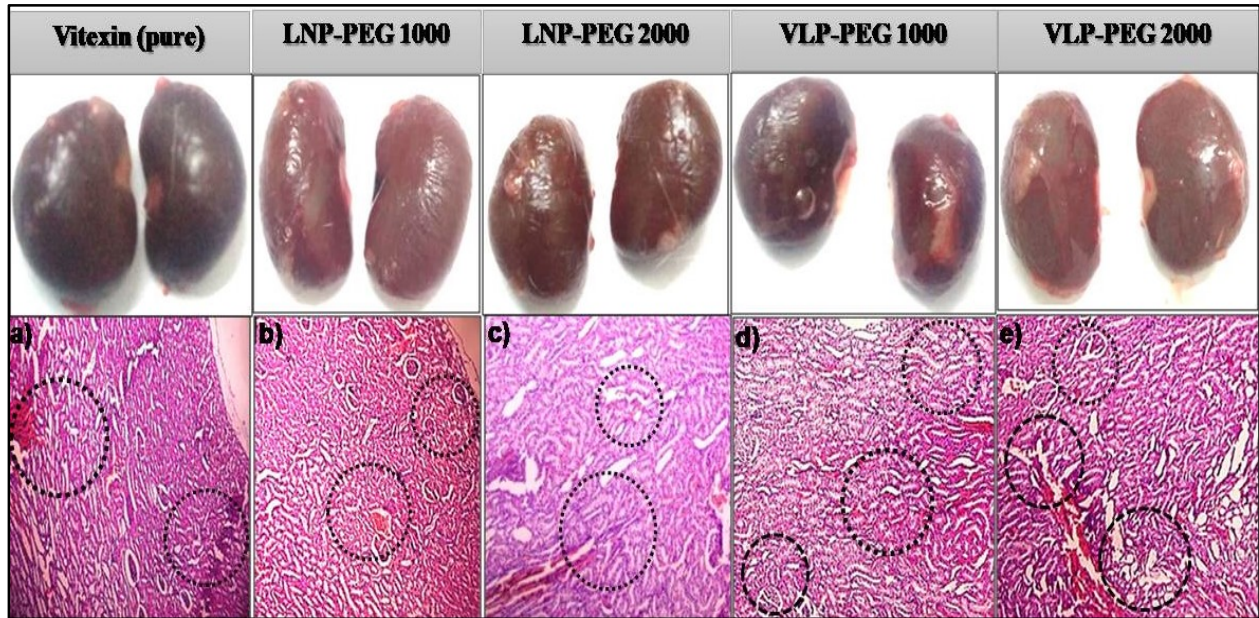
Figure: 13 *Kidney morphology along with respective histopathology of renal tissue*

The kidney section of the group treated with orally induced pure vitexin drug two fragments consisting of 10 glomeruli, tubules, vasculature, and interstitium. Glomeruli showed congestion and mesangial expansion. Tubules showed congestion and necrosis and vessels were also congested (Fig. 13a). LNP-PEG 1000 treated group showed a kidney section of two fragments consisting of 14 glomeruli, tubules, interstitium, and vasculature. Glomeruli showed capillary congestion and no membranous thickening or mesangial matrix expansion was noticed. Vessels were congested and the tubules showed tubular cell apoptosis. Mild cast thyroidization was also seen (Fig. 13b). Kidney section of LNP-PEG 2000 group showed two cores consisting of glomeruli and tubules. Mild mesangial hypercellularity and congestion was seen in the glomeruli. No membranous thickening was seen. Tubules exhibited single cell necrosis along with congested vessels (Fig. 13c). The group of rats treated with VLP-PEG 1000 showed a kidney section consisting of two fragments with 14 glomeruli, tubules, vasculature, interstitium, mild mesangial expansion, and congestion of the glomeruli, no membranous thickening, congestion in vessels and tubules as well as few single cell dropouts (Fig. 13d). VLP-PEG 2000 treated renal tissue showed two cores consisting of glomeruli and tubules. Glomeruli showed

mild mesangial hypercellularity and congestion. No membranous thickening was seen. Tubules showed single cell necrosis at various places along with congested vessels (Fig. 13e).

4.3.3   Spleen Histopathology
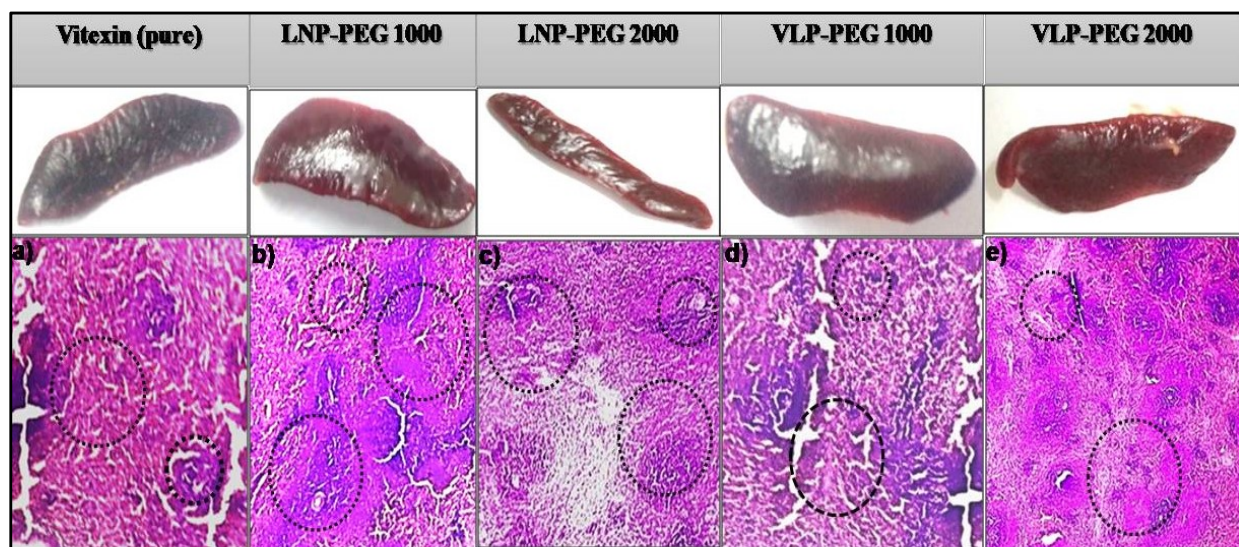
Figure: 14 *Spleen morphology along with respective histopathology of splenic tissue*

The group of rats treated with pure vitexin drug showed a spleen tissue of one fragment consisting of hyperplasia of red and white pulp and many hemosiderin cells were noticed in the red pulp (Fig. 14a). The spleen section of LNP-PEG 1000 had a single core consisting of red and white pulp. Red pulp consisted of splenic sinusoid and congestion of the cord was seen. White pulp showed lymphoid follicles with germinal centers around arterioles. Few trabeculae were seen transversing red pulp. No fibrosis or hyperplasia of the parenchyma was seen (Fig. 14b). Splenic tissue of LNP-PEG 2000 treated rats exhibited one fragment consisting of red and white pulp. Red pulp showed moderate to severe congestion and hemosiderin laden cells. White pulp showed hyperplasia whereas no dysplasia or hyalinization of vessels was seen (Fig. 14c). The spleen of VLP-PEG 1000 treated group displayed four fragments consisting of red and white pulp. Red pulp showed moderate to severe congestion and hemosiderin laden cells. White pulp showed hyperplasia with an absence of any dysplasia and hyalinization of vessels (Fig. 14d). VLP-PEG 2000 treated spleen section showed one fragment consisting of hyperplasia of both white and red pulp. Many hemosiderin laden cells were seen in the red pulp (Fig. 14e).

4.3.4 Serological Analysis
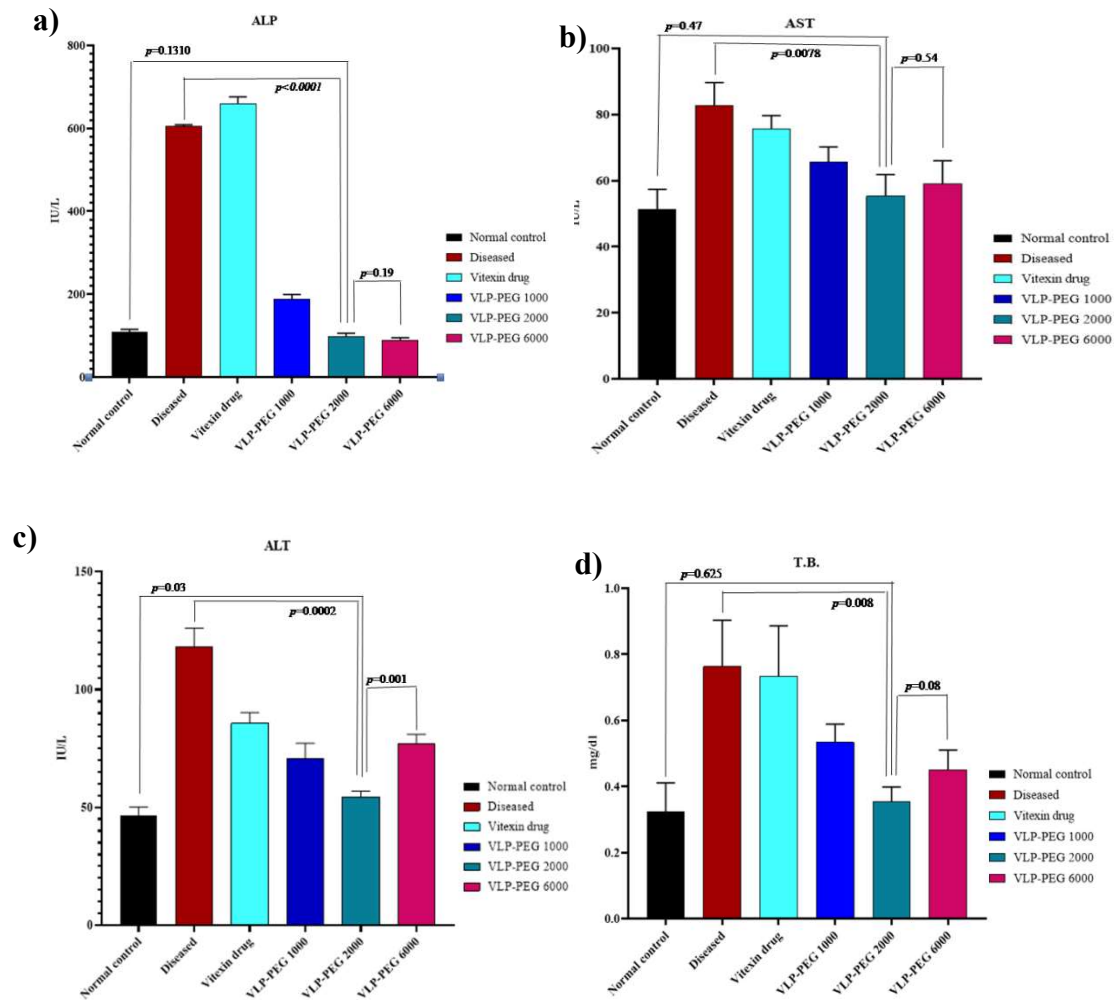
Figure: 15 *Serological indices after treatment (a) ALP, (b) AST, (c) ALT, (d) T.B.*

The results obtained from the serological testing of the various enzymes for liver functioning were analyzed statistically using t test and one way ANOVA which showed that the results obtained are of significance ($p < 0.05$). The two tailed test showed that the level of ALP in the group treated with VLP-PEG 2000 were significantly different from that of the diseased group ($p<0.0001$) though not much varying from VLP-PEG 6000 treated group ($p=0.192$), but it is similar to the normal control group ($p=0.13$). Whereas VLP-PEG 6000 is significantly different from that of the normal group ($p=0.0179$) (Fig. 15a) depicting that PEG-2000 coating is more effective in oral treatment as compared to PEG-6000. The results analyzed of AST, ALT, and T.B. also show a similar trend for the VLP coated with PEG-2000 oral treatment with the *p*

values differing significantly from the diseased sample ($p=0.0078$, $p=0.0002$, and $p=0.0085$ respectively) whereas similar to the normal control group ($p=0.47$, $p=0.037$, and $p=0.625$ respectively) (Fig. 15b,c & d). Further it was also noticed that the p-values of VLP-PEG 2000 in LFTs varied from that of VLP-PEG 1000 and vitexin drug depicting that it shows comparatively enhanced therapeutic effect than the other treatment formulations in the oral administration.

4.3.5 Body Weight, Ascites, and Food Intake
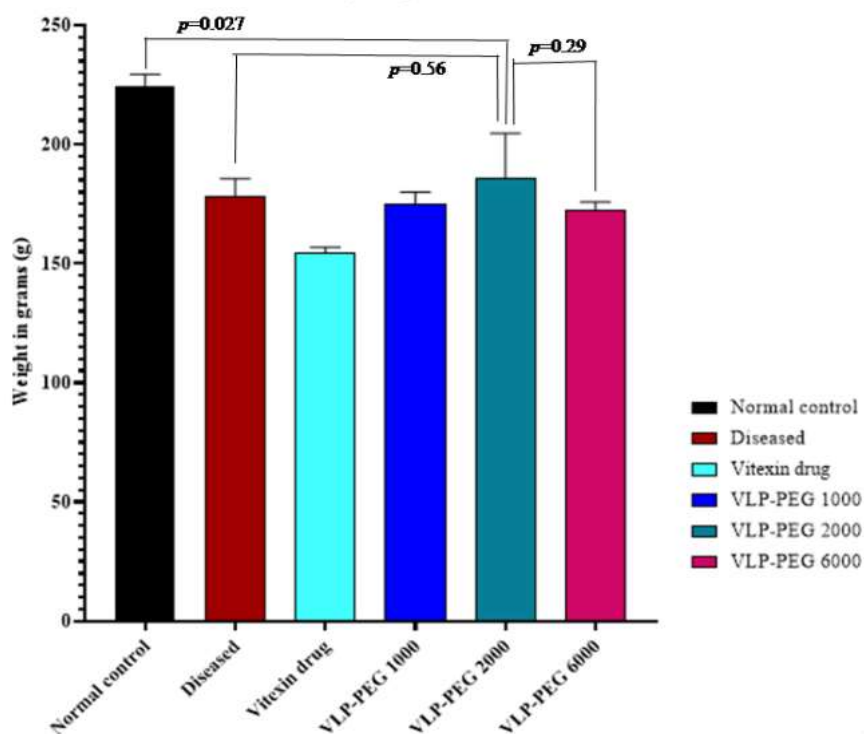
Figure: 16 *Body weight after treatment*

Table: 4 Ascites after treatment

Normal/ Control	Diseased	Vitexin drug	VLP-PEG 1000	VLP-PEG 2000
--	+++	++	+	--
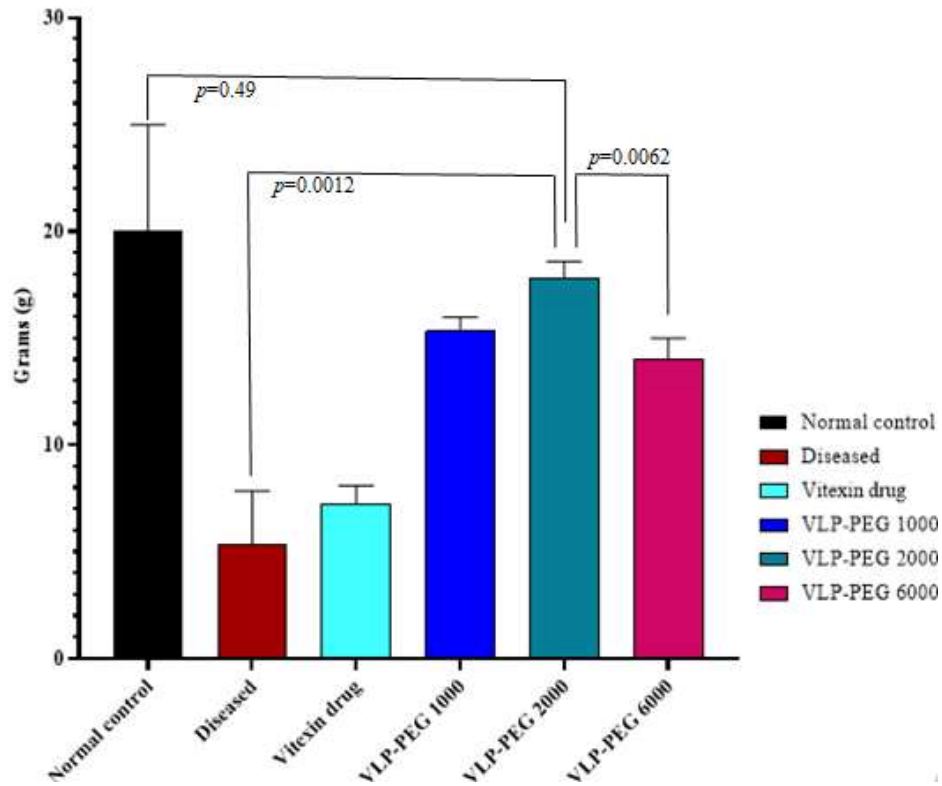
Figure: 17 *Variation in the food intake*

In the normal control group each rat consumed approx. 20g per day of feed. Disease control group consumption per rat was up to 5g per day. Approximately 15g, 18g, and 7g per day were consumed by each rat in VLP-PEG 1000, VLP-PEG 2000, and Vitexin group respectively (Fig. 17).

CHAPTER 5: DISSCUSSION

The nanoparticulate encapsulation of vitexin was shown to treat liver cirrhosis considerably better than pure drug when it was intravenously injected to the rat models of liver cirrhosis or as described the decompensated stage of ALD. Polyethylene glycol-6000 imparted even greater effectiveness by providing stealth and stability to the vitexin-loaded liposomes (Farooq et al., 2022). The drawback being the extensively larger size of the vitexin–loaded liposomes by the use of PEG-6000 coating has been taken into account in this research and different chain lengths of PEG coating such as PEG-1000 and PEG-2000 were used to examine their effect on the effectiveness, bioavailability, and a possible improvement in the sustainability of the drug release. These formulations of VLPs coated with PEG-1000 and PEG-2000 in the treatment of ALD by oral route of administration was studied for further improving the treatment for this final stage of liver disease to avoid the otherwise fate of morbidity of this disease.

The NASH/NAFLD-related chronic liver dysfunction prevails most abundantly at the global level (Makri et al., 2021). Mostly the patients of such chronic liver disease face such extensive complications related to the liver dysfunction that surgery becomes the only possibility for saving the patient and even then the rate of survival is quite low (Simon et al., 2021). The associated complication of ALD make it quite difficult to diagnose early hence rendering the chances of an early treatment i.e., in the compensated stage of liver disorder and surgery has its limitations due to other organs related complications such as portal hypertension, cardiovascular diseases, and liver cancer (Tacke & Weiskirchen, 2021). This burdens the health-care economically, making it 80% higher than other types of chronic liver diseases (Murag et al., 2021). The lack of such antifibrotic drug or any treatment strategy to overcome the drawbacks, limitations, and complications related to the invasive techniques has given rise to the use of nanomedicines as an alternative and a novel approach in regenerative field of medicine in the current century (Sampaziotis et al., 2017).

Owing to its versatility in curative nature, Vitexin has been recently preferred quite often for the treatment of many types of liver diseases and other related complications. Previous studies have introduced it to be effective in reversing the liver dysfunction and related metabolic discrepancies which have made it a suitable candidate for loading in nanoparticles with an aim to

coin the significance of using vitexin and liposomes in treatment of ALD. The preparation and characterization of the VLPs produced in the following research were carried out according to the methodology introduced by Farooq et al., while changes have been made by coating two different types of PEG for oral treatment. The liver disease model was also developed by the same methodology with few incorporated changes due to the use of Wistar rats for in vivo study. The average sizes of PEG-1000 coated VLPS and VLPs coated with PEG-2000 were observed to be 202nm and 234nm with drug release noted to be 40% and 32% respectively for 48 hours of examination which was better than the size of 458nm of PEG-6000 coating on vitexin-loaded liposomes and 44% of the drug release efficiency. The achieved size of the PEG-coated nanoparticles was found to be better effective in accordance with the study by Ma et al. in 2019 indicating that sizes 150-300nm of nanoparticles have a greater tendency of enrichment in the hepatic and splenic tissues which are the primary targets of treatment in ALD.

Due to the steric repulsion caused by PEG coating the stability of liposomal nanoparticles was enhanced and a stealth effect was induced making them capable of oral administration. Since this PEG coating also minimizes the release of vitexin the stomach (Shaedi et al., 2021) hence it has better chances to escape FPM and an early elimination increasing its circulation time in the body. This was analyzed in the *in vivo* results obtained from the serology tests of LFTs, histopathology, liver to body weight ratio, ascites, and food intake after the treatment completion. The food intake of the group treated with PEG-2000 was closest to the range of feed consumption by the normal control group. Similarly the liver to body weight ratio showed improvement both in VLP-PEG 1000 and VLP-PEG 2000 treated groups being predominantly better in the PEG-2000 treatment group. Serological indices and histopathological analysis showed variation with similar improvement in the results of the group treated by vitexin-loaded liposomal nanoparticles with PEG-2000 coating proving it to be the preferable delivery agent for oral route of ALD treatment.

CONCLUSION

PEG coating using PEG 1000 & PEG 2000 of liposomal nanoparticles contributes towards stealth effect and reduces the size of particles formed by PEG 6000 coating to the extent that is required for treating ALD. Pegylation enhances the bioavailability of vitexin encapsulated liposomal nanoparticles in oral route of administration for noticeable reversibility of liver damage. Better results were noticed when vitexin-loaded nanoparticles were coated with PEG-1000 and PEG-2000 in oral treatment as compared to uncoated vitexin liposomes treatment. Pegylation enhanced the stability that resulted in the long-term circulation in the body as compared to conventional drug and non-coated liposomes nanoparticles. After treatment of the rats with a decompensated stage of liver cirrhosis in ALD, the analysis done from histopathological, the serological test, and the results seen of liver to body weight ratio exhibited the significant improvement in PEG-2000 coated VLPs treated group. Hence, liposomes encapsulation of vitexin drug followed by PEG-2000 coating is proved as the better choice for enhancement of bioavailability and improvement in the pharmacokinetic behavior of vitexin drug when oral route of delivery is opted in treating ALD.

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