LIPOSOMAL ENCAPSULATION OF VITEXIN AND ITS IN VIVO ANALYSIS AGAINST LIVER CIRRHOSIS

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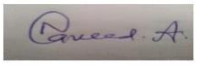
3. Name: Dr Naveed Ahmed Signature: 

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LIST OF ABBREVIATIONS

LNPs	**Liposomes Nanoparticles**
PEG-LNPs	**Pegylated Liposomes Nanoparticles**
DPPC	**Dipalmitoyl phosphatidylcholine**
NASH	**Non-Alcoholic SteatoHepatitis**
NAFLD	**Non-Alcoholic Fatty liver Disease**
ACLD	**Acute chronic Liver Disease**
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**
O.G	**Oral Gavage**

Abstract

Nanomedicine and nano delivery systems are relatively new, yet rapidly evolving sciences where nanoscale materials are used to act as diagnostic tools or to deliver therapeutic agents to specific targeted sites in a controlled manner. Nanomedicines and nano-based drug delivery systems enhance both the efficacy of new and old drugs through the comprehensive analysis of development and application of nanoparticles. Nanoparticulate structures with stimuli-sensitive polymers and liposomes have gained significant attention for the treatment of liver fibrosis. An advanced stage of fibrosis is liver cirrhosis, characterized by histological growth of regenerative nodules surrounded by dense fibrotic septa. Clinically, cirrhosis is considered as an end stage disease, that precedes to death, if the liver transplantation is not to be performed. Available anti-fibrotic therapies have focused against the reduction of hepatic inflammation rather than to vanquish fibrosis. Therefore, there is a need of significant therapeutic agent, capable of eradicating the fibrosis.

Vitexin is a natural flavonoid found in certain herbs. Recent study confirmed that vitexin inhibits NAFLD by activating AMPK which in turn inhibits lipogenesis and activates lipolysis and fatty acid oxidation. Due to hydrophobic nature, vitexin showed low bioavailability and less efficacy. Also, Reticulocyte endothelial system (RES) may causes the opsonization while systemic circulation. Different types of proteins in blood stream may bind to vitexin drug and ultimately lead to the formation of corona complex that changes its pharmacokinetic behavior.

To overcome the drawbacks, vitexin encapsulated liposome nanoparticles were synthesized by ‘thin film hydration method’ and used against liver cirrhosis for the first time. To enhance the stability, Polyethylene glycol (PEG) was used to enhance stability and for inducing the stealth effect, by coating the liposomes nanoparticles. Pegylation enhances the steric repulsion and hence known as better stabilizer for different types of nanoparticles. PEG follows the erosion -controlled release mechanism of drug that resulted in sustained release. Hence, it is noteworthy that encapsulating the vitexin drug within liposomes and tailoring these liposome nanoparticles by PEG, is a substantial strategy to combat liver cirrhosis.

Key Words: *Vitexin, Liposome Nanoparticles, Pegylation, Liver Cirrhosis, NASH, Fibrosis, Animal Model, Anti-Fibrotic agent, Histological Examination*

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CHAPTER 1:INTRODUCTION

1.1 Objective

The research work in dissertation is presented in two parts. The first part of this research emphasized upon the liposomal nano formulation of vitexin drug and its characterization. The selected drug bearing versatile nature has been used against NAFLD & fibrosis. But for the first time its nanoparticles are synthesized according to 'thin film hydration method' . Different aspects of formulated nanoparticles are characterized by using different characterizing techniques and eventually enabled them for liver cirrhosis in vivo analysis.

The second elaborated part emphasized upon the liver cirrhosis model development and in vivo analysis for encapsulated drug's improved pharmacokinetic behavior. Treatment proficiency was investigated among two different routes of administration in living system. In this way, the examination of anti-fibrotic and anti- cirrhotic activity of nanoparticles in well-established liver cirrhosis model was also the prime focus of our research and will be considered a significant step to uplift these liposome encapsulated vitexin nanoparticles to preclinical trials level.

1.2 Nanotechnology; An Introduction to new era

Nanotechnology is defined as the ability to restructure and manipulate the matter at the range of approximately 1-100nm at molecular or atomic level and exploiting the distinct properties and anomalies (Roco, 2011). The definition of nanotechnology is generally attributed to Feynman (1960) and his famous speech "There is plenty of room at the bottom" while Taniguchi (1974) apparently coined the word itself around 15 years later (Zingg & Fischer, 2018). After consultation with experts of more than 20 countries (Siegel et al . 1999) in 1998-1999, the concept of nanotechnology was agreed upon and attained certain degree of international recognition. It is conceptually distinct from the pre-1999 concepts that concentrated on either small feature under a given scale, ultra-precision engineering, ultra-dispersions, or atom and molecular patterns on surfaces (Roco, 2011).

1.2.1 Development in Nanotechnology

Nanotechnology is an emerging field that is developing at an elevated pace and capable to fetch the changes, stipulate innovative products, allow new and augmented human capacities, and generally reinvent relationships in several different sectors through innovation (Forloni, 2012).

Roco documented that, the protyping of first generation' nanoparticles at start, were passive (polymers, ceramics and nanostructured metals), then they became active (targeted drugs and 3D transistors etc.) with second generation in 2005, and then the development to shape the nano system (3D networking and guided assembly) attained as 3rd generation in 2010 and then eventually we will have the atomic design of molecular nano systems (Roco 2006, 2007).

Today, field of nanotechnology offers 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 (Sainz et al., 2015). Now the zone of environmental protection, medicines, green energy, and electronics are the key targets in nanotechnology's application while some nanotechnology tailored products have already entered the market like textiles, cosmetics, sports articles, housing & building (Forloni, 2012).

1.2.2 Nanotechnology As therapeutic weapon

Over the past two decades, nanotechnology has largely been investigated and emerging as a 'new technological revolution'. Significant engineered nanodevices, molecular and macromolecular nanoscale-level nanomedicines have been designed and developed for the last decades (Sainz et al., 2015).

Nanomedicine is the use of nanotechnology to bring about improvement in healthcare that could eventually offer significant breakthroughs in terms of efficient and cost-effective healthcare, a critical factor in making available and affordable drugs and treatments (Sainz et al., 2015). Nanomedicines offer advantages in several different respects in comparison with standard

low molecular weight drugs. For example, they (1) decrease the hepatic deterioration and renal excretion and cause the prolong circulation (2) modulate the reduction of volume distribution, resulting in non-targeted site avoidance (3) enhance the drugs' capacity to accumulate in pathological settings. Furthermore, nanomedicine formulation help low molecular weight chemotherapeutic agents to cross many barriers to pathological sites (Rizzo, Theek, Storm, Kiessling, & Lammers, 2013).

1.3 Liposomes and its Nanoparticulation

Liposomes are sphere shaped vesicles, comprising of one or more phospholipid bilayer and were first described in mid 60s (Akbarzadeh et al., 2013). The size of liposomes ranging from 30nm to several micrometers (Wagner & Vorauer-Uhl, 2011). The properties of the liposome considerably differ with lipid composition, size, surface charge and preparation process. Choice of bilayer contents also defines the fluidity or rigidity and surface charge. Unsaturated phosphatidylcholine species from natural sources like egg or soybean, stipulate less stable and permeable bilayers while saturated phospholipids having long acyl chain like dipalmitoyl choline form impermeable and rigid bilayer structure. Amid numerous capable modern drug delivery technologies, liposomes signify an advanced technology for the transportation of active molecule at the site of action (Sharma, Aara, Ali, & Trivedi, 2018). Currently, variety of liposomal formulations are in clinical use. Liposome technology has been emerged from conventional vesicles to 'second generation liposomes', whereby modulating the vesicles lipid composition, charge and size, long circulating liposomes are obtained. By using sialic acid or glycolipids, liposomes surfaces have been made modified (Akbarzadeh et al., 2013).

1.3.1 Liposomes as carrier

Liposomal encapsulation technology is the new delivery technique serve as curative promoters, used by medical researchers for drug delivery to the expected body organs. It is a form of submicroscopic foam generation called liposomes, which encapsulates various constituents (Akbarzadeh et al., 2013). Liposomes are widely used in the cosmetics and pharmaceutical industries as carriers for various molecules (Atrooz, 2011).

Liposomes are capable of encapsulating both hydrophilic and hydrophobic materials, preventing disintegration of the entrapped stuff and releasing at their destinations (Shehata, Ogawara, Higaki, & Kimura, 2008). Liposomes represent a mature technology with proven clinical efficacy and gained FDA approval recently (Meng et al., 2016).

1.4 Liver Cirrhosis

Fibrosis delineates the substitution of damaged tissue by collagenous scar, followed by the continuance of healing responses that commence fibrogenesis and Necroinflammation. An advanced stage of fibrosis is liver cirrhosis, characterized by histological growth of regenerative nodules surrounded by dense fibrotic septa, resulting in end stage liver malady. (Schuppan & Afdhal, 2008; Tsochatzis, Bosch, & Burroughs, 2014) These fibrotic septa prevent regular oxygen supply and disturbed the blood exchange in liver parenchyma. Such conditions consequently, cause portal hypertension complemented with hepatocellular dysfunction (Berzigotti, 2017). Portal hypertension is characterized by certain clinical complications corresponding variceal bleeding, peritonitis (Garcia-Tsao, Abraldes, Berzigotti, & Bosch, 2017), ascites (Liver, 2010), and hepatic encephalopathy(Hytiroglou et al., 2012). This advanced stage of fibrosis demonstrates the anatomical changes like; hepatocyte destruction, macro and micro vascular transformation, nodule development and formation of portosystemic shunts etc. (Berzigotti, 2017). Histopathologists suggested that histological term of cirrhosis should be swapped by “advanced liver disease”, to accentuate the active progression and varying degree of disease prognosis. (Hytiroglou et al., 2012; Tsochatzis et al., 2014)

One of the largest epidemiological study ("Global, regional, and national age–sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013," 2015) demonstrated liver cirrhosis as 4th leading cause of deaths in central Europe with estimated steady upsurge of approximately 50 million incidences for the last two decades worldwide. (Berzigotti, 2017) and 11th leading cause worldwide and accounts 3.5% of all deaths (Asrani, Devarbhavi, Eaton, & Kamath, 2019).

Etiological findings of cirrhosis can be done by histological and serological evaluation (Tsochatzis et al., 2014). Viral Hepatitis (B & C), (Wiegand & Berg, 2013) alcoholic liver

disease (Osna, Donohue, & Kharbanda, 2017) and nonalcoholic steatohepatitis (NASH) (Arrese & Feldstein, 2017) diabetes type 2 and obesity in old age (Farrell & Larter, 2006) are known to be common causes of liver cirrhosis. It is valuable to identify the cirrhosis etiology to expect complications and layout of treatment strategy.

1.4.1 Available Treatment for Liver Cirrhosis

Clinically, cirrhosis is considered as an end stage disease, that precedes to death if the liver transplantation is not to be performed (D'Amico, Garcia-Tsao, & Pagliaro, 2006). Hepatocytes transplantation ameliorates and reversed the liver physiology and advanced stage fibrosis(Cai et al., 2002; Nagata et al., 2003). Invitro expansion after isolating the progenitor or hepatocytes stem cells may had an optimism in transplantation paradigm (Malhi, Irani, Gagandeep, & Gupta, 2002; Nowak et al., 2005), but proficiency of progenitor or stem cells is considerably low and the essential manipulations for adequate engraftment in humans would sustain colossal risks of liver failure (Thorgeirsson & Grisham, 2006). Likewise, genetic restoration by telomerase can boost hepatocyte regeneration (Rudolph, Chang, Millard, Schreiber-Agus, & DePinho, 2000) but increased activity of telomerase may also likely to developed hepatocarcinogenesis (Martin & Dufour, 2008).

Matter of cirrhosis reversibility derived from indication achieved in animal cirrhosis models, by using putative anti-fibrotic agent or upon the cessation of agent responsible for liver damage (van Leerdam, 2008). Available anti-fibrotic therapies have focused against the reduction of hepatic inflammation rather than to vanquish fibrosis. Therefore, there is a need of significant therapeutic agent, capable of eradicating the fibrosis and reverse the liver cirrhosis.

CHAPTER:2 LITERATURE REVIEW

2.1 Vitexin: A Versatile Drug

In traditional Chinese medicines, vitexin is known as 'Mujingsu.' It is known by many synonyms such as Apigenin-8-C-glucoside and Orientoside. It is a c-glycosylated flavone found in a number of medicinal plants, including hawthorn, pearl millet, mung bean, pigeon pea, bamboo, mosses, passiflora, wheat leaves, mimosa, chasteberry or chaste tree etc. Flavonoids appear to exhibit many pharmacological activities (Basile, Sorbo, López-Sáez, & Castaldo Cobianchi, 2003; Cao et al., 2011; Edwards, Brown, Talent, Dickinson, & Shipley, 2012; Fu et al., 2007; Gaitan et al., 1989; He et al., 2016; Melchert & Alston, 1965; Nix, Paull, & Colgrave, 2015).

It exhibited potentially potent hypotensive, anti-inflammatory, anti-metastatic and anti-spasmodic properties. Vitexin has other benefits, such as anticonvulsant effects, anti-nociceptive effect, antiglycation and memory restoration stimulation (Aslam, Ahmad, & Mamat, 2015). Vitexin imposed antineoplastic activity in both in vitro and in vivo models via promoting apoptosis and autophagy and also inhibiting the proliferation and migration via multiple signaling pathways (Ganesan & Xu, 2017).

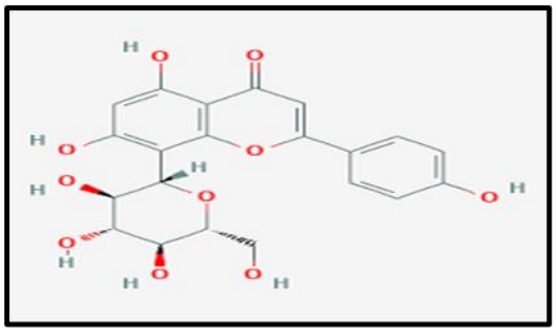


Figure 2.1: Structure of Vitexin Drug

2.1.2 Vitexin against Liver Inflammatory Diseases

Liver diseases, ranging from steatosis, hepatitis, and cirrhosis to hepatocellular carcinoma (HCC), are the world one of the leading cause of morbidity and mortality that result in immense socio-economic burden (Li et al., 2015). Different natural foods and herbs containing

frequent number of phytochemicals, have been suggested as nutritional treatment to patients having hepatic disorders for the last several years. An important group of phytochemicals containing natural polyphenols, gained mounting attention as capable agent for the treatment of liver diseases. The potential capabilities of polyphenols in oxidative stress remittance, insulin resistance, lipid metabolism and inflammation, captivated the attentions as liver disease therapy. Number of food and herbs having polyphenols have been documented that showed therapeutic potential on hepatic injuries through complex mechanisms (Tang et al., 2018).

The experimental results revealed the potential role of Alysicarpus monilifer's methanolic extract and its bioactive molecule i.e. vitexin in shielding liver function, lowering oxidative stress, and improving the histopathological structures in the CCl4-induced liver damage rat model (Ravan, Bahmani, Ghasemi Basir, Salehi, & Oshaghi, 2017).

Vitexin considerably reduced the protein expressions of TNF-α and TLR4, and suppressed the phosphorylation of p65, indicated that vitexin can inhibit the activation of TLR4/NF- κB signaling pathways to alleviate colitis-induced injury to the liver (Duan et al., 2020).

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 and vitexin-contained extract have been shown to be efficacious in reducing obesity, hepatoprotective and hepatic fibrosis. Recent research reported that vitexin inhibits NAFLD by AMPK activation that in consequently inhibits lipogenesis and stimulates lipolysis and oxidation of fatty acids. Additionally, insulin signaling in the liver of animals fed with HFD was improved by vitexin (Inamdar, Joshi, Malik, Boppana, & Ghaskadbi, 2019).

Anethum graveolense capacity L. (AGME) containing vitexin has been shown to inhibit TGF-β1 release and to reduce oxidative stress in the treatment and prevention of human hepatic

fibrosis and cirrhosis on the bile duct ligation (BDL) in rats (Sadik Ali, Anup Maiti and Vishal Kumar Vishwakarma, 2019)

Methanolic extract of Carissa opaca leaves (MCL) showed the protecting capability of CCl4-induced hepatic damage as an intrinsic amended property in the liver of rats. It has been documented that Phyto-constituents such as saponins, tannins, terpenoids, and flavonoids (isoquercetin, hyperoside, vitexin, myricetin, and kaempherol) present in MCL exert antioxidant activity by scavenging free radicals that cause lipid peroxidation (Sahreen, Khan, & Khan, 2011).

2.2.2 Carrier Necessity for Vitexin Delivery

Vitexin along with its therapeutics versatility, possess poor water solubility. Poor bioavailability and low aqueous solubility limit its clinical applications (Gu et al., 2017; Lai et al., 2014; Zu et al., 2012). Liang et al assessed the oral bioavailability and pharmacokinetic of vitexin rhamnoside after oral and intravenous administration to rats. Results confirmed the limited absorption and quick elimination of vitexin (Liang et al., 2007). Upon estimation, 40% of approved drugs and approximately 90% of drugs in developmental pipeline, contained poor soluble molecules (Loftsson & Brewster, 2010).

Number of marketed drugs suffer low bioavailability, poor solubility, and rapid elimination along with poor safety and tolerability (Hodgson, 2001). Poor solubility associated issues can lead to low bioavailability resulting in suboptimal drug delivery. Numerous drugs accompanying poor solubility and low bioavailability's have been reformulated to improve efficacy, safety, and patient compliance by nanoparticulation and nanonization (Kalepu & Nekkanti, 2015; Tanaka et al., 2009).

2.3 Significance of Liposome Nanoparticles Against Liver Fibrosis

Different researchers have proven the therapeutic capability of dexamethasone-loaded liposomes and made obvious the reduction of both liver inflammation and liver fibrosis by its use. Such nanoparticles have been shown to impact the Kupffer cells by reducing T cells via an immune response in liver, resulting in the reduction of fibrosis (Bartneck et al., 2015). Cationic

liposomes containing microbubbles for the successful delivery of artificial microRNA is also reported that was used to target the growth factor of connective tissue (CTGF) and can be useful for hepatic fibrosis inhibition. The delivery of artificial microRNA in a fibrotic mouse model induced by dimethyl nitrosamine resulted in a reduction of fibrotic marker collagen as well as α-smooth muscle actin (alpha-SMA) by targeting CTGF in their study (Yang et al., 2013). Peroxisome proliferator-activated receptor-gamma (PPAR-γ) ligand-loaded mannose-6-phosphate (M6P)–human serum albumin (HSA)-conjugated liposomes have been used successfully to target Hepatic stellate cells, thus commenced a new trend for the treatment of hepatic fibrosis. A size of 130 nm nanoparticles demonstrated M6P receptor targeting and thus minimized the liver fibrosis symptoms both in vitro and in vivo in a Ccl4-induced fibrosis mouse model (Zhang, Kong, Lu, & Zheng, 2013).

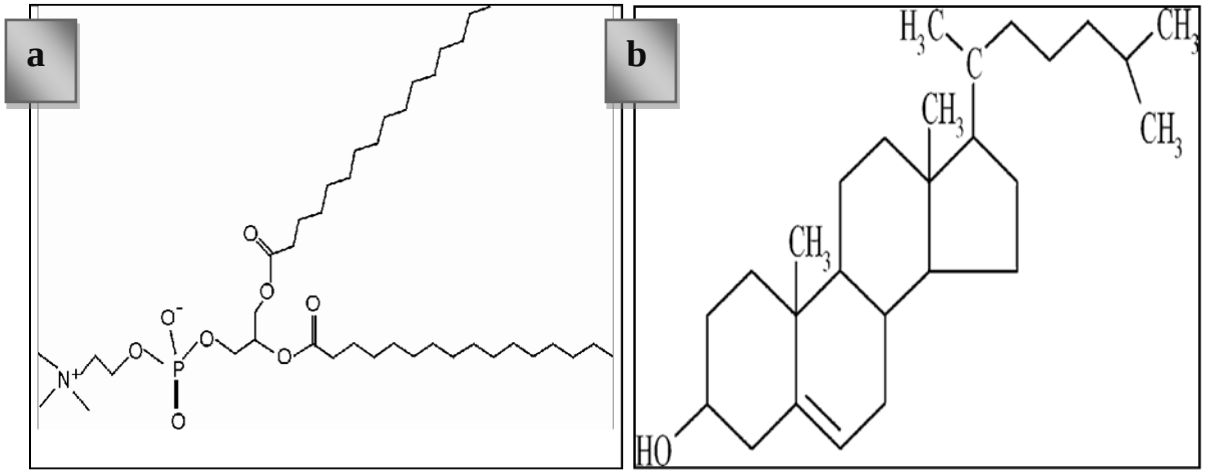


Figure 2.3: (a) Structure of DPPC (b) Structure of Cholesterol

2.4 Synthesis of Liposome Nanoparticles

All methods for preparing the liposomes include stages such as drying down lipids from organic solvent, dispersing the lipid in aqueous media, purifying the resultant liposome, and analyzing the final product. Different types of lipids that possess different properties like size, surface charge, biocompatibility, drug release kinetics and cell targeting, are used to formulate liposomes (Anderson & Omri, 2004; Tang et al., 2018).

Liposomes can be manufactured using several methods. These methods used to manufacture the liposomes influence their size and lamellarity (Dimov, Kastner, Hussain, Perrie, & Szita, 2017; Maeki, Kimura, Sato, Harashima, & Tokeshi, 2018; Pattni, Chupin, & Torchilin, 2015).

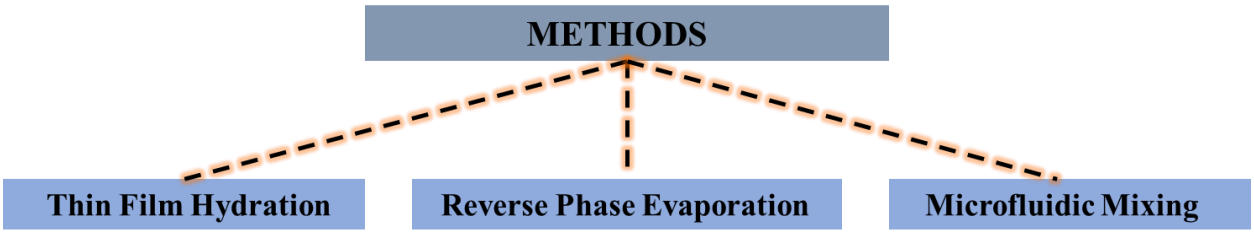


Figure 2.4: Different methods used for the synthesis of liposomes Nanoparticles

2.4.1 Drug loading

Two types of technique are used for drug loading

a) Passive loading

Passive loading specifies the process, in which the formation of liposomes and drug loading take place simultaneously. Hydrophilic molecules are homogeneously dispersed in the aqueous phase (both within and outside the liposomes), while hydrophobic drugs are maintained within the liposome bilayer, respectively. In particular, the drug and lipids are initially dissolved in appropriate solvent and then interact these with water, accompanied by the evaporation of solvent, thus obtaining a thin film, which is then hydrated to obtain liposomes. The lipid layer is spread in a drug-contained aqueous environment, when loading water-soluble drugs. Because of certain factors including lipid concentration, vesical size, drug solubility, and method of preparation, the trapping efficiency of passive loading differs. By passive loading technique, the average drug to lipid ratio (D/L) attained is <0.05 (w/w) in most of the cases (Gubernator, 2011; Zhao, May, Chen, Undzys, & Li, 2015).

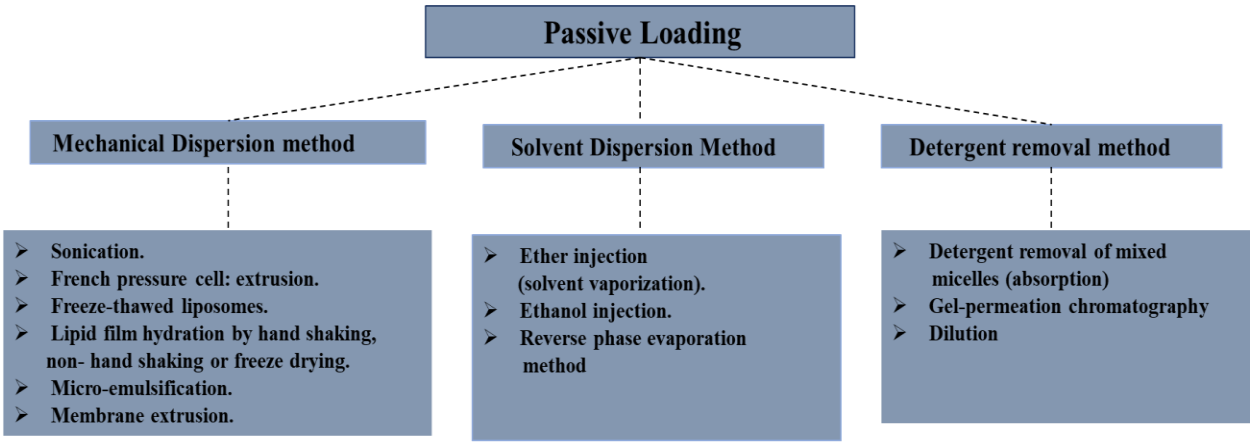


Figure 2.41: Different types of methods used in Passive loading

b) Active Loading

First, liposomes which contain a transmembrane gradient are created in active loading (the aqueous phase outside and inside the liposomes are different). After that, an amphipathic drug dissolved in the external aqueous phase can penetrate through the phospholipid bilayer(s), following the interactions with a trapping agent in the center to trap the drug in. Active loading remains an effective method that can be used to hold drugs efficiently and stably in the liposomes' core (Bhatt et al., 2018).

2.5 Stealth Liposomes Nanoparticles

Although liposomes are similar to bio membranes, but these are still foreign antigen 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 polyethylene glycol (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 these liposomes to leak out of circulation through endothelium.

Stealth liposomes' 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 to transport drugs or genetic material to targeted cells. New drug delivery for controlled release is developed by stealthening the liposomes. This stealth concept has been used to improve the popular doxorubicin-loaded liposomes, that 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).

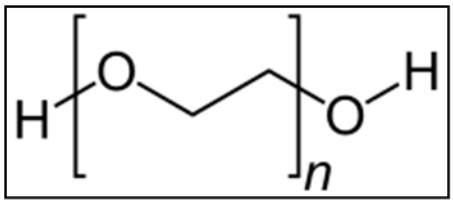


Figure 2.5: Structure of Polyethylene glycol (PEG)

2.6 Induction of Liver Cirrhosis by CCL4

The key methods for inducing liver cirrhosis in rats are the administration of CCL4 and bile duct ligation (BDL). Nowadays, CCL4 is commonly used, but is known as extremely toxic process. It damages hepatic tissues and linked with reactive free radical metabolites, metabolic activation, lipid peroxidation, disturbed calcium homeostasis, and covalent bonding. The administration of CCL4 results in inflammation, necrosis, and fibrosis, that spreads to connect the vascular structure that feed into and drain 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).

CCL4 induces toxicity in three or four distinct phases. Predominantly, first two or three weeks are described by 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, Trebicka, Liedtke, & Weiskirchen, 2015).

2.6.1 Mechanism behind the liver damage

CCL4 is metabolized to trichloromethyl radical (CCl3) in liver, by cytochrome P450 monooxygenases superfamily (CYP family). These radicals subsequently interacts with the nucleic acids, lipids, and proteins, thus damaging the major cellular functionalities resulting in lipid metabolism alteration i.e. fatty degeneration and causing steatosis and lowering the proteins level. Adduct formation between DNA and ccl3 additionally causes mutation. The oxygenation of ccl3 further initiates the lipid peroxidation and degradation of polyunsaturated fatty acid by the formation of trichloromethyl per oxy radicals (CCl3OO *). As a result, the permeability of membrane is lowered in the cellular organelles like mitochondria, endoplasmic reticulum, and plasma membrane, that causes the extensive hepatic damage that is eventually marked by inflammation, fibrosis, and cirrhosis and Hepatocellular carcinoma (HCC) (Weber, Boll, & Stampfl, 2003).

CHAPTER:3 MATERIAL AND METHODS

3.1 Experiment Design

3.1.1 Materials

Dipalmitoyl phosphatidyl choline (DPPC), Cholesterol, commercially available Vitexin drug, Polyethylene glycol (PEG- Molecular weight 6000) were purchased from Sigma-Aldrich USA. Urethane and carbon tetrachloride (Ccl4) were purchased from Strem chemicals. Sprague Dawley female rats were purchased from ASAB (Atta-ur-Rahman school of Applied Biosciences), National University of science & technology (NUST), Islamabad. Deionized water was used throughout the study.

3.1.2 Synthesis of vitexin loaded-liposomes Nanoparticles

For the synthesis, liposomes constituents i.e. DPPC and Cholesterol were used in 4:1 (percent molar ratio). At first lipid were weighed and dissolved in ethanol to form 100 μMolar solution. 200 μMolar solution of Vitexin drug was prepared in ethanol from which 500 μL drug solution was taken and mixed in lipid solution. Mixture was sonicated (at 80 MHz) for 40 minutes. Then 10mL water and lipid phase were allowed to be warmed in water bath individually until the temperature reached to 60°C. Lipid phase was mixed with water phase and this dispersion mixture was constantly mixed for ten minutes at 90 RPM. This new mixture was again sonicated (for 40 mins at 50 MHz) and then allowed to rotary evaporation (above the phase transition temperature i.e. 50 °C) to get rid of ethanol. Finally, unentrapped drug was removed via Minicolumn filtration (0.2μm size) by centrifugation (at 4500 RPM) for 1hr (Chorachoo, Amnuaikit, & Voravuthikunchai, 2013; Meng et al., 2016).

3.1.3 Pegylation of vitexin loaded- LNPs

The mixture of vitexin loaded- LNPs was made diluted up to 50ml, then 0.25% PEG was added drop wise, upon continues stirring and then allowed to rotary evaporation until 10ml solution was left behind. Unentrapped drug was removed via Minicolumn filtration by centrifugation (at 4500 RPM) for 1hr (Stiufiuc et al., 2013).

3.2 Physical Characterization

Characterization of PEG nanoparticles was done to evaluate and analyze their particle size, shape and surface charge, drug encapsulation, release efficiency and dispersity Index.

3.2.1 U.V-Vis Absorption Spectroscopy

UV-Vis spectroscopy is a technique mostly used in chemical and clinical laboratories. It measures the extent of absorption in the sample , when light beams pass through it and the absorption is measured from reflected beam. A light beam is split where one half of the beam is focused through the cuvette containing the measuring sample and the other half is guided to a cuvette containing only the solvent as control. Absorption can be measured at a given wavelength and a target range, and a spectrum is obtained that maps entire wavelength range versus its absorption at particular wavelengths. Maximum absorption is called as lambda max at specific wavelength. It analyses the electronic molecular transformation and obeys the Beer Lambert Law theory. The sample absorbance is proportional to the molar concentration in the sample cuvette, and the absorption value known as molar absorptivity is used when comparing different compound spectra. Beer-Lambert Law says

$$A=EcL$$

Molar absorptivity E= A/ cl (where A= absorbance, c= sample concentration in moles/ liter and L= length of light path through the cuvette in cm). This law enables UV-VIS spectroscopy as a useful tool for quantitative analysis. (Amendola & Meneghetti, 2009; Perkampus, 2013; Tomaszewska et al., 2013)

U.V-Vis spectra of vitexin loaded – LNPs and Pegylated vitexin loaded LNPs were measured by using Shimatzu UV-Vis 2800 BMS Scientific Technical Corporation (PVT) spectrophotometer, from 200-450nm at a resolution of 1nm. The de-ionized water was used as a reference for UV analysis. The UV spectra of Vitexin drug, LNPs loaded with and without drug, and PEG-Coated drug loaded LNPs were recorded.

3.2.2 Fourier transform infrared spectroscopy (FTIR) analysis

Fourier transforming infrared spectroscopy (FTIR) is an analytical technique used for the identification of organic and some inorganic materials. This technique measures the sample material by absorbing infrared radiation versus wavelength. Molecular components and structures are defined by the infrared absorption bands. When an infrared radiation irradiates a substance, the absorbed IR radiation normally excites molecules into a higher vibrational state. The wavelength of light that absorbed by single molecule is a function of the difference in energy between the excited vibrational and resting states. Wavelengths absorbed by the sample are the characteristic of its molecular structure (Khan, Saeed, & Khan, 2019; Mohamed, Jaafar, Ismail, Othman, & Rahman, 2017).

For FTIR analysis, samples were allowed to air dry and then prepared using compressed KBr discs. FTIR spectra were recorded between 4000-350 cm-1 using Bruker FTIR Spectrophotometer ALPHA II. FTIR analysis of all formulating constituents was done including DDPC, Cholesterol, PEG-6000, Blank liposomes, vitexin drug along with Liposomes nanoparticles and Pegylated-Liposomes nanoparticles.

3.2.3 Particle size and Area Distribution

To analyze the practical size, scanning electron microscopy (SEM) was used. The nanoparticles area distribution is calculated using 'image j software.' Analysis is performed on a chosen field. The 'Analyze Particles' command counts and measures objects in binary and/or threshold images. It works by scanning the image or range, until the edge of an item is identified. For particle size, values are given between 0 and 'Infinity' range. Particles with circularity values outside the specified range in this area will also be ignored. Analyzed the 8–bit binary image containing the best fit ellipse (cf. Edit. Range. Fit Ellipse) of measured particle(gray levels: Ellipses: 0; background: 255) (Goldstein et al., 2017).

Both types of nanoparticles were imaged by pouring small fraction of sample on cover slip by Micropipette. Slide surface was then coated with gold in sputter coater for 50 seconds at mA. Images were taken by using VEGA3 LMU Scanning Electron Microscope at National University

of Science and technology, Islamabad. Size distribution and dispersity of both type of NPs were evaluated by Dynamic Light Scattering (DLS) using Malvern Zeta Sizer Ver. 7.12

3.2.4 Zeta Potential

The zeta potential is the potential difference between solids and liquids, across phase boundaries. It is a measure of the particle's electrical charge which is suspended in liquid. Because zeta potential is not equal to the electrical surface potential of a double layer or to the Stern potential, it is often the only value that can be used to characterize the colloidal dispersion's double-layer properties. Zeta potential is expressed in millivolts (mV) and is also known as electro kinetic potential. Zeta potential analyzer was known to have surface charge and zeta potential. Zeta potential tells about the nanoparticles' stability, surface charge and average size. Zeta potential in colloids is the difference of the electrical potential through the ionic layer around a charged colloid ion. Put another way; it is the potential at the slipping plane in the double layer interface. The higher the zeta-potential, the more stable the colloid will usually be. Zeta potentials which are less negative than -15 mV typically represent the beginnings of particle agglomeration. The colloid will precipitate into a solid if zeta-potential equals zero (Glawdel & Ren, 2008).

The zeta potential (surface charge) of both type of LNPs was evaluated by Dynamic Light Scattering (DLS) using Malvern Zeta Sizer Ver. 7.12

3.2.5 Drug Encapsulation and Release Efficiency

Drug efficiency delineates the amount of drug to be entrapped with in the vesical of liposomes. To find drug encapsulation efficiency, different dilutions of the drug were made and analyzed by UV spectrophotometer at 330nm absorbance, to obtain the possible linear standard curve. An equation Y= mx+c was obtained. This standard curve value was further used in calculations to find out the unentrapped drug. Samples were centrifuged at 4500 rpm for 1hr and supernatants were analyzed under UV Vis spectrometry to find unentrapped drug fraction. (Nii & Ishii, 2005) After that, calculated values were used in given formula.

$$Encapsulation\ Efficiency = \frac{Total\ drug - Unentrapped\ drug}{Total\ drug} \times 100$$

3.2.6 Drug Release

The release behavior of drug from nanoparticles vector 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 LNPS and PEG-LNPs were examined up to 48 hours along with the addition of specified volume of phosphate buffer saline. From both 25 ml solutions of LNPs and PEG-LNPs, 3ml samples were placed into separate 15 ml centrifuge tube and allowed it for centrifugation for 10 mins at 4500 Rpm and 25ºc. while on other hand 3ml of Phosphate buffer saline was added to LNPs and PEG-LNPs solutions. Following centrifugation, supernatant was allowed for UV spectrophotometer analysis. Same procedure was followed after 1,2,4,6,12,24 and 48hr. At 330nm of wavelength, absorbance values were taken and used as cumulative drug release. Entire analysis was carried out by using empty nanoparticle solution as control.

3.3 Development of Liver Cirrhosis Model:

3.3.1 Animals

60 Sprague-Dawley female rats having weight of 85-105g and age of 4-6 weeks were used. The rats were kept under 12-hour light and dark cycle in separate cages with the access of water and food. Temperature was set around 27°C with 60-70% humidity. Chloroform was used to anesthetize the rats to follow histological examination. Handling and caring of rats were centered upon the regulation of good laboratory practice 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), peanut oil (a

delivery agent), Ethanol (de-contaminant) and 10% neutral formaldehyde buffer (dissolved in PBS).

3.3.3 Liver Cirrhosis Induction

Initially, the rats were set free for one week. A total of 60 rats were divided within 2 groups. 5 rats considered as positive control, without exposing them to any adverse reaction in entire procedure while 55 rats were exposed to detrimental chemicals via intraperitoneal injections. Initially, 2.5% Urethane was dissolved in DMSO (Dimethyl sulfoxide) and for the first two weeks, 1ml/kg dose of Urethane was injected intra-Peritoneally twice a week. The exposure with urethane is intended in order to cause great liver damage resulting in the reduction of induction time frame of cirrhosis. Then, Ccl4 was mixed with pure Peanut oil (50% v/v) and their intraperitoneal injections, having the dose of 1ml/kg were given twice a week for the rest of four weeks (Fortea et al., 2018; Gitiara et al., 2017).

3.3.4 Outcomes

Different conditions like; food and water consumption, body weight, liver weight and ascites were taken in consideration**.**

3.3.5 Serological Indices

Blood was extracted from heart for Serological liver function tests like, AST (Aspartate transaminase or aspartate aminotransferase test), ALP (Alkaline Phosphatase), ALT (Alanine transaminase) and T.B (Total Bilirubin) according to manufacturer's guidelines.

3.3.6 Histological Examination

After each week, one or two rats were sacrificed and organs like Liver, kidney and spleen were harvested. The size, shape and color of each diseased liver tissue were noted. Organs were obtained and placed immediately in 10% neutral-balanced formalin solution to avoid postmortem autolysis and decomposition. 5 µm serial sections of organs were taken, followed by the paraffin imbedding. Organs were stained with Hematoxylin and Eosin (HE) to observe the structural changes 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 (Tajima et al., 2013), but some amendments were made according to variation in our histological observation (Figure:3.36). Amendments were related to 'Piecemeal Necrosis' that include; score = 1,2,3,4 for Necro inflammation at Mild (few portal areas), Mild/moderate (most portal areas), Moderate (continuous around <50% of tracts or septa), Severe (continuous around >50% of tracts or septa) respectively (Knodell et al., 1981). Scoring was done by the METAVIR scoring system, which was developed in France in 1993, and has been adapted for histological staging of liver disease in most etiologies of chronic liver disease (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

In order to evaluate the anti-fibrotic/anti-cirrhotic effects of LNPs and Peg-LNPs, diseased rats were taken within experiment. Rats were categorized into different groups.

3.4.1 Negative Control Group

A set of eight diseased rats were isolated and assigned the tag as negative control. This group of rats were left untreated throughout the experiment, and the survived rats were sacrificed at the end of experiment for histopathological and serological analysis. Body and liver weight and ascites were noted.

3.4.2 Vitexin treated Intravenous (I.V) Group

Five rats were placed in this group. Vitexin drug at the dose of 10mg/kg were given intravenously for the duration of 15 days. Body and liver weight and ascites were noted at the end of experiment prior to dissection for histopathological and serological analysis.

3.4.3 Vitexin treated Oral Gavage (O.G) Group

Five rats were placed in this group. Vitexin drug at the dose of 10mg/kg were given via oral gavage for the duration of 15 days. Body and liver weight and ascites were noted at the end of experiment prior to dissection for histopathological and serological analysis.

3.4.4 Liposome Nanoparticles treated Intravenous (I.V) Group

Five rats were placed in this group. Liposomes Nanoparticles Dose at the dose of 500µg/kg were given intravenously for the duration of 15 days. Body and liver weight and ascites were noted at the end of experiment prior to dissection for histopathological and serological analysis.

3.4.5 Peg- Liposome Nanoparticles treated Oral Gavage (O.G) Group

Five rats were placed in this group. Liposomes Nanoparticles Dose at the dose of 500µg/kg were given intravenously for the duration of 15 days. Body and liver weight and ascites were noted at the end of experiment prior to dissection for histopathological and serological analysis.

CHAPTER 4: RESULTS

4.1 Physical Characterization of vitexin loaded – LNPs and Pegylated LNPs

Successful synthesis of both Pegylated and non-pegylated vitexin loaded liposomes nanoparticles, were justified by physical characterization.

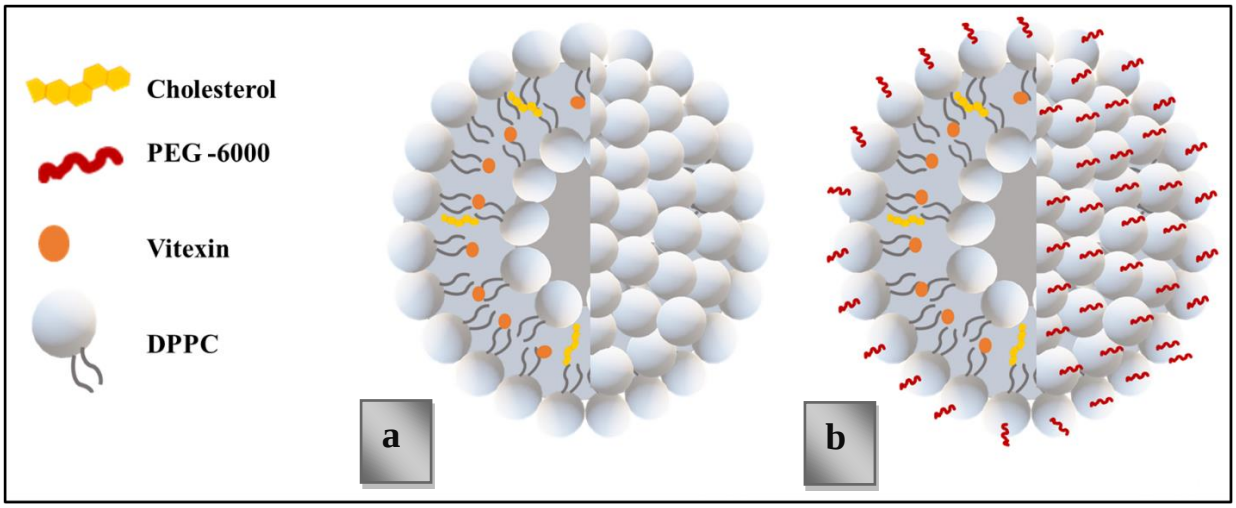


Figure 4.1: Pictorial depiction of Synthesized (a) LNPs (b) Peg coated LNPs

4.1.1 UV-VIS absorption spectroscopy

UV-VIS absorption spectroscopy of vitexin drug showed the surface plasmon resonance (SPR) peak mainly at 330nm, blank liposomes at 220nm & 239nm, Vitexin loaded-LNPs at 225nm & 340nm while pegylated vitexin loaded-LNPs showed absorption peaks at 270nm. The shift in the peaks delineate the sucessful conjugation of Vitexin, LNPs and PEG with each other.

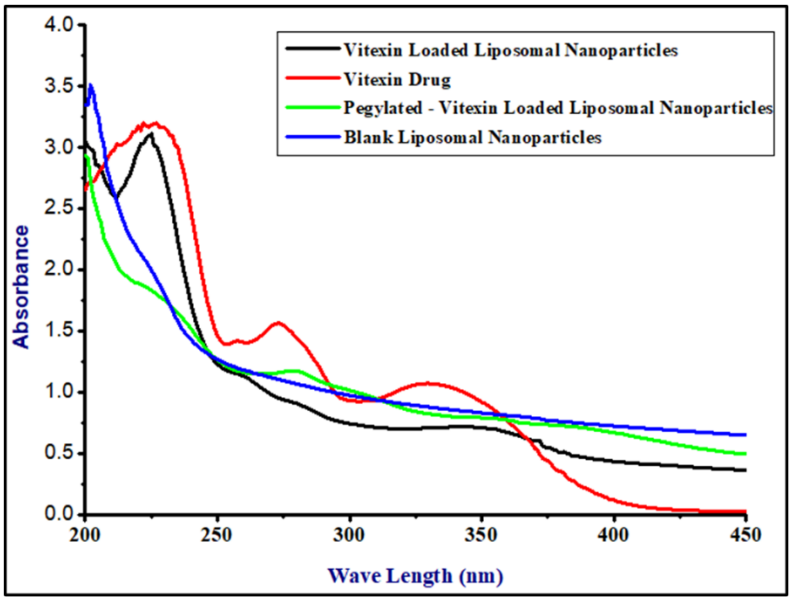


Figure 4.11: Comparative UV-VIS spectra of Vitexin, Blank LNPs, Vitexin-loaded LNPs, Pegylated- LNPs

4.1.2 Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectrum of Cholesterol indicated peaks or bands at 2850/cm (CH stretch, Alkanes), 873/cm (Tri-substituted Aromatics). DPPC spectrum indicated peaks at 2919/cm (CH stretch, Alkanes), 1632/cm (R-NH2, Amines), 1115/cm (C-O stretch, Ether), 720/cm (RCH2CH3, Bending mode). PEG-6000 spectrum delineated peaks at 3429/cm (O-H stretch, Alcohol), 2923/cm (CH stretch, Alkanes) and 1638/cm (C=C stretch, Alkenes). Vitexin drug spectrum exhibited peaks at 3391/cm (Ar O-H bonded), 1651/cm (C=C stretch, ketone), 1610/cm (C=C stretch, Alkene) and 1403/cm (Ar C-C, Aromatics). In distinction, the spectrum of Blank liposomes and Peg-liposomes depicted the disappearance of cholesterol's 2850/cm (CH stretch, Alkanes) and 1632/cm (R-NH2, Amines) of DPPC. Peg-liposomes spectrum illustrated the disappearance of DPPC's 1115/cm (C-O stretch, Ether). Vitexin's 1651/cm (C=C stretch, ketone) and 1403/cm (Ar C-C, Aromatics) has been disappeared in LNPs and PEG-LNPs spectra while DPPC'S 2919/cm (CH stretch, Alkanes) peak has been broaden. The observed changes in infrared bands proven the conformational changes in lipid biomolecules' by incorporating with vitexin drug and PEG 6000.

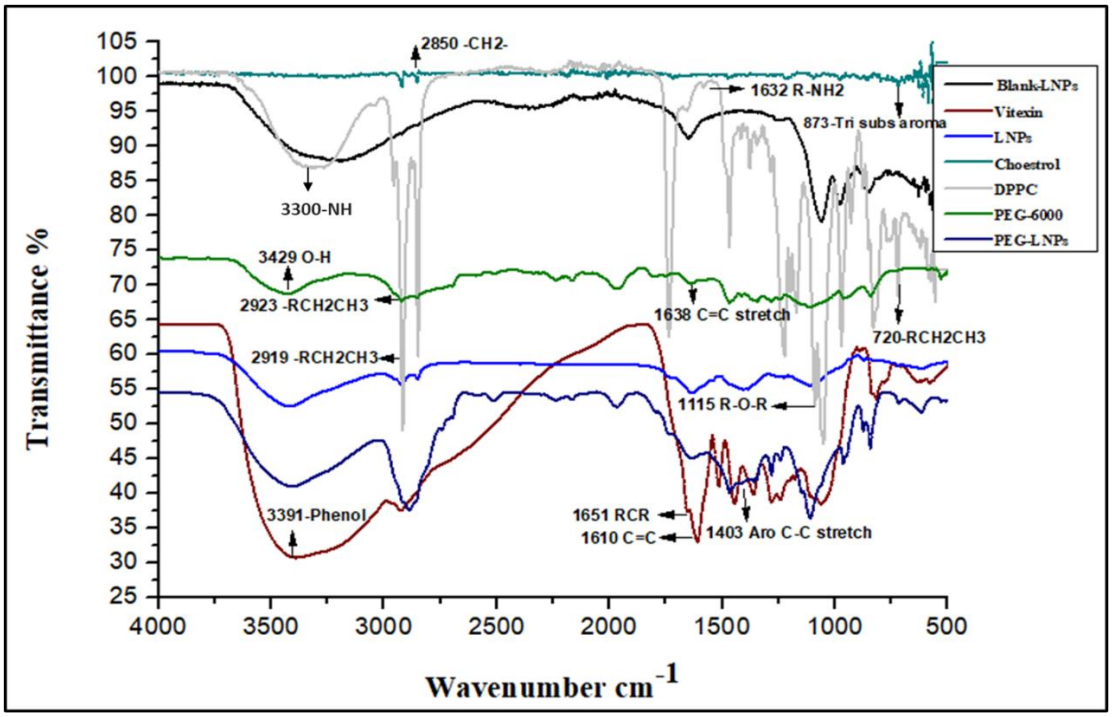


Figure 4.12: Comparative FTIR spectra of DPPC, Cholesterol, PEG-6000, Vitexin, Blank LNPs, Vitexin-loaded LNPs, Pegylated- LNPs

4.1.3 Particle size and Area Distribution

The vitexin loaded- LNPs' size is defined by the scanning electron microscopy and the area distribution of the nanoparticles is measured using image j software. Scanning image depicted the sphere shape nanoparticles with the average size of 155nm.

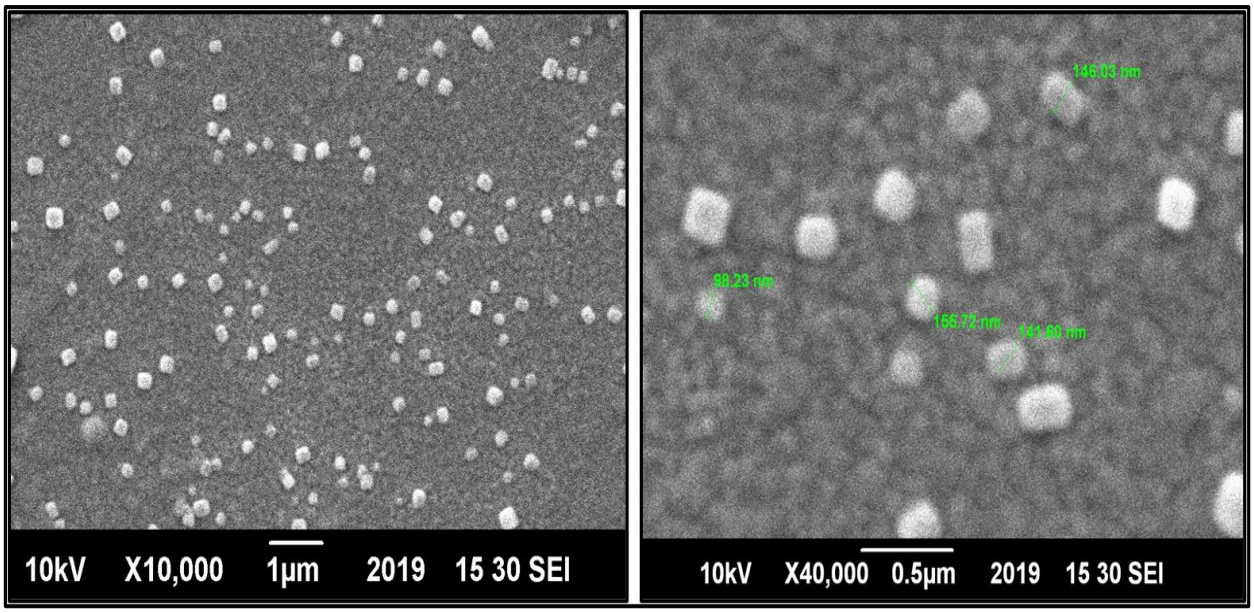


Figure 4.13a: SEM Image of Vitexin-loaded Liposomes Nanoparticles

The mean size of Vitexin, Blank Liposomes, LNPs and Peg – LNPs measured by Zeta sizer, were 64.9nm, 128nm, 168nm and 458nm respectively, measured by Zeta sizer. There was a significant increase in nanoparticle size while using PEG-6000.

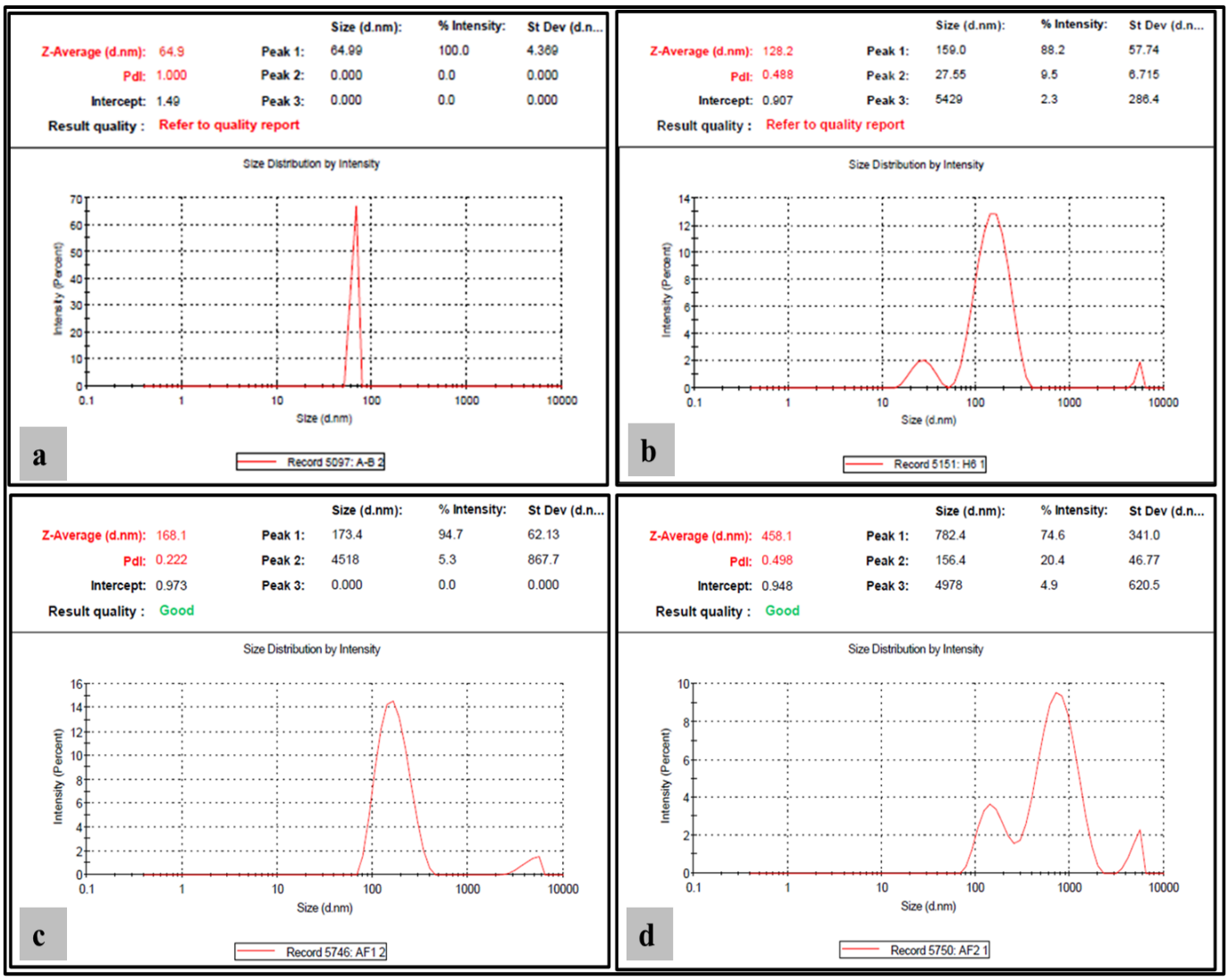


Figure 4.13 b: Size analysis by zeta sizer (a) Vitexin drug (b) Blank Liposomes (c) LNPs (d) Peg-LNPs

4.1.4 Zeta Potential & Poly Dispersity Index

The average Zeta potential of Vitexin, Blank Liposomes, LNPs and LNPs were -2.21 mV, -5.73 mV, -9.59 mV and -13.3 mV respectively with Polydispersity index (PDI) of 1.0, 0.488, 0.24 and 0.498 respectively. By using PEG-6000, there was a notable increase in zeta potential of LNPs, suggesting the enhanced stability than conventional LNPs. Both type of particles when observed under the Scanning Electron Microscope, seemed mainly spherical in shape with too little difference in size and shape between them.

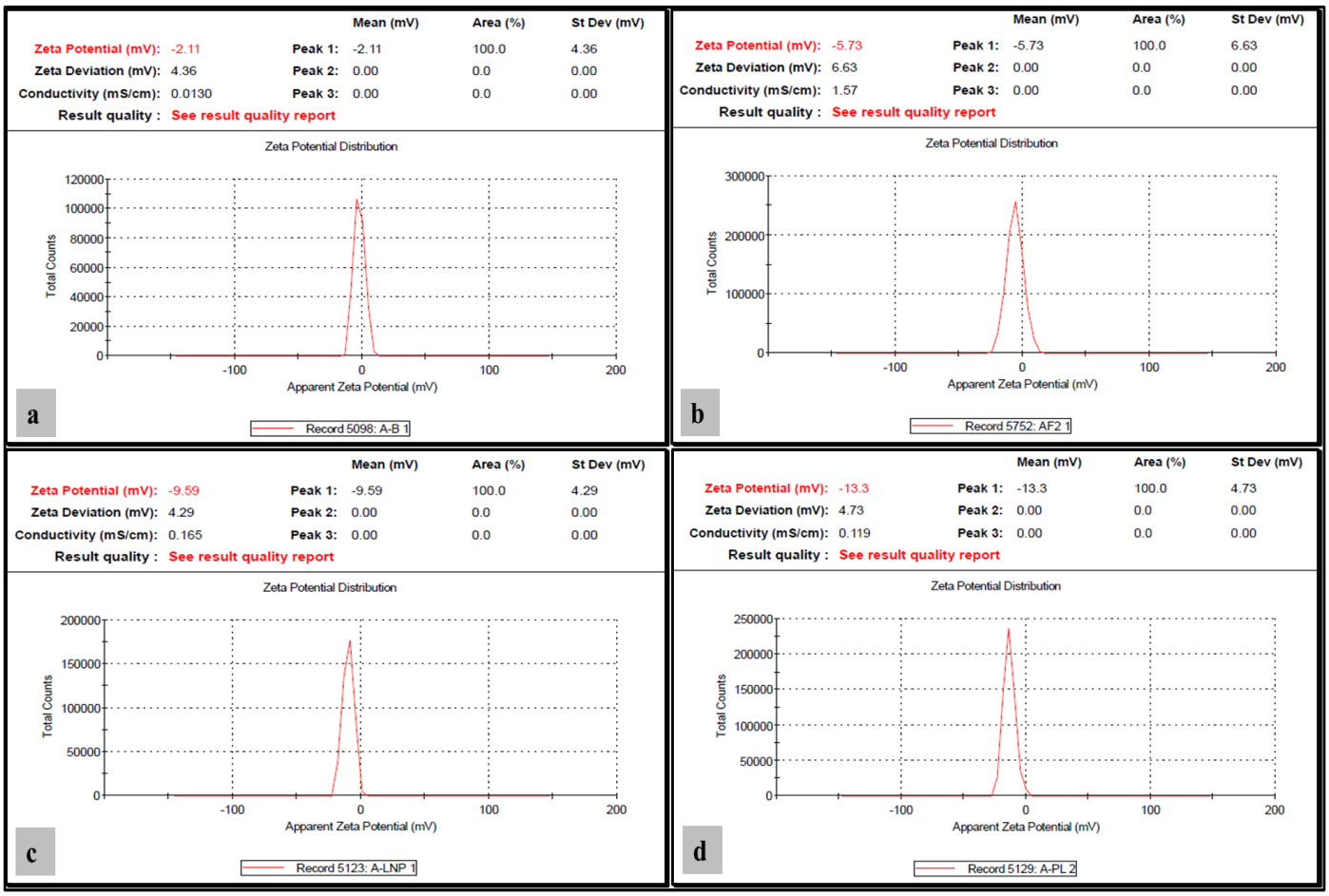


Figure 4.14: Zeta potential of (a) Vitexin drug (b) Blank Liposomes (c) LNPs (d) Peg-LNPs

4.1.5 Drug Encapsulation Efficiency

By calculating the aforementioned formula (in material Section), the Encapsulation Efficiency found to be 80%, that delineates the 80% entrapment of drug within Nanoparticles.

Calculation of unknown concentration of drug with the help of standard curve

$$\mathbf{Y = 10.017\ x - 0.3769}$$

UV analysis of 1st supernatant from mini column centrifuge tube, gave the absorption at 335 nm for vitexin.

Abs (330 nm)= **0.10689**

Y= Absorption value at specific point

X= Concentration of unknown

By putting value of abs **(330 nm)**, the concentration of drug was calculated which is **0.04829 mM**.

Amount of non entrapped drug = **0.02 mg/ml**

Total drug was **0.1 mg/ml** solution of drug

EE% = (Total drug – Non-Entraped)/total drug ×100

= 80%

4.1.6 Drug Release Kinetics

The release of drug from LNPs and PEG-LNPs were 72 % and 42 %, noted up to 48 hours suggesting the sustain released of drug with time from PEG-LNPs. Experiment demonstrated that the vitexin drug releases faster from LNPs as compared to Pegylated-LNPs which accordingly further improved the release profile of drug and increase its half-life. This long stay of drug results in attaining the increased bioavailability and ultimately leads to high efficacy in treating the diseases.

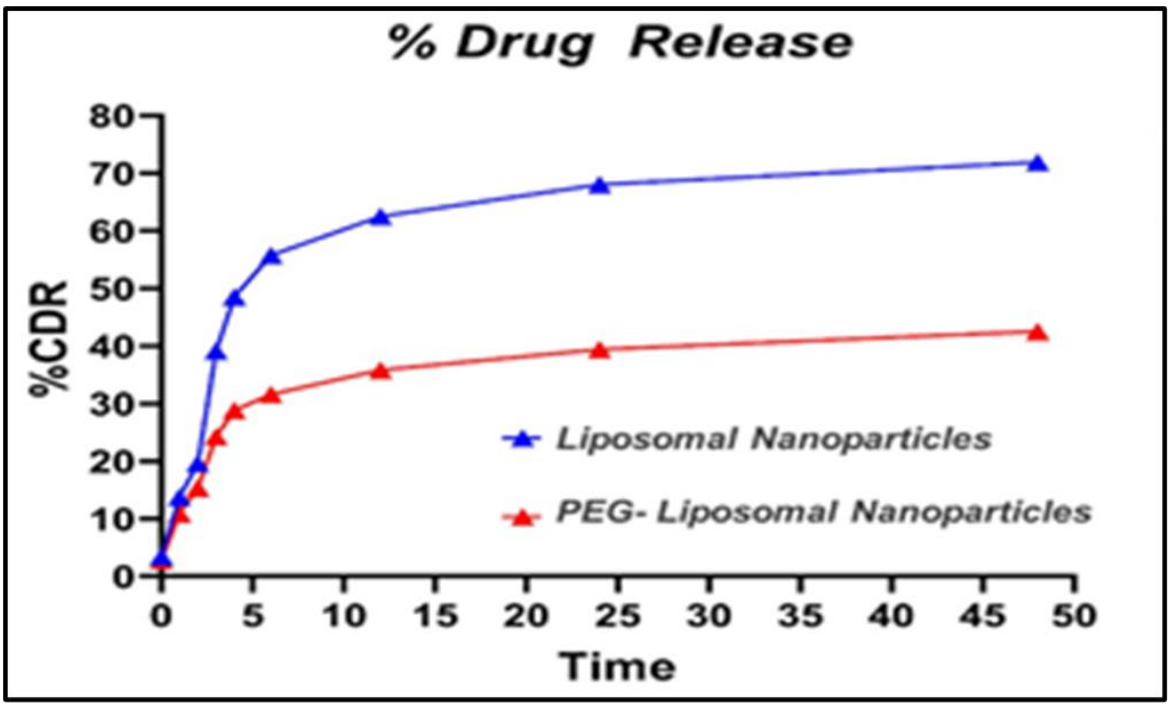


Figure 4.16: Comparative Drug release graph of LNPs and Pegylated-LNPs

4.2 Induction Of Liver Cirrhosis

4.2.1 Effects on Liver

Liver Per Week	Score
0	0/17
1st	3/17
2nd	7/17
3rd	8 /17
4th	9/17
5th	13/17
6th	11/17

Table 4.21: NASH/NAFLD Clinical Research Network Scoring System during induction.

4.2.2 Hepatic Histopathology

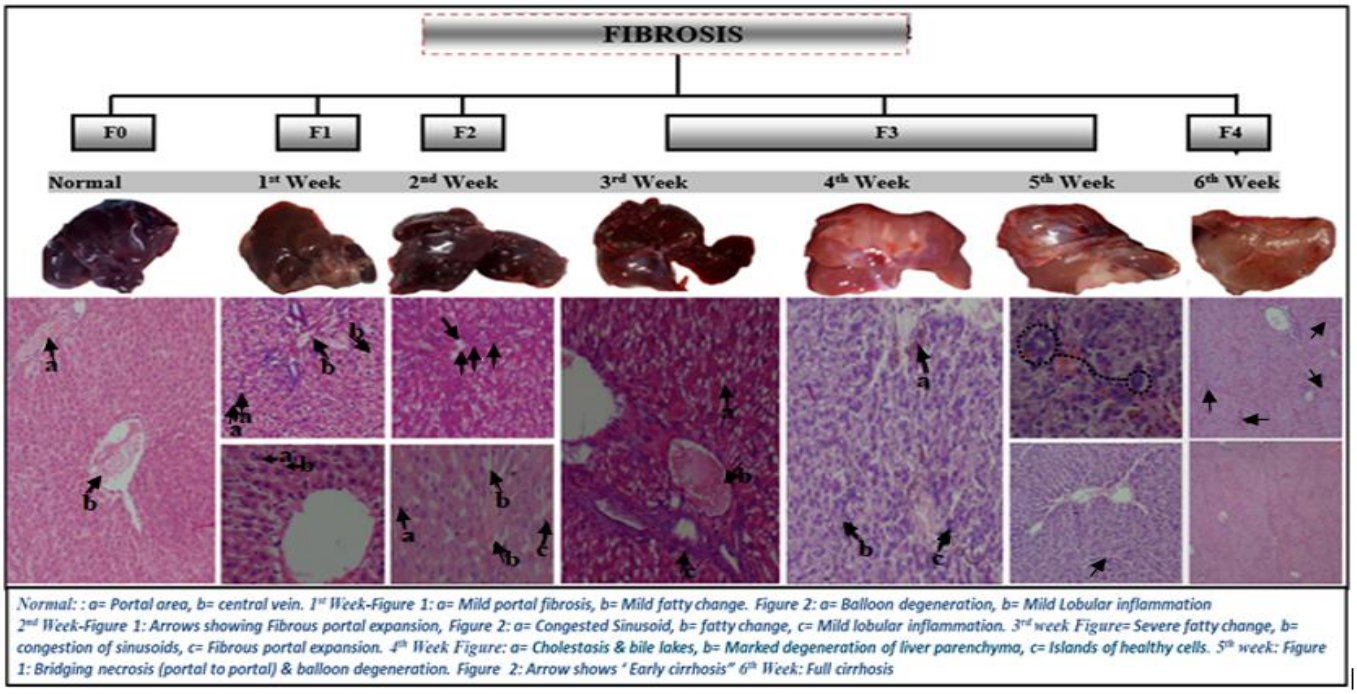


Figure 4.22: Metavir staging/classification for Fibrosis and per week histopathology of Liver during cirrhosis Induction

4.2.3 Renal Histopathology

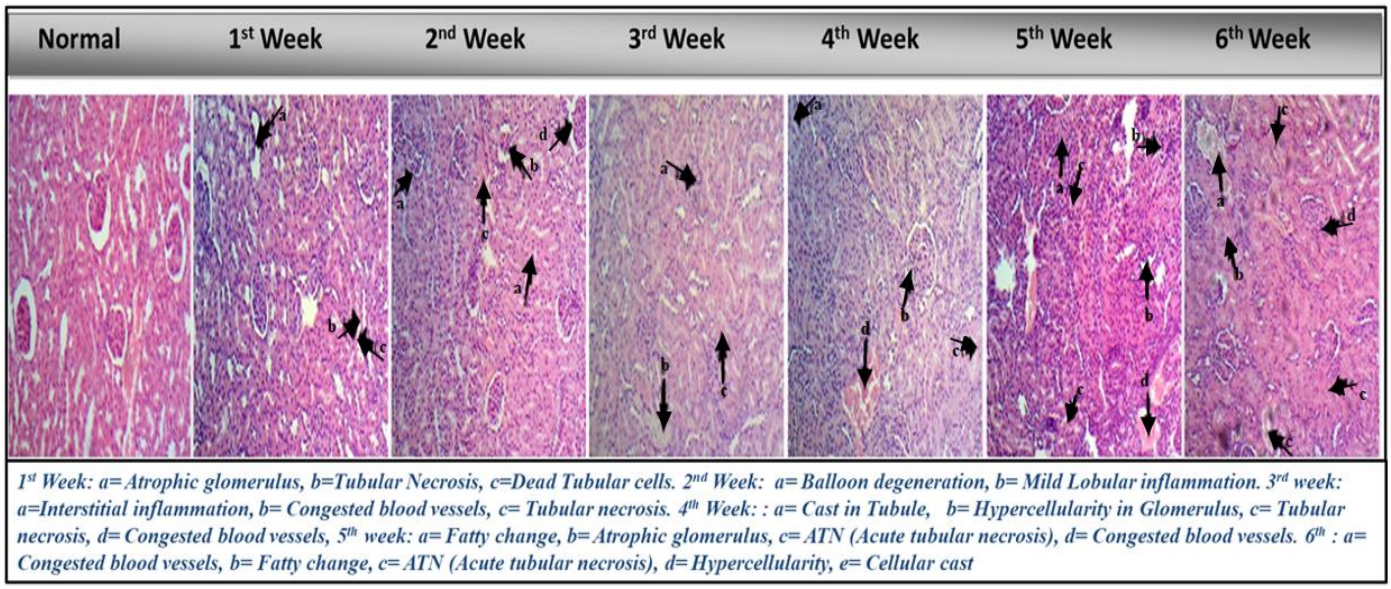


Figure 4.23: Per week histopathology of Kidney during cirrhosis induction

4.2.4 Spleen Histopathology

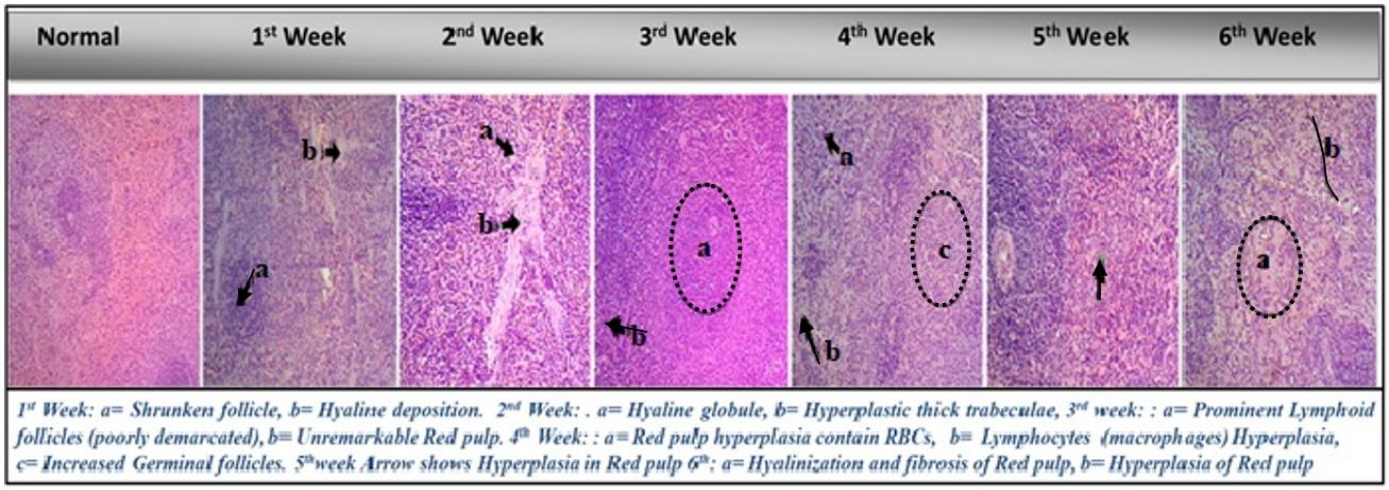


Figure 4.24: Per week histopathology of Spleen during cirrhosis induction

In the 1st week of induction, histological changes in kidney showed Tubular necrosis in 25% of tubules, Glomeruli showed mild Mesangial proliferation, Blood vessels were unremarkable, <20% of atrophic glomeruli, Interstitium was normal while Medullary tubules appeared normal. In spleen, Hyperplasia of white pulp, Germinal center increased in numbers and Red pulp showed mild hyperplasia were seen. In 2nd week, architectural changes include; Tubular necrosis in 30% of tubules, 20% of Glomeruli were atrophic, Blood vessels were unremarkable, Rare cellular casts and Medullary interstitium showed congestion while Germinal follicles were increased in numbers & germinal center was prominent and Red pulp showed congestion were seen in spleen. In 3rd week, kidney Glomeruli showed hypercellularity, 40% of cortical tubules showed necrosis, Medullary tubule were in normal limit and Blood vessels were congested and in spleen, Follicles were poorly demarcated, Follicle center was not prominent and Red pulp is unremarkable. In 4th week, Focal tubular necrosis, Focal Hyaline cast, few glomeruli showed necrosis (Hypocellularity) and Blood vessels were congested. In spleen, Follicles were normal in appearance but were ill defined, 50% of germinal centers were not prominent, Blood showed sub-epithelial Hyaline deposition, and deposition of pinkish material in blood vessels were seen. In 5th week, 20% of Glomeruli were shrunken, 20% Tubule showed necrosis & Hyaline casts, Cortical cells showed degeneration, Blood vessels were congested and Mild fatty change were seen in kidney while follicles were normal in number but germinal centers were not prominent and Red pulp showed shrinkage in spleen. 6th week of induction in

kidney, Glomeruli were atrophic with hypocellularity as well as some showed hyper cellularity, Tubular necrosis with few casts, Blood vessels were congested and Moderate fatty change were seen while in spleen, Follicles were decreased in number, Germinal centers were not prominent and Fibrosis of Red pulp were seen.

4.3.1 Serological Analysis (LFTs)

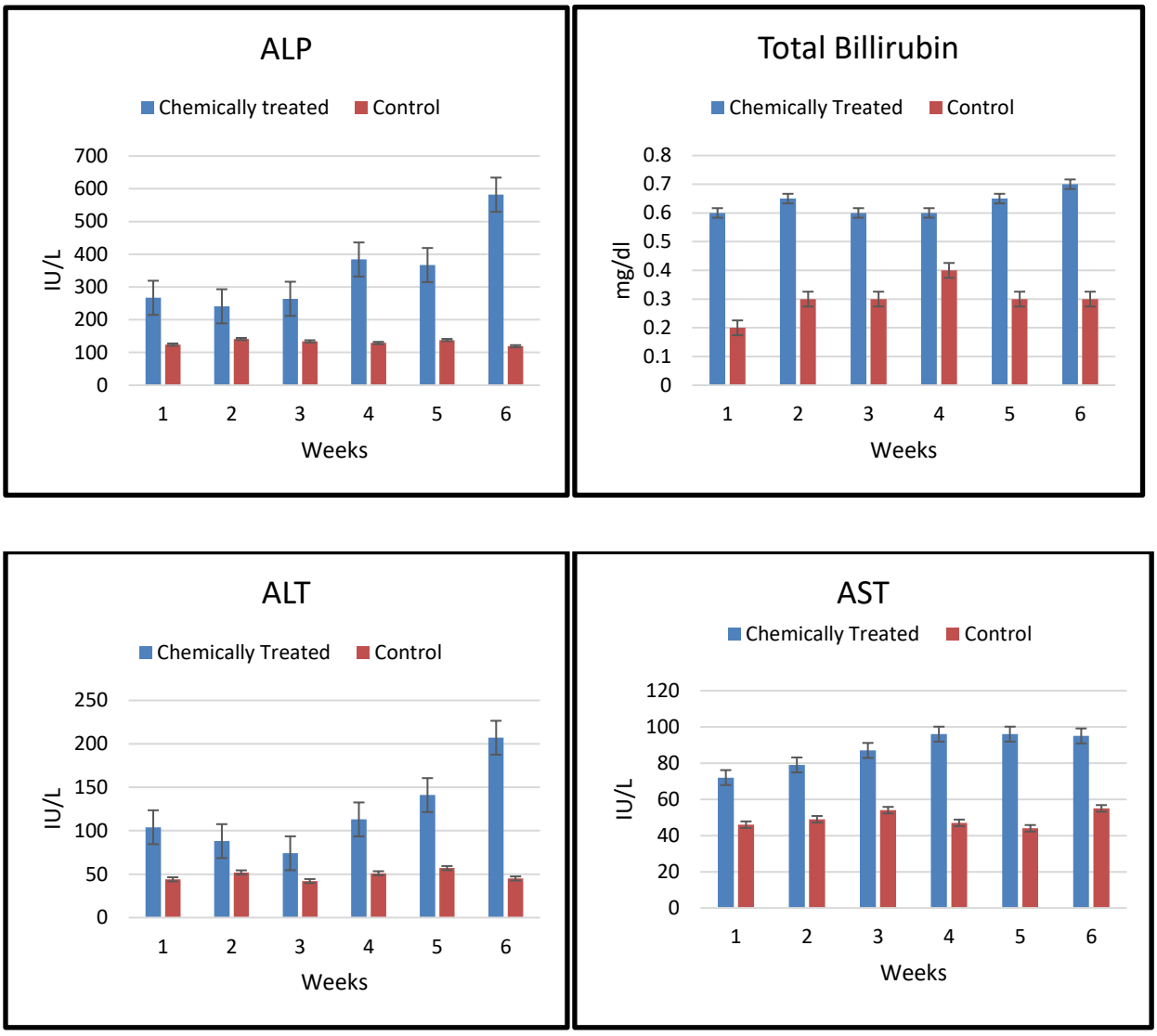


Figure 4.31: Serological indices during cirrhosis induction (a) ALP (b) T.B (c) ALT (d) AST

4.3.2 Body & Liver Weight and Ascites

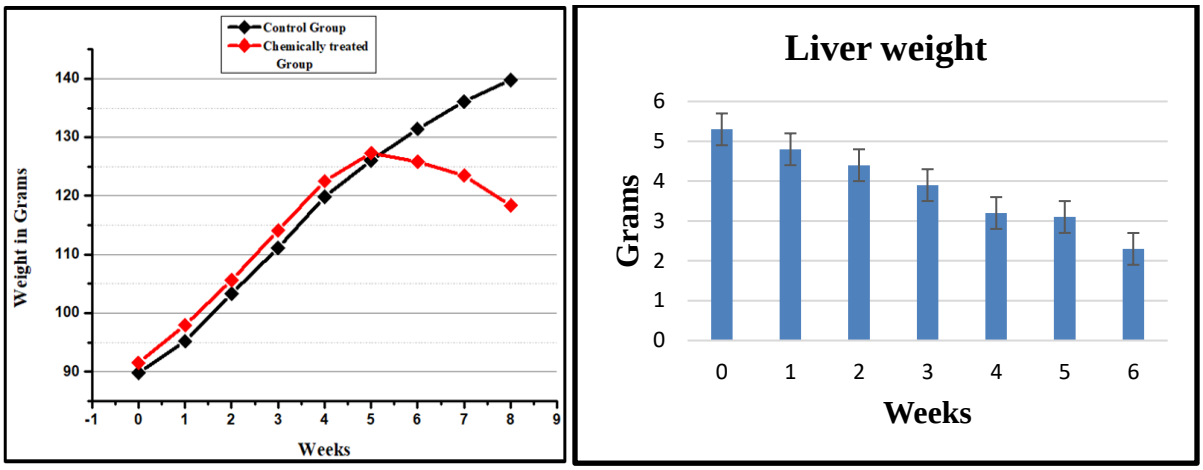


Figure 4.32: Weight Analysis during Cirrhosis induction (a) Body (b) Liver

WEEKS	1ST	2ND	3RD	4TH	5TH	6TH
ASCITES	--	--	+	++	+++	++

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

Table 4.32: Weekly Ascites observation during cirrhosis induction

4.4 Treatment

4.4.1 Hepatic Histopathology

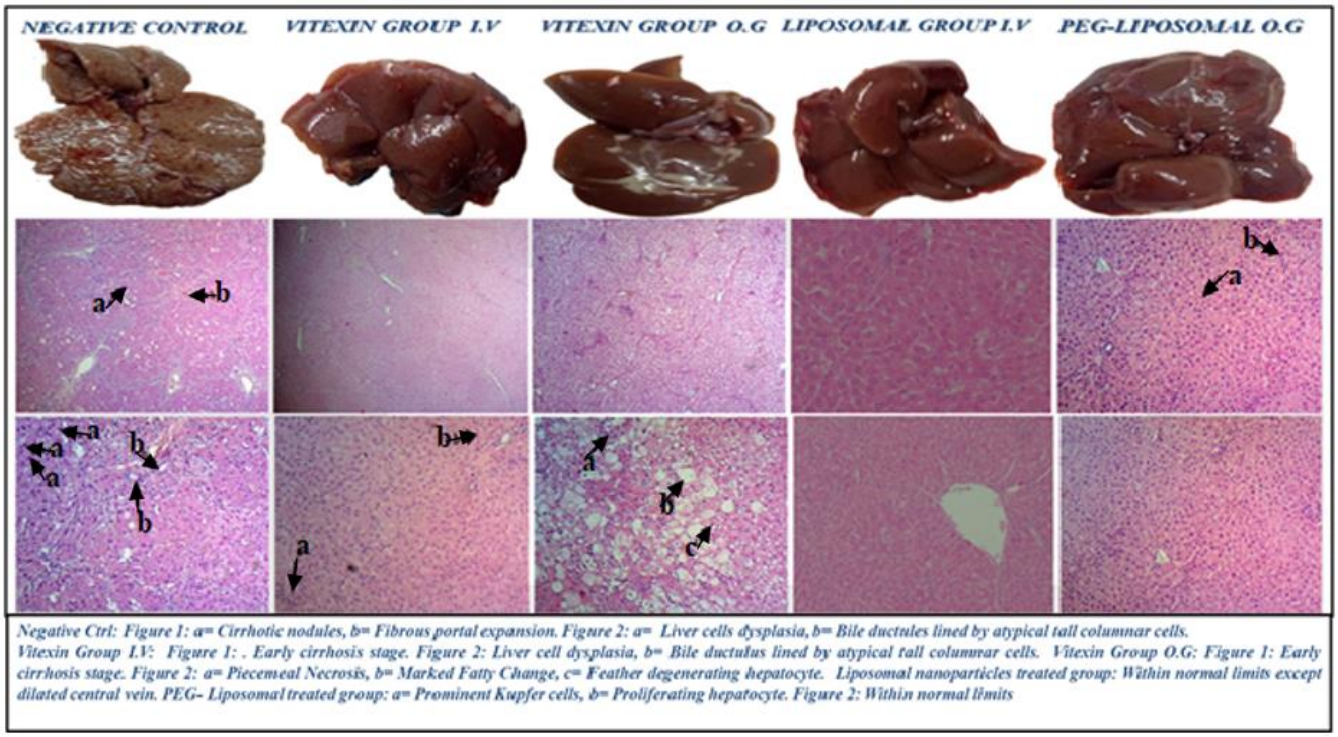


Figure 4.41: Liver histopathology after treatment (a) Negative ctrl (b) Vitexin intravenous group (c) Vitexin Oral Gavage group (d) LNPs treated group (e) PEG-LNPs treated group

Groups	Score
Negative control	12/17
Vitexin treated O.G group	9/17
Vitexin treated I.V group	8/17
Liposome treated I.V group	6/17
PEG- LNPs treated O.G group	3/17

Table 4.41: NASH/NAFLD Clinical Research Network Scoring System after treatment.

4.4.2 Renal Histopathology

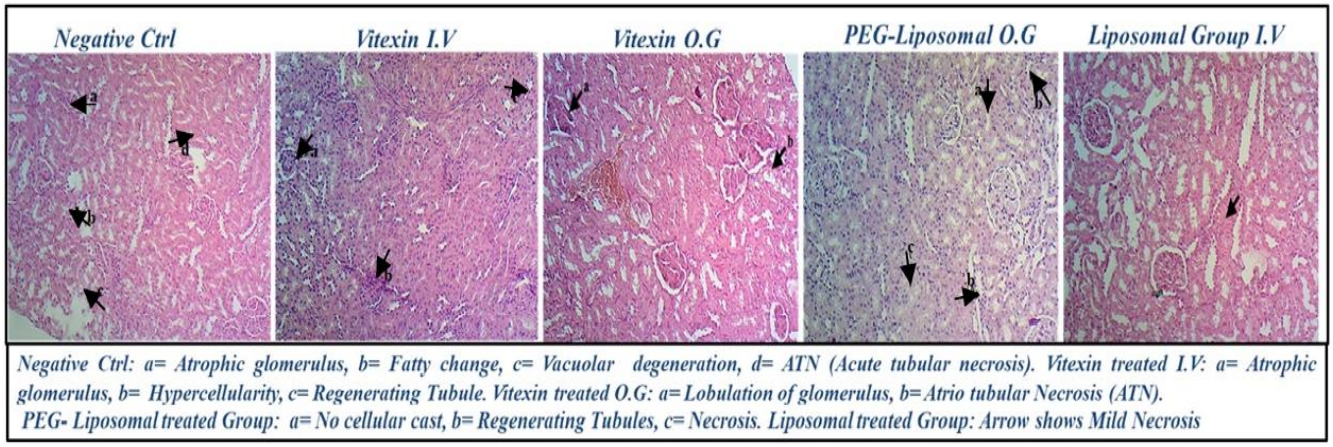


Figure 4.42: Kidney histopathology after treatment (a) Negative ctrl (b) Vitexin intravenous group (c) Vitexin Oral Gavage group (e) PEG-LNPs treated group (d) LNPs treated group

4.4.3 Spleen Histopathology

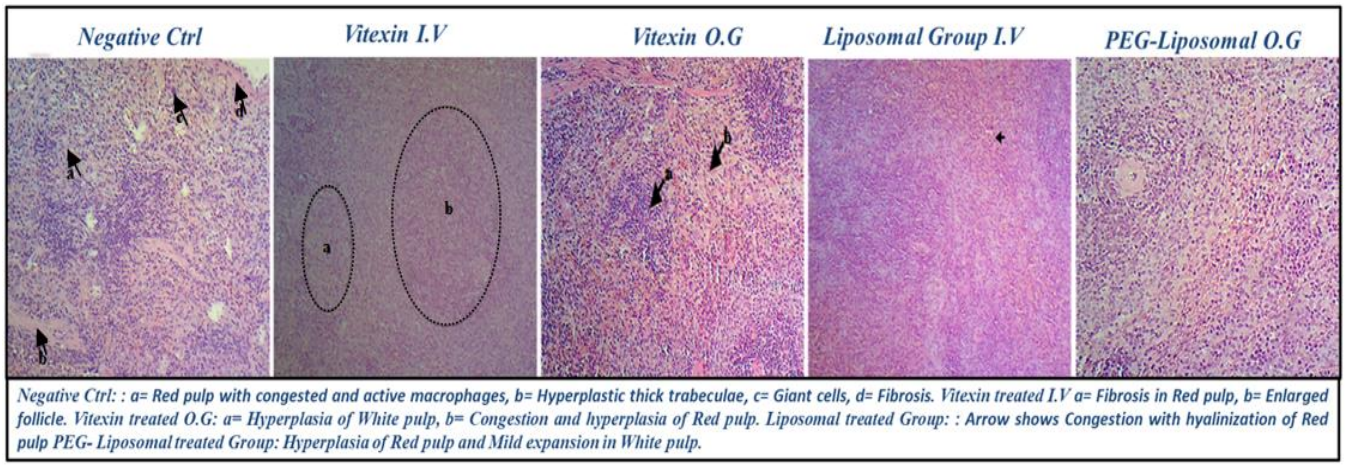
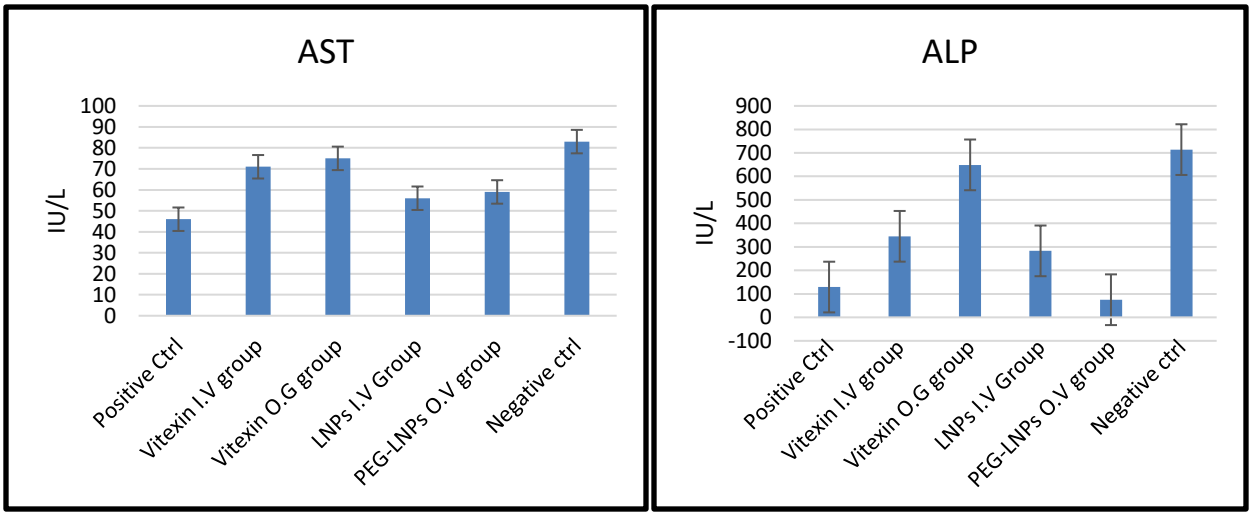


Figure 4.43: Spleen histopathology after treatment of (a) Negative ctrl (b) Vitexin intravenous group (c) Vitexin Oral Gavage group (d) LNPs treated group (e) PEG-LNPs treated group

Different types of formulated vitexin loaded liposomes were used as treatment and upon analyzing the histological and serological difference, conclusions were drawn. In Blank vitexin intravenously treated group, histological examinations of kidneys' Glomeruli showed mesangial proliferation, 25 – 50% of cortical sub-capsular tubules showed fatty change, necrosis & vacuolar degeneration, 30% of medullary tubules also showed vacuolar degeneration and blood vessels were congested while in spleen, Hyperplasia of White pulp, Congestion and hyperplasia of Red pulp were observed. In vitexin loaded liposomes treated group, histology of kidney showed the hypercellularity of glomeruli, atrophicity of glomeruli

decreased (rare atrophic Glomeruli i.e. <20%), <20% of tubules showed Necrosis & degeneration, No cellular casts were seen and Regenerating tubules were seen, while in spleen Follicles were of variable sizes & increased in number and 50% of Germinal centers were prominent. In blank vitexin orally treated group, in kidney >20% of Glomeruli were shrunken, Blood vessels were congested, 20% degeneration in Medullary tubule and 35% Cortical damaged tubules (ATN) were seen. In spleen Germinal centers were prominent and there was little fibrosis of red pulp but still decreased follicles and congestion of red pulp were seen. In Pegylated vitexin loaded liposomes treated group, there were <10% atrophicity of glomeruli, no cellular caste were seen, regenerating tubules were observed and there were lower extent of necrotic cells. In spleen there were no congestion and hyalinization but still hyperplasia of red pulp was observed.

4.4.4 Serological Analysis (LFTs)



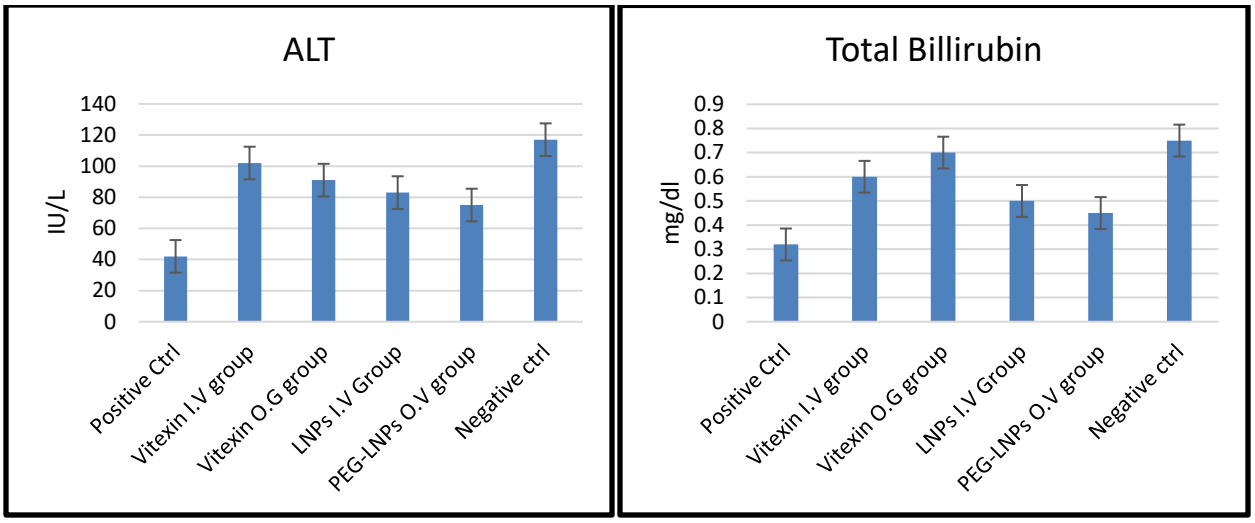


Figure 4.44: Serological indices after treatment (a) AST (b) ALP (c) ALT (d) T.B

4.4.5 Body & Liver Weight and Ascites

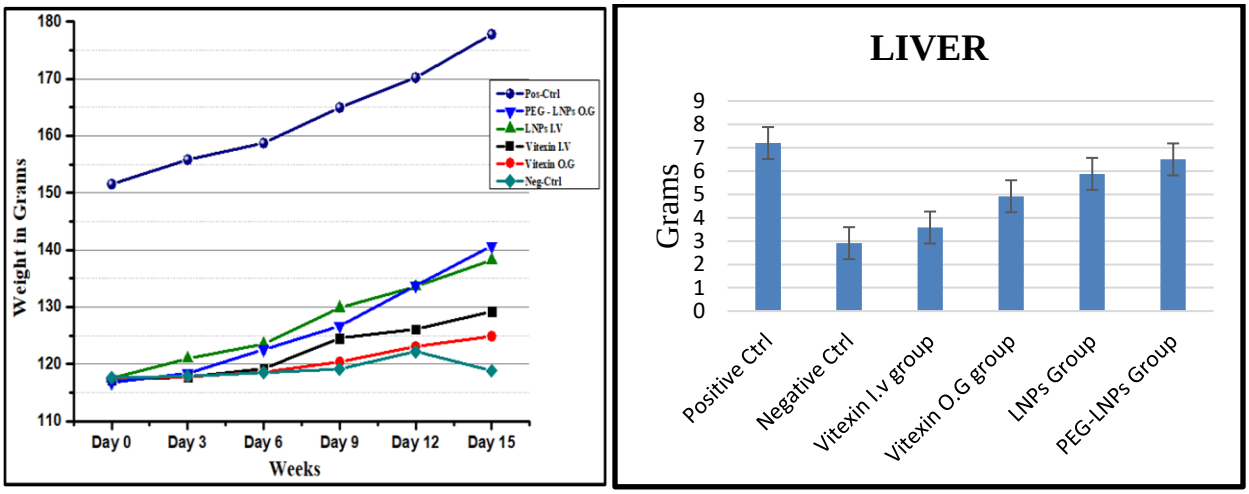


Figure 4.45: Weight Analysis after treatment (a) Body (b) Liver

WEEKS	Negative Ctrl	Vitexin (I.V)	Vitexin (O.G)	LNPs Group	PEG-LNPs Group
ASCITES	+++	++	++	+	--

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

Table 4.45: Analysis of Body, Liver weight and Ascites after treatment

CHAPTER: 5 DISCUSSION

Liver cirrhosis is generally considered a dynamic disorder bearing the advancing and regressive temperament. In this modern way of understanding the continuum of changes that characterize ACLD (advanced chronic liver disease), early diagnosis before decompensation occurs, is a significant step towards achieving a mortality reduction. Various pharmacological and non-pharmacological methods may be used to prevent decompensation (an alarming move in this disease's natural history) (Berzigotti, 2017). There is actually a lack of pure antifibrotic drugs (Nair, Berzigotti, & Bosch, 2016). Efficient artificial liver support remains an important unmet need for end-stage liver disease patients. The liver transplantation is still the only curative alternative available to date (in those without contraindications). Research in the field of regenerative medicine is indeed a big anticipated breakthrough of the 21st century that will offer a great promise (Sampaziotis et al., 2017). It is a challenge to 21st century, to cope the hepatic transplantation need with possible alternatives in cirrhosis patients. (Tsochatzis et al., 2014).

A recent published study demonstrated the potential of vitexin drug to alleviate the non-alcoholic fatty liver disease via activation of AMP activated protein kinase (AMPK) by binding to leptin receptors that resulted in lipolysis and inhibition of lipogenesis (Inamdar et al., 2019). The low aqueous solubility and poor bioavailability of vitexin limit its clinical application (Gu et al., 2017; Lai et al., 2014; Zu et al., 2012). Issues associated with poor solubility can lead to low bioavailability resulting in suboptimal drug delivery. Numerous drugs associated with poor solubility and low bioavailability's have been reformulated to improve efficacy, safety, and patient compliance by nanoparticulation and nanonization (Kalepu & Nekkanti, 2015; Tanaka et al., 2009).

In the present study, we successfully formulated the vitexin loaded liposomes nanoparticles (with and without PEG) and used as remedy against liver cirrhosis. Vitexin was loaded within liposomes by dissolving it into appropriate solvent and nano formulated by sonication. Due to the hydrophobic nature, vitexin is encapsulated in between the bilayer of liposomal vesicles. To achieve optimized vitexin loaded liposomes nanoparticles with desired features such as size, PDI, zeta potential and encapsulation efficiency, different parameters like DPPC & Cholesterol ratio, drug to lipids ratio, Sonication time and amplitude, temperature of

water bath during rotary evaporation were monitored. The smaller size nanoparticles were formulated, when DPPC and Cholesterol were used in 4:1 upon 80MHz amplitude of probe sonicator. Average size of blank liposomes and vitexin loaded liposomes nanoparticles were observed 128nm and 190nm While Zeta potential were observed -5.73mV and -8.34mV and PDI were 0.498 and 0.488 respectively. Drug release from liposomes nanoparticles was noted 72% for 48 hours examination. Due to less negative surface charge, the stability of nanoparticles was too low, and formulation was highly prone to agglomeration. To enhance the stability, Polyethylene glycol (PEG) was used to enhance stability and for inducing the stealth effect, by coating the liposomes nanoparticles. Pegylation enhances the steric repulsion and hence known as better stabilizer for different types of nanoparticles (D'Souza A & Shegokar, 2016). But one of the main disadvantages of using PEG is the increase in size of nanoparticles. The size, zeta potential and PDI of Pegylated vitexin loaded liposomes nanoparticles were 458nm, -13.4mV and 0.240 respectively. PEG follows the erosion -controlled release mechanism of drug that results in sustained release (Hu, Zhang, You, Yuan, & Du, 2012). 44% drug release was observed from Pegylated liposomes nanoparticles which delineates the sustained release. In the present study, comparative FTIR of Vitexin, vitexin loaded liposome nanoparticles and PEG coated liposomes showed the involvement of different functional groups which were conferred by the decrease of peaks intensities.

Liver cirrhosis model was successfully developed by using carbon tetrachloride. To reduce the time frame of liver cirrhosis induction, urethane was used initially that caused severe hepatic damage. Effects of aforementioned chemicals were analyzed on liver, kidney, and spleen by histological examination along with Liver function tests. Histological, serological, body and liver weight results exhibited the significant reversibility of collagenous scars to normal hepatic tissue paradigm by using the encapsulated vitexin drug contrary to blank drug. Better results were observed in Peg-LNPs treated group than LNPs group.

Conclusion

After the synthesis of nanoparticles, successful synthesis is proved by different characterization techniques. Pegylation enhanced the stability that resulted the long-term circulation in the body as compared to conventional drug and non-coated liposomes nanoparticles. After treating the diseased rats, the histological, serological, body and liver weight results exhibited the significant betterment in Peg-LNPs treated group. After that, LNPs treated group also showed better results up to some extent. So, for the reversibility of collagenous scars to normal hepatic tissue, the use of the encapsulated vitexin drug is better choice, contrary to blank drug. Hence, liposomes encapsulation along with PEG coating, is proved as the most effective stratagem in enhancing the bioavailability and pharmacokinetic behavior of vitexin drug and it has proven the anti-fibrotic activity or considerable therapeutics against liver cirrhosis. In comparison of route of administration for blank drug, the intravenous route is proved as better choice to use. Further studies should be implicated regarding the followed hypothesis to combat liver cirrhosis and approved this strategy to use at clinical level.

References:

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S. W., Zarghami, N., Hanifehpour, Y., . . . Nejati-Koshki, K. (2013). Liposome: classification, preparation, and applications. *Nanoscale Research Letters, 8*(1), 102. doi:10.1186/1556-276X-8-102

Amendola, V., & Meneghetti, M. (2009). Size evaluation of gold nanoparticles by UV− vis spectroscopy. *The Journal of Physical Chemistry C, 113*(11), 4277-4285.

Anderson, M., & Omri, A. (2004). The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. *Drug Deliv, 11*(1), 33-39. doi:10.1080/10717540490265243

Arrese, M., & Feldstein, A. E. (2017). NASH-RELATED CIRRHOSIS: AN OCCULT LIVER DISEASE BURDEN. *Hepatology communications, 1*(2), 84-86. doi:10.1002/hep4.1033

Aslam, M. S., Ahmad, M. S., & Mamat, A. S. (2015). Pharmacological potential of vitexin. *Indian Res. J. Pharm. Sci, 2*(2), 114-122.

Asrani, S. K., Devarbhavi, H., Eaton, J., & Kamath, P. S. (2019). Burden of liver diseases in the world. *Journal of hepatology, 70*(1), 151-171. doi:10.1016/j.jhep.2018.09.014

Atrooz, O. (2011). Effects of alkylresorcinolic lipids obtained from acetonic extract of Jordanian wheat grains on liposome properties. *Int J Biol Chem, 5*(5), 314-321.

Bartneck, M., Scheyda, K. M., Warzecha, K. T., Rizzo, L. Y., Hittatiya, K., Luedde, T., . . . Tacke, F. (2015). Fluorescent cell-traceable dexamethasone-loaded liposomes for the treatment of inflammatory liver diseases. *Biomaterials, 37*, 367-382. doi:10.1016/j.biomaterials.2014.10.030

Basile, A., Sorbo, S., López-Sáez, J. A., & Castaldo Cobianchi, R. (2003). Effects of seven pure flavonoids from mosses on germination and growth of Tortula muralis HEDW (Bryophyta) and Raphanus sativus L (Magnoliophyta). *Phytochemistry, 62*(7), 1145-1151. doi:10.1016/s0031-9422(02)00659-3

Bedossa, P. (1993). [Presentation of a grid for computer analysis for compilation of histopathologic lesions in chronic viral hepatitis C. Cooperative study of the METAVIR group]. *Ann Pathol, 13*(4), 260-265.

Bedossa, P., & Poynard, T. (1996). An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology, 24*(2), 289-293. doi:10.1002/hep.510240201

Berzigotti, A. (2017). Advances and challenges in cirrhosis and portal hypertension. *BMC Medicine, 15*(1), 200. doi:10.1186/s12916-017-0966-6

Bhatt, P., Lalani, R., Vhora, I., Patil, S., Amrutiya, J., Misra, A., & Mashru, R. (2018). Liposomes encapsulating native and cyclodextrin enclosed paclitaxel: Enhanced loading

efficiency and its pharmacokinetic evaluation. *Int J Pharm, 536*(1), 95-107. doi:10.1016/j.ijpharm.2017.11.048

Cai, J., Ito, M., Nagata, H., Westerman, K. A., Lafleur, D., Chowdhury, J. R., . . . Fox, I. J. (2002). Treatment of liver failure in rats with end-stage cirrhosis by transplantation of immortalized hepatocytes. *Hepatology, 36*(2), 386-394. doi:10.1053/jhep.2002.34614

Cao, D., Li, H., Yi, J., Zhang, J., Che, H., Cao, J., . . . Jiang, W. (2011). Antioxidant properties of the mung bean flavonoids on alleviating heat stress. *PLoS One, 6*(6), e21071. doi:10.1371/journal.pone.0021071

Chorachoo, J., Amnuaikit, T., & Voravuthikunchai, S. P. (2013). Liposomal encapsulated rhodomyrtone: a novel antiacne drug. *Evid Based Complement Alternat Med, 2013*, 157635. doi:10.1155/2013/157635

D'Amico, G., Garcia-Tsao, G., & Pagliaro, L. (2006). Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol, 44*(1), 217-231. doi:10.1016/j.jhep.2005.10.013

D'Souza A, A., & Shegokar, R. (2016). Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications. *Expert Opin Drug Deliv, 13*(9), 1257-1275. doi:10.1080/17425247.2016.1182485

Dimov, N., Kastner, E., Hussain, M., Perrie, Y., & Szita, N. (2017). Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system. *Scientific Reports, 7*(1), 12045. doi:10.1038/s41598-017-11533-1

Duan, S., Du, X., Chen, S., Liang, J., Huang, S., Hou, S., . . . Ding, P. (2020). Effect of vitexin on alleviating liver inflammation in a dextran sulfate sodium (DSS)-induced colitis model. *Biomed Pharmacother, 121*, 109683. doi:10.1016/j.biopha.2019.109683

Edwards, J. E., Brown, P. N., Talent, N., Dickinson, T. A., & Shipley, P. R. (2012). A review of the chemistry of the genus Crataegus. *Phytochemistry, 79*, 5-26. doi:10.1016/j.phytochem.2012.04.006

Farrell, G. C., & Larter, C. Z. (2006). Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology, 43*(2 Suppl 1), S99-s112. doi:10.1002/hep.20973

Forloni, G. (2012). Responsible nanotechnology development. *Journal of Nanoparticle Research, 14*(8), 1007. doi:10.1007/s11051-012-1007-1

Fortea, J. I., Fernández-Mena, C., Puerto, M., Ripoll, C., Almagro, J., Bañares, J., . . . Vaquero, J. (2018). Comparison of Two Protocols of Carbon Tetrachloride-Induced Cirrhosis in Rats – Improving Yield and Reproducibility. *Scientific Reports, 8*(1), 9163. doi:10.1038/s41598-018-27427-9

Fu, Y., Zu, Y., Liu, W., Hou, C., Chen, L., Li, S., . . . Tong, M. (2007). Preparative separation of vitexin and isovitexin from pigeonpea extracts with macroporous resins. *J Chromatogr A, 1139*(2), 206-213. doi:10.1016/j.chroma.2006.11.015

Gaitan, E., Lindsay, R. H., Reichert, R. D., Ingbar, S. H., Cooksey, R. C., Legan, J., . . . Kubota, K. (1989). Antithyroid and goitrogenic effects of millet: role of C-glycosylflavones. *J Clin Endocrinol Metab, 68*(4), 707-714. doi:10.1210/jcem-68-4-707

Ganesan, K., & Xu, B. (2017). Molecular targets of vitexin and isovitexin in cancer therapy: a critical review. *Ann N Y Acad Sci, 1401*(1), 102-113. doi:10.1111/nyas.13446

Garcia-Tsao, G., Abraldes, J. G., Berzigotti, A., & Bosch, J. (2017). Portal hypertensive bleeding in cirrhosis: Risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the study of liver diseases. *Hepatology, 65*(1), 310-335.

Gitiara, A., Tokhanbigli, S., Mazhari, S., Baghaei, K., Hatami, B., Hashemi, S. M., . . . Zali, M. R. (2017). Development of experimental fibrotic liver diseases animal model by Carbon Tetracholoride. *Gastroenterol Hepatol Bed Bench, 10*(Suppl1), S122-s128.

Glawdel, T., & Ren, C. (2008). Zeta Potential Measurement. In D. Li (Ed.), *Encyclopedia of Microfluidics and Nanofluidics* (pp. 2199-2207). Boston, MA: Springer US.

Global, regional, and national age–sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. (2015). *The Lancet, 385*(9963), 117-171. doi:10.1016/S0140-6736(14)61682-2

Goldstein, J. I., Newbury, D. E., Michael, J. R., Ritchie, N. W., Scott, J. H. J., & Joy, D. C. (2017). *Scanning electron microscopy and X-ray microanalysis*: Springer.

Gu, C., Liu, Z., Yuan, X., Li, W., Zu, Y., & Fu, Y. (2017). Preparation of Vitexin Nanoparticles by Combining the Antisolvent Precipitation and High Pressure Homogenization Approaches Followed by Lyophilization for Dissolution Rate Enhancement. *Molecules, 22*(11). doi:10.3390/molecules22112038

Gubernator, J. (2011). Active methods of drug loading into liposomes: recent strategies for stable drug entrapment and increased in vivo activity. *Expert Opin Drug Deliv, 8*(5), 565-580. doi:10.1517/17425247.2011.566552

He, M., Min, J. W., Kong, W. L., He, X. H., Li, J. X., & Peng, B. W. (2016). A review on the pharmacological effects of vitexin and isovitexin. *Fitoterapia, 115*, 74-85. doi:10.1016/j.fitote.2016.09.011

Hodgson, J. (2001). ADMET--turning chemicals into drugs. *Nat Biotechnol, 19*(8), 722-726. doi:10.1038/90761

Hu, F. Q., Zhang, Y. Y., You, J., Yuan, H., & Du, Y. Z. (2012). pH triggered doxorubicin delivery of PEGylated glycolipid conjugate micelles for tumor targeting therapy. *Mol Pharm, 9*(9), 2469-2478. doi:10.1021/mp300002v

Hytiroglou, P., Snover, D. C., Alves, V., Balabaud, C., Bhathal, P. S., Bioulac-Sage, P., . . . van Leeuwen, D. J. (2012). Beyond "cirrhosis": a proposal from the International Liver Pathology Study Group. *Am J Clin Pathol, 137*(1), 5-9. doi:10.1309/ajcp2t2ohtapbtmp

Inamdar, S., Joshi, A., Malik, S., Boppana, R., & Ghaskadbi, S. (2019). Vitexin alleviates non-alcoholic fatty liver disease by activating AMPK in high fat diet fed mice. *Biochem Biophys Res Commun, 519*(1), 106-112. doi:10.1016/j.bbrc.2019.08.139

Kalepu, S., & Nekkanti, V. (2015). Insoluble drug delivery strategies: review of recent advances and business prospects. *Acta Pharmaceutica Sinica B, 5*(5), 442-453. doi:https://doi.org/10.1016/j.apsb.2015.07.003

Khan, I., Saeed, K., & Khan, I. (2019). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry, 12*(7), 908-931. doi:https://doi.org/10.1016/j.arabjc.2017.05.011

Knodell, R. G., Ishak, K. G., Black, W. C., Chen, T. S., Craig, R., Kaplowitz, N., . . . Wollman, J. (1981). Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology, 1*(5), 431-435. doi:10.1002/hep.1840010511

Lai, F., Pini, E., Corrias, F., Perricci, J., Manconi, M., Fadda, A. M., & Sinico, C. (2014). Formulation strategy and evaluation of nanocrystal piroxicam orally disintegrating tablets manufacturing by freeze-drying. *Int J Pharm, 467*(1-2), 27-33. doi:10.1016/j.ijpharm.2014.03.047

Li, S., Tan, H.-Y., Wang, N., Zhang, Z.-J., Lao, L., Wong, C.-W., & Feng, Y. (2015). The Role of Oxidative Stress and Antioxidants in Liver Diseases. *International journal of molecular sciences, 16*(11), 26087-26124. doi:10.3390/ijms161125942

Liang, M., Xu, W., Zhang, W., Zhang, C., Liu, R., Shen, Y., . . . Chen, C. (2007). Quantitative LC/MS/MS method and in vivo pharmacokinetic studies of vitexin rhamnoside, a bioactive constituent on cardiovascular system from hawthorn. *Biomed Chromatogr, 21*(4), 422-429. doi:10.1002/bmc.777

Liver, E. A. F. T. S. O. T. (2010). EASL clinical practice guidelines on the management of ascites, spontaneous bacterial peritonitis, and hepatorenal syndrome in cirrhosis. *Journal of hepatology, 53*(3), 397-417.

Loftsson, T., & Brewster, M. E. (2010). Pharmaceutical applications of cyclodextrins: basic science and product development. *J Pharm Pharmacol, 62*(11), 1607-1621. doi:10.1111/j.2042-7158.2010.01030.x

Maeki, M., Kimura, N., Sato, Y., Harashima, H., & Tokeshi, M. (2018). Advances in microfluidics for lipid nanoparticles and extracellular vesicles and applications in drug delivery systems. *Adv Drug Deliv Rev, 128*, 84-100. doi:10.1016/j.addr.2018.03.008

Malhi, H., Irani, A. N., Gagandeep, S., & Gupta, S. (2002). Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. *J Cell Sci, 115*(Pt 13), 2679-2688.

Marques, T. G., Chaib, E., da Fonseca, J. H., Lourenço, A. C., Silva, F. D., Ribeiro, M. A., Jr., . . . D'Albuquerque, L. A. (2012). Review of experimental models for inducing hepatic cirrhosis by bile duct ligation and carbon tetrachloride injection. *Acta Cir Bras, 27*(8), 589-594. doi:10.1590/s0102-86502012000800013

Martin, J., & Dufour, J.-F. (2008). Tumor suppressor and hepatocellular carcinoma. *World journal of gastroenterology, 14*(11), 1720-1733. doi:10.3748/wjg.14.1720

Melchert, T. E., & Alston, R. E. (1965). Flavonoids from the moss Mnium affine Bland. *Science, 150*(3700), 1170-1171. doi:10.1126/science.150.3700.1170

Meng, J., Guo, F., Xu, H., Liang, W., Wang, C., & Yang, X. D. (2016). Combination Therapy using Co-encapsulated Resveratrol and Paclitaxel in Liposomes for Drug Resistance Reversal in Breast Cancer Cells in vivo. *Sci Rep, 6*, 22390. doi:10.1038/srep22390

Mohamed, M. A., Jaafar, J., Ismail, A. F., Othman, M. H. D., & Rahman, M. A. (2017). Chapter 1 - Fourier Transform Infrared (FTIR) Spectroscopy. In N. Hilal, A. F. Ismail, T. Matsuura, & D. Oatley-Radcliffe (Eds.), *Membrane Characterization* (pp. 3-29): Elsevier.

Nagata, H., Ito, M., Cai, J., Edge, A. S., Platt, J. L., & Fox, I. J. (2003). Treatment of cirrhosis and liver failure in rats by hepatocyte xenotransplantation. *Gastroenterology, 124*(2), 422-431. doi:10.1053/gast.2003.50065

Nair, H., Berzigotti, A., & Bosch, J. (2016). Emerging therapies for portal hypertension in cirrhosis. *Expert Opin Emerg Drugs, 21*(2), 167-181. doi:10.1080/14728214.2016.1184647

Nii, T., & Ishii, F. (2005). Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. *Int J Pharm, 298*(1), 198-205. doi:https://doi.org/10.1016/j.ijpharm.2005.04.029

Nix, A., Paull, C. A., & Colgrave, M. (2015). The flavonoid profile of pigeonpea, Cajanus cajan: a review. *SpringerPlus, 4*, 125-125. doi:10.1186/s40064-015-0906-x

Nowak, G., Ericzon, B. G., Nava, S., Jaksch, M., Westgren, M., & Sumitran-Holgersson, S. (2005). Identification of expandable human hepatic progenitors which differentiate into mature hepatic cells in vivo. *Gut, 54*(7), 972-979. doi:10.1136/gut.2005.064477

Osna, N. A., Donohue, T. M., Jr., & Kharbanda, K. K. (2017). Alcoholic Liver Disease: Pathogenesis and Current Management. *Alcohol Res, 38*(2), 147-161.

Paquet, K. J., & Kamphausen, U. (1975). The carbon-tetrachloride-hepatotoxicity as a model of liver damage. First report: Long-time biochemical changes. *Acta Hepatogastroenterol (Stuttg), 22*(2), 84-88.

Pattni, B. S., Chupin, V. V., & Torchilin, V. P. (2015). New Developments in Liposomal Drug Delivery. *Chemical Reviews, 115*(19), 10938-10966. doi:10.1021/acs.chemrev.5b00046

Perkampus, H.-H. (2013). *UV-VIS Spectroscopy and its Applications*: Springer Science & Business Media.

Ravan, A. P., Bahmani, M., Ghasemi Basir, H. R., Salehi, I., & Oshaghi, E. A. (2017). Hepatoprotective effects of Vaccinium arctostaphylos against CCl4-induced acute liver injury in rats. *Journal of basic and clinical physiology and pharmacology, 28*(5), 463-471. doi:10.1515/jbcpp-2016-0181

Rizzo, L. Y., Theek, B., Storm, G., Kiessling, F., & Lammers, T. (2013). Recent progress in nanomedicine: therapeutic, diagnostic and theranostic applications. *Curr Opin Biotechnol, 24*(6), 1159-1166. doi:10.1016/j.copbio.2013.02.020

Roco, M. C. (2011). The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *Journal of Nanoparticle Research, 13*(2), 427-445. doi:10.1007/s11051-010-0192-z

Rudolph, K. L., Chang, S., Millard, M., Schreiber-Agus, N., & DePinho, R. A. (2000). Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science, 287*(5456), 1253-1258. doi:10.1126/science.287.5456.1253

Sahreen, S., Khan, M. R., & Khan, R. A. (2011). Hepatoprotective effects of methanol extract of Carissa opaca leaves on CCl4-induced damage in rat. *BMC Complement Altern Med, 11*, 48. doi:10.1186/1472-6882-11-48

Sainz, V., Conniot, J., Matos, A. I., Peres, C., Zupancic, E., Moura, L., . . . Gaspar, R. S. (2015). Regulatory aspects on nanomedicines. *Biochem Biophys Res Commun, 468*(3), 504-510. doi:10.1016/j.bbrc.2015.08.023

Sampaziotis, F., Justin, A. W., Tysoe, O. C., Sawiak, S., Godfrey, E. M., Upponi, S. S., . . . Vallier, L. (2017). Reconstruction of the mouse extrahepatic biliary tree using primary human extrahepatic cholangiocyte organoids. *Nat Med, 23*(8), 954-963. doi:10.1038/nm.4360

Scholten, D., Trebicka, J., Liedtke, C., & Weiskirchen, R. (2015). The carbon tetrachloride model in mice. *Lab Anim, 49*(1 Suppl), 4-11. doi:10.1177/0023677215571192

Schuppan, D., & Afdhal, N. H. (2008). Liver cirrhosis. *Lancet (London, England), 371*(9615), 838-851. doi:10.1016/s0140-6736(08)60383-9

Shaheen, S. M., Shakil Ahmed, F., Hossen, M. N., Ahmed, M., Amran, M. S., & Ul-Islam, M. (2006). Liposome as a carrier for advanced drug delivery. *Pak J Biol Sci, 9*(6), 1181-1191.

Sharma, D., Aara, A., Ali, E., & Trivedi, L. (2018). An Updated Review On:Liposomes as Drug Delivery System. *Pharmatutor, 6*. doi:10.29161/PT.v6.i2.2018.50

Shehata, T., Ogawara, K., Higaki, K., & Kimura, T. (2008). Prolongation of residence time of liposome by surface-modification with mixture of hydrophilic polymers. *Int J Pharm, 359*(1-2), 272-279. doi:10.1016/j.ijpharm.2008.04.004

Stiufiuc, R., Iacovita, C., Nicoara, R., Stiufiuc, G., Florea, A., Achim, M., & Lucaciu, C. M. (2013). One-Step Synthesis of PEGylated Gold Nanoparticles with Tunable Surface Charge. *Journal of Nanomaterials, 2013*, 146031. doi:10.1155/2013/146031

Tajima, K., Nakamura, A., Shirakawa, J., Togashi, Y., Orime, K., Sato, K., . . . Terauchi, Y. (2013). Metformin prevents liver tumorigenesis induced by high-fat diet in C57Bl/6 mice. *Am J Physiol Endocrinol Metab, 305*(8), E987-998. doi:10.1152/ajpendo.00133.2013

Tanaka, Y., Inkyo, M., Yumoto, R., Nagai, J., Takano, M., & Nagata, S. (2009). Nanoparticulation of poorly water soluble drugs using a wet-mill process and physicochemical properties of the nanopowders. *Chem Pharm Bull (Tokyo), 57*(10), 1050-1057. doi:10.1248/cpb.57.1050

Tang, W. L., Tang, W. H., Szeitz, A., Kulkarni, J., Cullis, P., & Li, S. D. (2018). Systemic study of solvent-assisted active loading of gambogic acid into liposomes and its formulation optimization for improved delivery. *Biomaterials, 166*, 13-26. doi:10.1016/j.biomaterials.2018.03.004

Thorgeirsson, S. S., & Grisham, J. W. (2006). Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. *Hepatology, 43*(1), 2-8. doi:10.1002/hep.21015

Tomaszewska, E., Soliwoda, K., Kadziola, K., Tkacz-Szczesna, B., Celichowski, G., Cichomski, M., . . . Grobelny, J. (2013). Detection limits of DLS and UV-Vis spectroscopy in characterization of polydisperse nanoparticles colloids. *Journal of Nanomaterials, 2013*.

Tsochatzis, E. A., Bosch, J., & Burroughs, A. K. (2014). Liver cirrhosis. *The Lancet, 383*(9930), 1749-1761. doi:https://doi.org/10.1016/S0140-6736(14)60121-5

van Leerdam, M. E. (2008). Epidemiology of acute upper gastrointestinal bleeding. *Best Practice & Research Clinical Gastroenterology, 22*(2), 209-224. doi:https://doi.org/10.1016/j.bpg.2007.10.011

Wagner, A., & Vorauer-Uhl, K. (2011). Liposome Technology for Industrial Purposes. *Journal of Drug Delivery, 2011*, 591325. doi:10.1155/2011/591325

Weber, L. W., Boll, M., & Stampfl, A. (2003). Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol, 33*(2), 105-136. doi:10.1080/713611034

Wiegand, J., & Berg, T. (2013). The etiology, diagnosis and prevention of liver cirrhosis: part 1 of a series on liver cirrhosis. *Dtsch Arztebl Int, 110*(6), 85-91. doi:10.3238/arztebl.2013.0085

Wu, H., Zhang, G., Huang, L., Pang, H., Zhang, N., Chen, Y., & Wang, G. (2017). Hepatoprotective Effect of Polyphenol-Enriched Fraction from Folium Microcos on Oxidative Stress and Apoptosis in Acetaminophen-Induced Liver Injury in Mice. *Oxid Med Cell Longev, 2017*, 3631565. doi:10.1155/2017/3631565

Yang, D., Gao, Y. H., Tan, K. B., Zuo, Z. X., Yang, W. X., Hua, X., . . . Wang, G. (2013). Inhibition of hepatic fibrosis with artificial microRNA using ultrasound and cationic liposome-bearing microbubbles. *Gene Ther, 20*(12), 1140-1148. doi:10.1038/gt.2013.41

Zhang, F., Kong, D., Lu, Y., & Zheng, S. (2013). Peroxisome proliferator-activated receptor-γ as a therapeutic target for hepatic fibrosis: from bench to bedside. *Cellular and molecular life sciences : CMLS, 70*(2), 259-276. doi:10.1007/s00018-012-1046-x

Zhao, Y., May, J. P., Chen, I. W., Undzys, E., & Li, S. D. (2015). A Study of Liposomal Formulations to Improve the Delivery of Aquated Cisplatin to a Multidrug Resistant Tumor. *Pharm Res, 32*(10), 3261-3268. doi:10.1007/s11095-015-1702-6

Zingg, R., & Fischer, M. (2018). The nanotechnology patent thicket revisited. *Journal of Nanoparticle Research, 20*(10), 267. doi:10.1007/s11051-018-4372-6

Zu, Y., Zhang, Q., Zhao, X., Wang, D., Li, W., Sui, X., . . . Gu, C. (2012). Preparation and characterization of vitexin powder micronized by a supercritical antisolvent (SAS) process. *Powder Technology, 228*, 47-55. doi:https://doi.org/10.1016/j.powtec.2012.04.048

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