ANALYZING THE HEPATOPROTECTIVE EFFECTS OF SILYMARIN ENCAPSULATED PEGYLATED LIPOSOMAL NANOPARTICLES AND VITAMIN D & E FOR TARGETED NAFLD TREATMENT IN WISTAR RATS

Author

Farhat Batool

Regn Number

329991

Supervisor

Dr. Nosheen Fatima Rana

DEPARTMENT OF BIOMEDICAL ENGINEERING & SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

ISLAMABAD

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Author

Farhat Batool

Regn Number

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Thesis Supervisor:

Dr. Nosheen Fatima Rana

Thesis Supervisor's Signature: _____

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All praises to ALLAH Almighty, the most beneficent who blessed mankind with a beautiful life and explorative nature. Humble dedications are for the most perfect personality of the universe of all the times Prophet Hazrat Muhammad (PBUH) and his brother Imam Ali (A.S). The greatest scientist, whose moral and spiritual teachings enlightened our hearts, and flourished our thoughts towards achieving higher ideals of life.

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LIST OF ABBREVIATIONS

SNPs	**Silymarin loaded pegylated liposomes nanoparticles**
NASH	**Non-Alcoholic SteatoHepatitis**
NAFLD	**Non-Alcoholic Fatty liver Disease**
CCl_4	**Carbon tetrachloride**
PEG	**Polyethylene glycol**
AST	**Aspartate transaminase**
ALT	**Alanine transaminase**
ALP	**Alkaline phosphatase**
FTIR	**Fourier Transform Infrared**
I.V	**Intravenous**
DPPE	**1,2-Dipalmitoyl-sn-glycero-3-phosphorylethanolamine**
Vit.E	**Vitamin E**
Vit.D	**Vitamin D**
SNPE	**Silymarin-loaded liposomal nanoparticles and vitamin E**
SNPD	**Silymarin-loaded liposomal nanoparticles and vitamin D**
BNP	**Blank nanoparticles**
S	**Silymarin**
NP	**Nanoparticles**

ABSTRACT

Nanotechnology-based therapeutics have recently emerged as an inventive and optimistic replacement for traditional therapy. Currently, biocompatible materials are used to create nanoparticles and they have the potential to deliver drugs more precisely, either inactively by enhancing the drug nanocarriers' physicochemical characteristics or actively by applying homing technologies tailored to particular tissues or cells that enable disease site targeting while minimising side effects. Because of their ability to overcome a wide range of biomedical, biological or biophysical constraints, NPs can be developed as nanoplatforms for efficient drug delivery.

Silymarin has a diverse set of in vitro and in vivo actions, including antioxidant, anti-inflammatory, dose-dependent anti-apoptotic, and cell transporter altering properties. As a result, it has the potential to be a promising medication in alternative medicine. However, oral silymarin has a low bioavailability, which restricts its medical applications. But the bioavailability of silymarin can be increased by using liposomes as drug delivery systems. In the current study, the silymarin-loaded pegylated liposomal nanoparticle was successfully created and employed as a treatment for NAFLD. Liposomal NPs can be created as nanoplatforms for the effective and targeted delivery of drugs due to their ability to pass through a number of biological, biophysical, and biomedical barriers

To overcome the drawbacks, silymarin encapsulated liposome nanoparticles were synthesized utilizing DPPE by the ‘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 is hence known a as better stabilizer for different types of nanoparticles. PEG follows the erosion-controlled release mechanisthe m of drug that resulta ed in sustained release. Hence, it is noteworthy that encapsulating the silymarin drug within liposomes and tailoring these liposome nanoparticles by PEG, is a substantial strategy to combat NAFLD.

Keywords: Chronic liver disease; drug-induced liver injury; hepatoprotective and hepatotropic effects; metabolic-associated fatty liver disease; non-alcoholic fatty liver disease; oxidative stress; silymarin.

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CHAPTER:1 INTRODUCTION

1.1 Non-alcoholic fatty liver disease (NAFLD)

One of the most prevalent causes of chronic liver disease in the West is a non-alcoholic fatty liver disease (NAFLD), whose prevalence is predicted to increase along with that of diabetes, obesity, and other symptoms of the metabolic syndrome (Mengesha et al., 2021a). The range of liver damage caused by NAFLD is broad, starting with simple steatosis and progressing through inflammation, non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Lam & Younossi, n.d.). NAFLD is currently recognized as one of the most prevalent liver diseases in the world and is generally regarded as the hepatic symptom of the metabolic syndrome. According to estimates, 2-3% of individuals in most Westernized nations have NASH while 20–30% of the overall adult population has hepatic steatosis (Longato, 2013; Qiu & Chen, 2015).

NAFLD (nonalcoholic fatty liver disease) is the accumulation of fat (steatosis) in the liver without regard to the secondary causes of fatty liver, such as use of alcohol in excess, hepatitis due to virus or specific drugs. Diabetes, hypertension, obesity, hypertriglyceridemia and hyperlipidemia, are some of the metabolic disorders that are frequently linked to NAFLD (Neuschwander-Tetri, 2017). According to studies, the prevalence of liver illnesses linked to NAFLD is growing. NAFLD is becoming a significant factor of risk for liver cancer and liver disease at end-stage due to the elevated prevalence of type 2 diabetes obesity in the world. In the following ten years, NAFLD is predicted to be among the most prevalent causes of liver transplantation (Martins & Oliveira, 2018).

NAFLD includes a number of different liver conditions. Simple steatosis (grade 1), steatosis with inflammation of lobules and inflated liver cells (grade 2), inflammation of lobules, ballooned hepatocytes, and fibrosis (grade 3) are a few of the histological grades that have been documented NAFLD evaluation (grade 3). NAFLD can lead to liver failure, cirrhosis, and hepatocellular carcinoma (Dixon et al., 2001; Lindenmeyer & McCullough, 2018).

1.2 NAFLD pathogenesis

The aetiology of NAFLD is influenced by a variety of variables. Nutrition, hormones, and genes may all have a role in the emergence of NAFLD. It is well known that the development

of steatosis is correlated with hepatic fat accumulation and insulin resistance (Carr et al., 2016).

The pathophysiology of NAFLD and its growth is a difficult process with numerous open questions. The "two-hit hypothesis" is one popular theory for explaining the pathophysiology of NAFLD and NASH (nonalcoholic steatohepatitis) (Townsend & Newsome, 2016). The initial impact is linked to triglyceride buildup in the liver. Oxidative stress, cytokines, and mitochondrial dysfunction are a few of the factors that can cause NAFLD to proceed to serious liver damage (Fang et al., 2018).

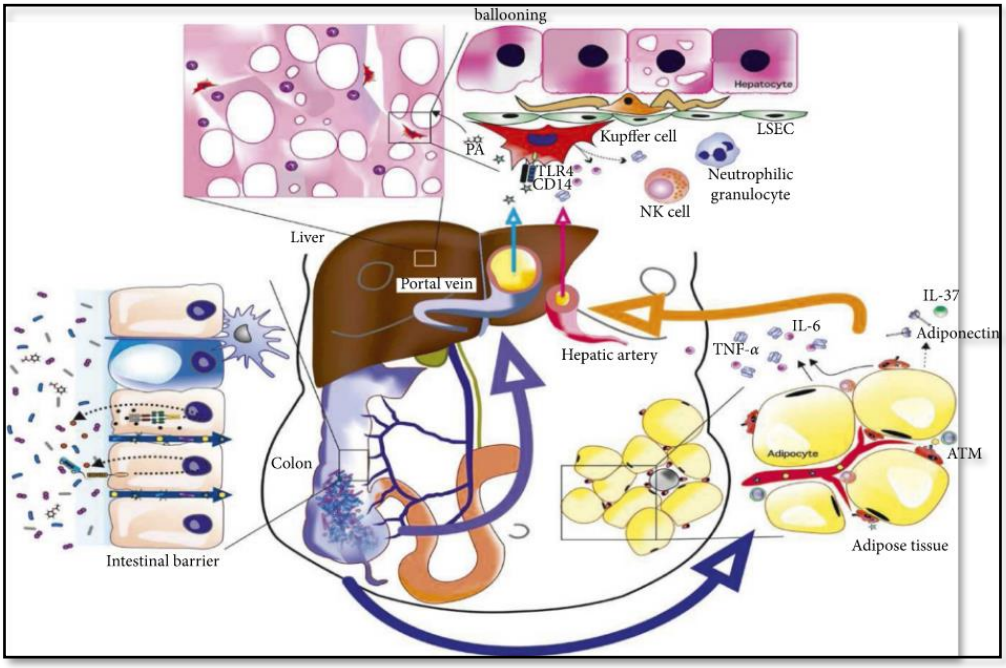

Figure 1.1: Hypothesis of many parallel impacts in non-alcoholic fatty liver disease

The pathophysiology of NAFLD, which takes into account a number of factors, cannot be entirely explained by the original "two-hit" strategy. In recent years, numerous researches have demonstrated that the gut-liver axis plays a critical role in the occurrence of NAFLD (GLA). Additionally, a lot of progress has been achieved over the past ten years, with the inflammatory role and diet with high sugar becoming important factors in the aetiology of NAFLD. The focus on genetic predispositions has increased as a result of technological advancements, and researchers have identified multiple variations in gene may affect how sugar and lipid metabolism are metabolised in the liver and as well as other organs, including adipose tissue (Figure 1.1) (Tilg et al., 2021).

1.3 Management of NAFLD

To stop the development of NAFLD into NASH and the advanced stages of hepatic fibrosis and cirrhosis, there is no effective pharmaceutical treatment currently available (Cassidy & Syed, n.d.) It is difficult to create a medicine for the treatment of NAFLD due to the complexity of pathophysiology of NAFLD, various severity levels of disease, and the heterogeneity of the patient population (Maev et al., 2020). In the last rounds of clinical testing, some projected effective medications failed (Malnick et al., 2020). Currently available treatments for NAFLD include dietary and lifestyle modifications, medicines to increase insulin sensitivity, lipid-lowering drugs, and therapies for related metabolic disorders. Additionally, research has been done on natural substances and antioxidant supplements to lessen NAFLD symptoms. The difficulty to attain therapeutic concentration in the hepatic tissue is the main disadvantage of pharmacotherapy in liver illnesses. Additionally, it can be difficult to tailor medications to certain liver tissue cells (Pettinelli et al., 2011).

The use of nanoparticles (NPs) as medication carriers has demonstrated to have enormous the possibility of managing NAFLD (Surendran et al., 2017). Because of their surface properties, size, increased absorption from gastrointestinal tract, protection from degradation and at target region increased cellular uptake, nanoparticles hold considerable potential for enhancing the bioavailability of medications. Additionally, NPs are made to collect in the desired tissue, such the liver, decrease the clearance of drug clearance, reduces the accumulation of drug in tissues other than the liver, and increases the cell specific absorption of liver (Dixon et al., 2001). In order to deliver drugs to the liver with precision, a large variety of NPs have been developed (Böttger et al., 2020). The most recent developments in the use of nanoparticles to treat NAFLD in addition to the therapeutic strategies currently being used to manage the condition (Wisse et al., 1985).

1.4 Silymarin

Silymarin is a naturally occurring substance that is found in species descended from milk thistle, or Silybum marianum. Silymarin is a member of the asteroid family (Asteraceae or Compositae). Large bright purple flowers and plenty of thorns are features of the mature shrub. The plant grows where it receives enough sunlight (Mokdad et al., 2014). Silymarin has the empirical formula $C_{25}H_{22}O_{10}$ and is a complex combination of the flavonolignan

isomers silybin, isosilybin, silydianin, and silychristin. Silymarin is the name given to its active ingredients as a whole (Khoonsari et al., 2017). The flavonoid taxifolin and at least seven flavolignans are present in the plant. Silybin, Silydianin, and Silychristin are the three most significant flavolignans that are present. Between 50 and 70 percent of the silymarin extract is silybin. Due to its favourable effects in the treatment of hepatic disorders, silymarin has been utilised as a supplementary and alternative medicine for many years (Charlton et al., 2001). However, because of its incredibly poor performance, the advantages are limited such as low bioavailability, low water solubility (50 g/mL), and inadequate intestinal absorption. In order to address these issues, nanotechnology methods seem to be a potential way to increase the effectiveness of therapeutic action (Bhattacharyya et al., 2021).

1.5 Nanotechnology

Nanotechnology-based therapeutics have recently emerged as an inventive and optimistic replacement for traditional therapy. Nanotechnology is a fast-growing field of study dealing with the the creation, exploitation, and use of materials with sizes ranging from 10 to 500 nanometers (nm), either through scaling up from single atomic groups, purification, or reduction of materials in bulk quantities into nanoparticles (NPs). Currently, biocompatible materials are used to create nanoparticles, and they have the potential to deliver drugs more precisely, either inactively by enhancing the drug nanocarriers' physicochemical characteristics (such as size and surface qualities), or actively by applying homing technologies tailored to particular tissues or cells that enable disease site targeting while minimising side effects. Because of their ability to overcome a wide range of biomedical, biological or biophysical constraints, NPs can be developed as nanoplatforms for efficient drug delivery. Approaches based on nanomedicine have recently been investigated for the treatment of liver disease (Giannitrapani et al., 2014).

1.6 Targeting Liver by Nanoparticles

Hepatocytes, Kupffer cells, and fenestrated endothelial cells make up the liver (Figure 1.2). It is known that the local hepatic macrophage population, known as kupffer cells, phagocytoses foreign particles. Negatively charged NPs are preferred by liver Kupffer cells for interaction, usually take up the majority of the nanoparticles. Kupffer cells control immunological and inflammatory reactions as well as illnesses of the liver, such as NAFLD. Liver cells are specialised epithelial cells that shows interaction with NPs, though less intensely than

macrophages. Additionally, to being involved in immunological and inflammatory responses, they also stimulate other liver cells. In contrast to Kupffer cells, the absorption of NPs in hepatocyte increases with a positive potential of zeta. Specialized endothelial cells known as liver sinusoidal endothelial cells create the interface between blood cells and liver cells (LSECs). The lack of basal lamina and open fenestrations in LSECs create a mesh-like structure that traps NPs in the liver. Stellate cells, which store fat, are essential for the development of liver fibrosis (Haute & Berlin, 2017).

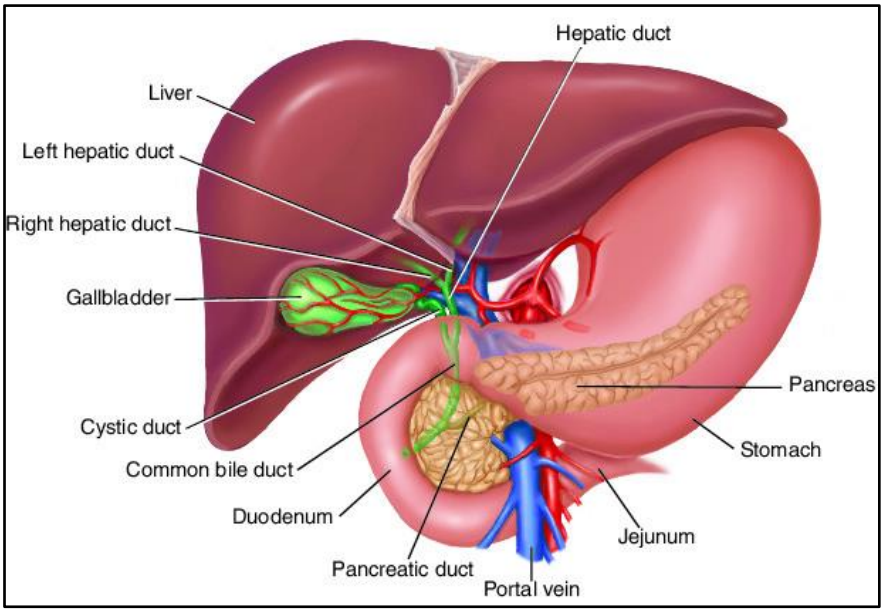

Figure 1.2: Anatomy of liver

1.6.1 Passive Targeting

A preferential buildup of NPs in the liver is known as passive targeting. This preference for the liver is explained by the fact that there are fenestrations along its endothelial barrier and no basal lamina. The bulk of NPs larger than 6 nm that are administered systemically end up in the liver. In order to transfer NPs taken orally to the liver, enterohepatic circulation is essential (Zhang et al., 2018)

Due to their placement in the liver sinusoid, nonparenchymal cells called Kupffer cells at the sinusoidal endothelium are in charge of passive targeting. Hepatocytes and hepatic stellate cells are passively targeted by smaller nanoparticles (under 100 nm), whereas nanoparticles larger than this generally congregate in sinusoidal endothelial cells and Kupffer cells. (Romero I et al., n.d.). For transport to the liver, NPs should be between 40 and 150 nm in size (Kasuya & Kuroda, 2009).

1.6.2 Active Targeting

After intravenous administration, the majority of NPs spontaneously build up in the liver thanks to RES. NPs build up in Kupffer cells, which are local macrophages of liver during passive targeting. PEGylation and active targeting are suitable tactics if the aim is other liver cells. PEGylation is the common method to prevent plasma opsonization and thus lessen non-specific macrophage entrapment (Moosavian et al., 2021).

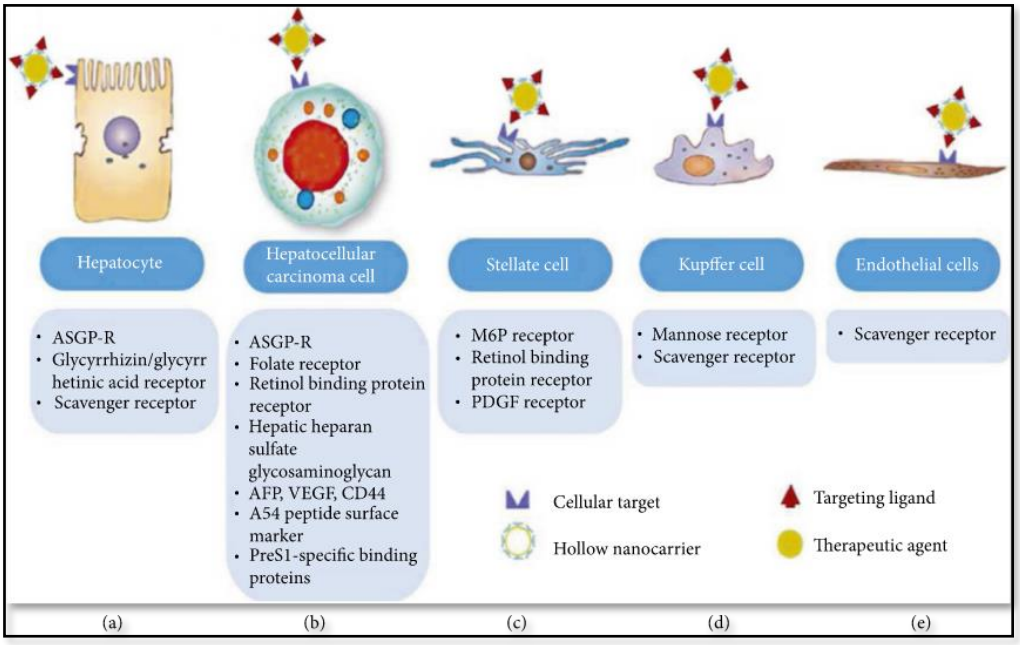

Figure 1.3: Liver cells have receptors targets for NP's that are actively targeted at the liver. Hepatocyte, hepatic stellate cells (HSC), hepatocellular carcinoma cell, endothelial cells and Kupffer cell are some examples of the types of liver cells (Wang et al., n.d.)

Utilizing affinity ligands on the nanoparticle surface for selective absorption by cells of liver is known as active targeting. The liver contains a variety of cell types with a range of distinct functions, as was previously noted. These cells express a variety of ligands with various pathogenic implications (Figure 1.3). Delivering nanoparticles to a particular population of liver cells through active targeting is a promising strategy. The treatment of liver illnesses has been studied using a variety of ligand-targeted strategies. The primary focus of treatment for NAFLD is the hepatocytes. Hepatocytes must be specifically targeted for high therapeutic effectiveness. Because different cells have receptors with similar activity, creating dynamically tailored nanoparticle carriers of drug for hepatocytes is difficult. Figure 1.3 shows a list of ligands that can be used delivery systems that are mediated by receptors (Moosavian et al., 2021).

1.7 Liposomal Nanoparticles for NAFLD Treatment

Various NPs have been used as delivery vehicles of drug for improving the therapy responsiveness in NAFLD. Several researches on the liver that targetting NPs are utilised in NAFLD treatment.

Liposomes are spherical lipid bilayers that surround an aqueous centre and can transport either hydrophobic or hydrophilic medicines (Moosavian & Sahebkar, 2019). Liposomes have been utilised in various ways as medication delivery systems ever since Bangham discovered them in the 1960s (Pantze et al., 2014). Liposomes have been used as a carrier of many compounds due to its many benefits, which include biodegradability, biocompatibility, and the capacity to carry a big payload.

To date, many nanoparticles have been used as vehicles for delivery of drug to improve therapeutic response in NAFLD. Silymarin-based formulations have logically developed along with the advancement of nanotechnologies and nanosystems used to deliver poorly water-soluble medicines and active principal ingredients (Akbarzadeh et al., 2013).

1.8 DPPE

1,2-Dipalmitoyl-sn-glycero-3-phosphorylethanolamine (DPPE), Phospholipid component with a molecular weight of 692.959 Da and empirical formula C37H74NO8P. Phospholipid component in cell membranes. For use in lipid bilayer studies and biological systems. 1,2-Dipalmitoyl-sn-glycero-3-phosphorylethanolamine is a glycerophospholipid in which a phosphorylethanolamine moiety occupies a terminal glycerol substitution site and palmitic acid occupies the other two substitution sites. Like most phospholipids PE usually has a saturated fatty acid on C-1 and an unsaturated fatty acid on C-2 of the glycerol backbone but the fatty acid distribution at the C-1 and C-2 positions of glycerol within all phospholipids is continually changing, owing to phospholipid degradation and the continuous phospholipid remodeling that occurs while these molecules are in membranes. PEs are neutral zwitterions at physiological pH. PE is frequently the main lipid component of microbial membranes and the second most abundant phospholipid in mammals, comprising as much as 45% of brain lipids. They are concentrated in mitochondria and are key building blocks of membrane bilayers where they are distributed asymmetrically with the majority confined to the inner leaflet. It appears that a primary role for PE, in bacterial membranes at least, is simply to dilute the high negative charge density of the anionic phospholipids. PE acts as a chaperone

in transport membrane folding (Bogdanov & Dowhan, 1999). In animals PE is involved in the secretion of very-low-density lipoproteins and aids in membrane fusion and fission (Vance, 2008). In plants lyso PE retards senescence by inhibiting phospholipase D. PE is the precursor to many important lipids, acts as a protein transport from the membrane to the vacuole, and is synthesized through the CDP-ethanolamine or the PS decarboxylation pathway. After being converted to diacyl glycerol PE acts as a second messenger (Lang et al., 1995).

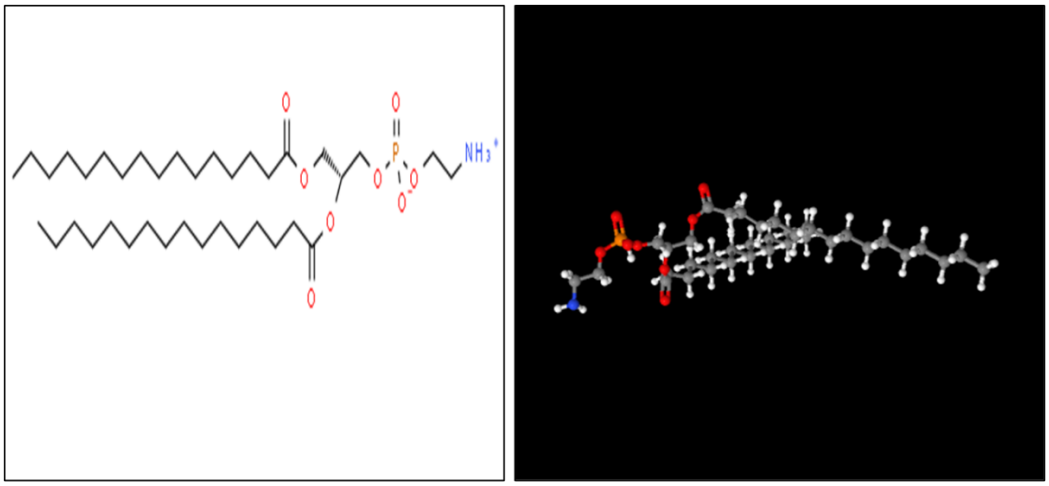

Figure 1.4: 2D and 3D structure of DPPE

CHAPTER:2 LITERATURE REVIEW

2.1 Silymarin

Silybum marianum L. commonly known as **Milk Thistle plant (کٹارہ اونٹ)** belongs to the family of **Carduus marianum**. It has proved its significance in viedic medicine and has a 2000 years old history of being used as a curative medicine against liver disorders and inflammation. It has proven its significance against multiple gallbladder disorders as well as against venoms such as snake bite, mushroom poisoning and insect stings. This plant has can be found abundantly in North America, Mexica and Canada. In Pakistan, it can be found in Kashmir. It has a thorny appearance with large leaves and purple – reddish flowers. Seeds and fruit are considered as major medicinal part of this plant. **Dioscorides (40 – 90 AD)**, a Greek physician and botanist discovered milk thistle's healing properties. It was titled as "best remedy against melancholy diseases" by **John Gerard** in 1597 (Siegel et al., 2013).

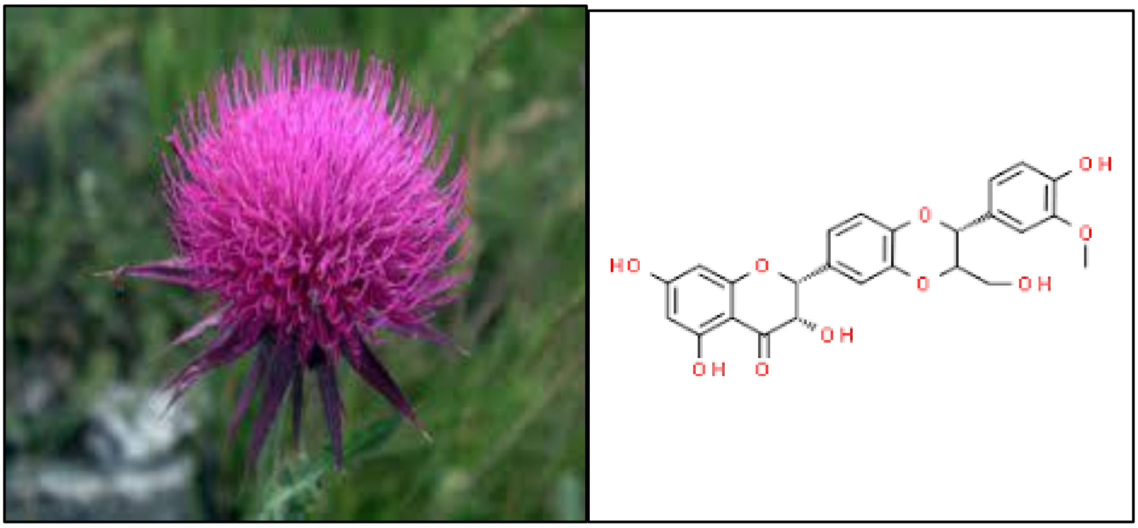

Figure 2.1: Structure of Silymarin

The antioxidant and hepatoprotection activities of milk thistle are mainly credited to the complex of flavonolignants present in it known as Silymarin. Silymarin is the active component of milk thistle plant, comprised of flavonolignants like silybin A, silybin B, isosilybin B, isosilybin A, silydianin, silyhermin, silychristin and neosilyhermin. This component is majorly present in fruit and seeds of this plant (Karimi et al., n.d.).

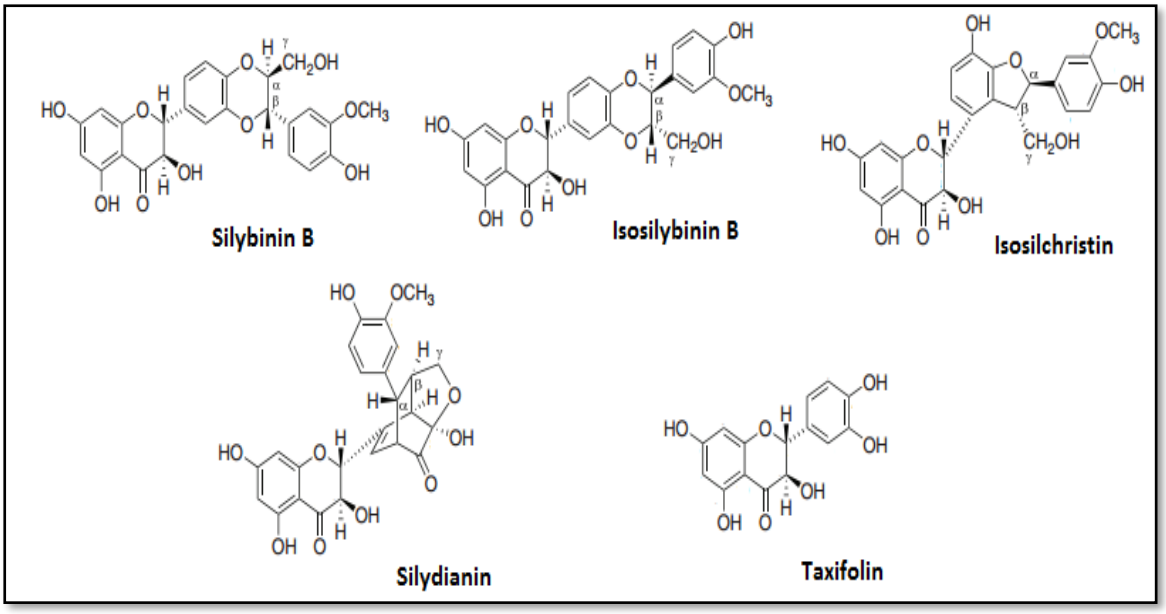

Figure 2.2: Structure of silymarin components

Silymarin has reported as an immunomodulating, anti-inflammatory, antifibrotic and as an antioxidant agent by proving its scavenging mechanism against free radicals. Multiple studies have accepted it as a safe herbal medicine, proving its therapeutic significance without causing toxicity (Kaur & Agarwal, 2007).

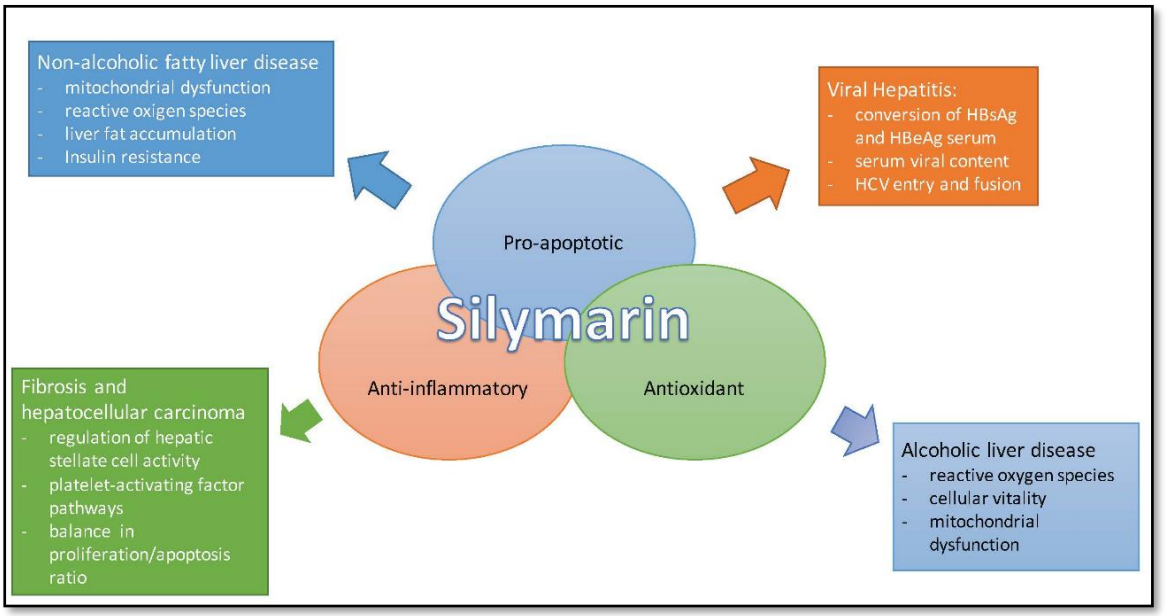

Figure 2.3: Multitargeted activity of Silymarin

2.2. Pharmacodynamics of Silymarin

The pharmacological effects of silibinin include anti-inflammatory, antioxidant and antifibrotic characteristics and regulation of insulin resistance.

2.2.1 Antioxidant properties of silymarin

The ability of silymarin to use scavengers, which enables the eradication of free radicals, gives birth to its antioxidant qualities(Tighe et al., 2020).

The antioxidant properties of silymarin may operate through a variety of mechanisms. These include scavenging of free radicals, intestinal ion chelation, encouraging the manufacture of protective molecules, and activating antioxidant enzymes. They also include the suppression of the enzymes that produce reactive oxygen species, which prevents free radical development (Surai, 2015). Sirtuin 1 activity, poly-(ADP-ribose)-polymerase function, and AMP-activated protein kinase activity—all significant regulatory processes associated with oxidative stress—have all been shown to be restored by silymarin's antioxidant characteristics (Akhtar et al., 2019). By inhibiting peroxisome proliferator-activated gamma receptor, fatty acid synthase, acetyl-CoA carboxylase and silymarin's antioxidant effects also improve hepatic lipid homeostasis by lowering de novo lipogenesis. (Salomone et al., 2017).

2.2.2. Antifibrotic properties of silymarin

Silymarin's antifibrotic effect is principally brought on by its capacity to prevent hepatic stellate cells from developing into myofibroblasts by blocking fibrogenic pathways involved in ETC, profibrogenic collagen and development of cytoskeleton. (Tighe et al., 2020). In particular, silymarin down-regulates TGF-ß1 mRNA, causes inhibition of NF-kB, and causes suppression of hepatic stellate cells activation. These results are supported by research using animal models, where silymarin was discovered to slow the development of early fibrosis. (Lieber et al., 2003; Trappoliere et al., 2009).

2.2.3. Anti-inflammatory properties of silymarin

Silymarin's immunomodulatory function reduces inflammation by preventing the inflammasomes and NF-kB from activating, which are crucial for controlling the immune response in inflammatory conditions (Tighe et al., 2020). Additionally, silymarin can repair the insulin receptor substrate-1/PI3K/Akt pathway and activate the farnesyl X receptor, both of which can lower steatosis and insulin resistance brought on by NAFLD. It has also been

demonstrated that silymarin's anti-inflammatory and antioxidant properties lessen virus-related liver damage in chronic HCV infection (Abenavoli et al., 2011)

2.2.4. Anti-toxin properties of silymarin

The principal methods by which silymarin prevents additional damage in cases hepatic injury caused by drug/toxin-related are through altering the permeability of membrane and the competitive inhibition of toxins at specific binding sites. This stops these hazardous substances from being absorbed, especially in the hepatic phalloidin-transporting system (Trakulsrichai et al., 2017).

2.2.5. Anti-cancer properties of silymarin

Additionally, silymarin exhibits anti-cancerous properties thought to be related to the oxidative stress supression, blocking of the mitochondrial pathway, stopping of the cell cycle and encouragement of apoptosis. At various stages of hepatocarcinogenesis, silymarin's anticancer effects have been demonstrated in in vitro and in vivo studies as well as animal models with HCC (initiation, promotion, and progression). Silymarin is ideally suited as a prospective therapy for CLD patients due to its capacity to promote hepatic regeneration, which is another crucial quality. Particularly, there is a connection to ribosomal RNA synthesis, perhaps involving the activation of polymerase I (Tighe et al., 2020).

2.3 Silymarin and NAFLD

The multifactorial liver injury known as non-alcoholic fatty liver disease (NAFLD), which poses a considerable risk for HCC, is one of the causes (Tighe et al., 2020). It comprises a broad range of liver damage in non-alcoholic consumers (20 g/day ethanol intake), which is defined by histological abnormalities of alcoholic cause (varying from fatty liver with no complications to steatohepatitis, fibrosis, and ultimately cirrhosis). While oxidative stress and insulin resistance are the key factors that contribute to its development, the precise cellular and metabolic pathways are still poorly known (Golovenko et al., 2016; Loria et al., 2003)

Researchers have discovered four potential contributing factors: insulin resistance, aberrant cytokine production, and fatty-acid metabolic disruption. Hepatocyte injury is brought on by the confluence of these processes and might take the form of direct oxidative damage, apoptosis or inflammation induced by tumour necrosis factor-alpha (TNF-) (Mehta et al., 2002). Pure steatosis is the first stage of this condition, and the second stage is characterised by steatohepatitis (non-alcoholic steatohepatitis, NASH) or fatty liver cirrhosis. Modifying

risk factors is the main goal of NAFLD treatment at the moment (obesity, diabetes mellitus, and hyperlipidemia). Several different therapy modalities have been tried, with various degrees of efficacy (Banini & Sanyal, 2017; Mccullough, 2002).

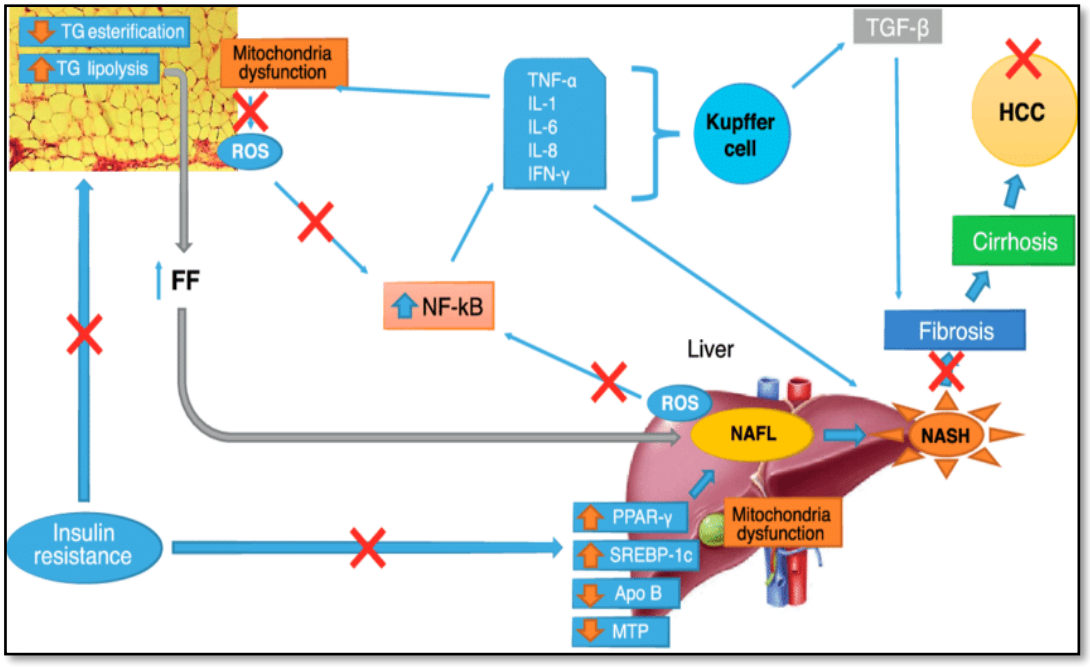

Figure 2.4: Mechanism of action of Silymarin

Oxidative stress and dysfunction of mitochondria are important factors in the pathogenesis of NAFLD. Silymarin has been discovered to have antioxidant, anti-inflammatory, and antifibrotic characteristics in cases of chronic liver disease (Serviddio et al., 2010). Various publications have shown the positive effects of silybinin, silybin-phospholipid complex, silymarin and silybin Vit.E-phospholipid complex in a variety of laboratory models and clinical trials.(Federico et al., 2017a).

These therapeutic strategies successfully avoided severe oxidative stress and maintained the bioenergetics of the hepatic mitochondria in NASH/NAFLD generated in several animal models or in human disease. The silybin-phospholipid complex has antifibrotic and anti-inflammatory characteristics because it partially prevents the changes in the fatty acid composition of mitochondrial membranes brought on by a diet low in methionine and choline. The silybin-phospholipid complex regulates the increased vulnerability of lipid membranes to oxidative degradation by preserving mitochondrial function. (Farrell & Larter, 2006).

2.4 Hepatoprotection

The term "hepatoprotective agent" refers to a substance that, when pretreated with silibinin, can delay or stop the progression of fibrosis or hepatic injury caused by toxins such ethanol, galactosamine, phalloidin, and CCl4. There is no evidence that silibinin can stop liver illness brought on by medications or chemicals, with the exception of a few case reports. This makes it difficult to translate these data into human disease.

The largest experience was probably preventing death cup (Amanita phalloides) intoxication using intravenous silibinin. A specific antidote for amanitin is called silibinin (Serviddio et al., 2010). The stimulation of nucleolar polymerase A, which boosts ribosomal protein synthesis and prevents lipid peroxidation, contributes to the effect of mushroom poisoning. Again, there are no available controlled data.

In conclusion, silibinin is a pharmacologically active substance with numerous features that may be able to treat liver conditions caused by a variety of different factors(Feher & Lengyel, 2012; Gillessen & Schmidt, 2020). Unfortunately, there aren't enough high-quality prospective trials to prove its clinical effectiveness. Furthermore, the use of silymarin in medicine is constrained by its low bioavailability. Better planning may avoid this issue (Farrell & Larter, 2006).

2.5 Vitamin D and NAFLD

The body uses vitamin D, a fat-soluble vitamin, for a variety of purposes. Numerous studies have demonstrated the value of vitamin D supplementation in treating NAFLD. Vitamin D supplementation's significance in NAFLD is still debatable, though. In certain studies, vitamin D has been encapsulated in nanoparticles to increase its stability and bioavailability, while in other studies, its antioxidant and anti-inflammatory properties have been examined.

El-Sherbiny et al. contrasted vitamin D NEs with traditional formulations in terms of chemical stability, solubility and bioavailability. They made vitamin D NEs from pea protein, and they found that rats responded better to them than the existing commercial formulation (Eliades & Spyrou, 2015).

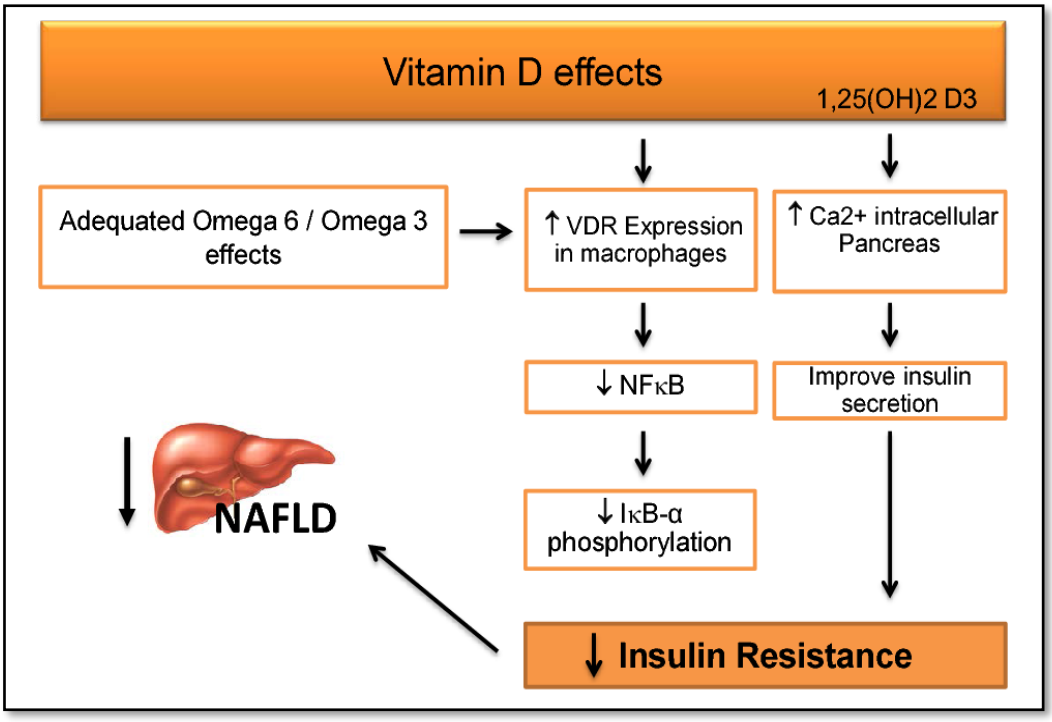

Figure 2.5: Vitamin D may limit the progression of NAFLD through multiple, potentially interacting mechanisms.

2.6 Vitamin E and NAFLD

Vitamin E plays a crucial role in the apoptotic and cell death processes (el Hadi et al., 2018; Perumpail et al., 2018). The potential of intracellular membrane of mitochondria was decreased, increased BCL-2, which is an anti-apoptotic protein and and reduction of pro-apoptotic proteins p53 and BAX, according to recent studies (Abd Ellatif et al., 2018).

Additionally, in the pathway of apoptosis in mitochondria (Fas/FasL), it reduces the activity of cytochrome C and caspase-9,8 and 3. In NAFLD, vitamin E can limit the expression of the cytokines TNF-alpha, IL-1, IL-2, IL-4, IL-6, and IL-8 as well as COX-2 and prevent NF-B from localising in the nucleus. (Oliveira et al., 2003; Tzanetakou et al., 2012).

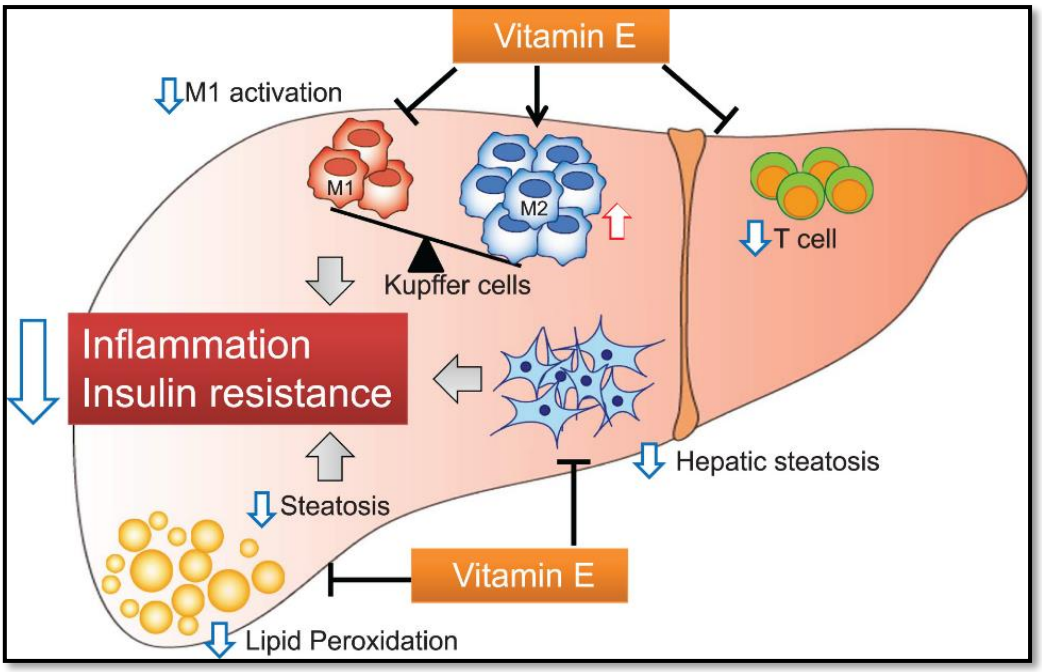

Figure 2.6: Role of Vitamin E in NAFLD

2.7 Liposomes, as a delivery system

Liposomes can increase the therapeutic activity and safety of medications as a delivery mechanism. Additionally, liposomes have been effectively used in drug delivery systems to increase some medicines' solubility and bioavailability (Yang et al., 2015). Water-soluble and lipophilic medications can be contained within lipid bilayer-containing liposomes, and their in vivo behaviour can be modulated by altering the surface features of the liposomes, such as by adding ligands for targeting and/or PEG to lengthen blood circulation duration. Consequently, a lot of research has been done on liposomes as drug delivery vehicles (Federico et al., 2017b). The reticular endothelial system (RES) frequently clears liposomes when given intravenously (Elmowafy et al., 2013). Liver, being the largest internal organ in human body contains kuppfer cells in which liposomes when injected, accumulates relatively heavily, especially in non-parenchymal cells. To provide a better therapeutic effect, liposomes must be preferentially taken up by liver parenchymal cells. Using receptor-mediated endocytosis to deliver medications to particular cell types is a promising technique (Zhong et al., 2017).

2.8 Synthesis of Liposome Nanoparticles

All methods for preparing the liposomes include stages such as drying the lipids from organic solvent and dispersing them in aqueous media followed by purification of the resulting liposome and then 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).

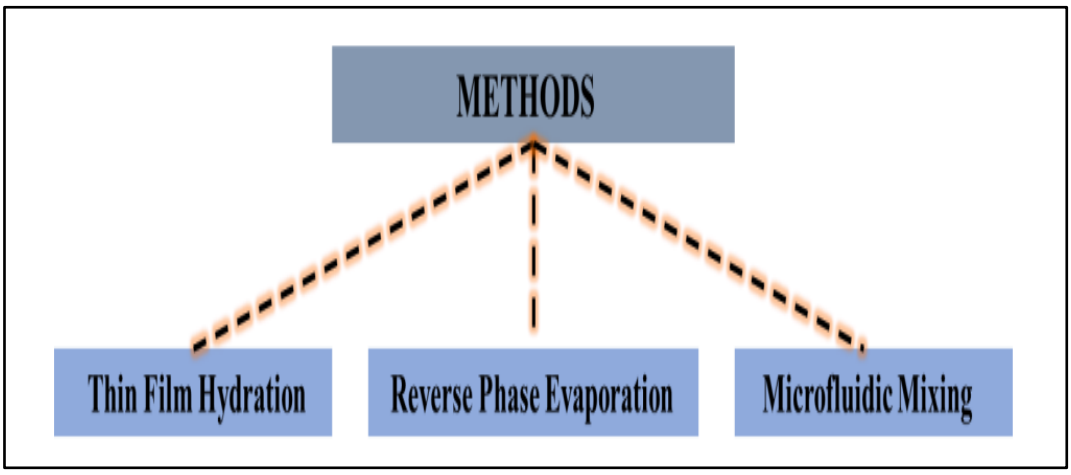

Figure 2.7: Different methods used for the synthesis of liposomes Nanoparticles

2.8.1 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).

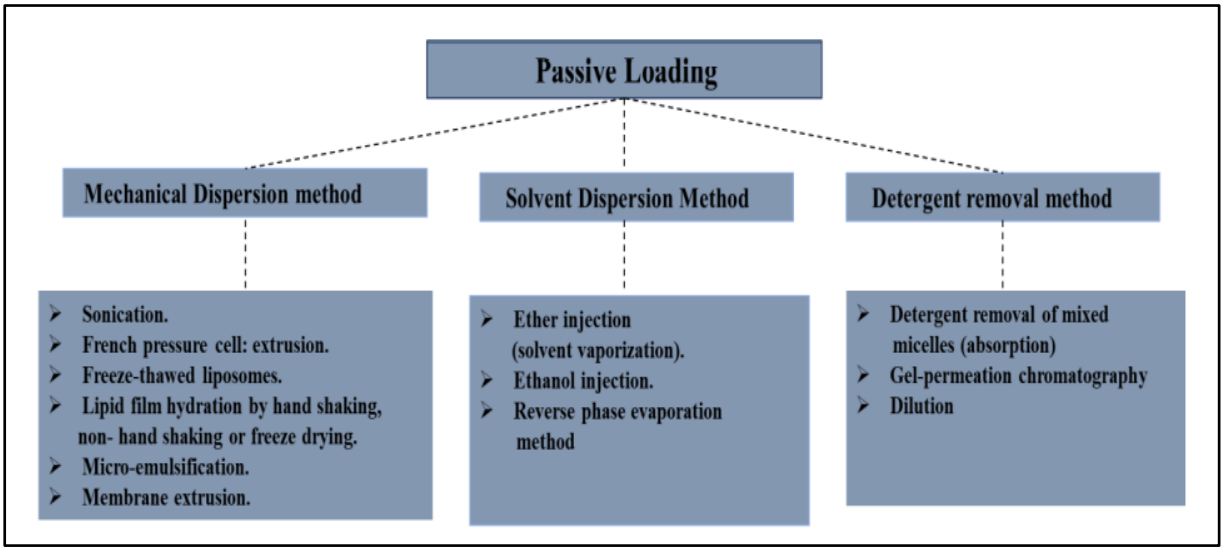

Figure 2.8: Different types of methods used in Passive loading

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).

2.9 Induction of NAFLD by CCL4

Acute liver injury can be brought on by the hepatotoxic compound CCl4, which was the first chemical utilised to create a toxic fatty liver model. By generating activated oxygen-free radicals, CCl4 speeds up the fatty acid degeneration and fibration processes and causes the breakdown of the hepatocellular structure and function. As a result, Kupffer cells are induced by CCl4 to produce pro-inflammatory liver damage is made worse by cytokines. In every two weeks, a peritoneal injection of CCl4 into mice causes severe liver toxicity with deteriorated, enlarged, and enhanced transaminase and lipid levels, as well as necrotic hepatocytes. When administered intragastrically to mice, similar outcomes were obtained (F. Zhong et al., 2020).

Model animals are vulnerable to poisoning, which can readily and quickly produce fatty liver in the model. Additionally, compared to human fatty liver, the pathophysiology, course of the

disease, and histomorphological alterations are different. Contrarily, CCl4 causes fibrosis but neither obesity nor IR. Therefore, when creating NALFD models, a different diet, such as the HFD or CDAA diet, can be combined with CCl4. When obese HFD-fed animals get numerous CCl4 administrations, the liver experiences fibrosis, apoptosis, and inflammation (F. Zhong et al., 2020).

2.10 Objective

The research work in dissertation is presented in two parts. The first part of this research emphasized upon the liposomal nano formulation of silymarin 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 non-alcoholic fatty liver disease in vivo analysis.

The second elaborated part emphasized upon the NAFLD 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- inflammatory activity of nanoparticles In well-established NAFLD model was also the prime focus of our research and will be considered a significant step to uplift these liposome encapsulated silymarin NP's to preclinical trial levels.

CHAPTER:3 MATERIAL AND METHODS

3.1 Experiment Design

3.1.1. Materials

1,2-bis(diphenylphosphino)ethane (DPPE), Cholesterol, commercially available Silymarin drug, Polyethylene glycol (PEG- Molecular weight 2000), and 100% pure vitamin E oil were purchased from Sigma-Aldrich USA. Urethane, peanut oil, vitamin D (2,00000 IU) and carbon tetrachloride (Ccl4) were purchased from Strem chemicals. Wistar 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 silymarin loaded-liposomes Nanoparticles

Liposome components DPPE and cholesterol were employed in a 4:1 ratio for the production (percent molar ratio). Weighted lipids were first dissolved in 100 mol of ethanol after being weighed. In ethanol, 200 molar solution of silymarin was produced, from which 500 µl of the drug solution was extracted and combined with the lipid solution. For 40 minutes at 80 MHz, the sonication of mixture was carried out. The lipid phase and 10mL of water were then each allowed to warm in a water bath until 60°C temperature was attained. Water phase and lipid phase were combined, and the resulting dispersion mixture was continuously stirred for 10 minutes at 90 RPM. To remove the ethanol, this new combination was once more sonicated for 40 minutes at 50 MHz before evaporation in rotary evaporator above the temperature of phase transition or 50 °C. Finally, a Dialysis tube was used to remove the unentrapped medication (Chorachoo et al., 2013; Meng et al., 2016)

3.1.3 Pegylation of Silymarin loaded- LNPs

The combination of silymarin-loaded LNPs was diluted to a volume of 50 ml, added drops at a time while being continuously stirred, and then subjected to rotational evaporation until 10 ml of solution remained. Unentrapped medication was released via a dialyzer tube. (Stiufiuc et al., 2013).

3.2 Physical Characterization

Characterization of PEG nanoparticles was done for the evaluation and analysis of size, surface charge, shape, drug release and encapsulation efficiency of NP's. (Farooq & Fatima Rana, 2020).

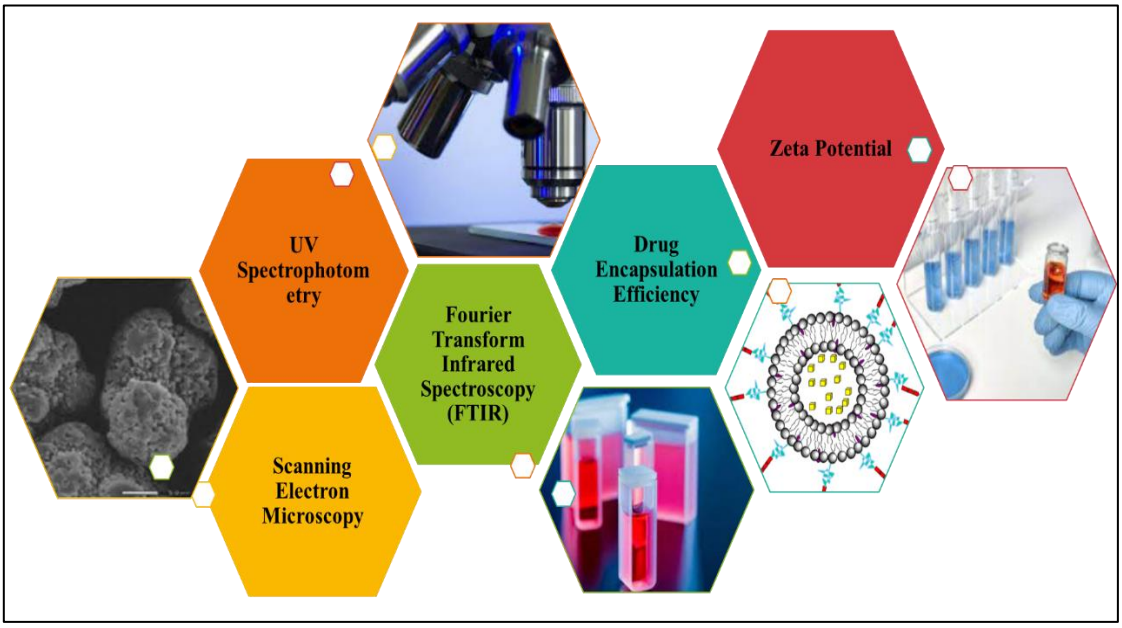

Figure 3.1: Physical Characterization of nanoparticles

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 silymarin loaded – LNPs and Pegylated silymarin loaded LNPs were measured by using Shimatzu UV-Vis 2800 BMS Scientific Technical Corporation (PVT) spectrophotometer, from 200-450nm at a resolution of 1nm. As a reference during UV analysis, the de-ionized water was used. The UV spectra of silymarin drug, LNPs loaded with and without drug, and PEG-Coated drug loaded LNPs were recorded (Farooq & Fatima Rana, 2020).

3.2.2. Fourier transform infrared spectroscopy (FTIR) analysis

Fourier transforming infrared spectroscopy (FTIR) is an analytical technique used for the identification of inorganic and some organic materials. This technique involves measuring of 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 IR radiation that is absorbed, normally causes excitation of molecules into a higher vibrational state. The absorbance of light wavelength by single molecule is a function of the difference in energy between the excited vibrational and resting states. Wavelengths that are absorbed by the sample are the characteristic of its molecular structure (Khan et al., 2019; Tran & Tran, 2020).

Samples were air dried before being processed for FTIR analysis using compressed KBr discs. FTIR spectra were captured using a Bruker FTIR Spectrophotometer ALPHA II between 400 and 350 cm-1. All formulation ingredients, including silymarin medication, blank liposomes, cholesterol, pegylated-liposome nanoparticles, DPPE underwent FTIR analysis (Farooq & Fatima Rana, 2020).

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. In binary and/or threshold pictures, the 'Analyze Particles' command counts and measures items. It operates by scanning an area or image until the edge of an object is found. Values for particle size are provided in the 0 to 'Infinity' range. In this region, particles with circularity values beyond the defined range will likewise be disregarded. examined the

8-bit binary image containing the best-fitting ellipse (cf. Edit. Of the observed particle, Fit Ellipse) (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. Gold was then spat over the slide surface for 50 seconds at mA. Images were captured at the National University of Science and Technology, Islamabad, using a VEGA3 LMU scanning electron microscope. Malvern Zeta Sizer Ver. 7.12 was used to measure the size distribution and dispersity of both types of NPs using Dynamic Light Scattering (DLS) (Farooq & Fatima Rana, 2020).

3.2.4. Zeta Potential

The difference of potential across phase boundaries between liquids and solids is known as the zeta potential. It is a measurement of the electrical charge of a suspended particle in liquid. Zeta potential is frequently the sole value that demonstrates the double-layer qualities of the colloidal dispersion because it is not equivalent to the electrical surface potential of a double layer or to the Stern potential. 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. A less negative than -15 mV potential of zeta typically represent the particle agglomeration beginning. The precipitation of colloid into a solid will occur 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. (Farooq & Fatima Rana, 2020).

3.2.5. Drug Encapsulation and Release Efficiency

Drug efficiency delineates the amount of drug to be entrapped with in the vesical of liposomes. A feasible linear standard curve was constructed by analysing various drug dilutions using a UV spectrophotometer at 250 nm absorbance in order to determine the effectiveness of drug encapsulation. A formula Y=mx+c was discovered. The unentrapped medication was calculated further using this standard curve value. To find the drug fraction

that was not entrapped, centrifugation of samples was carried out at 4500 rpm for 1 hour. The supernatants were then examined using UV-visible spectroscopy (Nii & Ishii, 2005). Following that, calculated values were incorporated into the formula.

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

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 in PEG-LNPs was examined up to 15 hours along with the addition of specified volume of phosphate buffer saline. From 25 ml solution of PEG LNPs, 3ml sample was placed into separate 15 ml centrifuge tube and allowed it for centrifugation for 10 mins at 4500 Rpm and 25ºc. PEG-LNPs solution received 3 ml of phosphate buffer saline in contrast. The supernatant was made available for UV spectrophotometer examination after centrifugation. The same process was carried out after 1, 2, 4, 6, 12 and 15 hours. Values of absorbance were measured at a wavelength of 250 nm and used to represent cumulative drug release. A control solution made up entirely of empty nanoparticles was used throughout the whole analysis (Farooq & Fatima Rana, 2020).

3.3 Development of Non-alcoholic fatty liver disease Model

3.3.1 Animals

45 Wistar female rats weighing 100-120g and aged 4-6 weeks were utilised in this study. The rats were maintained in separate cages with access to food and water for a 12-hour dark/light cycle. The temperature was set at roughly 27°C, with a humidity of 60-70%. To perform the histopathological study, the rats were anaesthetized using chloroform. The regulation of good laboratory practise established by the US FDA (Food and Drug Administration) in 1978 concentrated on rat handling and care (Farooq & Fatima Rana, 2020).

Figure 3.2: Female Wistar rats

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 (CCl4), peanut oil (a delivery agent), Ethanol (de-contaminant) and 10% neutral formaldehyde buffer (dissolved in PBS).

3.3.3. NAFLD Induction

The rats were initially released for two weeks (acclimatization phase). A total of 45 rats were split into two groups. 5 rats were used as a negative control, with no adverse reactions during the entire experimentation, whereas 40 rats were subjected to harmful compounds by intraperitoneal injections and then divided into eight treatment groups, each with five rats. Initially, 2.5% urethane was dissolved in DMSO (Dimethyl sulfoxide) and administered intra-peritoneally twice a week for the first two weeks of induction phase.

The purpose of the urethane exposure is to significantly damage the liver, shortening the time needed to induce nafld. Following that, Ccl4 was combined with pure Peanut oil (50% v/v), and intraperitoneal injections of 1ml/kg were administered twice weekly for the remaining four weeks of induction phase. (Fortea et al., 2018; Gitiara et al., 2017).

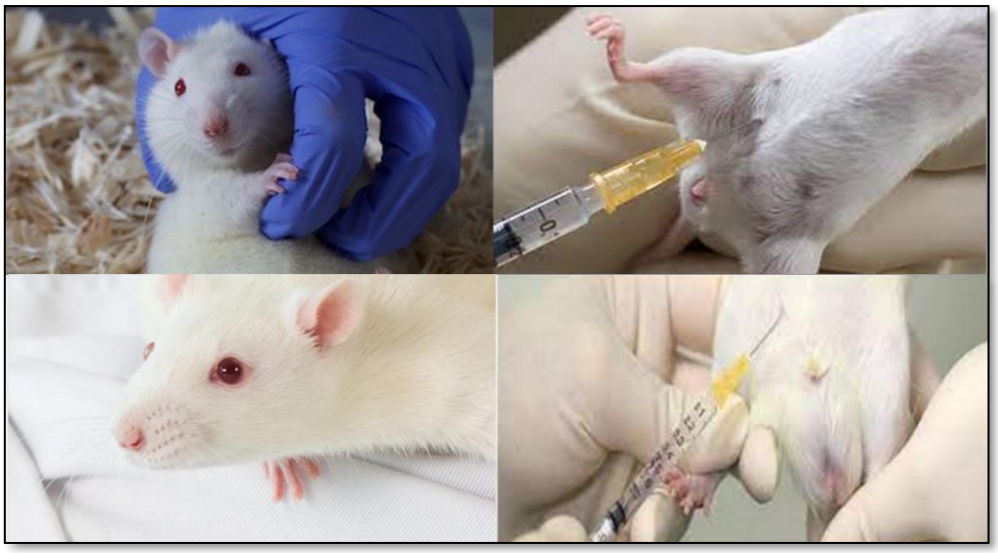

Figure 3.3: Induction of NAFLD in Wistar rats via intraperitoneal injection

3.3.4. Outcomes

Different conditions like; consumption of food and water, mortality rate, body weight and weight of liver were taken in consideration

3.3.5. Serological Indices

From the heart of rat, blood was extracted 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.

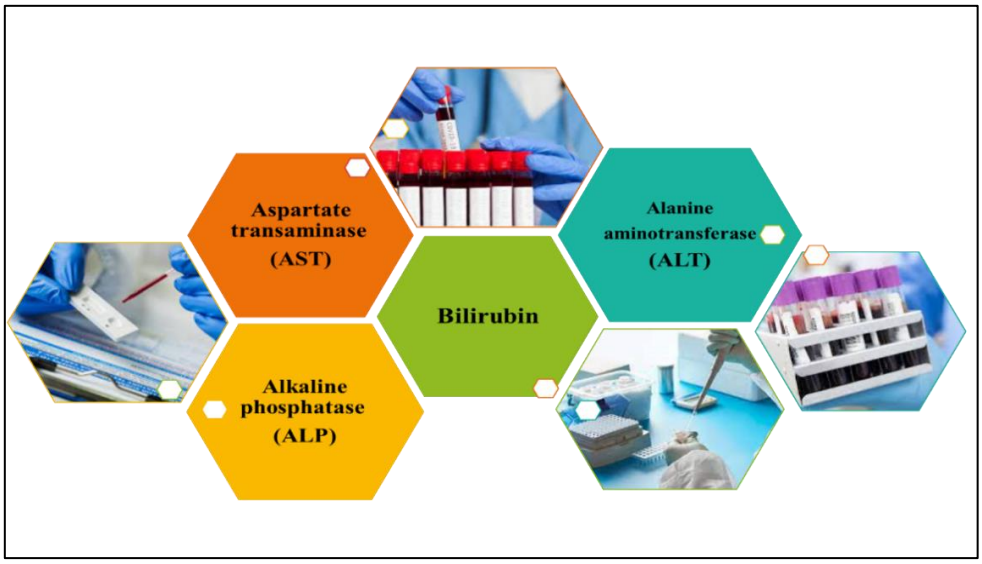

Figure 3.4: Serological testings

Clinical Research Network Scoring System- Definitions and Scores (Tajima et al., 2013) were modified in light of variations in our histology observations (Figure:3.36). Changes made in relation to "Piecemeal Necrosis" include scores of 1, 2, 3, and 4 for necro inflammation at mild (few portal areas), mild/moderate (most portal areas), moderate (constant around 50% of tracts or septa), and severe (constant around >50% of tracts or septa) (Knodell et al., 1981). The most common etiologies of chronic liver disease were scored using the METAVIR scoring system, which was created in France in 1993 and has been modified for histological staging of liver disease (Bedossa, 1993; Bedossa & Poynard, 1996).

Steatosis		
Grade	Parenchymal involvement	
	<5%	0
	5–33%	1
	33–66%	2
	>66%	3
Inflammation		
Lobular inflammation	Assessment of all inflammatory foci	
	No foci	0
	<2 foci per ×200 field	1
	2–4 foci per ×200 field	2
	>4 foci per ×200 field	3
Portal inflammation	Assessed under low magnification	
	None to minimal	0
	Greater than minimal	1
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
	Septal/bridging fibrosis	3
	Cirrhosis	4

Table: 3.1 NASH/NAFLD Clinical Research Network Scoring System (Definition and score)

3.4 Treatment Design

For evaluation of anti-NAFLD effects of SNPs, diseased rats were taken within experiment. Rats were categorized into different groups.

3.4.1. Positive Control Group

A set of five diseased rats were isolated and assigned the tag as positive control (disease group). This group of rats were left untreated throughout the experiment, and the survived rats were undergone ultrasound imaging and dissected at the end of experiment for histopathological and serological analysis. Body and liver weight and mortalities were noted.

Figure 3.5: Administration of dose via oral gavage

3.4.2. Negative Control group

A set of five diseased rats were isolated and assigned the tag as negative/normal control. Throughout the entire procedure, this group of rats was not subjected to any adverse chemicals and the survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation, and serological testing. Body and liver weight and mortalities were noted.

3.4.3. Silymarin treated Oral Gavage Group (S group)

Five rats were placed in this group. Silymarin drug at the dose of 1mg/kg were given intravenously for the duration of 15 days. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation, and serological testing. Body and liver weight and mortalities were noted.

3.4.4. Silymarin loaded Pegylated Liposomal Nanoparticles treated Oral Gavage Group (Group SNP)

Five rats were placed in this group. Liposomes Nanoparticles Dose at the dose of 500µg/kg were given orally for the duration of 15 days. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation and serological testing. Body and liver weight and mortalities were noted.

3.4.5. Vitamin E oral gavage group (Group Vit.E)

Five rats were placed in this group and were fed a standard laboratory chow for 12 weeks. For 15 days, rats were given vitamin E supplementation (100 mg/kg/day) dissolved in fresh milk with the help of oral gavage from day 1 till the end of the experiment at day 15. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation, and serological testing. Body and liver weight and mortalities were noted.

3.4.6. Vitamin E and Peg- Liposome Nanoparticles treated Oral Gavage Group (Group SNPE)

Five rats were placed in this group and were fed a standard laboratory chow. For 15 days, rats were given vitamin E supplementation (100 mg/kg/day) dissolved in fresh milk along with liposomal nanoparticle dose with the help of oral gavage from day 1 till the end of the experiment at day 15. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation and serological testing. Body and liver weight and mortalities were noted.

3.4.7. Vitamin D oral gavage group (Group Vit.D)

Five rats were placed in this group and were fed a standard laboratory chow for 12 weeks. For 15 days, rats were given vitamin E supplementation (2000 IU/day) dissolved in fresh milk with the help of oral gavage from day 1 till the end of the experiment at day 15. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation and liver function tests. Body and liver weight and mortalities were noted.

3.4.8. Vitamin D and Peg- Liposome Nanoparticles treated Oral Gavage Group (Group SNPD)

Five rats were placed in this group and were fed a standard laboratory chow. For 15 days, rats were given vitamin E supplementation (2000 IU/day) dissolved in fresh milk along with liposomal nanoparticle dose with the help of oral gavage from day 1 till the end of the experiment at day 15. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation, and serological testing. Body and liver weight and mortalities were noted.

3.4.9. Blank Pegylated Liposomal Nanoparticles treated Oral Gavage Group (Group BNP)

Five rats were placed in this group. Liposomes Nanoparticles Dose at the dose of 500µg/kg were given orally for the duration of 15 days. The survivors were undergone ultrasound imaging and dissected at the end of the experiment for histological investigation, and serological testing. Weights for the body, the liver, and ascites were noted.

3.4.10. Ultrasound Imaging of the Liver

Rats were starved for the whole night prior to the ultrasound scans. Rats were subsequently placed in the supine position after receiving an intraperitoneal injection of 4% chloral hydrate to induce anesthesia. Before each imaging session, using commercial hair removal cream (Veet), the abdomen of rat was depilated. A medical ultrasound gel (Doppler gel) was applied to the depilated skin. An ultrasound diagnostic probe (10MHz 128element Wireless Ultrasound Linear Probe; MSLPU42) was applied to examine the rat liver conditions. The rat liver was identified by wireless USG images of the liver that were collected through the ventral body wall in transverse and sagittal orientations.

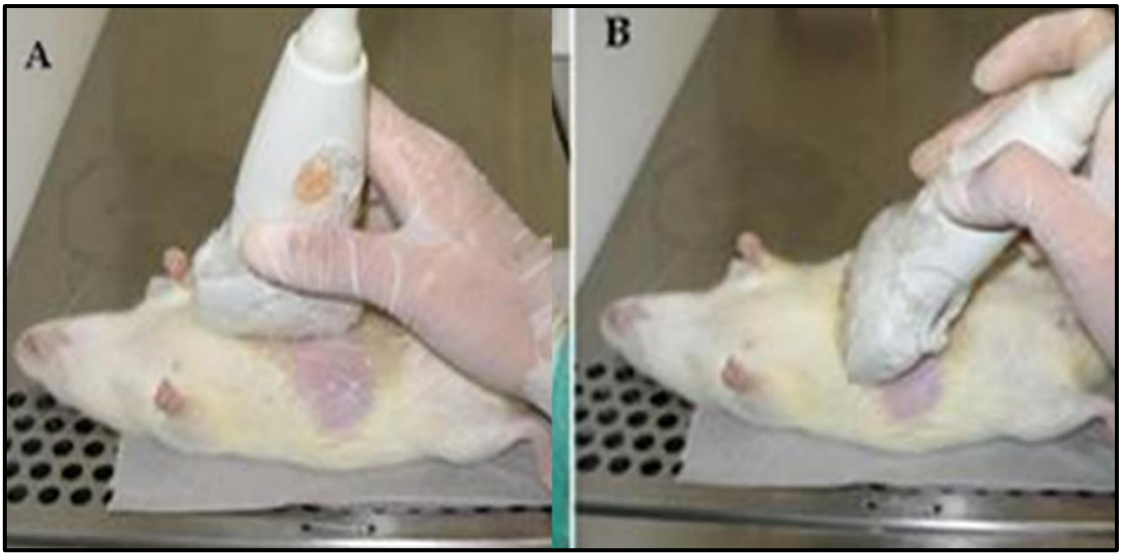

Figure 3.6: Ultrasound imaging of Rat liver

This whole procedure was carried out in the presence of a professionally certified radiologist. The liver was seen in the intercostal space transversely and longitudinally. The transducer transmits ultrasonic impulses at 10 MHz or 14 MHz and has a flat linear array with a 25-mm footprint. The probe vendor's proprietary "Wireless USG" software application, which is accessible as a free download from the Google Play Store and Apple App Store, was installed on an Android smartphone. The transducer was connected to Wi-Fi. The ultrasound images were then interpreted by the radiologist.

CHAPTER:4 RESULTS

4.1 Physical Characterization of silymarin loaded – LNPs and Pegylated LNPs

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

4.1.1. UV-VIS absorption spectroscopy

UV-VIS absorption spectroscopy of silymarin drug showed the surface plasmon resonance (SPR) peak mainly at 330nm, blank liposomes at 220nm & 280nm while pegylated silymarin loaded-LNPs showed absorption peaks at 210nm and 290nm. The shift in the peaks delineate the successful conjugation of Silymarin, LNPs and PEG with each other.

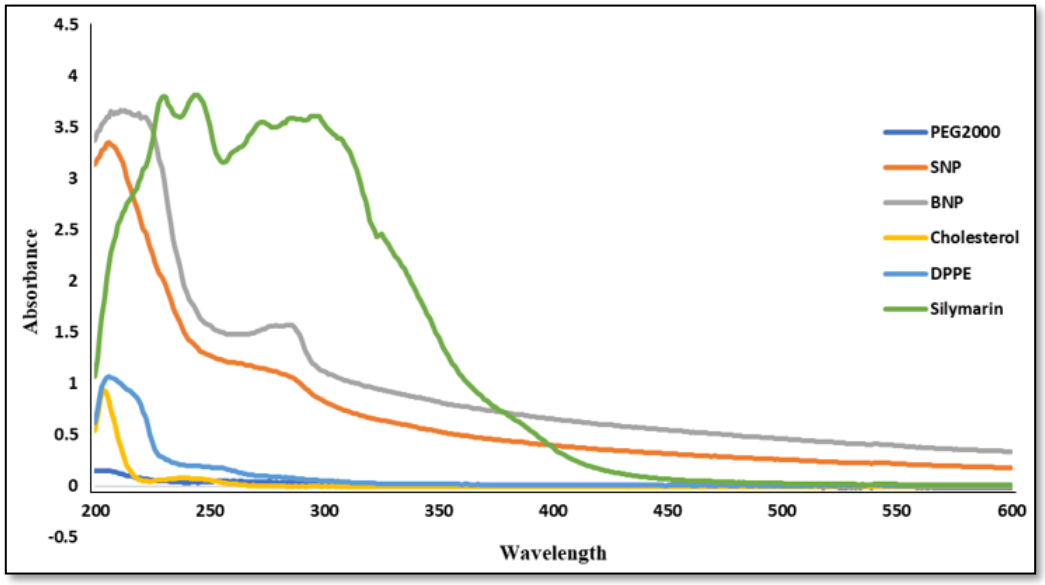

Figure 4.1: Comparative UV-VIS spectra of Silymarin, Blank LNPs

Silymarin-loaded LNPs

4.1.2. Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectrum of Cholesterol indicated peaks or bands at 1100/cm (C-O stretch, Ether), 2900/cm (C-H stretch, Alkane). DPPE spectrum indicated peaks at 1800/cm (C=O stretching, anhydride), 2900/cm (C-H stretching, Alkane), 2850/cm (C-H stretching, Alkane), 1050/cm (C-O stretching, primary alcohol). PEG-2000 spectrum delineated peaks at 850/cm (C=C bending, Alkene-trisubstituted), 1100/cm (C-O stretching, aliphatic ether), and 2900/cm (C-H stretching, Alkanes). Silymarin drug spectrum exhibited peaks at 800/cm (C-H bending 1,4-

disubstituted), 1150/cm (C-O stretching, aliphatic ether), 1500/cm (N-O stretching-nitro compound) and 1600/cm (C=C stretching, α,β-unsaturated ketone). In distinction, the spectrum of Blank liposomes and Peg-liposomes depicted the disappearance of cholesterol's 2900/cm (C-H stretch, Alkane) and 2850/cm (C-H stretching, Alkane) of DPPE. Peg-liposomes spectrum illustrated the disappearance of DPPE's 1800/cm (C=O stretching, anhydride). Silymarin's peaks has been disappeared in PEG-LNPs spectra as PEG-2000 masking. The observed changes in infrared bands proven the conformational changes in lipid biomolecules' by incorporating with silymarin drug and PEG 2000.

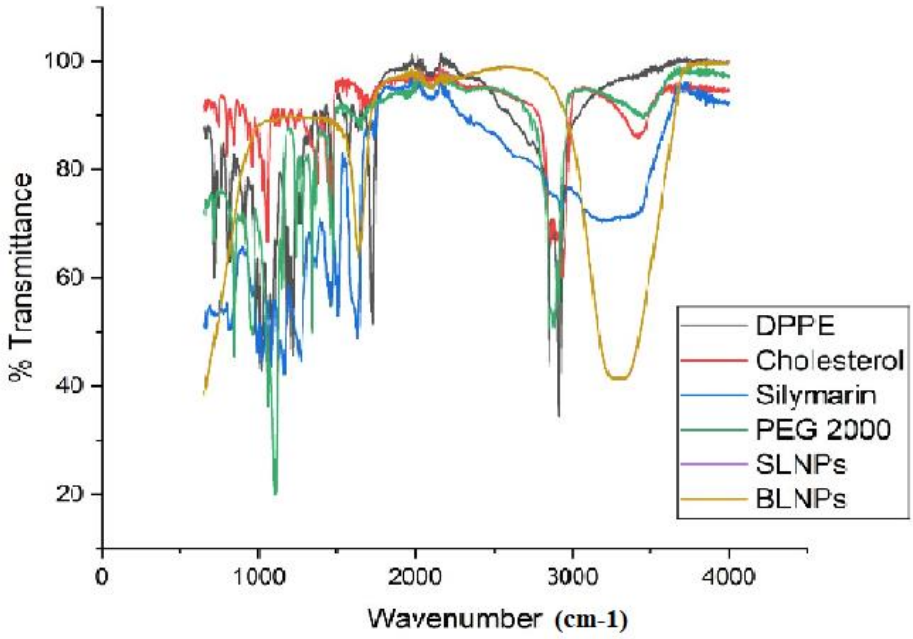

Figure 4.2: Comparative FTIR spectra of DPPE, Cholesterol, PEG-2000, Silymarin, Blank LNPs, Pegylated- Silymarin LNPs

4.1.3. Particle size and Area Distribution

The Silymarin 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 an average size of 125nm.

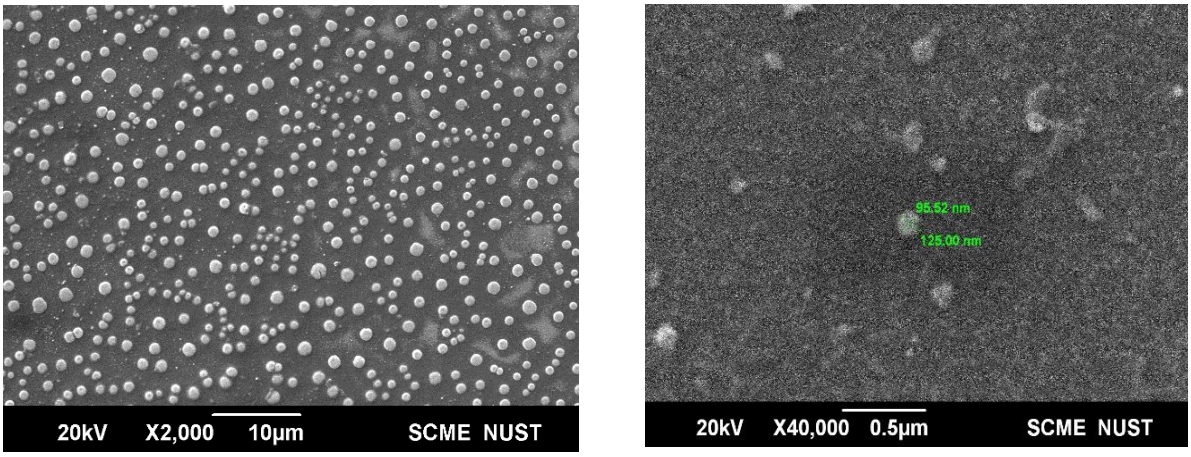

Figure 4.3: SEM Image of Silymarin-loaded Liposomes Nanoparticles a) Silymarin Loaded Liposomal Nanoparticles b) Blank Nanoparticles

4.1.4. Zeta Analysis

The average Zeta potential of Blank Liposomes was -16.8 mV and LNP's -23.4 mV. By using PEG-2000, 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.

4.1.5. Drug Encapsulation Efficiency

By calculating the aforementioned formula (in material Section), the Encapsulation

Efficiency found to be 72%, that delineates the 72% entrapment of drug within Nanoparticles.

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

$$y=6.5053x-0.2301$$

$$R^2 = 0.8711$$

UV analysis of 1st supernatant from mini column centrifuge tube, gave the absorption at 250 nm for silymarin.

Encapsulation efficiency (%)

$$= \frac{\text{Weight of drug in Nanoparticles}}{\text{Weight of feeding drug}} \times 100$$

$$72 = \frac{x}{0.482} \times 100$$

$$x = \frac{0.347}{0.482}$$

$$= 71.99\%$$

$$= \mathbf{72\%}$$

Drug Loaded Content(%)

$$= \frac{\text{Weight of drug in Nanoparticles}}{\text{Weight of Nanoparticle}} \times 100$$

$$= \frac{0.347}{0.75} \times 100$$

$$= \mathbf{46.26\%}$$

4.1.6. Drug Release Kinetics

The release of drug from PEG-LNPs was 72%, noted up to 15 hours suggesting the sustain released of drug with time from PEG-LNPs. Experiment demonstrated that the silymarin drug at a constant rate from 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 (Zhu et al., 2013).

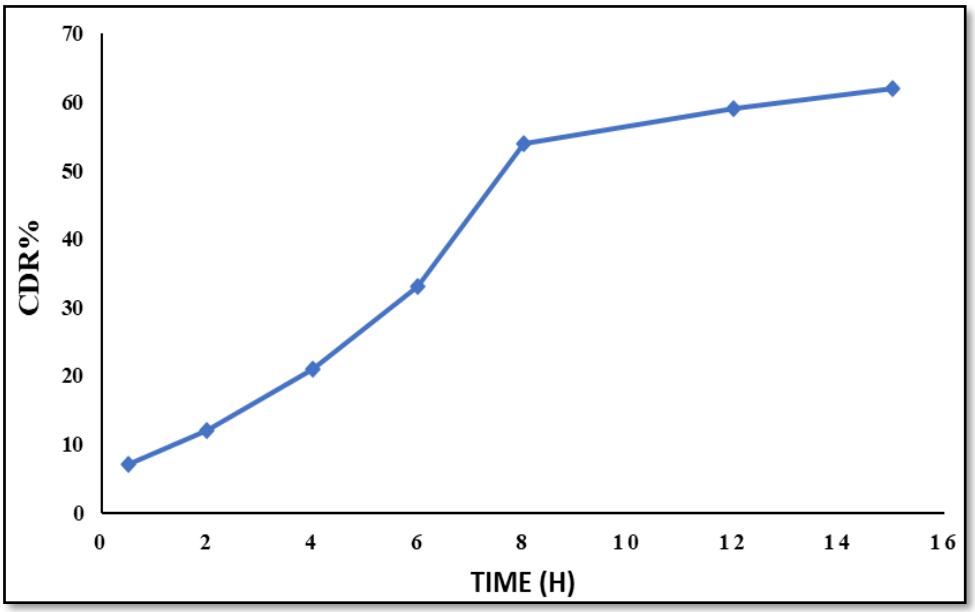

Figure 4.4: Percentage of cumulative drug release per hour

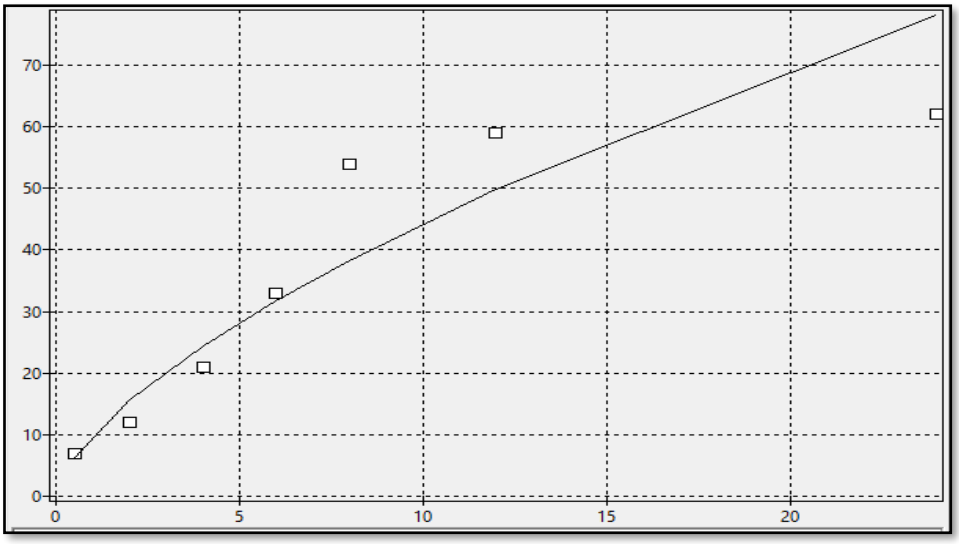

Figure 4.5: Drug release following zero order kinetics

4.2 Induction Of NAFLD

Even if it coexists with other liver illnesses, NAFLD is a unique pattern of liver damage that may be identified. In acinar zone 3, the most severe changes were observed in the early stages of the disease, when the histologic abnormalities have a unique distribution. The characteristics of steatohepatitis are not uniformly present in all biopsies, and no characteristic is diagnostic on its own. This makes the diagnosis challenging at times. Score systems were developed to help pathologists determine the severity of NAFLD due to the disease's inherent complexity and the vast range of findings.

4.2.1 Effects of NAFLD induction 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 4.1: NASH/NAFLD Clinical Research Network Scoring System during induction

4.2.2 Ultrasound Imaging of Liver after NAFLD induction

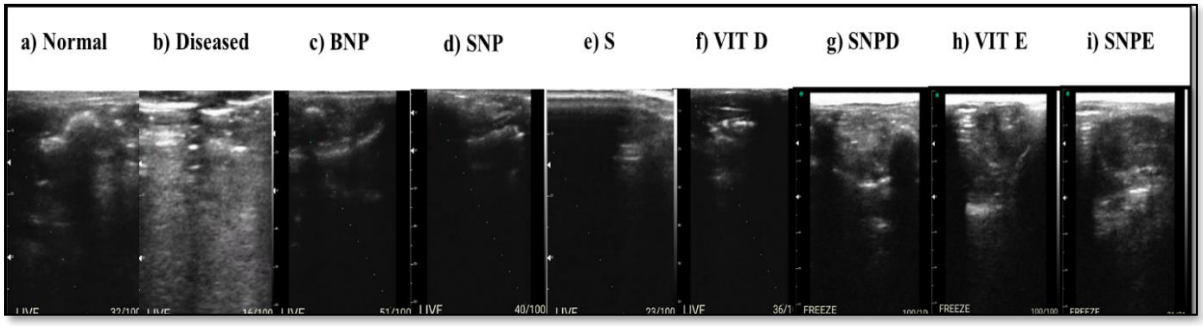

Figure 4.6: Liver histopathology after treatment a) Normal b) Diseased c) Blank nanoparticles d) Silymarin loaded pegylated nanoparticles e) Silymarin f) Vitamin D g) Silymarin loaded nanoparticles and vitamin D h) Vitamin E i) Silymarin loaded pegylated nanoparticle and Vitamin E.

a) In group normal, the liver was homogenous and showed normal echotexture. No focal lesion or pathology was observed b) In diseased group, slight ascites were observed with nodular liver surface, overall coarse and heterogeneous echotexture, fatty liver, demonstrating increased echogenicity ("bright liver") in comparison to normal liver c) In group BNP, nodular liver surface in addition to other fibrotic changes and inflammation was observed**,** enlarged liver filling of the entire field with livers looking brighter than normal liver d) In SNP group, nodular liver surface in addition to other fibrotic changes and inflammation was observed. Enlarged liver filling of the entire field with liver appearing brighter than normal liver e) Slight ascites observed and liver fills entire field with no edges visible f) In Vit.D group, nodular liver with slight ascites and inflammation was observed. Liver appeared lumpy and shrunken g) In SNPD group, nodular liver surface with coarsened echotexture of the liver and inflammed liver lobes h) In Vit.E group, decreased conspicuity of the intrahepatic vessels ("featureless liver") with nodular liver surface in addition to other fibrotic changes were observed i) In SNPE group, hepatocellular ballooning with surface nodularity and lobular inflammation was observed.

4.3 Treatment of NAFLD

4.3.1 Hepatic Histopathology after treatment

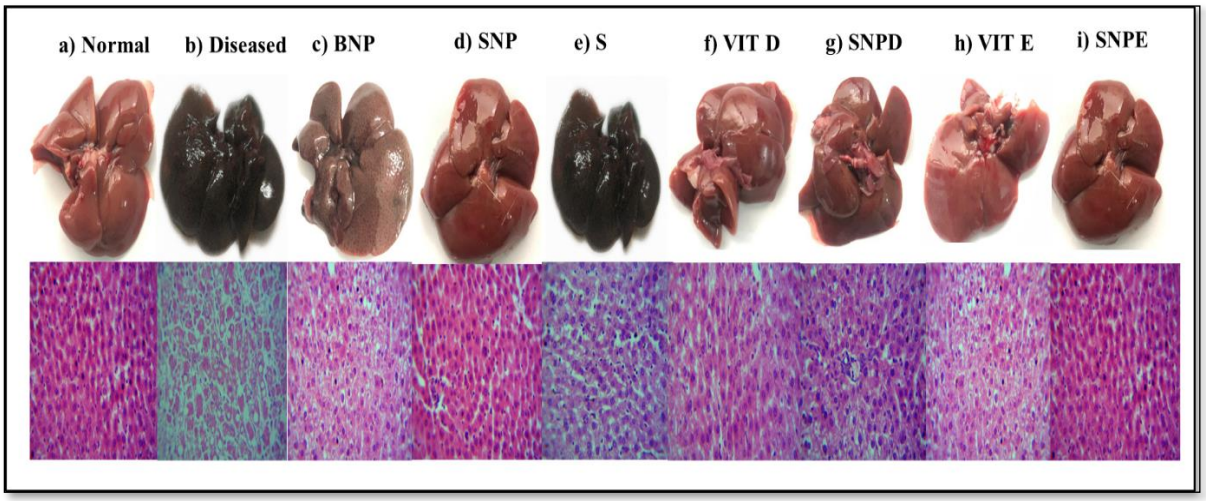

Figure 4.7: Liver histopathology after treatment a) Normal b) Diseased c) Blank nanoparticles d) Silymarin loaded pegylated nanoparticles e) Silymarin f) Vitamin D g) Silymarin loaded nanoparticles and vitamin D h) Vitamin E i) Silymarin loaded pegylated nanoparticle and Vitamin D

In NAFLD, mixed inflammatory infiltrates are primarily found in lobular distribution. The CD4-(+) and CD8-(+) lymphocyte combination and sporadic Kupffer cell aggregates make up the majority of the lobular infiltration (microgranulomas). Particularly close to Mallory-Denk bodies, polymorphonuclear leukocytes (PMNs) may be detected (MDBs). Although they are not always present, eosinophils may be observed in some cases (Brown & Kleiner, 2016).

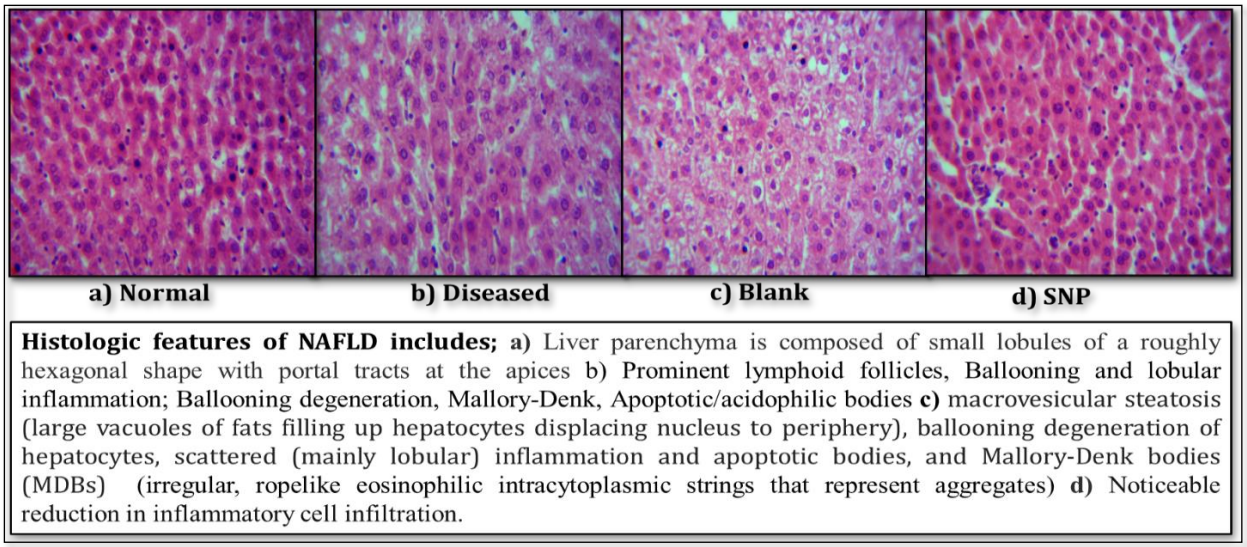

Figure 4.8: Liver histopathology after treatment

In close proximity to hepatic veins or in portal regions, lipogranulomas can be seen. Although it is frequently mild, portal inflammation in NAFLD is not uncommon. The intensity of portal inflammation has been linked to the level of fibrosis, and CD8-(+) T-cells and macrophages make up the majority of the portal infiltration. However, autoimmune or viral hepatitis must be taken into account if the portal tracts are extremely inflamed. Increased portal inflammation is frequently linked to disease development even though it is not necessary for the diagnosis of NAFLD (Brown & Kleiner, 2016; Brunt & Tiniakos, 2010a).

Hepatocellular damage in NAFLD can take many different forms, from apoptosis and ballooning degeneration to less obvious reactive alterations. An important aspect of NASH/NAFLD is ballooning injury because, in the appropriate situation, one convincing balloon cell might tip the diagnosis in favour of NASH/NAFLD (Brunt & Tiniakos, 2010b). Additionally, the presence of it carries a prognostic significance linked to a higher likelihood of cirrhosis development. Large (up to several times larger than a non-NAFLD hepatocyte), with pale or reasonably clear cytoplasm and wispy or feathery eosinophilic filaments, the typical balloon cell also frequently has a large, hyperchromatic nucleus with a conspicuous nucleolus. Only a few balloon cells may be present, or if they are not much larger than the hepatocytes around them, problems may occur (Kleiner et al., 2005).

The eosinophilic intracytoplasmic inclusions known as Mallory-Denk bodies—previously known as the Mallory bodies seen are made up of misfolded intermediate filaments (keratins), chaperone proteins, heat-shock proteins, and other components. Although not necessary for the histological detection of NASH/NAFLD, the presence of MDBs when combined with steatohepatitis and fibrosis indicates worse prognosis (Brunt, 2000; Brunt et al., 2011).

NAFLD may show a range of different histological abnormalities. Another indicator of liver damage is the presence of apoptotic or acidophil bodies, which are non-specific because they can also occur in many other liver disorders, including viral hepatitis and NASH/NAFLD. Hepatocytes that have experienced apoptosis, also known as programmed cell death, shrink and become eosinophilic. They may also have abnormal nuclei or dispersed nuclear fragments. They typically show up on H&E stain and have CK 18 fragments. Megamitochondria are eosinophilic intracytoplasmic inclusions that range in shape from round to irregular and are occasionally found in NASH. In the context of NASH/NAFLD, the development of megamitochondria may be caused by lipoprotein or oxidised phospholipid

damage. Megamitochondria are not specific, though, as they can also be found in drug-induced toxicity and alcoholic steatohepatitis. Glycogenated nuclei are vacuolated nuclei that are typically seen in periportal hepatocytes and are frequently found in NASH/NAFLD (Brunt, 2012; Brunt et al., 2011).

4.3.2 Ultrasound Imaging results of the Liver after treatment

In group normal, the liver was homogenous and showed normal echotexture. No focal lesion or pathology was observed. In the diseased group, slight ascites was observed with nodular liver surface and the overall echotexture of the liver was coarse and heterogeneous. Fatty liver demonstrated increased echogenicity ("bright liver") in comparison to normal liver.

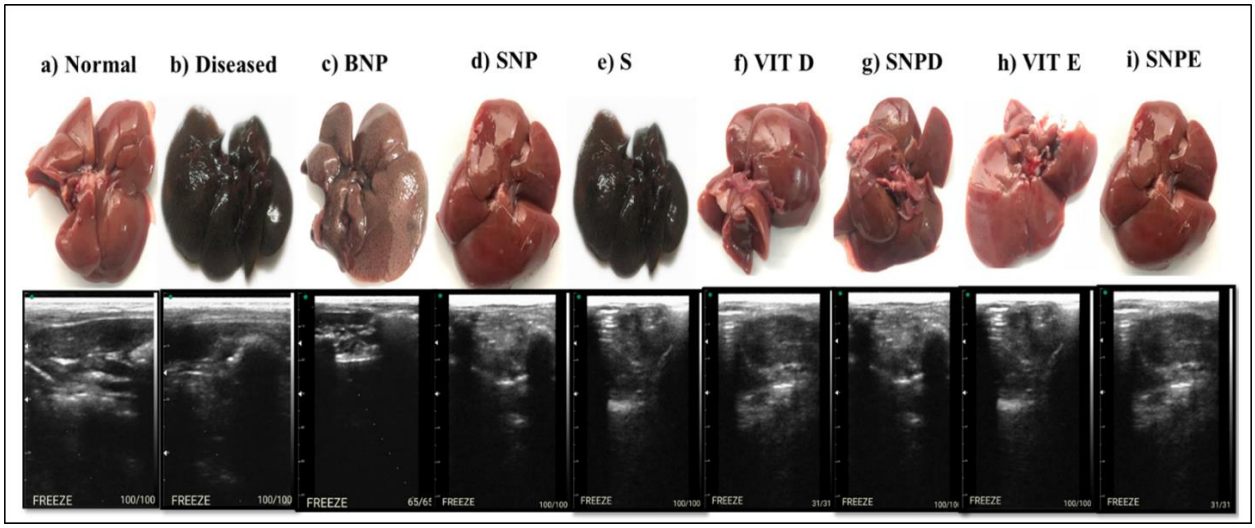

Figure 4.9: Liver ultrasound imaging after treatment a) Normal b) Diseased c) Blank nanoparticles d) Silymarin loaded pegylated nanoparticles e) Silymarin f) Vitamin D g) Silymarin loaded nanoparticles and vitamin D h) Vitamin E i) Silymarin loaded pegylated nanoparticle and Vitamin D

In group BNP, the rats were treated with unloaded nanoparticles and the ultrasound result were quite similar to the diseased group with a nodular liver surface in addition to other fibrotic changes and inflammation with an enlarged liver filling of the entire field. Moreover, the echotexture of the liver was coarsened. The SNP group, treated with silymarin-loaded pegylated nanoparticles was homogenous without free fluid and showed reduced echogenicity. In Silymarin treated group, nodular liver surface with slight ascites was observed. The liver was inflamed and looked lumpy and shrunken. In Vit.D group, a normal to a mild increase in echogenicity was observed.

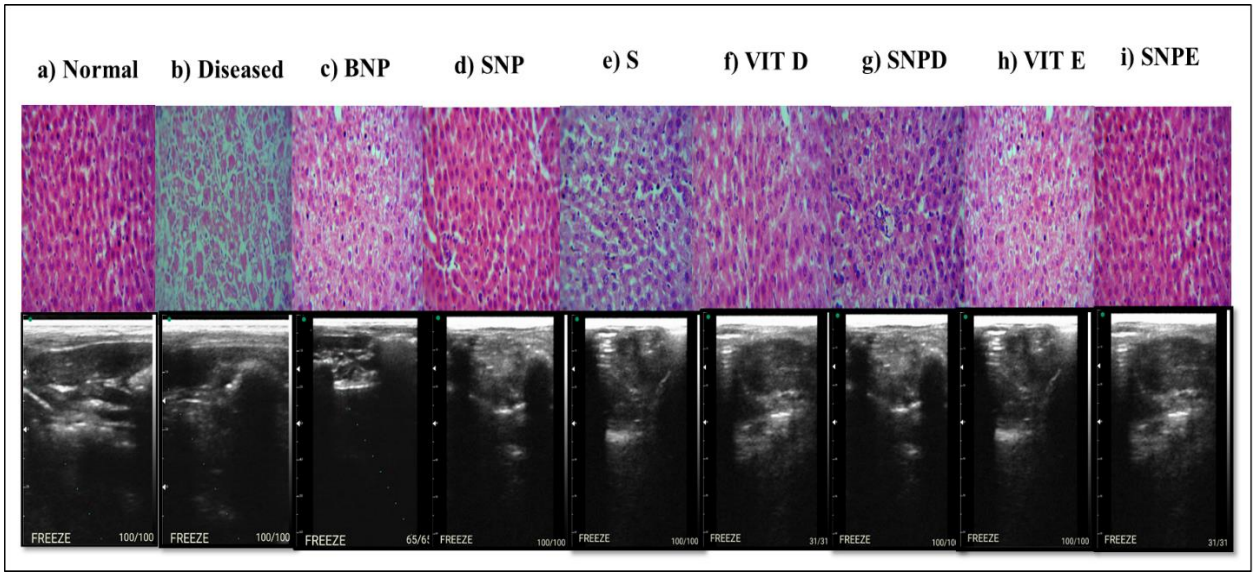

Figure 4.10: Ultrasound of Liver after treatment

In the SNPD group, reduced echogenicity with no free fluid and homogenous echotexture was observed. In Vit. E group, a mild increase in echogenicity was observed and the echotexture of the liver was normal (homogenous liver). In the SNPE group, echotexture of the liver was normal and it was homogenous. No nodular fluid was present.

4.3.3 Effects of NAFLD treatment on Liver

Score systems were developed to help pathologists determine the severity of NAFLD due to the disease's inherent complexity and the vast range of findings.

Groups	Score
Positive Control	12/17
Blank nanoparticle treated	11/17
Silymarin treated	10/17
Silymarin loaded nanoparticle treated	5/17
Vitamin E treated	7/17
Vitamin E + SNP treated	5/17
Vitamin D treated	8/17
Vitamin D + SNP treated	7/17

Table 4.2: NASH/NAFLD Clinical Research Network Scoring System after treatment.

4.3.4 Serological Analysis (LFTs)

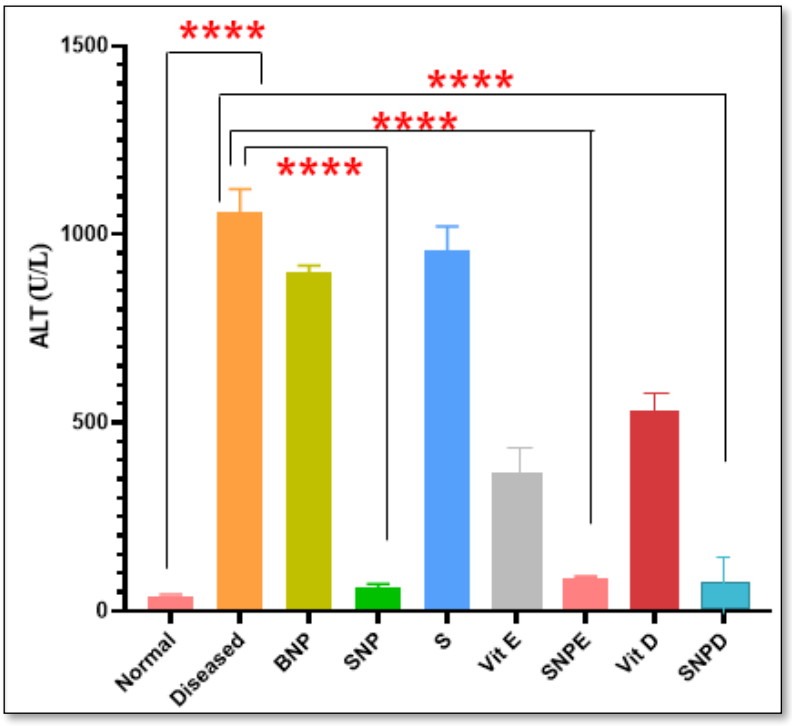

Figure 4.11: Serological indices after treatment; ALT

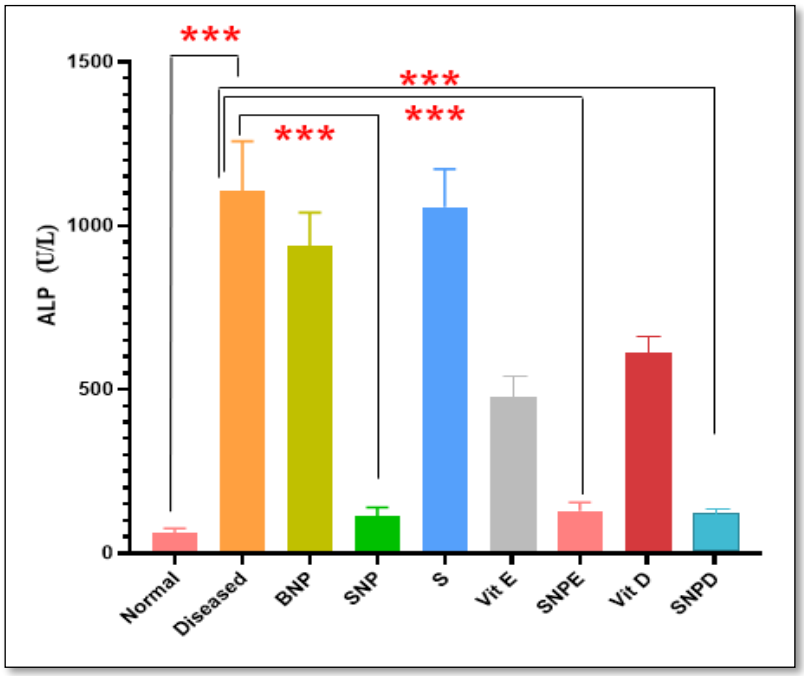

Figure 4.12: Serological indices after treatment; ALP

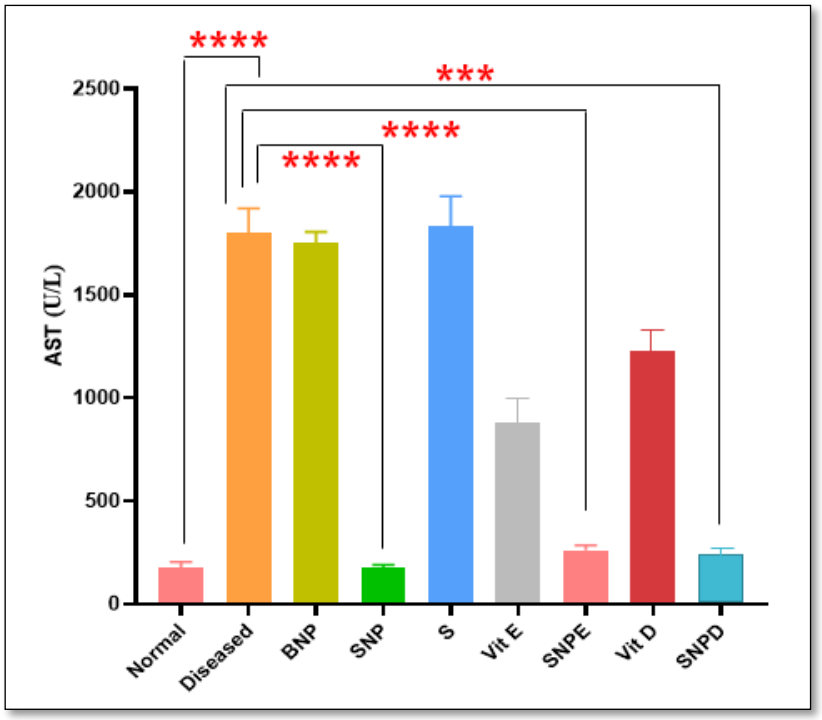

Figure 4.13: Serological indices after treatment; AST

4.3.5 Body Weight

This study showed that change of body weight was significantly associated with both the development and remission of NAFLD. During the first two weeks of acclimatization, the average weights of all groups were within the same range. However, with the beginning of induction phase of NAFLD from week 3rd until the end of treatment phase, a drastic change in average body weight was observed. In normal group, the weight increased with every passing week as no detrimental chemicals were induced to this group. In diseased group, the curve delinated indicating a sudden decline in body weight. In BNP group, a similar curve was observed as the treatment includes blank (unloaded) nanoparticles. In S group, a decline in body weight was observed from the 3rd week with a non-significant improvement during the treatment phase. A similar pattern was observed in case of Vit.E and Vit.D groups. However, SNP, SNPE and SNPD groups showed a significant increase in body weight during the treatment phase.

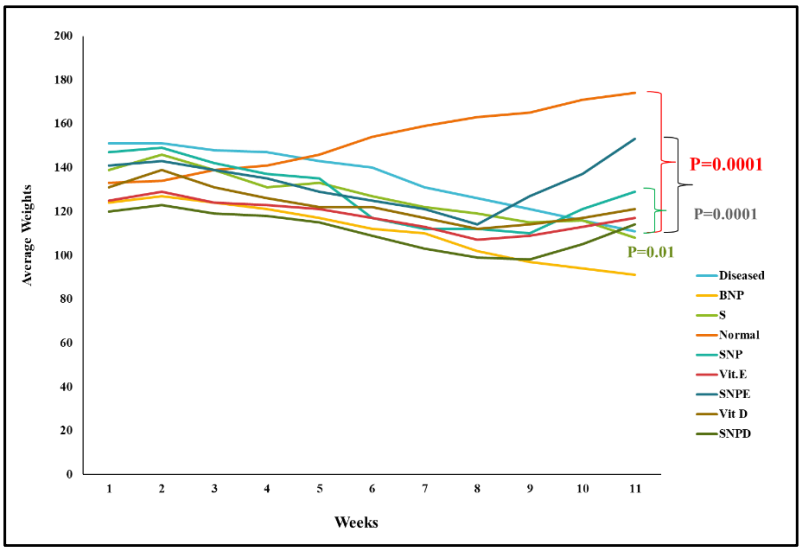

Figure 4.14: Body weight Analysis during experimental procedure

4.3.6 Liver Weight

Following the dissection of NAFLD/NASH animal models, the liver weights were calculated, and the average weights were recorded. The liver in the normal group had a uniform weight of 6.48g. The liver weights in the diseased and BNP groups were 11.12g and 11.02g, respectively, with a little difference between the two groups. The liver weights in the S group, vitamin E group, and vitamin D group were 10.66g, 9.78g, and 10.11g, respectively. The measured liver weights in the SNP and SNPE groups were 6.98g and 6.91g, which were virtually similar; whereas in the SNPD group, it was 8.81g.

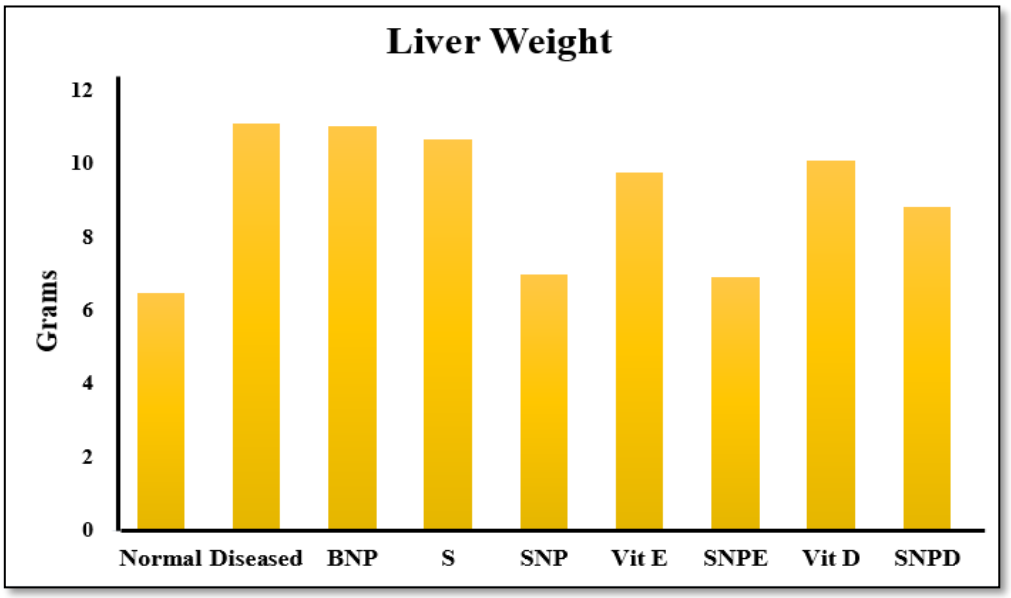

Figure 4.15: Liver weight Analysis after treatment

4.3.7 Mortality rate

Animal models of NAFLD/NASH were divided into nine groups ; normal, diseased, blank liposomal nanoparticles (BNP), silymarin (S), silymarin loaded pegylated liposomal nanoparticles (SNP), vitamin E (Vit.E), silymarin loaded pegylated liposomal nanoparticles with vitamin E (SNPE), Vitamin D (Vit.D) and silymarin loaded pegylated liposomal nanoparticles with vitamin D (SNPD). During the acclimatization and induction phase, all animal models were alive and survived the diseased stage with variation in their body weights. However, during treatment phase, a sudden mortality rate was observed. Due to portal hypertension, bleeding from mouth of animals was observed.

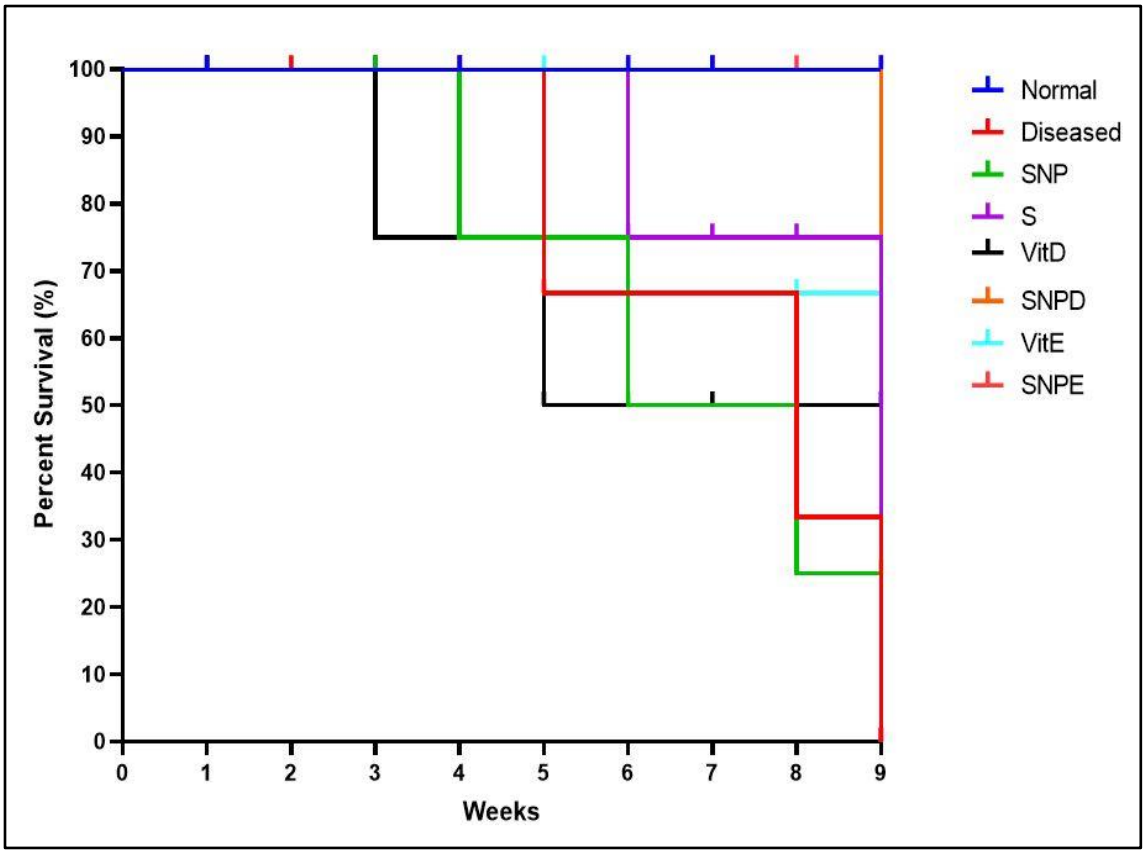

Figure 4.16: Kaplin meier graph representing survival rate of NAFLD rat model

In diseased group, 3/5 rats were dead as they were not given any treatment during the treatment phase. In BNP group, a similar mortality rate as that of diseased group was observed. In S group, vit.E and vit.D group, 2/5 rats were dead by the completion of treatment phase. In SNP and SNPD group, 1/5 rats were dead whereas in SNPE group, no mortality was observed.

CHAPTER:5 DISCUSSION

NAFLD; the second most common cause of liver transplantation; is a serious and expanding problem throughout the world (Lindenmeyer & McCullough, 2018). Because it can lead to cirrhosis and have side effects from portal hypertension, which is mostly caused by extensive fibrosis and irregular tissue remodelling. Higher portal venous pressure, however, has also been observed in clinical NAFLD and experimental fatty liver models when fibrosis is much less progressed and cirrhosis is absent. Early increases in intrahepatic vascular resistance may facilitate the onset of liver disease (Iwakiri, 2014).

The term "portal hypertension" refers to an increase in blood pressure within the portal venous system, a network of veins. The portal vein, which divides into smaller vessels and passes through the liver, is formed when the veins from the stomach, intestine, spleen, and pancreas combine. Blood cannot adequately flow through the liver if the liver's blood arteries are obstructed as a result of liver disease (Iwakiri, 2014). As a result, the portal system experiences significant pressure. Varices may form in the oesophagus, stomach, rectum, or umbilical region as a result of this elevated pressure in the portal vein (belly button). Varices are prone to bleeding and rupturing, which can lead to consequences that could be fatal (Ryou et al., 2020).

According to preclinical evidence, silymarin, an extract from milk thistle seeds, has been used to lessen oxidative stress and the resulting cytotoxicity, sparing liver cells that are still healthy or that have not yet sustained irreparable damage (Elmowafy et al., 2013; S. Zhong et al., 2017). Oral silymarin has a low bioavailability, which restricts its medical applications. But the bioavailability of silymarin can be increased by using liposomes as drug delivery systems. In the current study, the silymarin-loaded pegylated liposomal nanoparticle was successfully created and employed as a treatment for NAFLD (Federico et al., 2017b). Liposomal NPs can be created as nanoplatforms for the effective and targeted drug delivery due to their ability to pass through a number of biological, biophysical, and biomedical barriers (Yang et al., 2015).

Average liposome size and silymarin-loaded liposome size were 95.52 nm and 125 nm, respectively, with corresponding zeta potential readings of -16.8 mV and -23.4 mV. For a 15-hour study, 72% of the drug was released from liposome nanoparticles. The stability of nanoparticles was too low due to the lower negative surface charge, and the formulation was

very prone to agglomeration. Polyethylene glycol (PEG) was employed to cover the nanosized liposomes in order to increase stability and induce the stealth effect. Pegylation improves steric repulsion and is therefore recognised as a superior stabiliser for several types of nanoparticles (D'Souza A & Shegokar, 2016). But the expansion of nanoparticles in size is one of the main drawbacks of utilising PEG. PEG uses a drug's erosion-controlled release mechanism to produce a sustained release (Hu, Zhang, You, Yuan, & Du, 2012). Pegylated liposome nanoparticles showed a sustained release of 56% of the drugs when tested. Comparative FTIR of Silymarin and Silymarin-loaded Pegylated Liposomal Nanoparticles in the current investigation demonstrated the participation of several functional groups, as indicated by the reduction in peak intensities.

Carbon tetrachloride was effectively used to establish the NAFLD model. Initially, urethane was employed to shorten the period of illness induction, but this resulted in serious liver damage (Asrani et al., 2019). In addition to liver function tests and ultrasound imaging, a histological investigation was used to examine the effects of the aforementioned substances on the liver (Farooq & Fatima Rana, 2020). Results from histological, serological, body composition, and liver weight analyses showed that utilising the encapsulated silymarin medicine coupled with Vitamin E and Vitamin D as opposed to a blank nanoparticle significantly reversed collagenous scars to normal hepatic tissue model. In comparison to the Peg-LNPs group, the Peg-LNPs+Vitamin E group showed better results.

The use of silymarin nanoparticles as supportive therapy for the majority of liver diseases, including NAFLD/NASH, fibrosis, cirrhosis, and liver damage brought on by excessive alcohol consumption, has demonstrated promising results (Pradeep et al., 2007). There was a significant drop in liver function tests in clinical trials with NAFLD patients (ALP, AST and ALT). Silymarin's antioxidant activity is thought to be the mechanism of action that causes these therapeutic outcomes. By scavenging the free radicals that cause lipid peroxidation and interfering with the enzyme systems linked to the cellular damage that causes fibrosis and cirrhosis, it has an antioxidant effect (Freitag et al., 2015; Mengesha et al., 2021b). A few mortalities were seen during this treatment, nevertheless.

It is well accepted that the body's primary lipid-soluble chain-breaking antioxidant is vitamin E. Molecules in the vitamin E family also have anti-inflammatory and anti-atherogenic characteristics in addition to their anti-oxidative qualities (Ellatif et al., 2018). Although the aetiology of NAFLD and how it develops into fibrosis remains to be fully understood, it is

thought that oxidative stress is a major factor in the deadly hepatocyte injury linked to NAFLD (Rinella, 2015). It functions through the glutathione peroxidase pathway protecting cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. α-tocopherol contributes significantly to the removal of free radical intermediates and the interruption of the oxidation reaction. Vitamin E therefore, seems to be a promising therapeutic strategy in NASH/NAFLD patients by targeting oxidative stress components. In contrast, when Vitamin E was added to SNP, it produced better results than SNP, in which mortalities were noted. Low serum Vitamin D levels may result in nonalcoholic fatty liver disease (NAFLD). NAFLD severity and incidence are linked to hypovitaminosis D. When compared to controls, those with NAFLD have a 26% higher risk of vitamin D insufficiency (Pacana & Sanyal, 2012).

The liver is susceptible to vitamin D's effects via the vitamin D receptor (VDR). VDR is present in hepatic cells, and its expression can lessen inflammation in conditions that affect the liver chronically. According to in vitro research, VDR enhanced insulin sensitivity by boosting the expression of the muscle-specific glucose transporter-4 and controlling free fatty acids (FFAs). Additionally, vitamin D has effects on the liver that are antifibrotic, antiproliferative, and anti-inflammatory. Additionally, vitamin D can lower levels of cytokeratin 18 apoptotic fragment M30, which is a sign of liver damage. The results of the subsequent investigation showed that anthropometric measurements and glycemic index variables among animal models of NAFLD may not be improved by vitamin D administration. However, it alleviates NAFLD symptoms, and when combined with SNP and given orally, it had better outcomes with fewer fatalities (Hariri & Zohdi, 2019).

CHAPTER 6: FUTURE PROSPECTS

This study allows us to understand the real effects of silymarin along with vitamin E on ALT, AST and ALP levels, from patients with liver diseases, in addition to signaling to the need of new clinical trials with more appropriate methodological designs.

For maximum benefit, treatment with silymarin and vitamin E should be initiated as early as possible in patients with fatty liver disease and other distinct liver disease manifestations such as acute liver failure, when the regenerative potential of the liver is still high and when removal of oxidative stress, the cause of cytotoxicity, can achieve the best results.

In 2021, liver biopsy remains the only diagnostic procedure that can reliably assess the presence of NASH and early fibrosis but increasing efforts are made towards non-invasive testing and molecular classification of NAFLD subtypes.

Identifying more homogenous cohorts of patients with NAFLD to address natural history and evaluate novel treatment strategies would enlighten our knowledge of the disease and contribute to implementing the practice of precision medicine in NAFLD diagnosis and management.

There is a need for extensive longitudinal human trials investigating the reversal effect of SNPE administration on advanced fatty liver disease in humans. Furthermore, the appropriate dosage effect needs further investigation as well as the effect of the route of administration. Children with NAFLD have been reported to have a diet that is insufficient in vitamin E which may contribute to the pathophysiology of NAFLD. Thus, prevention of NAFLD with adequate amounts of vitamin E+SNP (i.e. dietary consumption or supplementation)from an early age needs further investigation especially since it is an easy and low cost intervention

CHAPTER 7: CONCLUSION

Silymarin has a diverse set of in vitro and in vivo actions, including antioxidant, anti-inflammatory, dose-dependent anti-apoptotic, and cell transporter altering properties. As a result, it has the potential to be a promising medication in alternative medicine. While an extract containing 200 to 400 mg/day of silymarin is considered effective in various liver disorders, this research focuses on liver treatment with relatively lower dose of silymarin that effectively targets the liver. For this purpose, liposomal nanoparticles were synthesized to increase the bioavailability of silymarin. Various characterization approaches are used to demonstrate the success of nanoparticle creation. When compared to traditional medications and non-coated liposome NP's, pegylation increased the stability, resulting in long-term circulation in the body. This silymarin coated liposomal nanoparticle formulation with other combinations treat liver disease with 1mg/kg dose rather than 400mg/day. Histological, serological, body, and liver weight outcomes improved significantly in the Peg-LNPs+Vitamin E (SNPE) treatment group. Following that, the SNPs and SNP+Vitamin D treated groups also showed improved results to some extent, owing to the deaths recorded in these two treatment groups. In contrast to the blank drug, the usage of the encapsulated silymarin drug is therefore preferable for reversing scars of collagen to normal tissues of liver. Thus, liposome encapsulation with PEG coating along with vitamin E is demonstrated to be the most effective strategy in boosting the bioavailability and pharmacokinetic behaviour of silymarin medication, and it has demonstrated the anti-fibrotic activity or significant therapy against NAFLD. The following hypothesis should be investigated further in order to validate this treatment approach for application in clinical settings.

The present study adds to our understanding of the effect of vitamin E on increased lipid parameters in rats and the changes of fat deposition in the liver it may cause. The data support individualised supplementation of SNP with vitamin E, as it may prevent or reverse NAFLD and NASH. SNPE may be effective as a monotherapy for dietary-induced NAFLD and the metabolic disturbances that accompany it. However, further clinical studies are needed before making-any-definitive-recommendations.

CHAPTER 8: REFERENCES

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