Investigation of Silymarin Encapsulated Liposomal Nanoparticles Against Copper Toxicity Associated Liver Dysfunction and Neurobehavioral Abnormalities in Wistar Rats

Author

Tuba Maryam

Regn Number

00000328808

Supervisor

Dr. Nosheen Fatima Rana

DEPARTMENT OF BIOMEDICAL ENGINEERING & SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

ISLAMABAD

2022

Investigation of Silymarin Encapsulated Liposomal Nanoparticles Against Copper Toxicity Associated Liver Dysfunction and Neurobehavioral Abnormalities in Wistar Rats

Author

Tuba Maryam

Regn Number

00000328808

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

MS Biomedical Science

Thesis Supervisor:

Dr. Nosheen Fatima Rana

Thesis Supervisor's Signature: _____

DEPARTMENT OF BIOMEDICAL ENGINEERING & SCIENCES SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY, ISLAMABAD

2022

Declaration

I certify that this research work titled *"Investigation of Silymarin Encapsulated Liposomal Nanoparticles Against Copper Toxicity Associated Liver Dysfunction and Neurobehavioral Abnormalities in Wistar Rats"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Signature of Student

Tuba Maryam

2020-NUST-MS-BMS-00000328808

Certificate for Plagiarism

It is certified that MS Thesis Titled **"*Investigation of Silymarin Encapsulated Liposomal Nanoparticles Against Copper Toxicity Associated Liver Dysfunction and Neurobehavioral Abnormalities in Wistar Rats"*** by **Tuba Maryam** has been examined by us. We undertake the follows:

a. Thesis has significant new work/knowledge as compared already published or are under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.
b. The work presented is original and own work of the author (i.e. there is no plagiarism). No ideas, processes, results or words of others have been presented as Author own work.
c. There is no fabrication of data or results which have been compiled/analyzed.
d. There is no falsification by manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
e. The thesis has been checked using TURNITIN (copy of originality report attached) and found within limits as per HEC plagiarism Policy and instructions issued from time to time.

Name & Signature of Supervisor

Dr. Nosheen Fatima Rana

Signature **:** _____

Copyright Statement

Acknowledgements

I am thankful to my Creator Allah Subhana-Watala to have guided me throughout this work at every step and for every new thought which You setup in my mind to improve it. Indeed, I could have done nothing without Your priceless help and guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was Your will, so indeed none be worthy of praise but You.

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

I would also like to express special thanks to my supervisor Dr. Nosheen Fatima Rana for her help throughout my thesis and also for the Nano and Micro Drug Delivery course which she has taught me. That course was the motivation behind this research work.

I would also like to pay special thanks to Farhat Batool and Misha Fatima for their tremendous support and cooperation. Each time I got stuck in something; they came up with the solution. Without their help, I wouldn't have been able to complete my thesis. I appreciate their patience and guidance throughout the whole thesis.

I would also like to thank Dr. Asim Waris, Dr. Faheem Amin and Dr. Nabeel Anwar for being on my thesis guidance and evaluation committee. I am also thankful to my senior Tehreem Tanveer for her support and cooperation.

Finally, I would like to express my gratitude to all the individuals who have rendered valuable assistance to my study.

Dedicated to my exceptional parents and siblings whose tremendous support and cooperation led me to this wonderful accomplishment.

List of Abbreviations

ALP	**Alkaline phosphatase**
ALT	**Alanine transaminase**
AST	**Aspartate transaminase**
ATP	**Adenosine triphosphate**
BLNP	**Blank liposome nanoparticle**
CYP	**Cytochrome P-450**
DI	**Deionized**
DPPC	**Dipalmitoylphosphatidylcholine**
FDA	**Food and Drug Administration**
FTIR	**Fourier Transform Infra-Red**
GSH	**Glutathione**
HCC	**Hepatocellular carcinoma**
HE	**Hematoxylin and eosin**
IV	**Intravenous**
LNP	**Liposome Nanoparticle**
LPO	**Lipid peroxidation**
MT	**Metallothionein**
PBS	**Phosphate buffered saline**
PEG	**Polyethylene glycol**
RES	**Reticuloendothelial system**
SEM	**Scanning Electron Microscopy**
SLNP	**Silymarin Liposome Nanoparticle**
SOD	**Superoxide dismutase**
TBH	**Tert-butyl hydroperoxide**
UV	**Ultraviolet**
WD	**Wilson's Disease**
ZSLNP	**Zinc and Silymarin Liposome Nanoparticle**

Abstract

The fields of nanomedicine and nano delivery systems, in which nanoscale materials are utilized as diagnostic instruments or to administer therapeutic medicines to precisely targeted areas, are new but rapidly developing fields. Through a thorough examination of nanoparticle production and use, nanomedicines and nano-based drug delivery systems improve the effectiveness of both new and existing treatments. Recent years have seen a surge in interest in the use of nanoparticulate structures including stimuli-sensitive polymers and liposomes for the treatment of liver disorders. Wilson disease is characterized by copper accumulation in both the liver and extrahepatic organs. The liver is particularly vulnerable to chronic copper poisoning because it is the first organ to absorb copper from the circulation. Copper's toxicity manifests in several ways, including liver cirrhosis, hemolytic anemia, renal tubule injury, damage to the brain and other systems. The available therapies aim to lower copper levels by various means. However, a potent therapeutic drug that can repair the damaged brain and liver tissue is desperately needed.

Milk thistle (Silybum marianum L.), a member of the Carduus marianum family, has been used for decades to treat liver and gallbladder problems. Medical researchers have shown silymarin and silibinin to have hepatoprotective, antioxidant, and cytoprotective properties. The effectiveness of silymarin as a medication for the liver is diminished by its poor water solubility and low oral bioavailability.

In order to get around these problems, the "thin film hydration method" was used for synthesizing liposome nanoparticles that are encapsulated with silymarin and may be used to combat copper toxicity. Polyethylene glycol (PEG) was employed to coat the liposome nanoparticles to increase their stability and to induce the stealth effect. After the induction of copper toxicity in rats, various methods such as serological analysis and behavioral tests were carried out to assess the effectiveness of the different treatment plans. The silymarin liposome nanoparticles showed improved treatment as compared to silymarin. The combination therapy of the liposomes along with zinc proved to be a more effective treatment plan than zinc therapy.

Key Words: *Silymarin, Liposome Nanoparticles, Copper Toxicity, Wilson's Disease, Animal Model, Histological Examination, Serological Indices, Cognitive impairment*

Contents

List of Figures

Chapter 1: Introduction

1.1. Objective:

This research study is divided into two sections. The liposomal nano formulation of silymarin and its characterization were the focus of the first section of this study. The hepatoprotective properties of the chosen drug have made it useful in the treatment of liver ailments. A "thin film hydration technique" is utilized to create the nanoparticles and increase the bioavailability of the drug. Using a variety of characterization methodologies, distinct characteristics of nanoparticle formulations are assessed and ultimately allowed for *in-vivo* examination of liver copper toxicity.

As a phenotypic pathological state for Wilson's illness and an *in-vivo* investigation for enhanced pharmacokinetic performance of the encapsulated medication, the second section focused on developing and treating a liver copper toxicity model. The effectiveness of the treatment was evaluated by comparing the nano-formulation with the drug that was taken orally. These liposome encapsulated silymarin liposome nanoparticles will be regarded as an important step towards preclinical trials when they are tested in the well-established animal model for liver copper toxicity.

1.2 Nanotechnology in Therapeutics

In nanotechnology, functional systems are designed at the molecular level. Physical, optical, and electrical properties of these systems make them appealing to a wide range of fields, from materials science to biology. In nanomedicine, nanotechnology is used to provide highly targeted medicinal treatments for the prevention, diagnosis, and treatment of illness (Bamrungsap et al., 2012; Wagner et al., 2006). Commercialization efforts throughout the world have been boosted by the recent boom in nanomedicine research, with a wide range of medicines in the industry and an increasing number in the queue (Lobatto et al., 2011).

New drug delivery technologies that use nanotechnology have the potential to broaden the scope of the pharmaceutical industry. Drugs that are selected for full-scale development based on their safety and efficacy data but fail to reach clinical development due to a lack of bio pharmacological properties may have poor bioavailability and undesirable pharmacokinetic properties. These problems can be caused by solubility and permeability issues in the intestinal epithelium. As a result of nanomedicine, pharmaceutical firms will be able to reformulate current pharmaceuticals on the market to prolong their shelf life and

improve their performance by boosting efficacy, safety, and patient adherence. This will ultimately reduce healthcare expenditures (Hughes, 2005; Rathbone et al., 2003).

1.3 Liposomes and their Nanoparticulation

The pharmaceutical industry has been transformed by the invention of the liposomal drug delivery method. Liposomes were initially reported by Alec Bangham in 1961 (Bangham et al., 1965). Since then, liposome research has been ongoing, and its applications in a variety of fields, such as medication, biomolecule, and gene delivery, are now well-established. Lipid bilayers and an interior aqueous cavity characterize liposomes, which are vesicles that form into spheres. Sterols, such as cholesterol, may be added to phospholipids or synthetic amphiphiles to alter membrane permeability in liposomes (Bulbake et al., 2017). For liposome synthesis, thin-film hydration is the most common approach, which involves dissolving lipid components in an organic solvent. The film will be rehydrated in an aqueous solvent after the solvent has been evaporated using rotary evaporation (H. Zhang, 2017). Methods such as freeze-drying, and the infusion of ethanol may also be used. Techniques such as homogenization, sonication, membrane extrusion, freeze-thawing, or membrane extrusion may be used to adjust the size and distribution of the particles. Liposomal formulations and processing may produce liposomes with a wide range of morphological and functional properties (Bulbake et al., 2017).

1.4 Liposomes as Carriers

Liposomes are artificial vesicles made from amphiphilic phospholipids. These vesicles may range in size from 50 nm to several micrometers and are made up of a sphere bilayer structure around an aqueous core domain. In addition to their desirable biological features, liposomes have the capacity to encapsulate both hydrophilic and hydrophobic medicines, making them ideal for a variety of applications. The features of liposomes, such as their size, surface charge, and functionality, may be readily altered by the insertion of compounds to the lipid membrane or by altering the surface chemistry. In terms of medication delivery, liposomes represent the most established nano systems. In terms of decreasing systemic effects and cytotoxicity, as well as slowing down drug clearance, they have been shown to be effective (Blume & Cevc, 1990; Torchilin, 2005). DNA, antisense oligonucleotide, siRNA, proteins, and chemotherapeutic drugs have been found to have favorable pharmacokinetic characteristics in nanoscale liposomes (Papahadjopoulos et al., 1991). Figure 1 provides

examples of liposomal medications that are more effective and less hazardous than their non liposomal competitors (Bulbake et al., 2017).

Figure 0.1: Commercially available liposomal formulations (Bulbake et al., 2017)

Using liposomal encapsulation technology, medical researchers can transport drugs to the intended target organs. Liposomes are a kind of submicroscopic production that encapsulates a variety of substances. Liposomes are frequently employed as carriers for numerous compounds in the cosmetics and pharmaceutical sectors. Hydrophilic and hydrophobic compounds may be encapsulated in liposomes, preventing their disintegration, and releasing them at their destinations. Liposomes are a well-established technology that has been clinically proved and has received FDA clearance (Akbarzadeh et al., 2013a). In recent years, liposomes have gained widespread acceptance as a delivery mechanism for medicinal medicines. Liposomes may prolong the release of the encapsulated medicinal ingredients, resulting in a better therapeutic result (Khan et al., 2013).

1.5 Wilson's Disease

Wilson disease (WD) is an autosomal recessive disorder of copper transport that is distinguished by reduced excretion of copper into the bile and integration into the transporter copper protein ceruloplasmin. The accumulation of copper in the liver and extrahepatic organs is the defining characteristic of Wilson disease (Behari & Pardasani, 2010a). The toxic buildup of copper in the liver, brain, cornea, and kidney causes the symptoms of Wilson's disease (WD). Bradykinesia, rigidity tremor, dystonia, dysarthria, ataxia, and less often,

chorea and seizures are all neurologic manifestations. Psychological symptoms include depression, personality disorders, cognitive decline, and sometimes psychosis, while hepatic manifestations include acute and chronic hepatitis, cirrhosis, and hepatic failure. In the United States, one in 30,000 people has WD, with a carrier rate of one in 90 and a gene frequency of 0.56 percent. It is more prevalent in populations where there is a high prevalence of consanguineous marriages (Ala et al., 2007).

Considering the wide range of possible symptoms, a low threshold for diagnosis is critical. A positive prognosis is linked to early diagnosis and treatment, but treatment delays may lead to partial recovery and even death. Doctors must look for signs of neurological or liver disease, as well as low serum ceruloplasmin and increased copper excretion in urine, to make the diagnosis of WD. Ceruloplasmin, a copper-dependent enzyme, requires a little amount of copper as a trace element. However, the creation of reactive oxygen species in cells that have too much intracellular copper is hazardous (Gitlin, 2003).

The P type ATPase gene is the target of the hereditary abnormality (ATP7B). Wilson's illness is thought to be caused by more than 500 different types of genetic abnormalities. H1069Q is the most frequently occurring mutation in Central Europe's population (Behari & Pardasani, 2010a). Steatosis, acute or chronic hepatitis, or cirrhosis are all signs of a liver disease that may develop due to Wilson’s disease. It is severe extrapyramidal syndrome, characterized by rigidity, dysarthria, and muscular spasms, may develop from neurological diseases that most typically appear after the age of 20 as motor impairments (tremors, speech, and writing difficulties) (Scheiber et al., 2017). Clinical and laboratory tests serve as the foundation for the diagnosis (neurological signs, liver lesions, low ceruloplasmin, increased free serum copper, high Cu volumes in urine, Kayser Fleischer ring) (Kelly & Pericleous, 2018). Genetic evidence or a high Cu level in liver tissue serve to confirm the diagnosis. Wilson's illness is fatal if left untreated. If cared after appropriately, the survival rate is comparable to the general population's survival rate (Mulligan & Bronstein, 2020).

1.6 Available Treatments for Wilson’s Disease

In order to prevent or reverse the toxic effects of copper, pharmacological treatment for Wilson's Disease aims to reduce the amount of copper being absorbed, induce the production of endogenous cell proteins, promote the excretion of copper in the urine or bile, or a combination of these (Brewer et al., 2001). D-penicillamine, trientine and tetrathiomolybdate (chelating agents) are all examples of pharmacological drugs that remove copper. An increase

in zinc promotes enterocyte metallothionein, which inhibits copper absorption. For long-term maintenance, zinc treatment is the safest and simplest regimen. The experience of using zinc in paediatric patients was recently described and it is usually a good idea to start with Trientine. It has recently been determined which of these drugs has the most important function in the treatment of WD (Schilsky, 2001). Most individuals with abnormal liver function recover after six to 12 months of treatment with medical therapy for this condition. Therapy must continue throughout the patient's whole life (Liu et al., 2017).

1.6.1 Penicillamine (D-PCA)

Due to its ability to *in-vivo* chelate copper, facilitate its elimination, and significantly lessen the disease's severity, D-PCA is a preferred therapy option for WD. Although D-PCA induces a significant degree of copper excretion in the urine, it does so slowly. Patients with advanced or severe WD should not use D-PCA. Adverse responses to D-PCA, such as cognitive degeneration, early indications of gastrointestinal disorders and allergic reactions, leukopenia, thrombocytopenia, hemolytic anemia, and autoimmune diseases, are prevalent when D-PCA is taken for a lengthy period (Xu et al., 2019). This may cause damage of the neurological system, hence D-PCA should be used with care when administered to mice. Hydroxyl radicals and free copper in the striatum rise when D-PCA is administered.

A patient should not be given D-PCA if they are experiencing significant neurological symptoms, notably muscular stiffness. Anaphylaxis, the most dangerous side effect, manifests as a high temperature and a skin rash in the first several days after exposure. When a major adverse response develops, the use of PCA should be promptly ceased (Xie & Wu, 2017). In the same way, people with liver illness should not be given D-PCA since it increases the strain on the liver and may lead to transitory symptoms of early therapy. Up to 20% to 50% of patients may have side effects or neurological abnormalities as a result of their first therapy, and these effects may be permanent in certain cases. PCA, according to some specialists, is not the best therapy for WD (Ala et al., 2007).

1.6.2 Zinc

Zinc has been shown to be a beneficial supplement for people with WD who are trying to limit copper absorption. WD patients were given zinc sulphate or zinc gluconate by mouth, and Yang discovered that this dramatically enhanced the excretion of copper in the urine (Wang et al., 2010). In the third year of follow-up, patients who utilized zinc sulphate as maintenance medication had substantial improvements in their clinical complaints. Zn has

high clinical effectiveness in asymptomatic and preclinical patients, as well as in the maintenance phase after the use of copper chelators (Brewer et al., 1998).

The pharmaceutical company Gate Pharmaceuticals has created a kind of zinc acetate called Galzin specifically for the treatment of Wilson's disease. The Food and Drug Administration (FDA) in the United States has authorized zinc acetate for use in maintenance therapy for adults and children with Wilson's disease. This medication is also effective in the treatment of pregnant women and those who have yet to show any symptoms of the condition. Some patients have trouble adhering to treatment plans over the long term. Hence, routine checks for copper and zinc levels in urine over the course of 24 hours are important. Overtreatment and copper deficit induction may occur with prolonged use of any anti-copper therapy (Brewer, 2001).

Chapter 2: Literature Review

2.1 Silymarin

Many studies have demonstrated that herbal medicines are widely used because of their preventive benefits on a variety of organ toxicities. Neurotoxicity, depression, hepatitis, cancer, hepatitis B, and nephrotoxicity are only a few of the conditions where these protective effects have been shown (Ernst, 2005). Liver and gallbladder issues; protecting the liver from snake and bug stings, mushroom poisoning, and alcohol misuse have all been treated using milk thistle (Silybum marianum L.), an old medicinal plant from the Carduus marianum family. The seeds or fruits of this plant, which may be found in Kashmir, North America, Canada, and Mexico, have a reddish-purple bloom and big thorny leaves (Karimi et al., 2011).

Milk thistle was initially cultivated in Europe, where it was used to treat jaundice since it was believed to be able to open the liver's blockages. As a result, this herb has been utilized for millennia as an alternative therapy for gastrointestinal system and digestive issues, liver and biliary system ailments, menstruation difficulties, and varicose veins (Saller et al., 2001). Milk thistle, on the other hand, was originally used for its antioxidant and hepatoprotective properties. In addition to silymarin, other flavonoids including silychristin, neosilyhermin, silyhermin, and silydianin may also be found in the herb's fruit and seeds, but the active ingredient is a complex mixture of silymarin and these other flavonoids. Since it scavenges free radicals and boosts glutathione levels, silymarin may be used to treat hepatitis, hepatic cirrhosis, and mushroom poisoning in addition to its antifibrotic, immunomodulating, anti-inflammatory benefits (Fraschini et al., 2002a).

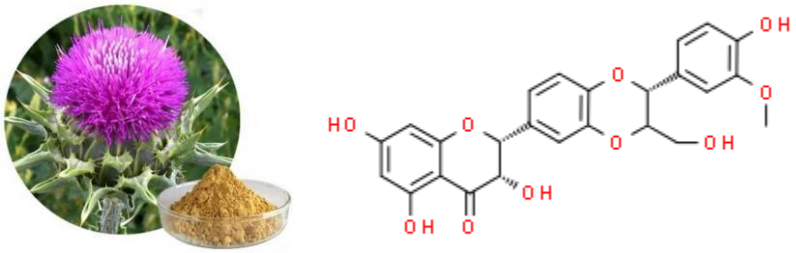

Figure 0.1: Milk thistle and its extract, the chemical structure of silymarin (Fraschini et al., 2002a)

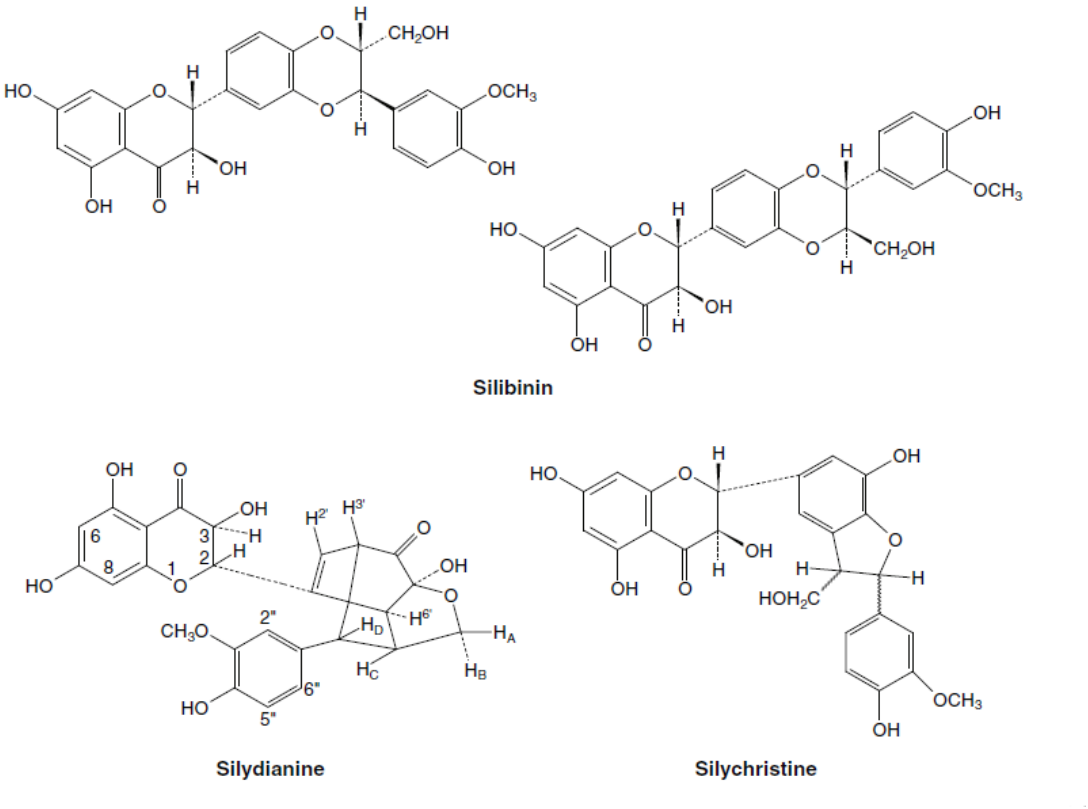

Figure 0.2: The three structural components of silymarin: silibinin, silydianine, and silychristine

2.1.2 Silymarin as a Hepatoprotectant

In terms of silymarin's three isomers, silibinin is the most potent. Silymarin and silibinin have been discovered to be cytoprotective and, above all, hepatoprotective in the medical community. Numerous liver illnesses with degenerative necrosis and impaired function are treated with silymarin (Cacciapuoti et al., 2013). The toxin of Amanita phalloides may also be antagonized by this flavonoid, as well as the toxicity of galactosamine, thioacetamide, halothane, and carbon dioxide. Histamine, radiation, iron overload, and even viral hepatitis cause minimal injury to the hepatocytes while the chemical is present. The drug silymarin is frequently prescribed as a supportive therapy in cases of food poisoning caused by fungus and chronic liver problems such as steatosis and alcohol-related liver disease. The drug is included in the pharmacopoeias of several countries under the brand names LegalonTM or HepatronTM (Fraschini et al., 2002a).

In terms of liver health, silymarin is a hepatoprotective as well as a regenerative agent. Lipid peroxidation (LPO) and hepatocyte external cell membrane alteration are the two mechanisms of action that reduce the oxidative stress generated by toxins that disrupt cell

membranes (Vargas-Mendoza et al., 2014). Toxins cannot get into the cells of the liver because of a compound formed by silymarin. The metabolic stimulation of hepatic cells and the activation of RNA iosynthesis by ribosomes by silymarin are additional benefits of this compound (Pietrangelo et al., 1995). Researchers found that silymarin had an impact on rat hepatic cells when employed as a reference factor to evaluate liver weight/animal body weight percent. The incidence of hepatomegaly was lower in this group than in other groups that received antioxidant supplements. Between the silymarin and silymarin-alcohol groups, no differences were found. This implies that silymarin protects the liver (Sonnenbichler et al., 1986).

By increasing cysteine availability and promoting cysteine synthesis, silymarin boosts hepatic glutathione production, while suppressing the conversion of cysteine to taurine (Kwon et al., 2013). The control of cysteine production may thus play a role in antioxidant defense. When silymarin was given to rats with bile fibrosis, it decreased collagen accumulation by 30% (Boigk et al., 1997). Patients with cirrhotic alcoholism had a marginally better survival rate than those in the untreated control group in a human study (Ferenci' et al., 1989). Because of its antioxidant, anti-inflammatory, and anti-fibrotic properties, silymarin is one of the most often utilized natural compounds for the treatment of liver diseases (Bergheim et al., 2006).

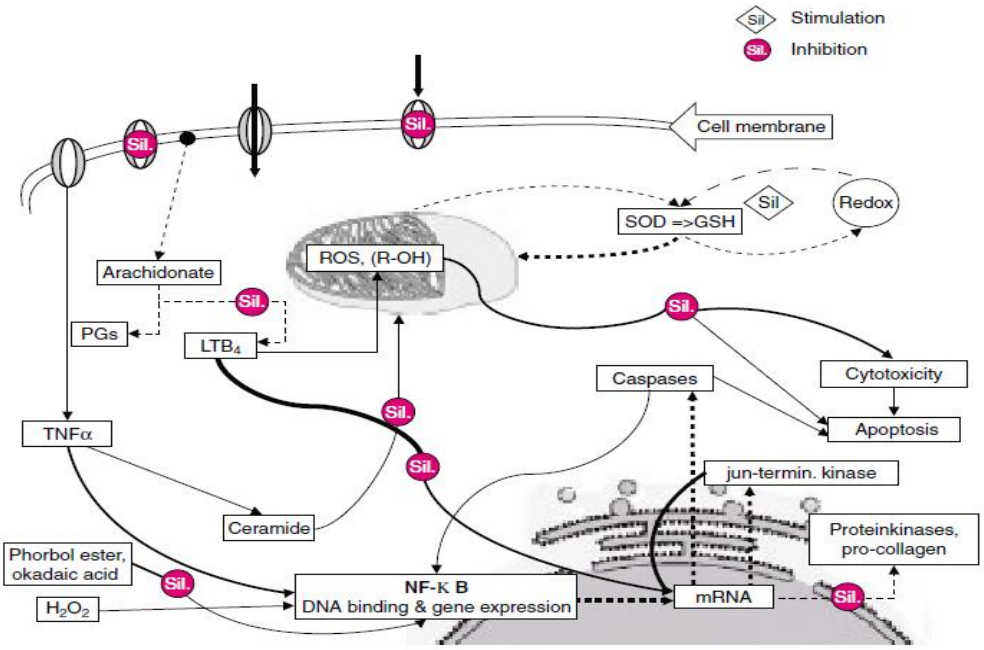

Figure 0.3: Schematic overview of the main pharmacological effects of silymarin relevant to its hepatoprotective properties. (Saller et al., 2001)

2.2 Pharmacodynamics of Silymarin

2.2.1 Antioxidant Properties

Antioxidant activity is often high in flavonoids. As silibinin's dehydrosuccinate sodium salt, dehydrosuccinate sodium salt of silbinin, which is hydrophilic, acts as an effective inhibitor of the oxidation of linoleic acid in water by Fe2+ ions (Ferenci' et al., 1989). A well-known experimental system for the generation of hydroxy radicals, the NADPH-Fe2+-ADP microsomal peroxidation, is likewise inhibited in a concentration-dependent manner (Kondeva-Burdina et al., 2013). Lipid peroxidation caused by Fe(III)/ascorbate is suppressed by silibinin dihemisuccinate in rat hepatic microsome experiments; the suppression is concentration-dependent (Bosisio et al., 1992; Mira et al., 1994). Regardless of the model used to induce peroxidation, silymarin has been found to be as effective as quercetin and dihydroquercetin and even more effective than quercitrin as an antiperoxidant (Cavallini et al., 1978).

Tert-butyl hydroperoxide (TBH)-treated rat hepatocytes showed a reduction in lactate dehydrogenase loss, increased oxygen consumption, decreased generation of lipid peroxides, and increased urea synthesis when silymarin was added to the perfusion medium. TBH produces a rise in Ca2+, which silymarin may counteract by lowering ion levels to below 300 nmol/L. The suppression of lipid peroxidation and the modification of hepatocyte Ca2+ concentration seem to play a key role in silymarin's protective action (Farghali et al., 2000).

2.2.2 Protective Effects in Models of Oxidative Stress

Structural and functional damage to tissues may result from the unregulated production of prooxidant free radicals in the body. An inducer's prooxidant effect surpasses the antioxidant potential of the cell defense mechanism, resulting in oxidative stress. When administered to newborn rats, silibinin was shown to protect neonatal hepatocytes against damage caused by erythromycin and other antidepressant medications (Davila ' et al., 1989). Rat hepatocytes exposed to hypotonic saccharose solutions, which induce osmotic stress, similarly showed silymarin's cytoprotective effect (Farghali et al., 2000). Oxidative stress in the liver caused by ethanol or paracetamol may be countered by silymarins and silibinins (Carnpos et al., 1989). Additionally, it has been shown that silibinin therapy reduces the rise in plasma levels of AST, ALT, and GGT following paracetamol intoxication, as has been previously documented (Valenzuela & Garrido, 1994).

2.2.3 Activity against Lipid Peroxidation

Lipid peroxidation is brought on by the presence of both free radicals and unsaturated fatty acids in a given environment. There is a potential for a variety of illnesses of lipoprotein metabolism to manifest in the liver and in other organs as a consequence of the degeneration of cell membranes brought on by lipid peroxidation. Silymarin works as an antioxidant not only due to the fact that it functions as a free radical scavenger, but also due to the fact that it has an effect on enzyme systems that are related with glutathione and superoxide dismutase (Valenzuela et al., 1989). It has been discovered that all components of silymarin are capable of inhibiting lipid peroxidation, and silymarin protects the mitochondria and microsomes of rat liver cells against chemicals that are responsible for the generation of lipid peroxide *in-vitro* (Bindoli Cavalln & Siliprandi, 1977; Fiebrich & Koch, 1979).

2.2.4 Stimulation of Liver Regeneration

Protein synthesis in the wounded liver is one of the processes that might explain silymarin's ability to induce liver tissue regeneration. Silibinin increased the production of ribosomes and DNA synthesis, as well as protein synthesis, in the livers of rats that had had a portion of their organs removed in trials. Intriguingly, silibinin only increased protein production in wounded livers, not in healthy ones (Bahmani et al., 2015). Since RNA polymerase I may be controlled physiologically at particular binding sites, the mechanism by which silibinin increases protein production in the liver has yet to be fully elucidated(Luper, 1998). Galactosamine's inhibitory impact on the production of liver proteins and glycoproteins was totally eliminated in rats treated intraperitoneally with silymarin 140 mg/kg for four days (Fraschini et al., 2002b).

2.2.5 Inhibition of Cytochrome P450

Cytochrome P450 (CYP) detoxification may be inhibited by silymarin, which has anti-inflammatory properties (phase I metabolism). Recent studies in mice have demonstrated that silibinin is capable of inhibiting a number of hepatic CYP enzyme activities, (Baer-dubow Ska et al., 1998) however silymarin has not been shown to have any impact on the CYP system at all (Miguez et al., 1994).

Silymarin's hepatoprotective benefits, notably against A. phalloides poisoning, may be attributable to this action. Only until the CYP system activates the Amanita toxin does it become harmful to hepatocytes. Anti-bioactivation of toxins may help reduce their harmful consequences. The ability of silymarin and other antioxidants to scavenge free radicals,

which are generated by enzymes in the CYP system, may be another benefit of antioxidant supplementation (Fraschini et al., 2002b).

2.3 Mechanisms of Action of Silymarin

α-amanitin and phalloidin are inhibited from adhering to the cell surface by silymarin and silibinin, which also prevents their absorption by the body's membrane transport mechanisms. As a result, silymarin and silibinin have the ability to alter the chemical and physical characteristics of cell membranes. Many types of cells have been studied, including erythrocyte, mast and leucocyte as well as macrophage and hepatocyte, and the results reveal that silymarin increases the resistance of cell membranes to damage (Valenzuela & Garrido, 1994) (MOURELLE et al., 1989).

Furthermore, the protection provided by silymarin and silibinin against hepatotoxic chemicals may be explained by the well-documented scavenging action of these compounds. It is possible that the antioxidant properties of silymarin and silibinin may have a role in the treatment of hepatic damage caused by toxic substances. As free radicals are suppressed, GSH levels are elevated, and SOD activity is stimulated, it is likely that silymarin and silibinin may counteract the reduction of the two primary detoxification systems, GSH and superoxide dismutase (SOD) (Fraschini et al., 2002b). Simultaneously, silibinin seems to stimulate RNA polymerase I and the transcription of rRNA in the nucleus, increasing ribosomal protein production in the cell membrane and the nucleus, respectively (Luper, 1998). Hepatotoxin-damaged structural proteins and enzymes must be restored, which is why stimulating protein synthesis is such a crucial part of the healing process (Valenzuela & Garrido, 1994).

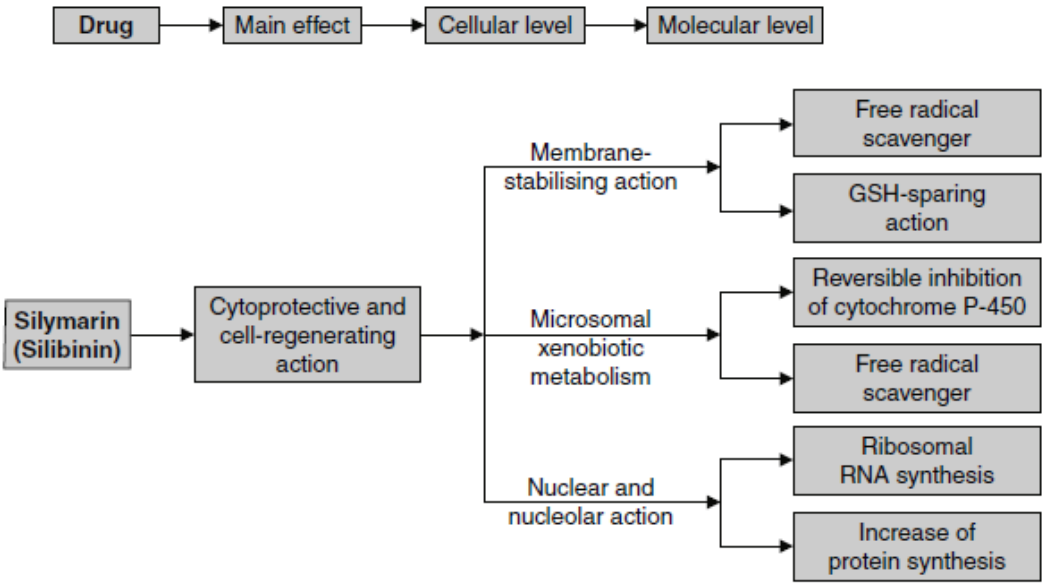

Figure 0.4: Mechanism of action of silymarin as proposed by Valenzuela and Garrido (Valenzuela & Garrido, 1994)

2.4 Pharmacokinetics of Silymarin

Typically, silymarin is supplied in capsule form as a standard extract (70 to 80 percent silymarin), as it is not water-soluble. After oral treatment, just 2% to 3% of the drug is reabsorbed in the bile of rats. Peak plasma levels are reached in animals and humans within four to six hours. Most of silymarin's excretion occurs in the bile and the urine. Its half-life in the body is between 6 and 8 hours (Morazzoni et al., 1993). As silibinin, silymarin or pure S. marianum extract in mice produced plasma levels of 500 mg/L (as silibinin) 90 minutes after oral treatment of 200 mg/kg. The liver quickly conjugates silibinin and other silymarin components with sulphate and glucuronic acid. Conjugates are excreted in the bile and plasma, where they account for 80% of the dosage supplied to patients. Only a very little amount is excreted in the urine. Enterohepatic circulation is suggested by these findings: absorption in the gut, conjugation in the liver, bile excretion, hydrolysis by the flora, and reuptake in the intestine (Schrieber et al., 2011).

In SENCAR mice (6 to 7 weeks old), 50 mg/kg of silibinin was administered orally and the tissue distribution of the drug was investigated. A half-hour after intake, silibinin concentrations peaked in the liver, lungs, stomach, and pancreas, with values of 8.8 ± 1.6, 4.3 ± 0.8, 123 ± 21, 5.8 ±1.1 µg/g of tissue, respectively. Silibinin concentrations in the epidermis and prostate peaked at 1.4 ± 0.5 and 2.5 ± 0.4 µg/g, respectively, one hour after ingestion. The highest concentrations of sulphate conjugates and -glucuronides of silibinin

were found in all other tissues except the lungs and stomach, which had peak values after 0.5 hours. After 0.5 or 1 hour of treatment, there was an exponential decline in concentrations of free and conjugated silibinin, with an elimination half-life ranging from 45 to 94 minutes for the conjugated component. When silibinin was administered orally at doses of 100 and 200 mg/kg/day, there was a considerable increase in the amount of phase II enzyme activity found in the liver, small intestine, lungs, stomach and skin (Zhao & Agarwal, 1999).

2.5 Carrier Necessity for Silymarin Delivery

Silymarin is a biopharmaceutical class IV molecule that must be delivered in a manner that meets certain parameters. A lack of water solubility for the active components in phytomedicines might adversely influence their bioavailability, which is an issue for development and engineering of drug delivery systems (Piazzini et al., 2018). Alternatively, researchers may now use a variety of solubilization methods to entrap weakly water-soluble active plant components such as silymarin in aqueous nanovehicles. It is thus necessary to use nanostructured lipid carriers to circumvent these restrictions. In order to enhance its bioavailability and give a continuous delivery of the active herbal extract, nanotechnology looks to be a potential way to enhance therapeutic activity and promote sustained release (di Costanzo & Angelico, 2019).

2.6 Liposome Nanoparticles for Liver Diseases

It is possible to use liposomes as a drug delivery vehicle by encapsulating medicines that are hydrophobic (stick to membranes) or hydrophilic (stick to water) in the aqueous core (Allen & Cullis, 2013). Passive and active (remote) loading of medicines into liposomes are the two most often used strategies. This method depends on dissolving the hydrophobic medication in organic solvent, followed by solvent removal, or hydration with water that contains a water-soluble drug in order to achieve passive loading. Liposomes are then created concurrently with the loading of the medication. An active loading method uses a gradient to load pharmaceuticals into liposomes that have already been created. As a result of limited drug-liposome interaction, "burst release events" are more likely to occur in passively loaded liposomes (Gubernator, 2011). Active loading, on the other hand, often results in a high drug-to-lipid ratio and stable particles. A large range of formerly "unloadable" substances may now be loaded using active loading techniques, including amphipathic compounds.

Hepatocytes make up most of the liver parenchyma and are implicated in a wide range of disorders, including HCC, hepatitis B/C, Wilson's disease, and many more. The hepatocyte

has been a target for liposomal medication delivery because of its participation in a wide range of disease processes. Liposomal compositions have therefore been tested for their ability to target hepatocytes, with variable degrees of effectiveness. There are several receptors that may be employed to target liposomes to hepatocytes, including ASGPR, heparan sulphate proteoglycans, and the folate receptor (FR) (Böttger et al., 2020).

2.6.2 Stealth Liposome Nanoparticles

Even though liposomes and biomembranes are closely related, the body views them as alien antigens. In this way, they are identified by the body's Reticulocyte endothelial system (RES) after contact with plasma proteins. As a result, they are flushed out of the body (Akbarzadeh et al., 2013b). Synthetic phospholipids and polyethylene glycol (PEG) coating of the liposome particle, chitin derivatives, freeze drying, polymerization, and ganglioside micro-encapsulation solve these stability restrictions (Shaheen et al., 2006). Reduced liposomal phagocytosis and long-term circulation result in frequent time for these liposomes to leak out of circulation via endothelium, which is why PEG coating is beneficial.

To deliver medications or genetic material to specific cells, stealth liposome vesicles have a sphere-shaped bilayer membrane made of phospholipids and a variety of lipid chains that are stabilized or coated with PEG or colloidal polymers. Liposomes may be disguised to create new forms of controlled-release medication delivery (Akbarzadeh et al., 2013b).

2.7 Copper in The Human Body

Cu, or copper, is an important mineral required for a wide variety of functions in the human body. Enzyme prosthetic groups and protein binding account for the great majority of Cu in healthy persons. The complicated system of Cu transporters and chaperone proteins strictly controls Cu homeostasis. The liver and the brain have the greatest quantities of copper (Cu) of any organ or tissue in the body. The cupric form of copper (Cu++) is the most common in living things, however enzymes that include copper may have many bonded cation forms, sometimes even in the same molecule (Gaetke et al., 2014). Ceruloplasmin, also known as ferroxidase I, is a Cu protein that helps shuttle copper from the interstitial lumen and retention locations to the cells that carry out erythropoiesis. Cu is essential in the development and upkeep of the myelin sheath that surrounds neurons, and it plays a role in the production of the melanin pigment that colors the skin, hair, and eyes. Copper is also included in copper, zinc-superoxide dismutase (Cu, Zn-SOD), which scavenges the free radical superoxide, and in cytochrome c oxidase, which catalyzes the reduction of oxygen to

water, the fundamental step in cellular respiration (R Uauy, 1998). Cu is also present in the catecholamine biosynthesis enzyme dopamine-beta-hydroxylase. Errors in Cu-enzyme/-protein activity likely contribute to the etiology of a wide range of diseases, including those affecting the liver, the nervous system, and other organs (de Romaña et al., 2011).

In normal circumstances, the body can regulate the quantity of Cu by either decreasing absorption or increasing excretion, since the Cu quantity consumed via food and water is fairly modest (Evans & Halliwell, 1994). Acute and chronic Cu poisoning are uncommon because the body's Cu homeostasis is well-regulated, preventing excess buildup of Cu. However, adrenal gland insufficiency, inherited abnormalities in Cu metabolism, and accidental or occupational exposure to high levels of Cu may all lead to hazardous effects. The pathophysiology, therapeutics, and preventative measures for health issues related with Cu toxicity have been improved by recent research on how copper imbalance develops and changes metabolic activities (Rosenzweig, 2001).

2.7.2 Copper Status and Markers

Vitamin and mineral supplementation, as well as adrenal gland function, may all have a role in determining a person's Cu status beyond what they eat and other exposures. Hormones released by the adrenal glands, for instance, stimulate the liver to produce ceruloplasmin, the body's primary Cu-binding protein (Harris, 1993). A buildup of Cu in the body might result from a dysfunctional liver or an inadequate adrenal gland. Bioavailability of Cu is reduced when the liver is unable to secrete ceruloplasmin. Zinc (Zn) and other minerals in the diet may also affect Cu status. Zn shortage is linked to Cu accumulation in several storage organs because Zn acts as Cu's antagonist. In spite of the fact that Cu toxicity is relatively rare under normal living settings, there is mounting evidence to indicate that copper insufficiency is more common than what was thought before. Mild Cu shortage or excess Cu subjection is not readily identified. However, there are no reliable diagnostic tests for Cu poisoning, hence blood, urine, and hair analyses are utilized instead (Harris, 1992). The most often used markers are serum Cu concentration and ceruloplasmin. While both methods may detect little shifts in Cu status, the most noticeable shifts are only picked up by the more sensitive indicator. Because ceruloplasmin production is controlled by the quantity of accessible Cu in the liver, it is considered as a method of identifying the status of copper in the body. Additionally, the consequences of intermediate levels of Cu shortage and superfluous Cu subjection are poorly understood (M Olivares, 2008).

Several cuproenzyme activities are reduced in moderate Cu deficiency. Their use is limited, however, by a dearth of standardized tests and a high degree of inter- and intra-subject variability. In addition, there are no known laboratory tests that may be used as early indicators of Cu overload. Since copper is put away for the most part in the cerebrum, liver, and different organs as opposed to in the blood or urine, there is an interest for biomarkers that are precise, delicate, and painless to distinguish an expansion in copper levels in the body before the beginning of useful or clinical outcomes (MC Linder, 1996). Out of the many proteins investigated as potential markers of copper status, the copper chaperone, zinc superoxide dismutase, seems to provide data that are encouraging (Rodriguez-Granillo et al., 2010). The reactant working of Cu, Zn-SOD in tissues was exhibited to be debilitated by dietary limitation of Cu, and Cu supplementation reestablished the protein action in Cu-lacking rodents. Also, unobtrusive Cu shortage in rodents causes an expansion in the protein creation of the Cu chaperone for SOD in the erythrocytes, though Cu supplementation significantly lessens the amount of the Cu chaperone for SOD mRNA in the mononuclear blood cells. Notwithstanding the requirement for more examination under different settings to demonstrate its viability as a sign of early Cu deficiency, the consequences of these examinations show that adjustments of the action as well as articulation of Cu, Zn-SOD might act as an appropriate marker of the amount of copper in the body (MA Levy, 2001).

Because both insufficient and excessive Cu consumption are associated with negative health outcomes, establishing adequate intake levels and threshold levels for Cu is a difficult task. Physiological systems provide proper Cu homeostasis at this consumption level, and detrimental effects are undetectable. By definition, toxic levels are intakes that exceed the maximum safe limit. Poisonous outcomes from overabundant Cu are for the most part subject to the intriguing rate of clinical sickness, like liver cirrhosis for reasons unknown. This is on the grounds that there are no painless, delicate biomarkers of capacity or early harm from overabundant Cu (de Romaña et al., 2011). Predictive biomarkers for increased hepatic Cu storage and, by extension, the development of illness remains elusive. Increases in liver enzyme levels, and scattered, dotted hepatocytic necrosis were seen in male Wistar rats exposed to a high dosage of Cu, as reported by Yang et al. They further show that subjection to low, sub-harmful portions of Cu created changed gene expression levels and that genes associated with oxidoreductase action, digestion, and signal transduction added to the advancement of the noticed side effects. In view of these outcomes, it appears to be that

adjustments of quality articulation might be more delicate signs of conceivable pernicious impacts of Cu than ordinary proportions of toxicity (Yang et al., 1997).

2.7.3 Copper Homeostasis

In order to meet the demands of the cells while limiting the detrimental consequences associated with shortage or excess, organisms have developed a range of mechanisms for the effective absorption, intracellular transit, protein stacking, and copper capacity. Digestive assimilation, biliary discharge, and intrahepatic stockpiling keep Cu levels steady in the body, and low to moderate Cu exposure has little to no effect on Cu homeostasis. The liver assumes a critical part in the catch, dispersion, and discharge of Cu in warm blooded creatures (JR et al., n.d.).

The rate of Cu absorption is affected by the individual's Cu status as well as their Cu consumption, compound structure, and the presence of different components in the food that might energize or hinder its retention. The human body cannot keep a substantial supply of Cu. About 30–50% of copper that is taken is absorbed in the small intestine, largely in the form of Cu++; only limited quantity absorbed in the stomach. Absorption of Cu in the small intestine results in its transit in the blood, where it is mostly coupled to albumin but also to transcuprein (ZL & JD, n.d.). Hepatocyte storage, plasma secretion, and biliary excretion are all possible destinations for ingested Cu. Metallothionein and cuproenzyme synthesis account for the vast majority of Cu in hepatocytes. Ceruloplasmin is the primary carrier of Cu from the liver to the tissues, while albumin, transcuprein, and histidine may also bind to Cu. Up to eight Cu atoms, in both the cupric and cuprous phases, may be found in ceruloplasmin, the primary Cu-binding protein and acute-phase protein. Ceruloplasmin is the major form in which copper being carried in the blood exists (Harris, 1993).

Tissue-expressed Cu chaperones and Cu-transporting ATPases (Cu-ATPases) are responsible for facilitating the transport of copper to freshly generated cuproenzymes and for removing excess copper from the body, respectively. Cu homeostasis is maintained in part via Cu trafficking routes, which provide Cu for vital enzymes and proteins while limiting accumulation of the metal to dangerous levels (Prohaska, 2008). The role of copper-handling proteins, also known as copper chaperones, in regulating copper transport inside cells, has been established. Specifically, Cu chaperones are involved in three of the most important routes for copper (Cu) trafficking. The Cu chaperone expressed by the Cu chaperone for superoxide dismutase (CCS) gene transports Cu to Cu, Zn-SOD, and Cu delivered to the

mitochondria for activation of cytochrome c oxidase, whereas the Atox1 Cu chaperone transports Cu to transport ATPases in the secretory route (Fields et al., 2001).

In yeast without superoxide dismutase, the Cu transport protein Atox1 goes about as a Cu-subordinate silencer of oxidative harm. Neuronal cell lines transfected with the Atox1 quality to support endogenous Atox1 articulation are safeguarded against serum starvation and oxidative pressure, and Kelner et al. (2000) further showed that Atox1 might assume a part in safeguarding neuronal cells against oxidative harm produced by Cu (Kelner et al., 2000). By examining the capability of Cu conveyance to the secretory course in Cu usage and homeostatic upkeep, Hatori et al. (2012) showed that the glutathione/glutathione disulfide (GSH/GSSG) match manages the Cu transport pathway by regulating the redox condition of a Cu chaperone Atox1. By shaping an intramolecular disulfide, GSSG oxidizes the Cu-organizing cysteines in Atox1. The disulfide may be reduced by GSH alone, restoring Atox1's copper-binding capacity. Lack of Atox1 is lethal to cells, yet high GSH levels are essential for their survival. When glutathione (GSH) levels drop, atox1's redox potential is comparable to that of glutaredoxin, making it critical for cellular survival. The outcomes raise the likelihood that GSH homeostasis and Cu homeostasis are practically associated and cooperate to support circumstances for Cu removal and cell expansion (Hatori & Lutsenko, 2013).

Cu-ATPases ATP7A and ATP7B are fundamental for the biosynthetic consolidation of copper into secretory and plasma film bound proteins. Synergist ATPase action, Cu-actuated dealing, posttranslational changes, and protein connections are just a portion of the manners by which ATP7A and ATP7B apply their exercises in Cu transport. Both ATP7A and ATP7B play urgent capabilities in human physiology, and they are developmentally moderated polytopic layer proteins (Prohaska, 2008). There is widespread expression of Cu-ATPases, and their transport activity is essential for numerous cellular processes, including those involved in brain maturation, liver function, and connective tissue creation. CTR1 and CTR2 are two homologous transmembrane solute carrier transporters that regulate the uptake of reduced copper ions. Cu transporters CTR1 (encoded by SLC31A1) and CTR2 (encoded by SLC31A2), as well as ATP7A and ATP7B, have been studied for their molecular properties, involvement in mammalian Cu homeostasis, and the physiological repercussions of their inactivation. Expression levels and subcellular localization of these Cu transporters are very variable (Lutsenko, 2021). CTR1 is involved in several stages of mammalian development, from early embryonic through adult homeostasis regulation. In contrast, very little is known

about CTR2 and how it is regulated, expressed, or even supposed to operate. Both are the main transporters for platinum-based chemotherapy medicines like cisplatin and may carry other divalent metal ions as well. Many eukaryotic organisms, from yeast to humans, rely on the Cu transporters of the SLC31 (CTR) family (Wee et al., 2013). It has become evident that organic entities have advanced captivating systems for tight control of Cu digestion through the portrayal of the capability, methods of activity, and guideline of CTR and other sub-atomic elements that practically help out CTR for Cu transport, compartmentalization, fuse into cuproproteins, and detoxification (Kim et al., 2013).

Both ATP7A and ATP7B, which are tracked down in the Golgi complex, are copper-shipping ATPases that move copper particles from the cytosol to the Golgi lumen, where they might be utilized by Cu-subordinate chemicals. Golgi, a vital compartment for fitting Cu homeostasis, is where Cu is integrated into the secretory and plasma layer focused on cuproenzymes. The Golgi complex additionally conveys these ATPases to appropriate post-Golgi objections to ensure legitimate Cu motions in the body and to forestall possibly dangerous Cu development (R. Polishchuk & Lutsenko, 2013). Transformations in ATP7A or ATP7B or in the proteins that administer their dealing change their departure from Golgi or resulting recovery to this organelle. This might cause Cu lack (Menkes sickness) or Cu overabundance by disturbing the homeostatic Cu balance (Wilson illness). Changes in the ATP7B carrier, which loads Cu(I) onto recently created cuproenzymes in the trans-Golgi organization (TGN) and sends out abundance copper by means of dealing from the TGN to the plasma film, cause Wilson sickness (WD). While we presently find out about the enzymatic qualities and cell science of the Cu-ATPases because of these exploration, we actually don't have the foggiest idea how the Golgi controls the dealing of ATP7A/7B to keep Cu levels stable (Strausak et al., 2001).

2.7.4 Mechanisms of Copper Toxicity

Protective mechanisms against metal-induced toxicity are often a trifecta of import regulation, sequestration, and boosted export in most species. These processes control metal status at the transcriptional, translational, and enzymatic levels through metal-binding proteins. As referenced above, Cu homeostasis is firmly controlled by an organization of metal particle carriers and chaperones to guarantee that Cu is provided to basic proteins without harm to the cells (Bleackley & MacGillivray, 2011). Tissue injury and illness are linked to Cu homeostasis disruptions. Several processes, including free radical prompted oxidative harm, have been proposed to make sense of Cu-actuated cell poisonousness,

notwithstanding the immediate connection with basic macromolecules and minerals (de Romaña et al., 2011).

As a result of their ability to participate in redox cycling events, transition metal ions like Fe and Cu encourage the creation of ROS. Numerous studies have linked Cu toxicity to Cu ions' ability to produce ROS, which modify the design or potentially capability of crucial biomolecules. Reducing Cu++ to Cu+ with superoxide or reducing agents like ascorbic acid or GSH allows Cu+ to catalyze the Haber-Weiss process, which produces hydroxyl radicals from hydrogen peroxide (B Halliwell, 1984). Considering that reducing Cu++ only requires one electron, the second electron could be involved in the generation of hydroxyl radicals. In biological processes, the hydroxyl radical is the most potent oxidizing radical that can form. It may cause DNA strand breakage and base oxidation by drawing hydrogen from amino carbons to produce carbon-centered protein radicals and from unsaturated fatty acids to form lipid radicals, respectively (Bremner, 1998). The response result of nitric oxide and superoxide, peroxynitrite, may also facilitate the liberation of Cu ions from protein complexes like ceruloplasmin. In order to exert their effects, transition metal ions may generate ROS such superoxide and hydroxyl radical, which in turn generate DNA adducts like malondialdehyde and 4-hydroxynonenal (Gaetke et al., 2014).

Many experimental investigations have shown evidence that oxidative harm brought about by ROS contributes altogether to Cu toxicity. Examples include Cu's ability to adavnce atherogenesis by promoting the conversion of macrophages into foam cells and by developing vasoconstrictor and prothrombotic qualities, and by involving oxidative alteration of low-density lipoprotein (LDL) (Powell, 2000). Peroxidation in the membranes of hepatocyte lysosomes may be caused by excess Cu due to the interaction of lipid radicals with oxygen to create peroxy radicals (RJ Sokol, 1990). Furthermore, Cu overload greatly lowers the activities of catalase and GSH peroxidase, and it inhibits mitochondrial respiration and increases chemiluminescence in rat liver. Malignant growth, cardiovascular infection, diabetes, atherosclerosis, neurological issues, and constant aggravation may be generally brought about by ROS since they might overpower the body's cell reinforcement components and cause DNA harm, lipid peroxidation, protein alteration, and different effects (L. Zhang et al., 2019).

Metal chelators, for example, ammonium tetrathiomolybdate and ethylenediaminetetraacetic corrosive, lessen neuronal demise in rodents after intrahippocampal infusions of cupric

sulfate, providing evidence to a job of oxidative damage in Cu poisonousness (Myers et al., 1993). In mice expressing different quantities of Cu, Zn-SOD, hyperoxia increases the metal-binding proteins ceruloplasmin and metallothionein in the lungs, but it has no impact on Cu, Fe, or Zn tissue levels. Ceruloplasmin may serve as a storage of Cu for Cu, Zn-SOD production, since mice with enhanced expression of Cu, Zn-SOD show a considerable drop in circulating ceruloplasmin and Cu concentrations. In addition, dietary Cu may influence endothelium-dependent arterial relaxation because persistent decrease in Cu, Zn-SOD degrades vascular tone. This is likely mediated by a direct inactivation of nitric oxide synthesis and an increase in lipid peroxidation (MA Levy, 2001).

Menkes disease and Wilson's disease are two serious conditions that affect humans and are linked to abnormal Cu absorption and excretion. The deficiency of Cu has been related to the inactivation of critical metabolic enzymes; however other, non-enzymatic mechanisms may also be at play in Menkes disease (Song et al., 2011). In contrast, Cu-induced oxygen radical-mediated damage is often blamed for the effects of Cu buildup in WD. Depending on the individual, Wilson's illness may appear mostly in the liver or the nervous system. Lipid peroxidation in the mitochondria of the liver is present in WD patients, and vitamin E levels in the liver and blood are lower than they should be (BM Myers, 1993).

Genetic aberration of Cu metabolism (WD) and the assumed environmental illness Indian Childhood Cirrhosis both show signs of chronic Cu poisoning in the form of liver cirrhosis and harm to other organs (ICC). Consumption of milk that has been cooked or kept in corroded Cu or brass containers seems to be the source of ICC, non-ICC, and potentially idiopathic Cu toxicity; however, a genetic predisposition has also been related to ICC-like sickness (Strausak et al., 2001). In addition to its role in the development of ALS, Alzheimer's, Parkinson's, and Huntington's disorders have all been linked to Cu in the literature. Neuronal death, elevated levels of Cu, Fe, and Zn, and amyloid-beta protein deposits are all hallmarks of Alzheimer's disease. The gene molecule for amyloid precursor protein in early-onset Alzheimer's disease has a Cu-binding site, and amyloid- protein binding to Cu and Zn may enhance ROS formation in the brain (Vulpe et al., 1993).

2.7.5 Copper Toxicity as a Pathological Condition of Wilson’s Disease

The transition metal copper is a trace element that is necessary for the body. To facilitate electron transfers to oxygen, it has a very high redox potential. As a cofactor for enzymes, Cu must be sequestered to prevent the formation of reactive oxygen species. In part because of

the liver's microcirculatory system and its unique position in the body, copper metabolism is tightly controlled by the liver (Barber et al., 2021). Because oxidative cell damage, reduced immunological function, and organ dysfunction are linked to both too much and too little Cu, its absorption, uptake, export, and transport are strictly managed. Copper (Cu) is used for mitochondrial respiration and free radical detoxification in liver parenchymal cells, and it is also actively transported into the trans-Golgi network through ATP7B transporters (Bhattacharjee et al., 2017). Metalothionein (MT) proteins and ATP7B transport excess Cu to endo- or lysosomal-derived compartments and the apical (bile canalicular) membrane, respectively (Nyasae et al., 2007; E. v. Polishchuk et al., 2014).

Viral infection and cholestatic illness have been linked to elevated Cu levels. However, it is not obvious whether oxidative damage is the major mechanism of toxicity from Cu. Over-accumulation of copper causes acute oxidative stress in animals, but studies in animals show that cells' redox buffering can reduce this stress but cannot compensate for other biochemical effects, such as lipid metabolism changes, transcriptional activation impairments, or mitochondrial fragmentation (Barber et al., 2021).

With an estimated worldwide allele frequency of 1:90, Wilson Disease is the most widely recognized form of copper toxicity and is an autosomal recessive illness of copper metabolism. A great deal of focus should be given to the disease's neuropathology. However, the connection between hepatic and brain Cu toxicity and neurologic WD is poorly recognized. Cu concentrations in the WD brain may be up to 10 times higher than normal due to non-specific accumulation (Litwin et al., 2013).

Chapter 3: Materials and Methods

3.1 Experiment Design

3.1.1 Materials

Purchased from Sigma-Aldrich USA: Dipalmitoyl phosphatidyl choline (DPPC), Cholesterol, commercially available Silymarin medication, Polyethylene glycol (PEG- Molecular weight 2000). Additionally, copper sulphate and zinc sulphate were also acquired from Sigma-Aldrich USA. Islamabad's ASAB (Atta-ur-Rahman school of Applied Biosciences), National University of science & technology (NUST), sold the female Wistar rats used in the study. Throughout the investigation, ethanol, deionized, and distilled water were used.

3.1.2 Synthesis of PEGylated Silymarin-loaded Liposome Nanoparticles

Liposomes contents, the lipids dipalmitoylphosphatidylcholine (DPPC) and cholesterol, were used for synthesis. 0.5 mM stock solutions of both the lipids were prepared, out of which 100 µM solutions were used by taking 1.6 mL of DPPC stock and 0.4 mL of cholesterol stock. These were mixed with 8 mL ethanol to make a final solution of 10 mL. The ratio of DPPC and Cholesterol was thus 4:1. The silymarin medication was dissolved in ethanol to form a 1mM stock solution, and 0.1 mL of that solution was then mixed with 0.4 mL ethanol before being added to the lipid solution. For blank nanoparticles, this step was skipped. Then, bath sonication of the solution was carried out for 40 minutes. Next, 10 mL of the lipid and water phases were allowed to warm up in a water bath until 60°C was attained. The DI water and lipid solution was mixed, and probe sonication was carried out for 40 minutes. Ethanol was removed from this solution by rotary evaporation.

The nanoparticle mixture was diluted to 50 mL by adding DI water. A 0.25% PEG 2000 solution was prepared and 20 mL of this was added in the nanoparticle mixture drop-wise while it was being constantly stirred. Rotary evaporation was carried out again to remove the excess DI water till 10 mL solution was left. This solution was passed through a dialysis membrane in PBS solution for 40 minutes to remove the unentrapped drug (Farooq et all., 2022).

3.2 Physical Characterization

In order to assess and examine the particle size, shape, surface charge, drug encapsulation, and release efficiency of PEG nanoparticles, multiple analysis were conducted for characterization.

3.2.1 U.V-Vis Absorption Spectroscopy

Ultraviolet-visible spectroscopy is concerned with the interaction between ultraviolet-visible radiation and matter. The ultraviolet (UV) sector of the electromagnetic spectrum includes wavelengths between 10 and 380 nanometers. As a rule of thumb, it is separated into three primary categories: UVA, which is in the 320–380 nm range, UVB (280–320 nm), and UVC (100–280 nm). The wavelength range of 10–200 nm is often known as vacuum ultraviolet (VUV), due to the fact that it can only be studied in vacuum. 380–750 nm is the spectral range that is included in the term "visible" (Vis). As a result of its association with the stimulation of atoms' outermost electrons, UV-Vis spectroscopy is often referred to as "electronic spectroscopy" (Picollo et al., 2019). UV-vis spectrophotometers transport light from a light source through a sample to a detector on the other side of the sample. Typically, data graphs have the baseline at the bottom and the peaks going upward, with wavelength in nanometers along the x-axis and absorbance (A) along the y-axis (Rocha et al., 2018).

A Shimatzu UV-Vis 2800 BMS Scientific Technical Corporation (PVT) spectrophotometer was used to analyze the U.V.-Vis spectra of Pegylated silymarin loaded lipid nanoparticles and blank lipid nanoparticles from 200-600 nm, at a resolution of 1 nm. As a standard for UV spectroscopy, de-ionized water was utilized. Silymarin medication, the lipids DPPC and cholesterol were also measured in the UV spectrum.

3.2.2 Fourier transform infrared spectroscopy (FTIR) analysis

Infrared (IR) spectroscopy, also known as Fourier transform infrared (FTIR) spectroscopy, may be used for a broad range of purposes, including the examination of tiny molecules or chemical complexes as well as the investigation of cells and tissues. (Berthomieu & Hienerwadel, 2009). The apparatus emits an infrared (IR) radiation beam from a blazing black-body source. The shaft then goes by means of the interferometer, where the ghostly encoding happens. In an interferometer, the recombination of shafts with fluctuating way lengths produces valuable and horrendous obstruction, otherwise called an interferogram. The indicator then estimates the energy versus season of the specific interferogram signal for all frequencies simultaneously. In the interim, a beam is overlaid to serve as a reference (background) for the functioning of the device (Mohamed et al., 2017).

Liquid samples for the nanoparticles were used for FTIR analysis. Using a Bruker FTIR Spectrophotometer ALPHA II, FTIR spectra were obtained between 4000-350 cm^{-1}. All

formulation components, including DDPC, Cholesterol, PEG 2000 and silymarin medication were also analyzed in their powder form by FTIR.

3.2.3 Particle size

SEM (scanning electron microscopy) was utilized to examine the practical size. Typically, the incident electrons (from an electron gun) in a SEM have energy between 2 and 40 keV. Two or three electromagnetic condenser lenses reduce the electron beam to a tiny probe that is raster-scanned over a particular region of the specimen surface by scan coils. The atomic masses of the elements in the sample, the angle at which the beam of electrons impacts the sample, and the energy of the electron beam all play a role in determining the dimensions of the teardrop-shaped volume through which the electrons enter the sample. The interaction of the electron beam with the specimen causes the production of secondary electrons, backscattered electrons, Auger electrons, x-rays, and possibly light. The sample holder has many detectors that collect this data. It is possible for the signal produced by each detector to be sent to a display that can rasterize the image in sync with the electron beam (Vernon-Parry, 2000).

By pouring a tiny portion of material onto a cover slip using a Micropipette, both the unloaded and drug-loaded nanoparticles were photographed. The surface of the slide was then coated with gold in a sputter coater at mA for 50 seconds. Using the VEGA3 LMU Scanning Electron Microscope at the National University of Science and Technology in Islamabad, images were captured.

3.2.4 Zeta Potential

The electrical double layer hypothesis makes possible the explanation of phenomena associated with the surface charge of a solid phase in interaction with a liquid. The electrokinetic or zeta-potential is a quantifiable feature of an electrified contact, according to this hypothesis. Under the effect of an external electrical field operating parallel to the interface surface, an electrified interface between two phases causes one phase to move relative to the other. The empirically measurable potential of the sliding plane is the electrokinetic or zeta potential. Zeta potential indicates the stability, surface charge, and average size of nanoparticles (Salopek et al., 1992).

Using Dynamic Light Scattering (DLS) and Malvern Zeta Sizer Version 7.12, the zeta potential (surface charge) of both types of LNPs was determined at Quaid-i-Azam University, Islamabad.

3.2.5 Drug Encapsulation Efficiency

Efficacy defines the quantity of medication encapsulated in the liposome vesicles. UV spectrophotometer at 330nm absorbance was used to test several dilutions of the medication to generate a feasible linear standard curve for determining drug encapsulation efficiency. It was possible to arrive at the equation Y = mx + c. The unentrapped silymarin was then calculated using this standard curve value. To discover the unentrapped drug fraction, samples were centrifuged at 4500 rpm for 1 hour and the supernatants were examined by UV Vis spectrometry (Nii & Ishii, 2005). This was followed by plugging the results into the following formula.

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

3.2.6 Drug Release Efficiency

In nanomedicines, the behavior of drug release from nanoparticle vectors is of major relevance. Release of drug cargo to the intended location in a time-dependent way is the primary focus of nano formulations that result in controlled or sustained release.

With the addition of a certain amount of phosphate buffered saline, the drug release of SLNPs were evaluated for up to 15 hours. 3 ml samples of SLNPs were put in a 15 ml centrifuge tube and centrifuged for 10 minutes at 4500 RPM and 25^{o}C, while 3 ml of phosphate buffer saline was added to the SLNPs solution. After centrifugation, the supernatant was analyzed by UV spectrophotometer. Following the same technique after 0.5, 1, 2, 4, 6, 8, 12 and 15 hours. At a wavelength of 230 nm, absorbance values were measured and utilized as a measure of cumulative drug release. As a control for the whole investigation, a blank nanoparticle solution was used.

3.3 Development of Copper Toxicity Model:

3.3.1 Animals

For this experiment, 35 female Wistar rats were employed, each weighing 80-120 g and being between the ages of 5-7 weeks. Separate cages with water and food were used to house the rats, who were maintained on a 12-hour light/dark cycle. 27°C and 60-70% humidity were the conditions. Rats were anesthetized with chloroform before being subjected to a histological investigation. Good laboratory practices given by the US Food and Drug Administration (FDA) in 1978 dictated how rats were cared for and how they were handled.

3.3.2 Copper Toxicity Induction

The 35 rats were randomly allocated in 7 groups of 5 rats each. One group was the positive control in which the rats were not given any chemicals throughout the study. The rats were acclimatized for 7 days before the induction was started. The rest of 30 rats were given copper sulfate in a dose of 200 mg/kg Body Weight daily up to 90 days through oral gavage (V. Kumar et al., 2015).

3.3.4 Serological Indices

AST (Aspartate transaminase or aspartate aminotransferase test), ALP (Alkaline Phosphatase), ALT (Alanine transaminase) and T.B (Total Bilirubin) tests were performed on blood taken from the heart in accordance with the manufacturer's specifications.

3.3.5 Histological Examination

The liver and brain were collected from the rats when they were sacrificed at the conclusion of the research. Each diseased liver tissue's size, shape, and color were recorded. To prevent postmortem autolysis and decomposition, the organs were removed and promptly put in a 10% neutral-balanced formalin solution. Following the paraffin embedding, 5 µm serial slices of organs were collected. Structural alterations in organs were observed using Hematoxylin and Eosin (HE) staining on histological slides.

3.3.6 Forced Swim Test

With regard to depression, the forced swim test is primarily used. A cylindrical tank filled with 24 + 1°C water was used, and each animal was put in it alone. The immobility test lasted for six minutes, according to the study. It was determined that an animal was immobile if it ceased struggling and remained still while afloat, or if it only made the minimal movements required to maintain its head above water. The animals were then taken out of the water, dried with a towel, and returned to their cages (Quamar et al., 2019).

3.3.7 Y Maze Test

The Y maze test was used to examine the working memory condition of rats by observing their spontaneous alternation behavior. Y-shaped with three arms, 45 x 35 x 12 cm, the device was constructed of black painted wood. At the intersection of three arms of the device, rats were placed, and they were allowed to make arm choices for six minutes about which arm they moved to. The % of alterations was calculated using the following equation (Lamtai et al., 2020).

$$\%\ Alteration\ = \frac{Spontaneous\ alteration}{Total\ number\ of\ arm\ enteries\ -2} \times 100$$

3.4 Treatment Design

The antioxidant properties of silymarin Peg-LNPs were examined in experiments with diseased rats. The 30 rats were divided in 6 groups of 5 rats each, based on the treatment plans.

3.4.1 Negative Control Group (Diseased)

Five ill rats were separated and tagged as a diseased group. This group of rats was kept undisturbed and untreated throughout the study and were sacrificed for histological and serological investigation at the conclusion of the study. Body and liver weights of the rats were recorded.

3.4.2 Silymarin Treated Group

Five rats were allocated in this group. They were administered 10 mg/kg Body Weight zinc silymarin for a period of 20 days through oral gavages. At the conclusion of the experiment, the body and liver weights were recorded prior to dissection for histological and serological investigation.

3.4.3 Silymarin Nanoparticles Treated Group

There were five rats in this group. The Silymarin Liposomes Nanoparticles Dose was administered orally for twenty days at a dose of 500 µg/kg Body Weight. At the conclusion of the experiment, the body and liver weights were recorded prior to dissection for histological and serological investigation.

3.4.4 Blank Nanoparticles Treated Group

There were five rats in this group. The Liposomes Nanoparticles Dose was administered orally for twenty days at a dose of 500 µg/kg Body Weight. At the conclusion of the experiment, the body and liver weights were recorded prior to dissection for histological and serological investigation.

3.4.5 Zinc Sulfate Treated Group

Five rats were allocated in this group. They were administered 10 mg/kg Body Weight zinc sulfate for a period of 20 days through oral gavages. At the conclusion of the experiment, the body and liver weights were recorded prior to dissection for histological and serological investigation.

3.4.6 Zinc sulfate and Silymarin Nanoparticles Treated Group

Five rats were allocated in this group. They were administered 10 mg/kg Body Weight zinc sulfate and 500 µg/kg Body Weight Silymarin nanoparticles for a period of 20 days through oral gavages. At the conclusion of the experiment, the body and liver weights were recorded prior to dissection for histological and serological investigation.

Chapter 4: Results

4.1 Physical Characterization of Silymarin Loaded LNPs and Blank LNPs

Both silymarin loaded liposomes and blank liposome nanoparticles were successfully synthesized, as shown by physical characterization.

4.1.1 UV-VIS absorption spectroscopy

UV-VIS absorption spectroscopy of silymarin medication exhibited the surface plasmon resonance (SPR) peak primarily at 230 nm, blank liposomes at 207 nm and Silymarin loaded-LNPs at 206 nm. The peaks of cholesterol was at 204 nm, DPPC at 202 nm and PEG 2000 at 205 nm.

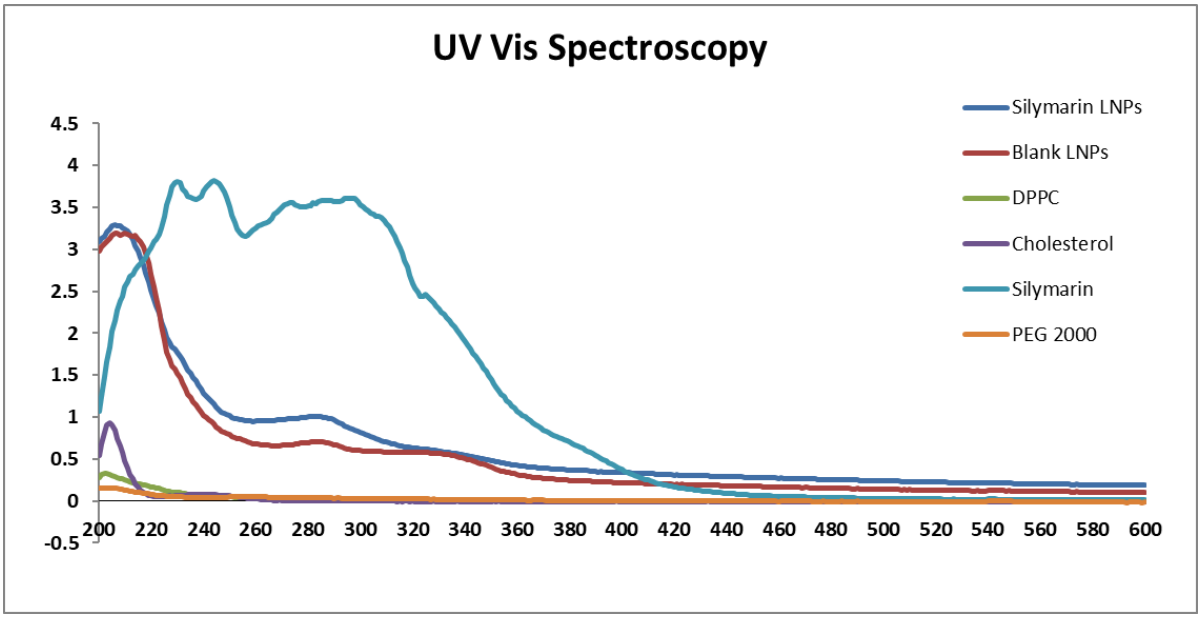

Figure 0.1: Comparative UV-VIS spectra of Blank and Silymarin LNPs along with their components

4.1.2 Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectrum of Cholesterol indicated peaks or bands at 2850/cm (CH stretch, Alkanes), 873/cm (Tri-substituted Aromatics). DPPC spectrum indicated peaks at 2919/cm (CH stretch, Alkanes), 1632/cm (R-NH2, Amines), 1115/cm (C-O stretch, Ether), 720/cm (RCH2CH3, Bending mode). PEG-2000 spectrum delineated peaks at 3429/cm (O-H stretch, Alcohol), 2923/cm (CH stretch, Alkanes) and 1638/cm (C=C stretch, Alkenes). The observed changes in infrared bands proven the conformational changes in lipid biomolecules' by incorporating with silymarin drug and PEG 2000, which masked the peaks due to coating.

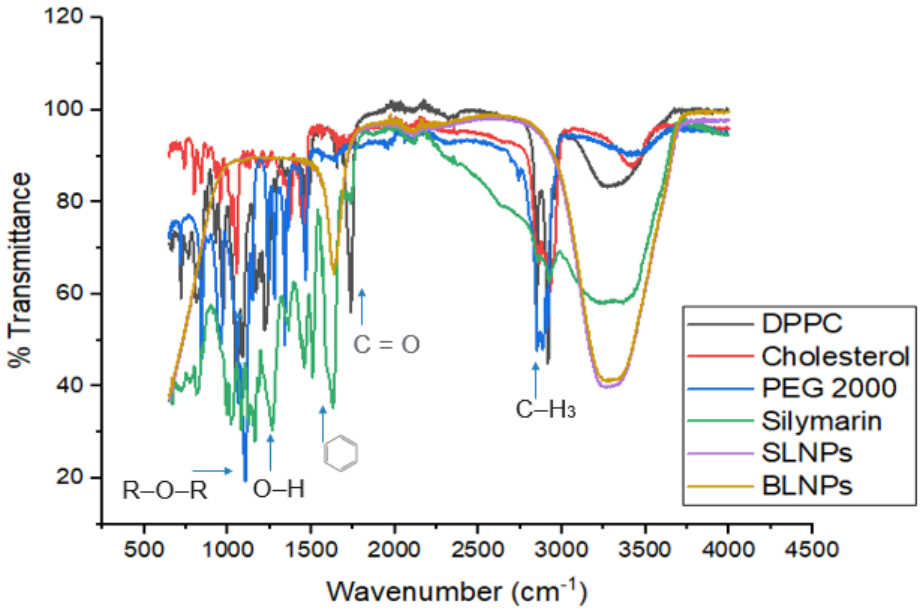

Figure 0.2: Comparative FTIR spectra of DPPC, Cholesterol, PEG-2000, Silymarin, Silymarin LNPs and Blank LNPs

4.1.3 Particle size

The scanning electron microscopy is used to determine the size of the silymarin loaded-LNPs, and blank LNPs. A scanning picture revealed that the silymarin loaded liposome nanoparticles were spherical in form and had an average size of 67 nm, while the blank nanoparticles had an average size of 24 nm.

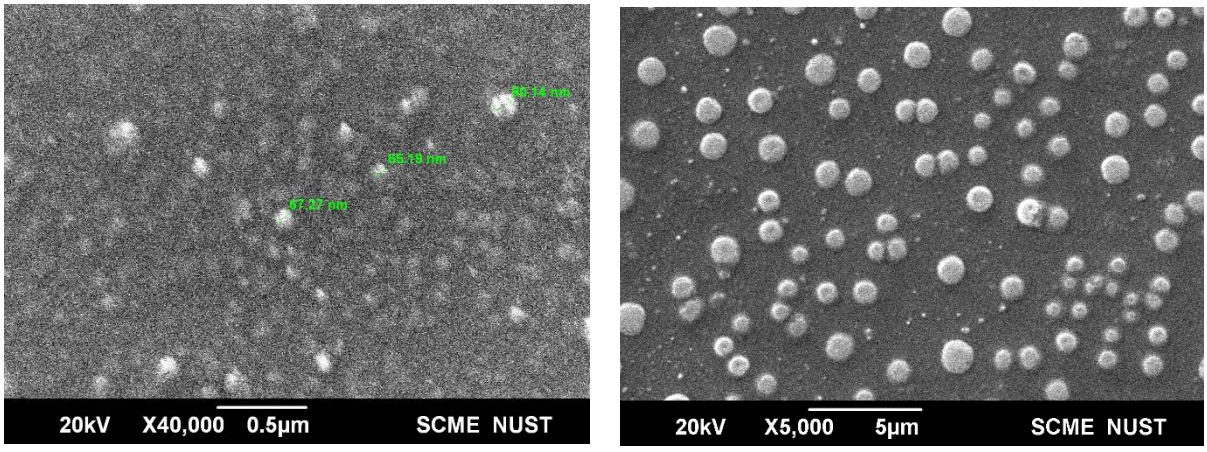

Figure 0.3: SEM Images of Silymarin LNPs

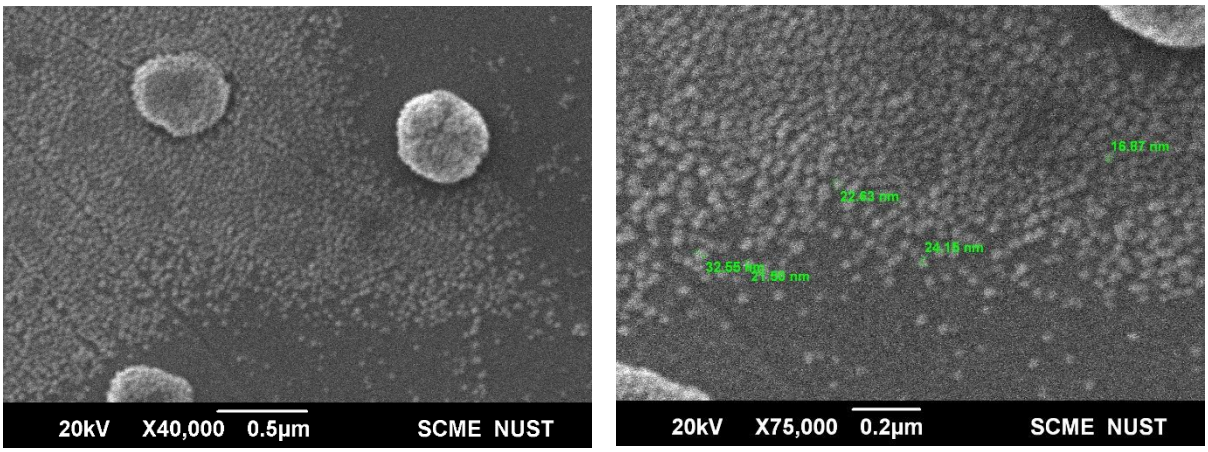

Figure 0.4: SEM Images of Blank LNPs

4.1.4 Zeta Potential

The average Zeta potential of silymarin LNPs and blank LNPs were -14.8 mV and -17.8 mV respectively.

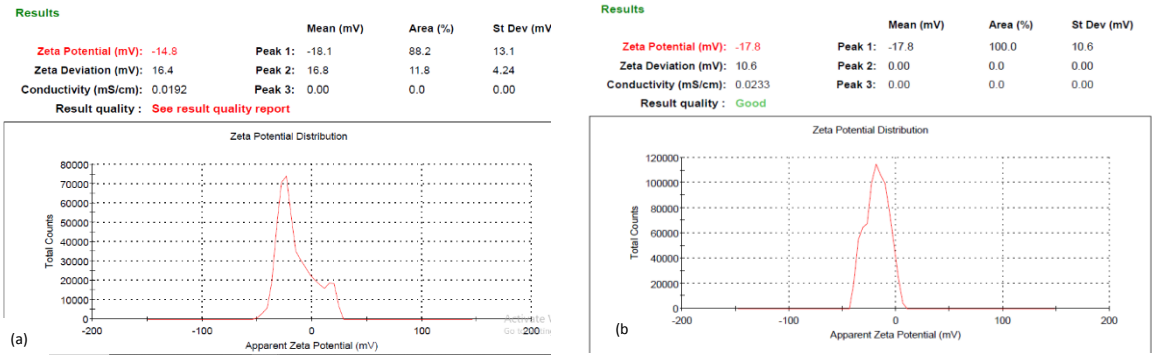

Figure 0.3: Zeta Potential of (a) Blank LNPs and (b) Silymarin LNPs

4.1.5 Drug Encapsulation Efficiency and Drug Loading Capacity

The previous formula, which can be found in the material section, was used to determine the encapsulation efficiency, which was calculated to be 74%. This indicates that there was 74% of the medicine encapsulated inside the nanoparticles.

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

$y = 6.7404x - 0.0195$

UV analysis of 1st supernatant from mini column centrifuge tube, gave the absorption at 230 nm for silymarin.

Abs (230 nm) = 1.7333

Y= Absorption value at specific point
X= Concentration of unknown

By putting value of absorbance (230 nm), the concentration of drug was calculated which is **0.26 mM.**

Amount of unentrapped drug = **0.1254 mg/ml**

Total drug was **0.4824 mg/ml** solution of drug.

Encapsulation Efficiency % = (Total drug – Unentrapped drug)/total drug ×100

= 74%

The same values were put in the formula for drug loading capacity:

Drug Loading Capacity % = (Total drug – Unentrapped drug)/Nanoparticle weight ×100

The weight of the nanoparticles was found to be 0.75mg per 10ml solution.

Hence, the drug loading capacity was 47.6%.

4.1.6 Drug Release Kinetics

The fact that 56% of the medicine was released from the liposome nanoparticles after being monitored for up to 15 hours suggests that silymarin LNPs maintain a steady release of the drug throughout time. Because the medicine is kept in the body for such a long period of time, its bioavailability is improved, which eventually results in a greater degree of success in treating illness.

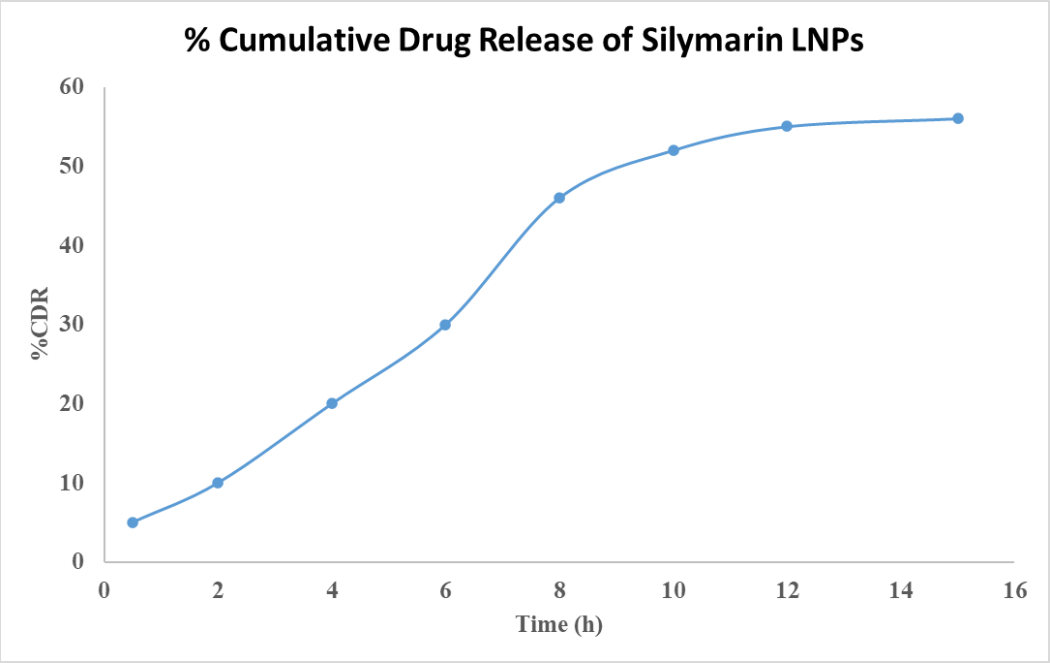

Figure 0.4: Drug Release of Silymarin LNPs

4.2 Treatment of Copper Toxicity

4.2.1 Body and Liver Weights

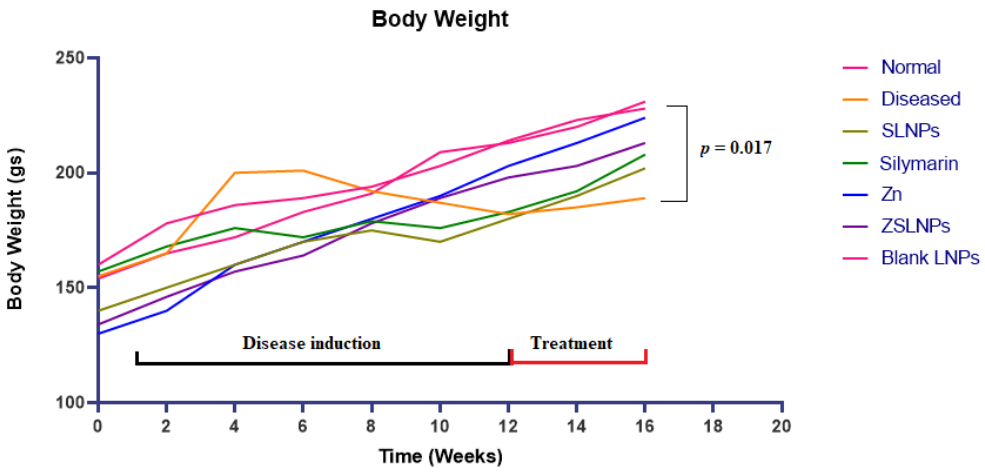

Figure 0.5: Body Weights of Rats

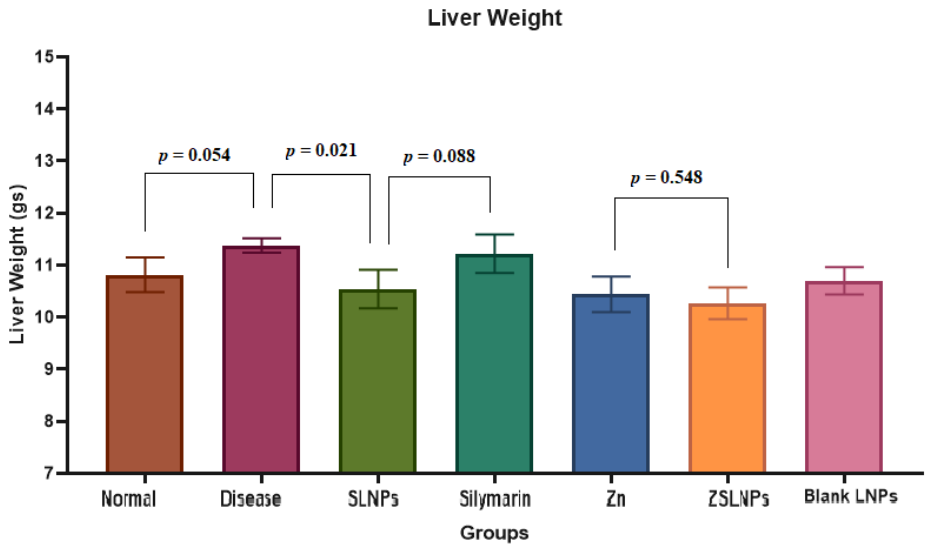

Figure 0.6: Liver Weights of Rats

There was a significant decrease in the body weight of diseased rats as compared to the normal group (p = 0.017). There was a significant effect of Silymarin nanoparticles on decreasing the liver weight (p = 0.021).

4.2.2 Hepatic Histopathology

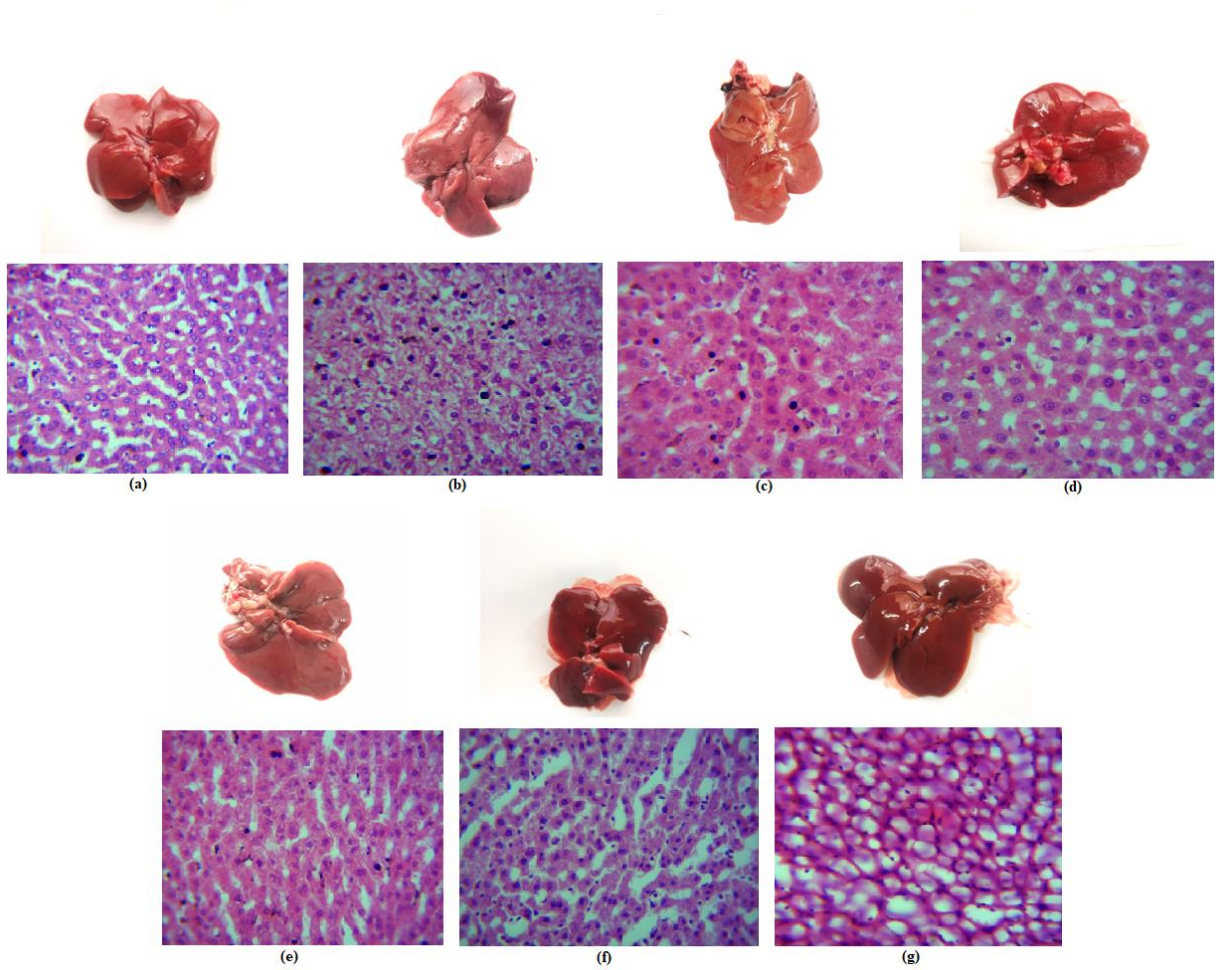

Figure 0.7: Liver Histopathology of (a) Normal (b) Diseased (c) BLNPs Treated (d) Silymarin Treated (e) SLNPs Treated (f) Zn Treated (g) ZSLNPs Treated

The number of hepatic cells with granular and vacuolar degeneration was increased in diseased and blank LNPs treated group. This number was slightly decreased in the silymarin, and zinc treated groups. This degeneration decreased significantly in the SLNPs and ZSLNPs group.

Karyolysis, which is the total disintegration of the chromatin of a dying cell owing to the enzymatic destruction by endonucleases, was seen in the diseased and BLNPs group. This was likewise improved in the groups that had been treated with SLNPs and ZSLNPs.

4.2.3 Brain Histopathology

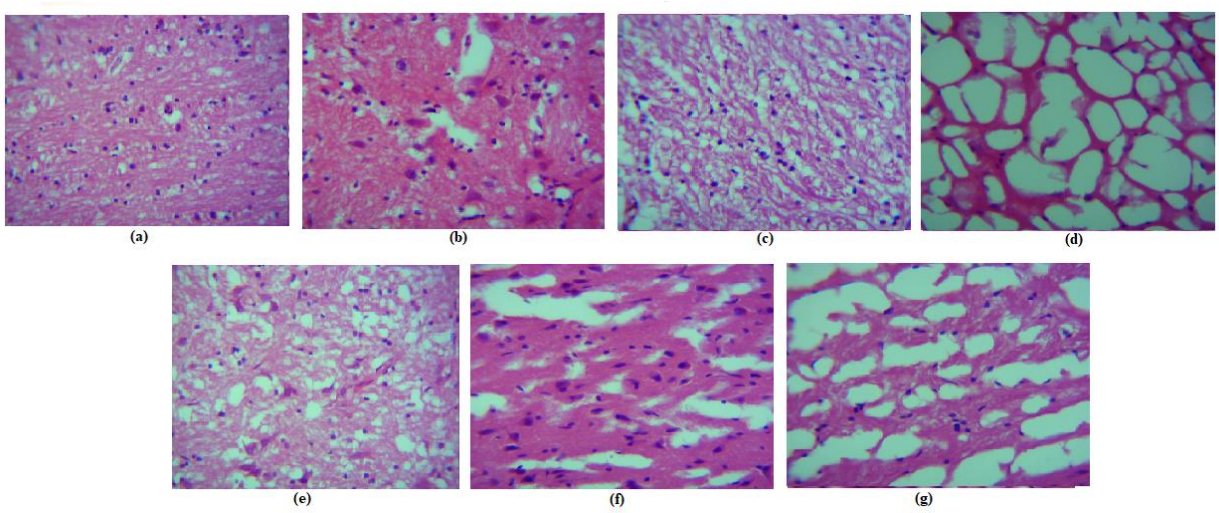

Figure 0.8: Brain Histopathology of (a) Normal Brain (b) Diseased Brain (c) BLNPs Treated (d) Silymarin Treated (e) SLNPs Treated (f) Zn Treated (g) ZSLNPs Treated

Cell swelling, neuronal vacuolation, demyelination, and neuronophagia were seen in the areas of the sick and blank LNPs rats' brains, respectively. In these groups, there were neurons that had shrunk, had pyknotic nuclei that had condensed chromatin, and had sparse amounts of eosinophilic cytoplasm, all of which were indicative of apoptosis.

These pathologies improved in the treatment groups of SLNPs and ZSLNPs.

4.2.4 Serological Analysis (Liver Function Tests)

The bilirubin levels were significantly increased in the diseased group (p = 0.022) and blank LNPs group (p = 0.024). With treatment with SLNPs, the bilirubin levels decreased significantly as compared to the diseased group (p = 0.002). SLNPs treatment also showed better results than silymarin treatment (p = 0.011) and the combination therapy of zinc and SLNPs performed better than zinc therapy (p = 0.072).

There was a significant increase in aspartate transaminase levels of diseased rats as compared to the normal group (p = 0.00006). Treatment with Silymarin LNPs significantly decreased AST as compared to no treatment (p = 0.0003). SLNPs also performed significantly better than silymarin in decreasing AST (p = 0.0014) and the combined treatment of Zinc sulfate and SLNPs decreased AST levels significantly as compared to zinc therapy (p = 0.00007).

There was a significant increase in alanine transaminase and alkaline phosphatase levels of diseased rats as compared to the normal group (p = 0.0002, p = 0.0002). Treatment with

Silymarin LNPs significantly decreased ALT and ALP as compared to no treatment (p = 0.0007, p = 0.0083).

SLNPs also performed significantly better than silymarin (p = 0.0011, p = 0.0456) and the combined treatment of Zinc sulfate and SLNPs decreased ALT and ALP levels significantly as compared to zinc therapy (p = 0.0085, p = 0.0003).

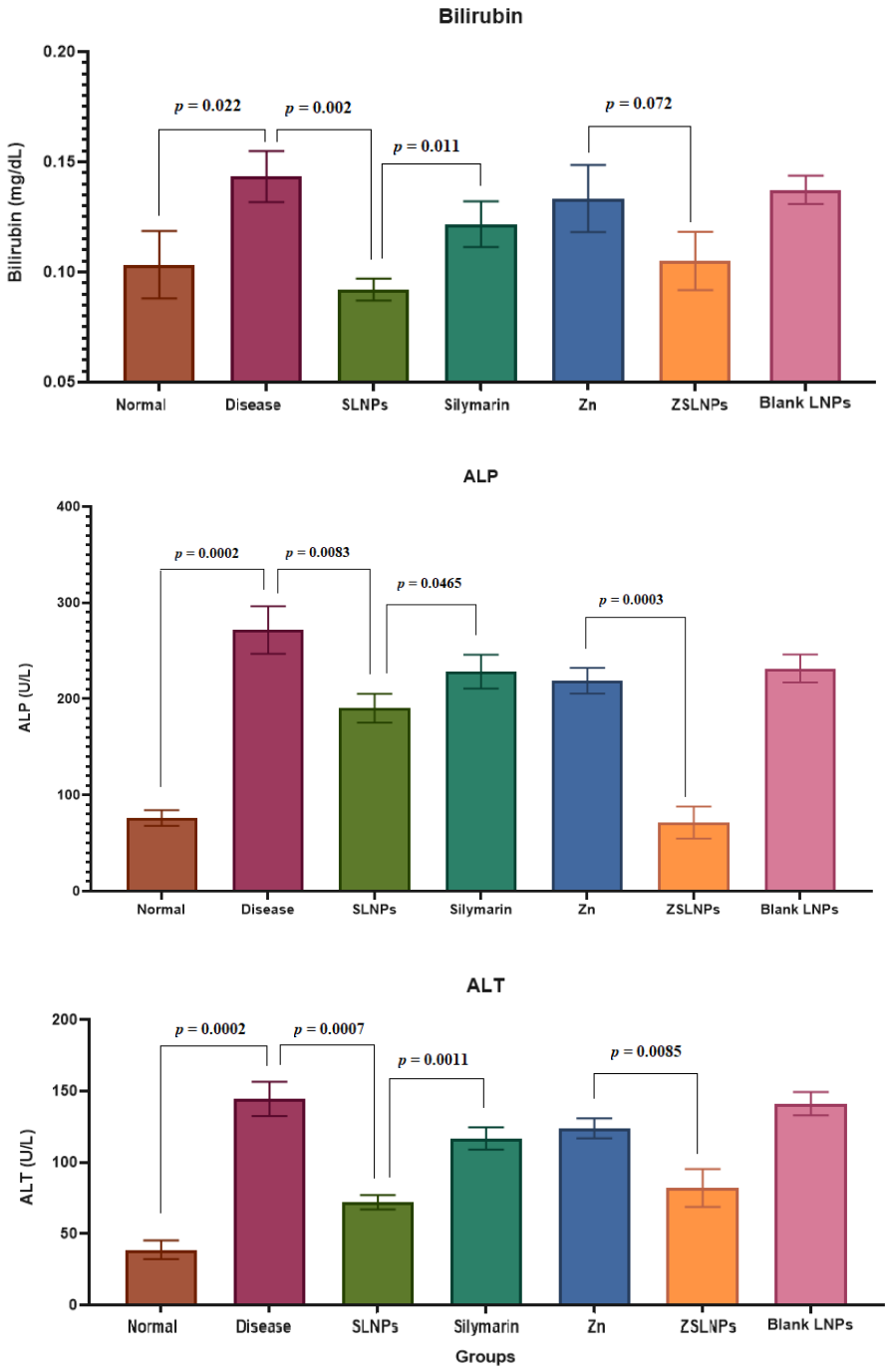

Figure 0.9: Serological Indices (Bilirubin, ALP, ALT)

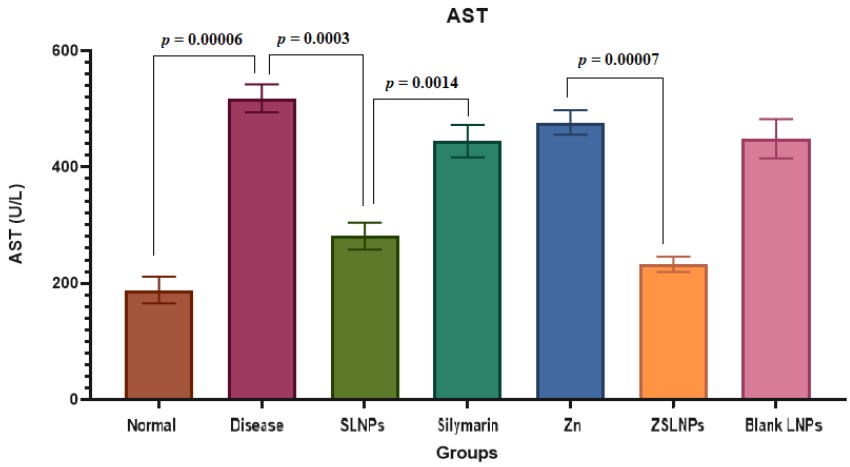

Figure 0.12: Serological Indices (AST)

4.2.5 Forced Swim Test

There was a significant increase in immobility time of diseased rats as compared to the normal group *(p* = 0.0002*)*. Treatment with Silymarin LNPs significantly decreased this time as compared to no treatment (*p* = 0.0004). SLNPs also performed significantly better than silymarin *(p* = 0.002*)* and the combined treatment of Zinc sulfate and SLNPs decreased immobility time significantly as compared to zinc therapy *(p*= 0.0008*)*.

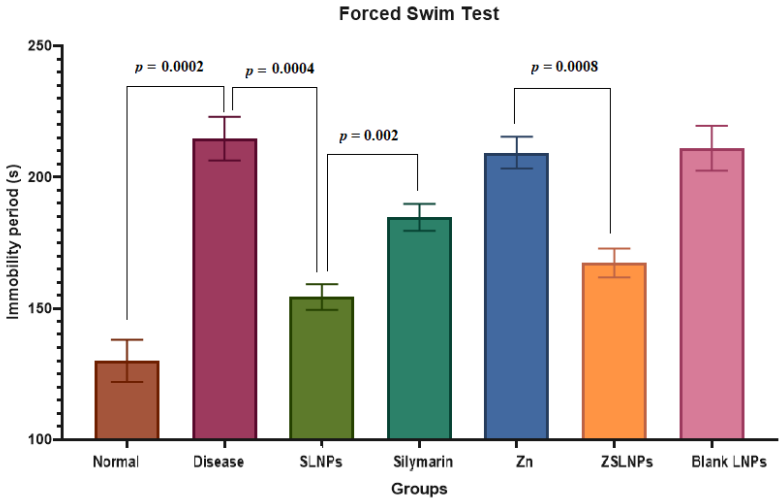

Figure 0.13: Immobility Time of Rats

4.2.6 Y Maze Test

There was a significant decrease in % spatial memory of diseased rats as compared to the normal group *(p* = 0.0009*)*. Treatment with Silymarin LNPs significantly increased spatial

memory as compared to no treatment *(p* = 0.006*)*. SLNPs also performed significantly better than silymarin *(p* = 0.023*)*.

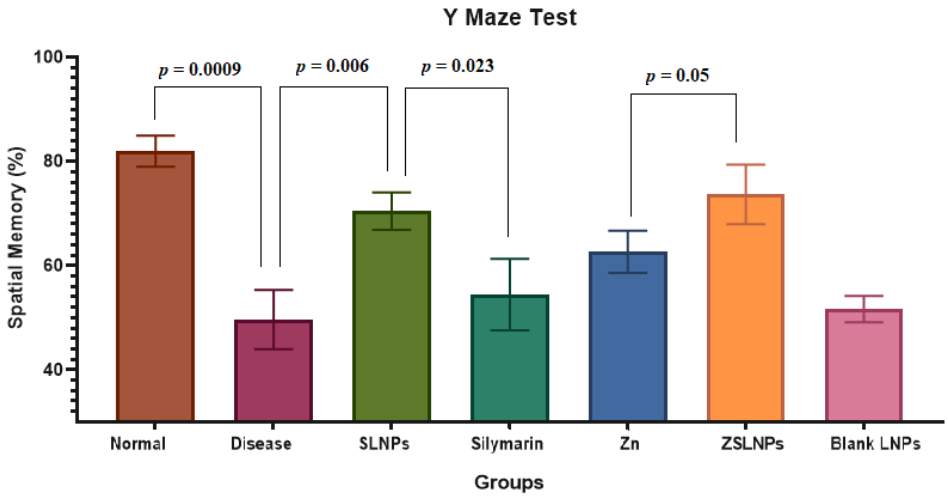

Figure 0.14: Spatial Memory of Rats

Chapter 5: Discussion

In Wilson disease (WD), an autosomal recessive condition of copper transport, copper accumulates in the liver and other extrahepatic tissues due to impaired copper excretion through the bile and diminished incorporation into the ceruloplasmin copper protein transporter (Behari & Pardasani, 2010b). The frequency of the disease's causing allele is believed to be 1:90 on a global scale, Wilson Disease is the most widely recognized form of copper toxicity (Litwin et al., 2013). Mutations in ATP7B cause decreased conversion of apoceruloplasmin, leading to low ceruloplasmin levels in Wilson's disease patients. Due to a lack of excretion into the biliary canaliculi, copper also builds up in the hepatocytes (Schilsky, 2017; Sherlock & Dooley, 2008). Because the liver is the first organ to accumulate copper when it enters the bloodstream, chronic copper toxicity largely affects it. Toxic effects of copper include liver cirrhosis as well as hemolytic anemia and injury to renal tubules as well as the brain and other organs (Winge & Mehra, 1990).

Liver and gallbladder issues have been treated for years using milk thistle (Silybum marianum L.), an old medicinal plant from the Carduus marianum family (Karimi et al., 2011). In addition to silymarin, other flavonoids including silychristin, neosilyhermin, silyhermin, and silydianin may also be found in the herb's fruit and seeds, but the active ingredient is a complex mixture of silymarin and these other flavonoids. Silymarin and silibinin have been discovered to be cytoprotective, antioxidant and, above all, hepatoprotective in the medical community (Fraschini et al., 2002b). Because of its weak solubility in water and limited oral bioavailability, silymarin's usefulness as a liver medicine reduces. Silybin is only absorbed by the gastrointestinal system in 20-50% of cases. Silybin has a 0.95% absolute oral bioavailability (Sornsuvit et al., 2018).

Using PEG 2000, we were able to effectively manufacture stealth silymarin-loaded liposomes nanoparticles for the treatment of copper toxicity in the current investigation. Liposomes containing silymarin were loaded by dissolving the compound in a suitable solvent and then using sonication to create a nano-sized formulation. Silymarin is hydrophobic, thus it is encased in the bilayer of a liposomal vesicle. Nanoparticle size was measured to be 24nm for unloaded liposomes and 67nm for silymarin-loaded liposomes, respectively. However, measurements of Zeta potential showed values of -14.8 mV and -17.8 mV. After monitoring liposomes nanoparticles for 15 hours, we found that 56 percent of the drug had been released. The liposomal components, silymarin loaded liposome nanoparticles, and blank liposomes

were analyzed using Fourier transform infrared spectroscopy (FTIR), and the results demonstrated that distinct functional groups were involved, as shown by a drop in peak intensities.

The use of copper sulphate in a toxicity model proved to be very effective. Though it is essential for many bodily functions, copper may be poisonous at high enough concentrations to damage cells. It's possible that chronic Cu exposure may harm the kidney, spleen, and thymus in mice, and the basal ganglia in humans (Mitra et al., 2012). It is believed that hepatocytes are one of the primary cell types affected by Cu poisoning. Several investigations have shown that Cu causes hepatocytes to undergo apoptosis, mitochondrial abnormalities, and oxidative stress (Haywood et al., 2005). The cellular toxicity of Cu exposure is poorly understood. However, many mechanisms have been suggested to explain it. One common mechanism is that it may cause oxidative stress by increasing ROS production. It is widely established that reactive oxygen species (ROS) and oxidative stress may trigger apoptosis. Once within the hepatocytes, some of the Cu will be taken to the mitochondria to be incorporated into cytochrome c oxidase. Overproduction of reactive oxygen species (ROS) is induced by high Cu concentrations; this further affects mitochondrial electron transport, leading to mitochondrial impairment and, ultimately, apoptosis (Li et al., 2015).

Effects of this toxin were examined on liver and brain by histological examination combined with Liver function testing. The effects of copper poisoning on cognitive performance were also investigated using behavioral testing. There was an increase in liver enzymes which corresponds to liver disease and the spatial memory of the rats decreased, suggesting an impairment in their cognitive ability. Kumar et all (2014) reported increased liver enzyme levels in rats that were administered copper sulfate in the doses 100 and 200 mg/kg BW. The latter group had higher liver enzyme levels than the group given the lower dose (V. Kumar et al., 2015).

Moreover, the depression like symptoms of rats increased as well. Histological examination proved the detrimental effects of copper on liver and brain cells of the rats. There was significant degeneration in both the hepatocytes and neurons. All these findings correspond to the phenotypic manifestations of copper toxicity. Behavioral alterations related with a greater copper content in the brain have been shown in previous investigations on copper toxicity. Quamar et all (2019) reported that the immobility period of rats that were administered copper sulfate for 6 weeks was significantly increased (Quamar et al., 2019).

In this study, silymarin was chosen to treat the abnormalities discussed above. Because of its antioxidant, anti-inflammatory, and antifibrotic properties, silymarin is used to treat a variety of liver ailments, including cirrhosis, hepatocellular carcinoma, and chronic liver disease. Virus-related liver damage may be mitigated with the help of silymarin's antioxidant and anti-inflammatory properties, which work by dampening the inflammatory cascade and adjusting the immune system. In hepatitis C virus infection, its intravenous injection is accompanied by a direct antiviral action. For alcoholism, silymarin improves cellular vitality and decreases lipid peroxidation and cellular necrosis. Further, the usage of silymarin/silybin has significant biological effects in non-alcoholic fatty liver disease. By counteracting oxidative stress, insulin resistance, liver fat buildup, and mitochondrial dysfunction, these drugs are useful in the treatment of non-alcoholic fatty liver disease. Liver cirrhosis and hepatocellular carcinoma are typical terminal phases of several hepatopathies, and silymarin is employed in both of these conditions by altering certain molecular patterns (Federico et al., 2017).

Experts in colloidal and pharmaceutical sciences have proposed silymarin-based formulations with better stability and solubility, increased bioavailability, and efficient therapeutic performances, and there is a growing body of scientific literature attesting to their efforts. Existing research towards increasing silymarin bioavailability has focused on using nanocarriers. Early reports of silymarin in liposomes may be found in the scientific literature, dating back to the early 2000s. In order to optimize the formulation made by ethanol injection, Maheshwari et al. looked at a variety of variables, including the drug-to-lipid ratio, the fraction of CHOL, and the presence of the charge inducer DCP (Maheshwari et al., 2003). Silymarin has low water solubility, however El-Samaligy et al. explored the possibility of encapsulating the medication in a liposomal dosage-form for buccal delivery through spray to circumvent instability issues that frequently arise in the GIT (di Costanzo & Angelico, 2019). Comparing silymarin liposomes with a phytosomal form of silymarin revealed improved hepatoprotection and anti-inflammatory properties. Aside from increasing medication absorption, this formulation was meant to enhance hepatocyte regeneration and avoid liver inflammation (N. Kumar et al., 2014).

In this research the use of silymarin LNPs as opposed to silymarin medication significantly reversed the diseased to normal hepatic tissue as shown by histological and serological outcomes. The effects of zinc and SLNPs combination treatment were superior to those of zinc therapy alone.

Conclusion

Different characterization methods are used to verify the success of silymarin liposomal nanoparticle production. Histological, serological, body, and liver weight data shown significantly improvement in the SLNPs treated group after treatment of the diseased rats. The findings for the group treated with both zinc and SLNPs were even more impressive than those for the zinc-only group. SLNPs also improved the cognitive behavior and depressive symptoms of the diseased rats. Therefore, liposome encapsulation with PEG coating has been shown to be the most successful strategy for improving the bioavailability and pharmacokinetic behavior of the silymarin medication, which has shown antioxidant action or significant therapy against copper toxicity.

Silymarin liposomal nanoparticles proved to be an effective treatment for managing the phenotypic effects of copper toxicity. SLNPs significantly reduced liver enzymes AST, ALP, ALT and bilirubin. SLNPs also significantly improved the cognitive behavior of rats along with treating depression symptoms. Hence, SLNPs exhibited good oral delivery of silymarin. As compared to the traditional therapy of zinc, a combined therapy of zinc and supplementary silymarin LNPs proved to be a more effective treatment plan. Involving more research into the following hypothesis to prevent copper toxicity and authorize this method for usage at the clinical level is needed.

Future Prospects

This research was a first step in the synthesis and characterization of liposomal nanoparticles for enhanced silymarin bioavailability. Further investigations of drug absorbance and biodistribution in the body can be carried out by using similar animal models. This will enable further optimizations of the liposomes for better drug delivery and targeting. Different PEG molecules can also be tested for improved bioavailability. In this research, DPPC was employed as the lipid for nanoparticle synthesis. Other phospholipids can also be tested or copolymeric liposomal nanoparticles can be synthesized and evaluated for better efficacy.

Further evaluations of copper toxicity and the treatment can be carried out by measuring oxidative and antioxidant parameters, detection of mRNA and protein expression levels, analysis of mitochondrial membrane potential and assessment of hepatocyte apoptosis.

References

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

Ala, A., Walker, A. P., Ashkan, K., Dooley, J. S., & Schilsky, M. L. (2007). Wilson's disease. In *www.thelancet.com* (Vol. 369). http://www.

Allen, T. M., & Cullis, P. R. (2013). Liposomal drug delivery systems: From concept to clinical applications. In *Advanced Drug Delivery Reviews* (Vol. 65, Issue 1, pp. 36–48). https://doi.org/10.1016/j.addr.2012.09.037

B Halliwell, J. G. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J*, *219*, 1–14.

Baer-dubow Ska, W., Szaefer, H., & KRAJKA-KUZNIAK Marcinkowski, V. K. (1998). *Inhibition of m urine hepatic cytochrom e P450 activities by natural and synthetic phenolic com pounds*.

Bahmani, M., Shirzad, H., Rafieian, S., & Rafieian-Kopaei, M. (2015). Silybum marianum: Beyond Hepatoprotection. *Journal of Evidence-Based Complementary and Alternative Medicine*, *20*(4), 292–301. https://doi.org/10.1177/2156587215571116

Bamrungsap, S., Zhao, Z., Chen, T., Wang, L., Li, C., Fu, T., & Tan, W. (2012). Nanotechnology in therapeutics: A focus on nanoparticles as a drug delivery system. In *Nanomedicine* (Vol. 7, Issue 8, pp. 1253–1271). https://doi.org/10.2217/nnm.12.87

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, *13*(1), 238–252. https://doi.org/10.1016/S0022-2836(65)80093-6

Barber, R. G., Grenier, Z. A., & Burkhead, J. L. (2021). Copper toxicity is not just oxidative damage: Zinc systems and insight from wilson disease. In *Biomedicines* (Vol. 9, Issue 3). MDPI AG. https://doi.org/10.3390/biomedicines9030316

Behari, M., & Pardasani, V. (2010a). Genetics of Wilsons disease. In *Parkinsonism and Related Disorders* (Vol. 16, Issue 10, pp. 639–644). https://doi.org/10.1016/j.parkreldis.2010.07.007

Bergheim, I., McClain, C. J., & Arteel, G. E. (2006). Treatment of alcoholic liver disease. *Digestive Diseases*, *23*(3–4), 275–284. https://doi.org/10.1159/000090175

Berthomieu, C., & Hienerwadel, R. (2009). Fourier transform infrared (FTIR) spectroscopy. In *Photosynthesis Research* (Vol. 101, Issues 2–3, pp. 157–170). https://doi.org/10.1007/s11120-009-9439-x

Bhattacharjee, A., Chakraborty, K., & Shukla, A. (2017). Cellular copper homeostasis: Current concepts on its interplay with glutathione homeostasis and its implication in physiology and human diseases. In *Metallomics* (Vol. 9, Issue 10, pp. 1376–1388). Royal Society of Chemistry. https://doi.org/10.1039/c7mt00066a

Bindoli Cavalln, A. L., & Siliprandi, N. (1977). *INHIBITORY ACTION OF SILYMARIN OF LIPID PEROXIDE FORMATION IN RAT LIVER MITOCHONDRIA AND MICROSOMES*.

Bleackley, M. R., & MacGillivray, R. T. A. (2011). Transition metal homeostasis: from yeast to human disease. *Biometals*, *24*(5), 785–809. https://doi.org/10.1007/s10534-011-9451-4

Blume, G., & Cevc, G. (1990). Liposomes for the sustained drug release in vivo. In *Biochimica et Biophysica Acta*.

BM Myers, F. P. R. H. S. K. N. L. (1993). Alterations in hepatocytes lysosomes in experimental hepatic copper overload in rats. *Gastroenterology*, *105*, 1814–1823.

Boigk, G., Stroedter, L., Herbst, H., Ju¨, J., Waldschmidt, J., Riecken, E. O., & Schuppan, D. (1997). *Silymarin Retards Collagen Accumulation in Early and Advanced Biliary Fibrosis Secondary to Complete Bile Duct Obliteration in Rats It increases protein synthesis via stimulation of RNA synthe*.

Bosisio, E., Benelli, C., & Pirola, O. (1992). EFFECT OF THE FLAVANOLIGNANS OF Silybum marianum L. ON LIPID PEROXIDATION IN RAT LIVER MICROSOMES AND FRESHLY ISOLATED HEPATOCYTES. In *Pharmacological Research* (Vol. 25, Issue 2).

Böttger, R., Pauli, G., Chao, P. H., al Fayez, N., Hohenwarter, L., & Li, S. D. (2020). Lipid-based nanoparticle technologies for liver targeting. In *Advanced Drug Delivery Reviews* (Vols. 154–155, pp. 79–101). Elsevier B.V. https://doi.org/10.1016/j.addr.2020.06.017

Bremner, I. (1998). Manifestations of copper excess. *Am J Clin Nutr*, *67*, 1069S-1073S.

Brewer, G. J. (2001). Zinc acetate for the treatment of Wilson's disease. *Expert Opinion on Pharmacotherapy*, *2*(9), 1473–1477. https://doi.org/10.1517/14656566.2.9.1473

Brewer, G. J., Dick, R. D., Johnson, V. D., Brunberg, J. A., Kluin, K. J., & Fink, J. K. (1998). *Treatment of Wilson's disease with zinc: XV Long-term follow-up studies*.

Brewer, G. J., Dick, R. D., Johnson, V. D., Fink, J. K., Kluin, K. J., & Daniels, S. (2001). Treatment of Wilson's disease with zinc XVI: Treatment during the pediatric years. *Journal of Laboratory and Clinical Medicine*, *137*(3), 191–198. https://doi.org/10.1067/mlc.2001.113037

Bulbake, U., Doppalapudi, S., Kommineni, N., & Khan, W. (2017). Liposomal formulations in clinical use: An updated review. In *Pharmaceutics* (Vol. 9, Issue 2). MDPI AG. https://doi.org/10.3390/pharmaceutics9020012

Cacciapuoti, F., Scognamiglio, A., Palumbo, R., Forte, R., & Cacciapuoti, F. (2013). Silymarin in non alcoholic fatty liver disease. *World Journal of Hepatology*, *5*(3), 109–113. https://doi.org/10.4254/wjh.v5.i3.109

Carnpos, R., Guerra', A. G. R., & Valenzuela, A. (1989). *Silybin Dihemisuccinate Protects Against Glutathione Depletion and Lipid Peroxidation Induced by Acetaminophen on Rat Liver*.

Cavallini, L., Bindoli, A., & Siliprandi, N. (1978). COMPARATIVE EVALUATION OF ANTIPEROXIDATIVE ACTION OF SILYMARIN AND OTHER FLAVONOIDS. In *Pharmacological Research Communications* (Vol. 10, Issue 2).

Davila ’, J. C., Lenherr, A., & Acosta, D. (1989). PROTECTIVE EFFECT OF FLAVONOIDS ON DRUG-INDUCED HEPATOTOXICITY IN VITRO*. In *Toxicology* (Vol. 57).

de Romaña, D. L., Olivares, M., Uauy, R., & Araya, M. (2011). Risks and benefits of copper in light of new insights of copper homeostasis. *J Trace Elem Med Biol*, *25*(1), 3–13. https://doi.org/10.1016/j.jtemb.2010.11.004

di Costanzo, A., & Angelico, R. (2019). Formulation strategies for enhancing the bioavailability of silymarin: The state of the art. In *Molecules* (Vol. 24, Issue 11). MDPI AG. https://doi.org/10.3390/molecules24112155

Ernst, E. (2005). The efficacy of herbal medicine - An overview. In *Fundamental and Clinical Pharmacology* (Vol. 19, Issue 4, pp. 405–409). https://doi.org/10.1111/j.1472-8206.2005.00335.x

Evans, P. J., & Halliwell, B. (1994). Measurement of iron and copper in biological settings: bleomycin and Cu-phenanthroline assays. *Methods Enzymol*, *233*(C), 82–92. https://doi.org/10.1016/s0076-6879(94)33010-7

Farghali, H., Kamenikova, L., Hynie, S., & Kmonickova, E. (2000). SILYMARIN EFFECTS ON INTRACELLULAR CALCIUM AND CYTOTOXICITY: A STUDY IN PERFUSED RAT HEPATOCYTES AFTER OXIDATIVE STRESS INJURY U ´ ´. In *Pharmacological Research* (Vol. 41, Issue 2).

Farooq A, Iqbal A, Rana NF, Fatima M, Maryam T, Batool F, Rehman Z, Menaa F, Azhar S, Nawaz A, Amin F, Mohammedsaleh ZM, Alrdahe SS. A Novel Sprague-Dawley Rat Model Presents Improved NASH/NAFLD Symptoms with PEG Coated Vitexin Liposomes. International Journal of Molecular Sciences. 2022; 23(6):3131. https://doi.org/10.3390/ijms23063131

Federico, A., Dallio, M., & Loguercio, C. (2017). Silymarin/Silybin and Chronic Liver Disease: A Marriage of Many Years. *Molecules 2017, Vol. 22, Page 191*, *22*(2), 191. https://doi.org/10.3390/MOLECULES22020191

Ferenci’, P., Drapsirs, B., Dittrich, H., Fraok3, H., Benda, L., Lochs, ’ H, Me&, ’ S, Base’, W., & Schneidcg ’is1, B. (1989). *Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver*.

Fiebrich, F., & Koch, H. (1979). Silymarin, an inhibitor of lipoxygenase. In *P.S. Fitt and P.I. Peterkin, Biochem. J* (Vol. 35). Clarendon Press.

Fields, M., Lewis, C. G., & Bureau, I. (2001). Aspirin reduces blood cholesterol in copper-deficient rats: a potential antioxidant agent. *Metabolism*, *50*(5), 558–561. https://doi.org/10.1053/meta.2001.22513

Fraschini, F., Demartini, G., & Esposti, D. (2002a). *Pharmacology of Silymarin*.

Gaetke, L. M., Chow-Johnson, H. S., & Chow, C. K. (2014). Copper: toxicological relevance and mechanisms. *Archives of Toxicology 2014 88:11*, *88*(11), 1929–1938. https://doi.org/10.1007/S00204-014-1355-Y

Gitlin, J. D. (2003). Wilson Disease. *Gastroenterology*, *125*(6), 1868–1877. https://doi.org/10.1053/j.gastro.2003.05.010

Gubernator, J. (2011). Active methods of drug loading into liposomes: Recent strategies for stable drug entrapment and increased in vivo activity. In *Expert Opinion on Drug Delivery* (Vol. 8, Issue 5, pp. 565–580). https://doi.org/10.1517/17425247.2011.566552

Harris, E. (1992). Copper as a cofactor and regulator of Cu, zinc superoxide dismutase. *J Nutr*, *122*, 636S-640S.

Harris, E. (1993). *The transport of copper* (pp. 163–179). Wiley-Liss.

Hatori, Y., & Lutsenko, S. (2013). An expanding range of functions for the copper chaperone/antioxidant protein atox1. *Antioxidants and Redox Signaling*, *19*(9), 945–957. https://doi.org/10.1089/ars.2012.5086

Haywood, S., Simpson, D. M., Ross, G., & Beynon, R. J. (2005). The Greater Susceptibility of North Ronaldsay Sheep Compared with Cambridge Sheep to Copper-induced Oxidative Stress, Mitochondrial Damage and Hepatic Stellate Cell Activation. *Journal of Comparative Pathology*, *133*(2–3), 114–127. https://doi.org/10.1016/J.JCPA.2005.02.001

Hughes, G. A. (2005). Nanostructure-mediated drug delivery. In *Nanomedicine: Nanotechnology, Biology, and Medicine* (Vol. 1, Issue 1, pp. 22–30). https://doi.org/10.1016/j.nano.2004.11.009

JR, T., WR, K., HL, A., & LL, A. (n.d.). Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope 65Cu. *Am J Clin Nutr*, *49*, 870–878.

Karimi, G., Vahabzadeh, M., Lari, P., Rashedinia, M., & Moshiri, M. (2011). "Silymarin", a Promising Pharmacological Agent for Treatment of Diseases. In *Promising Pharmacological Agent Iran J Basic Med Sci* (Vol. 14, Issue 4).

Kelly, C., & Pericleous, M. (2018). Wilson disease: more than meets the eye. In *Postgraduate medical journal* (Vol. 94, Issue 1112, pp. 335–347). https://doi.org/10.1136/postgradmedj-2017-135381

Kelner, G. S., Lee, M. H., Clark, M. E., Maciejewski, D., McGrath, D., Rabizadeh, S., Lyons, T., Bredesen, D., Jenner, P., & Maki, R. A. (2000). The copper transport protein Atox1 promotes neuronal survival. *J Biol Chem*, *275*(1), 580–584. https://doi.org/10.1074/jbc.275.1.580

Khan, I., Elhissi, A., Shah, M., Alhnan, M. A., & Ahmed, W. (2013). Liposome-based carrier systems and devices used for pulmonary drug delivery. In *Biomaterials and Medical Tribology: Research and Development* (pp. 395–443). Elsevier Inc. https://doi.org/10.1533/9780857092205.395

Kim, H., Wu, X., & Lee, J. (2013). SLC31 (CTR) family of copper transporters in health and disease. *Molecular Aspects of Medicine*, *34*(2–3), 561–570. https://doi.org/10.1016/J.MAM.2012.07.011

Kondeva-Burdina, M., Zheleva-Dimitrova, D., Nedialkov, P., Girreser, U., & Mitcheva, M. (2013). Cytoprotective and antioxidant effects of phenolic compounds from Haberlea rhodopensis Friv. (Gesneriaceae). *Pharmacognosy Magazine*, *9*(36), 294–301. https://doi.org/10.4103/0973-1296.117822

Kumar, N., Rai, A., Reddy, N. D., Raj, P. V., Jain, P., Deshpande, P., Mathew, G., Kutty, N. G., Udupa, N., & Rao, C. M. (2014). Silymarin liposomes improves oral bioavailability of silybin

besides targeting hepatocytes, and immune cells. *Pharmacological Reports*, *66*(5), 788–798. https://doi.org/10.1016/J.PHAREP.2014.04.007

Kumar, V., Kalita, J., Misra, U. K., & Bora, H. K. (2015). A study of dose response and organ susceptibility of copper toxicity in a rat model. *Journal of Trace Elements in Medicine and Biology*, *29*, 269–274. https://doi.org/10.1016/j.jtemb.2014.06.004

Kwon, D. Y., Jung, Y. S., Kim, S. J., Kim, Y. S., Choi, D. W., & Kim, Y. C. (2013). Alterations in sulfur amino acid metabolism in mice treated with silymarin: A novel mechanism of its action involved in enhancement of the antioxidant defense in liver. *Planta Medica*, *79*(12), 997–1002. https://doi.org/10.1055/s-0032-1328704

Lamtai, M., Zghari, O., Ouakki, S., Marmouzi, I., Mesfioui, A., el Hessni, A., & Ouichou, A. (2020). Chronic copper exposure leads to hippocampus oxidative stress and impaired learning and memory in male and female rats. *Toxicological Research*, *36*(4), 359–366. https://doi.org/10.1007/s43188-020-00043-4

Li, X. L., Wong, Y. S., Xu, G., & Chan, J. C. N. (2015). Selenium-enriched Spirulina protects INS-1E pancreatic beta cells from human islet amyloid polypeptide-induced apoptosis through suppression of ROS-mediated mitochondrial dysfunction and PI3/AKT pathway. *European Journal of Nutrition*, *54*(4), 509–522. https://doi.org/10.1007/S00394-014-0732-X/FIGURES/8

Litwin, T., Gromadzka, G., Szpak, G. M., Jabłonka-Salach, K., Bulska, E., & Członkowska, A. (2013). Brain metal accumulation in Wilson's disease. *Journal of the Neurological Sciences*, *329*(1–2), 55–58. https://doi.org/10.1016/j.jns.2013.03.021

Liu, J., Luan, J., Zhou, X., Cui, Y., & Han, J. (2017). Epidemiology, diagnosis, and treatment of Wilson's disease. In *Intractable and Rare Diseases Research* (Vol. 6, Issue 4, pp. 249–255). International Advancement Center for Medicine and Health Research. https://doi.org/10.5582/irdr.2017.01057

Lobatto, M. E., Fuster, V., Fayad, Z. A., & Mulder, W. J. M. (2011). Perspectives and opportunities for nanomedicine in the management of atherosclerosis. In *Nature Reviews Drug Discovery* (Vol. 10, Issue 11, pp. 835–852). Nature Publishing Group. https://doi.org/10.1038/nrd3578

Luper, S. (1998). A Review of Plants Used in the Treatment of Liver Disease: Part 1. In *Alternative Medicine Review* ◆(Vol. 3, Issue 6).

Lutsenko, S. (2021). Dynamic and cell-specific transport networks for intracellular copper ions. *Journal of Cell Science*, *134*(21). https://doi.org/10.1242/jcs.240523

M Olivares, M. M. P. A. F. P. (2008). Present situation of biomarkers for copper status. *Am J Clin Nutr*, *88*, 859S-862S.

MA Levy, Y. T. A. R. T. B. (2001). Cellular response of antioxidant metalloproteins in Cu/Zn SOD transgenic mice exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol*, *281*, L172–L182.

Maheshwari, H., Agarwal, R., Patil, C., & Katare, O. P. (2003). Preparation and pharmacological evaluation of silibinin liposomes. *Arzneimittel-Forschung*, *53*(6), 420–427. https://doi.org/10.1055/S-0031-1297130

MC Linder, M. H.-A. (1996). Copper biochemistry and molecular biology. *Am J Clin Nutr*, *63*, 797S-811S.

Miguez, M.-P., Anundi, I., Sainz-Pardo, L. A., & Lindros, K. O. (1994). Hepatoprotective mechanism of silymarin: no evidence for involvement of cytochrome P450 2El. In *Chem.-Biol. Interact* (Vol. 91).

Mira, L., Silva, M., & Manso, C. F. (1994). SCAVENGING OF REACTIVE OXYGEN SPECIES BY SILIBININ DIHEMISUCCINATE. In *Biochemical Pharmacology* (Vol. 48, Issue 4).

Mitra, S., Keswani, T., Dey, M., Bhattacharya, S., Sarkar, S., Goswami, S., Ghosh, N., Dutta, A., & Bhattacharyya, A. (2012). Copper-induced immunotoxicity involves cell cycle arrest and cell death in the spleen and thymus. *Toxicology*, *293*(1–3), 78–88. https://doi.org/10.1016/J.TOX.2011.12.013

Mohamed, M. A., Jaafar, J., Ismail, A. F., Othman, M. H. D., & Rahman, M. A. (2017). Fourier Transform Infrared (FTIR) Spectroscopy. In *Membrane Characterization* (pp. 3–29). Elsevier Inc. https://doi.org/10.1016/B978-0-444-63776-5.00001-2

Morazzoni, P., Montalbetti, A., Malandrin0, S., & Pifferi, G. (1993). Comparative pharmacokinetics of silipide and silymarin in rats. In *EUROPEAN JOURNAL OF DRUG METABOUSM AND PHARMACOKINETICS* (Vol. 18, Issue 3).

MOURELLE, M., MURIEL, P., FAVARI, L., & FRANCO, T. (1989). PREVENTION OF CCL4-INDUCED LIVER CIRRHOSIS BY SILYMARIN. *Fundamental & Clinical Pharmacology*, *3*(3), 183–191. https://doi.org/10.1111/j.1472-8206.1989.tb00449.x

Mulligan, C., & Bronstein, J. M. (2020). Wilson Disease: An Overview and Approach to Management. In *Neurologic Clinics* (Vol. 38, Issue 2, pp. 417–432). W.B. Saunders. https://doi.org/10.1016/j.ncl.2020.01.005

Myers, B. M., Prendergast, F. G., Holman, R., Kuntz, S. M., & Larusso, N. F. (1993). Alterations in hepatocyte lysosomes in experimental hepatic copper overload in rats. *Gastroenterology*, *105*(6), 1814–1823. https://doi.org/10.1016/0016-5085(93)91080-2

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

Nyasae, L., Bustos, R., Braiterman, L., Eipper, B., & Hubbard, A. (2007). Dynamics of endogenous ATP7A (Menkes protein) in intestinal epithelial cells: copper-dependent redistribution between two intracellular sites. *Am J Physiol Gastrointest Liver Physiol*, *292*(7), 1181–1194. https://doi.org/10.1152/ajpgi.00472.2006.-We

Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K., Woodle, M. C., Lasic, D. D., Redemann, C., & Martin, F. J. (1991). Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. In *Proc. Natl. Acad. Sci. USA* (Vol. 88).

Piazzini, V., Lemmi, B., D'Ambrosio, M., Cinci, L., Luceri, C., Bilia, A. R., & Bergonzi, M. C. (2018). Nanostructured lipid carriers as promising delivery systems for plant extracts: The case of silymarin. *Applied Sciences (Switzerland)*, *8*(7). https://doi.org/10.3390/app8071163

Picollo, M., Aceto, M., & Vitorino, T. (2019). UV-Vis spectroscopy. *Physical Sciences Reviews*, *4*(4). https://doi.org/10.1515/psr-2018-0008

Pietrangelo, A., Borella, F., Casalgrandi, G., Montosi, G., Ceccarelli, D., Gallesi, D., Giovannini, F., Gasparetto, A., & Masini, A. (1995). Antioxidant Activity of Silybin In Vivo During Long-term Iron Overload in Rats. In *GASTROENTEROLOGY* (Vol. 109).

Polishchuk, R., & Lutsenko, S. (2013). Golgi in copper homeostasis: a view from the membrane trafficking field. *Histochem Cell Biol*, *140*(3), 285–295. https://doi.org/10.1007/s00418-013-1123-8

Polishchuk, E. v., Concilli, M., Iacobacci, S., Chesi, G., Pastore, N., Piccolo, P., Paladino, S., Baldantoni, D., vanIJzendoorn, S. C. D., Chan, J., Chang, C. J., Amoresano, A., Pane, F., Pucci, P., Tarallo, A., Parenti, G., Brunetti-Pierri, N., Settembre, C., Ballabio, A., & Polishchuk, R. S. (2014). Wilson Disease Protein ATP7B Utilizes Lysosomal Exocytosis to Maintain Copper Homeostasis. *Developmental Cell*, *29*(6), 686–700. https://doi.org/10.1016/j.devcel.2014.04.033

Powell, S. (2000). The antioxidant properties of zinc. *J Nutr*, *130*, 1447S-1454S.

Prohaska, J. (2008). Role of copper transporters in copper homeostasis. *Am J Clin Nutr*, *88*, 826S-829S.

Quamar, S., Kumar, J., Mishra, A., & Flora, S. (2019). Oxidative stress and neurobehavioural changes in rats following copper exposure and their response to MiADMSA and d - penicillamine . *Toxicology Research and Application*, *3*, 239784731984478. https://doi.org/10.1177/2397847319844782

R Uauy, M. O. M. G. (1998). Essentiality of copper in humans. *Am J Clin Nutr*, *67*, 952S-959S.

Rathbone, M. J., Hadgraft, J., & Roberts, M. S. (2003). *Modified-release drug delivery technology*. Marcel Dekker.

RJ Sokol, M. D. G. M. K. H. H. S. (1990). Oxidant injury to hepatic mitochondrial lipids in rats with dietary copper overload. *Gastroenterology*, *90*, 1061–1071.

Rocha, F. S., Gomes, A. J., Lunardi, C. N., Kaliaguine, S., & Patience, G. S. (2018). Experimental methods in chemical engineering: Ultraviolet visible spectroscopy—UV-Vis. *Canadian Journal of Chemical Engineering*, *96*(12), 2512–2517. https://doi.org/10.1002/cjce.23344

Rodriguez-Granillo, A., Crespo, A., Estrin, D. A., & Wittung-Stafshede, P. (2010). Copper-transfer mechanism from the human chaperone Atox1 to a metal-binding domain of Wilson disease protein. *J Phys Chem B*, *114*(10), 3698–3706. https://doi.org/10.1021/jp911208z

Rosenzweig, A. C. (2001). Copper delivery by metallochaperone proteins. *Acc Chem Res*, *34*(2), 119–128. https://doi.org/10.1021/ar000012p

Saller, R., Meier, R., & Brignoli, R. (2001). *The Use of Silymarin in the Treatment of Liver Diseases*.

Salopek, B., Krasi, D., & Filipovi, S. (1992). *St&ni Clanak I MEASUREMENT AND APPLICATION OF ZETA-POTENTIAL Rudarsko-geoloiko-naftni zbornik*.

Scheiber, I. F., Brůha, R., & Dušek, P. (2017). Pathogenesis of Wilson disease. In *Handbook of Clinical Neurology* (Vol. 142, pp. 43–55). Elsevier B.V. https://doi.org/10.1016/B978-0-444-63625-6.00005-7

Schilsky, M. L. (2001). *Treatment of Wilson's Disease: What Are the Relative Roles of Penicillamine, Trientine, and Zinc Supplementation?*

Schilsky, M. L. (2017). Wilson Disease: Diagnosis, Treatment, and Follow-up. *Clinics in Liver Disease*, *21*(4), 755–767. https://doi.org/10.1016/J.CLD.2017.06.011

Schrieber, S. J., Hawke, R. L., Wen, Z., Smith, P. C., Reddy, K. R., Wahed, A. S., Belle, S. H., Afdhal, N. H., Navarro, V. J., Meyers, C. M., Doo, E., & Fried, M. W. (2011). Differences in the disposition of silymarin between patients with nonalcoholic fatty liver disease and chronic hepatitis C. *Drug Metabolism and Disposition*, *39*(12), 2182–2190. https://doi.org/10.1124/dmd.111.040212

Shaheen, N. J., Hansen, R. A., Morgan, D. R., Gangarosa, L. M., Ringel, Y., Thiny, M. T., Russo, M. W., & Sandler, R. S. (2006). The burden of gastrointestinal and liver diseases, 2006. *American Journal of Gastroenterology*, *101*(9), 2128–2138. https://doi.org/10.1111/j.1572-0241.2006.00723.x

Sherlock, Shiela., & Dooley, James. (2008). *Diseases of the Liver and Biliary System.* 724.

Song, M., Zhou, Z., Chen, T., Zhang, J., & McClain, C. J. (2011). Copper deficiency exacerbates bile duct ligation-induced liver injury and fibrosis in rats. *J Pharmacol Exp Ther*, *339*(1), 298–306. https://doi.org/10.1124/jpet.111.184325

Sonnenbichler, J., Goldberg, M., Hane, L., Madubunyi, I., Vogl, S., & Zetl. I. (1986). *Stimulatory effect of Silibinin on the DNA synthesis in partially hepatectomized rat livers: non-response in hepatoma and other malign cell lines*.

Sornsuvit, C., Hongwiset, D., Yotsawimonwat, S., Toonkum, M., Thongsawat, S., & Taesotikul, W. (2018). The Bioavailability and Pharmacokinetics of Silymarin SMEDDS Formulation Study in Healthy Thai Volunteers. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2018*. https://doi.org/10.1155/2018/1507834

Strausak, D., Mercer, J. F. B., Dieter, H. H., Stremmel, W., & Multhaup, G. (2001). Copper in disorders with neurological symptoms: Alzheimer's, Menkes and Wilson diseases. *Brain Res Bull*, *55*(2), 175–185. https://doi.org/10.1016/s0361-9230(01)00454-3

Torchilin, V. P. (2005). Recent advances with liposomes as pharmaceutical carriers. In *Nature Reviews Drug Discovery* (Vol. 4, Issue 2, pp. 145–160). https://doi.org/10.1038/nrd1632

Valenzuela, A., & Garrido, A. (1994). Biochemical bases of the pharmacological action of the flavonoid silymarin and of its structural isomer silibinin. In *Biol Res*.

Valenzuela, A., Vial, S., & Guerra, R. (1989). Selectivity of Silymarin on the Increase of the Glutathione Content in Different Tissues of the Rat. In *420 PlantaMedica* (Vol. 55).

Vargas-Mendoza, N., Madrigal-Santillán, E., Morales-González, Á., Esquivel-Soto, J., Esquivel-Chirino, C., García-Luna y González-Rubio, M. G., Gayosso-de-Lucio, J. A., & Morales-González, J. A. (2014). Hepatoprotective effect of silymarin. In *World Journal of Hepatology* (Vol. 6, Issue 3, pp. 144–149). Baishideng Publishing Group Co. https://doi.org/10.4254/wjh.v6.i3.144

Vernon-Parry, K. D. (2000). *Scanning Electron Microscopy: an introduction*.

Vulpe, C., Levinson, B., Whitney, S., Packman, S., & Gitschier, J. (1993). Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nature Genetics*, *3*(1), 7–13. https://doi.org/10.1038/NG0193-7

Wagner, V., Dullaart, A., Bock, A.-K., & Zweck, A. (2006). The emerging nanomedicine landscape. In *NATURE BIOTECHNOLOGY VOLUME* (Vol. 24, Issue 10). http://scientific.thomson.com/products/sci/

Wang, X. P., Zhang, W. F., Huang, H. Y., & Preter, M. (2010). Neurology in the People's Republic of China - An update. *European Neurology*, *64*(6), 320–324. https://doi.org/10.1159/000321648

Wee, N. K. Y., Weinstein, D. C., Fraser, S. T., & Assinder, S. J. (2013). The mammalian copper transporters CTR1 and CTR2 and their roles in development and disease. *The International Journal of Biochemistry & Cell Biology*, *45*(5), 960–963. https://doi.org/10.1016/J.BIOCEL.2013.01.018

Winge, D. R., & Mehra, R. K. (1990). Host Defenses against Copper Toxicity. *International Review of Experimental Pathology*, *31*(C), 47–83. https://doi.org/10.1016/B978-0-12-364931-7.50007-0

Xie, J. J., & Wu, Z. Y. (2017). Wilson's Disease in China. In *Neuroscience Bulletin* (Vol. 33, Issue 3, pp. 323–330). Science Press. https://doi.org/10.1007/s12264-017-0107-4

Xu, M. B., Rong, P. Q., Jin, T. Y., Zhang, P. P., Liang, H. Y., & Zheng, G. Q. (2019). Chinese herbal medicine for wilson's disease: A systematic review and meta-analysis. In *Frontiers in Pharmacology* (Vol. 10, Issue MAR). Frontiers Media S.A. https://doi.org/10.3389/fphar.2019.00277

Yang, X. L., Miura, N., Kawarada, Y., Terada, K., Petrukhin, K., Gilliam, T. C., & Sugiyama, T. (1997). Two forms of Wilson disease protein produced by alternative splicing are localized in distinct cellular compartments. *Biochemical Journal*, *326*(Pt 3), 897. https://doi.org/10.1042/BJ3260897

Zhang, H. (2017). Thin-film hydration followed by extrusion method for liposome preparation. In *Methods in Molecular Biology* (Vol. 1522, pp. 17–22). Humana Press Inc. https://doi.org/10.1007/978-1-4939-6591-5_2

Zhang, L., Liu, Y., Hu, X., Wang, Y., & Xu, M. (2019). Metal ion interactions with methyl gallate characterized by UV spectroscopic and computational methods. *Food Chemistry*, *293*, 66–73. https://doi.org/10.1016/j.foodchem.2019.04.083

Zhao, J., & Agarwal, R. (1999). Tissue distribution of silibinin, the major active constituent of silymarin, in mice and its association with enhancement of phase II enzymes: implications in cancer chemoprevention II enzyme induction by systemically administered silibinin in different tissues, including skin, where silymarin has been shown to be a strong cancer chemopreventive agent, and suggest further studies to assess the cancer preven-tive and anticarcinogenic effects of silibinin in different cancer models. In *Carcinogenesis* (Vol. 20, Issue 11).

ZL, H., & JD, G. (n.d.). Genetic and molecular basis for copper toxicity. *Am J Clin Nutr*, *63*, 836S-41S.