IN-VIVO EVALUATION OF SILYMARIN ENCAPSULATED LIPOSOMAL NANOPARTICLES IN CHRONIC MILD STRESS (CMS) MODEL AND DEPRESSION INDUCED LIVER DISORDERS

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

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Praise to be Allah Almighty, the most benevolent and the most merciful. Without His will, nothing is possible. My ideas and dreams regarding this research work would not have materialized, had it not been for Allah's blessings on me. My utmost humble dedications to the Holy Prophet (S.A.W), who is the city of knowledge and to His brother Ali (A.S) who is the door of the city of knowledge and to the household of Holy Progeny from where knowledge took birth.

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LIST OF ABBREVIATIONS

SLNPs	**Silymarin loaded pegylated liposomes nanoparticles**
CMS	**Chronic mild stress**
NAFLD	**Non-Alcoholic Fatty liver Disease**
CNS	**Central nervous system**
PEG	**Polyethylene glycol**
BBB	**Blood brain barrier**
OFT	**Open field test**
TST	**Tail suspension test**
EPMT	**Elevated plus maze test**
I.V	**Intravenous**
DMPC	**1,2-Dimyristoyl-sn-glycero-3-phosphocholine**
HC	**Hippocampus**
PFC	**Prefrontal cortex**
RES	**Reticulo-endothelial system**
ACTH	**Adreno cortico trophic hormone**
BNP	**Blank nanoparticles**
HPLC	**High performance liquid chromatography**
NP	**Nanoparticles**

ABSTRACT

Depression is categorized as one of the most prevalent psychological disorders that affect personal wellbeing and social life of individuals. Symptoms vary from anhedonia to suicide commitment. The molecular mechanism behind is the low concentration of neurotransmitters serotonin, dopamine and norepinephrine in central nervous system. These are primarily responsible for regulating and alleviating mood. In the chronic mild stress (CMS) model of depression, silymarin, a plant-derived polyphenolic flavonoid of Silybum marianum, elicited strong antidepressant-like action. It increased the levels of monoamines, particularly 5-hydroxytryptamine (5-HT) in the cortex and dopamine (DA) in the mice hippocampal region and prefrontal cortex. The objective of the current research was to investigate silymarin's antidepressant potential in CMS-induced depressive-like behavior in mice and to identify its potential mechanism(s) of action. The mice were given silymarin and silymarin loaded liposomal nanoparticles (SLNPs) for two weeks after following CMS protocol for 28 days (4 weeks). Animals were assessed for behavioral alterations, including exploratory activity in an open field test, behavioral despair in a forced swim test, and anxiety-like behaviors in an elevated plus maze test. There lies a close relationship between depression and inflammatory liver diseases. Hence the effect of depression on liver has also been checked. Silymarin is a commercially available hepatoprotective drug but due to its antioxidant properties, research has been conducted to evaluate its neuroprotective effect and hence its prescription as antidepressant drug. However, due to its poor solubility and bioavailability there is delay in the onset of treatment outcomes in many individuals. Certain side effects and contraindications are also important regimen opponents. In this study, Silymarin loaded liposomal nanoparticles (SLNPs) are prepared, characterized, and realized for the depression treatment in Chronic Mild Stress (CMS) mice model of depression and its treatment efficiency on symptoms of inflammatory liver diseases in mice as well. It presented face construct and validity response. As such the SLNPs present improvement in depression measurement parameters as compared to the simple silymarin. The SLNPs also positively impacted the aggression, anhedonia and rearing in mice, however simple silymarin treated mice did not show improvement in social and personal behavior. As such SLNPs compensated for delayed onset of fluoxetine response.

Key words: depression, mice model of depression, chronic mild stress liposomal nanoparticle

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CHAPTER 1: INTRODUCTION

1.1 Depression

Depression is a chronic, disabling condition with elevated lifetime prevalence (Nemeroff, 2007a). Usually, its occurrence is accompanied by feelings of inadequacy, a persistently depressed mood, anhedonia, and hopelessness, and lack of interest. (Wiersma et al., 2011). Anhedonia is a condition in which a person is unable to feel pleasure and gets entrapped in a depressed mood that can interfere with the thoughts of a person and sleep patterns, is a fundamental feature of depression, a severe central nervous system (CNS) disorder (Ashraf et al., 2019). The stages of depression are similar to those of many other illnesses: i. Major depression is characterized by a variety of symptoms that make it difficult for a person to work, sleep, eat, and engage in once-pleasurable activities. These incapacitating depressive episodes can happen just once, twice, or multiple times in a lifetime. ii. Dysthymia, a less severe form of depression, is characterized by long-lasting, persistent symptoms (Nemeroff, 2007b) that do not disarm a person but prevent from operating at full capacity or from contentment. Bipolar disorder and manic-depressive disease are not nearly as common as other types of depression. Cycles of mania or exhilaration and despair are involved in it. The mood swings can be abrupt and intense at times, but most of the time they are moderate.

1.2 Prevalence

Millions of people worldwide struggle with depression, an illness that has the potential to be fatal and can strike at any age, from infancy to old age. Depression has a significant negative impact on society by eroding hope, aspiration, and occasionally even the will to live (Nemeroff, 2007b). In terms of pervasiveness, dysfunction, economic cost, misery, and morbidity, depression is an ailment of considerable public health importance. Women experience depression more frequently than males. According to the Global Burden of Disease report, men have unipolar depression episodes at a point prevalence of 1.9 percent compared to women (Kessler et al., 2012) at a point prevalence of 3.2 percent, while men experience unipolar depressive episodes at a one-year prevalence of 5.8 percent compared to women at 9.5 percent. By another ten years, depression is predicted by the WHO to overtake heart disease as the second most common disease in terms of morbidity (Perona et al., 2008). Not just adults, but 2% of school children and 5% of teenagers

experience depression as well, with the majority of cases going unreported. Depression is one of the most common and widespread disorder in developing countries including Pakistan. Lack of resources, poverty, socioeconomic instability, unemployment, and high inflation are the possible reasons behind the high occurrence of depressive ailments in Pakistani community. A systematic review revealed that an indigenous random community sample's mean prevalence for anxiety and depression was 33.62 percent according to (Husain et al., 2000). Among these depressive symptoms were present in 10–33% of men and 29–66% of women. It is such an alarming situation since it has a substantial role in the emergence of anxiety, stress as well as cognitive dysfunctions (Kanter et al., n.d.).

1.3 Neurobiology of depression

Depression continues to be one of the most often diagnosed psychiatric conditions. It is still extant in the society as a challenge which has not been resolved by the doctors and patients, despite the fact that medical practitioners have considerably expanded their knowledge about the condition, and its treatment. (Duman & Monteggia, 2006; Maletic et al., 2007). The effect of depression on the functional and anatomical processes taking place inside the brain has come under increasing scrutiny in recent years. It is generally believed that depression was brought on by a "chemical imbalance" (Nestler et al., 2002a) in the brain. From past three decades it has been hypothesized that monoamine levels, such as serotonin, norepinephrine, and dopamine,(Meyer et al., 2006) are usually thought to be low in the brain following untreated major depression episodes (MDEs). Although the neuroscience of depression is not fully understood, it is known that genetic, environmental, psychological, and biological variables contribute to its aetiology (Nestler et al., 2002b). A growing body of research suggests that in the neuropathology of psychiatric disorders such as major depression, could have been caused because of the involvement of oxidative stress. (Ng et al., 2008). Hence dopaminergic and serotonergic neurotransmitter system impairment, as well as an imbalance in the oxidant-antioxidant system, are the primary causes of depression and its associated symptoms.

1.4 Monoamine theory of depression

Depression is a prevalent psychiatric ailment, however despite extensive prior research, its aetiology is still unknown. As a result, numerous theories have been put out to clarify the pathophysiology of depression. The most popular of this pathophysiology is the **'monoamine hypothesis'**. Diminished concentration of monoamines including dopamine, serotonin and noradrenaline in synaptic gaps when someone is in the state of depression, is the main idea behind this hypothesis. According to this hypothesis the concentrations of monoamines, such as serotonin, noradrenaline, and dopamine, in synaptic gaps are diminished in the depressive state(Lee et al., 2010) . As a result, the monoamine hypothesis has guided the development of the majority of antidepressants, which are widely used today. There is other evidence to support this hypothesis in addition to the fact that antidepressants are all monoamine agonists. Firstly, depression is a common side effect, when the monoamine antagonist reserpine, was used to treat conditions including high blood pressure. That's why it is now rarely prescribed. As a result, monoamine agonists prevent depression but the antagonists of monoamine (reserpine) cause depression.

The finding that levels of 5-HT, as measured by its metabolites, appear to be linked with depression is another evidence in favor of the monoamine hypothesis. For instance, it has been discovered that patients who have low levels of a 5-HT metabolite are more likely to have died by suicide(Hall, n.d.).

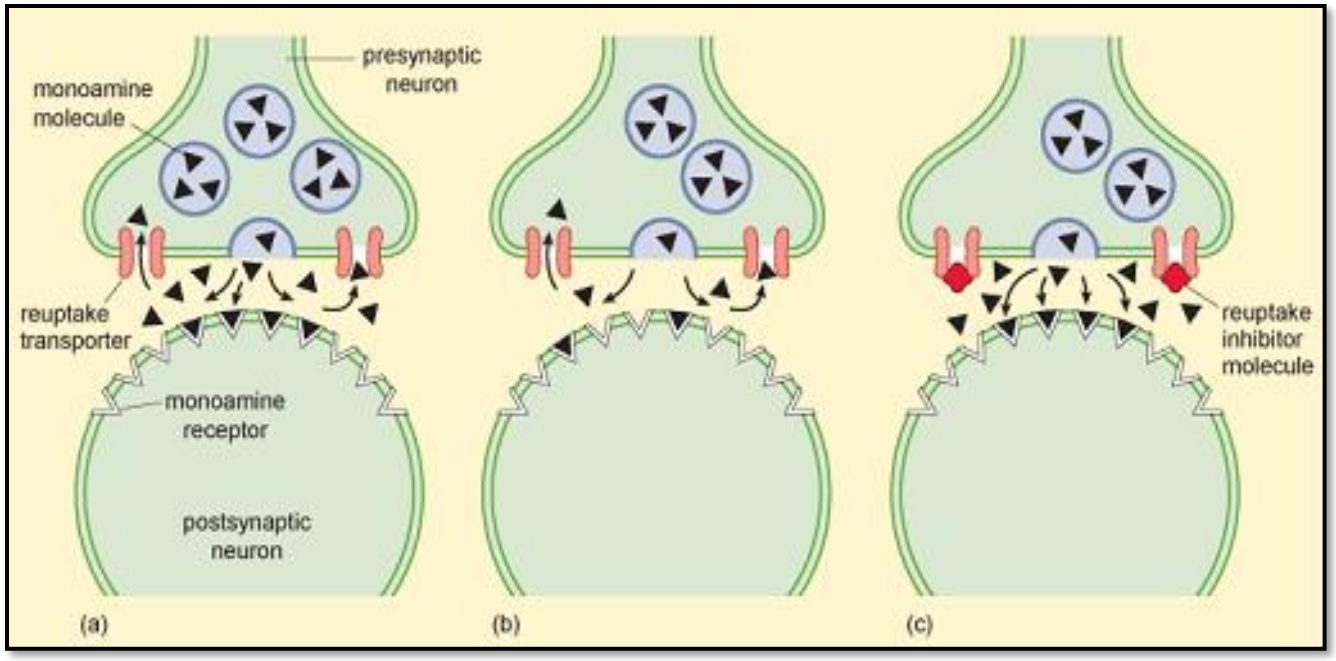


Figure 1.4: Monoamine hypothesis of depression

1.5 Effect of depression on liver

Liver diseases and depression are intimately related. Every third patient with hepatitis or liver cirrhosis has depressive symptoms. On the other side, every third patient with a depressive (Kai Kahl et al., 2017)problem will eventually acquire an alcohol disorder. Inflammatory processes, in which the microbiota and increased intestinal permeability of the gut play a vital role, appear to be a significant relationship between depression and hepatic illness. Dysregulation of the hypothalamic-pituitary-adrenal axis, which is associated with liver diseases, could be caused by psychological distress according to a study. (Russ et al., 2015) which causes the release of pro-inflammatory factors in the liver (such as interleukin-6 and tumour necrosis factor alpha)17 and contribute to the emergence of Non-Alcoholic Fatty Liver Disease (NAFLD). Therefore, this research also includes the effect of depression on liver and hence provide a broader spectrum of depression study.

1.6 Management of depression

Due to complex neuropathology of drug resistance in depression, therapy failures, and even more adverse repercussions are common with contemporary depression medications(Sarko, n.d.).

Therefore, it is essential to treat depression effectively in its early phases in order to stop morphological and functional problems. There hasn't been much research on the efficacy of various treatments for depression, despite the fact that data suggests that patients who are severely depressed require antidepressant drug therapy and those who are not severely depressed may benefit from alternative methods (i.e., "nonbiological"). Those drugs that have already been approved by the US Food and Drug Administration (FDA) for the treatment of depression are the SSRIs sertraline and paroxetine (Brady et al., n.d.). However, not every patient responds well to the pharmacotherapy and psychotherapy that is offered. Resistance to the related adverse effects, non-adherence, and fear of addiction are major obstacles to effective pharmacological therapy of depression (Anestopoulos et al., 2013). In order to account for the limitations of the therapeutic choices that are currently accessible, efforts should be made to develop more efficient and/or safe therapy options for the prevention and/or treatment of depression. Therefore, several herbal remedies have been demonstrated to be antidepressant in nature. Additionally, some herbal products are said to be adaptogens since they have the power to raise the body's resistance to stress. These medicinal herbs could be useful in easing the symptoms of depression, based on the neuropharmacological activities they exhibit. Owing to these properties Silymarin (Thakare et al., 2017)was tested for its neuro- and cardioprotective properties because of its strong antioxidant activity (Milić et al., n.d.; Nencini et al., 2007). Silymarin is a potent drug being commercially used for the treating the liver diseases specifically NAFLD. Additionally, this plant has a long history of usage as a natural treatment option for varicose veins, ailments of the biliary tract and liver, difficulties with the upper gastrointestinal tract, and digestive issues(Saller et al., 2007a). Due to its low toxicity, strong antioxidant properties, and regenerating properties, silymarin has garnered interest for medicinal usage (Liu et al., 2014). Chemically, silymarin is an amalgamation of at least seven flavonolignans, including taxifolin and the flavonoids silybin A, silybin B, isosilybin A, and isosilybin B. Little is known about silymarin's ability to reduce symptoms similar to depression. But it has been reported recently that silymarin could be a neuroprotective agent because of its effect against different neurodegenerative disease.

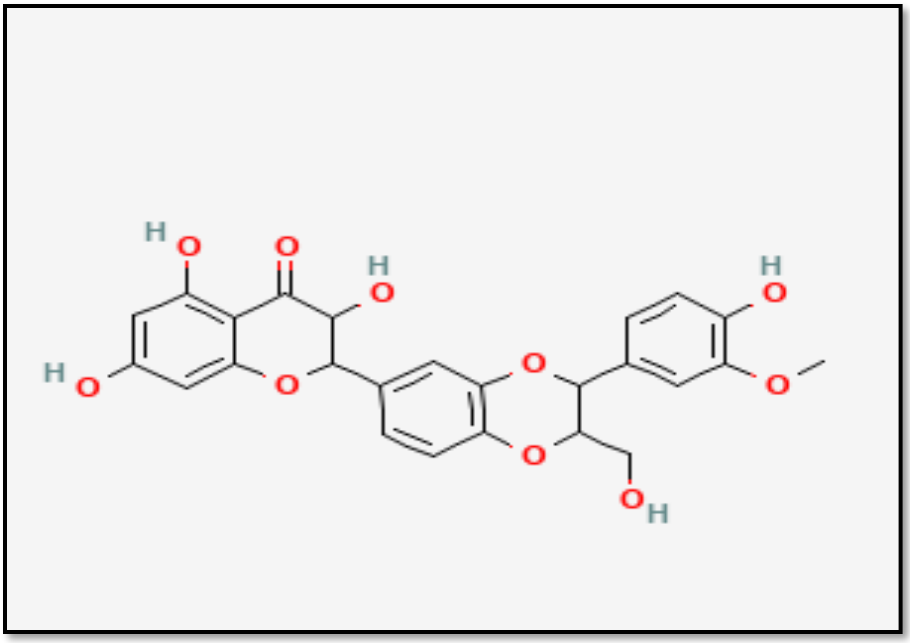


Figure 1.5: Structure of Silymarin

Despite it possesses the therapeutic benefits, but due to its poor solubility (Nasr, 2016), it has a limited bioavailability, which prevents it from producing the desired therapeutic results. In this aspect, nanoparticles have demonstrated their viability as a source for improving drug bioavailability across the oral administration route, resulting in optimized therapeutic effects(El-Ghazaly et al., n.d.; Zorkina et al., 2020)

1.7 Role of nanotechnology

Despite substantial advancements in knowledge of the cellular and molecular underpinnings of brain illnesses and in the design of therapeutic approaches, the efficient transport of drugs to the central nervous system continues to be a significant challenge (Villabona-Rueda et al., 2019). The blood brain barrier (BBB) is one of the most critical impediments to deliver potent molecules in the central nervous system. Its functions include protecting the central nervous system (CNS) against neurotoxic agents in addition to delivering nutrients and oxygen from the bloodstream to the brain (van Tellingen et al., 2015). The tight connections present between adjacent endothelial cells, producing the proteins claudins, occludins, and different adhesion molecules, cause the BBB to be impermeable to many chemicals.

Systems with dimensions of 10 to 100 nm and distinct chemical and physiological characteristics fall under the umbrella of nanotherapy. Nanopreparations are differentiated because of their plasticity, altered surface characteristics, flexibility, and controlled release of the potent bioactive ingredient. Drugs intended to treat mental disease require the precise transportation of central nervous system (CNS) active medications to the brain and this objective is achieved via nanotherapeutics (Agrahari, 2017). Hence drug loading into nanocontainers, which have a minute size and the potential to cross the BBB, is one of the most promising approaches to overcome the BBB.

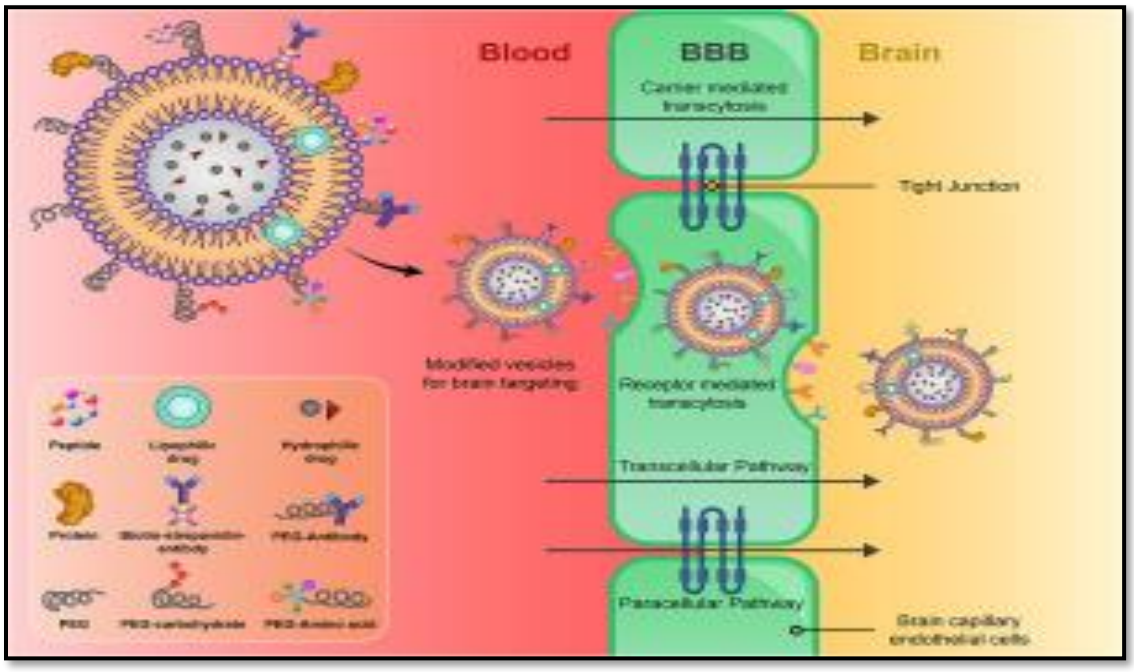


Figure 1.6: Schematic representation of liposomal nanoparticles crossing the BBB

This form of drug delivery greatly improves the bioavailability of the potent agent and minimizes the associated side effects. Therefore, contemporary research offers the development of nano-vehicle system to deliver the drug at targeted area. Several delivery systems are therefore developed by researchers to improve the efficacy of the drug. This study focuses on Lipid based Nanoparticles specifically liposomes.

1.8 Liposomal nanoparticles

The characteristic features of liposomes include their spherical shape and lipid bilayer. The range of ideal diameters is 100 to 200 nm. These nanocarriers enable for increased drug loading percentages and balanced nanocontainer sizes. The excellent biocompatibility, biodegradability, increased bioavailability, and stability of therapeutic substances are the main characteristics of liposomes (Uhde et al., 1989). Additionally, they can also alter the surfaces to allow for active targeting. The use of liposomes for BBB crossing has been studied for many years (Brambilla et al., 1995). Liposomes are altered to target transcytosis via the CNS endothelium by adding biologically active ligands to the liposomal surface, such as peptides, antibodies, or small molecules. Liposomes are thus intriguing alternatives for therapeutic drug delivery that can penetrate the BBB. The present study therefore sought to broaden the area of pertinent investigations by examining the potential protective impact of silymarin when it is administered in the form of a nanoparticle (encapsulated in liposomes) against the biochemical as well as behavioral changes brought on by Chronic Mild Stress (CMS) and ultimately its impact on liver.

CHAPTER 2: LITERATURE REVIEW

2.1 Silymarin: A versatile drug

An ancient medicinal plant known as *Silybum marianum L*. (Milk Thistle) has been used for centuries to treat a various illnesses. It hails from the Carduus marianum family, and is effective against liver and gallbladder disorders, protecting the liver from snakebites and insect stings, mushroom poisoning, and alcoholism(Křen & Walterová, n.d.). However, the hepatoprotective and antioxidant properties of milk thistle were its early uses. The active ingredient of this herb is silymarin. Various substances, namely silybin A, silybin B, isosilybin A, and isosilybin B, flavonolignants like silychristin, neosilyhermin, silyhermin, and silydianin and make up this active ingredient. (Mayer et al., 2005). These ingredients of the active substances are more abundant in the fruit and seeds of this plant than the other parts.

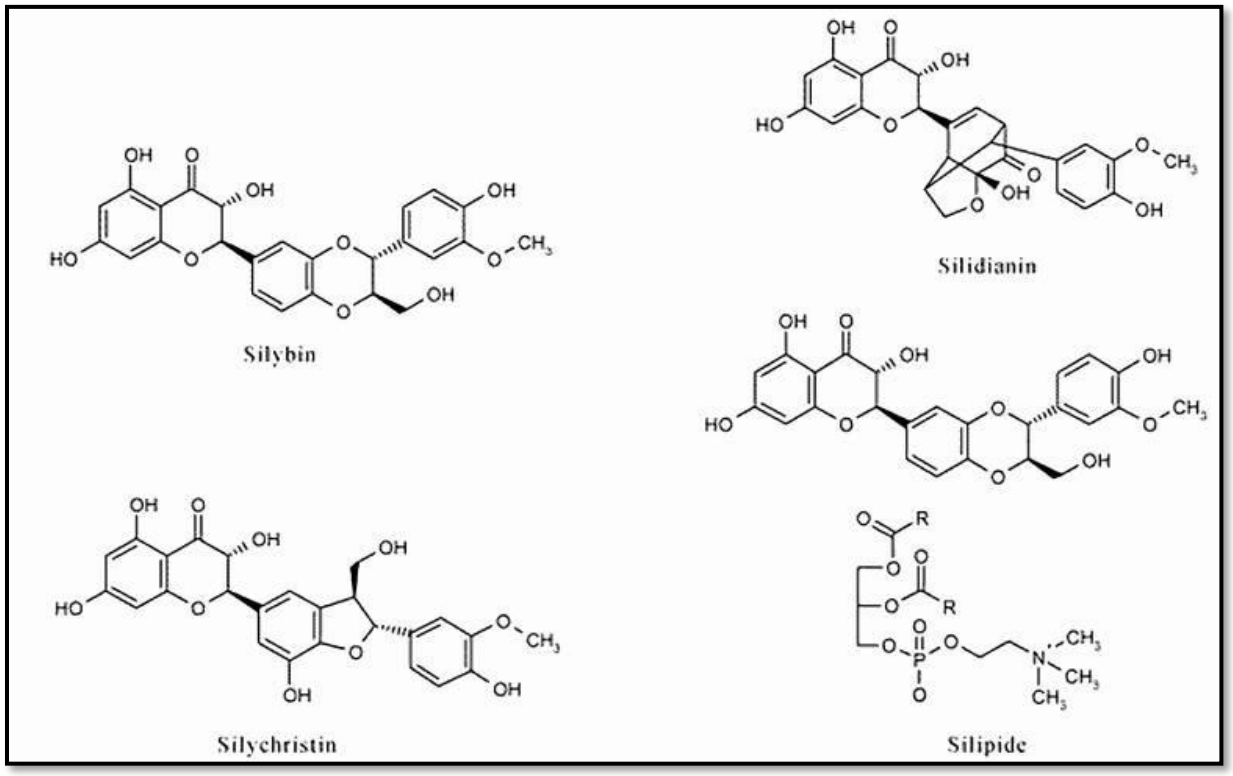


Figure 2.1: Chemical structure of some of the silymarin components

Additionally, silymarin's effectiveness is linked to disorders of many organs. These include the prostate, lungs, CNS, kidneys, skin and pancreas(Gazak et al., 2007) . Silymarin possesses

antioxidant properties, which include a free radical scavenger role plus raising glutathione concentrations. Furthermore, it also has antifibrotic, immunomodulating, anti-inflammatory, and anti-fibrotic activities, making it useful for treating hepatitis, hepatic cirrhosis, and mushroom poisoning (Karimi et al., 2005). Pharmacological investigations have established silymarin as a safe herbal product because it is not poisonous at physiological concentrations unless it is administered incorrectly at therapeutic levels (Wu et al., 2009).

2.1.1 Neuroprotective effect of silymarin

Biological predisposition, exposure to traumatic and stressful life experiences, and predisposing temperament and personality factors are deemed critical in depression development.(Thakare et al., 2018). Physicians, herbalists, and traditional healers around the world have employed herbal medicines for treatment and prevention of diseases since ages. Milk thistle (Silybum marianum) has been interested investigators as a homeopathic treatment for inflammatory ailments for a long time. Due to its wider range of therapeutic applications, silymarin is now commercially available as a standardized blend of 4 flavonolignan isomers, which was once utilized as a raw extract (Saller et al., n.d.) from the Silybum marianum's seeds. (cardui mariae fructus).

Silymarin, a plant-derived polyphenolic flavonoid of Silybum marianum, elicited strong antidepressant-like action in an acute restraint stress model of depression. It increased the levels of monoamines in mice, particularly 5-hydroxytryptamine (5-HT), dopamine (DA) and norepinephrine (NE) in the cerebellum(Thakare et al., 2018).

Brain tissues are susceptible to reactive oxygen species (ROS) damages, primarily because of the high oxygen utilization coupled with large amounts of polyunsaturated fatty acids, enhanced free iron ions, and weak antioxidant defenses(Galhardi et al., 2009) . Silymarin significantly decreased protein oxidation in the cortex and hippocampus of aged rats when given in the quantities of 200 mg/kg/day as compared to young rats.

In hemi-parkinsonian rats, 200 mg/kg of silymarin decreased the 6-hydroxydopamine (6-OHDA)-induced rotational behavior. Furthermore, it protected the substantia nigra pars compacta neurons from its toxicity. This is an indication for a dose-dependent neuroprotective effect of silymarin

against 6-OHDA toxicity. This particular neuroprotective effect is primarily courtesy of oxidative stress reduction and an estrogenic pathway (Baluchnejadmojarad et al., 2010).

Additionally, silymarin can increase the concentration of a few neurotransmitters in the brain. Ethanolic and aqueous extracts of silymarin utilized in a study on the modified forced swimming test in mice indicated that silymarin's aqueous extract had an antidepressant impact in animal models. The deductions were made since the ethanolic extract had no effect on mice's immobility duration while the latter was dramatically reduced (Gharagozloo et al., 2010).

It has been demonstrated that the most active silymarin compound, silibinin, reduces the reduction in hippocampus serotonin levels brought on by methamphetamine, and it has been hypothesized that this inhibition of serotonin loss may contribute to silibinin's protective effects on methamphetamine-induced cognitive impairment (Yaghmaei et al., 2012).

Despite being well known for its antioxidant properties, sylimarin has recently been shown to interact with central neurotransmitters, such as those in the serotonergic system, and may help to control mood disorders including anxiety and depression (Yaghmaei et al., 2012).

2.1.2 Hepatoprotective effect of silymarin

Liver is the primary organ responsible for metabolic and excretory processes. Because of its favourable location in the body, it is constantly and frequently exposed to xenobiotics. Toxins that are absorbed from the gastrointestinal system enter liver first, where they cause a range of hepatic illnesses. As a result, liver illnesses continue to be a severe health issue. Liver damage is brought on by inflammation, apoptosis, necrosis, immunological response ischemia, fibrosis, ischemia, altered gene expression, and regeneration. It spans from acute hepatitis to hepatocellular cancer (Shaker et al., 2010).

Silymarin has been utilized as a "hepatoprotectant" for many years. It has an antioxidant, immunomodulatory, antifibrotic, antiviral and antiproliferative activities, while the exact mechanism of action has not yet been fully established. Silymarin is primarily excreted in bile. A short half-life and rapid hepatic conjugation are its key features. Concerning its administration, high or repeated oral doses are prescribed in management of hepatic inflammation in vivo (Morishima et al., 2010).

According to in vitro tests, silybin A and silybin B, two silymarin components, can quell T-cell proliferation and pro-inflammatory cytokines release in a dose-dependent manner. Silymarin at increased oral doses reduced liver inflammation in patients with the chronic liver disease ((Morishima et al., 2010).

In studies on animals, silymarin and silybinin were shown to exhibit anti-hepatotoxic properties against acute ethanol intoxication, CCl4, thioacetamide, D-galactosamine, cisplatin, thallium and acetaminophen toxicity in the rat or mouse liver (Gharagozloo et al., 2010).

Elevated levels of free fatty acids circulating in blood, and their concentrations are correlated with non-alcoholic fatty liver disease's (NAFLD) severity. Potential treatments for NAFLD include substances that inhibit or lessen the destruction of hepatocytes brought on by free fatty acids(Z. Song et al., 2007).

2.2Mechanism of action of silymarin in brain

The amelioration of monoaminergic, neurogenesis (improving 5-HT, NE, and BDNF levels), and attenuation of inflammatory cytokines system (Thakare et al., 2018)and oxidative stress via control of corticosterone response are associated with the probable mechanisms implicated in the antidepressant-like effect of silymarin. These antioxidant defense systems are restored in the cerebral cortex and hippocampus.

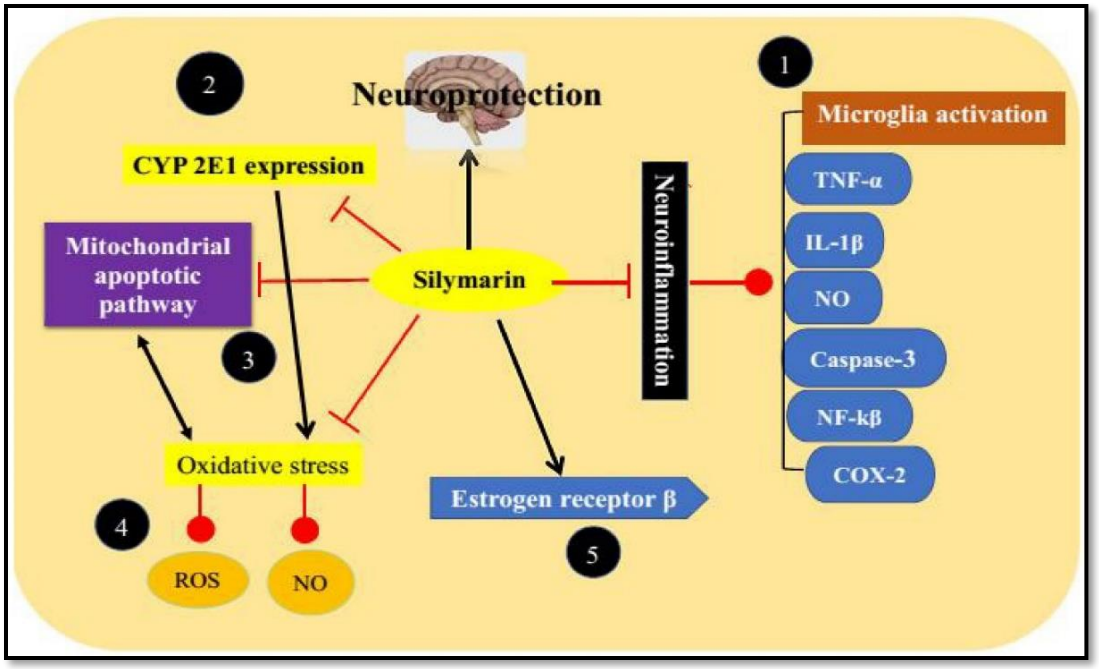


Figure 2.2: Mechanism: Silymarin in Brain

2.3 Mechanism of action: Silymarin in liver

The numerous processes through which silymarin works are as follow: It circulates through the enterohepatic system, changing from plasma to bile, and then concentrating in the hepatocytes. silymarin, has a steroid structure, improves DNA and RNA synthesis, hence improving the liver cells' capacity for regeneration; altering the hepatocyte external membrane's structure to inhibit the entry of xenobiotics into the cell ; removing free radicals from the body and elevating glutathione levels inside cells to stop lipid peroxidation; Another way that silymarin works is by altering the cell membrane's transporters and receptors, including the bile salt export pump, organic anion uptake transporter peptides (OATP), ABC transporters (P-gp), and TNF-dependent transporters(Saller et al., 2007b).

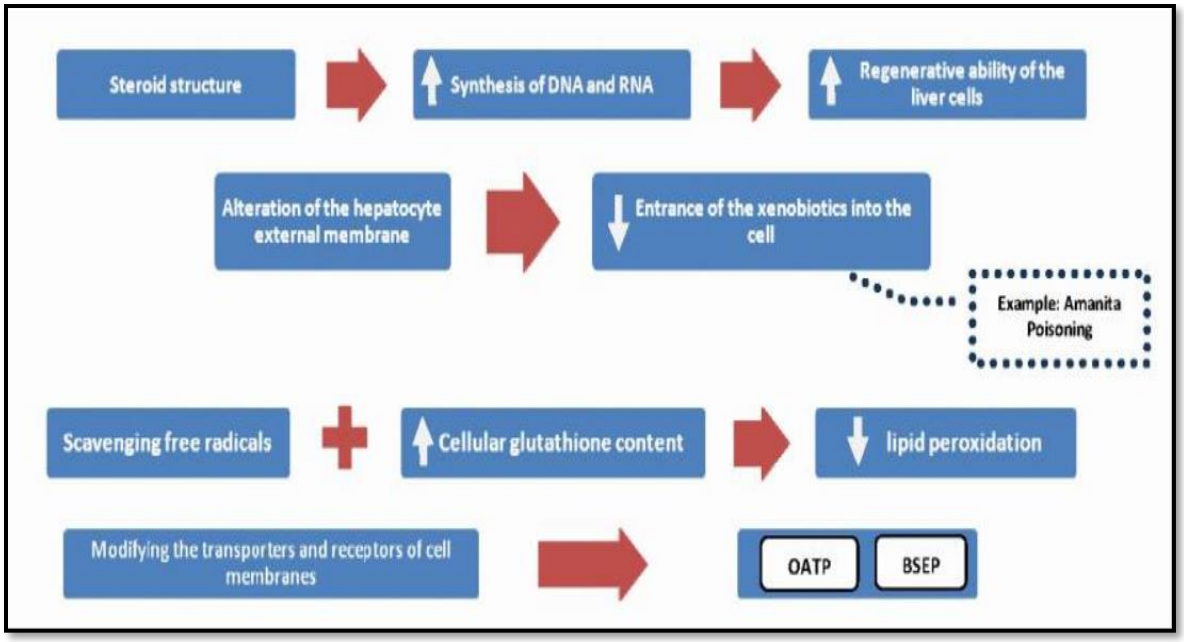


Figure 2.3: Different mechanism of action of silymarin

2.4 Need of formulation strategies of Silymarin

Silymarin is a legally recognized drug that has certain hepatoprotective, antiviral, antioxidant and anticancer activities. It has low water solubility and features ineffective intestinal absorption coupled with the inclusion of numerous components, and accelerated metabolism (Abenavoli et al., 2018) (Bijak, 2017). These undesirable characteristics generally raise several questions. These questions are primarily focused on formulation and design of the drug delivery systems since insufficient water solubility of the active components in phytomedicines might impair the corresponding bioavailability (Dressman et al., 2007). Conversely, various solubilization technologies are accessible to investigators, allowing them to entrap active plant components in nano-vehicles that are weakly water-soluble, such as silymarin (Patra et al., 2018).

For instance, effective nanoencapsulation enables phenolics and antioxidants to passively enter the lymphatic and blood circulatory systems from the gut lumen, significantly increasing their bioavailability (Li et al., 2015; Obeid et al., 2017). As a result, silymarin-based formulations have already been commenced using cutting-edge methods, and it has been discovered that doing so increases their therapeutic efficacy against a variety of disorders (Agarwal et al., n.d.).

2.4.1 Significance of liposomal nanoparticles in targeted delivery

Liposomes are hollow, spherical nanoparticles. They have a closed lipid membrane shell (mono- or multi-layer) which contains an aqueous solution. Beneficial features that make these supramolecular aggregates a better choice over other carriers of therapeutic drugs include:

1) Their ability to encapsulate both hydrophilic and lipophilic drugs

2) Targeting and controlled release properties

3) Cell affinity

4) Tissue compatibility

5) Decreased drug toxicity,

6) Improved drug stability (Li et al., 2015).

Furthermore, liposomal systems can quickly access reticulo-endothelial system (RES) rich tissues, just like the liver and spleen. It is this specific, self-targeted characteristic of liposomal carriers, which makes them a suitable choice for an effective drug delivery to the hepatic region.

Research suggests that Silymarin has a potential neuroprotective effect against various neurodegenerative diseases, particularly when incorporated in nanovehicles. In the lipidic matrix of nanostructured lipid carriers, the essential lipid-based nanoparticle systems, (Bseiso et al., 2015; Müller et al., 2002), the medication is solubilized, resulting in improved encapsulation, stability, and bioavailability(Islam & Amran, n.d.).

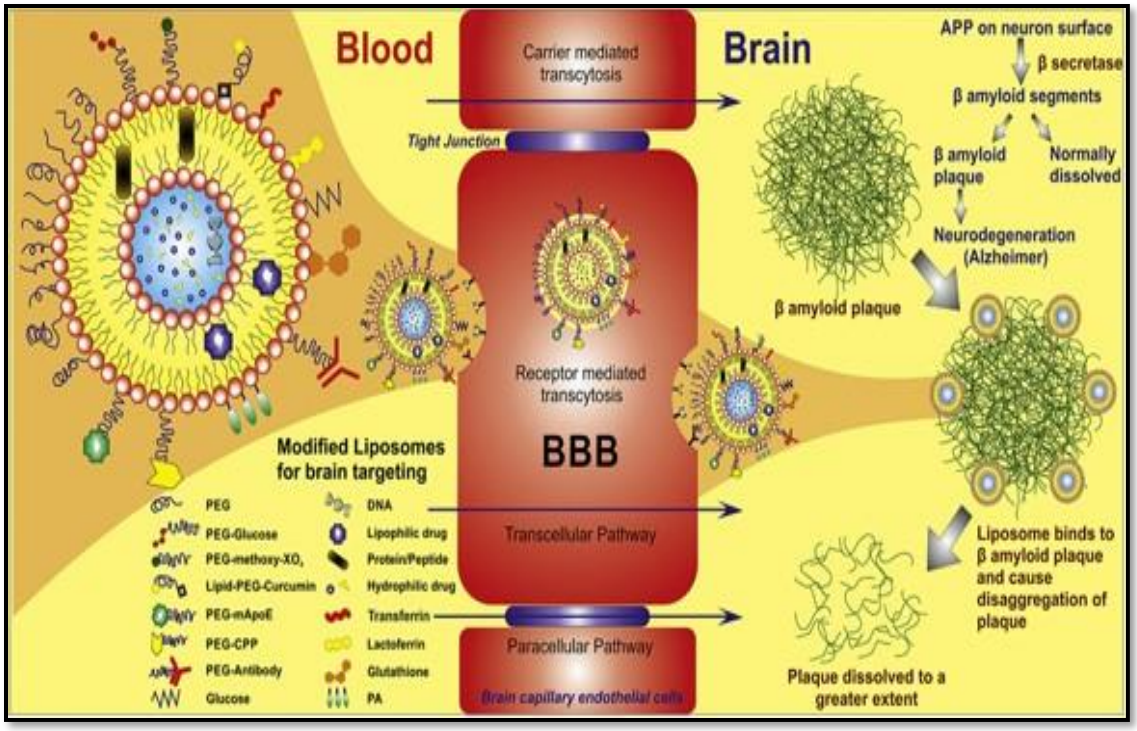


Figure 2.4: Liposomal targeted delivery in brain

2.4.2 Synthesis of liposomes

All techniques for making liposomes involve following basic steps:

1-Drying lipids from organic solvents

2- Dispersing the lipid in aqueous media

3- Purifying the resulting liposome

4- Evaluating the finished product.

Liposomes are made from a variety of lipids with various characteristics, including size, surface charge, biocompatibility, drug release kinetics, and cell targeting (Islam & Amran, n.d.).

There are numerous ways to make liposomes. The techniques employed to create the liposomes have an impact on their lamellarity and size(Akbarzadeh et al., 2013).

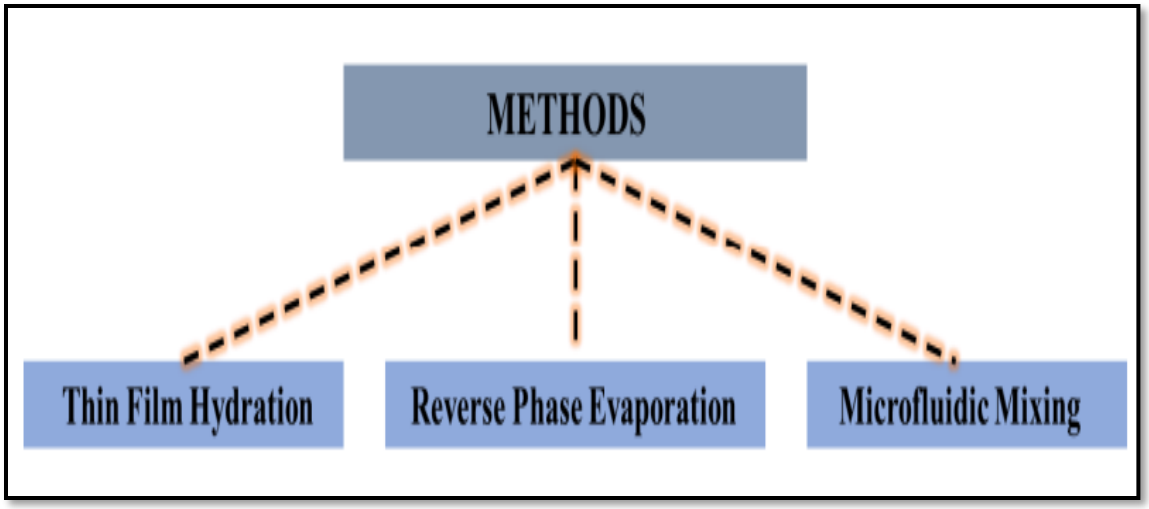


Figure 2.5: Preparation methods of liposomes

2.4.3 Drug loading

Drug loading Two types of technique are used for drug loading.

a) Passive loading

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

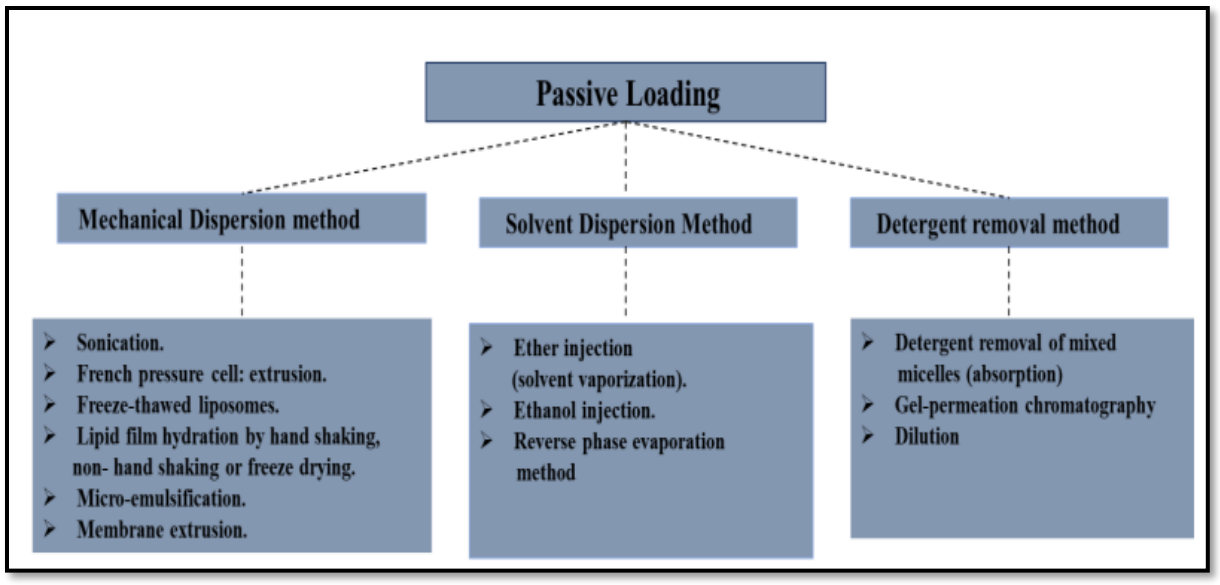


Figure 2.6: Methods use in Passive Loading

b) Active loading

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

2.4.4 Stealth Liposomes Nanoparticles

Although liposomes are similar to bio membranes, but these are still foreign antigen for the body. Hence, after interaction with plasma proteins, these are recognized by body's Reticulocyte endothelial system (RES). Consequently, these are eliminated from blood stream (Islam & Amran, n.d.). Such limitations linked with stability, can be overcome with the use of synthetic phospholipids. Furthermore, coating the liposome particle by either polyethylene glycol (PEG) or chitin derivatives can make a difference too. Freeze drying, polymerization, ganglioside micro-encapsulationare also effective. (Szoka & Papahadjopoulos, 1978a) . PEG coating reduces the percentage of liposomal phagocytosis and results in a long-term circulation and thus provides frequent time to these liposomes to leak out of circulation through endothelium. Stealth liposomes'

vesicles are sphere shaped with bilayer membrane, that consist of phospholipids with assorted lipid chains, stabilized or coated with PEG or colloidal polymers, that are used to transport drugs or genetic material to targeted cells. New drug delivery for controlled release is developed by stealthening the liposomes. This stealth concept has been used to improve the popular doxorubicin-loaded liposomes, (Romberg et al., 2008)that are currently marketed for treating solid tumors as Doxil or caelyx (H. Song et al., 2011).

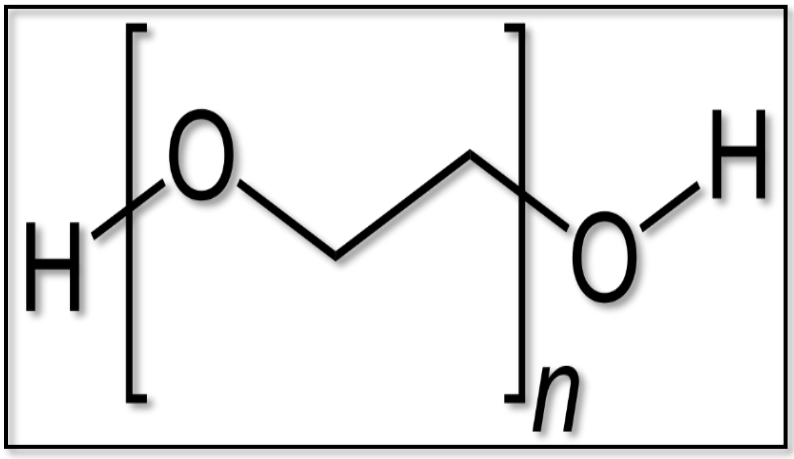


Figure 2.7: Structure of Polyethylene Glycol (PEG)

2.4.5 Relationship between depression and Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD happens to be the most frequent form of chronic liver diseases. It is believed that it affects 25% of the world's population. Additionally, it is anticipated that this will rise along with the prevalence of metabolic syndrome and obesity(Neuschwander-Tetri, 2017). However, a thorough description of how depression affects NAFLD is still requires more research. At least one in five people will experience depression at some point in their lives, according to the Global Burden of Disease research conducted between 1990 and 2017 (Cho et al., 2021). Depression frequently coexists with other chronic conditions, which progressively affects health consequences (Goldbacher et al., 2009). There is a 2-fold increased risk of developing metabolic syndrome in people with depression, according to numerous community- and population-based research.

The pathophysiology of both NAFLD and depression may include systemic inflammation, and both disorders proceed in conditions of elevated oxidative stress (Labenz et al., 2020). Increased levels of depressive symptoms may be caused by systemic inflammation brought on by

proinflammatory cytokines such interleukin-6, interleukin-1 beta, and tumour necrosis factor alpha leading towards NAFLD (Szoka & Papahadjopoulos, 1978b). Because serotonin catalysing enzymes are expressed more frequently in people with NAFLD, this is another possible explanation for the link between NAFLD and depression (Labenz et al., 2020).

2.4 Objectives of research

- The primary aim of the current research study was to explore the antidepressant potential of silymarin in mice showing depressive-like behavior as a result of Chronic Mild Stress (CMS).
- Furthermore, the study aimed at identifying its potential mechanism(s) of action as well. These include primarily neurogenesis, neuroinflammation, and/or oxidative stress. Lastly, depression's effect on liver was also an objective of this study.
- The research work in dissertation is presented in two parts. The first part of this research emphasized upon the liposomal nano formulation of silymarin drug and its characterization. The selected drug bearing versatile nature has been used against neurological disorders specifically CMS and liver inflammation induced because of depression.
- But for the first time its nanoparticles are synthesized according to 'thin film hydration method'. Different aspects of formulated nanoparticles are characterized by using different characterizing techniques and eventually enabled them for depression and liver inflammation via in vivo analysis.
- The second elaborated part emphasized upon the development of CMS model of depression and in vivo analysis for encapsulated drug's improved pharmacokinetic behavior.
- Treatment proficiency was investigated using oral route of administration in living system. In this way, the examination of neuroprotective and hepatoprotective activity of nanoparticles in well-established CMS depression model was also the prime focus of this research
- It will be considered a significant step to uplift these liposome encapsulated silymarin nanoparticles to preclinical trials level

CHAPTER 3: METHODOLOGY

3.1 Experiment Design

3.1.1 Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipid, Cholesterol, commercially available Silymarin drug, Polyethylene glycol (PEG- with Molecular weight 1000) were purchased from Sigma-Aldrich USA. Balb/c female mice were purchased from Animal House of ASAB (Atta-ur-Rahman school of Applied Biosciences), National University of science & technology (NUST), Islamabad. Deionized water was used throughout the study.

3.1.2 Synthesis of Silymarin loaded-liposomes Nanoparticles

For the synthesis, liposomes constituents i.e., DMPC and Cholesterol were used in percent molar ratio of 4:1. Firstly, lipid were measured and dissolved in ethanol to form 100 μMolar solution. 200 μMolar of silymarin drug solution was formulated in ethanol from which 500 μL of solution was measured and then added to lipid solution. For 40 minutes mixture was sonicated at 80 MHz. Then 10mL water and lipid phase were heated in water bath independently till the temperature touched 60°C. Lipid and water phase were mixed and this dispersion mixture was continuously agitated for 10 minutes at 90 RPM. Again, this new mixture was sonicated at 50 MHz for 40 mins and then allowed for rotary evaporation (greater than 50 °C which is the phase transition temperature) to get rid of the excess ethanol (Chorachoo et al., 2013). Finally, unentrapped drug was removed via dialysis tube with size 2 Inf Dia 18/32 – 14.3mm and with a pore size of 12-14000 Daltons.

3.1.3 Pegylation of silymarin loaded- LNPs

The mixture of silymarin-loaded nanoparticles (SLNPs) was diluted to a volume of 50 ml, at this point 0.25% PEG (1000) was added drop by drop, stirred continuously, and allowed for rotary evaporation until only 10 ml of solution remained. A dialysis tube was used to remove any unentrapped drug.

3.2 Physical Characterization

To assess and study the particle size, shape, surface charge, drug encapsulation, release efficiency, and dispersity Index of SLNPs, characterization was conducted through different characterization techniques were carried out.

3.2.1 U.V-Vis Absorption Spectroscopy

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

$$A=EcL$$

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

U.V-Vis spectra of SLNPs Blank were measured by using Shimatzu UV-Vis 2800 BMS Scientific Technical Corporation (PVT) spectrophotometer, from 200-450nm at a resolution of 1nm. For reference purpose, deionized water was used. The UV spectra of silymarin drug, PEG-Coated SLNPs and BNPs were recorded.

3.2.2 Fourier transform infrared spectroscopy (FTIR) analysis

It is a systematic technique used for the detection of mostly organic and a few inorganic substances. The sample material is measured by absorbing infrared radiation (IR) versus wavelength. The

absorption bands in the IR describe the molecular components and structures of the sample. When an infrared radiation irradiates a substance, the molecules go to the exciting state with greater vibration due to the absorbed IR radiation. The difference in energy between the excited vibrational state and the resting state determines the wavelength of light that a single molecule absorbs. The molecular structure of the sample is defined by the wavelengths it absorbs (Zeeshan et al., 2019) Samples were air dried before being processed for FTIR analysis using compressed KBr discs. FTIR spectra were captured using the Bruker FTIR Spectrophotometer ALPHA II between 4000 and 350 cm^{-1}. FTIR analysis of all formulating constituents was done including DMPC, Cholesterol, Silymarin, Blank liposomes (BNPs), and Pegylated- silymarin loaded liposomal nanoparticles (SLNPs).

3.2.3 Particle size and Area Distribution

Scanning electron microscopy (SEM) was utilized to examine the practical size. Using the "image j software," the nanoparticles' area distribution is determined. An analysis of the chosen field is conducted. In binary or threshold pictures, the 'Analyze Particles' command counts and measures items. It operates by scanning an area or image until the edge of an object is detected. Values for particle size are provided in the 0 to 'Infinity' range. In this region, particles with circularity values beyond the defined range will likewise be disregarded. Analyzed the 8-bit binary image of the best-fitting ellipse of the observed particle (cf. Edit. Range. Fit Ellipse; grey levels: Ellipses: 0; background: 255). (Zhou et al., n.d.). By using a micropipette to place a little portion of the sample on a cover slip, both types of nanoparticles (BNPs, and SLNPs) were photographed. Gold was then spat over the slide surface for 50 seconds at mA. The National University of Science and Technology, Islamabad's VEGA3 LMU scanning electron microscope was used to capture the images. Malvern Zeta Sizer Ver. 7.12 was used to measure the size distribution and dispersity of both types of NPs using Dynamic Light Scattering (DLS).

3.2.4 Zeta Potential

The potential difference across phase boundaries between solids and liquids is known as the zeta potential. It is a measurement of the electrical charge of a suspended particle in liquid. Zeta potential is frequently the sole value that can be used to describe the double-layer characteristics of the colloidal dispersion because it is not equivalent to the electrical surface potential of a double

layer or to the Stern potential. Zeta potential is expressed in millivolts (mV) and is also known as electro kinetic potential. Zeta potential analyzer was known to have surface charge and zeta potential. Zeta potential explains about the nanoparticles' stability, surface charge and average size. Zeta potential in colloids is the differential in the electrical potential via the ionic layer around a charged colloid ion. It is, in other words, the potential at the slipping plane of the double layer interface. The stability of the colloid will typically increase with the zeta-potential. Particle agglomeration often starts at zeta potentials that are less negative. If the colloid's zeta-potential is equal to zero, it will solidify (Z Zeta-Potential, n.d.).

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

3.2.5 Drug Encapsulation and Release Efficiency

Drug efficiency delineates the amount of drug to be entrapped with in the vesical of liposomes. A feasible linear standard curve was constructed by analyzing various drug dilutions using a UV spectrophotometer at 280 nm absorbance to determine the effectiveness of drug encapsulation. An equation Y=mx+c was established. The unentrapped drug was calculated further using this standard curve value. To find the drug fraction that was not entrapped, samples were centrifuged at 4500 rpm for 1 hour. The supernatants were then examined using UV-visible spectroscopy. (2005) Nii & Ishii Following that, the given formula was employed with the calculated data.

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

3.2.6 Drug Release

The release behavior of drug from nanoparticles vector has great importance in treating with nanomedicines. Release of drug cargo in time dependent manner at targeted site is the main concern of nano formulation that results in controlled or sustained release. Drug release LNPS and PEG-LNPs were examined up to 48 hours along with the addition of specified volume of Phosphate Buffer Saline (PBS). From both 25 ml solutions of BNPs and SLNPs, 3ml samples were placed into separate centrifuge tubes and allowed for 10 min centrifugation at 4500 rpm at room

temperature. While on other hand 3ml of PBS was poured to SNPs and SLNPs solutions. After this, supernatants were collected and allowed for UV spectrophotometer analysis. Same procedure was conducted after 1,2,4,6,12, and 15hr. At 280nm of wavelength, absorbance values were taken and used as cumulative drug release. Entire analysis was performed by using empty nanoparticle solution as control.

3.3 Development of Depression Model

3.3.1 Animals

Female BALB/c mice with the age of 7 weeks and weight 24-28g were purchased from ASAB animal house. The animals were made into groups randomly and subjected to an acclimatization period for about 1 week with free access to food and water as per the requirement. Mice were kept in Standard homcages (42 × 26 × 18 cm). Home cages were filled with fresh sawdust that was replaced after every 2 days and a consistent 9:15 h light/dark cycle was kept. Temperature was maintained at 27°C ± 2°C with humidity 600% ± 5%. Mice were divided into five groups for each type of depression model with five mice per group. Weights of mice were recorded twice a week throughout the depression and treatment timeline. The average weight, per week, is presented in results. Mice handling and care were governed by US FDA guidelines for proper laboratory practise. (Food and Drug Administration) in 1978.

3.3.2 Depression induction through Chronic Mild Stress Model (CMS) of depression

Initially, Chronic Mild Stress (CMS) model followed the strategy of decreasing stressors and inducing anhedonia. Typically, various stressors such as water deprivation, food deprivation, the cages tilting, and related stressors were used in different days for four weeks (Son et al., 2019). The details of each day of week are provided in table 1.

Days	Mild Stressors	Condition
1	Wet bedding stressor	• 04 hours • Food and water deprivation
2	Cage tilting stressor	• 04 hours • Food and water deprivation • Tilting at 45º angle
3	Without bedding stressor	• 4 hours • Empty cage
4	Heat stressor (48ºC)	• 4 hours • Temperature maintained using electric heaters
5	Water containing cage stressor	• 4 hours • 1/4th of cage filled with water
6	Restraint stress	• 4 hours • Each individual mouse was placed in a falcon tube with a small front opening for proper breathing

Table 3.1 Induction of depression in mice using CMS depression model

Figure 3.1: Induction of CMS in mice

3.4 Behavioral tests

For analysis of depression, standard behavioral tests were performed twice throughout the experimentation. First round of tests was conducted before the treatment to assess the stress level of mice confirming the successful induction of depression like symptoms in mice. The second round of tests was performed right after the treatment to investigate and analyze the effectiveness of the administered therapeutic agents i.e., Silymarin and Silymarin Loaded Nanoparticles (SLM). After the development of depression mice started showing depressive like phenotype as, tail biting, jumping out of cages, huddling in corner, hiding under bedding, aggressive behavior, excessive grooming, and anhedonia.

3.4.1 Forced Swim Test (FST)

All mice were subjected to forced swim test using the standard protocol that has been described previously (Can, Dao, Arad, et al., 2011). For this purpose, a large glass tank was filled with clean water at room temperature. All the mice were placed in water tank one by one and video recording

was conducted simultaneously. A recording of 6 minutes was saved for the purpose of analysis. Later on, their behavior was analyzed, and results were generated.

3.4.2 Tail Suspension Test (TST)

All the mice were subjected to the tail suspension test using standard protocol (Can, Dao, Terrillion, et al., 2011). For this purpose, tail of each mouse was wrapped in an adhesive tape and attached to table surface in a manner that their bodies were hung towards the ground. A video recording of 6 minutes was saved for the purpose of analysis. Later on, their behavior was analyzed, and results were generated.

3.4.3 Elevated Plus Maze Test

The Elevated Plus Maze (EPM) test is used to gauge anxiety-related behaviour in animal models of CNS disorders (Rodgers et al., n.d.). A central area, two oppositely positioned open arms, two oppositely positioned closed arms, and an elevated "+"-shaped maze makes up the EPM device. Each mouse was put into the maze and given free reign to explore it. Later, using a video tracking device and a video camera positioned above the maze, the activity of the mice was observed and recorded.

3.4.4 Drug dosage and routes of administration

Both BNPs, SLNPs and simple silymarin solution followed oral route of administration and the dosage was 10µl.

3.4.5 Serological indices

After induction and treatment, mice were euthanized using standard surgical procedure and blood was directly collected from heart via cardiac puncture.

3.4.6 Adreno Cortico Trophic Hormone (ACTH) testing

Mice from all groups were sacrificed early in the morning using 5ml of chloroform and blood sample was taken via cardiac puncture and stored in purple top tubes (containing anticoagulant EDTA agent). The blood samples were then centrifuged at 3000rpm for 6 minutes and temperature

was maintained at 4° C. The plasma was then stored at -20° C until delivered to lab for ACTH testing.

3.4.7 Histological Examination

Mice from all groups were dissected and the size, appearance and color of each diseased liver tissue were noted. Organs (liver and brain) were obtained and placed immediately in 10% neutral-balanced formalin solution to avoid postmortem autolysis and decomposition. 5 µm serial sections of organs were taken, followed by the paraffin imbedding. Organs were stained with Hematoxylin and Eosin (HE) to observe the structural changes as mentioned in histological slides. These structural changes were analyzed under LB-200 Biological Microscope. Images were captured with magnification 100x using Pixel Pro software for a Labomed biological microscope. The pathological grading and scoring were done, centered upon the criteria of Histological grading. Moreover, half of the brain were placed immediately on dry ice and stored at -80 for neurotransmitter analysis. 3.4.7 Detection and quantification of Neurotransmitter in Brain, prefrontal cortex and hippocampus by RP-HPLC analysis

3.5 Quantification of Monoamine Neurotransmitters (Dopamine and Serotonin) via RP-HPLC

3.5.1 Sample preparation

The brain parts hippocampus and prefrontal cortex were separated from cranium by dissecting the mice brain on an ice-cold petri plate. The dissected areas (hippocampus, prefrontal cortex and remaining portion) were separated and placed in PBS (pH 7.4). The volume of PBS used for homogenization was 0.2 ml for hippocampus and prefrontal cortex while 0.5 ml was used for other brain parts. Samples were homogenized and centrifuged at 15,000 rpm and 4° C for 17 min. Supernatants collected were stored at −80°C till analysis.

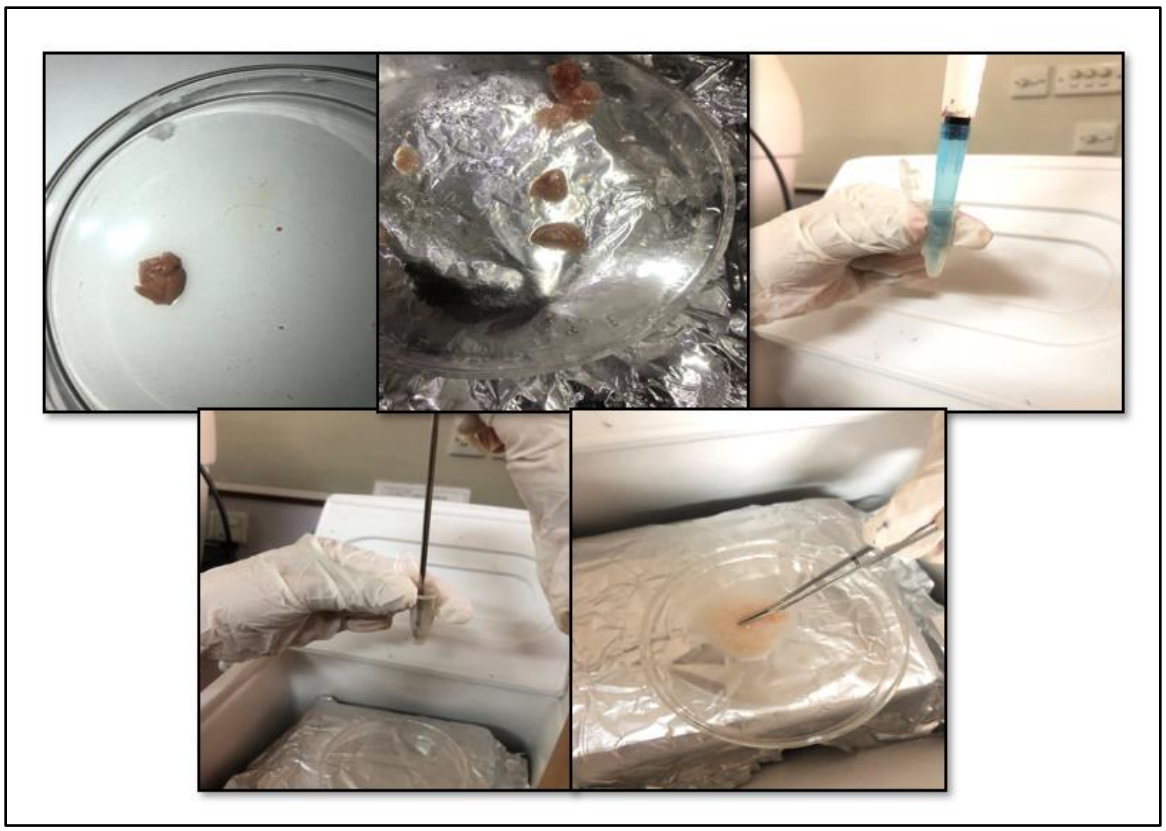


Figure 3.2: Sample preparation for HPLC analysis

3.5.2 HPLC

The RP-HPLC based detection and quantification of monoamines (dopamine and serotonin) was conducted via Shimadzu HPLC structure encompassed on SCL-10A VP controller, DGU-14A degasser, FCV-10AL VP low pressure mixer, LC-10AT VP pump coupled with 3D-PDA detector (SPD- M10A VP) and LC solution software. The column used was Beckman Ultrasphere C18 (5µm; 4.6 × 250 mm) at Department of Pharmacy, Quaid e Azam University, Islamabad. Using a mobile phase made of phosphate buffer (6.8 g of potassium dihydrogen phosphate per 1000 ml of distilled water), the elution was carried out isocratically at a pH of 3.5 using orthophosphoric acid. The injection volume was 50 µl, and the flow rate was 1.5 ml/min.

3.6 Treatment Design

To evaluate the neuroprotective and hepatoprotective effects of SLNPs and Peg-SLN, diseased mice were taken within experiment. Mice were categorized into different groups.

3.6.1 Negative Control Group

A set of five diseased mice were isolated and assigned the tag as negative control. This group of mice were left untreated throughout the experiment, and they were sacrificed at the end of experiment for histopathological and serological analysis. Body and organ weights were noted.

3.6.2 Silymarin treated Oral Group

Five mice were placed in this group. Silymarin drug with the dose of 10mg/kg were given orally for the duration of 02 weeks. Body and organ weight were noted at the end of experiment prior to dissection for histopathological and serological analysis.

3.6.3 Blank pegylated Liposomal Nanoparticles treated Oral Group

Five mice were placed in this group. Liposomes Nanoparticles dose of 500µg/kg were given orally for the duration of 02 weeks. Body and organ weight were noted at the end of experiment before dissecting them for histopathological and serological assessment.

3.6.4 Silymarin loaded Pegylated Liposomal Nanoparticles treated Oral Group

Five mice were placed in this group. Drug loaded and pegylated Liposomal Nanoparticles with the dosage of 500µg/kg were given orally for the duration of 02 weeks. Body and organ weight were noted before conducting dissections for serological and histopathological examination.

3.6.5 Positive Control Group

Five mice were placed in this group. No disease induction and treatment were conducted in this group of mice. They were sacrificed at the end of experiment along with other mice and their body and organ weight were noted accordingly

CHAPTER 4: RESULTS

4.1 Physical characterization of Silymarin Loaded SPLs and PEGylated SLPs

SLPs were formed by the thin-film hydration method. A phospholipid bilayer vesicle was formed using 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and cholesterol. Dimyristoyl Lecithin is a myristoylated phosphatidylcholine, and a synthetic phospholipid used in the formation of liposomes (*1,2-Dimyristoyl-Sn-Glycero-3-Phosphocholine | C36H72NO8P - PubChem*, n.d.) and lipid bilayers specifically with the application of drug delivery.

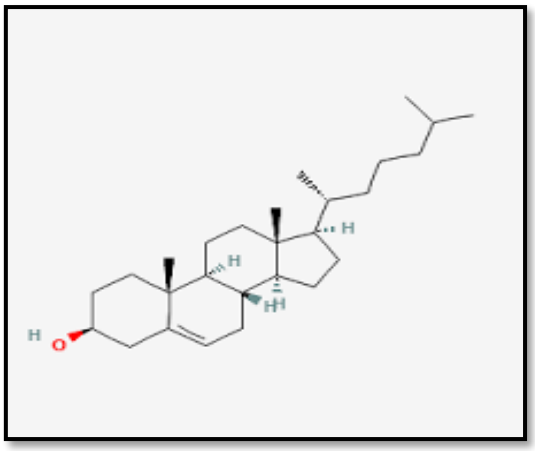
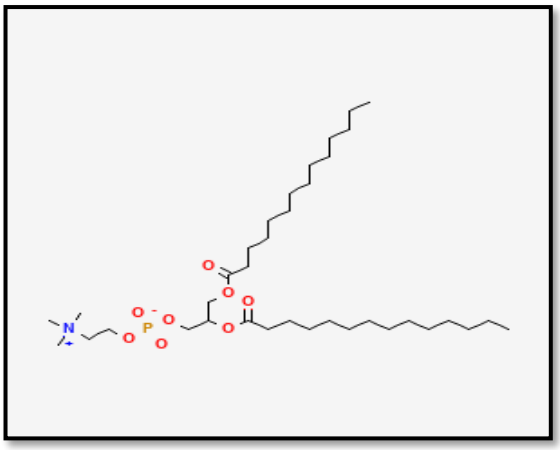
a)  b) 

Figure 4.1: structure of (a) Cholesterol and (b)DMPC

4.1.1 UV-VIS Absorption Spectroscopy

Absorption spectroscopy of silymarin drug showed the surface plasmon resonance (SPR) peak mainly at 330nm, blank liposomes at 220nm & 230nm, Silymarin loaded SNPs at 230nm & 290nm. PEG 1000 showed a peak at 225nm and 250 nm. The Lipid DMPC showed a peak at 210 and 250 nm. Cholesterol exhibited the peak at 220 and 230nm whereas silymarin showed a peak at 230nm and 270nm respectively. The shift in the peaks delineate the successful conjugation of Silymarin, SNPs and PEG with each other.

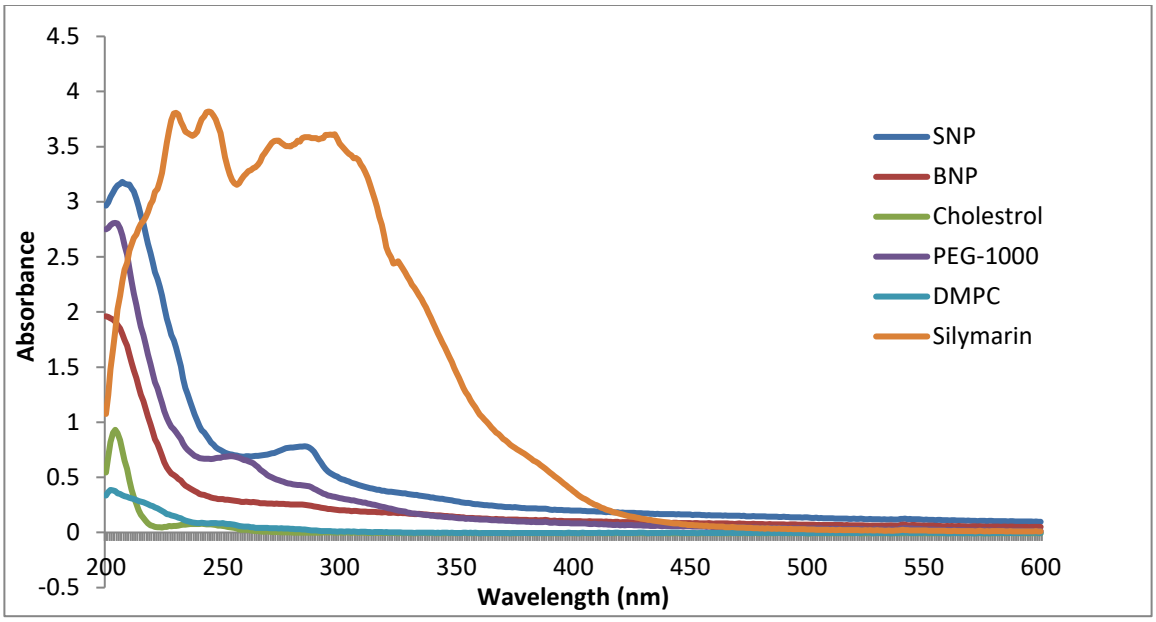


Figure 4.2: Comparative UV-Vis spectra of Silymarin, SNPs, BNPs, Cholesterol, Peg 1000 and DMPC

4.1.2 Fourier Transform Infrared Spectroscopy (FTIR) analysis

The FTIR spectrum of PEG 1000 indicated a peak at around 1200/cm (C-O stretch, exhibiting ether group) and 2850/cm (showing alkanes). DMPC spectra indicated peaks at 1500/cm (O-H stretch, hydroxyl group) and 2830/cm (C-H stretch, indicating alkane). The cholesterol gave peaks at 1300/cm (showing a C-H bending with alkane group) and 3000/cm (C-H stretching with alkene group). The spectra of Silymarin drug showed peaks at 980/cm (C=C bending with alkene group) 1750/cm (presenting a ketone group with C=O stretch) and 3300/cm (alkyne group with C-H stretching). The spectra peaks of both Silymarin loaded liposomal nanoparticles (SLNPs) and Blank liposomal nanoparticles are coated with Peg 1000. Hence, the polymer coating has masked their individual peaks. By integrating silymarin drug and PEG 1000, the structural changes in lipid biomolecules were demonstrated by changes in infrared bands.

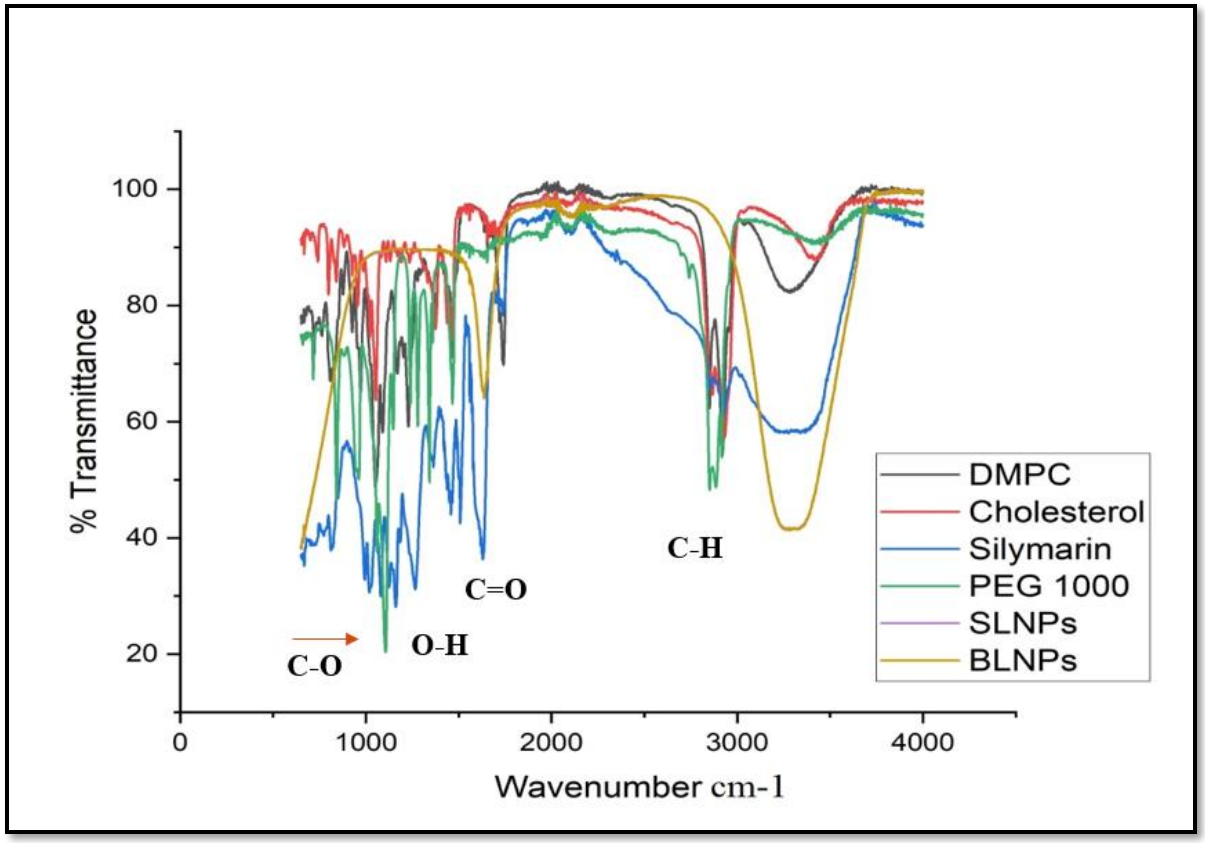


Figure 4.3: FTIR spectra of DMPC, Cholesterol, Silymarin, PEG 1000, SLNPs and BNPs

4.1.3 Particle Size and Area Distribution

The size of Silymarin loaded nanoparticles SNPs is determined by the Scanning Electron Microscopy (SEM) and the area distribution of the nanoparticles is measured using image j software. Scanning image illustrated the sphere shaped nanoparticles with the standard size of 116nm.

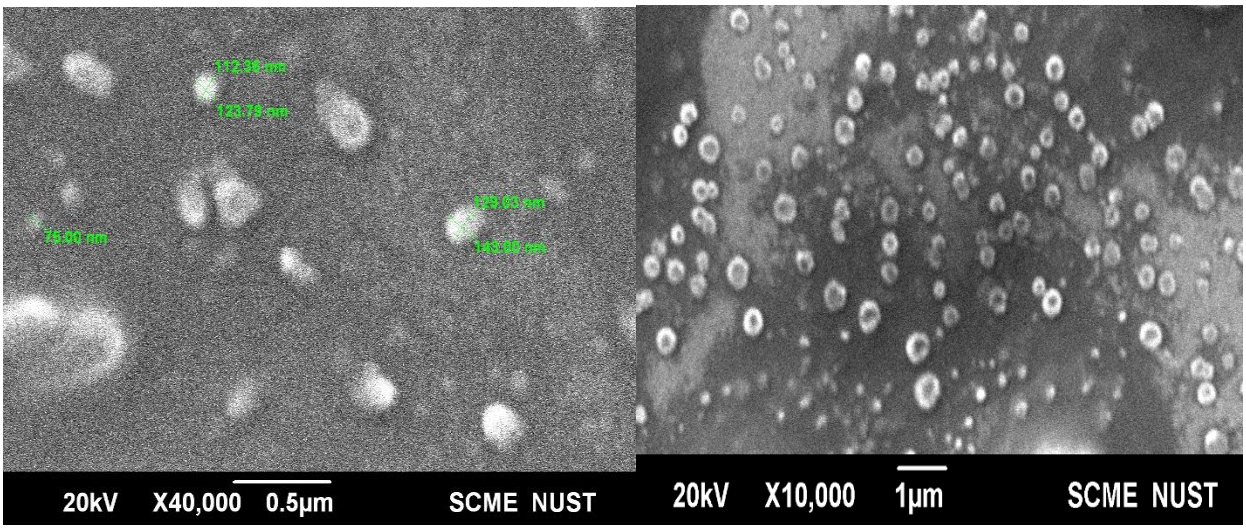


Figure 4.4: SEM image of Silymarin Loaded Liposomal Nanoparticles (SNPs)

The average size of BNPs, and SNPs were 11.06 and 118.3nm respectively.

4.1.4 Zeta Potential and Poly Dispersity Index

The average Zeta potential of Blank Liposomes, and SNPs were -10.6 mV and -11.8mV respectively. with Polydispersity index (PDI) of 0.635 and 0.643 respectively. By using PEG-1000, there was a significant surge in zeta potential of SNPs, implying the increased stability than conventional SNPs. Both type of particles when observed under the Scanning Electron Microscope, seemed mainly spherical in shape with too little difference in size and shape between them.

4.1.5 Drug Encapsulation Efficiency and Drug Loading Capacity

By using the formula (in material Section), the Encapsulation Efficiency found to be 75.15%, that delineates almost 75% entrapment of drug within nanoparticles. Moreover, drug loading capacity of liposomal nanoparticles calculated is 48.2%.

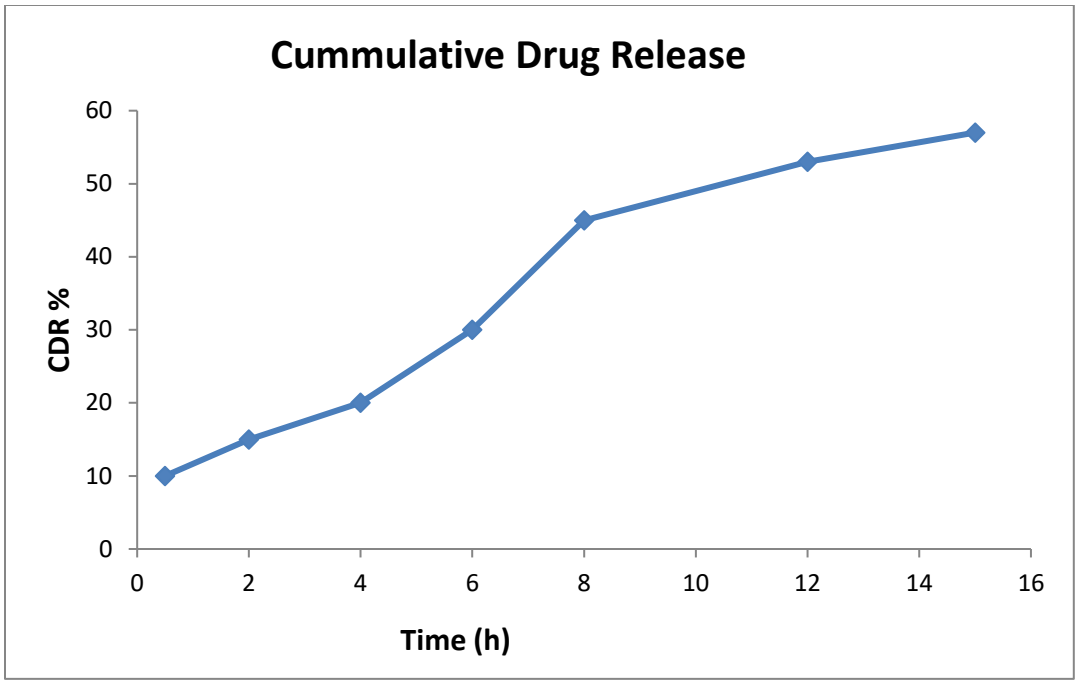


Figure 4.5: Cumulative drug release of Peg 1000 coated SNPs

4.1.6 Drug Release Kinetics

The release of drug from SNPs were noted up to 15 hours suggesting the sustain released of drug with time. This long stay of drug results in attaining the increased bioavailability and ultimately leads to high efficacy in treating the diseases.

4.2 Induction of Depression in Balb/c Mice

The standard CMS model as mentioned above was followed to induce depression in female balb/s mice. The depression induction was confirmed via general observation, and behavioral testing. The general observations include rearing, tail biting, hiding beneath the bedding, decreased food and water consumption and increased aggression.

4.3 Behavioral Test Assessment

Behavioral tests were conducted twice throughout the experimentation. Firstly, after the induction of depression and secondly after the treatment.

4.3.1 Forced Swim Test (FST)

The FST is used to examine depressive-like behavior in rodents and is based on the supposition that immobility exhibits a measure of behavioral despair. In current study FST was carried out initially after two weeks of depression induction. When mice were subjected to the forced swim test, a recording of 6 minutes is made from which the last four minutes are analyzed for results as in the initial two minutes mice are very active and behave aggressively. Therefore, the depression induction can be better observed and investigated during the last four minutes. For the purpose of analysis, the immobility time of each mouse is noted. Greater the immobility time, more depressed the mice was and vice versa(Cryan & Holmes, 2005). From the results it can be observed that depressed mice exhibited the greatest immobility time. Moreover, after completing the treatment successfully FST was conducted again to evaluate the efficiency of treatment.

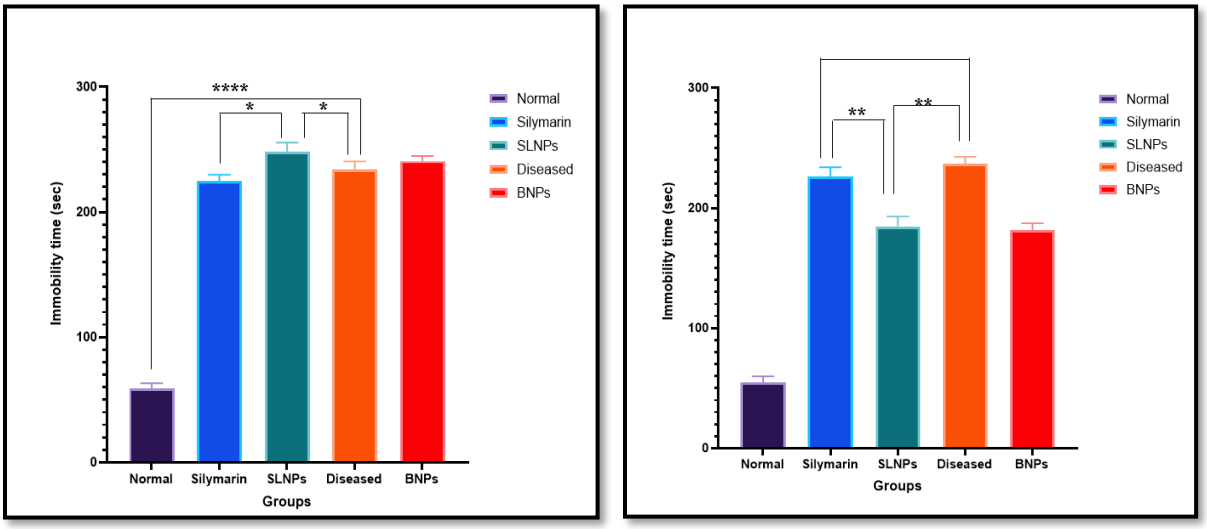


Figure 4.6: (a) Forced Swim Test after depression induction. (b) Forced Swim Test after treatment

4.3.2 Tail Suspension Test (TST)

The TST is a mouse standard behavioral test specifically measuring “depression-like” behavior and learned helplessness. In this test, usually 6 minutes in duration, the consequential escape leaning behaviors of rodents (mice) are quantified(Mayorga & Lucki, 2001). Tail climbing behavior and the overall activity of mice is noticed which depicts the stress and depression state of the mice. Again, the test was performed after treatment with Silymarin and SLMs. The results

are recorded in terms of the mean immobility time of each mouse. From the results of the tail suspension test it can be observed that the depressed mice exhibited the highest immobility time and treated mice showed less immobility time.

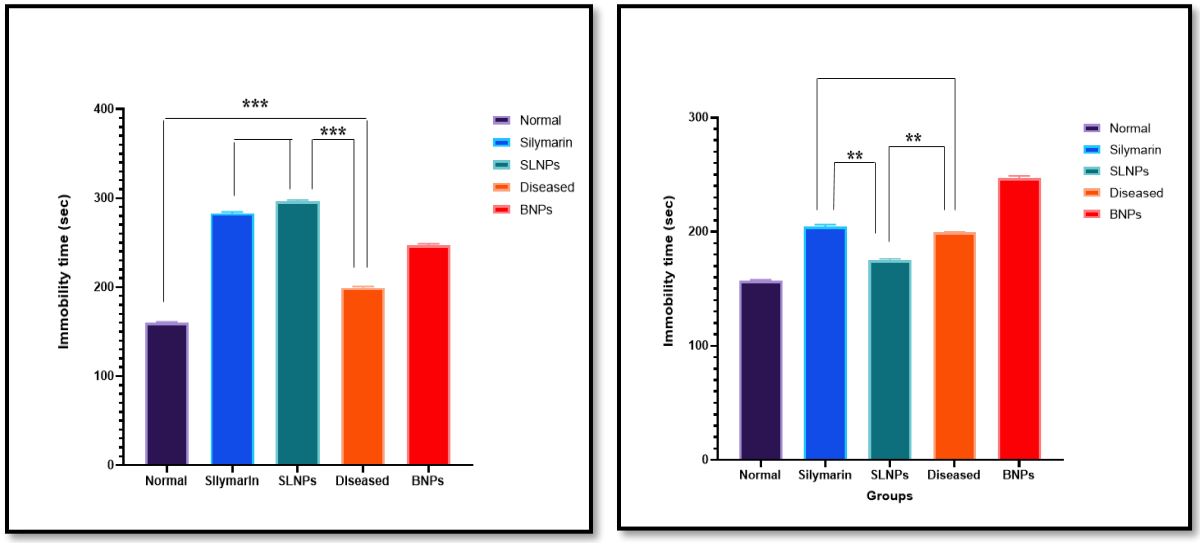


Figure 4.7: (a) Tail Suspension Test after depression induction. b) Tail Suspension Test after treatment

4.3.3 Elevated Plus Maze Test (EPMT)

One of the most recognized tests for determining anxiety-like behavior is the EPMT. Mice are given access to every arm and let to roam freely throughout it for five minutes. The ratio of time spent in the open arms to time spent in the closed arms is used to measure the anxiety behavior of mice, and an increase in open arm activity (duration and/or entries) shows anti-anxiety behaviour (Walf & Frye, 2007). The findings suggest that treated mice spent more time in the open arm while depressed mice spent more time in the closed arm.

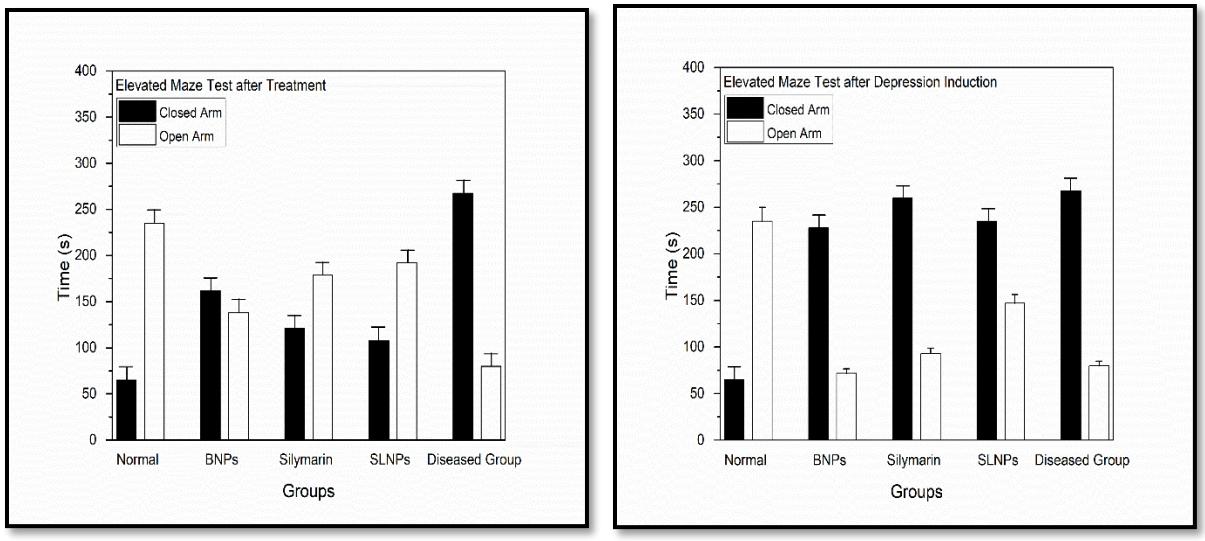


Figure 4.8: (a) Elevated Plus Maze Test after depression induction. (b) Elevated Plus Maze test after treatment

4.4 Serological Indices

To assess the depression induction in depressed mice group and effectiveness of treatment in treated groups, blood samples were drawn via cardiac puncture and mice were then sacrificed.

4.4.1 ACTH Testing

An increased secretion of ACTH and cortisol is referred as the neuroendocrine response (Douglas et al., 2003) to stressor exposure. Hence ACTH is considered as the blood biomarker of depression. A significant higher ACTH levels from normal were observed in depressed mice group and in the group which received no treatment. A lower level of ACTH was reflected in SLNPs treated group in comparison of simple silymarin treated group.

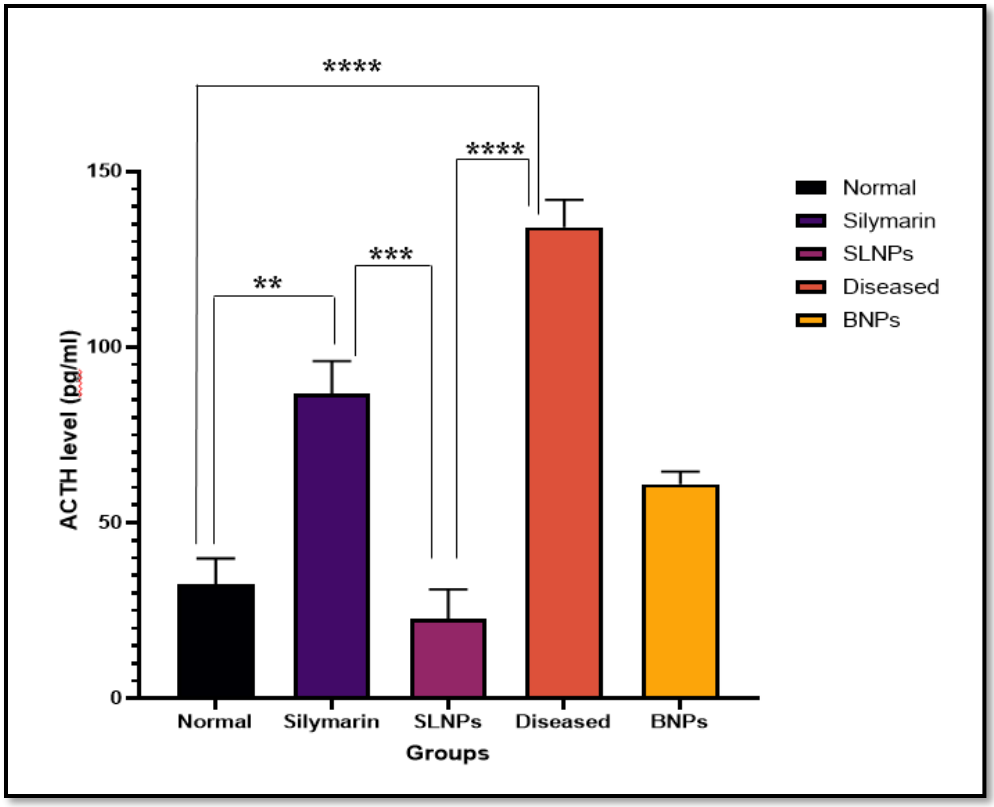


Figure 4.9: ACTH levels

4.4.2 Body Weights

During the acclimatization period of 01 week almost all mice groups have weights within the same range. During the induction phase of chronic depression which lasted for 04 weeks a significant drop in body weights were observed. The diseased mice groups which afterwards were treated with SNPs showed a substantial increase in body weights as it could be represented by graph.

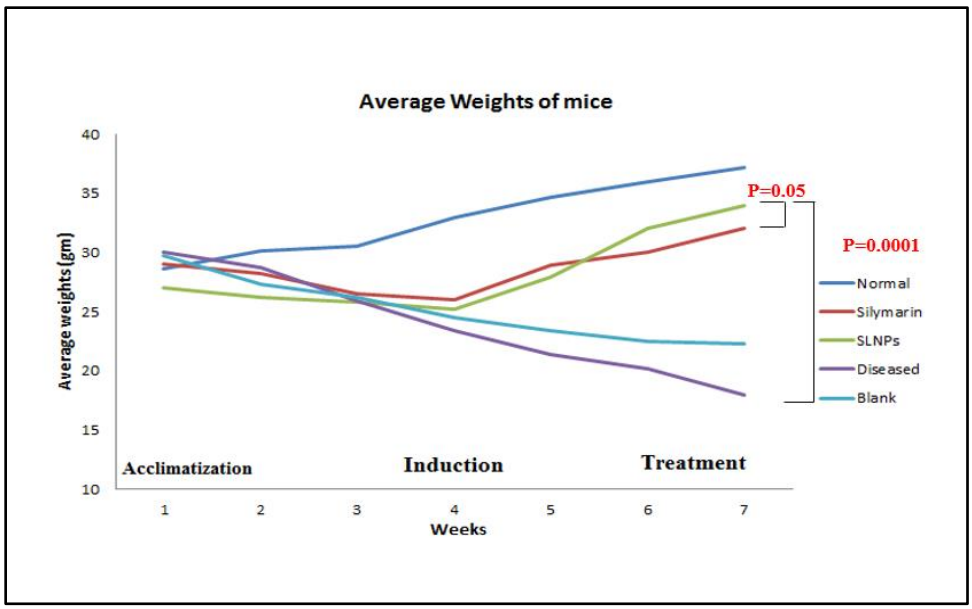
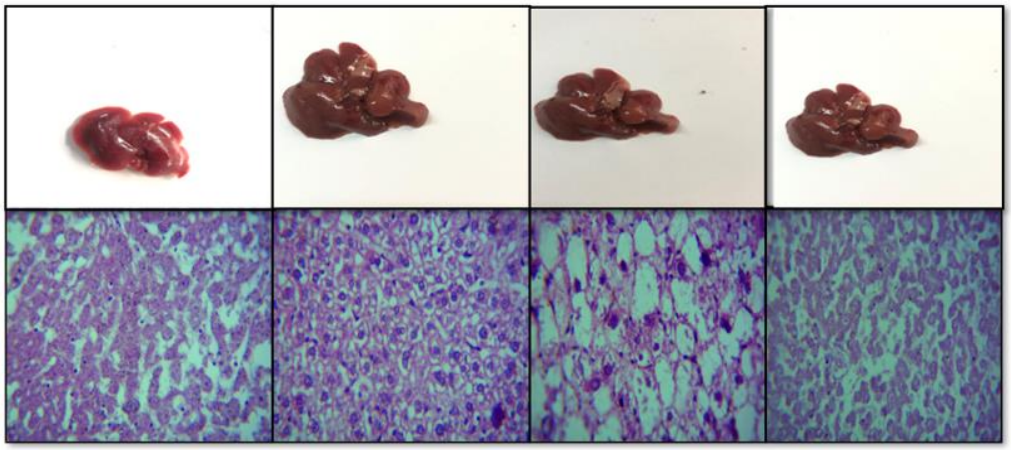


Figure 4.10: Body weights of mice

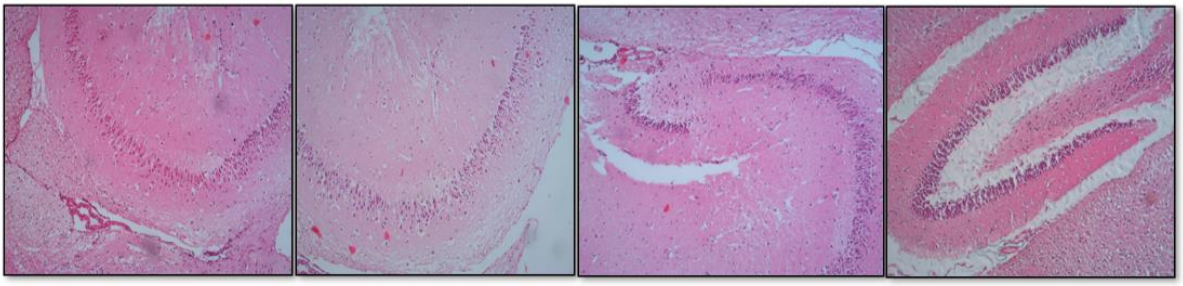
4.5 Histopathology of Liver



a) Normal hepatic architecture with central vein and surrounding hepatocytes, sinusoids and nucleus **b)** Normal histological appearance, reduced cytoplasmic vacuolation, and Hepatocytes with abundant granular, eosinophilic cytoplasm and centrally placed, round nuclei with prominent nucleoli. **c)** Hepatic centrilobular mononuclear cell infiltration, congestion of central vein and blood sinusoids, fatty changes, and foci of hepatic cell necrosis **d)** Binucleated hepatocytes, showing increased infiltration of inflammatory cells in pericentral areas, cytoplasmic vacuolation, fatty changes of hepatocytes, necrotic cells, and congested blood sinusoids.

Figure 4.11: Histopathology of Liver

4.6 Histopathology of Brain



a) Regular size, shape, and arrangement of brain tissue **b)** Cellular nuclei morphology was clear and reduction in cellular atrophy and neuronal swelling observed **c)** Large cells which are mostly multipolar, neuronal swelling, chromatolysis and nuclear margination, vacuolated cells and vascular congestion were observed. **d)** focal inflammatory cellular infiltration, hyperchromatic cells, vascular congestion cellular atrophy, shrinkage and vacuolated cells in the cerebral cortex

Figure 4.12: Histopathology of Brain

4.7 HPLC Analysis:

The chromatogram was obtained at 211 nm. The two-fold serial dilutions of dopamine and serotonin (100, 50, 25, 12.5, 6.25, 3.125 and µg/ml) were used to quantify neurotransmitter level in brain, hippocampus and prefrontal cortex of mice using the calibration curve equations. The R^2 value of dopamine and serotonin were 0.9939 and 0.9957 respectively. Utilizing UV-spectrum, 3D-graph, and retention time, the standard and sample were contrasted. The retention time of dopamine and serotonin was 7.084 and 25.221 respectively.

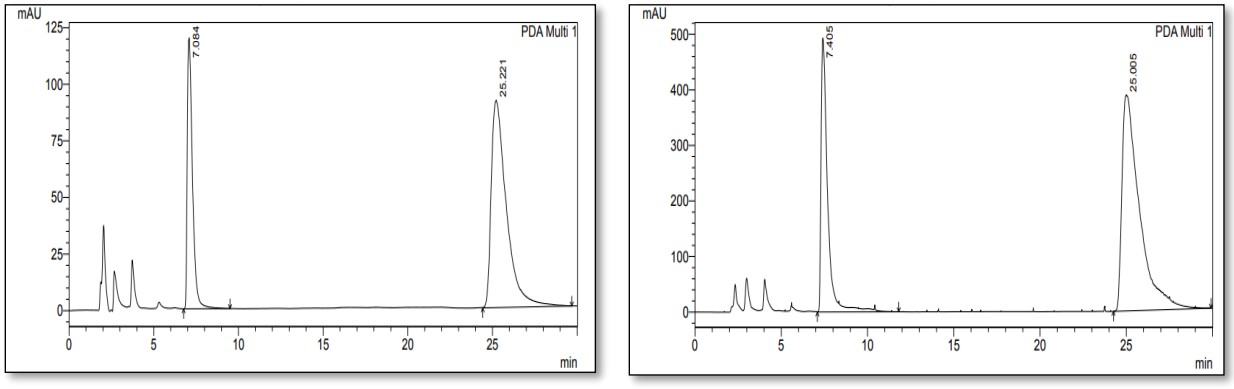


Figure 4.13: Retention time curves of Dopamine and Serotonin standards

4.7.1 Dopamine level in Hippocampus, Prefrontal Cortex, and Whole brain

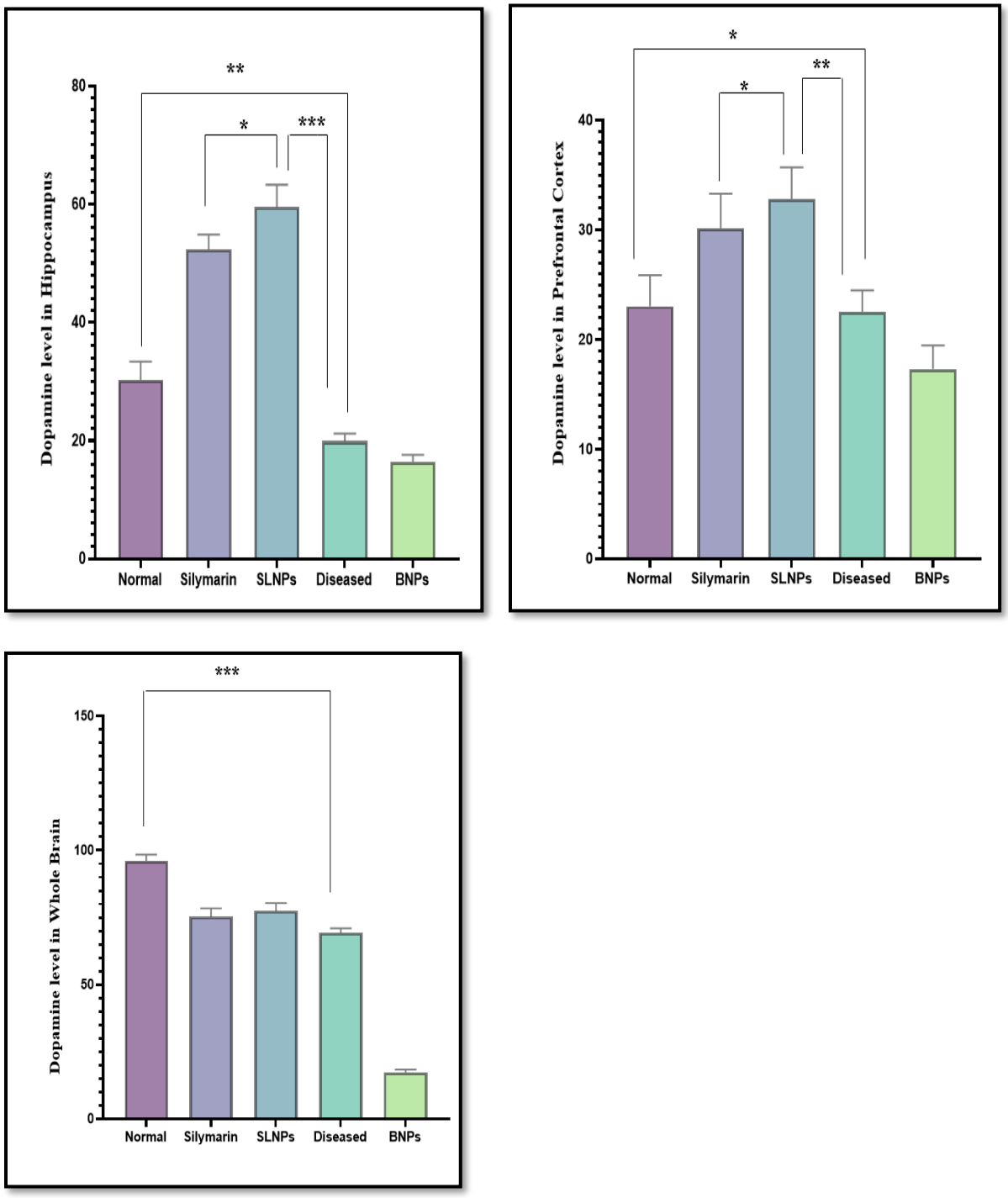
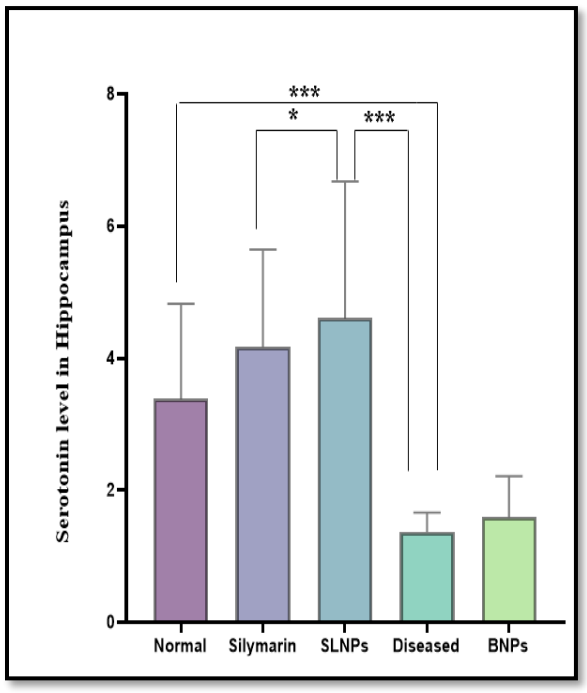
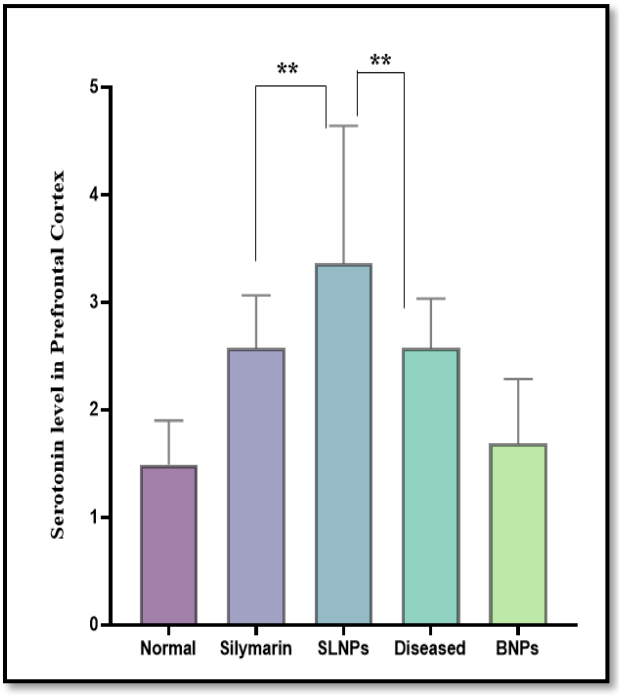


Figure 4.14: Dopamine level in Hippocampus (HC), Prefrontal cortex (PFC), and Whole brain

The aforementioned figure suggests that comparatively high level of dopamine is present in hippocampus and then prefrontal cortex as compared to whole brain. Compared to the diseased group a significant increase in dopamine level in HC and PFC is observed in SLNPs as compared to the group treated with Silymarin only. Compared to normal group and treated group (Silymarin and SLNPs) the dopamine level showed a slight increase. Whereas no significant increase in dopamine level was observed in whole brain when diseased and treated (Silymarin and SLNPs) groups were compared. Anyhow, a significant correlation was observed between diseased and normal group.

4.7.2 Serotonin level in Hippocampus, Prefrontal Cortex, and Whole brain





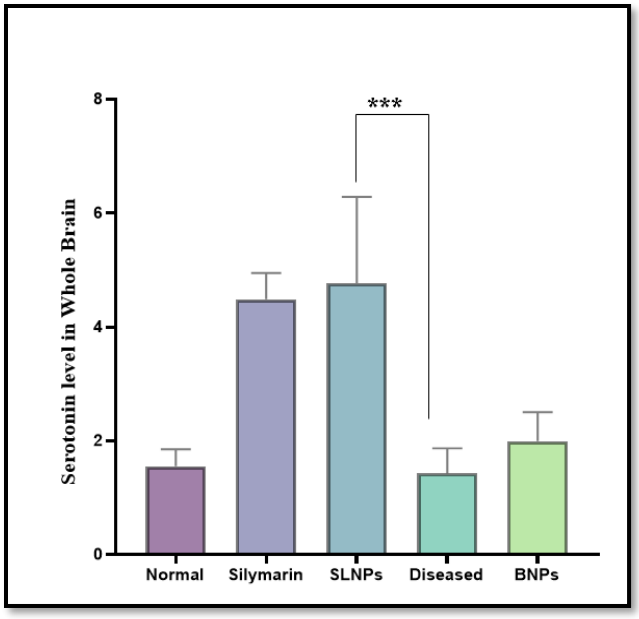


Figure 4.15: Serotonin level in Hippocampus (HC), Prefrontal Cortex (PFC), and Whole brain

The figure exhibits comparatively high level of serotonin is present in hippocampus and then prefrontal cortex as compared to whole brain. Compared to the diseased group a substantial rise in dopamine level in HC and PFC is observed in SLNPs. Compared to normal group and treated group (Silymarin and SLNPs) the serotonin level showed a slight increase. A higher significance of two hysterics is observed between groups treated with Silymarin and SLNPs which proves the efficiency of the system as SLNPs treated group shows high level of serotonin as compared to silymarin treated group. Moreover, much significant increase in serotonin level is observed in whole brain when diseased and treated (SLNPs) groups are compared. Anyhow, no considerable correlation was observed between diseased and normal group and Silymarin and SLNPs group.

CHAPTER 5: DISCUSSION

Neuropsychiatric disorders are complex set of symptoms with major public health importance, in terms of its prevalence. It has been reported that there exists a significant commonality between anxiety, depression, and cognitive impairment, and about 90% of patients with depression experience comorbid anxiety. A contemporary study has revealed that mild cognitive impairment also increases the risk for anxiety and depression. A depressed individual can also have somatic disorders, along with other psychiatric disorders. The mice models and the parameter for the measurement of neuropsychiatric behavior have acquired importance in terms of studying the molecular basis of depression as well as to look for acceptable and practicable treatment option. In this regard, it has been demonstrated that CMS was a suitable model for the pre-clinical assessment of antidepressants. This method's theoretical justification is that depression ultimately results from an incapacity to handle numerous unpleasant stimuli presented by the environment. Stressors are employed to replicate this effect in animals by inducing behavioral impairments that can be corrected with antidepressant medications. In order to find out whether prolonged administration of silymarin or silymarin nanoparticles might lessen or reverse the stress-induced depressive-like behavior in mice, a CMS model was adopted in the current work. In behavioural despair tests, particularly the TST and FST mice exposed to CMS displayed depressive-like behavior, which is demonstrated by decreased weight gain and increased immobility periods. Additionally, this model reduces self-care and motivated behavior, which is thought to mirror anhedonia in animals, one of the two main diagnostic criteria for a major depressive episode in humans. For validity of depression symptoms aggressive behavior, tail biting, and rearing as a marker for depression were also observed. These findings represented that the protocols used have face construct and validity. Treatment efficiency was determined by conducting FST, TST, and OFT after two weeks of treatment. An increase in wight gain along with regular food intake was observed. The results of former two test were quite similar where depressed mice showed highest immobility rate followed by simple silymarin treated and the ones treated by SLNPs as compared to the control.

In the present research, the Silymarin loaded liposomes nanoparticles (with PEG 1000) were successfully formulated and used as remedy against depression and its associated symptoms and

side effects on liver. Silymarin was loaded within liposomes by liquifying it into appropriate solvent and nano formulated by sonication. Different parameters, including the DMPC and Cholesterol ratio, the drug to lipids ratio, the sonication time and amplitude, and the temperature of the water bath during rotary evaporation, were monitored in order to achieve optimized silymarin loaded liposomes nanoparticles with the desired features, such as size, PDI, zeta potential, and encapsulation efficiency. When DMPC and cholesterol were mixed in a 4:1 ratio, the smaller size nanoparticles were created upon 80MHz amplitude of probe sonicator. Average size of blank liposomes and silymarin loaded liposomes nanoparticles observed was 90nm and 155nm While Zeta potential observed were -10.6mV and -11.8mV and PDI were 0.635 and 0.643 respectively. Drug release from liposomes nanoparticles was noted for 24 hours examination. To enhance the stability, and to avoid the chance of agglomeration, Polyethylene glycol (PEG) was used which enhanced the stability by inducing the stealth effect. Pegylation enhances the steric repulsion and hence known as better stabilizer for different types of nanoparticles (D'Souza A & Shegokar, 2016). But one of the main disadvantages of using PEG is the increase in size of nanoparticles. PEG follows the erosion -controlled release mechanism of drug that results in sustained release (Hu, Zhang, You, Yuan, & Du, 2012). Drug loading capacity and encapsulation efficiency of liposomal nanoparticles is **48.2%** and **75.15%** and the graph of cumulative drug release kinetics showed zero order kinetics. Hence it implies the sustained drug release. In the present study, comparative FTIR of Silymarin, silymarin loaded liposome nanoparticles and blank liposomes showed the involvement of different functional groups which were deliberated by the decrease of peaks intensities.

Pro-inflammatory cytokines and cytokine inducers like lipopolysaccharide and CMS have been attributed to depressive-like behavior in experimental animals. The current investigation revealed elevated levels of TNF- and IL-1 in the PFC and hippocampus of stressed mice, which is consistent with earlier studies. This may be due to decrease in the production of pro-inflammatory cytokines after treatment with SLNPs. This conclusion is backed by earlier research that shown silymarin inhibited neuroinflammation in many experimental paradigms. Pro-inflammatory cytokines can harm the PFC and hippocampus's neuronal plasticity, which is linked to decreased neurogenesis and changed neurotrophic factor signaling.

CMS model of depression successfully established by inducing various stressors. The effect of depression related symptoms on liver were also analyzed via histopathology of liver tissues as it has been reported depression and liver diseases are interrelated. Moreover, the levels of monoamine neurotransmitters (Dopamine, and Serotonin) in each mice group were also quantified via RP-HPLC. The serotonin and dopamine levels that were lowered by CMS exposure were returned to control values after administration of SLNPs; this effect may be achieved via inhibiting neuroinflammation. However, a few variations in the level of dopamine and serotonin in whole brain were observed as the study is based on animal model and variations are quite common in living system. Other than that Adreno Cortico Trophic Hormone (ACTH) which is a stress hormone, its level was also checked. The level of ACTH was significantly reduced after being treated with SLNPs. Moreover, histopathological findings also supported the narrative that treating depressed mice with silymarin and SLNPs decrease the symptoms of inflammatory liver diseases. Liver histopathological, results exhibited the substantial reversing of collagenous scabs to normal hepatic tissue pattern by using the encapsulated silymarin drug in liposomal nanoparticles along with its pegylation via PEG 1000. Hence, both inflammatory liver diseases and neurodegenerative diseases could be managed via silymarin.

CHAPTER 6: FUTURE PROSPECTS

Despite decades of neurobiological research and major improvements in our understanding of its pathogenesis, depression is still a common and relatively challenging condition. Our understanding of the biochemical causes of depression is expected to grow as a result of ongoing and future research, which is also expected to lead to the development of several new antidepressant medications. These potential treatments include a number of cutting-edge medications that target neuromodulatory systems other than monoamines as well as approaches for localized brain stimulation that specifically target the neural networks implicated in depression. Therapy that lasts a long time obviously has side effects. When compared to pure antidepressants, studies of various combination therapies for the treatment of neuropsychiatric illnesses have revealed less side effects. So combinational therapy for treating depression can also be checked. Novel therapeutic agents, such as peptides, nucleic acids (DNA and RNA), and genes, in addition to medications and chemicals, have demonstrated potential for usage as nanomedicines for the treatment of a number of chronic illnesses. Research should be conducted for their utilization in treatment of depression. To ensure the short- and long-term effects of nanomedicines on people, numerous, substantial clinical trials are still required.

There is still room for more investigation on silymarin's biodistribution and absorption in the treatment of neuropsychiatric diseases. This research focuses on oral drug delivery of Silymarin and SLNPs. Hence other routes of administration including Intravenous (I.V), and trans epidermal for the treatment of neurological diseases should also be evaluated. Moreover, efforts should be put up in the synthesis of multifunctional liposomes with the ability to tackle multiple pathologies. Different methods of nanoparticle formation can also be looked upon along with uniformity and variance in liposomal composition.

CHAPTER 7: CONCLUSION

The current experimental results show that silymarin has antidepressant-like activity in Balb/c mice and is associated with numerous changes in neurotransmitter, immune, and inflammatory systems, including 5-HT, DA, IL-6, and TNF- in the hippocampus and cerebral cortex, as well as serum corticosterone. Despite the therapeutic benefits listed above, silymarin has a low bioavailability due to its poor solubility, which prevents it from demonstrating the desired therapeutic result. Referring to this, nanoparticles have demonstrated their viability as a platform for improving drug bioavailability via a variety of administration routes, resulting in optimal therapeutic effects. Because of this, the current study sought to broaden the scope of pertinent research by examining silymarin's possible protective effects, particularly in the form of nanoparticles, against the behavioral and biochemical changes brought on by chronic mild stress (CMS). Hence liposomal nanoparticles were synthesized using ‘thin film hydration technique’. The successful synthesis of liposomal nanoparticles was confirmed by different characterization techniques. Pegylation enhanced the stability that resulted the long-term circulation in the body as compared to simple silymarin. After treating the diseased mice, the histological, serological, body weights and neurotransmitter quantification results demonstrated the substantial betterment in Peg-SLNPs treated group. The FST and OFT behavioral results showed that the administration of SLNPs decreased anxiety- and depression-like behaviors all together. The findings also suggested that ACTH involvement as well as regulation of the monoamine system were likely involved in these behavioral alterations. When considered collectively, these results imply that SLNPs may be a useful clinical intervention for the treatment of neurological illnesses, such as depression. Given that silymarin is a natural product with fewer side effects and the potential to be used in the long-term management of anxiety and depression, it is also a viable alternative to selective serotonin reuptake inhibitors (SSRIs). Moreover, simple silymarin treated group also indicated positive results to some extent. So, for the reversibility of inflammatory liver disease to normal hepatic tissue, and to achieve the normal ACTH, and monoamine levels, the use of the encapsulated silymarin drug in nanoparticles along with Peg 1000 is a superior choice, contrary to simple drug. Thus, the combination of liposome encapsulation and PEG coating has been demonstrated to be the most successful strategy for increasing the bioavailability and pharmacokinetic behavior of silymarin drug, and it has also showed significant therapy against inflammatory liver ailments.

Due to its anti-inflammatory and antioxidant properties as well as increased neurogenesis in the prefrontal cortex and hippocampus, silymarin exhibits antidepressant-like effects. This establishes silymarin, particularly in nanoparticle state, as a prospective method for treating depression. To validate silymarin's antidepressant activity for the treatment of depression and related mood disorders, additional clinical research is needed. Moreover, the route of administration of drug was oral, so further studies should be implicated regarding its route to combat depression and symptoms of inflammatory liver diseases as result of depression to approve this approach at clinical level.

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