'Administration of Fluoxetine incorporated Liposomal Nanoparticles in Chronic Mild Stress (CMS) Animal Model for the treatment of Depression'

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'Administration of Fluoxetine incorporated Liposomal Nanoparticles in Repeated Social Defeat Stress Animal Model for the treatment of Depression'

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Abstract

Depression is one of the most severe disorder that is affecting people all over the world. Stomach upsetting, headache, nausea, breathing problems all linked to depression symptoms. Depression must be addressed because it leads to a low energy level and reduced work productivity in a person. Antidepressants such as Fluoxetine are commonly used medication to change chemicals in the brain known as serotonin, dopamine, and norepinephrine. These chemicals are involved in monitoring and controlling mood swings in a person. However, the Blood-Brain Barrier (BBB) plays an essential and significant hurdle in transporting antidepressants to the nervous system. Nanotechnology has provided the solution and enhances the targeted drug delivery to the nervous system. In this research work, Fluoxetine incorporated liposome nanoparticles were fabricated and developed to transport the drug (Fluoxetine in this case) across the BBB to the nervous system with high efficiency. A mice model of depression, 'Chronic Mild Stress (CMS),' was made, and depression was induced in mice to test the drug delivery efficacy. Drug was successfully targeted to the brain. Hence, it's proved to be the best treatment procedure because of testing.

Chapter 1

1. Introduction

1.1. Depression

Depression is one of the primary and critical mental health disorders that involves mood swings, loss of interest, loss of pleasure, low energy level, guilt feelings, disturbed emotions, sleep deprivation or loss of appetite, and low concentration. These mental health issues can lead to chronic stages and impairments in different abilities during daily life routine. In addition to it, depression can also lead to a suicide attempt (Lam, 2018). According to WHO, approximately 1 million lives are lost per year due to suicide. These 3000 leading deaths from suicide attempts are because of depression. Every person can lead to 20 or more than 20 suicide attempts after completing one suicide. After depressive disorders, Bipolar disorder includes both chronic as well as depressive episodes. Chronic episodes involve increased mood swings and increased energy consumption resulting in deprivation of sleep and hyperactivity of brain functioning. (WHO, 2012)

According to WHO, depression is the known leading cause of disability in both females and males. The probability of depression in females is 50% greater than that of males in middle and upper-class countries (WHO, 2008). Research studies show that the risk key factor depression in developed countries is more significant, especially in young children. This risk key factor or component can be related to the mental health and growth of young children having some effects of depression in the next generations. (Rahman et al., 2013)

1.2. Global Prevalence of Depression

Depression is one of the critical parameters to estimate mental health in an individual or a society. The cross-sectional analysis shows the prevalence of depression in men (5.7%) and women (9.4%). The prevalence of mood swings in Europe and the US is higher than the Major Depressive Disorder (MDD) ranges from 7.7% to 11.8% and 3.0% to 10.4%. According to a nationwide survey, the US population and Estonia prevalence vary from 6.5% to 5.5% (Kleinberg et al., 2010). The prevalence of depression in China is much higher, up to 27.2%, compared to Greece (38.5%). There exists a difference between mood swings and

depression prevalence in different countries. Croatia comes under the lower level for depression prevalence compared to other major countries (Yunming et al., 2012).

According to some recent Chinese research, the prevalence of depression in patients pointed out globally due to the enhanced risks, symptoms, disorders in different patients. The Government has started awareness campaigns and sessions to raise treatment and recognition of depressed symptoms in patients. The screening method for depression should be done to improve the management of disorders and diagnosis during practice. There was a considerable risk of developing depression symptoms in older, unmarried, and unemployed people. The Zung depression scale was useful to help with population screening and further treatment after getting diagnosed. (Milanović et al., 2015)

1.3. Prevalence of Depression in Pakistan

Women in Pakistan are at greater risk of getting depression as compared to men. According to the latest research, women suffer from depression at approximately 41.8% and men at approximately 29.1%. Different varieties of diseases attack women due to some gender-specific responsibilities and other socio-cultural factors. Stress is more common in Pakistan due to poverty, hunger, domestic issues, malnutrition, socio-cultural factors, and many more that refer to depression later. (Okasha, 2016)

Depression is found in pregnant women as an independent factor for developing low birth weight babies. The mortality rate is higher for long-term mental health effects for developing children. Children of a depressed mother must face more failures and other depressive disorders in future lives than normal mothers. The main global health concern is to identify and treat maternal depression successfully. (Khan et al., 2006) Moreover, there exists a relation between self-esteem and improved pregnancy results. Like family, many other factors, such as family, socio-cultural factors, status, and income, also play an important role in affecting both males' and females' mental health. (Reblin & Uchino, 2008)

1.4. Biochemical basis of depression

The nature of mental health procedures gains more attention due to the development of the neuroscience field in the 20th century. Neuroscience covers many biological fields that involve molecular biology, cell biology, brain imaging technology and gene functional studies. Nobel prize was given to many scientists to investigate psychiatric disorders based on neuroscience. (Nestler, 2018)

1.4.1. Mechanism of Depression

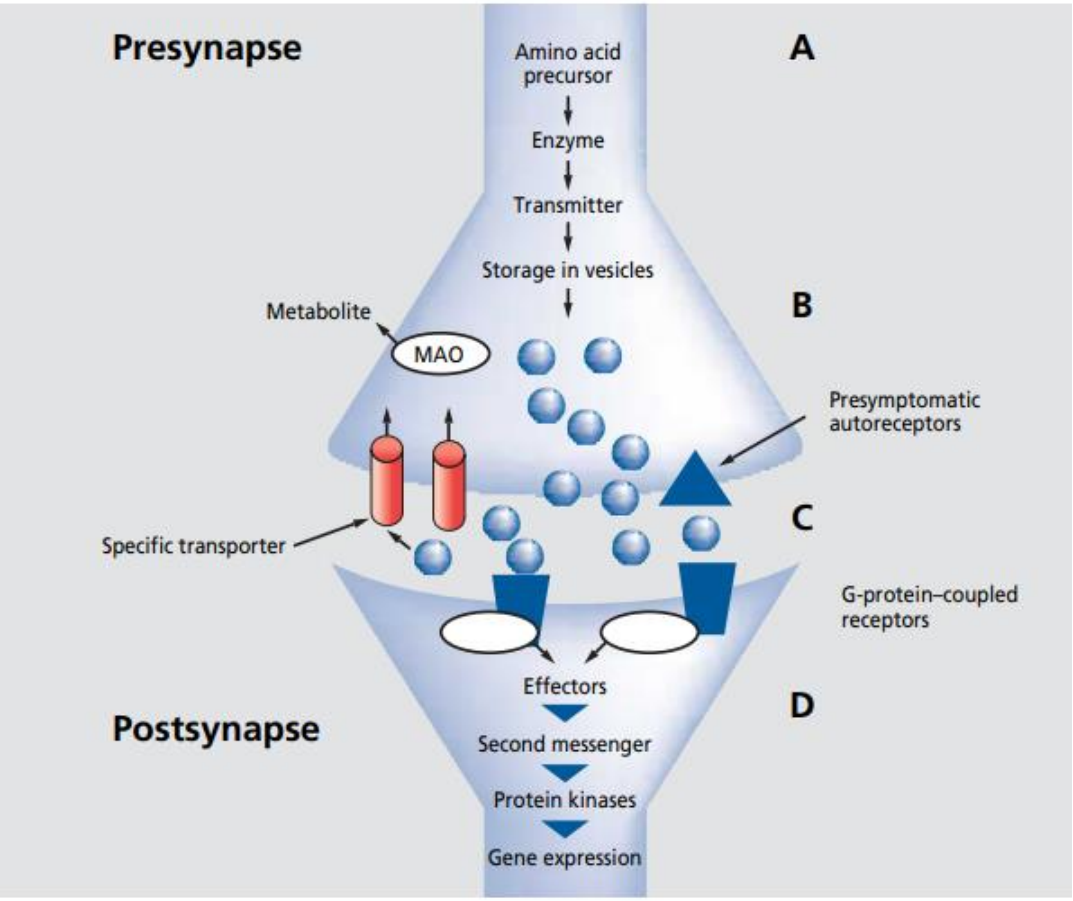


Figure 1: Diagrammatic representation of the mechanism of depression. (A), converted into transmitters via enzymatic processes, and stored in synaptic vesicles (B). The transmitters are released into the synaptic cleft (C), where they either react with presynaptic auto receptors to regulate synthesis and release or with postsynaptic receptors to induce the downstream events signal transduction cascade (D). MAO, monoamine oxidase

1.4.2. The Mediating role of Monoamines

Dopaminergic neurons are found in the midbrain and extend towards large areas of the complete brain. Monoaminergic systems monitor the brain functions such as mood, sleep, appetite, cognition and reward by exploring the brain's anatomy. The concentration and number of monoamines increase the uptake of monoamine at the synaptic cleft. The antidepressants are most efficient in the treatment of depression. (Belmaker & Agam, 2008) Prohibiting the enzyme monoamine oxidase prompts an enhanced level of monoamines at presynaptic neurons and has some antidepressant effects. This is known as the monoamine deficiency hypothesis. This

hypothesis covers the pathophysiology of depression in a place where neurotransmitters like serotonin, dopamine, and norepinephrine are present in the central nervous system. Serotonin is an essential neurotransmitter in the case of depression. The lower serotonin levels are linked with mood swings, memory loss, and other related behavioral changes. This process refers to the serotonin deficiency hypothesis. The lower level of serotonin receptor has been found many times in patients having depression with major depressive disorder (MDD). (Burkhouse et al., 2017)

The enhanced concentration of monoamine oxidase enzymes metabolizes serotonin and affects the serotonin concentration, thus causing serotonin deficiency. The pathophysiology of Major Depressive Disorder (MDD) is based on the lower level of norepinephrine metabolism and enhanced level of tyrosine hydroxylase. The classical theories about depression are based on serotonin and norepinephrine, and dopamine role. Approximately all antidepressants target the monoamine systems. The monoamine hypothesis is the most relevant neurobiological theory of depression. (Boueiz, 2015)

1.4.3. Neurotrophic hypothesis of depression

The first depressive episode is reactive social stressors that stimulate that. The next episodes enhance the minor stressors that occurred voluntarily. During the depression, the hippocampus and other brain parts get lost their volume. Hence, sensitivity to stress has become enhanced. Brain-derived neurotrophic factor (BDNF) has more attention for preclinical studies between stress-induced depression and lessens the hippocampus levels along with an increased expression of BDNF. (Carroll, 2004)

1.5. Treatment of Depression

There are many options available for the treatment of depression in the literature. Researchers have found psychotherapies, psychopharmacology, and different combinations of therapies. Hollon et al., 2005). The depression process and pathophysiology mechanism are still under investigation due to the complexity of this disease. This results from an imbalance in brain chemicals like neurotransmitters and a lack of signals in the brain. Treatment of different kinds is available according to its severity level. Different therapies such as behavioral therapy, cognitive-behavioral therapy (CBT), and antidepressants (Selective Serotonin reuptake Inhibitors) provides treatment against depression. Antidepressants work by changing the

signaling pathway of neurotransmitters in the central nervous system, namely serotonin, dopamine, and norepinephrine, thus; increasing our mood and making us feel better.

1.6. Antidepressants

There are many types of antidepressants, but mostly available antidepressants work by targeting the monoamine neurotransmitters. Antidepressants are grouped into the following five groups based on the mode of action:

1. Tricyclic antidepressants (TCAs)
2. Selective serotonin reuptake inhibitors (SSRIs)
3. Monoamine oxidase inhibitors (MAOIs)
4. Serotonin-norepinephrine reuptake inhibitor (SNRI)
5. Non-TCA antidepressants

Antidepressants are drugs that help treat depression by changing the chemical imbalances of neurotransmitters in the brain. The chemical imbalance causes mood swings and changes in behavior in the brain. Neurotransmitters are found in nerve cells as a vesicle. One of the antidepressants is a selective serotonin reuptake inhibitor (SSRIs) that affects the serotonin level and regresses the selective level of serotonin in the brain but has some side effects. Every antidepressant has its mode of action in the brain. (Pinna, 2015) the selective serotonin reuptake inhibitors (SSRIs) are the most prescribed antidepressants for treatment and as a medication for major depressive disorder (MDD). Their mode of action work by increasing serotonin's availability in the synaptic region by blocking the serotonin reuptake transporter. For example, an innovative antidepressant, Mirtazapine, blocks the α-2 adrenoreceptors found on the noradrenergic neurons and 5-HT2A and 5-HT2C receptors, thus increasing dopamine norepinephrine release in cortical areas. (Harmer et al., 2017)

1.7. Nanotechnology

The central nervous system and brain are covered by a complicated system of barriers protecting it from the surrounding environment. The **blood-brain barrier** contains an endothelial membrane that controls the specific passage of different selective substances to and from the CNS. (Sweeney et al., 2018) The selective permeability capacity of the blood-brain barrier (BBB) is the foremost hurdle to make patients resistant to the conventional mode of treatment and side effects having delayed onset of action. BBB is made up of continuous non-fenestrated

vessels that just allow ions, cells, and specific chemicals. The BBB's restraining nature refers to a significant hurdle for drug delivery to the CNS. (Daneman and Prat, 2015)

Recently, the field of nanotechnology has provided a solution to the BBB hurdle. Nanomedicine has offered many opportunities to develop unique treatment procedures for several psychiatric and neurological disorders by using fine nano-sized particles. Nanoparticles can be modified according to size, specific area for targeting for drug delivery. Different nanoformulations are made to treat depression disorders. Among them, liposomal nanoparticles are the most widely used and safe nanoparticles. Liposomes are nano-sized vesicles consists of one or more bilayers, phospholipids and separated by the aqueous partition. Hence, liposomes can incorporate a lot of hydrophobic, hydrophilic, along with lipophilic therapeutic agents. This property makes liposomes more attractive and best for delivery as a vehicle. (Vieira and Gamarra, 2016).

1.8. Animal Models

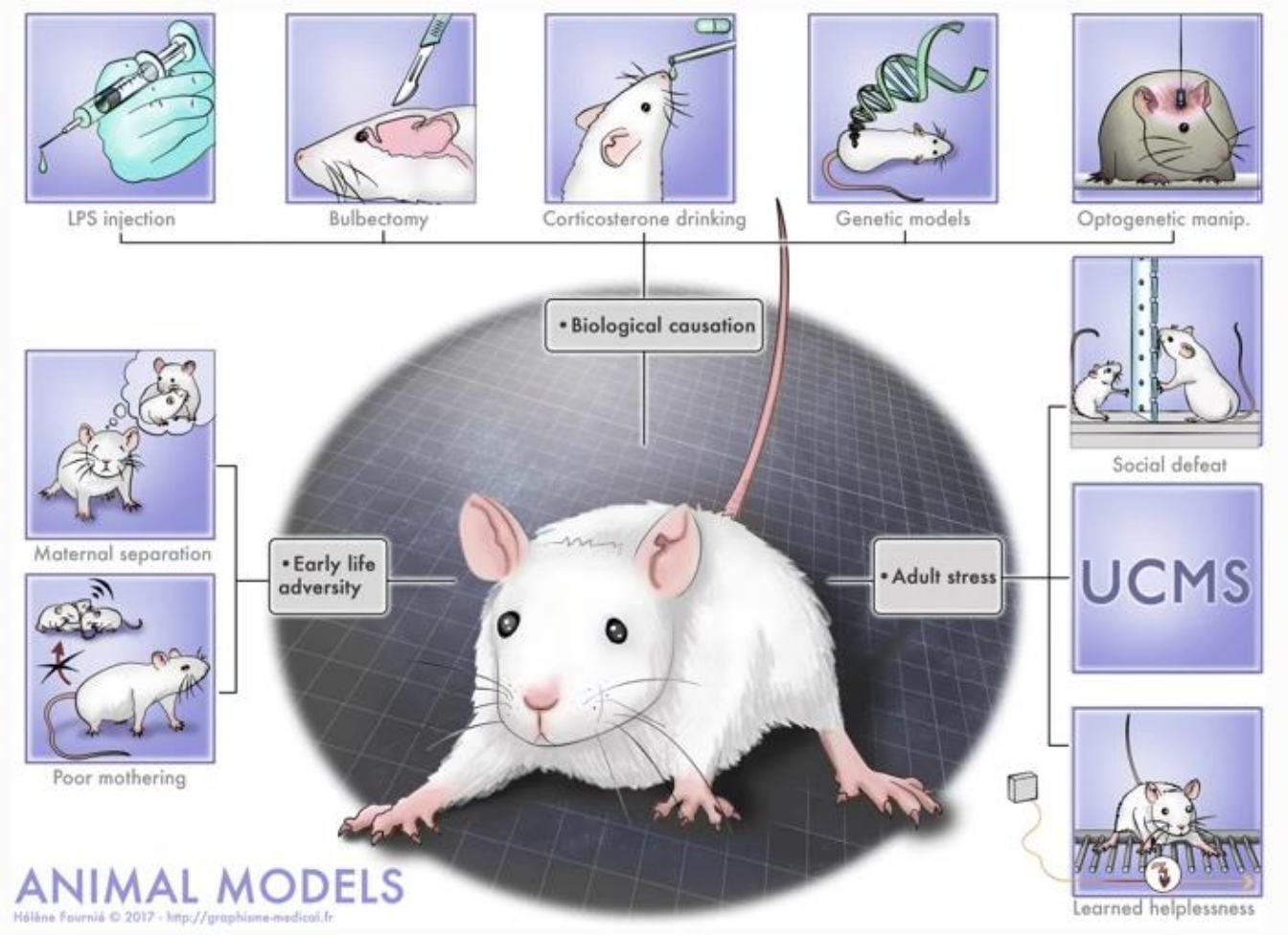


Figure 2: Animal models of depression. The models are mimicking different causes: early life adversity, biological causation, the stress in adulthood

Since three main categories, animal models are made: construct validity, face validity and predictive validity. (Nestler and Hyman, 2010) The animal models can be developed based on chronic stress disclosure and genetic variations. A lot of animal models worked because of the type of stressors applied. Some models can directly target the significant depressive disorder biological substrates, such as changes and variations in the brain circuit or the immune system. (Uher and McGuffin, 2010).

1.9. Objectives

2. Fabrication and development of Fluoxetine incorporated liposome nanoparticles for the treatment of depression
3. Characterization of Fluoxetine incorporated liposome nanoparticles
4. Induction of depression in Chronic Mild Stress (CMS) mice model
5. Behavioral test analysis after treatment with Fluoxetine incorporated liposome nanoparticles

The treatment with fluoxetine incorporated liposome nanoparticles is most effective and has fewer side effects and rapid onset of action.

Chapter 2

2. Literature Review

2.1. Fluoxetine Hydrochloride (Prozac) as an Antidepressant

Fluoxetine is FDA approved antidepressant for major depressive disorder, panic disorder, bipolar disorder, etc. Fluoxetine is also used in combination with olanzapine for the treatment of depression. (Mikocka-Walus et al., 2019) Non-FDA approved Fluoxetine is used to treat social anxiety disorder, post-traumatic effects, and selective mutism. (Slee et al., 2019)

2.1.1. Mechanism of Action

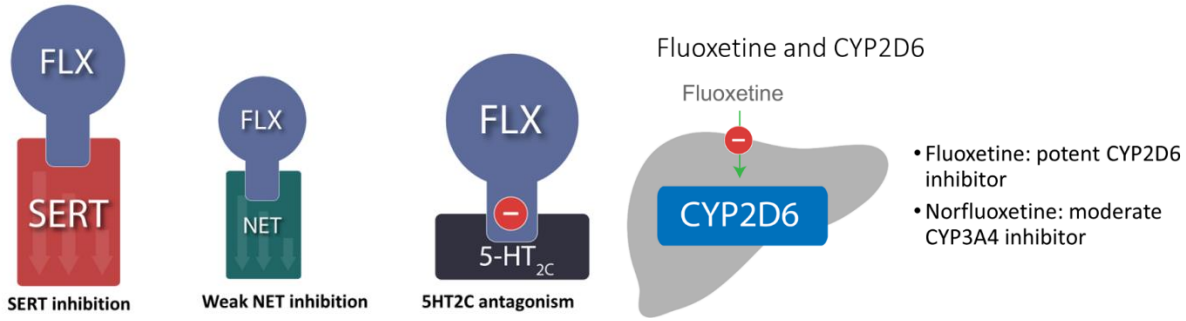


Figure 3: Fluoxetine Mechanism of Action (Cao et al., 2019)

Biological amines such as serotonin and norepinephrine impart a vital role in depression. The level and concentration of serotonin are low in the cerebrospinal fluid in the case of depressed patients. The prefrontal cortex's projection at the dorsal side of the nucleus shows presynaptic serotonin receptors such as 5HT1A. The working principle of Fluoxetine depends on blockage of the reuptake of serotonin in the presynaptic serotonin present in the presynaptic side terminal. Fluoxetine acts on two receptors, slightly 5HT2A and 5HT2C receptors. Fluoxetine activates because of its reuptake of serotonin, and its half life span was of almost 2 to 4 days. The antidepressant effect becomes visible within 2 to 4 weeks. The active metabolite of Fluoxetine is norfluoxetine that is produced after the action of an enzyme CYP2D6 (cytochrome P450). The metabolism property of Fluoxetine plays an essential role in the drug to drug interactions at the isoenzyme (CYP2D6). CYP3A4 can be inhibited by Fluoxetine (half-life 7 to 9 days). (Cao et al., 2019)

2.1.1. Limitations of using Fluoxetine

Like other medicines, Fluoxetine also has some contradictions and adverse side effects due to hypersensitivity of Fluoxetine or its related component in composition and monoamine oxidase inhibitors (MAOI) usage for the treatment of psychiatric disorders like Major Depressive Disorder (MDD), panic disorder, anxiety, diarrhea, sweating, dry mouth, insomnia or vasodilation. Fluoxetine has some contradictions when giving along with pimozide, tamoxifen, linezolid or thioridazine. (Sohel et al., 2020)

The most found side effects of Fluoxetine involve anorexia, headache, diarrhea, yawning, insomnia, nervousness, bleeding, or seizures. 5HT2C antagonism contributes towards anxiety, agitation or insomnia in a patient who is taking Fluoxetine. Patients must educate about panic attacks when administered with Fluoxetine. The adverse side effects are time and dose-dependent and disappear after passing the time. The side effects can last longer in the case of Fluoxetine. To minimize the adverse side effects of Fluoxetine, it is necessary to take some benzodiazepines or bupropion or Mirtazapine. (Bahar et al., 2018)

2.2. Liposomes as an Effective Drug Delivery System

Colloidal structures having one or more spherical shaped bilayers of lipid having an inner water part are known as liposomes. Liposomes have an increasing advantage and attraction due to their role as drug carriers for different drug delivery systems (DDSs). Liposomes were made in England in 1961 by Bangham (Bangham et al., 1964), who showed the phospholipids are hydrated in water solution and make closed structures. Phospholipids have one or more bilayers and membranes that can catch water or lipid drugs, depending on nature.

Phospholipids are amphiphilic molecules having a water-soluble hydrophilic head part and a lipid-soluble hydrophobic tail. Liposomes have both phospholipids as well as cholesterol. The unique form of liposomes having phospholipid and cholesterol makes unique properties like self-healing in water solution and makes the liposomes ideal carriers with applications in different fields such as medicine, cosmetics, ecology, immunology, cleansing, diagnostics and the food industry. The properties of liposomes are affected by many factors like lipid composition, size, a charge on the surface, and preparation method. The lipid bilayer components measure the rigidity or charge of the bilayer. (Mishra et al., 2018)

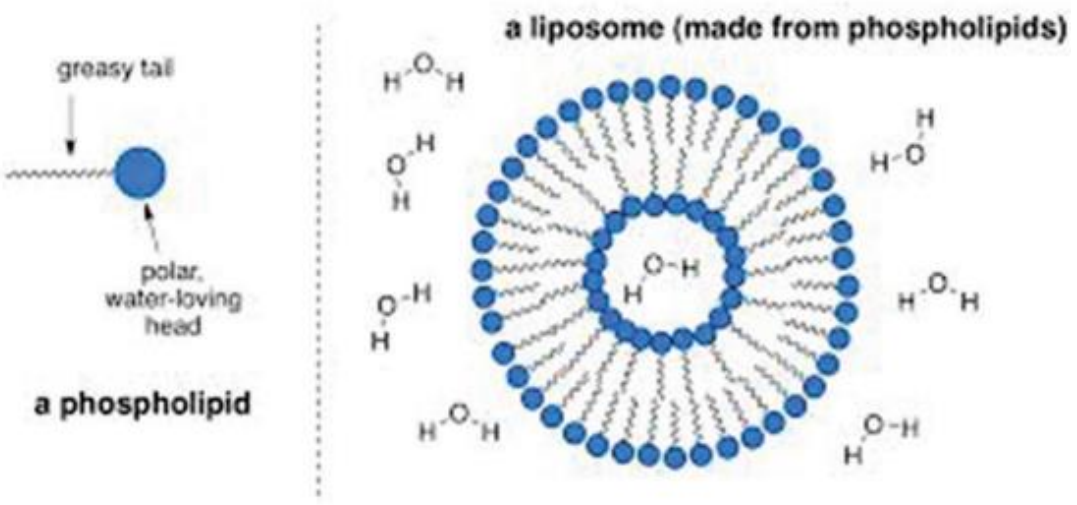


Figure 4: Liposomes are made by phospholipid in a water solution (Karami et al., 2018)

Liposomes interact with cells using different unique interactions such as cell surface and electrostatic interactions and adsorption mechanisms. The second interaction that takes place by phagocytic cells of macrophages and neutrophils is endocytosis. The fusion with plasma cell membrane occurs by insertion of the lipid bilayer of the liposome into the plasma membrane, and liposomal content is released into the cytoplasm. Bilayer components swap with each other, and another type of mechanism takes place at a specific time. (Nikam et al., 2020)

2.2.1. Role of Liposome Nanoparticles in Research and Development

Nanotechnology is a multi-disciplinary and diverse field of research and development that involves the processing and production of different materials or devices on a nanometer scale. Nanotechnology has influenced many other fields of medicine, food, and cosmetics as well as in nanomedicine. Liposomes are the most critical delivery vehicle for bioactive agents. Biomembranes and cells are unique models called liposomes due to the likeness of liposomes with biological membranes. Liposomes are used as drug delivery vehicles or carriers. Liposome nanoparticles have more surface area, increased solubility, enhanced bioavailability, biodegradable, biocompatible, and accurate targeting of the encapsulated material compared to simple liposomes. (Panahi et al., 2017)

The targeting ability of liposome nanoparticles is one of the most critical and beneficial properties to fulfill enough amount or concentration of drugs at the target site for more efficacy and efficient results. Liposome nanoparticles have more encapsulation efficiency and release

lipid-soluble amphiphilic components, drugs, and biological molecules such as peptidase or genes. This property is used in food industries, genetic engineering, drug delivery systems, and gene therapy systems. (Mehrabi et al., 2016) Additionally, drugs having a variety of lipophilicity can easily be encapsulated into liposomes, and lipophilic drugs can be entrapped in the lipid bilayer. Hydrophilic drugs are found in the water compartment, and drugs have little separation between the water and the lipid phase, both in the lipid bilayer and water core. (Akbarzadeh et al., 2013)

2.2.2. Cholesterol

Liposomes are made up of other molecules in their structure, along with phospholipids known as sterols. Sterols are one of the main parts of the biological membranes. Sterols incorporate in the bilayers monitor the properties of the liposome nanoparticles. (Luo et al., 2016) Among sterols, cholesterol is the most utilized component in the synthesis of the nanoliposome vesicles. Cholesterol monitors the fluidity of the lipid bilayer and prohibits the crystallization of the acyl chains of phospholipids. Cholesterol also plays as a creation of steric hindrance and lessen the permeability of lipid membranes to different solutes. This phenomenon in liposome structures is the enhanced stability of the vesicles by different mechanisms like mentioned above. (Daraee et al., 2016)

2.2.3. The proposed structure of Fluoxetine loaded DPPC Nanoparticles

The proposed model of Fluoxetine loaded DPPC nanoparticles has shown in the below figure. It has shown that our drug (hydrophilic) will be attracted towards the water compartment and will be encapsulated in the core part of the liposomal formulation. The cholesterol molecules will be entrapped in the lipid bilayer of the phospholipids due to their hydrophobic nature.

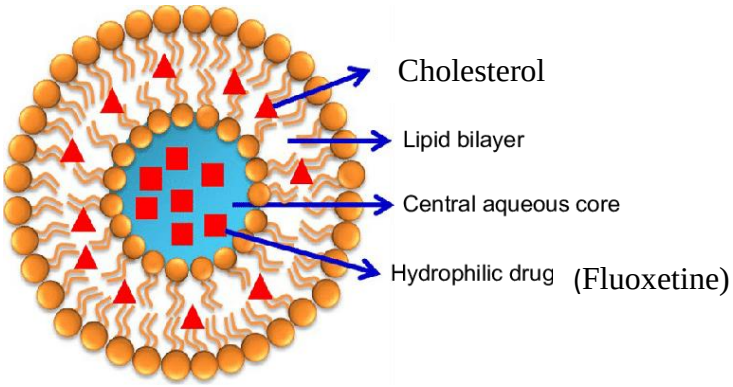


Figure 5: Proposed structure of Fluoxetine loaded DPPC liposomal nanoparticles. The polar drug will be encapsulated in the core and non-polar cholesterol molecules embedded in the bilayer.

2.3. Depression an Alarming Public Health Issue

Depression is one of the commonly found disorders in all age groups and genders. According to the World Health Organization (WHO), depression is the 4th leading cause of disability globally and rise to 2020. Depression has many signs and symptoms, and this problem is increasing day by day. Depression has become chronic or acute and recurrent, which leads to unique impairments in an individual's life. It can also lead to a suicide attempt or death or loss of an individual every year. Depression can enhance the risk for other diseases like Alzheimer's, epilepsy, diabetes, cancer, and cardiovascular diseases shown in below figure 6. 80% of patients recover from depression with antidepressant medications. Less than 25% of people have access to effective treatment. Resistant to depression treatment takes place in almost 40% of the patients.

Antidepressants can cause unwanted side effects like weight increase, low blood pressure, digestion, etc. This may also lead to the reoccurrence of depressive signs and enhanced suicidal risk. The neurotransmitter hypothesis helps us to understand and focus on relevant processes to prohibit depressive episodes. Treatment of serotonin reuptake inhibitor and other noradrenaline reuptake inhibition is an important factor in psychiatric care. (Lang & Borgwardt, 2013) Depression is a life-threatening disorder that affects hundreds of millions of people worldwide and causes disruption in an individual's life if left untreated. Depression is a non-homogeneous disorder and a complicated process that involve many subtypes and more than one etiology or causes. A depressed individual can also have somatic disorders, along with other psychiatric disorders. (Duman & Voleti, 2012)

2.4. Animal Model of Depression

Animal models are made on three basic constructs: face validity, predictive validity, and construct validity. (Nestler & Hyman, 2010) The other constructs involve homological validity, pathogenic validity and mechanistic validity based on other features. (Belzung & Lemoine, 2011)

The animal models are established or developed based on chronic stress exposure, genetic monitoring, glucocorticoid control, and gene interactive environmental factors. Every model has its pros and cons. The most used animal model for depression is rodents and rats. (Uher & McGuffin, 2010)

2.5. Chronic Mild Stress Model for Depression Studies

Firstly, Chronic Mild Stress (CMS) model was established by Katz and colleagues in 1981. (Katz et al., 1981) Then, Willner lab had developed another model paradigm based on two strategies. One of them is decreasing stressors, and another one is to induce anhedonia. Typically, many weeks have various stressors such as water deprivation, food deprivation, the cage's tilting, and other related stressors. The Chronic Mild Stress (CMS) method aims to develop the depressed state in response to stressors or stress stimuli chronically. This model has low reward sensitivity and the development of anhedonia. The signs of chronic mild stress model involve a lower sugar level, the loss of weight and appetite, etc. The high level of glucocorticoids results in atrophy and apoptosis in the prefrontal cortex and hippocampus parts. The release of corticosterone and the low level of glucocorticoid expression results in cognitive impairment linked with depression. A low level of endogenous ATP in the mice brain is responsible for abnormal gap junction, and antidepressants reverse this gap junction to increase gene expression of connexin 43. (Quesseveur et al., 2015) A chronic mild stress model study shows that ATP's stimulation and release in the astrocyte cells induce antidepressant-like behavior. (Crema et al., 2010)

Chapter 3

3. Materials and Methods

3.1. Synthesis of Fluoxetine loaded Liposomal Nanoparticles

3.1.1. Materials

Different chemicals are used for experimental work, such as Fluoxetine hydrochloride, Dipalmitoylphosphatidylcholine (DPPC), Absolute alcohol (ethanol), Cholesterol, and Milli-Q water. These chemicals were ordered from Sigma-Aldrich (USA).

3.1.2. Methodology

Fluoxetine-loaded liposomal nanoparticles were synthesized by using a method known as the "modified ethanol injection" method. (Chorachoo et al., 2013) First of all, took fluoxetine hydrochloride, and it was dissolved in enough quantity of absolute ethanol to gain a final concentration of 0.1 mg/ml. The lipid phase was prepared at a specific concentration of 100 µmo*l*/ml. Cholesterol and Dipalmitoylphosphatidylcholine (DPPC) were prepared and dissolved in 10 ml of ethanol in a specific ratio of 4:1. After that, Fluoxetine from above mentioned lipid suspension solution was added around 500µl. Sonicated the mixture for 45 minutes. 10ml of Milli-Q water was placed in a water bath that was previously set at a temperature of 60oC and sonicated lipid and drug suspension. The water phase was merged with the lipid phase after heating up to the temperature of 60^{o}C. The resulting suspension was shaken in a water bath for 15 minutes to allow even mixing and phase inversion. The resultant mixture was poured into a round bottle flask linked to a rotary evaporator (Eyela Rotary Vacuum Evaporator N-100 series, Japan) to evaporate ethanol. Therefore, the cloudy suspension of Fluoxetine incorporated liposomal nanoparticles were transferred into a glass vial, sealed, and put in storage till further use.

3.2. Characterization of Fluoxetine loaded Liposomal Nanoparticles

Characterization of Fluoxetine loaded liposomal nanoparticles was carried out to determine and evaluate their size, the net charge on their surface, their aggregation, drug encapsulation and release kinetics to ensure they are of the right size and nature to be used for the treatment of depression in mice models.

3.2.1. Determination of particle size and area Distribution

Fluoxetine loaded liposomes were investigated under a scanning electron microscope (SEM) to identify their morphology. For this purpose, glass slides having loaded liposomes were subjected to coating with gold (30nm) to make them operative and conductive for SEM analysis. SEM analysis determines the physical distribution of the nanoparticles, particularly SEM, to confirm the nanoparticles' spherical morphology.

3.2.2. FTIR Analysis

FTIR stands for Fourier Transform InfraRed, the most useful procedure of infrared spectroscopy. In this procedure, infrared (IR) radiation is passed through a sample, and the sample absorbs some part of infrared radiation, and some part is transmitted through the sample. Hence, the resulting spectrum shows the molecular absorption and transmission, making a molecular fingerprint of the sample to identify unknown material and determine the quality and amount of the material present in the sample. (Litipijiang et al., 2020)

3.2.3. Surface charge and Zeta potential

The value of zeta potential typically determines the net charge on the nanoparticles in terms of the magnitude of attraction or repulsion between them and their stability. Zeta potential of Fluoxetine loaded. Liposomal nanoparticles were measured by dynamic light scattering technique (Zetasizer-Nano Malvern (Germany)).

3.3. Introduction of depression using Chronic Mild Stress (CMS) model in Mice

Animal models of depression are used to understand human psychopathology. In the chronic mild stress model of depression, mice are revealed acutely to a continuous micro stressor that results in mice's variations in behavior involving anhedonia, less response towards food or reward, etc. Different tests and predictions can check out the behavioral changes in mice. The

chronic mild stress is then treated with antidepressant drugs. Chronic mild stress is induced one by one, as shown in the table below. (Willner, 2017)

Table 1: Induction of Chronic Mild Stress (CMS) in Mice in two weeks

No. of Days	APPLIED MILD STRESSORS (Shifted the mice groups into another new cage from home cages)	Conditions
Day 1	Wet Bedding Stressor	▪ 4 hours ▪ Added Wet Bedding ▪ No food, No water
Day 2	Empty Cage Stressor	▪ 3-4 hours ▪ Removed Bedding ▪ No water, No food
Day 3	Without Bedding Stressor	▪ 3 hours ▪ No Bedding ▪ Tilted the cages at 45º
Day 4	Water containing Cage Stressor	▪ 4-5 hours ▪ Added 0.25 cm Water (no bedding) ▪ Leave them alone with no food and water
Day 5	Social Stressor	▪ Separate each mice and shifted into neighboring cages, and leave them alone for

		3 to 4 hours
Day 6	Day/Night Cycle Stressor	▪ Disturbed the 24-hour Day/Night cycle of each mice group and leave them 24 hours in light or 24 hours in the darkroom

3.3.1. Animals and Cages

20 Male mice aged between 6-7 weeks and weight 23-29g were purchased from ASAB Animal house lab, NUST H-12. According to the requirement, the mice were made into groups and exposed to an acclimatization time for about two weeks and a portion of food and water supply. Standard home cages had mice and a size of about 42 × 26 × 18 cm. Home cages were filled with fresh sawdust that was replaced after every 2-3 days. The temperature was maintained at 27°C ±2°C with humidity 50% ±5%. All the animals were closely monitored and observed. Several studies have demonstrated that exposure to chronic stress reduces food intake in animals, resulting in weight loss. For this purpose, weights of mice were recorded every three days throughout the depression and treatment timeline.

3.3.2. Grouping of Mice

20 mice were divided into 4 different groups. Each group contains 5 mice, and total groups were four in number. This grouping is based on the way of treatment that the mice will undergo.

Table 2: Grouping of Mice

Sr. no	Groups	Size	Label
1.	Group 1	5	CMS-1
2.	Group 2	5	CMS-2
3.	Group 3	5	Control
4.	Group 4	5	Simple Depression

3.3.3. Chronic Mild Stress (CMS) Procedure

One group of 10-15 mice animals was exposed to 4 weeks of Chronic Mild Stress, and a control group was subjected to normal handling for the same period. Another group of the same 10-15 mice animals was exposed to 4 weeks of chronic mild stress compared to the control group. Two independent experiments and procedures were run to confirm the findings.

3.3.4. Behavioral Test Assessment

Behavioral tests were performed to assess the performance of mice throughout the experiment. First, tests were performed before the treatment to check out mice's stress level to confirm the induction of depression-like behavior in mice. After that, these tests were performed again right after treatment to analyze the administered therapeutic agents' efficacy.

(i) Tail Suspension Test (TST)

All the mice were taken and performed tail suspension test by using standard procedure. (Can et al., 2012b) The tail of every mice was fixed with adhesive tape and attached to the table surface so that their bodies were hung towards the ground. The video was made for 6 minutes and analyzed by software to obtain the results.

(ii) Open Field Test (OFT)

Open field test was performed by using standard procedure. (Seibenhener and Wooten, 2015) Mice from every group were taken and placed in a cubic glass box keeping the box uncovered from the top and analyzed mice's activities. The video was made for 6 minutes, and analyzed the results by using the software.

(iii) Forced Swim Test (FST)

A forced swim test was performed using the standard procedure. (Can et al., 2012a) For this purpose, a large glass tank was filled with clean water at room temperature, and all the mice were placed in a water-filled tank one by one. The video was made along with it of about 6 minutes for analysis. After that, the behavior of mice was analyzed by using software and results were generated.

3.3.5. Drug Dosage and Route of Administration

Specific drug dosage was given to the mice that are mentioned in table 3. The administration route was intravenous through the tail of mice using a standard syringe of 1ml for 2 weeks.

Table 3: Mice groups and Drug dosage

Sr.no	Groups	Treatment	Dosage
1.	CMS-1	Fluoxetine loaded liposomal nanoparticles	500µg/kg
2.	CMS-2	Simple Fluoxetine	10mg/kg
3.	Control	-	-
4.	Simple Depression	-	-

3.3.6. Treatment with Fluoxetine incorporated Liposomal Nanoparticles

Hence, treatment was given CMS-1 and CMS-2 groups and was given Fluoxetine incorporated liposomal nanoparticle by 500ug/kg via IV. One depressed group was only given simple Fluoxetine by 10mg/kg via IV, and one depressed group was not treated but remained depressed. Treatment was given for 2 weeks. Each dose was given after 24 hours.

3.3.7. Outcome

The immobility time from the forced swim test and tail suspension test showed depression-like symptoms in mice. The majority of the time, in the last 4 four minutes, mice were immobile. In an open field test, mice explored the outer region of the box. They did not come in the central region, which explains depressed mice were more likely to hide in the outer region than explore the open places (center region). All these behavioral tests showed under stressed conditions, and mice developed chronic mild stress like depression.

Chapter 4

4. Results and Discussion

4.1. Characterization of Fluoxetine incorporated Liposome Nanoparticles

After the fabrication of fluoxetine liposome nanoparticles, characterization was done to confirm the drug entrapment within the nanoparticles and the drug-loaded particles with the right properties and morphological features for targeted drug delivery to the brain effectively and efficiently for the treatment of depression.

4.1.1. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) results show that the Fluoxetine incorporated liposome nanoparticles have spherical shaped boundaries with a diameter of 194 nm.

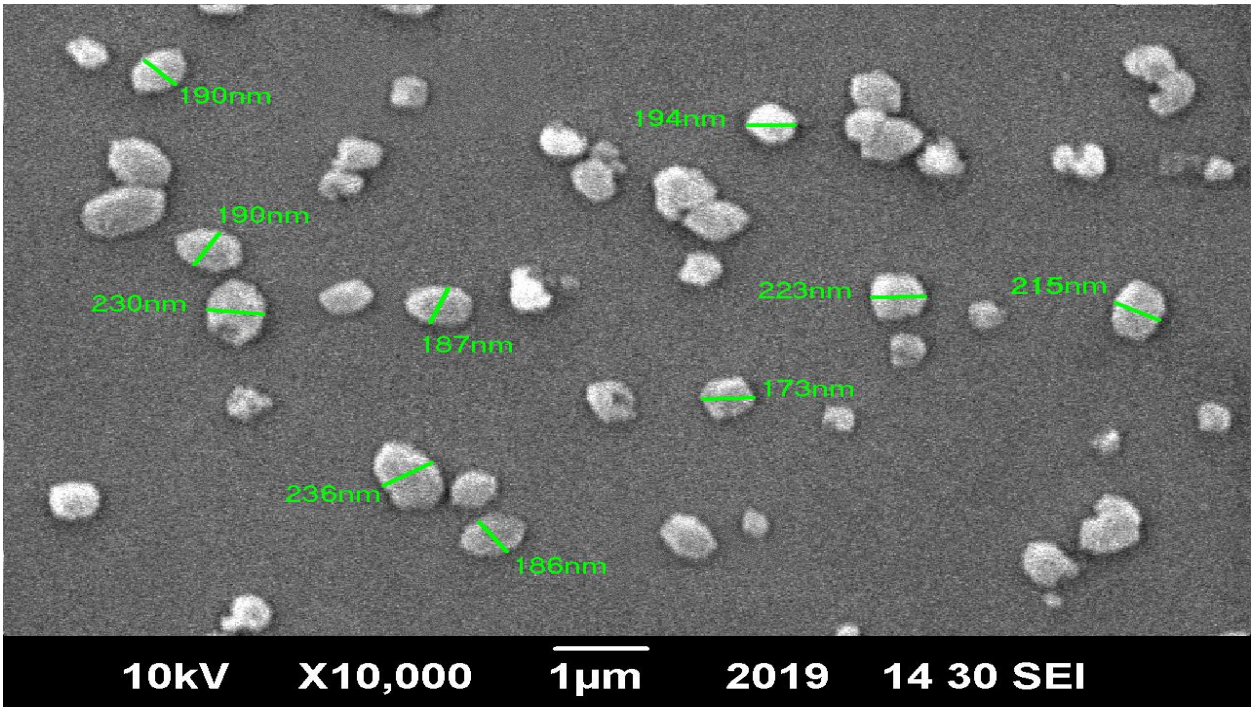


Figure 6: Scanning Electron Micrograph shows well-dispersed nanoparticles in a size range of 173-236nm.

4.1.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is significant to find out different functional groups and chemical bonds involved in nanoparticles by measuring vibrational frequencies. How many functional groups are absorbed on the surface of nanoparticles are measured and determined by FTIR spectroscopy? The theory behind it depends on the bonds present between different elements that can absorb light at multiple frequencies under FTIR spectroscopy. The light is determined by using an infrared spectrometer that provides the output of the infrared spectrum. FTIR analysis is done at multiple wavelengths to measure and determine the materials' structure and molecular composition by determining a material's absorbance of infrared light. FTIR analysis of non-pegylated liposome nanoparticles showed different peaks having different functional groups present in the material. Different peaks represent different functional groups, and a detailed overview is given in table 4. FTIR analysis is preferring to use for fast data collection, easy to use, and reproduce the data soon.

Table 4: Functional Groups in Liposome Nanoparticles

Peaks	Functional Groups	Peaks	Functional Groups
2959	Carboxylic acid (DPPC)	1238	R-CO-O stretching
1613	Benzene Ring (F/Chl.)	1162	CN Stretch (F)
1515	Nitro Group (DPPC)	1106	P=O (DPPC)
1324	Alkyl Halide (F)	764	Mono Substitute Benzene (F)
		696	Meta Substitute Benzene (F)

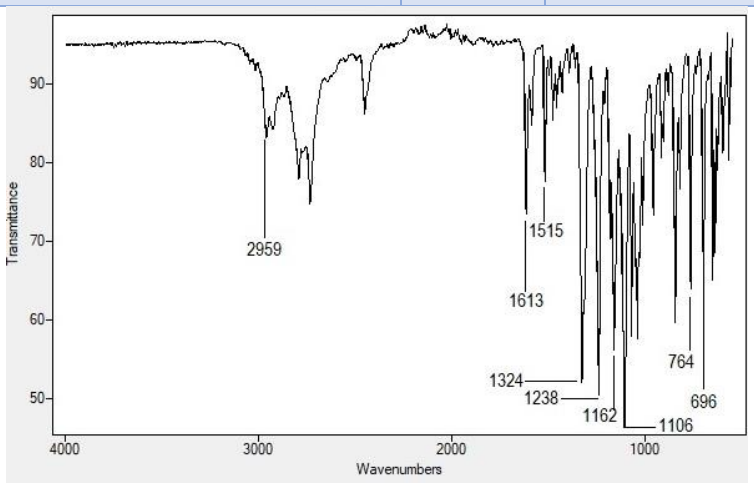


Figure 7: FTIR Analysis of non-pegylated Liposome Nanoparticles

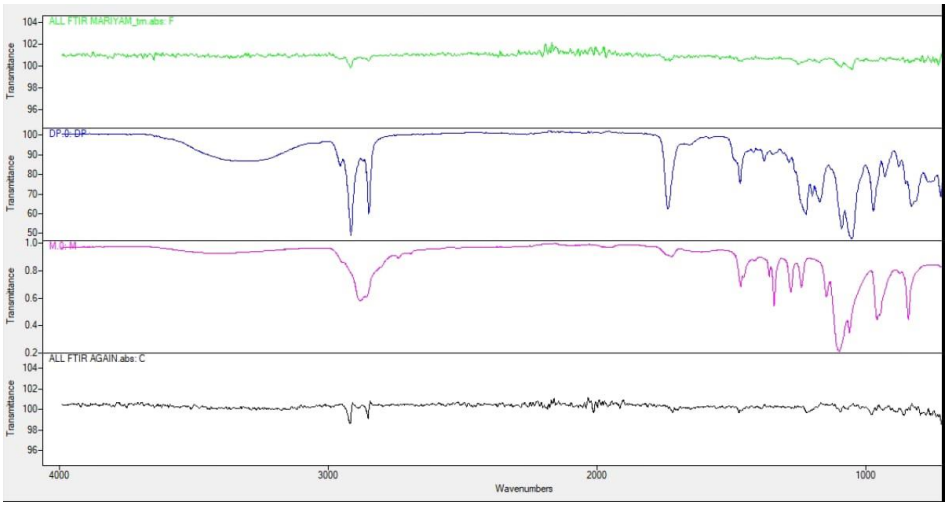


Figure 8: FTIR analysis of Fluoxetine, DPPC, Cholesterol

4.1.3. Zeta Potential and Zeta average

Zeta potential was recorded, and the value was -17mV, as shown in the figure. The Fluoxetine incorporated liposome nanoparticles have an average particle size (diameter hydrodynamically) around 205.2 nm, as shown in the figure. The result of zeta potential shows the charge on nanoparticles' surface that can affect the stability because of electrostatic attraction and repulsion forces present among the nanoparticles. Zeta potential also measures the particle's interaction with one another in-vivo. The value of zeta potential is higher, indicating the stability of particles having fewer aggregation problems. The negative sign shows the charge on the surface of nanoparticles. The surface charge helps the nanoparticles to attract a layer of oppositely charged ions towards it.

Moreover, the physical stability of nanosuspensions can easily be understood by using the knowledge of zeta potential. The value other than -30 mV to +30 mV in zeta potential is considered to have enough repulsive force to gain better physical colloidal stability. The small zeta potential value can result in coagulation and aggregation of nanoparticles because of van der Waal forces acting on it. In both cases, particles result in instability. Zeta potential and zeta average are significant because they affect the pH of the medium, ionic strength, temperature, and concentration of any additive.

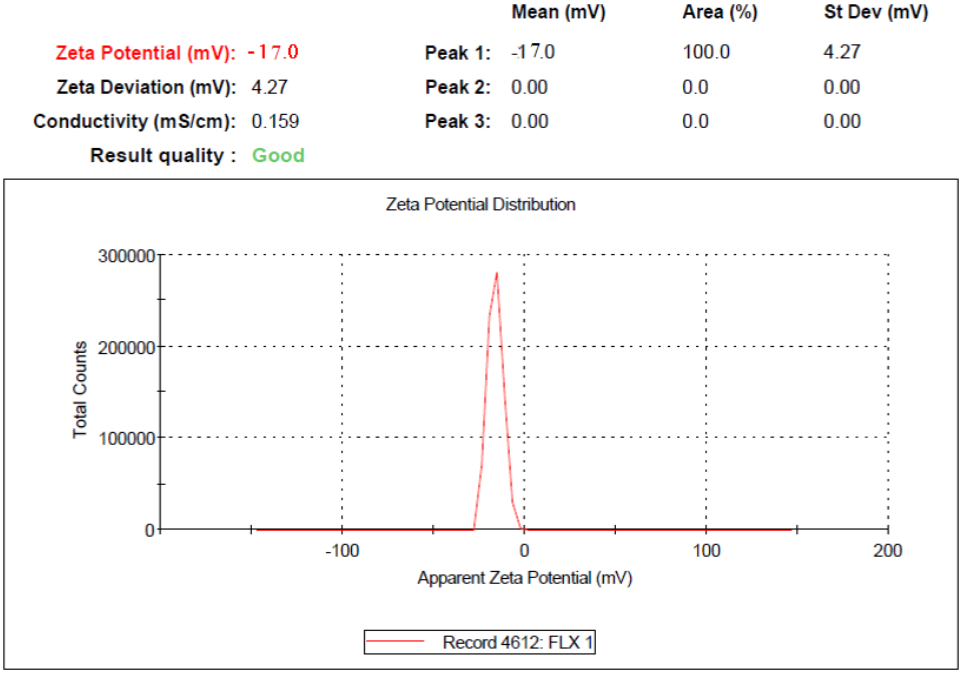


Figure 9: Zeta potential plot of Fluoxetine loaded liposomal suspension indicates a liposome net charge of -17mV.

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 205.2	Peak 1:	205.2	95.3	97.07
PdI: 0.245	Peak 2:	4963	4.7	627.2
Intercept: 0.951	Peak 3:	0.000	0.0	0.000

Result quality : Good

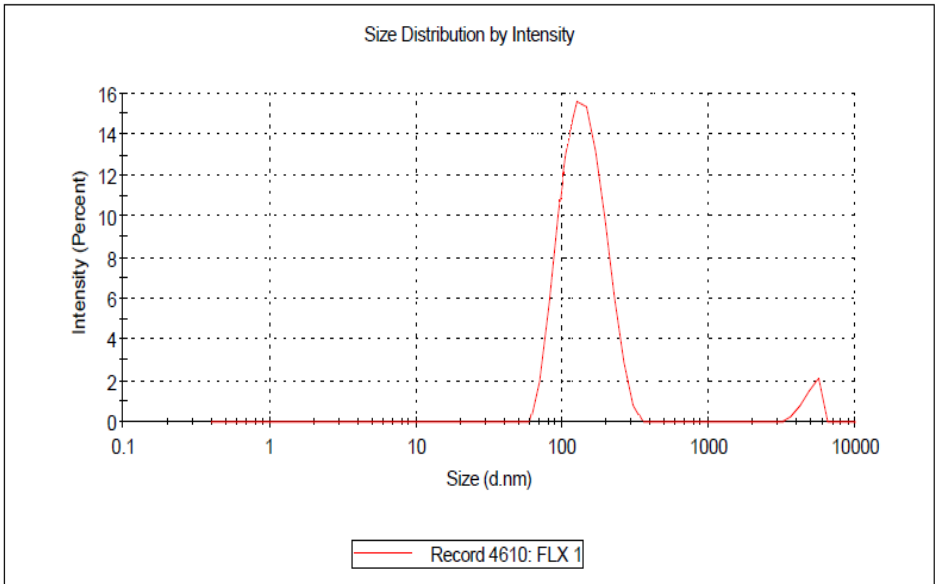


Figure 10: Particle size distribution profile and polydispersity index (PDI) of Fluoxetine loaded DPPC liposomes.

4.1.4. Drug release Kinetics

The analysis of the drug release profile of Fluoxetine incorporated liposome nanoparticles was performed using a UV spectrophotometer. The drug release kinetics is a significant area of interest for developing an ideal brain targeted drug delivery system for the treatment of depression in-vitro. The results were calculated, and the drug release graph was made, as shown in the figure. The graph shows a sustained release of Fluoxetine from the liposome nanoparticles over an entire 48h time. It can also be observed after 5-6 hours when all the drugs left the system and are released from the system.

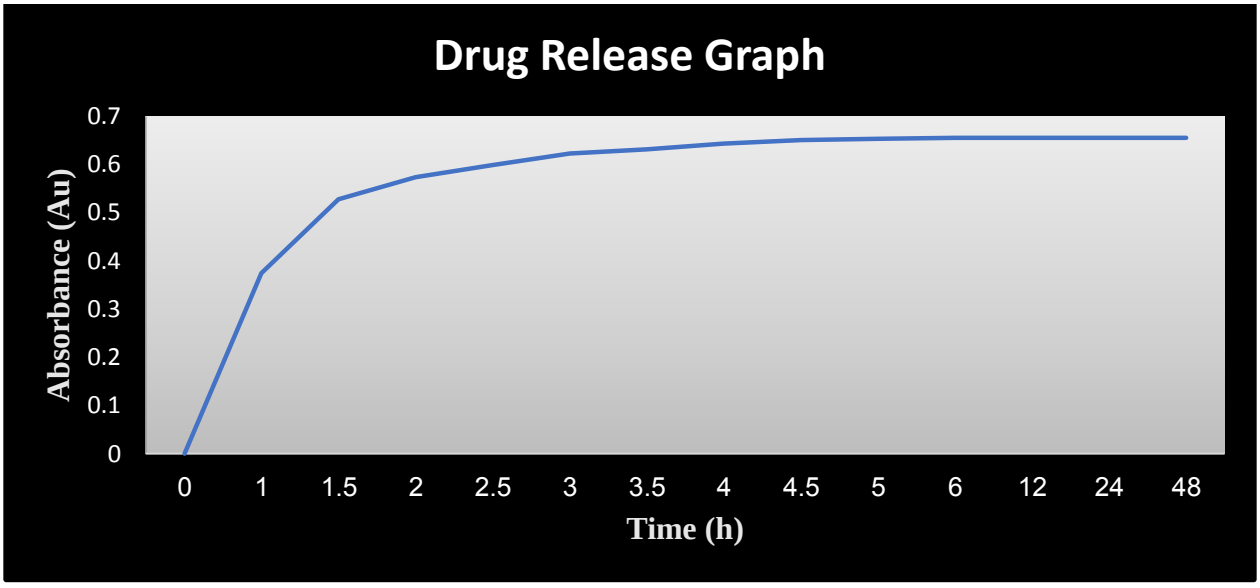
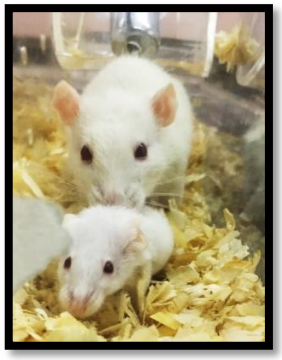


Figure 11: Drug Release Graph Results

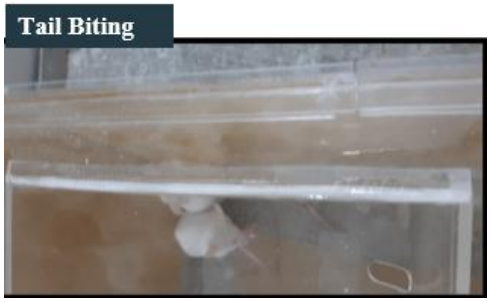
4.2. Mice Model of Depression (Induction of Chronic Mild Stress)

ACCLIMITIZATION PERIOD



INDUCTION OF CHRONIC MILD STRESS

BEHAVIORAL OBSERVATIONS



BEHAVIORAL TESTS

TAIL SUSPENSION TEST

FORCED SWIM TEST

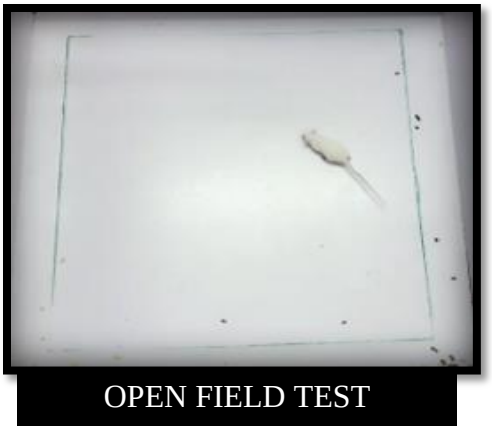


Figure 12: Induction of CHRONIC MILD STRESS in mice

4.2.1. Weight analysis

Depression often results in weight gain or weight loss. The bodyweight of each mice group was monitored every week for study. The bodyweight of mice become decreases because of chronic mild stress. The body weight was determined after the induction of the CMS depression model in the mice. The control group's body weight is increasing with time while CMS G-1, CMS G-2 and SD groups bodyweight is decreasing due to the induction of depression and stress as well as harsh environmental conditions. After treatment, the bodyweight got better with time. Moreover, the treatment with simple Fluoxetine and Fluoxetine incorporated liposome nanoparticles results in weight gain in mice groups named CMS G-1 and CMS G-2.

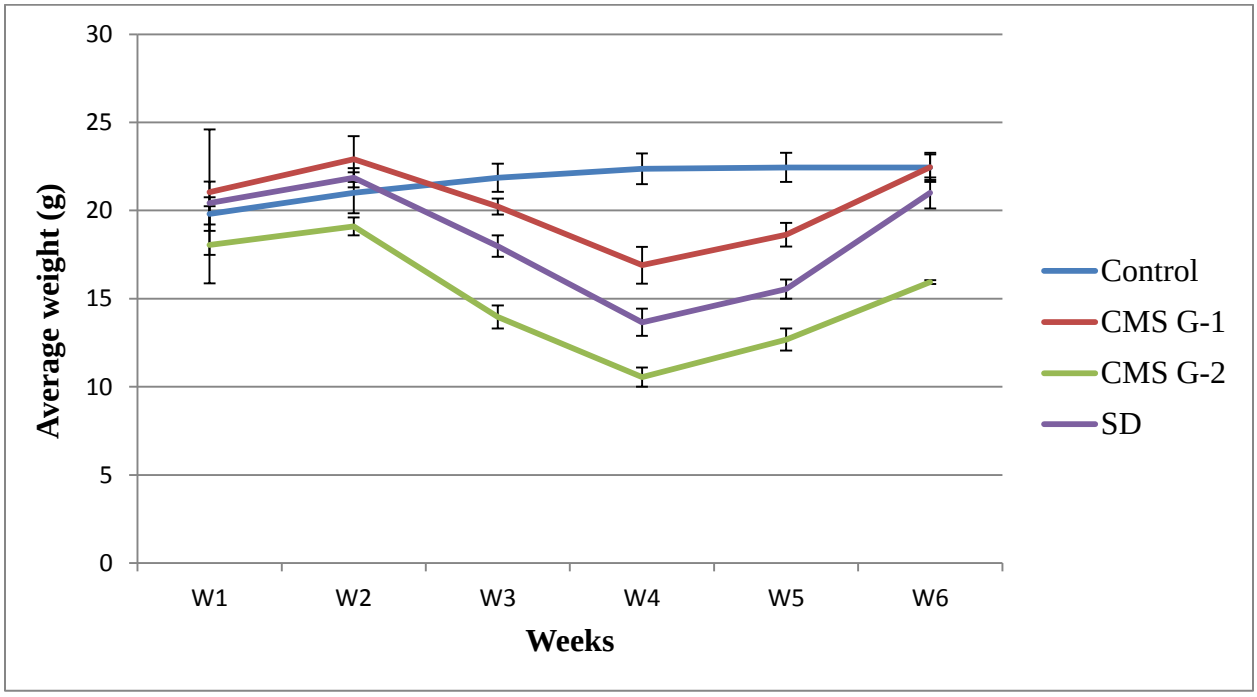


Figure 13: Body Weight Analysis of Mice subjected to Chronic Mild Stress Model of depression

4.2.2. Behavioral and Treatment Graph Test Results

Behavioral tests were performed to confirm the induction of depression and anxiety-like behaviors in mice after applying a chronic mild stress procedure. These tests were also used to confirm the reversal of depression and anxiety-like behavior in mice after one-week treatment. Treatment is done with Fluoxetine and Fluoxetine incorporated liposome nanoparticles in mice groups individually.

(i) Open Field Test (OFT)

Open field test is performed after treatment of mice groups. This test is used to locate the locomotor activity and mice's willingness to explore the environment; hence, it shows the stress and anxiety-like behavior in mice. Depressed mice show a low level of locomotor activity, and mice prefer to stay along with the arena's walls and in darker areas. An observation exhibits that stressful mice show anxiety-like behavior and spend minimum time in bright and central area zones. These depressed mice think of themselves as safe in the center zone. Treatment of mice with Fluoxetine and Fluoxetine incorporated liposome nanoparticles show a remarkable change in mice's anxiety-like behavior. The treated mice spend more time in central and brighter zones as compared to depressed mice. This can be seen in the figure. The figure shows a positive response having nanoparticles treatment, and it gives the best results.

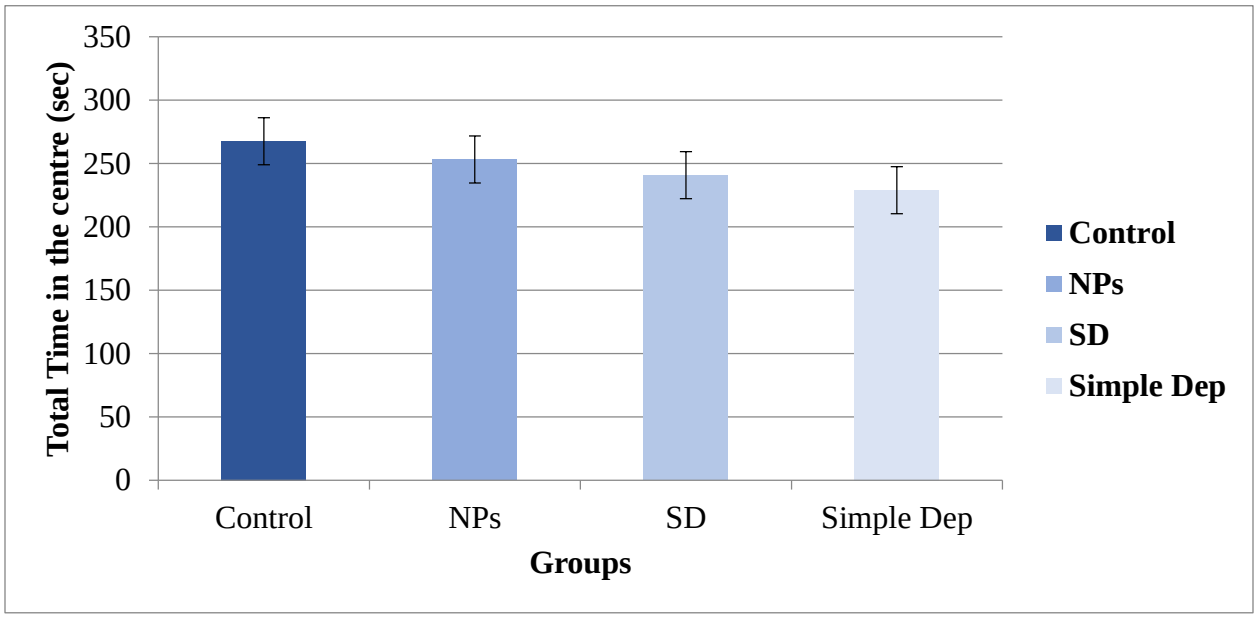
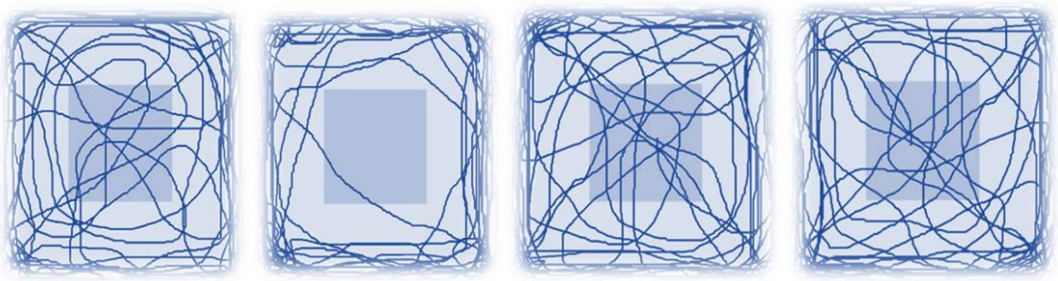


Figure 14: Open Field Test (OFT) results of mice subjected to Chronic Mild Stress (CMS). Graphical Results Format



Control Mice Depressed mice NPs treated Mice SD treated drug

Figure 15: Open Field Test (OFT) results of mice subjected to Chronic Mild Stress (CMS). Results of the mice tracking software

This software-generated shows the path taken by mice. The box is divided into two zones, inner and outer zone. Any depressed mice would not like to explore the box's inner zone or the open field area. Any normal mice would like to explore the inner zone/open field as much as the outer zone. Here it can be seen that only depressed mice did not explore the inner zone/open field; instead, all the other groups were normal enough to explore the open field area.

(ii) Forced Swim Test (FST)

A forced swim test is performed to access the anxiety-like behavior and stressful condition of mice. The recording of almost 5 minutes is made after the mice are subjected to the forced swim test and analyzed the last 3 minutes of mice activity. The First 2 minutes are too active for mice, and the last 1 minute was very aggressive. This showed the effects of treatment. Moreover, mobility time and immobility time is also observed during this test. The higher immobility time shows the more depressed mice, and higher mobility time shows the more active mice. It can also be seen that the mice treated with fluoxetine and liposome nanoparticles shows more mobility time and positive response as compared to depressed ones.

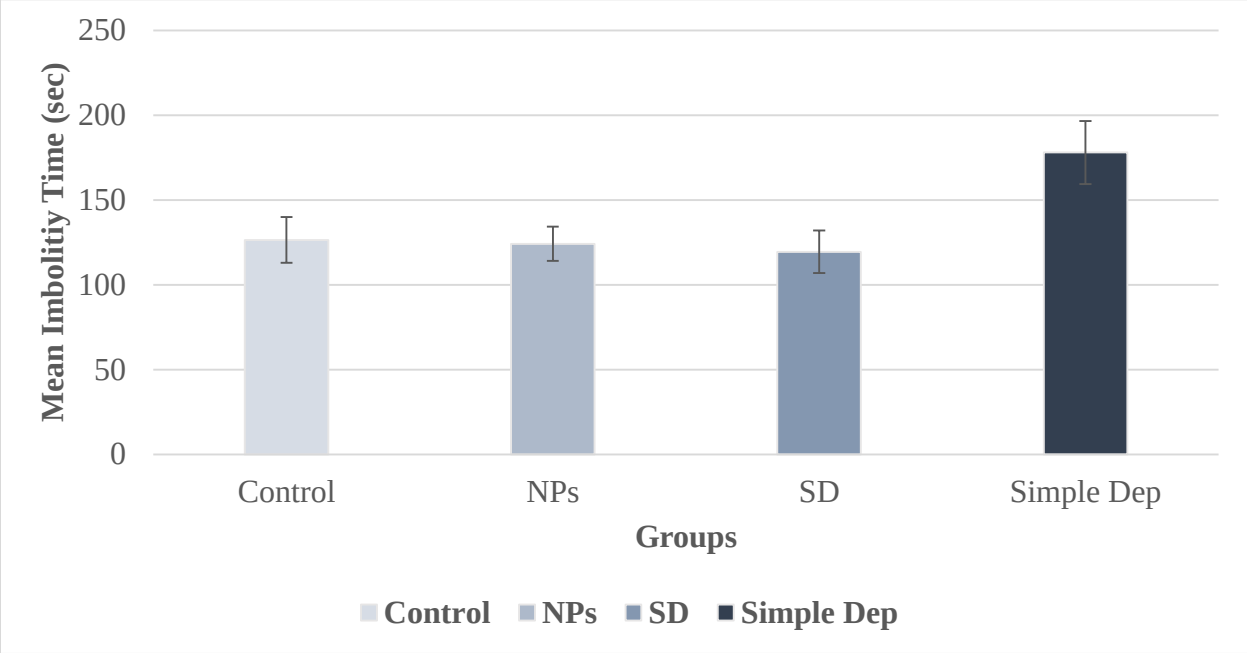


Figure 16: Forced Swim Test results subjected to Chronic Mild Stress (CMS)

(iii) Tail Suspension Test (TST)

A tail suspension test is performed to determine stress and anxiety-like behavior in animals. Tail climbing behavior and the whole activity during the tail suspension test show the mice's stress and depression-like condition. The results are recorded by using the average immobility time of each mice. The more active mice have higher mobility and low-stress levels and vice versa. The results of the tail suspension test can be observed in depressed mice too. The depressed mice show higher immobility time as compared to treated mice and control group mice. Moreover, the results exhibit a positive treatment response having nanoparticles treated mice compared to other non-treated mice.

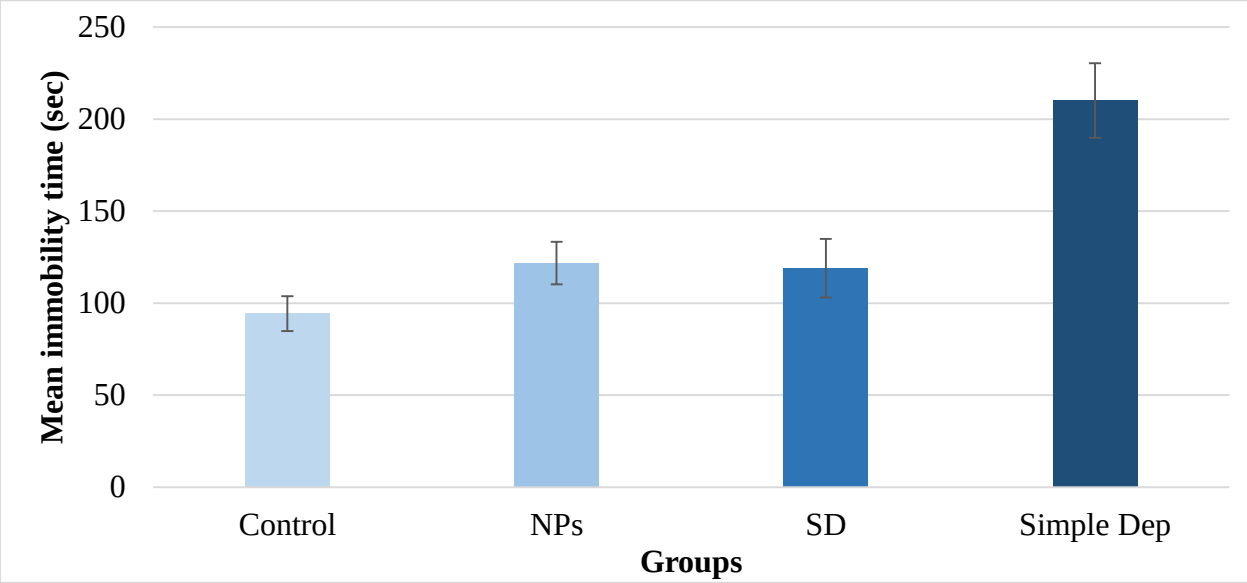


Figure 17: Tail Suspension Test results subjected to Chronic Mild Stress (CMS)

CONCLUSION

Depression is one of the most critical mental health conditions affecting almost every person once in their whole lifetime. This research's main objective and purpose were to design liposome nanoparticles that can act as an efficient tool for the treatment of this critical mental health condition, depression. Many antidepressants are used overall globally, but the selective permeability of the blood-brain barrier makes a hurdle in the treatment of depression; therefore, limiting the benefits of these medications. The solution for this ailment is provided by Nanotechnology that has changed the world of science and medicine. Nanotechnology involves developing and fabricating an excellent nano-sized structure that can pass through the selective membrane of a biological system. Thus, it delivers the required quantity of medication during the treatment.

One of the most used antidepressants known as fluoxetine hydrochloride was used in this research after being encapsulated inside DPPC liposome nanoparticles. Liposomes are preferred to be used and the best choice due to the resemblance with the natural biological system. After that, the chronic mild stress (CMS) model was developed by inducing depression and anxiety-like behavior in which mice were subjected to different stressors day by day. Behavioral tests were performed to confirm the results of the depression model and check out the treatment test results. The results concluded that Fluoxetine incorporated liposome nanoparticles exhibited a reversal of anxiety-like behavior and symptoms; therefore, serving as the best treatment option for depression.

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