Administration of Fluoxetine incorporated Liposomal Nanoparticles in the treatment of Learned Helplessness model of Depression

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2019

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I certify that this research work titled "*Administration of Fluoxetine incorporated Liposomal Nanoparticles for the treatment of Learned Helplessness model of Depression"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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ABSTRACT

Depression is one of the common leading mental health disorders that does not have a specific criterion of symptoms but negatively affects a persons' mood, way of thinking and living. According to WHO few symptoms of depression can be described as sadness, loss of interest, being miserable, or suicidal thoughts. Depression is treatable with a number of different kind of antidepressants but sometimes these antidepressants can cause more adverse effect rather than treating the depression, blood brain barrier is one of the main hindrance that antidepressants face, it's a semipermeable membrane separating the blood from the cerebrospinal fluid that doesn't let certain molecules or drug pass through blood brain barrier hence certain antidepressants fail to work.

The aim of this research was to see the effects of fluoxetine and fluoxetine incorporated DPPCnanoparticles in Learned Helplessness mice model of depression. Peg coated and uncoated Fluoxetine incorporated Liposomal nanoparticles using DPPC, cholesterol, PEG 6000 were made via Thin Film Hydration method. Nanoparticles were subjected to characterizations before being injected into the depressed mice via intravenous route.

This research concludes that liposomal nanoparticle containing fluoxetine can be an effective for treatment of depression with lesser side effects than only fluoxetine. Therefore, these liposomal nanoparticles could be an effective way to treat depression with lesser side effects and having early onset of action.

Key words: *depression, mice model of depression, learned helplessness, liposomal nanoparticle*

CHAPTER 1: INTRODUCTION

1.1. Major depression disorder

Major Depression Disorder (MDD) is a draining mental disorder, that affects mainly a persons' ability to think and act accordingly, mostly categorized by hopelessness, low and gloomy mood, disturbed sleep, disesteem and hallucination suicidal thoughts or mood swings. When feelings of sadness, helplessness, and worthlessness and mainly hopelessness, when it all becomes lifestyle and keeps a person from participating in life, it may be something more than sadness. It may turn into clinical depression. It's a serious condition that affects physical and mental health. (Kurlowicz, 2007)

Different people experience depression in different ways because depression cannot only be described in a number of symptoms. Depression can interfere with daily work which results in no productivity and being lazy, overall no interest in regular activities like before. It can also interfere with relationships and can affect physical and especially mental health. The successive changes of mood are a significant characteristic sign of depression. (Gaynes *et al.*, 2019)

Many people may not feel these highs and lows of mood at all and live a normal life as to be seen from the outside but still can develop anxiety and depression. As, anxiety and depression go hand in hand anxiety leads to development of depression, suicidal thoughts. It is more than just a cycle of the blues, depression isn't a weakness or mood and a patient can't simply snap out of it or make themselves feel about their situation. Depression may require life-long treatment. Most people with depression feel better with trial and error medication, or with therapy or both (Davis *et al.*, 2008) According to FDA almost 45 studies' showed that 52% of new antidepressants failed to work because patients failed to deliver all the symptoms that were required for prescribing that key antidepressants for that particular set of symptoms. (Kobak *et al.*, 2007)

1.2. Treatment of Depression

Developments in the drug treatment of depression has been actively pursued.

Grouped into various classes of drugs with slightly different mechanisms of action, antidepressants are widely used as a treatment option(Harmer et al., 2017). Four classes of antidepressant drugs are widely known i.e. monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), serotonin reuptake inhibitors (SSRIs) and norepinephrine (NE)–serotonin (5HT) reuptake inhibitors (Gundersen et al., 2013). Among these drugs, SSRIs are the most excessively prescribed treatment medications for major depressive disorders. They are thought to relieve depression symptoms by a number of mechanisms that include increasing serotonin levels in the brain. SSRIs inhibit serotonin reuptake transporters at presynaptic terminals and thereby sustain levels of serotonergic neurotransmitter levels at postsynaptic clefts (Benfield et al., 1986; Murdoch and McTavish, 1992; Perez-Caballero et al., 2014).

Regardless of class, antidepressants need to penetrate the BBB to reach their site of action within the brain. Despite of having various advantages, treatment by antidepressents is often constrained by reduced permeation of therapeutic agents into the central nervous system (CNS). A vast majority of bioactive agents do not readily permeate into the brain tissue due to the existence of the blood-brain barrier (BBB) and the associated P-glycoprotein efflux transporter (O'Brien, 2011). Apart from this, high expression of P-gp on intestinal epithelium may decrease rate and concentration of drug diffusing across the basolateral membrane and entrance into general circulation from the intestine (Nasar 2009). Since oral route is the most preffered route for administration of antidepressant drugs, it is vital to overcome the absorption barrier posed by the P-gp efflux transporter(Nasar 2009; Hoosain 2015).

1.3. Polymer coated Liposomal nanoparticles and depression

To treat depression is to cross the blood brain barrier, blood brain barrier (BBB) is a complex system made up of closed linked cells, tight junctions, endothelial cells. All these junctions does not allow the entry of any drug in high concentrations only water, few gasses cross this barrier. In order to treat depression a drug must cross this BBB barrier, nanoparticles provide a better solution in this regard due to their small size they are able to cross blood brain barrier with drug trapped inside them and basically target the

area where drug is needed to be delivered. Different anti-depressants combine with different nanomaterial for their specific affects. (Dimitrijevic and Pantic, 2014)

To deliver drugs in CNS system lipid based nanoparticles, solid lipid nanoparticles, cationic nanoparticles, Nano-emulsions and polymeric nanoparticles are frequently used. These nanoparticles are able to cross blood brain barrier via the positive charge to negative charge attraction and deliver drug right at the target without the loss of drug. Nanoparticles provide a great deal of flexibility protein, peptides and other molecules can also be bound to the surface of nanoparticles. Oil, triglycerides and water is also transported to CNS region via nano-emulsions. Polymers like polylactic acid, poly D, polylactide-co-glycolide (PLGA), L-glycolide (PLG) and poly cyano-acrylate (PCA) these polymeric nanoparticles may provide better results. (Deore, Shahi and Dabir, 2016)

Different nanoparticles with different antidepressants provide vast results against depression all these antidepressants nanoparticles has been tested on mice and rats model and they have reversed the depression like symptoms.

The research work in this dissertation has been presented in two parts. The first part of the research focuses on the 'Learned Helplessness Model of Depression' in mice. Learned helplessness is a commonly used model of depression in which animal/mice is subjected to uncontrollable stress. It mainly focuses on changes in behavior analysis of depressed animal. In this study Depressed mice via learned helplessness model seems to not avail the opportunity to escape a difficult situation even if it is presented to them hence concluding the mice has been successfully depressed and their treatment via liposomal nanoparticles could help them reverse the effects of learned helplessness depression. Herein, we describe the preparation and characterization of two fluoxatine incorporated liposomal formulations one of which was coated with PEG (PEG6000) and the other without polymer coating. Liposomal formulations were than characterized using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Drug loading and encapsulation efficiency, and drug release.

Finally these nanoparticles were delivered in depressed mice to reverse the learned helplessness analysis. Depressive symptoms were monitored by weight comparisons and behavioral tests. The main objective of this dissertation is to provide a safer, more

effective way to treat depression with lesser side effects by using nanoparticles for depression.

1.4. Aims & objectives:

The main aim of this study is to make sure the fluoxetine incorporated liposomal nanoparticles are able to cross blood brain barrier. As crossing the blood brain barrier ensures the targeted drug delivery that will have an early onset of effect.

Main objectives are provided below:

- Preparation and characterization of PEG coated and uncoated Fluoxetine loaded liposomal nanoparticles.
- Developing mice model of depression (learned helplessness) via heat stress that will exhibit good face validity, construct validity and predictive validity.
- Intravenous administration of Fluoxetine loaded liposomal nanoparticles in depressed mice.

CHAPTER 2: LITERATURE REVIEW

2.1. Fluoxetine

Fluoxetine is one of the most successful anti-depressant. It belongs to the class selective serotonin reuptake inhibitor (SSRI) that is approved by Food and Drug administration. Fluoxetine has higher efficacy and lesser side effects than the other classes of anti-depressants; tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), it can be given for a number of reasons including panic disorder, bulimia and binge eating problems. (Haque *et al.*, 2014)

The main reason that fluoxetine was and is still preferred over other anti-depressants is its safety profile. It is suitable for children above 8 years old to older people and even in during pregnancy. Fluoxetine under brand name 'Prozac' has been globally known and helped a great deal in order to raise awareness against depression. (Wenthur, Bennett and Lindsley, 2014) Uses of Non FDA approved fluoxetine are for social phobia, borderline personality disorder, PTSD (post-traumatic stress disorder). (Abouhussein *et al.*, 2018)

Fluoxetine has been studied vastly in animal models of depression and has been able to reverse the depression like symptoms from rodents and other animals

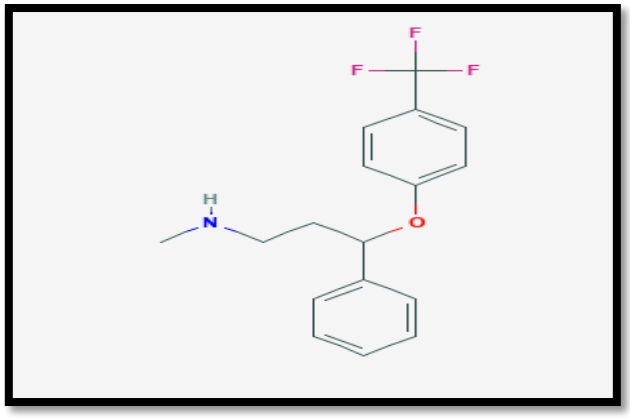


Figure 1 : Chemical structure of Fluoxetine

2.2. Mechanism of action

Biological amines serotonin and norepinephrine plays a major role in depression development. Patients with depression have low level of serotonin in cerebrospinal fluid and even lower serotonin uptake sites in depressed people. Pre-synaptic terminal of serotonin (5HT1A) is located in prefrontal cortex.

Fluoxetine works by blocking the reuptake transported protein in the pre-synaptic terminal which in turn blocks the reuptake of serotonin in pre-synaptic and enhances the action of serotonin on $5HT_{IA}$. (Cao *et al.*, 2019) fluoxetine shows quick result than other antidepressants because of its activating effect which happens by the reuptake of serotonin, its half-life is 2-4 days hence results can be seen in 2-4 weeks. Norfluoxetine; is an active metabolite of fluoxetine which has a bigger half-life of 4-9 days and is it produced when cytochrome P450 enzyme (CYP2D6) acts on it. (Robertson and Dodd, 2019)

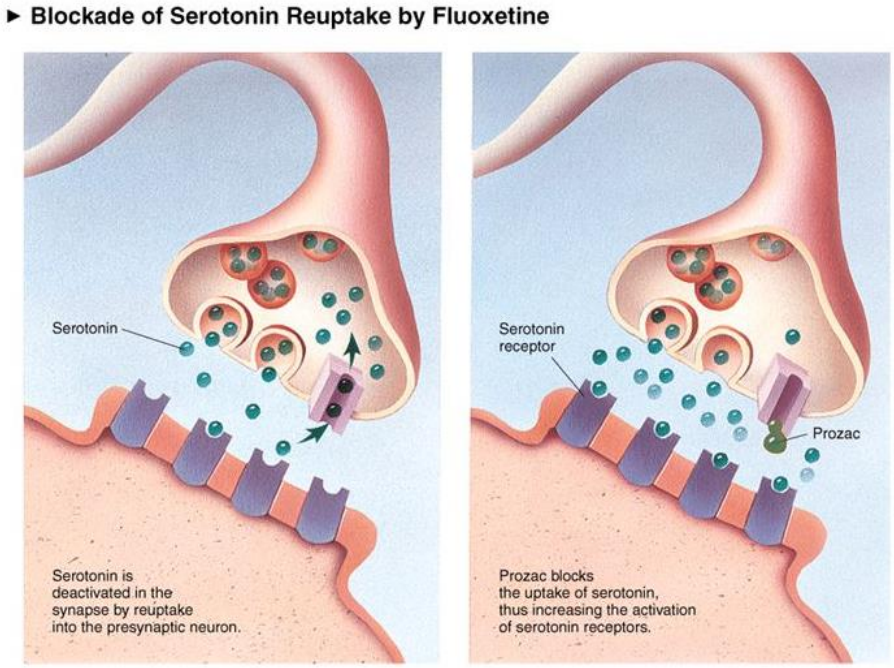


Figure 2: Mechanism of fluoxetine(Wenthur, Bennett and Lindsley, 2014)

2.3. Application of nanoparticles in biomedicine

` Nanoparticles are of fundamental importance in nanofabrication because of their great scientific impact, their intense life changing affects on biomedical, optical, electronic fields and many other fields. Nanoparticles have unique properties such as a large specific surface area and consequently greater reactivity than macro-sized particles. They encompass wide vaierty of products under a one dimension and atleast less than 1-100nm in size. They have different physiochemical properties and colours depending on the material they are synthesized with. (Khan, Saeed and Khan, 2017) With these nanostructures drug delivery has been changed for the better more focused and controlled drug release drug delivery to a targeted area. Due to their small size penetration between and into the cell, blood brain barrier makes them feasible for brain targeting as well.

2.4. Nanomedicine and treatment of depression

The success of nanotechnology has made a significant impact on clinical therapeutics in the last two decades (Rajesh *et al.*, 2016)and huge advancements have been done in developing the field of Nano medicine in brain disorder's studies to detect, diagnose and effectively treat brain related disorder. (Bozzuto, 2015)

Nano medicine as per national institute of health is a formulation of drug whose end product's size is less than a micron (Dimitrijevic and Pantic, 2014) Nano medicine has gained much advantage because of its ability to overcome biological barriers, enhances the bio-availability of drug, by even crossing blood brain barrier (Dong, 2018) it effectively deliver hydrophobic therapies, and preferentially target disease sites (Dailly *et al.*, 2004)

The nanoparticles are the small unit whose dimensions almost resembles to the building blocks of biological macromolecules such as proteins and DNA, this feature give a benefit to nanoparticles of being used for therapeutic purpose. Surface functionalization of nanoparticles can be done by various functional groups, signaling molecules, targeted molecules to make it target specific.(Mittal *et al.*, 2014) It can also be made biocompatible by binding with various functional groups and also it is conjugated with drug to be used as drug delivery vehicle. (Timbie, Mead and Price, 2015) The

surfaces of nanoparticles can be modified in such a manner so as it can bind to various functional groups that defines the fate of the nanoparticles that where should it be targeted.

One of the biggest applications of using nanomaterials as biomedicine is the it has an internal core or void where the drug or the material to be targeted is encapsulated. So, not only the toxicity caused by the drug is minimized but also sustained release of the drug is achieved. Nanoparticles encapsulate radiolabelled molecules and other small molecules in its internal core or void to be used in imaging techniques. Such molecule encapsulated by nanoparticles does not cause harmful effects in the rest of the body due to its target specificity and is also biocompatible. (Kaur *et al.*, 2014)

2.5. Liposomes as an efficient drug delivery system

Liposomes are special delivery vehicle, microscopic closed spherical vesicles; that consist of an internal aqueous part surrounded by one or multiple lipid bilayers. Lipid bilayer mimics biological membrane and save the drug from being attacked till reaching the desired target. These lipid bilayers that surround the aqueous phase in which drugs can be present. The liposome diameter varies from 400 nm to 2.5 mm along with nanoparticles (NPs) varying in size from 1 to 100 nm, together they exhibit exceptional chemical and physical properties that are subjugated for drug delivery by conjugation with drugs. (Mura *et al.*, 2018) Liposomes have been broadly studied and currently are used in the treatment of several diseases.

Liposomes membrane have unique bilayer-structure like properties, they can sort out and solubilize both hydrophilic and hydrophobic materials they can be used as transferors for both water-soluble, lipophilic molecules is composed of natural with combination with synthetic lipids which are moderately biocompatible and biodegradable, non-immunogenic substance. (De Jong and Borm, 2008)

Lipophilic drugs because of their nature, usually conjugates within lipid bilayers. This major factor helps in the bio-distribution and pharmacokinetics of the drug. Liposomes are of great valued for their biological and technological benefits, and are considered to be the most successful drug vehicle up till now. (Bozzuto, 2015)

Liposomes maximizes the drug absorption which leads to better pharmacokinetics and pharmacodynamics along with minimizing rapid degradation and controlling many side effects and reducing toxicity, prolonging the half-life of entrapped drug. (Mura *et al.*, 2018)

Along these properties of liposomes and their surface modification make it the most effective drug delivery system. High interstitial pressure causes low solubility of drug and rapid clearance of intravenously administered drugs from the blood circulation hinders the acceptable uptake of drugs in diseased region. Liposomes provide a better way for the drug delivery system because of the flexibility of their chemical composition, structure. (Lamichhane *et al.*, 2018)

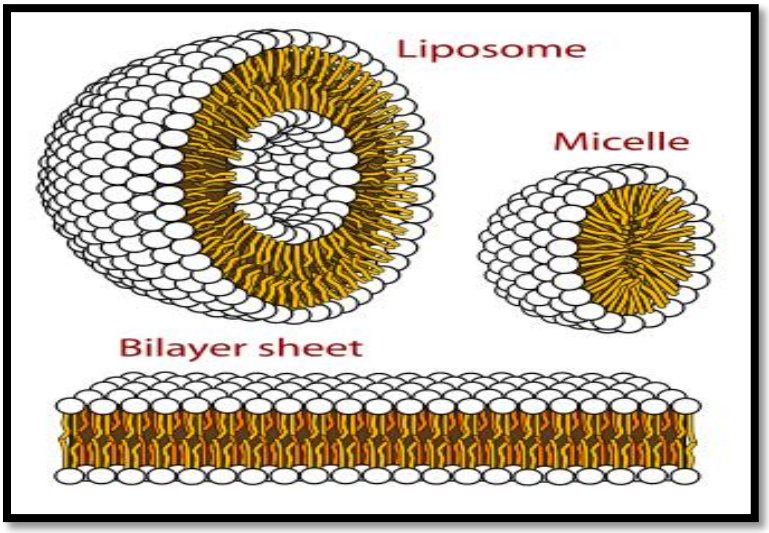


Figure 3: Types of liposomes

2.6. Conventional and non-conventional liposomes

Liposomes and lipid nanoparticles (LNPs) are similar in nanofabrication, but slightly different in function and composition. Both are lipid nanoformulations and excellent at drug delivery system, transporting drug/gene/protein of interest within a protective, outer layer of lipids mimicking membrane bilayer for the safety of substance inside. In application LNPs can take a variety of forms.(Journal *et al.*, 2017)

LNPs are liposome-like structures encapsulating a wide-ranging variety of nucleic acids DNA,RNA and as such they are the most popular non-viral gene delivery system.

Conventional liposomes include one or more layer of lipid bilayer surrounding an aqueous region, but not all LNPs have an attached bilayer that would make them as lipid vesicles or liposomes. Some LNPs have a micelle-like structure, encapsulating drug molecules in a non-aqueous core. (Malam, Loizidou and Seifalian, 2009) liposomes have many advantages that include high efficiency encapsulation efficiency regardless of drug solubility, low toxicity, drug protection against degradation factors like pH and light and the reduction of tissue irritation. (Laouini *et al.*, 2012)

Pharmaceuticals, cosmetics and food like other many bioactive substances can be incorporated into liposomes. (Malam, Loizidou and Seifalian, 2009) Liposomes properties such as biodegradability, biocompatibility combined with their nano size increases efficiency in nanomedicine, food industry and cosmetics. Liposomal nanoparticles can carry both hydrous phases and lipid in the structure of liposomes, which in return leads to vast variety of modification in the drug delivery, encapsulation and release of lipid-soluble, amphiphilic ingredients, drugs and biological molecules like peptides or genes. Hence they have many applications from cosmetics to medicine .(Journal *et al.*, 2017).

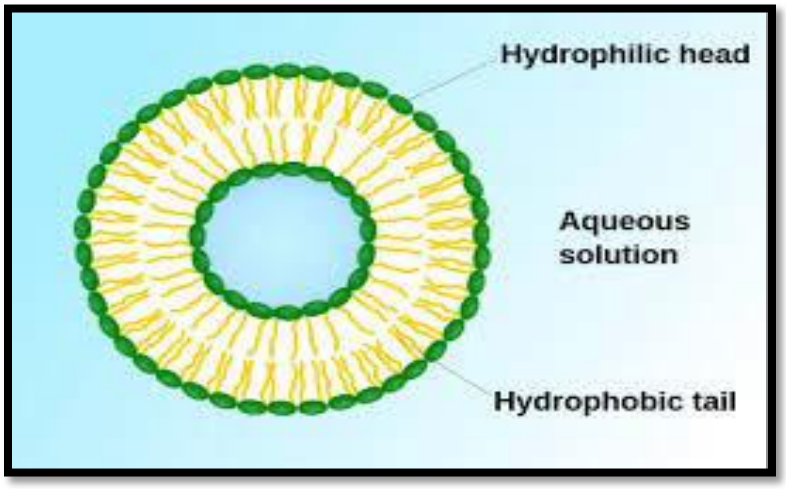


Figure 4: Liposomal nanoparticle (Bozzuto, 2015)

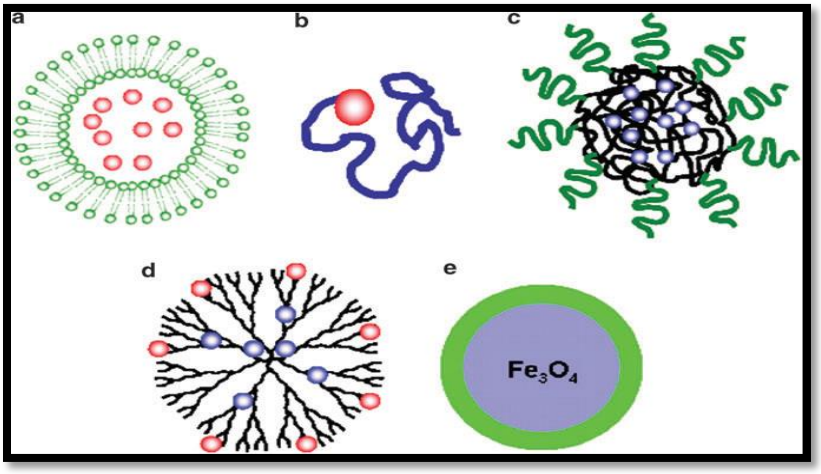


Figure 5: Example of Polymer coated Liposomal nanoparticle (Bozzuto, 2015)
(a) liposome, (b) polymer–drug conjugate, (c) polymeric nanoparticle, (d) dendrimer, and (e) iron oxide nanoparticle. Red dots are hydrophilic drugs and the blue dots are hydrophobic drugs. (Zhang *et al.*, 2008)

The most important property of using nanoparticles in medicine and diagnostics is its biocompatible nature. (Couvreur, 2013) The outer surface of nanoparticles are modified by binding small functional group molecule or encapsulating it with polyethylene glycol (PEG) to make it biocompatible and so it do not awake the immune reactions and so other inflammatory processes as well and is considered as self-molecule.

Surface functionalization of nanoparticle with small functional group molecules or with other ligands make nanoparticle highly targeted, also the controlled and sustained release of drug is because the surface group attached.(Tong, Qin and Sun, 2017)

Furthermore, the functionalization has a lot to do with the bio-distribution of drug and plays an important role in its pharmacokinetic behavior. Nanoparticle surface modified by small functional group plays an important role in the mode of excretion of nanoparticle from the body and also its bio-distribution gives an idea of the type of clearance the nanoparticle follows (Swati Deore, S. R. Shahi, 2018).

2.7. Current treatment methods for depression

Antidepressants are majorly used treatment method for depression as they are able to cross the blood brain barrier but sometimes they gets expelled by PGP in brain, leaving very little amount of antidepressant at targeted place (Lamichhane *et al.*, 2018). Other methods include nanoparticles as they have more targeted delivery and many other methods as descried below in table 1.

S. no	Active transporters	• Transportation of drug through endogenous amino acids • They are able to cross blood brain barrier with ease. • Prodrugs can be attached to them as can be transported across blood brain barrier. (Tong, Qin and Sun, 2017)
1	**Passive transport**	• Through the hydrophobic effect drugs are fused within the inner cavity of the structure. • Nanostructure materials are targeted to particular sites in brain, the specifically calculated amount of the drug is released to the targeted area.(Dailly *et al.*, 2004)
2	**Self-delivery**	• The drugs that are needed to be delivered in the system are openly conjugated to the carrier nanostructure material for easy release. (Dailly *et al.*, 2004)
3	**Exosomes**	• Their non-immunogenic nature is the most beneficial aspect, resulting in a long and stable circulation. • Exosomes deliver proteins, small molecules and nucleic acids to cross the blood brain barrier. (Bozzuto, 2015)
4	**Electroporation**	• Electroporation uses electrical pulse to generate temporary pores within the plasma membrane permitting gene transfer DNA into cells. (Rajesh *et al.*, 2016)
5	**Nanostructures**	• It has the benefits of multifunctionalization, ability to carry drug to the targeted area , controlled drug release. (Journal *et al.*, 2017).
6	**Intranasal**	• Direct delivery is through nose to brain's olfactory region • It crosses barrier of blood brain easily and is target specific • It could be in form of nano emulsion or in-situ gel form: they both prevent the drug from first pass metabolism hence higher bioavailability. (Bozzuto, 2015)
7	**Non-invasive**	Self-emulsifying drug delivery is a mixture of oil, drug, surfactant, and co-surfactant. Self-micro emulsifying drug delivery system (SMEDDS) is indeed SEDDS that can form fine oil-in-water droplets usually less than 50 nm in diameter size, without being digested, under mild agitation of the gastrointestinal tract. (Dimitrijevic and Pantic, 2014)

Table 1: Different current methods for treatment of depression

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials:

16:0 PC (DPPC) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine powder, Cholesterol Sigma Grade, ≥99%, Fluoxetine hydrochloride, MilliQ water, Ethanol. Double distilled water. All chemicals were purchased from Sigma-Aldrich (USA).

3.2. Part 1: Synthesis and characterization of Drug loaded liposomal nanoparticles

3.2.1. Preparation of uncoated liposomal nanoparticle

Fluoxetine was dissolved in ethanol by the concentration of 0.1mg/ml. lipid phase was prepared by concentration of 100 *u*mo*l*/ml by dissolving DPPC and cholesterol in ethanol as 4:1 in 10ml ethanol. 500 ul of fluoxetine from previous mixture was added in lipid phase and then sonicated for 45 minutes. 10ml of MilliQ water was placed in water bath to reach the temperature of 60 ^{o}C along with sonicated suspension. After warming up to the temperature of 60 ^{o}C both phases was mix with each other and the mixture was shaken in water bath for further 15 minutes. The mixture was poured into round bottom flask and rotatory evaporated to get rid of ethanol. Nanoparticles were ready after this evaporation and stored at 4^{o}C in glass vial.

3.2.2. Preparation of PEG coated nanoparticles:

PEG coated FLX -liposomal nanoparticles were also prepared by using modified ethanol injection method (Chorachoo et al., 2013). Fluoxetine hydrochloride was dissolved in enough amounts to absolute ethanol to attain a final concentration of 0.1mg/ml. For the preparation of the lipid phase at a concentration of 100 µmol/ml , DPPC and cholesterol were dissolved in 10ml ethanol in a ratio of 4:0.75. 500µl of fluoxetine from previous mixture was added in lipid phase. 2.5 mg/ml PEG 6000 was added drop wise to the solution and solution was stirred for 1 hour on magnetic stirring. The mixture was then subjected to sonication (Branson Sonifier® SFX250, Danbury, USA) for 45 min. 10ml of Milli-Q water was placed in water bath that was previously set at a temperature of 60oC

along with sonicated lipid and drug suspension. After warming up to the temperature of 60oC, the water phase was emulsified with the lipid phase and the resulting suspension was shaken in water bath for 15 minutes to allow even mixing and phase inversion. The resultant mixture was then transferred into a round bottle flask which was then connected to a rotary evaporator (Eyela Rotary Vacuum Evaporator N-100 series, Jeol, Japan) to evaporate ethanol. Subsequently, the cloudy suspension of fluoxetine incorporated liposomal nanoparticles was then poured into a glass vial, sealed, and put in storage till further use.

3.3. Characterization of Fluoxetine incorporated liposomal nanoparticles:

The characterization of uncoated and PEG coated Fluoxitine-liposomal nanoparticles was done to evaluate and analyze their particle size and surface charge, drug encapsulation efficiency and release efficiency, conduct further characterization tests for nanoparticles.

3.3.1. UV-Vis absorption spectroscopy (UV-Vis)

UV-Vis absorption spectroscopy is one of the most widely used techniques in both clinical and chemical laboratories. It actually is the measurement of extent of absorption that occurs in sample when a beam of light passes through it and from the reflected beam the absorption is measured. In UV-Vis spectrophotometer, a beam of light is split where one half of the beam is directed through the cuvette containing the sample being analyzed and the other half is directed to a cuvette containing the solvent only (reference). Absorption can be measured both at specific wavelength and at a desired range and a spectrum is obtained that plots entire range of wavelength versus its absorption at specific wavelength. The maximum absorption at specific wavelength is called as lambda max. It measures the electronic transition of molecules and obeys the principle of Beer Lambert Law. The absorbance of the sample is proportional to the molar concentration in the sample cuvette, the absorption value known as the molar absorptivity is used when comparing the spectra of different compounds. 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 makes UV-Vs absorption spectroscopy useful for quantitative analysis.

3.3.2. Particle Size and Area Distribution:

To analyze the practical size, scanning electron microscopy (SEM) was used. The particle size as determined by the scanning electron microscopy was further analyzed and area distribution of the nanoparticles was calculated by using image j software. Analysis was performed on a selected area. The command 'Analyze Particles' counts and measures objects in binary or threshold images. It works by scanning the image or selection until it finds the edge of an object. It then outlines the object using the Wand Tool, measures it using the Measure. . . [m] command, and then resumes scanning until it reaches the end of the image or selection. Features of threshold images can be extracted by specifying suitable Size and Circularity ranges and by choosing if particles should be traced by their outer edge or by flood filling. For Size of particles, values were given between the range of 0 and 'Infinity'. Particles with size (area) outside the range specified in this field are ignored. Circularity ranges were given from 0 (infinitely elongated polygon) to 1 (perfect circle). Particles with size circularity values outside the range specified in this field are also ignored. 8–bit binary image containing the best fit ellipse (cf. Edit . Selection . Fit Ellipse) of each measured particle (gray levels: Ellipses: 0; Background: 255) was analyzed.

3.3.3. Fourier transforms infrared spectroscopy analysis (FTIR) analysis:

Fourier Transform-Infrared Spectroscopy (FTIR) is an analytical technique used to identify organic (and in some cases inorganic) materials. This technique measures the absorption of infrared radiation by the sample material versus wavelength. The infrared absorption bands identify molecular components and structures.

When a material is irradiated with infrared radiation, absorbed IR radiation usually excites molecules into a higher vibrational state. The wavelength of light absorbed by a particular molecule is a function of the energy difference between the at-rest and excited vibrational

states. The wavelengths that are absorbed by the sample are characteristic of its molecular structure. (Khan, Saeed and Khan, 2017)

FTIR analysis of DPPC, cholesterol, FLX, and PEG coated FLX-liposomal nanoparticle was carried out. KBr pellet were prepared for each sample and analyzed by using FTIR Spectrophotometer, equipped with software OMNIC™ Version 6.0 a; Thermo Fisher Scientific, Waltham, MA, USA). Infrared spectra was than recorded between 4000–400 cm−1.

3.3.4. Zeta Potential:

The zeta potential is the potential difference across phase boundaries between solids and liquids. It's a measure of the electrical charge of particles are that are suspended in liquid. Since zeta potential is not equal to the electric surface potential in a double layer or to the Stern potential, it is often the only value that can be used to describe double-layer properties of a colloidal dispersion. Zeta potential, also known as electrokinetic potential, is measured in millivolts (mV). Surface charge and zeta potential was known by zeta potential analyzer. Zeta potential tells about the stability, surface charge and average size of the nanoparticles.

In colloids, zeta potential is the electric potential difference across the ionic layer around a charged colloid ion. Put another way; it's the potential in the interface double layer at the slipping plane. Typically, the higher the zeta-potential, the more stable the colloid. Zeta potential that is less negative than -15 mV typically represents the beginnings of agglomeration of particles. When the zeta-potential equals zero, the colloid will precipitate into a solid.

3.3.5. Drug loading and encapsulation efficiency

3.3.5.1. Standard curve:

Different millimolar concentrations of fluoxetine were prepared by dissolving fluoxetine into ethanol. Standard curve was made by taking uv of these different dilutions and by plotting 263nm value against these millimolar concentrations. Y=mx+c equation's value was obtained.

3.3.5.2. Drug efficiency:

Drug efficiency tells about the drug entrapment within liposomal vesicles. After the preparation of nanoparticles, UV spectrum was taken and at 263 nm absorbance was observed. This absorbance was plotted in y=mx+c equation to get the concentration value which was then compared to initial drug concentration, in result it gave free drug present in medium.

3.3.5.3. Drug loading:

After the preparation of nanoparticles, they were centrifuged at 4500rpm in single column filter at 30 ^{o}C for 2.5 hours. Nanoparticle filterate was stored. Column was again refilled by PBS solution and centrifuged at 4500rpm at 30 ^{o}C for 2 hours. PBS filterate was stored at 4^{o}C. Column was again filled with distilled water and centriguged at 4500rpm at 30^{o}C. 500 *ul* of column's nanoparticles were put in pre-weighed eppindorf and weighed again.

3.4. Development of learned helplessness mice model of depression

Learned helplessness develops when a person or animal is subjected to adverse conditions and after trying to escape for quite some time they tend to think escape isn't possible and condition is uncontrollable so they do not try to escape or change the condition even if an escape is presented later on.

3.4.1. Subjects

Subjects were female 6 weeks old mice Adolescents Blab/c mice were purchased from NIH, Islamabad. These mice were made into groups randomly and acclimatized for about 2 weeks with constant supply of clean water and food. Mice were kept in Standard home cages (30 × 15 × 14 cm). Home cages were filled with fresh sawdust and a consistent 9:15 h light/dark cycle was kept. Temperature was maintained at 27°C ±2°C with humidity 50% ±5%.

3.4.2. Groups

These mice were divided into 5 groups after letting them acclimatize for 2 weeks with supply of water and food. Mice were divided into 5 groups. Control (group C),group

1(only fluoxetine), group 2(fluoxetine nanoparticles), group 3 (peg coated nanoparticles), group 4(no drug),

GROUPS	TREATMENT	
LH G-1	Nanoparticle	500µg/kg
LH G-2	PEG coated Nanoparticle	500µg/kg
LH G-3	Fluoxetine	10mg/kg
Control	No treatment	
SIMPLE DPRESSION	No treatment	-

Table 2: Groups of Mice based on treatment

3.4.3. Cages

Cardboard box of (18x18x30 cm^3) were used as an experimental box, divided into further two compartments with that were separated by gate with an opening built in. Mice could pass through it even provide an opening. The rest of the time they could not cross the partition that separated the hot and cold box.

3.4.4. Protocol:

I. Mice were divided into 5 groups. Control (group C), group 1(only fluoxetine), group 2(fluoxetine nanoparticles), group 3(Peg coated liposomes), group 4 (no treatment)

II. Mice were labeled by markers on their tails. One compartment of box was heated via hot plate. Temperature was allowed to reach till 32-37.5^oC (temperature was being raised by 2 degrees after every 3 days).One mouse out of group was placed in that high temperature for almost an hour in dark active period and was allowed to leave the high temperature to the normal one for about 15 minutes out of 50-60 minutes randomly, followed by a light beam indicating the opening, the rest of the time it was not allowed to leave. All the other mice were also went through the same rough condition/high temperature one by one. This protocol was carried out for almost 2 weeks. Group 1, 2, 3,4 were all passed through this protocol.

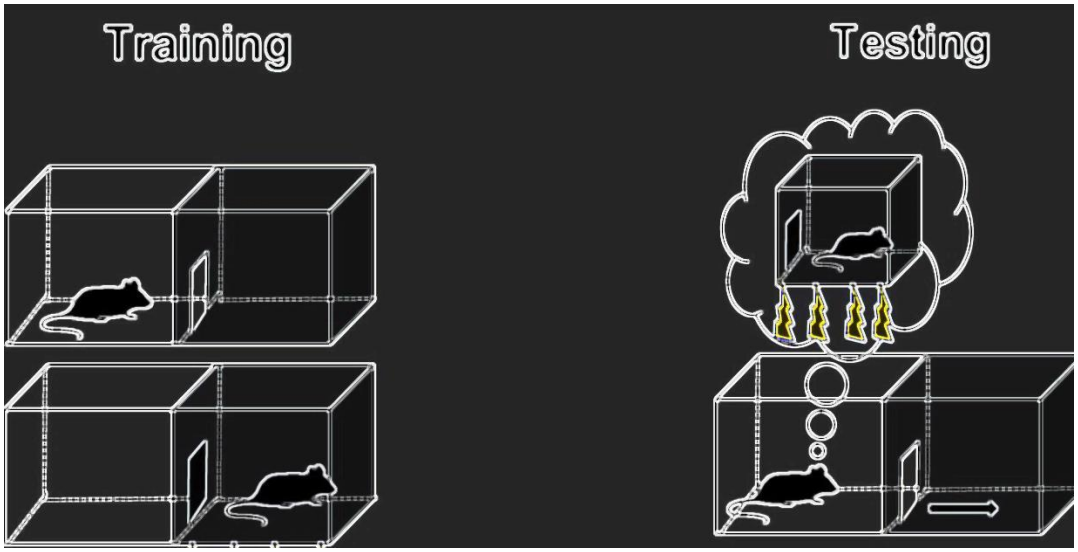


Figure 6: Learned helplessness protocol(Dailly et al., 2004)

3.4.4.1. Learned helplessness

I. In the beginning of protocol mice took the opportunity and left the opening provided but with the passage of time they started depressive like phenotype and showing avoidance, escape latency and failure.

II. Avoidance as mice were shown the light beam they immediately changed the compartment. Escape latency they were slow in escaping the hot environment. Failure being they were unable to escape the hot environment means those who failed had developed depression. Majority of mice were fallen into the escape latency and failure category.

After the development of depression mice started showing depressive like phenotype which included these and many other:

- Tail biting
- Jumping out of cages
- Huddling in corner
- Hiding under bedding
- Aggressive behavior
- Excessive grooming

Figure 7: Mice Huddled in corner

Figure 8: Excessive grooming

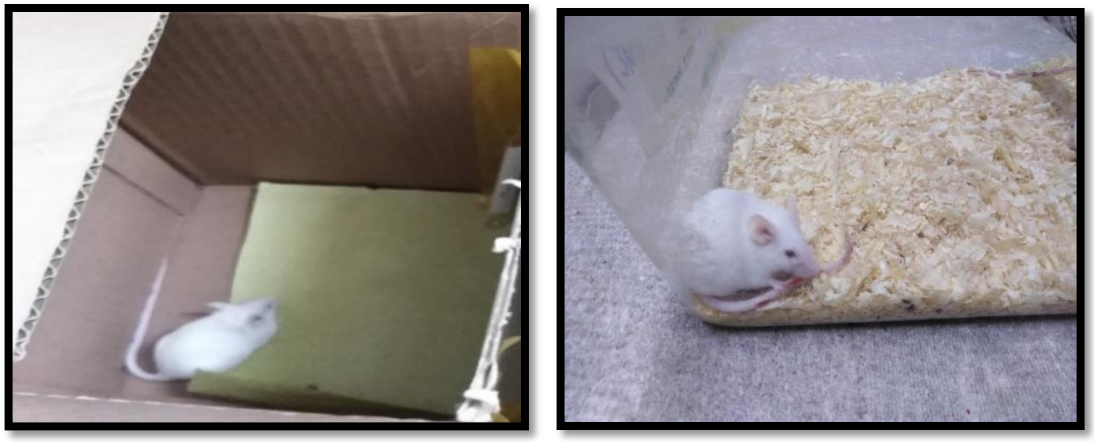


Figure 9: No escaping and tail biting

3.5. Behavioral Assessment:

Mice that showed depressive like phenotype were assessed through behavioral tests; forced swim test, tail suspension test and open field test.

3.5.1. Forced swim test:

A large glass tank was used to assess depression from forced swim test. Tank was half filled with clean water and let it reach room temperature. All the mice one by one were placed in water tank for total of 6 minutes, alongside control mice in different tank but with screen in between tanks. The last 4 minutes were assessed, control group mice stayed actively floating and swimming of all 6 minutes.

3.5.2. Tail suspension test:

All the mice were subjects to this test. Mice's tail were wrapped in adhesive tape and attached to metal wire for all of 6 minutes, last 4 minutes were observed for immobility. Mice that exhibit depressive like phenotype could not struggle to get themselves free, but mice that did not show depressive like phenotype were categorize as not depressed hence they struggled to get free and climbed their tail towards safety.

3.5.3. Open field test:

A large cubic box was used for this purpose. Each group's mice were placed on the bottom surface of box keeping the box uncovered from top and noted their movements. Later that movement is analyzed by software and result is generated.

3.5.4. Sucrose preference test:

Mice were given simple water and sucrose water to drink. Mice that showed depressive-like phenotype preferred simple water.

3.6. Treatment of depressed mice

As behavioral tests concluded mice were showing depressive like phenotype was indeed depressed. Hence, treatment was started LH G-1, LH G-3 and LH G-3 were given fluoxetine incorporated liposomal nanoparticle and PEG coated liposomal nanoparticles 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 hour.

Figure 10: Mice being injected (IV)

3.7. Outcome:

The immobility time from forced swim test and tail suspension test indicated depression-like symptoms in mice. Majority of the time in last 4 four minutes mice were immobile. And in open field test mice explored the outer region of the box they did not come in center region which explains depressed mice were more likely to hide in outer region

rather than exploring the open places (center region). All these behavioral tests showed under stressed conditions mice developed learned helplessness phenotype-like depression.

3.8. Histology

Mice were dissected and organs were collected brain, heart, spleen, kidney and liver. Slides of brain, liver, kidney, heart and spleen of H&E stain were prepared and organ embedded paraffin plates were obtained from Ali pathology lab, Islamabad. Slides were examined under a Labomed LB-200 Binocular Biological Microscope. Images were captured with magnification 40x, 100x and 400x by using Pixel Pro software for a Labomed biological microscope

CHAPTER 4: RESULTS

4.1. Particle size and Scanning Electron Microscopy

SEM results showed the uncoated nanoparticles size within 277nm and that size were further verified by image J software. Analysis was performed on a selected area. 8–bit binary image containing the best fit ellipse (cf. Edit . Selection . Fit Ellipse) of each measured particle and results were obtained.

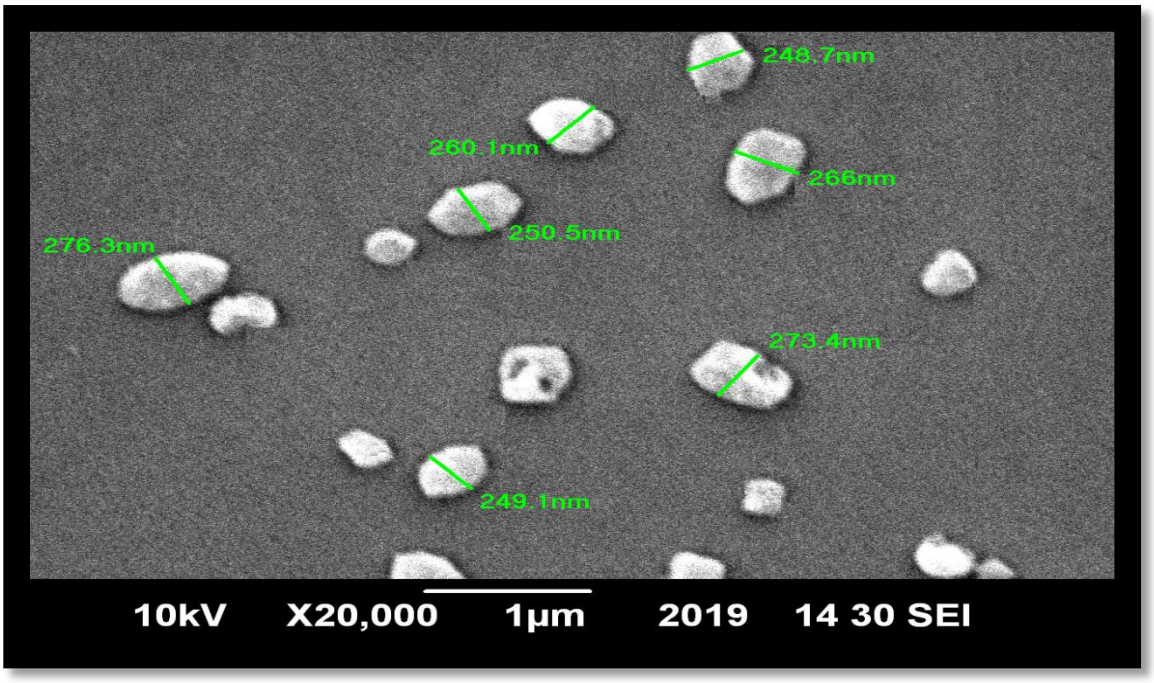


Figure 11: Nanoparticle; obtained from Scanning electron microscopy

4.2. Drug release kinetics

4.2.1. Standard Curve

As fluoxetine was dissolved in ethanol and different concentrations of fluoxetine was obtained. By taking UV of those concentration and collecting the UV's at 263nm standard curve's equation was obtained.

y=mx+c

y = 0.88x + 0.3704

4.2.2. Drug release

After uncoated nanoparticles being made they were mix with PBS (Ph 7.0) by equal volume. After every 30 minutes sample was taken from the mixture, centrifuged and UV was checked. After 48 hours all the points for 263nm were collected and graph was developed. It can be seen from the drug release graph drug released from nanoparticles over the course of 4 hours.

Drug release graph of PEG coated liposomes showed that drug released from the liposomes over the course of 8 hours.

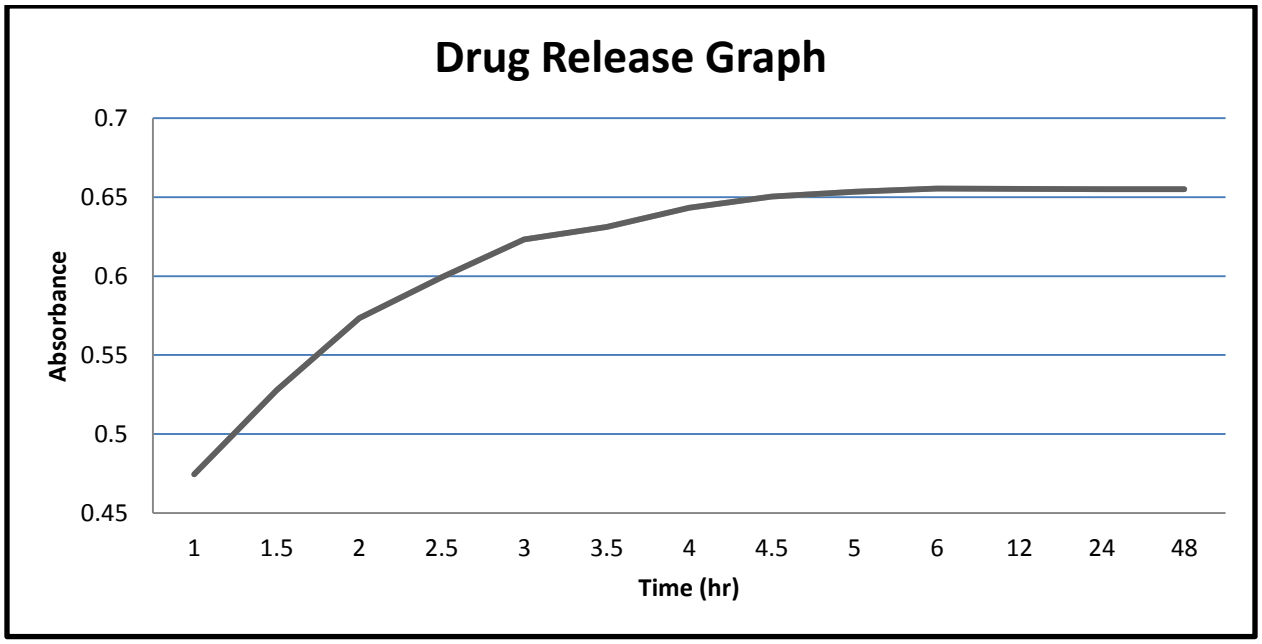


Figure 12: Drug release graph

4.2.3. Drug encapsulation efficiency:

After the preparation of nanoparticles UV was taken and after putting the values in standard curve the amount of free drug was calculated which was further put in encapsulation efficiency formula as:

According to the formula:

$$\text{E.E (\%)} = \frac{Total\ Drug\ added - Free\ non-entrapped\ drug}{Total\ Drug\ added}\ x\ 100$$

Therefore drug encapsulation efficiency by this formula is **70%**

by drug loading capacity's formula:

- $$\text{LC (\%)} = \frac{entrapped\ drug}{nanoparticles\ weight}\ x\ 100$$

Loading capacity is calculated to be **34%.**

70% EE tells us about the amount of drug successfully being incorporated inside the both coated and uncoated liposomal nanoparticles which will release in the body over the course 8 hours and 4.5 hours respectively.

4.2.4. Zeta potential:

Zeta potential is the charge that exists on the interface between solid particle in liquid medium. The average charge on the surface of nanoparticle is -16.0 and the zeta average 220.5 nm.

The value of zeta potential was recorded to be -16.0 mV .The average particle size also known as the hydrodynamic diameter of fluoxetine loaded liposomal. The value of zeta potential defines the charge on the surface of the nanoparticles and has a marked affect the stability of particles present in the suspension that is a result of the electrostatic attraction and repulsion between the nanoparticles. Moreover, it also determines how the particles will interact with each other in-vivo. For charged nanoparticles, the magnitude of interactions between the particles will be more as the zeta potential value increases thereby resulting in the formation of stable nanoparticles with a highly uniform size

distribution. This value of zeta potential (mV) indicates formation of uniform nanoparticles with minimum aggregation.

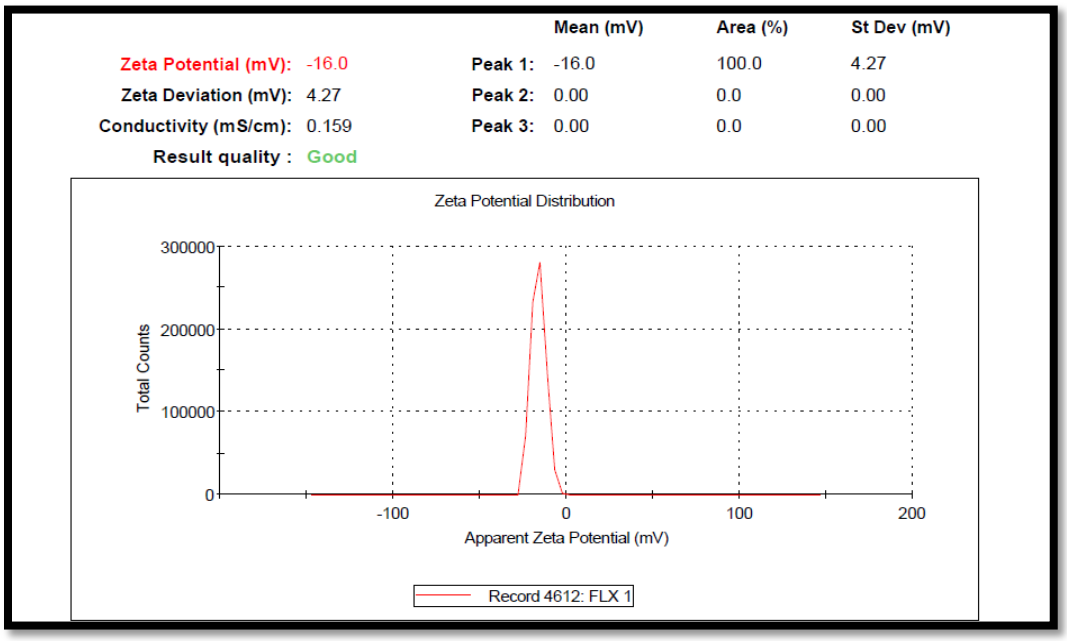


Figure 13: Zeta Potential values for liposomal nanoparticles

All these characterization results proved nanoparticle were made successfully and because of their suitable size and charge they were able to cross blood brain barrier and deliver targeted nanoparticle to brain to treat depression.

4.2.5. Fourier transform infrared spectroscopy analysis (FTIR) analysis

FTIR analysis of DPPC, cholesterol, FLX, and PEG coated and uncoated FLX-liposomal nanoparticle was carried out. KBr pellet were prepared for each sample and analyzed by using FTIR Spectrophotometer, equipped with software OMNIC™ Version 6.0 a; Thermo Fisher Scientific, Waltham, MA, USA). Infrared spectra was than recorded between 4000–400 cm−1.

Infrared spectra for cholesterol, DPPC, FLX, coated and uncoated FLX loaded liposomal nanoparticles and PEG coated FLX-liposomal nanoparticles are presented in Figure.

Important vibrations detected in the spectrum of PEG 6000 are the C–H stretching at 2,890 cm−1,C–O stretching at 1,110 cm−1and–OH stretching at 3,350 cm−1 (Biswal et al., 2008). FTIR spectra of cholesterol showed bands between 2800–3000 cm−1 which were characterized due to presence of asymmetric and symmetric stretching vibrations of CH2and CH3 groups (Liu et al., 2002; Zhou et al 1997). The characteristic strong peak at 2899 cm−1is due to CH2 symmetric stretching vibration. Cholesterol has one double band (CC) in the second ring. This was observed at 1674 cm-1(Gupta, Singh, Kumar & Khajuria, 2014).

The pure DPPC spectra showed the characteristic vibration of DPPC for the methylene(CH2) stretching i.e. 2915 cm-1 for asymmetric vibration and 2850 cm-1 for symmetric vibration). Carbonyl group (C=O) was observed at 1750 cm-1 while symmetric PO2-stretching vibration at 1095 cm-1 (Mahato et al., 2015). Fluoxetine spectrum presents characteristic secondary amine groups at 3340 cm1 and to chains and rings C–H vibrations together with vibrational modes detected at 2900–2975 cm1 of CH2, CH groups. In the same way, the band at 1635 cm1 can be attributed to the bending N–H and stretching C–F modes at 1045 cm1 (González et al., 2011).

In PEG-FLX loaded liposomal nanoparticles conformational changes were observed within the lipid structure which were clarified through the CH2 stretching frequency regions of the acyl chain (2800-3000 cm-1), the C=O stretching regions (1700-1760 cm-1) and the PO2 - stretching bands of the lipid head group (1000-1300 cm-1). The reported change of the IR frequencies confirms lipid conformational changes in the lipids' structure upon the incorporation of fluoxetine and PEG coating. (Pham et al,2018).

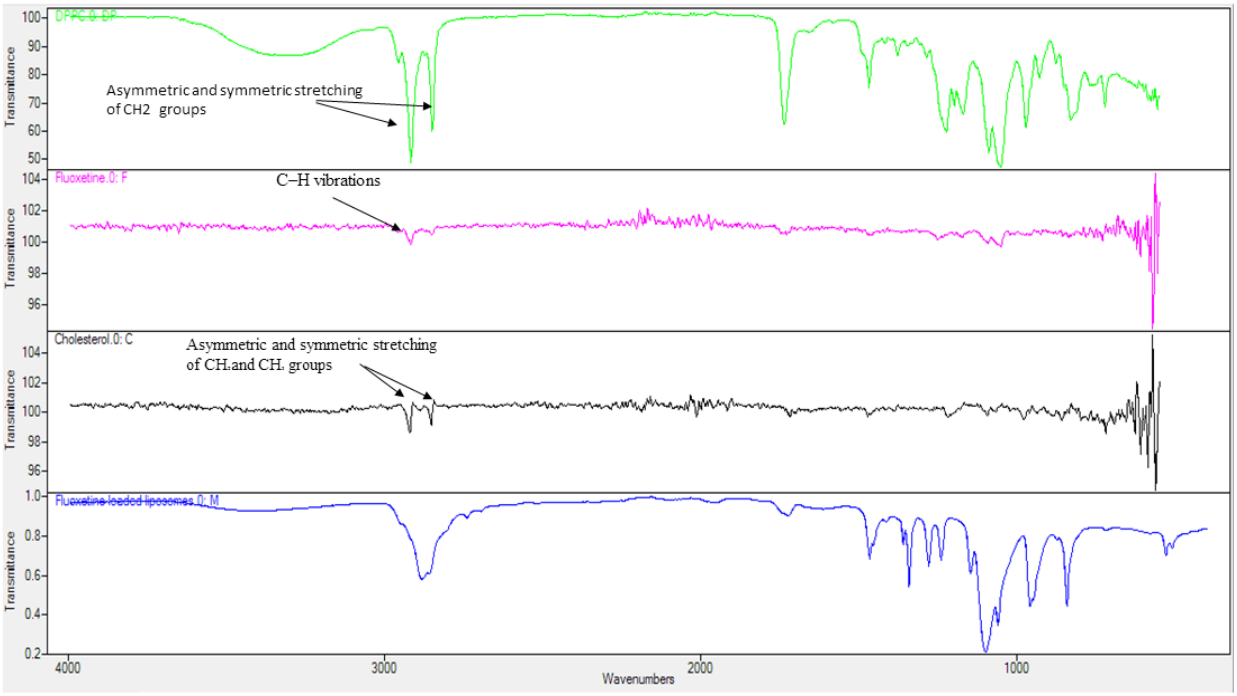


Figure 14: FTIR spectra of Cholesterol, DPPC, Fluoxetine and PEG coated Fluoxetine loaded liposomal nanoparticles

4.3. Animal mice model of depression

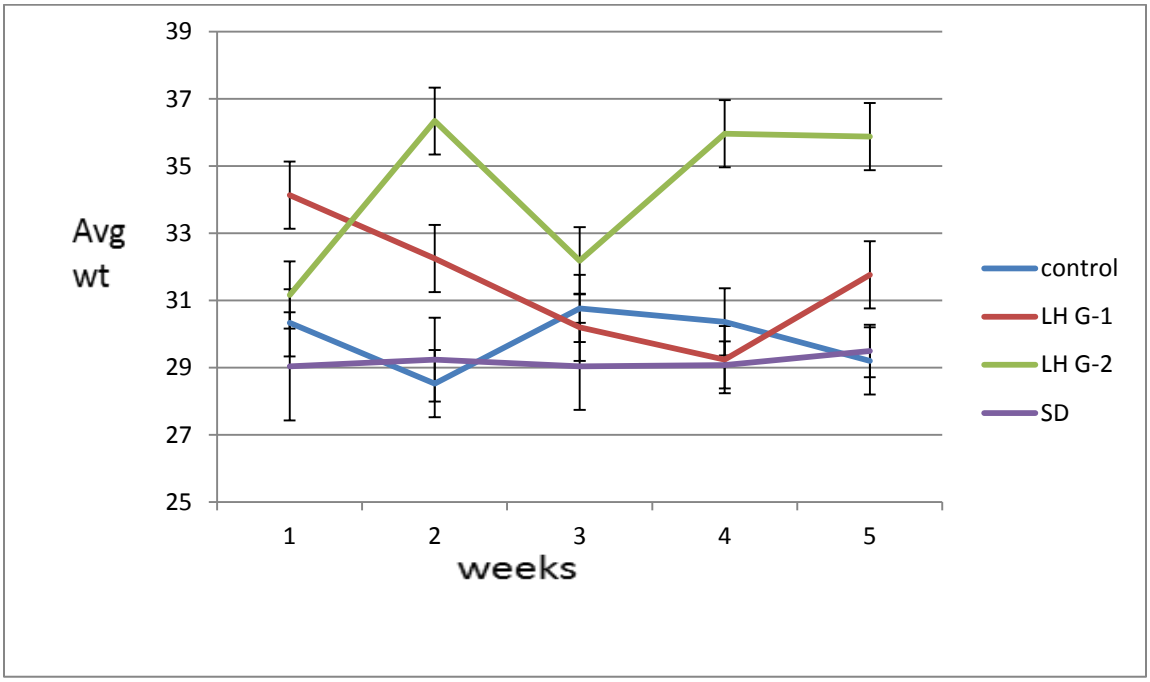
4.3.1. Weight:

Weight of the control group has been increased with the passage of time. Weight of LH G-1, LH G-2, LH-G3 decreased during depression weeks because of the the harsh environment an conditions but in treatment their weight average got better. The group that was given no treatment for their depression their weight got less and less with the passage of time no treatment and depression.

Figure 14: Line representation of mice weights

4.4. Behavioral tests:

After the development of depression (learned helplessness) mice were to test their behavioral analysis. Forced swim test, tail suspension test and open field test are good indicators of mice depressive like phenotype. In these tests mice are put in adverse conditions to which they try to escape if they do not have depression but if they show depressive like phenotype then they cannot escape these adverse conditions.



4.4.1. Forced swim test

Forced swim test proved mice were depressed or atleast showed depressive like phenotype. The behavioural despair test is a test, centered on a rodent's response to the threat of drowning, whose result has been interpreted as measuring susceptibility to negative mood. It is commonly used to measure the effectiveness of antidepressants, although significant criticisms of its interpretation have been made. Here in this graph of forced swim test it can be seen the immobility time was higher for depression group as it was not treated or given any relief from their depression. Control group has the lowest immobility time exhibiting their normal behavior. These results show the antidepressant fluoxetine and fluoxetine incorporated liposomal nanoparticles were able to treat

depression in mice and nanoparticle may have better onset of reaction than simple fluoxetine.

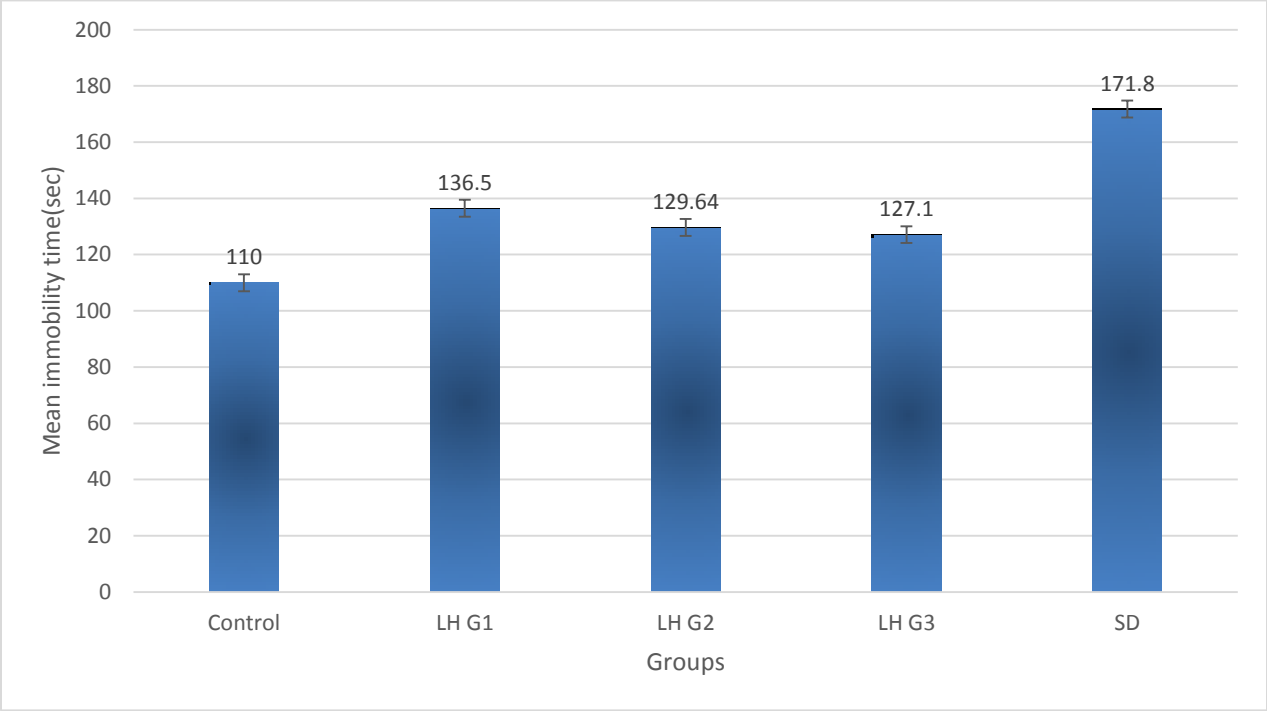


Figure 15: Graphical representation of forced swim test

4.4.2. Tail suspension test

The tail suspension test (TST) is an experimental method used in scientific research to measure stress in rodents. It is based on the observation that if a rat is subjected to short term inescapable stress then the rat will become immobile.

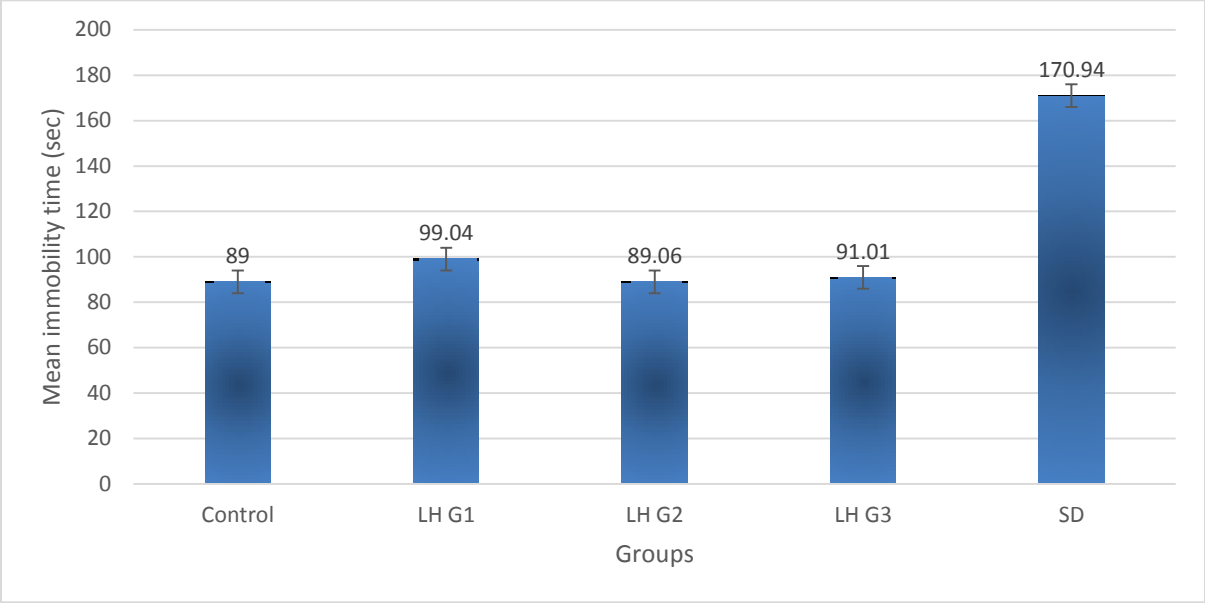
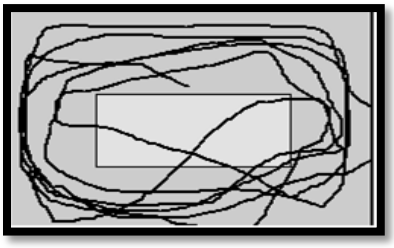


Figure 16: Graphical representation of tail suspension test

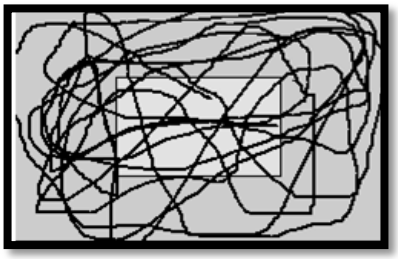
In this graph it is visible the immobility time for control is the lowest which signifies their normal behavior and the highest immobility time is of simple depression group. Coated and uncoated Nanoparticles incorporated fluoxetine and simple drug has immobility time lesser than simple depression which tells fluoxetine and nanoparticle reverted the depressive like phenotype in mice and treated them.

4.4.3. Open field test:

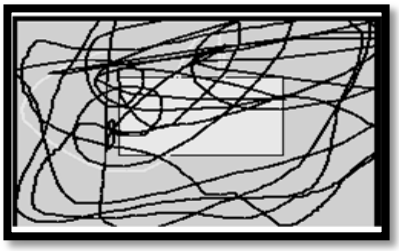
Control



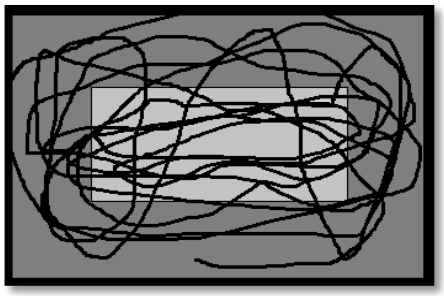
Depression



Uncoated Nanoparticles



Fluoxetine treated



PEG coated Nanoparticles

Figure 17: Software images of open field test

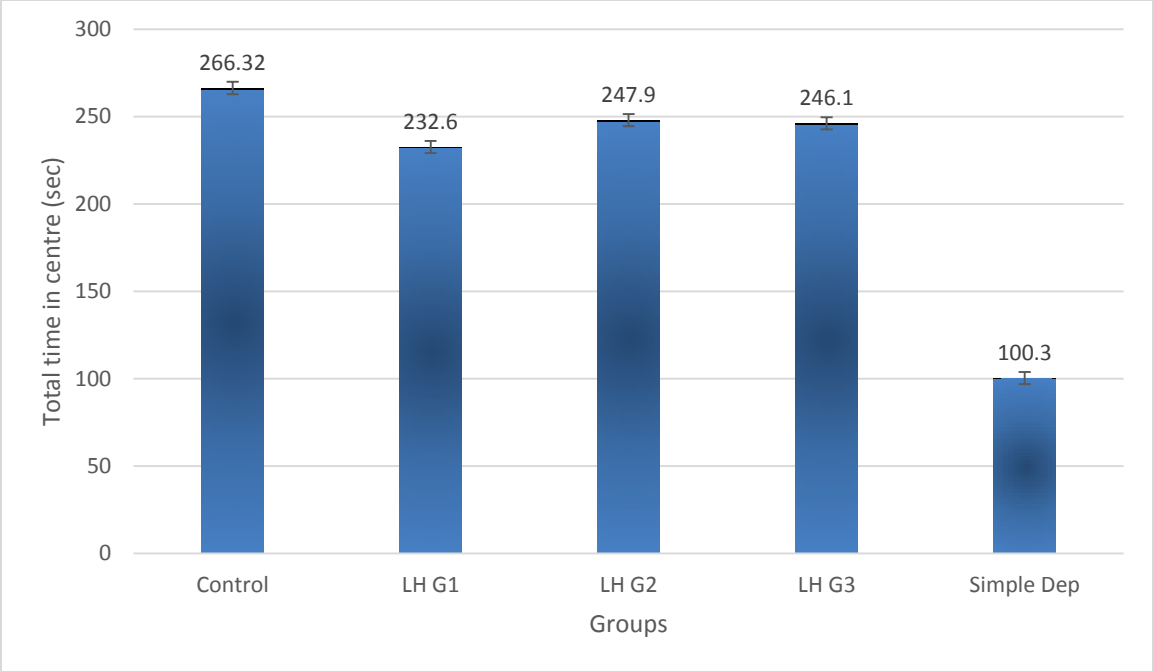


Figure 18: Graphical representation of open field test

All these software generated shows the path taken by mice the box is divided into two zones inner and outer zone. Any depressed mice will not like to explore the inner zone of box or the open field area any normal mice will like to explore the inner zone/open field as much as outer zone. Here it can be seen only depressed mice did not explore the inner zone/open field instead all the other groups were normal enough to explore open field area.

4.5. HISTOLOGY

4.5.1. Liver Histology

4.5.1.1. Control Group

- Liver from a control mice hepatocytes has feathery cytoplasm containing glycogen.
- Hepatocytes are arranged in one cell layer thick plates

4.5.1.2. Untreated/ Depressed Group

Untreated or depressed mice had stressed cells that are increased in matrix density, crystalline inclusions, swollen cristae that are separated due to flocculent material in cyst-like dilations. Necrosis and apoptosis is also observable**.**

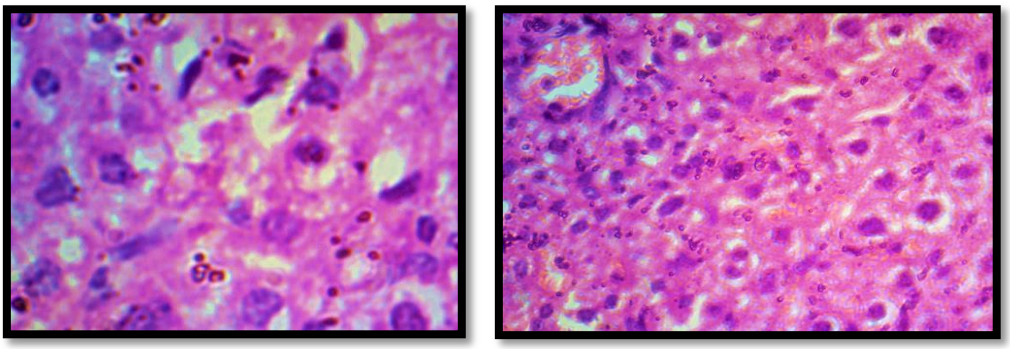


Figure 19: Liver histology (untreated group)

4.5.1.3. Simple Fluoxetine Group

- Liver cells hepatocytes appear normal in histology after the administration of fluoxetine via IV.
- Cytoplasm is also normal closer to control group mice

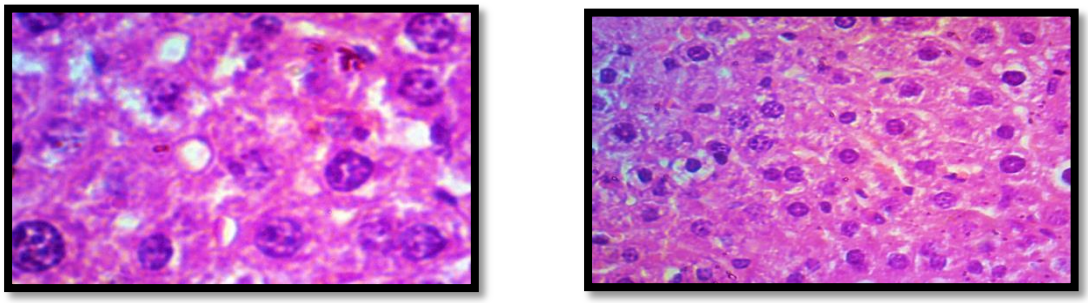


Figure 20: Liver histology (simple fluoxetine group

4.5.1.4. Uncoated Nanoparticles Treated Group

Group that was given fluoxetine incorporated nanoparticles also seems to appear normal hepatocytes and sinusoids.

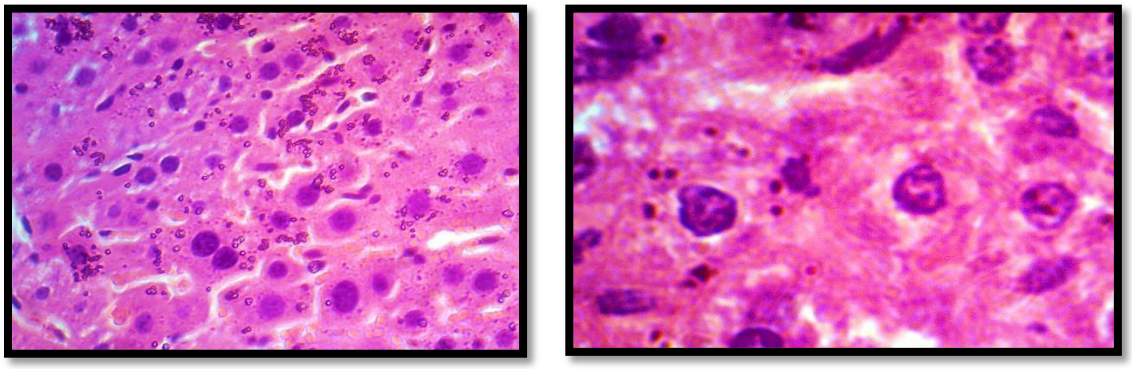
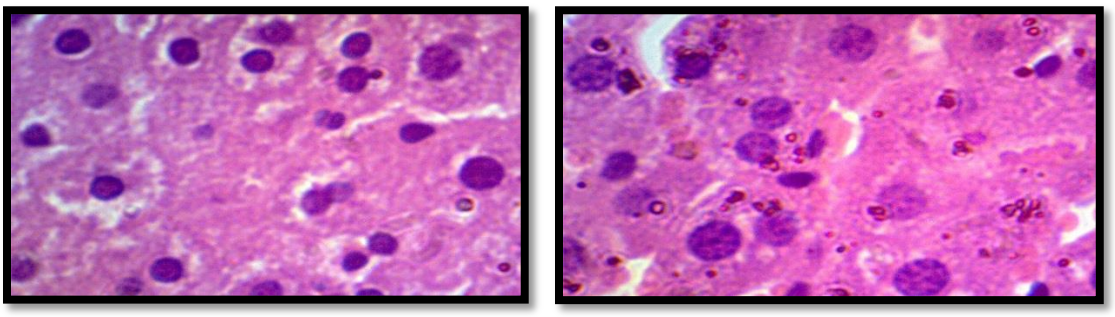


Figure 21: Liver histology (un coated nanoparticle treated group)

4.5.1.5. PEG coated Nanoparticles Treated Group

Group that was given peg coated fluoxetine incorporated nanoparticles also seems to appear normal hepatocytes and sinusoids.



4.5.2. Kidneys histology:

Capillaries, proximal tubules ,bowman's capsule all is intact over the three groups. Mesengial cells has well intact cell membrane. Podocytes is also presents. The kidney is organised into many lobes, organised in a pyramidal structure, where the outer portion is made up of cortex, and the inner portion is made up of the medulla. The kidney contains about 1 million functional units called nephrons, which are continuous with a system of collecting tubules

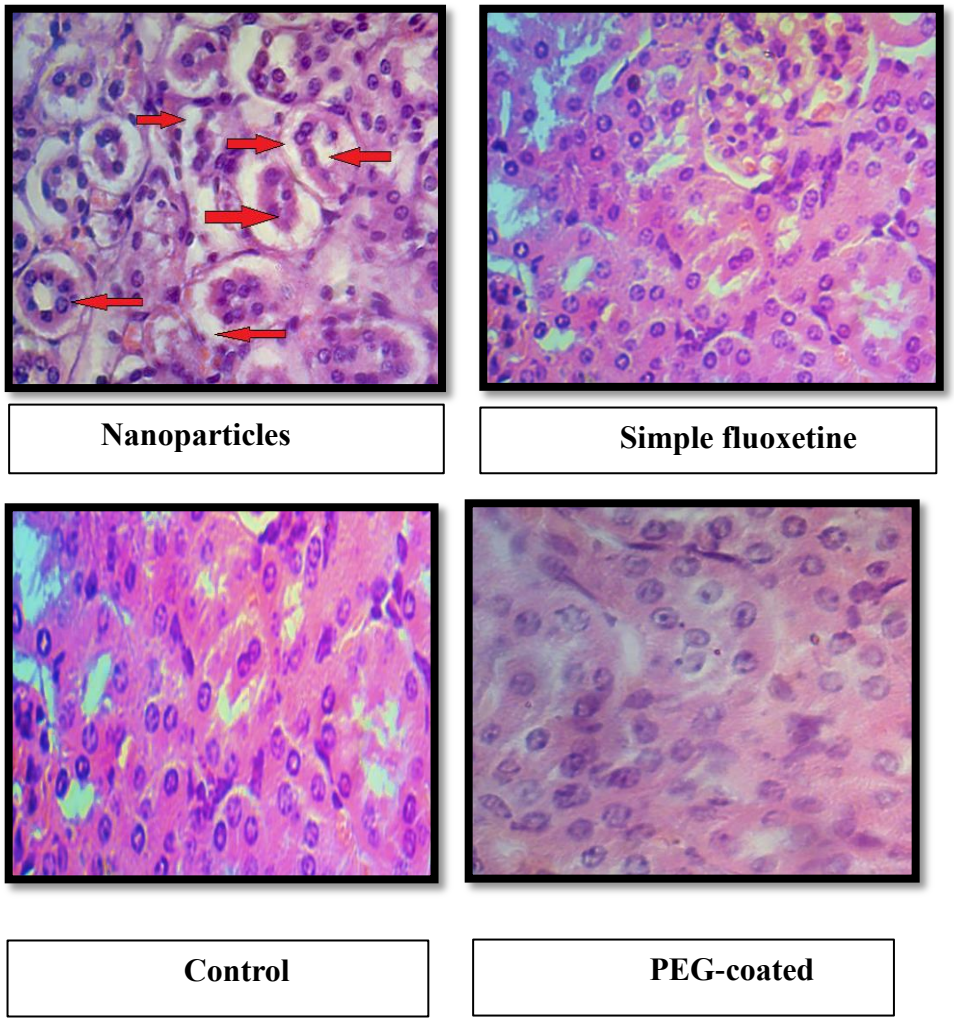


Figure 22: Kidney histology

4.5.3. Heart Histology:

Intercalated discs, myofibrils, nuclei are normal among all the groups. After treatment cells seems normal nuclei are intact. The heart is composed of cardiac muscle, specialised conductive tissue, valves, blood vessels and connective tissue. Cardiac muscle, the myocardium, consists of cross-striated muscle cells, cardiomyocytes, with one centrally placed nucleus

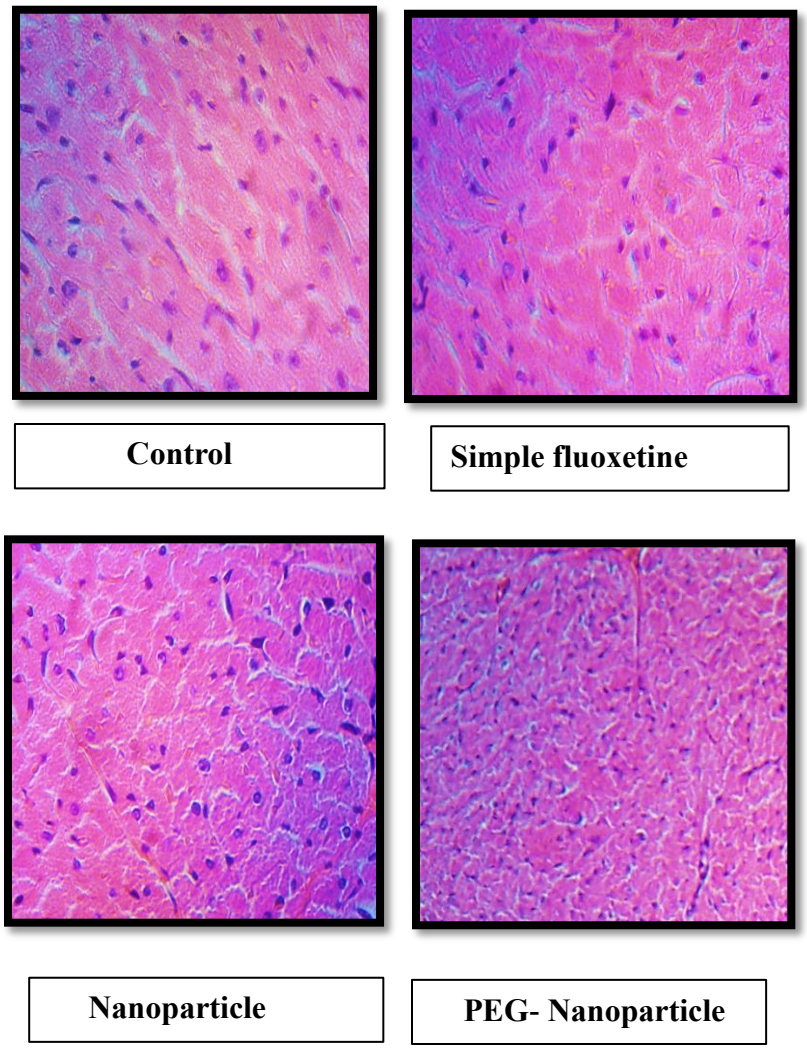


Figure 23: Heart histology

4.5.4. Spleen Histology

Red and white pulp is evenly distributed. Sheathed capillaries are present. Splenic cords are meshwork of erythrocytes, macrophages, plasma cells and granulocytes**.** Marginal zone of lymphocytes evolved from B-lymphoid follicle, in the outer rim there are loosely arranged lymphocytes whereas in the mental zone there are tightly packed lymphocytes are present.

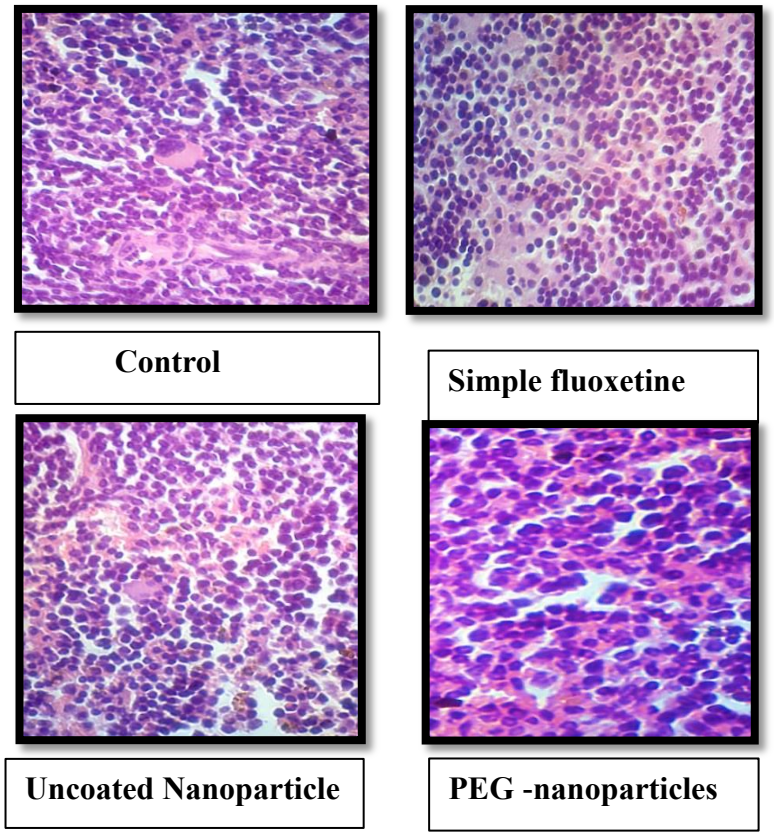


Figure 24: Spleen histology

4.5.5. Brain histology

From these brain microscopic slides it can be seen there is no cytotoxicity, all cell members are intact no cell debris are found. Cells seems normal in nature even after long term usage of fluoxetine incorporated nanoparticles, Nucleus, glial cells and oligodendroglia seems normal. Cells structure is intact. No cytotoxicity can be seen.

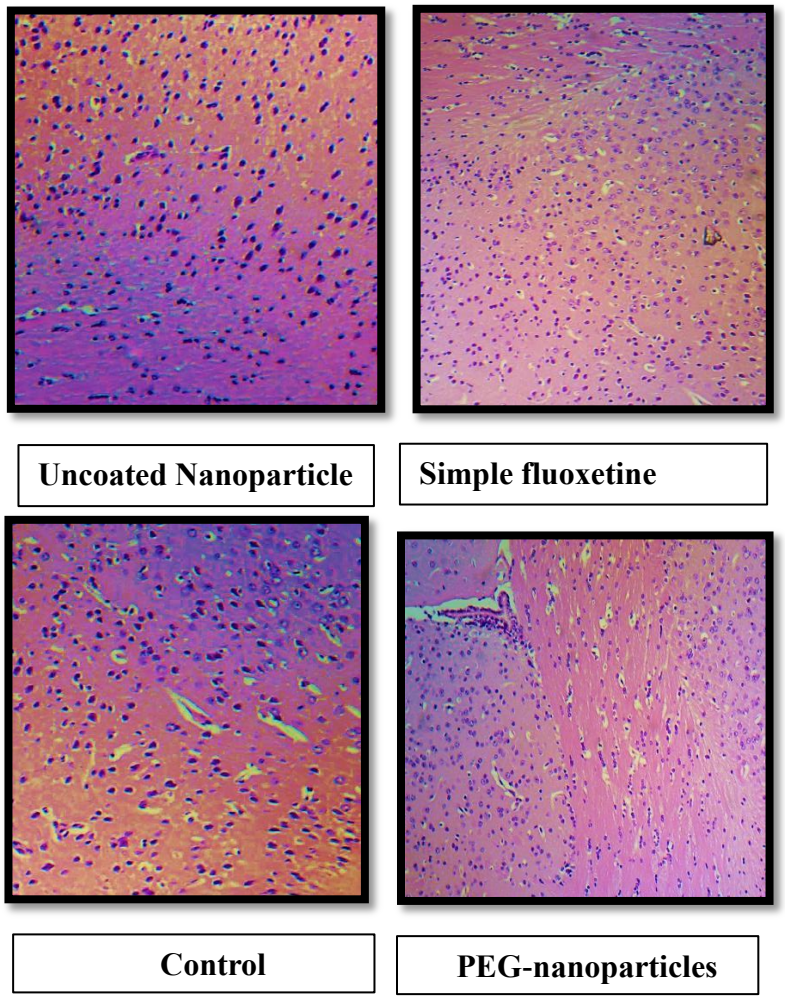


Figure 25: Brain Histology

CHAPTER 5: DISCUSSION

Major depressive disorder characterized by at least two weeks of low mood that is present across most situations. It is often accompanied by low self-esteem, loss of interest in normally enjoyable activities, low energy, and pain without a clear cause. To treat depression many anti-depressants and other methods are used. Many antidepressants aren't able to stay in blood rain barrier because of PGP. Although a variety of antidepressant drugs are currently available but the selective permeability of the blood brain barrier possesses a significant hurdle thereby limiting the advantages offered by these medications. It is made up of continuous non fenestrated vessels that cautiously regulate passage of ions, chemicals and cells to and from the membrane thus regulating homeostasis resulting in protection of the CNS from pathogens, toxins, injury and disease. This restraining nature of the BBB poses to be a major obstacle for drug delivery to the CNS. In this research PEG coated and un coated fluoxetine incorporated liposomal nanoparticles were successfully made and particle size showed that it was able to cross blood brain barrier and treat depressive like phenotype.

Drug release kinetics and encapsulation efficiency, zeta potential showed nanoparticles were not aggregated and well made. Nanotechnology has revolutionized the world of science and medicine enabling the development of fine nano-sized structure that can pass through any minute biological system delivering the required beneficial service. Learned helplessness model of depression is closed related to human adverse environment as a person, they try to escape it for so long in the end they tend to give up hence they develop learned helplessness of depression. This model of depression has better face validity. Forced swim test, open field test and tail suspension test showed mice developed depressive-like phenotype and fluoxetine incorporated liposomal nanoparticle were able to cross blood brain barrier and treat the depressed mice hence it can be concluded from all these test results that mice developed depression and nanoparticles and simple fluoxetine were able to treat depression. Nanoparticles may have faster onset of drug

rather than simple anti-depressant and drug delivery was more targeted than simple fluoxetine. Histology of all the organs (brain, heart, spleen, kidney and liver) showed longer use of simple fluoxetine or liposomal nanoparticle showed no signs of cytotoxicity these drugs are safe to use. Future prospect of this research are quite vast. Effects of chronic depression model on their protein level can be studied and effects of liposomal nanoparticles can also be studied. Hence, this discussion concludes this study and its future prospect.

All those histology images of slides show after two weeks of treatment with simple fluoxetine and fluoxetine incorporated liposomal nanoparticles all the cells in organs are intact. No signs of cytotoxicity are present hence it can be concluded these drugs are safe to use for longer period of time and nanoparticles may have early onset of activation and gave better results because of their targeted delivery and lesser side effects.

CHAPTER 6: CONCLUSION

These results show that liposomal nanoparticles coating with polymers i.e. PEG has targeted drug delivery and they were able to treat learned helplessness model of depression after two weeks of intravenous treatment in mice by crossing the blood brain barrier and giving rather better results than being treated by fluoxetine antidepressants. Histology results also showed no side effects of using fluoxetine incorporated liposomal nanoparticles has no side effects on cells structure. Future aspects can do research on the long term use of nanoparticles on depressed mice and their long term effects.

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