Role of Vitexin Liposomes in Treatment of Depression

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

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Abstract

In this study, the antidepressant impact of vitexin liposomes is inspected in depression models in vivo. Depression is a very predominant and incapacitating condition, which is not completely manageable by the drugs currently available in the market. Numerous patients do not respond to available treatments or show partial response which arises the need for other better therapeutic choices. Vitexin is inadequately absorbed by the gastrointestinal tract and therefore to increase bioavailability and for vitexin to show any effectiveness against depression we synthesize liposomes. To test the ability of these synthesized liposomes, animal model is used. First, depression is induced through the unpredictable chronic mild stress (UCMS) protocol, in mice. In this procedure, young mice are incessantly presented with unpredictable mild stressors. UCMS can be utilized for screenings of antidepressants on an assortment of depressive-like behaviors. Then, the tail suspension test (TST), the forced swim test (FST) and open field test (OFT), are utilized to determine depressive symptoms in two stages, after induction of depression and after treatment. UCMS is the preferred protocol for inducing depression because of its capacity to impel long-term behavioral discrepancies and then allowing these behavioral short-comings to be reversed by chronic therapy. Following fruitful induction of depression, simple vitexin and vitexin liposomes (250 µg and 500 µg) are injected intravenously, testing and investigation is performed which demonstrated that vitexin loaded liposomes are a superior method of treatment for depression. The results show that neither simple vitexin nor vitexin liposomes caused any cytotoxicity at any given dose. The 500 µg dose gave the best results, almost close to those of control, followed by the 250 µg dosage which gave positive results but not as good as the higher dose.

Key Words: *Depression, Vitexin, Vitexin liposomes, UCMS*

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List of Acronyms

UCMS- Unpredictable Chronic Mild Stress

FST- Forced Swim Test

TST- Tail Suspension Test

OFT- Open Field Test

DPPC- 1, 2 – dipalmitoyl – sn – glycerol – 3 – phosphocholine

SEM- Scanning Electron Microscopy

EE- Encapsulation Efficiency

CNS- Central Nervous System

Serotonergic 5-HT1A- Serotonergic 5-hydroxytryptamine 1A receptor

Noradrenergic α2- Noradrenergic Alpha 2

DRD1- Dopaminergic receptors D1

DRD2- Dopaminergic receptors D2

DRD3- Dopaminergic receptors D3

Chapter 1

Introduction

Depression is a classic mental disorder. More than 300 million human beings of varying age groups struggle with the adverse effects of depression. Depressive disorders are portrayed by mental, social and physiological adjustments, including anhedonia, sentiments of desperation and regret, self-destructive thoughts, unsettled sleep, reduced appetite, and cognitive changes. Stressful life occasions regularly go before the beginning of an affective episode, suggesting that stress plays a significant part in the advancement of human depression (Detanico et al., 2009).

Background

Vitexin (apigenin-8-C-glucoside), is a c-glycosylated flavone, present in several medicinal plants. It has a broad spectrum of pharmacological effects, which include anti-cancer (Yang et al., 2013), anti-oxidant, anti-nociceptive, anti-inflammatory (Borghi et al., 2013), anti-Alzheimer's disease (Choi et al., 2014), anti-spasmodic (Ragone, Sella, Conforti, Volonté, & Consolini, 2007), and anti-hypoxia/ischemia injury (Min et al., 2015). In addition, an ongoing report demonstrated that vitexin shows an antidepressant-like effect. It gave primary proof that the antidepressant–like impact of vitexin relied upon an increase in catecholamine levels in the synaptic cleft and catecholamine further interacted with serotonergic 5-HT1A, noradrenergic α2 and dopaminergic D1 (DRD1), D2 (DRD2), and D3 receptors (DRD3) (Ö. D. Can, Demir Özkay, & Üçel, 2013). Uncommonly, vitexin is inadequately absorbed in the gastrointestinal tract. It straightforwardly arrived at the colon where the gut microflora hydrolyzes it by ring opening and deglycosylation of the heterocyclic C ring (Zhang, Tie, Bao, Wu, & Zhang, 2007). The first-pass impacts of vitexin are mostly in the intestinal (roughly 94%), the gastric (30%) and the hepatic (50%), which results in low bioavailability of vitexin (Xue et al., 2014). Oral and intravenous administration to mice results in vitexin being quickly and broadly disseminated to different tissues (Yin et al., 2014), despite the fact that the bioavailability of vitexin given orally was very low, roughly 5% (Wang, 2012). Proceeding administration through oral route, vitexin demonstrated most noteworthy levels in the stomach and the digestive tract after just half an hour, which identifies with residual drug and enterohepatic dissemination (Wang, 2012), with the liver demonstrating a high take–up then observing that most elevated concentrations showed up in liver (Yin et al., 2014). Equivalently, apigenin enterohepatic pathway additionally impacts the accumulation of these apigenins in the body and these apigenin for the most part discharge in urinary rought (Therapeutics, 2005). By administration through the intravenous route to rodents, vitexin was appropriated for the most part to the kidneys and liver, least to fat and brain, and after that discharged most in bile and urine (Yin et al., 2014).

The utilization of liposomes as carriers of drugs have been documented thoroughly (Fan et al., 2015). There are a few reports demonstrating the upsides of utilizing liposomes as carriers. . Liposomes are made out of lipids similar to those seen in organic layers; in this manner, they are required to be biocompatible, biodegradable, for all intents and purposes nonimmunogenic, and nontoxic (Kelly, Jefferies, & Cryan, 2011). Moreover, liposomes are appropriate for conveyance of therapeutic agents since those liposomes typically give sustained drug levels, their content being released gradually, and drug efficacy is increased.

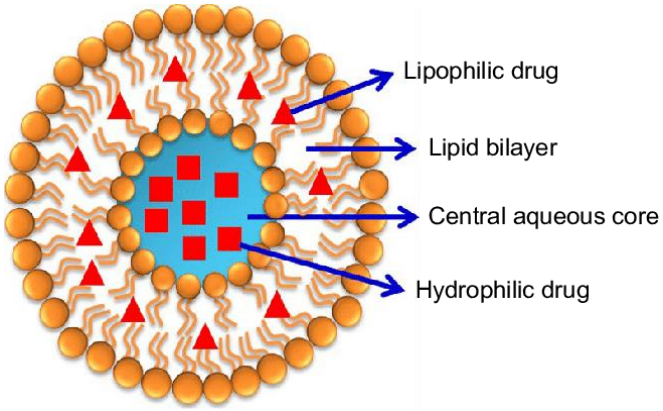

Figure 1 Structure of a liposome

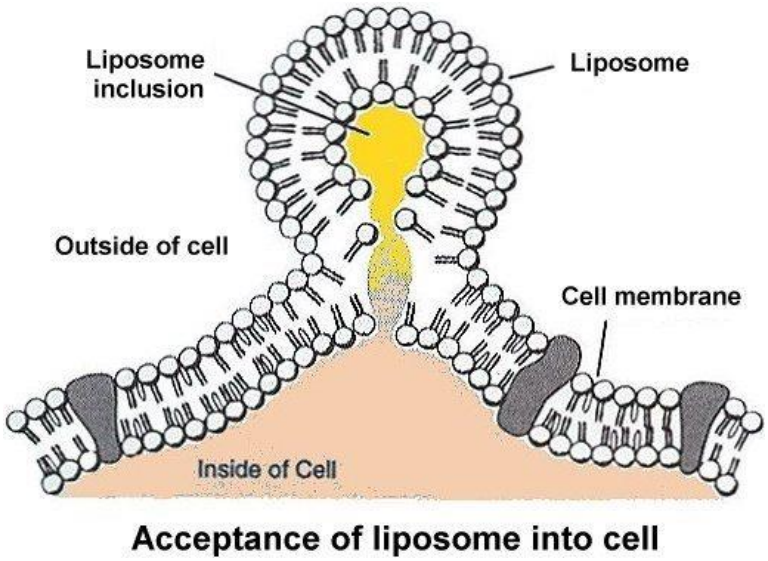

Figure 2 how liposome interacts with the lipid bilayer of the cell

A famous rodent paradigm is the unpredictable chronic mild stress (UCMS) which is used to cause depressive and anxiety-like behaviors. The fundamental goal of UCMS is to create behavioral deficits in rats and mice. It also promotes screening for potential therapeutic pharmacological agents (Hoffman, 2016). The UCMS model has been employed by multiple studies to generate anxiogenic effects. The tests that are highly used hand in hand with the UCMS protocol are the tail suspension test (TST), the forced swim test

(FST), (both measuring stress coping/social despondency), the open field test (OFT; measuring exploratory behavior, anxiety-like behavior and locomotor activity). UCMS empowers to evaluate wide assortment of depressive-like behaviors, for example, behavioral despair, diminished social behavior, and that's only the tip of the iceberg; and chronic (2–4 weeks), yet not acute, administration of antidepressants following stress exposure could deliver an extended remedial impact parallel with the impact got in human patients by the same agents (Burstein & Doron, 2018).

Problem Statement

Despite vitexin's potent biological activity, it's partly not soluble in water and not efficiently absorbed by the gastrointestinal tract and so it is hard for it to reach its target destinations. Since we are targeting anti-depressant abilities of vitexin, it needs to pass the blood brain barrier, hence the synthesis of Vitexin liposomes.

Objectives of Thesis

The objectives of this study are

- to develop novel liposomal encapsulated vitexin formulations and
- to investigate the antidepressant-like effects of vitexin liposomes,
- examining their effects on the unpredictable chronic mild stress-induced behavioral changes in mice.

Significance of Study

The basic aim of this research is to develop an efficient and biocompatible nanocarrier that can cross the BBB and enable us to study the anti-depressant effects of vitexin.

- How effective any herbal medication is, depends on the delivery of useful level of the therapeutically active compound.
- Vitexin shows less bioactivity in the body as it is poorly absorbed by the gastrointestinal tract.
- Liposomes improve absorption and bioavailability of biomaterials.
- Due to their composition, variability and structural properties, liposomes are extremely versatile.
- They have good ability to move from a hydrophilic environment to the lipid friendly environment.
- Since we are targeting antidepressant abilities of vitexin, it should be able to pass the blood brain barrier, hence the synthesis of Vitexin liposomes.

Thesis Overview

Chapter 1 deals with the motivation and background of the work done. In the Chapter 2, there is literature review and previous study. The Chapter 3 includes the methodology part to design the device and experimental protocol being studied. The next Chapter 4 displays the results acquired and Chapter 5 has discussion and in the last Chapter 6 conclusion, future works and limitations has been discussed.

Chapter 2

LITERATURE REVIEW:

Major depressive disorder is one of very enfeebling conditions that has been recognized as the 11th most causing disease globally and is a burden to the global environment (Bromet et al., 2011). Serious impairments have been associated with this disorder that affect the social and occupational ability of the patient, reduce the quality of life, including the onset of many physical and mental diseases. The mortality rate is also found to be high in this case that resulted in the life time prevalence of the disorder to be 11-16% globally (Otte et al., 2016). Although many therapeutic interventions have been introduced so far and a lot pharmacological therapies are being utilized for the purpose but still no effective results have been seen by using these medication on one third of the patients. More than one third of the patients usually do not have abeyance from this chronic disorder by the application of the existing therapies (Cuijpers, Karyotaki, Weitz, & Andersson, 2014). For that purpose, the development of novel drugs for the treatment and also the study of neuro physiological pathways are still required. It is the need of hour to do the correct mapping of the pathophysiology of the major depressive disorder. For serving this issue, the work on animal models is highly recommended and is under consideration to validate a novel therapy for the disorder.

Vitexin is a therapeutic agent found in many medicinal plants such as the hawthorn, pigeon pea, mung bean, mosses, mimosa, in seeds, fruits, flowers, leaves, roots etc. The flavonoids is a vast class of compounds that are being used as the pharmacological agents so vitexin recently gained interest due to its therapeutic benefits regarding many conditions. It can be used as the anti-inflammatory, antidepressant, anti-oxidant and anti-cancer (He et al., 2016).

It also has its efficacy as anti-hypoxia/ ischemia injury, antiviral infections (Rusak, 2008). The vitexin was also found to be useful in the treatment of Alzheimer disease. It should results on central nervous system and cardiovascular systems which are multi linked effects that can be used as a therapeutic effect in many screenings. It was used in vitro and in vivo model in the neurological disorders such as scopolamine-induced memory impairment.

In order to use vitexin and its isomer isovitexin as a therapeutic drug it is important to examine the cytotoxicity of the two drugs. The safety of the drug is a basic parameter to use to for the in vivo

studies as treatment agent. Till date a lot of studies are done on vitexin only and not much work done related to the isovitexin. So technically, vitexin has no cytotoxic effect observed so far if it is used 200ug/mL in vitro (Rosa, Rios-Santos, Balogun, & Martins, 2016). In an in vitro study which was done on the leaf extracts of the Ficus deltoidea, no genotoxic or cytotoxic effects either acute or chronic were observed, although the concentration of vitexin and isovitexin used were relatively high (Farsi et al., 2013).

The cytotoxicity was also examined for the liver and gastro intestinal tract. It was found that even if high dose is administered (10mg/kg) and treatment is repeated several times, that is still safe for the period of seven days (Rosa et al., 2016). So with some preventive and precautionary measures, the vitexin and isovitexin can be used as potential drug for the treatment of many syndromes.

Since ancient days, the use of biomaterials in conjugation with the natural plant extracts for the therapeutic purpose is common. This is done in order to improve the efficacy of the natural extract and to make them available for the uptake by cells efficiently. From lasts few decades, a lot of work is being done on the natural products by plants to find out the health benefits of the extracts and also the chemical characteristics of the natural products. in past few years, good initiatives are made to develop the novel therapeutic drug delivery systems that can be used to deliver the active agents and extracts of medicinal plants which further can be used as drugs (Edwards, Brown, Talent, Dickinson, & Shipley, 2012).

For that purpose, the liposomes are widely been used. The advantage of using liposomes is that they can encapsulate the plant extract and improve the uptake of that active agent making it more available to the cells. These are been utilized in the food industry as well. The encapsulation of the extract with the biomaterial enhances its absorption if they are administered orally or through the skin (topically). Encapsulation is basically the process of entrapping the substance with another substance that result in the formation of particles which are very small in size usually few nanometers in diameters.

The liposomes and phytosomes are the examples of the encapsulation systems that have advantages in the fields of therapeutics, nanomedicine, food industry and pharmacokinetics. One of the most used carrier system is liposomes. Liposomes are the systems composed of the bilayer and are termed as the double membranes system. These are the lipid molecules that are made by the phospholipids and cholesterol. The phospholipids used for this purpose can be lecithin. The

liposomes are formed when the phospholipids are dissolved in the aqueous medium. The phospholipids disperse in the aqueous media. The basic mechanism of the liposome formation is though hydrophobic and hydrophilic interactions that is between the water molecules and phospholipids (Hoffman, 2016).

Liposomes play an important role in the distribution of many different types of chemicals, compounds and genetic material to a wide range of cells and tissues of the body mainly by stabilizing the therapeutic compounds, protecting them from the body's natural response to foreign agents. Their ability to stabilize the compound helps the desired product to overcome obstacles it wouldn't have been able to circumvent on its own. This helps in improving the bio distribution of the therapeutic compound as well, since it can now reach tissues it wasn't able to before, like overcoming the blood brain barrier to reach the brain.

Liposomes also have the ability to entrap both hydrophilic and hydrophobic materials in their aqueous center and lipid bilayer respectively, this also plays a role in stability of the compound as materials which were insoluble in the body on their own will now be able to circulate in the system for longer periods of time improving bioavailability.

The ability of the liposome to entrap a variety of types of compounds permits the delivery of a wide range of macromolecules e.g. DNA and proteins, whose delivery would have been problematic otherwise.

The reason for using vitexin by encapsulation system is hydrophobic nature of the flavonoid. The vitexin has poor solubility in the water and as a result it is difficult to target the drug to the active sites. The vitexin is widely been used in combination with the liposomes because it was observed that it enhances the solubility of the drug and also makes it convenient to target it to the required target sites. Also liposomes resemble in structure to the biological membranes. The structure of the cell membrane is also double membrane just like liposome.

So it is expected that these liposomal particles will depict a biocompatible behavior in the cell. Another advantage of using liposomal particles is their ability of sustain drug release that solves the issue regarding the low stay rate of drug in the body of patients. If the drug release will be slow the efficacy and therapeutic effect will be increased and less dose will be required to treat a certain disease. Also the study is to investigate the anti-depressant ability of the vitexin, so the drug should

pass the blood brain barrier, so the vitexin liposomal particles will prove to be better choice for this study.

Also, liposomes are a better choice than any other type of carrier as they are biocompatible with all the cells and tissues in the body and therefore elicit no immune response that would trigger unpleasant side effects. They are also able to carry large payloads ensuring a large quantity of the drug is available to the body for a longer period of time, increasing effectiveness of the drug and making sure that the desired therapeutic results are achieved.

Another advantage of liposomes is their ability to self-assemble, making synthesis a not so complicated process compared to other drug delivery systems which require complex techniques to synthesize. Also their biological characteristics; like charge, particle size, number of lamellae, lipid composition and the option to modify their surface with the desired polymers and ligands, play a vital role in liposome stability *in vitro* and *in vivo.* These biological characteristics can also be modified to achieve a range of biophysical and physiochemical properties to help the liposome reach its desired target. Modification of the biological characteristics paves the way for targeted drug delivery systems which will ensure minimal toxicity (Sercombe et al., 2015).

To test the effectiveness of vitexin liposomes and also to test the difference between different dosages of vitexin liposomes and simple vitexin, an animal model will be used. Animal models have been used extensively to understand the complexity of depression, more commonly mice have been used since they are readily available, inexpensive, give predictable and reliable results, lab environment is easy to maintain to ensure no environmental factors interfere with the study, and easy to handle and most importantly have similar genetic pathways to humans when it comes to depression and display similar depressive-like behavior to humans. In the many strains of mice that are available studies have shown that Balb/c mice are preferred for depression models due to their increased susceptibility to the disorder. Moreover female mice are preferred over males since they are even more susceptible (de Sá-Calçada et al., 2015).

To check the effectiveness of our liposomal drug compound, first depression needs to be induced in mice and for that UCMS model has been chosen since it is easily reciprocatable, gives predictable desired results and works best on Balb/c mice.

The unpredictable chronic mild stress (UCMS) is a widely known rodent pattern and model that is frequently being used to induce the anxiety and depression like behavior in the rodents. The basic aim of using this model is to create the behavioral deficiencies in the rodent (mice, rodents) such as anhedonia and behavioral hopelessness (Hoffman, 2016). This practice is done in order to do the screening for the all possible therapeutic agents that can be helpful for the treatment of respective disorder. This model was initially introduced by a scientist named Katz in 1981 and then was basically develop by the other biologist Willner in 1984. The model gave huge benefits in terms of screening the neurological and behavioral results for the disorder of depression and anxiety. At first it was only been used for the study of rats, later it was also recommended for the study of mice and was utilized for the mice model development also (Ducottet & Belzung, 2004).

UCMS has many advantages compared to other protocols that use extreme stressors or acute stress. UCMS protocol includes daily exposure to seven different stressors for 4 hours, which are given in a random order weekly, these include empty cage, dampened sawdust, tilted cage, hot air stream, wet cage, social stress and mouse restraint. Significant changes in behavior can be seen after the completion of the protocol. It allows us to investigate any significant changes in behavioral, neurological and physiological properties that result from chronic stress exposure. Using this protocol we can also test potential therapeutic agents against depression (Frisbee, Brooks, Stanley, & D'Audiffret, 2015a).

The procedure is simple and helpful in understanding the pharmacokinetics of the therapeutic interventions. In the procedure, the young mice are given the conditions with chronic unpredictable mild stressor and the therapeutic agents were administered sequentially. After the treatment was terminated, the physiological and behavioral indications were observed as a result of the treatment and were analyzed accordingly.

Behavioral tests are done in order to analyze depressive behavior and behavior after treatment to see effectiveness of treatment. One of the tests that is included in this procedure is the forced swim test (FST). This test involves placing a mouse in a water bath for 5 minutes and check its level of activity and inactivity. It is assumed that the more aggressively a mouse tries to escape and the more time it spends to try to escape reflects normal behavior indicating that the mouse is not depressed in any way, on the other hand if the mouse gives up just making enough movements not to drown and less time is spent in trying to escape than this reflects despair that is a common

symptom of depression. The advantages of FST are that it is easy to set up as only a container large enough to fit the mouse is required and a stopwatch to note the time, it is also easy to perform. Another advantage is that you can get the results quickly and easily and quickly analyze them. Moreover it is sensitive to a broad range of anti-depressants that make it a desired screening test that has a high predictive validity (Yankelevitch-Yahav, Franko, Huly, & Doron, 2015).

Another test is the tail suspension test (TST) used to screen potential antidepressants. In this test mice are hung, for six minutes, by their tails in such a way that they cannot hold onto anything for support or climb their tails. This test also like the FST is based on the escaping and giving up behavior of the mouse, in that the more time spent trying to escape reflects normal, undepressed behavior and more time spent just being suspended and not trying to escape reflects despair. The advantages of the TST are that only some tape, camera and stopwatch is required, it is easy to set up and easy to perform, and results can be analyzed quickly and easily (A. Can et al., 2011).

The open field test (FST) is another test used commonly to measure animal behavior. It is relatively easy to perform and gives quick results that can be easily analyzed. A variety of behavioral information can be gleaned from it, like the emotionality of the test subject and general ambulatory ability. The fecal matter collected during the test period is also an indication of the animals' behavioral state, more pellets reflect more anxious-like behavior. In this test the mouse is left in the middle of the field, sticking to the corners shows depressive-like behavior while exploring represents normal behavior (Seibenhener & Wooten, 2015).

At the end, to analyze the safety of simple vitexin and vitexin liposomes, it is important to check cytotoxicity, which is done by performing histology tests on different mice organs, like, brain, liver, heart, spleen and kidneys, to check if depression or treatment of depression has caused any physical damage to the cells and organs.

Chapter 3

Material and Methods

Experimental Design

Vitexin liposomes are synthesized and characterized. To test the efficiency of these liposomes 5 groups of 5 mice were taken (Control, Untreated, Vitexin only [10mg/kg], 250 µg/kg Vitexin liposomes and 500 µg/kg of vitexin liposomes) and the UCMS protocol is applied on all groups except the control group. Adolescent animals are persistently put under unpredictable but mild/gentle stress. Behavioral tests i.e. forced swim test, open field test and tail suspension test are done to ensure successful induction of depression. 2 of the mice from the untreated group are sacrificed and their organs histologically analyzed to see the effect depression had, if any, at a cellular level, after two weeks of UCMS protocol. These two mice were sacrificed in order to find out the effects of depression on mice and the rest of the 3 mice in the untreated group were left to see if being left in a normal, controlled environment, for the 2 weeks the rest of the groups were being treated by vitexin, has any affect or any reduction in symptoms of depression. After that simple vitexin and both dosages of vitexin liposomes are given. Behavioral and biological indices are acquired at the end of treatment to identify effects of vitexin liposomes on depression. Mice were weighed at the end of every week to keep track of any significant changes or health issues. At the end of the protocol mice were sacrificed and their organs analyzed histologically to check for any anomaly caused by depression. Results were compared with the 2 mice that were sacrificed after depression, the 3 mice that were left untreated and the 3 groups of mice that were treated with different dosages of simple vitexin and vitexin liposomes, in order to get a clear picture of the effect of vitexin and whether it was the drug or the control environment that had that effect. Cytotoxicity tests were also done to check any ill effects of vitexin on those mice organs.

Liposome synthesis and characterization	Induction of depression	Behavioral tests to check for depression	Treatment with drug	Behavioral tests to check level of treatment	Histological studies	Cytotoxcity studies

Table 1 Experimental design

Animals used

4 week old female BALB/c mice, kept in standard cages (30 cm×19 cm×13 cm). A 9:15 hour light: dark cycle was maintained, temperature at 27°C ± 3°C, humidity at 50% ± 5% and unlimited access to food and water, except during 4 hours of stressor application. 5 groups (control, untreated, vitexin only, 250 µg liposomes, 500 µg liposomes) of 5 mice per cage, were kept together under identical conditions. All procedures were done as per institutional approaches on the treatment of experimental animals.

Figure 3 Groups of mice, 5 mice per cage

Chemicals used

- Vitexin,
- 1, 2 – dipalmitoyl – sn – glycerol – 3 – phosphocholine (DPPC),
- Cholesterol,
- Ethanol,
- MilliQ water,
- PBS.

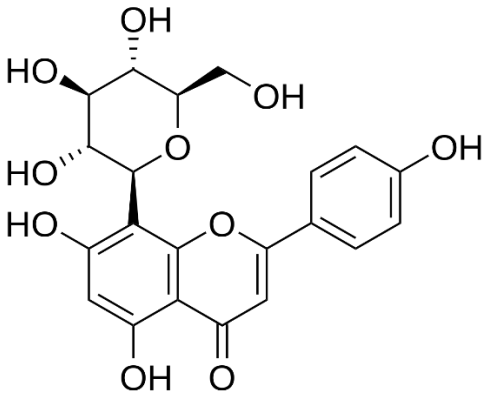

Figure 4 Structure of vitexin

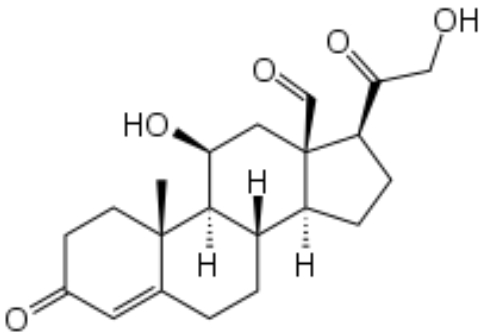

Figure 5 Structure of Cholesterol

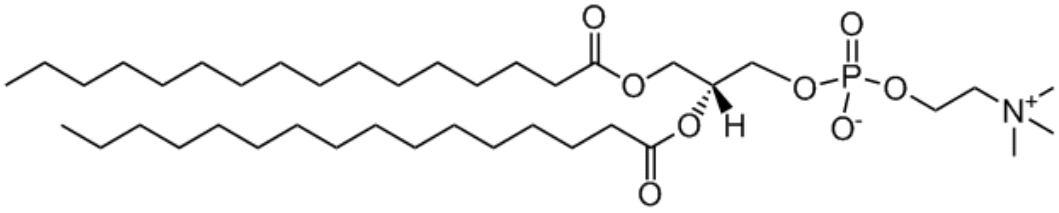

Figure 6 Structure of DPPC

Preparation of Liposomes

Vitexin liposomes were prepared by modified ethanol injection method. Vitexin solution was prepared by adding 1 mg of vitexin in 10 mL of absolute ethanol to get a concentration of 0.1 mg/mL. DPPC and cholesterol were dissolved in a ratio of 4:1 in 10 mL of ethanol to prepare the lipid phase. 500 μl of the vitexin solution was then added to the above solution of lipids and sonicated for half an hour. The lipid phase and 10mL of sonicate MilliQ water were warmed separately in water baths till they reached a temperature of 60°C. Both these phases were then mixed together continuously for 5 minutes and put in a

round bottle flask. A rotary evaporator was then used to let the ethanol evaporate. The final product was refrigerated and stored till further use (Chorachoo, Amnuaikit, & Voravuthikunchai, 2013).

Figure 7 Probe Sonication

Figure 8 Rotary Evaporator

Size Measurement and ζ-Potential

ζ-potential analyzer was used to measure size and ζ-potential of the liposomes. 100 μl of prepared liposomes was taken and diluted with a ratio of 1:150 in distilled water, and ζ-potential and z-average mean were measured.

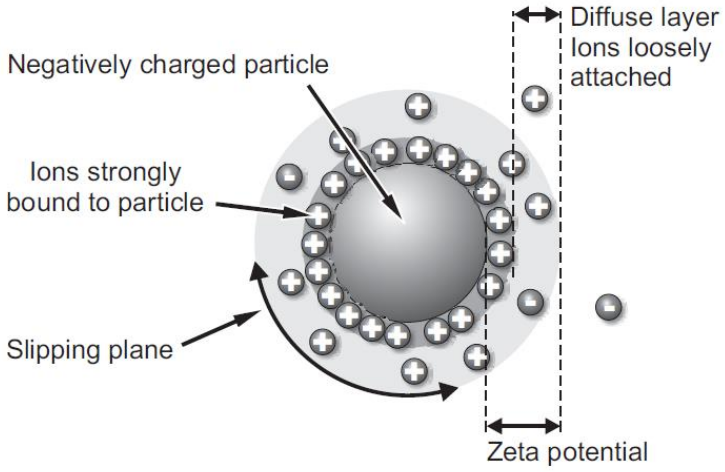

Figure 9 How zeta potential is calculated

Scanning Electron Microscope (SEM)

Particle size and morphological data of prepared vitexin liposomes have been investigated by SEM. 3 mL MilliQ was added to 200 μl of liposome formulation. After drying this sample of diluted liposomes on a cover slip, they were stained with crystal violet for a minute. Water was used for rinsing off the excess dye and then fixing was done by Gram's iodine solution for a minute. When dry, gold sputtering was done on the cover slip and the sample was then investigated.

Standard curve

Different concentrations of vitexin solution were prepared in ethanol to get a standard curve which would help us calculate unknown concentrations. It is useful for finding out the encapsulation efficacy, where we have an absorbance value and with the help of the standard curve, can find out the unknown concentration and subsequently the encapsulation efficacy. The concentrations we prepared were 0.025mM, 0.05mM, 0.075mM, 0.1mM, 0.25mM, 0.5mM and 1 mM. UV Spectra was done of these solutions and the standard curve plotted with concentrations against absorbance values.

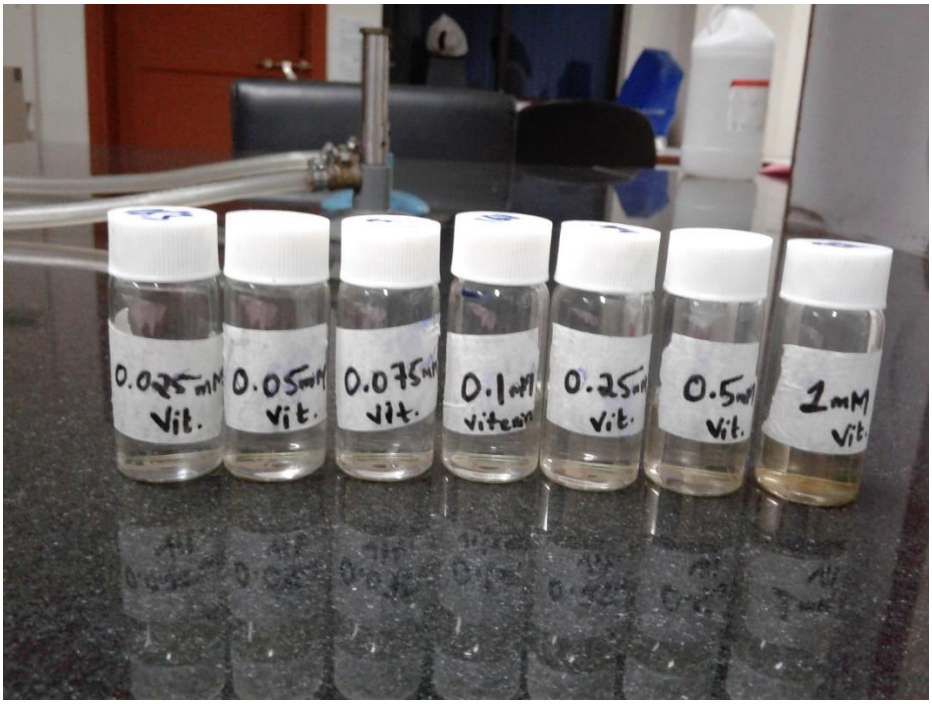

Figure 10 Solutions of different concentrations of vitexin to plot standard curve

Encapsulation Efficacy

Encapsulation efficacy is calcukated as the percentage of free vitexin to the original amount of vitexin added. Centrifugal ultrafiltration was performed using YM30 (MWCO 30 kDa) regenerated cellulose ultrafiltration device (Millipore Corp., MS, USA). 1-mL of vitexin liposomes were loaded into the centrifuge ultrafiltration unit and centrifuged at 25°C at 4,500×g using a microcentrifuge. The filtrate was collected and the scattering by the filtrate was measured using UV-Vis. The formula for calculating Encapsulation efficacy is as below:

$$EE\% = \left\{\frac{(x_2 - x_1)}{x_2}\right\} \times 100$$

x_2 = total vitexin added, and x_1 = free drug (Wallace, Li, Nation, & Boyd, 2012).

Unpredictable Chronic Mild Stress

In the beginning of UCMS protocol mice were left for a period of 1 week to acclimatize. In the subsequent 2 weeks, mice were daily exposed to one of the following stressors: dampened sawdust (4 hours), empty cage (4 hours), wet cage (2 cm water in a sawdust free cage for 4 hours), tilted cage (home cages were tilted at a 45° angle for 4 hours), social stress (switching the cage for 4 hours), mouse restraint (mice were placed in a 50mL plastic tube (Falcon) with openings in both sides for breathing, for 4 hours) (Frisbee, Brooks, Stanley, & D'Audiffret, 2015b), hot airstream (mice were submitteded to a hot air stream from a hair dryer

for 10 min) (Monteiro et al., 2015). In order to maintain unpredictability and prevent habituation, the stressors and sequence of stressors took place at different times (Detanico et al., 2009).

Week 1	Week 2 & 3		Week 4 & 5		
Acclimatization period	UCMS protocol	Behavioral tests	Treatment	Behavioral tests	Dissected

Table 2 Animal Model

Day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	Social stress	Empty cage	Tilted cage	Dampened sawdust	Wet cage	Hot air stream	Mice restraint
Week 2	Tilted cage	Social stress	Wet cage	Hot air stream	Dampened sawdust	Mice restraint	Empty cage

Table 3 Two weeks of UCMS protocol – Timetable

Wet cage

Figure 11 Wet Cage – water at 25°C was added in an empty cage, and the mice were left in that cage for 4 hours

Empty cage

Figure 12 Empty cage – mice were left in a sterile empty cage for 4 hours

Dampened Sawdust

Figure 13 Dampened sawdust – the bedding of the mice was dampened and they were left in these conditions for 4 hours

Tilted Cage

Figure 14 Tilted cage – mice were left in a cage tilted at an angle of 45°C for 4 hours

Mice Restraint

Figure 15 Mice restraint – mice were placed in 50mL falcon tubes with holes cut for breathing, for 4 hours

Hot Air Stream

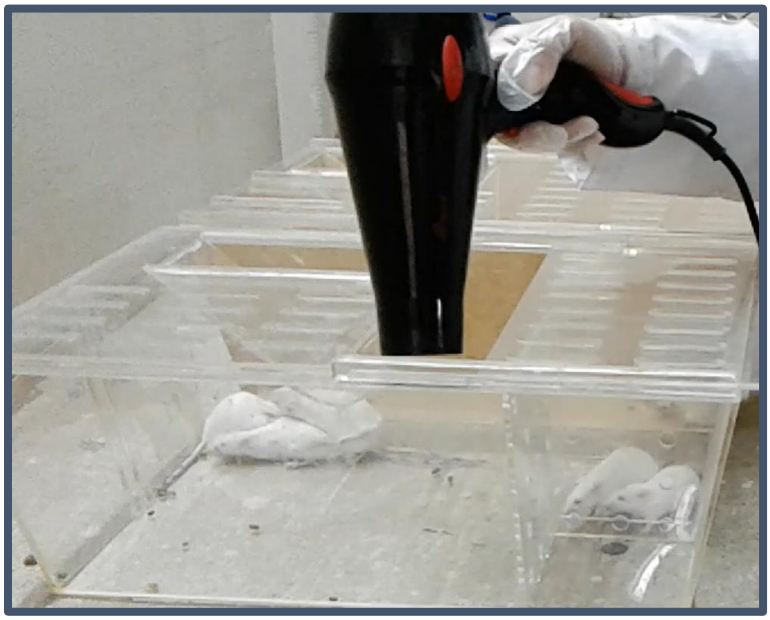

Figure 16 Hot air stream – mice were exposed to a hot air stream with the help of a blow dryer, for 10 minutes

Behavioural Tests

A solitary essential eyewitness oblivious in regards to the trial conditions makes the observations regarding behavioral changes.

Forced Swim Test

Forced swimming tests (FSTs) were performed as described by (Can et al.,2009). Individually, each mice was forced to swim in a glass tank containing water at 25 ± 1°C. The climbing, swimming and immobility time durations were measured with a stopwatch. Time for swimming, the horizontal movement on the surface of the water, time for climbing, the upward-directed movements of the forepaws along the sides of the tank, and immobility time, the movement required to just keep the head above the water. (Xue et al.,2012).

New, fresh water was used in the chamber for every mouse to evade the impact of caution substances. The mice were towel dried after the preparation and the test sessions.

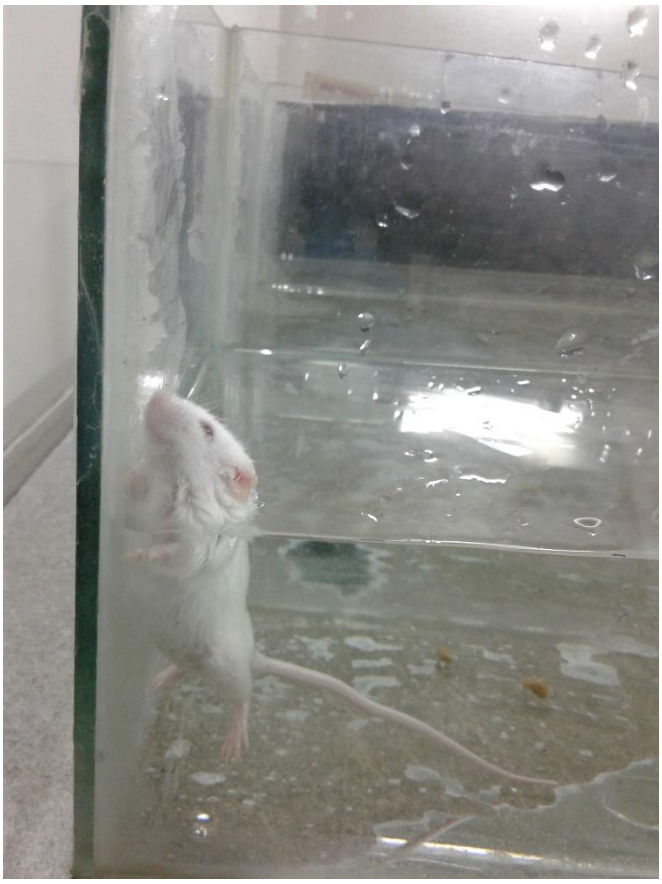

Figure 17 Forced swim test – mouse is displaying climbing behavior

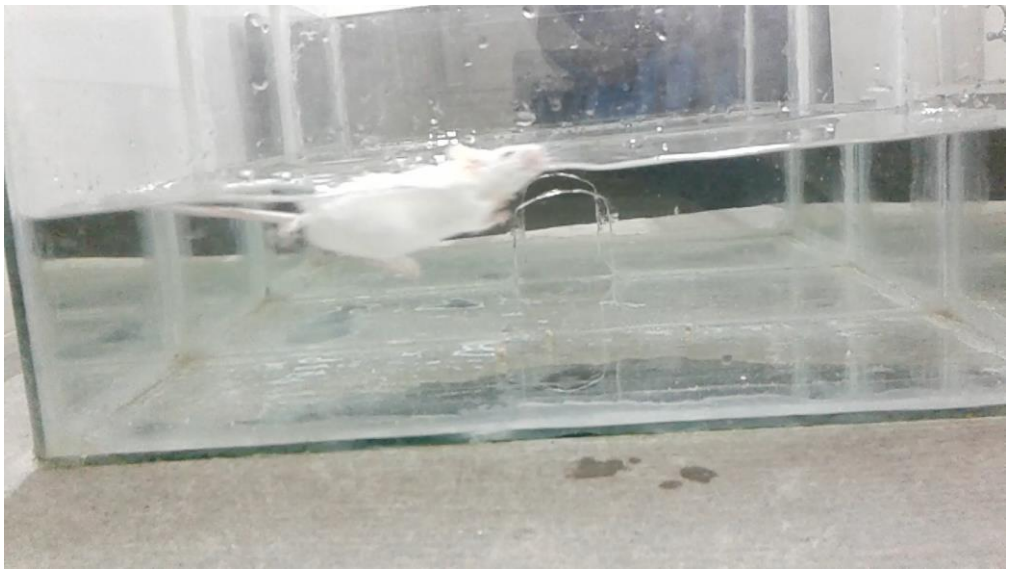

Figure 18 Forced swim test – mouse is swimming

Tail Suspension Test (TST)

Potential antidepressant-like impacts of vitexin were surveyed utilizing the tail suspension test (TST). The mice were suspended by taping their tails up. Time of immobility of the mice was estimated during the last 4 minutes of 6 minutes test term (Kwon etal., 2010; M¨ uller etal.,2012).

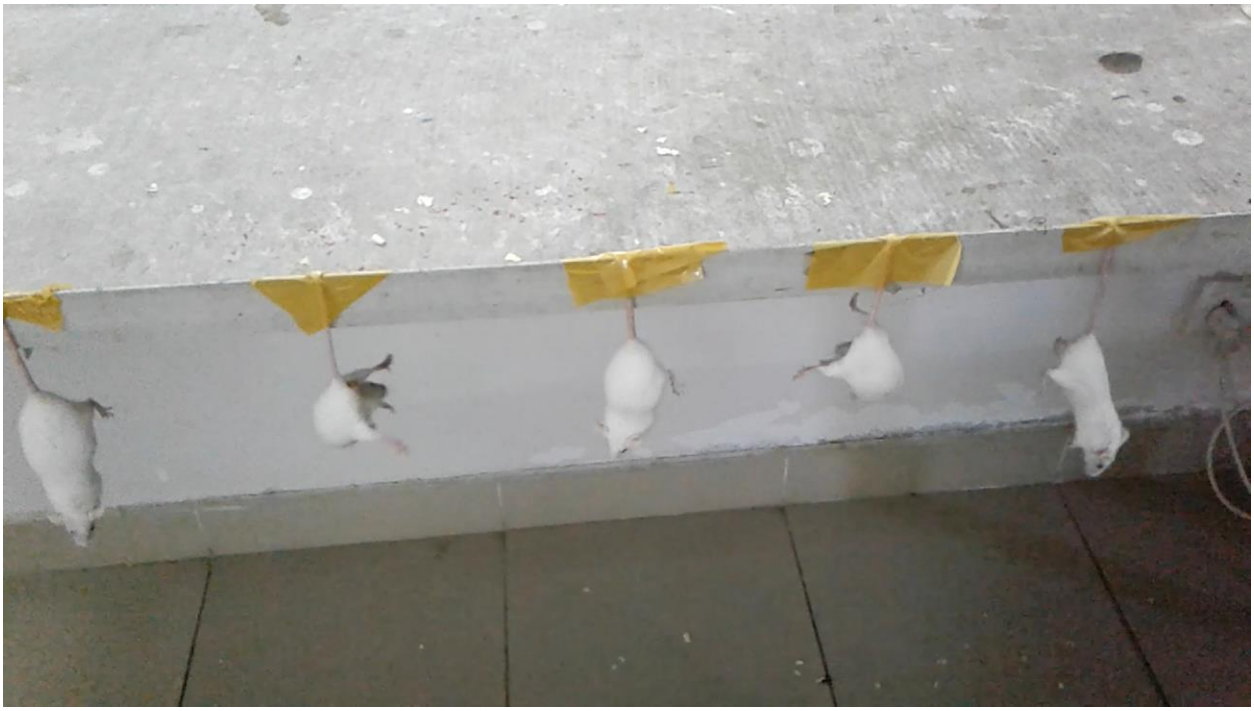

Figure 19 Tail suspension test

Open Field Test

Calvin S. Hall developed the open field test in 1932 to test animal emotionality. Toward the beginning of the test, the mouse is set in the focal zone, for about five minutes, and factors like horizontal locomotion (number of floor line crossings), recurrence of vertical action and grooming are assessed. The degree of nervousness is determined with OFT periphery and central time/entries ratio (Belovicova, Bogi, Csatlosova, & Dubovicky, 2017).

Figure 20 Open field test

Cytotoxicity

It is crucial to analyze safety of vitexin and vitexin liposomes in the human body. After evaluation from behavioral tests was complete, the mice were sacrificed and their organs (brain, heart, liver, spleen and kidneys) were histologically analyzed. Samples for histology were stored in formaldehyde solution. Blood samples were taken from all the mice and stored in the fridge in EDTA tubes. Organs from mice were cryo-preserved at a temperature of -90°C and stored for later analysis.

Statistical analyses

Data of 5 animals from each group was analyzed statistically using Statistica software. One–way ANOVA followed by Tukey's test was performed to compare between the experimental groups. The results were stated as mean ± standard error of mean (S.E.M.). If $P < 0.05$ then the differences between the datasets were considered significant.

Chapter 4

Results

Vitexin liposomes were successfully synthesized using the modified ethanol injection method. Successful synthesis was inferred by the results of the different characterization techniques done, which demonstrated that the liposomes were of the right size. Their zeta potential told us that they won't aggregate and were stable. They had a significant Encapsulation Efficiency. These liposomes were then given to depressed mice, the results demonstrated that vitexin liposomes had a significant effect on depression.

Size Measurement and ζ-Potential.

Average particle size was 1233nm. Surface charge of liposomes was −12.6, which demonstrates that the particles are stable enough to not aggregate much.

System

Temperature (°C):	25.0	**Zeta Runs:**	12
Count Rate (kcps):	35.4	**Measurement Position (mm):**	4.50
Cell Description:	Zeta dip cell	**Attenuator:**	6

Results

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-12.6	**Peak 1:**	-12.6	100.0	4.61
Zeta Deviation (mV):	4.61	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.163	**Peak 3:**	0.00	0.0	0.00
Result quality :	Good				

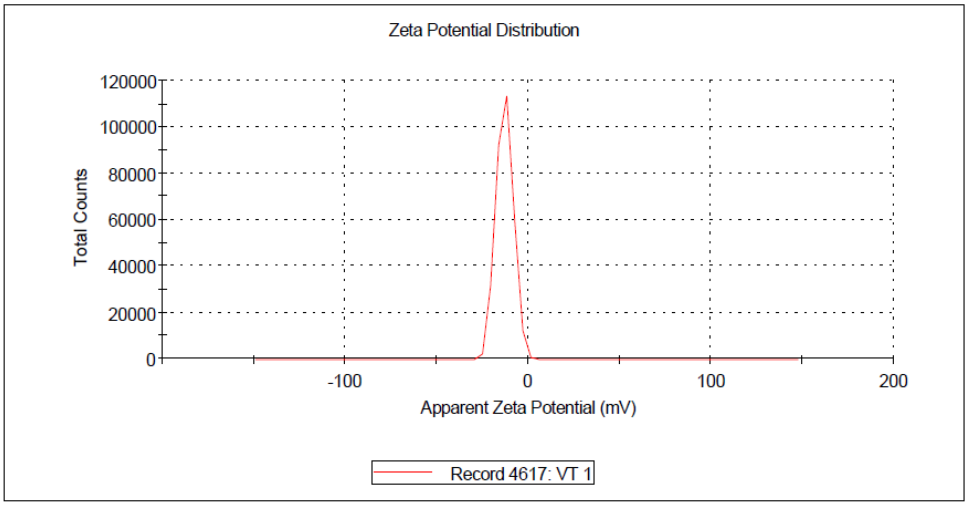

Figure 21 Zeta Potential

Results

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	1233	Peak 1:	1741	68.7	470.0
PdI:	0.674	Peak 2:	424.6	29.2	81.74
Intercept:	0.944	Peak 3:	5560	2.2	8.632e-5

Result quality : Good

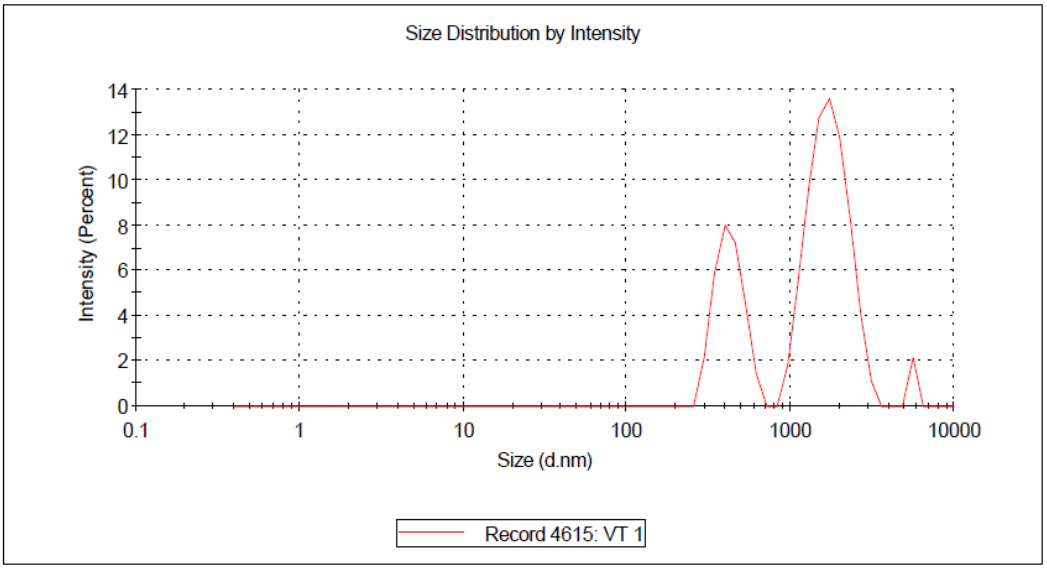

Figure 22 Z- Average – average size of liposomes

Scanning Electron Microscope Observations

The liposomal formulation possesses a fine spherical shape with relatively monodispersed distributed size. The particles do not show any sign of aggregation.

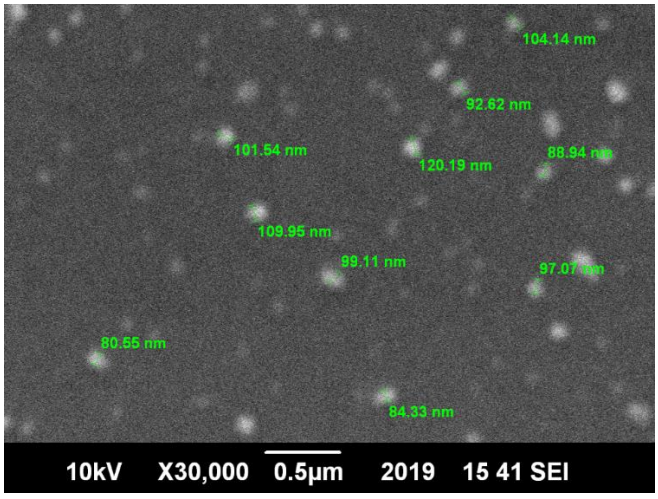

Figure 23 SEM image at X 30,000

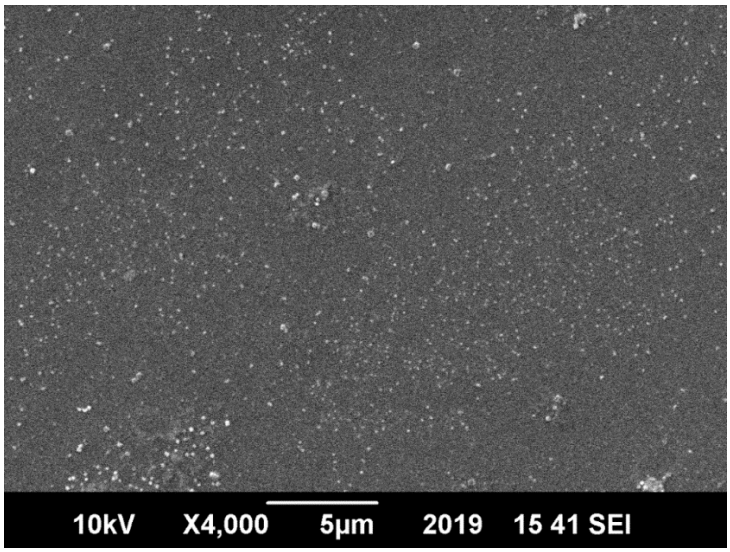

Figure 24 SEM image at X 4,000

Standard Curve

By plotting the standard curve, we got the equation y=10.017x – 0.3769, where y is the absorbance and x is the concentration in mM. This equation can be used to find out unknown concentrations if the absorbance value is known. The R^2 value gives us an idea of the goodness of fit to the regression model, here the value of 0.9934 tells us that the data is a good fit for the regression model.

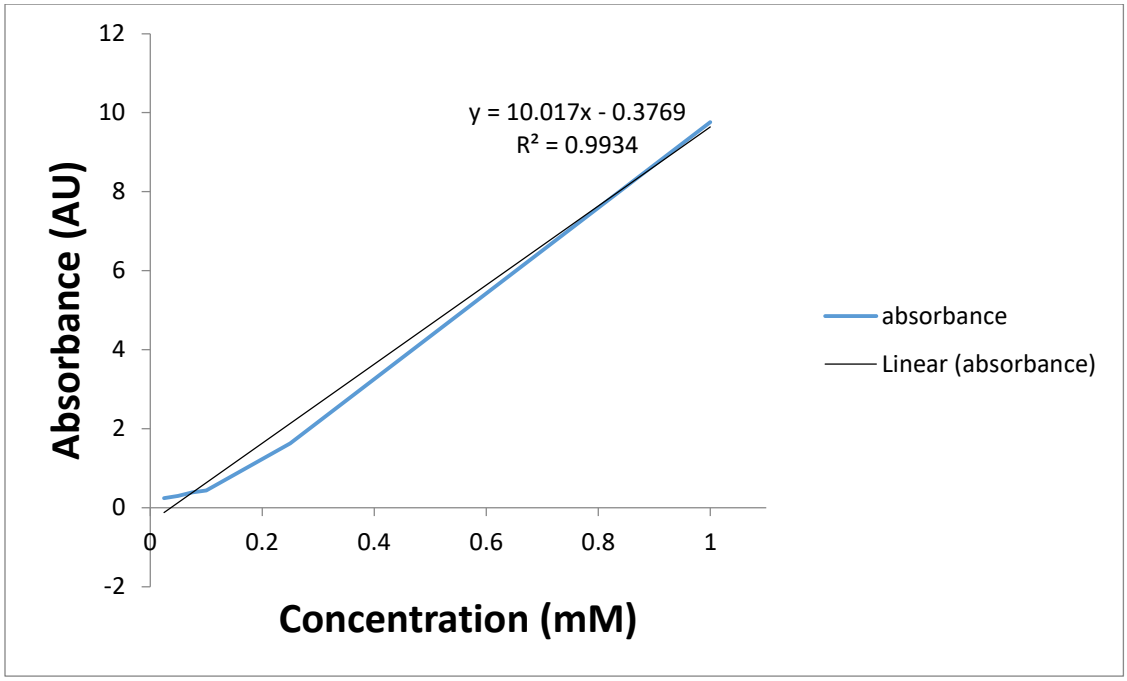

Figure 25 Standard curve of Vitexin

Encapsulation Efficiency

After ultracentrifugation of the liposomal solution, the filtrate, which contains all the unbound vitexin is analyzed by UV Spectroscopy. Vitexin displays a peak at 335nm, this point is used to read the absorbance value of vitexin from the curve of the filtrate solution which was 0.13679. Using the equation derived from the standard curve, the absorbance value of 0.13679 was put in the equation and the unknown concentration was calculated, which was 0.051mM which is 0.022mg/mL. Originally, 0.05mg/mL of vitexin was used to make the liposomal formulation. Using the EE formula, Encapsulation Efficiency of vitexin content was calculated to be 56%.

Symptoms of anxiety

- Tail biting
- Excessive facial grooming
- Digging
- Isolation
- Change in sleeping patterns
- Changes in weight

Tail Biting

Tail biting is a sign of anxiousness, done as a self-soothing behavior, under stressful conditions.

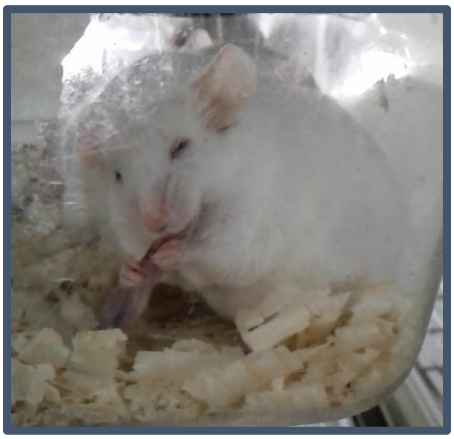

Figure 26 Tail biting

Excessive Grooming

Mice like to keep themselves clean and will groom themselves periodically throughout the day. However, a mouse that spends an inordinate amount of time vigorously grooming himself might be a nervous or stressed-out mouse. A mouse might find a new/unfamiliar environment stressful, or he can become nervous if he is suddenly housed with other mice he does not know. He might even resort to this self-soothing behavior (constant, vigorous grooming) if he has no respite from bright lights; no den to retreat to or because of many other factors.

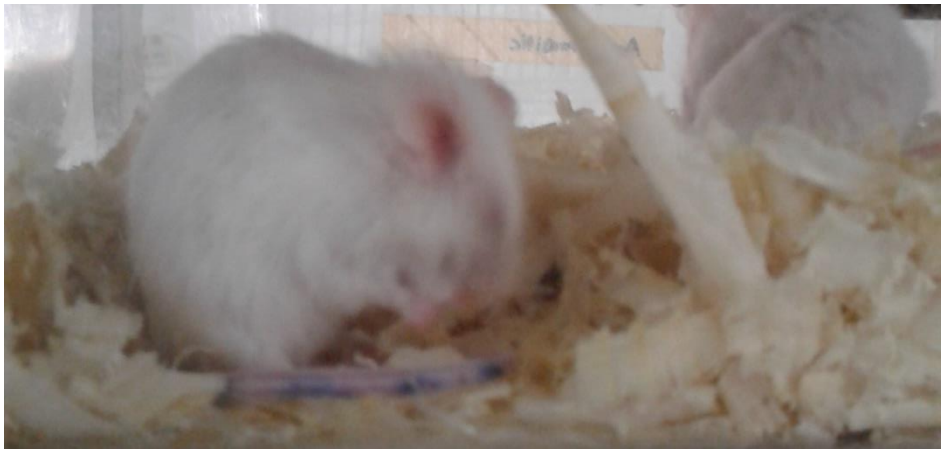

Figure 27 Excessive grooming

Change in sleeping patterns

Mice, especially female mice, are social creatures, they tend to huddle together while sleeping. A change in this sleeping pattern signifies a change in their mental condition i.e. if they sleep in isolation, it is a sign of anxiety-like behavior or depression.

Figure 28 Sleeping pattern of healthy, non-depressed mice, they huddle together

Figure 29 Sleeping pattern of a depressed or anxious mouse, they are isolated

Changes in weight

Depression is signified by either a drastic increase or decrease in weight. Any significant change in weight demonstrates anxiousness. The graph below shows that the mice increased in weight during the first week of acclimatization, and after the first week of depression there was a drastic decrease in weight, and this trend continued at the end of the second week of stressor application. Treatment with the different dosages of vitexin showed an overall increase in weights after two weeks of treatment.

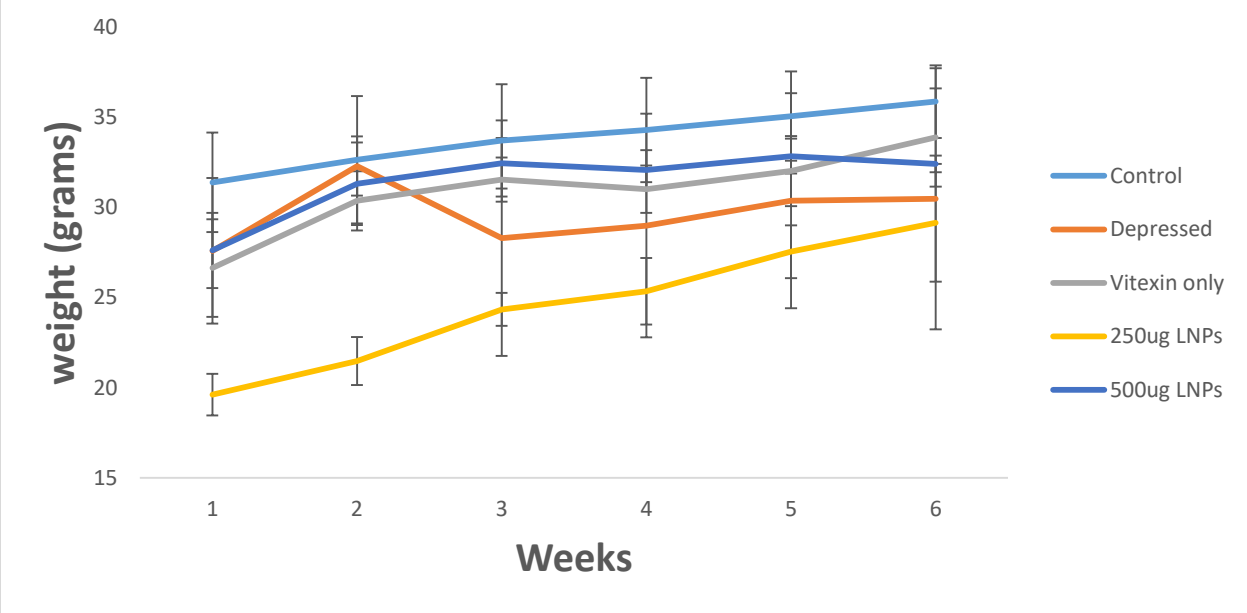

Figure 30 Changes in weight

Forced swim test

In the FST, vitexin liposomes, at both of the applied doses, induced a significant reduction in the immobility duration of the mice, 500 µg dose displayed better results than 250 µg dose which were still better than those of vitexin only. Furthermore, administration of vitexin liposomes increased the climbing and decreased the swimming duration.

Swimming time

One-way ANOVA test performed for the data obtained from the FST indicated that compared to the depressed group, mice administered with vitexin and vitexin liposomes significantly increased the swimming duration of the mice (F(4,20)=114.35, P=.00000). Post-hoc Tuckey's test confirmed that the difference between the data of all the groups was statistically significant. The graph of mean swimming time shows that simple vitexin increased the swimming time, while 250 µg dosage further increased it and the 500 µg dosage almost brought the swimming time back to normal.

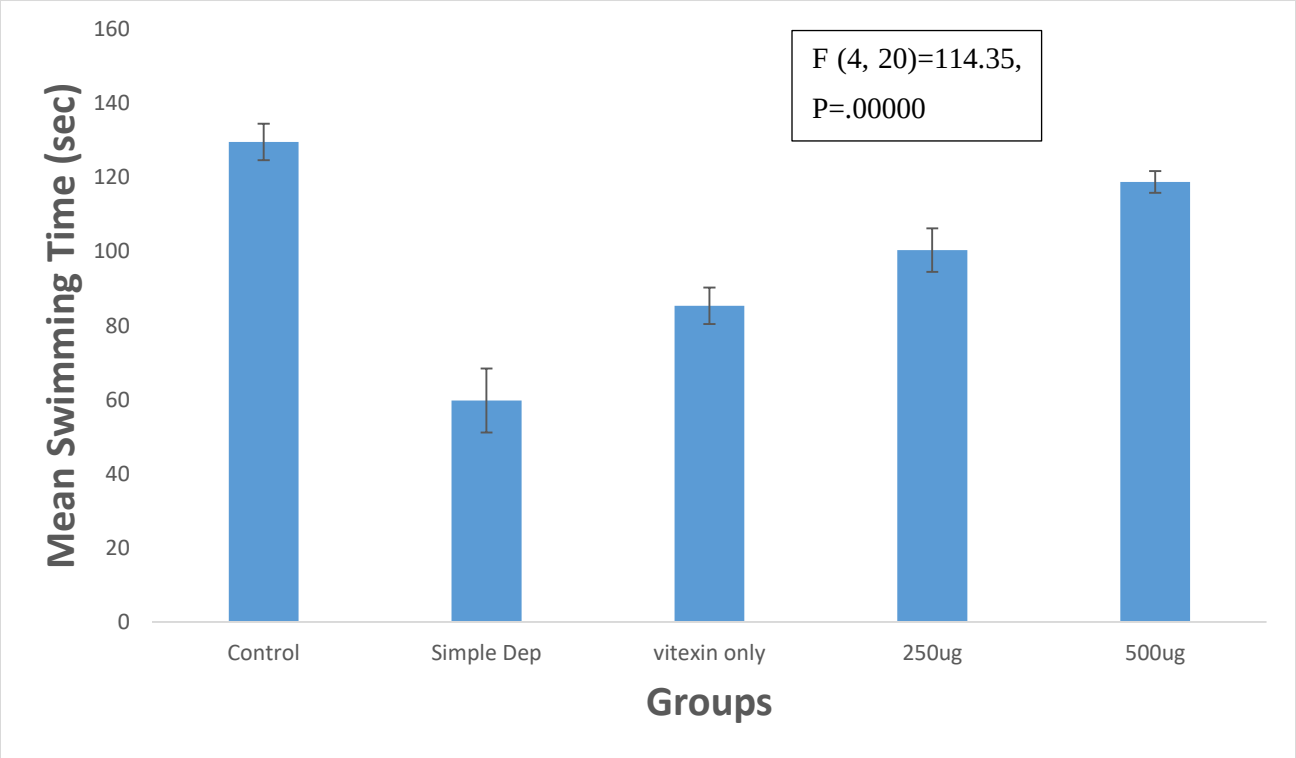

Figure 31Effect of vitexin administered at different dosages on mean swimming time

Climbing time

One-way ANOVA test performed for the data obtained from the FST indicated that compared to the depressed group, mice administered with vitexin and vitexin liposomes significantly increased the climbing duration of the mice (F(4,20)=9.7999, P=.00015). Post-hoc Tuckey's test confirmed that the difference between the data of all the groups was statistically significant. The graph of mean climbing time shows that simple vitexin increased the climbing time, while 250 µg dosage results were same as simple vitexin and the 500 µg dosage almost brought the climbing time back to normal.

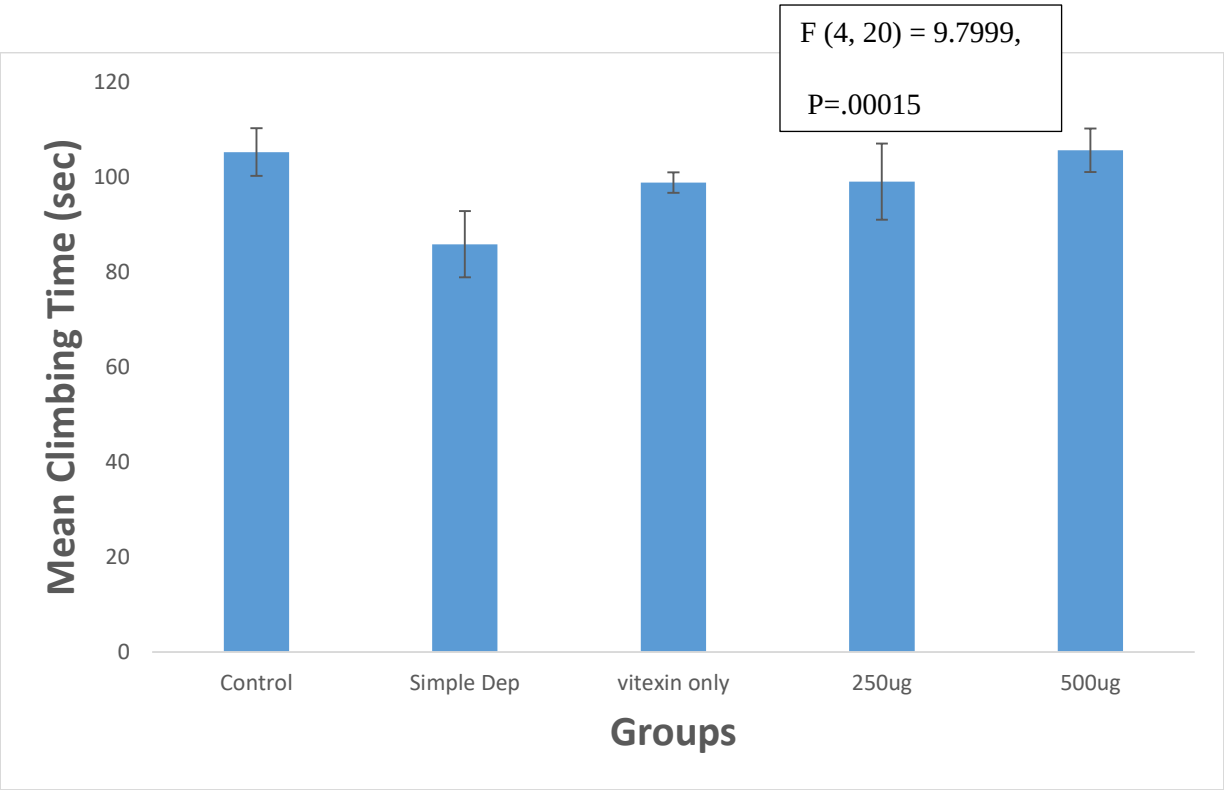

Figure 32 Effect of vitexin administered at different dosages on mean climbing time

Immobility time

One-way ANOVA test performed for the data obtained from the FST indicated that compared to the depressed group, mice administered with vitexin and vitexin liposomes significantly decreased the immobility duration of the mice (F(4,20)=368.91, P=.0000). Post-hoc Tuckey's test confirmed that the difference between the data of all the groups was statistically significant. The graph of mean immobility time shows that simple vitexin decreased the swimming time, while 250 µg dosage further decreased it and the 500 µg dosage almost brought the immobility time back to normal.

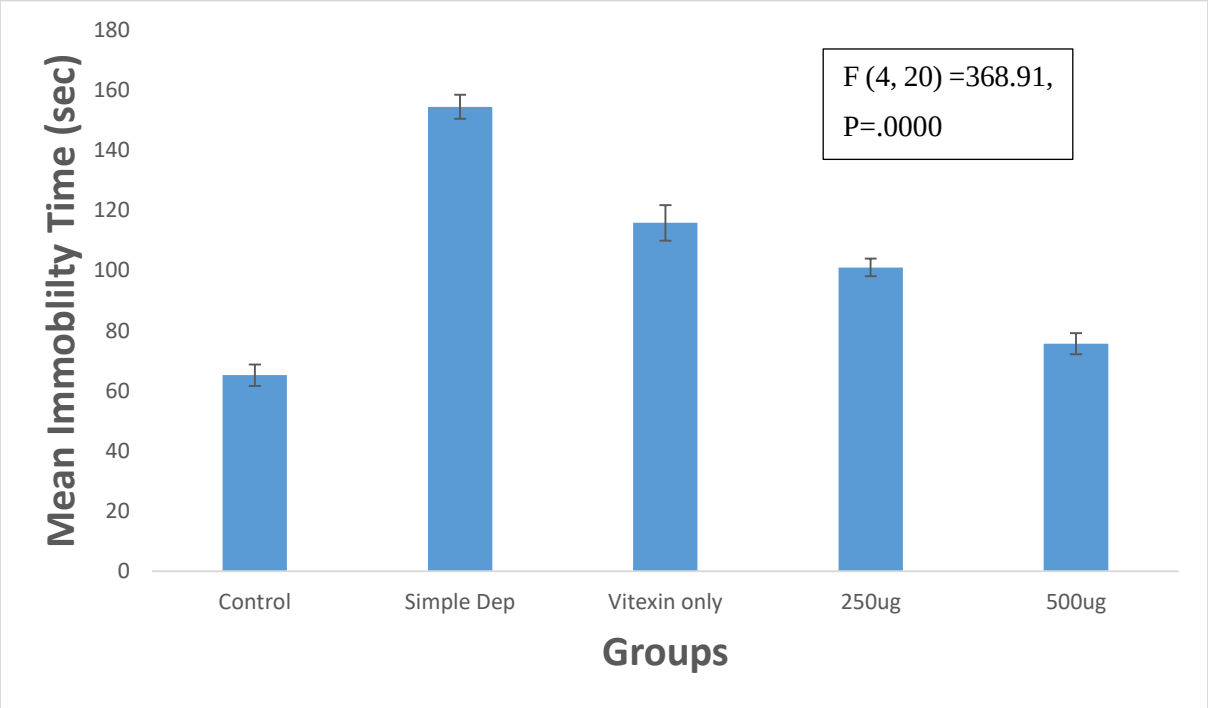

Figure 33 Effect of vitexin administered at different dosages on mean immobility time in FST

Tail suspension test

One-way ANOVA test performed for the data obtained from the TST indicated that compared to the untreated group, mice administered vitexin (10mg/kg) and 250 µg of vitexin liposomes showed significantly decreased immobility time and those administered with 500 µg of vitexin liposomes showed similar results to those of control group (F(4,20)=456.09, P=.0000). Post-hoc Tuckey's test confirmed that the difference between the data of all the groups was statistically significant.

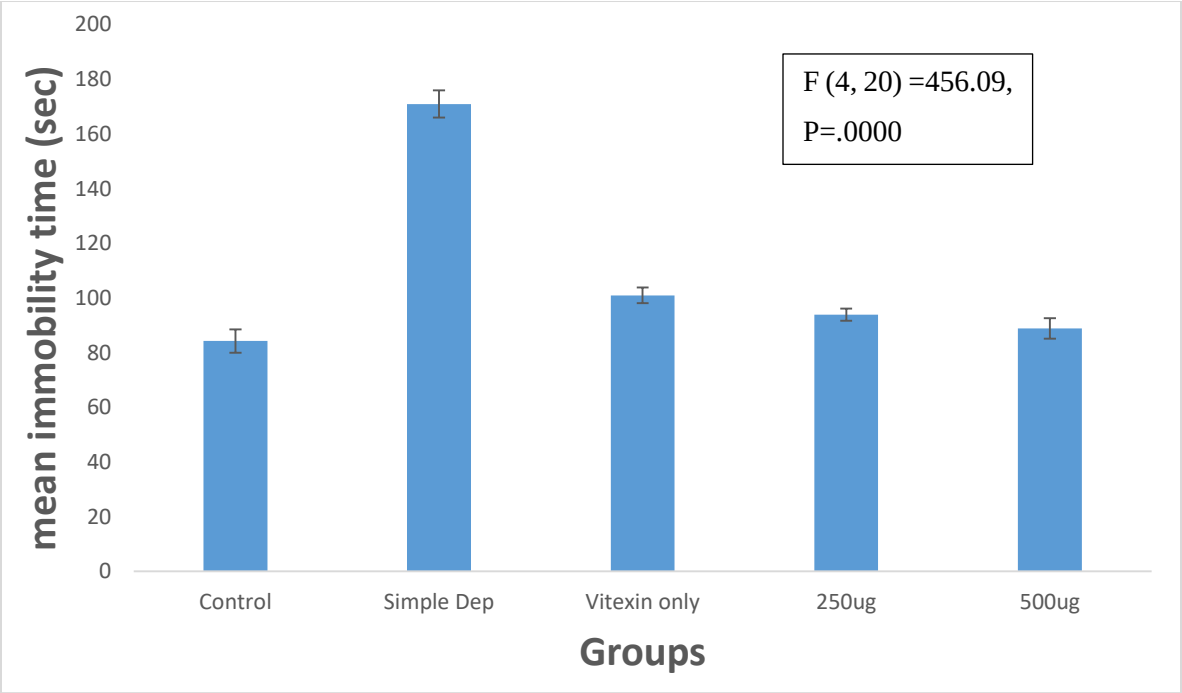

Figure 34 Effect of vitexin administered at different dosages on mean immobility time in TST

Open field test

There was a statistical trend toward stress modulating the number of center square entries in this behavioral test, with untreated, stressed mice showing the lowest amount. Chronic stress also decreased (an anxiogenic-like effect) the percent center square time. Treatment with vitexin only improved those times while treatment with vitexin liposomes significantly increased both center square entries and the amount of time spent there.

One-way ANOVA test performed for the data obtained from the OFT indicated that compared to the untreated group, mice administered vitexin (10mg/kg) and 250 µg of vitexin liposomes showed significantly increased time spent in the center zone and those administered with 500 µg of vitexin liposomes showed similar results to those of control group (F(4,20)=992.80, P=.0000). Post-hoc Tuckey's test confirmed that the difference between the data of all the groups was statistically significant.

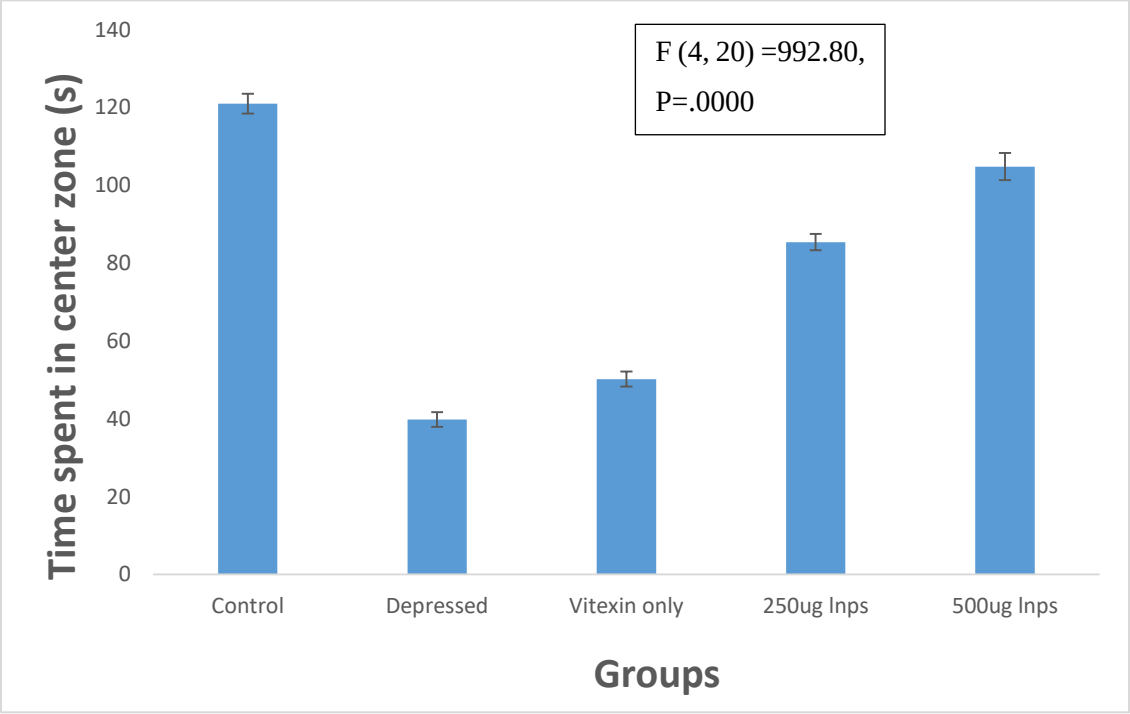

Figure 35 Effect of vitexin administered at different dosages on mean time spent in center zone

Cytotoxicity

Vitexin presents no cytotoxicity (IC_{50} N 200 µg/mL) in vitro (Rosa et al., 2016). In-vivo studies on mice, showed no significant acute and sub-chronic toxicity. Furthermore, even repeated treatment with high dose of vitexin (10 mg/kg, iv) and vitexin liposomes (250 µg/mL and 500 µg/mL) over a long period of fourteen-days is safe and showed no cytotoxic effects.

Liver

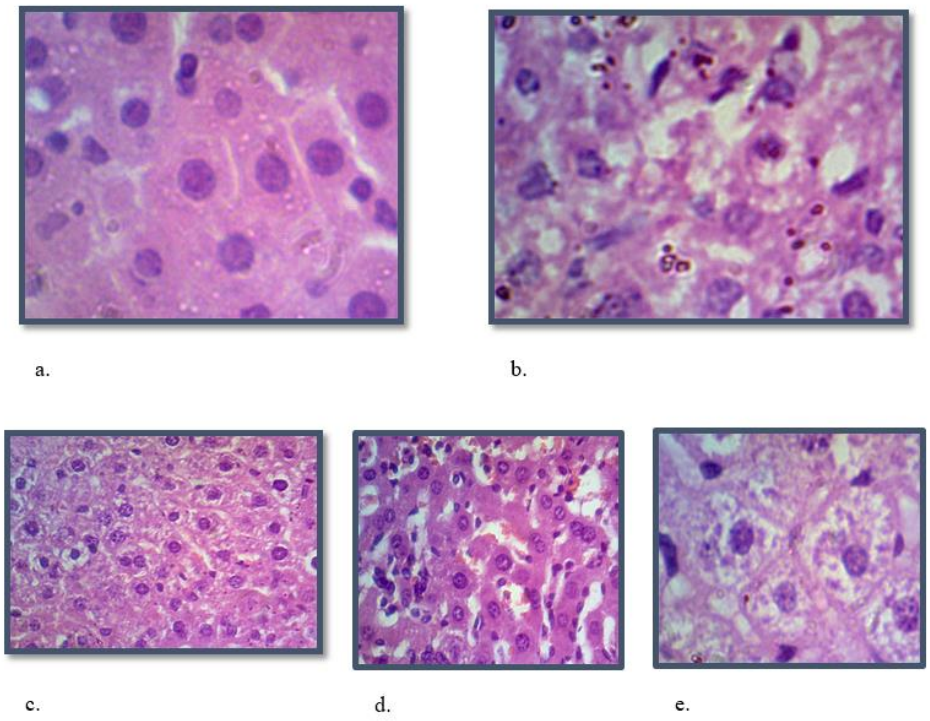

Figure 36 (a) Liver cells of a normal mouse (b) Liver cells of a depressed mouse (c) Liver cells of a mouse treated with vitexin only, (d) Liver cells of a mouse treated with 250 µg dosage of vitexin liposomes, (e) Liver cells of a mouse treated with 500 µg dosage of vitexin liposomes

Figure 36(a) shows liver from a control mice hepatocytes has feathery cytoplasm containing glycogen. Hepatocytes are arranged in one cell layer thick plate. Figure 36(b) shows 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 36(c) shows that liver cells, hepatocytes, appear normal in histology after the administration of vitexin via IV. Cytoplasm is also normal, closer to those in control group mice. Figure 36(d) and Figure 36(e) shows that the cells of the group that were given vitexin incorporated nanoparticles also seem to appear normal, with normal hepatocytes and sinusoids.

Brain

Figure 37 shows histological images of control and the 3 dosages of vitexin show that vitexin did not show any cytotoxic effect on brain cells even after repeated exposure of 14 days. Caudate nucleus, glial cells and oligodendroglia seem normal. Cells structure is intact. No cytotoxicity can be seen.

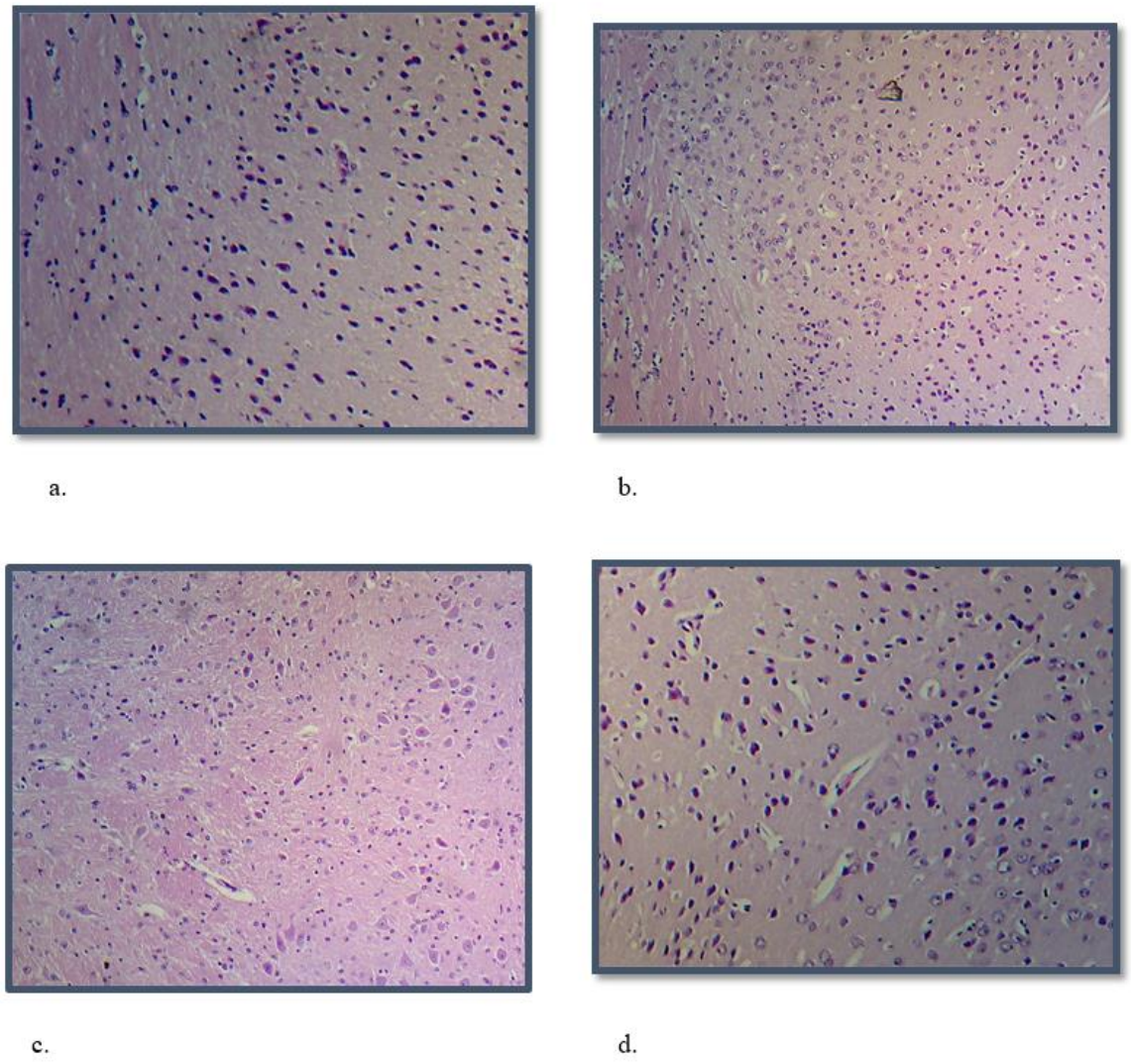

Figure 37 (a) Brain cells of a normal mouse (b) Brain cells of a mouse treated with vitexin only, (c) Brain cells of a mouse treated with 250 µg dosage of vitexin liposomes, (d) Brain cells of a mouse treated with 500 µg dosage of vitexin liposomes

Heart

Figure 38 shows histological images of control and the 3 dosages of vitexin show that vitexin did not show any cytotoxic effect on heart cells even after repeated exposure of 14 days. Intercalated discs, myofibrils, nuclei are normal among all the groups. After treatment cells seems normal, nuclei are intact.

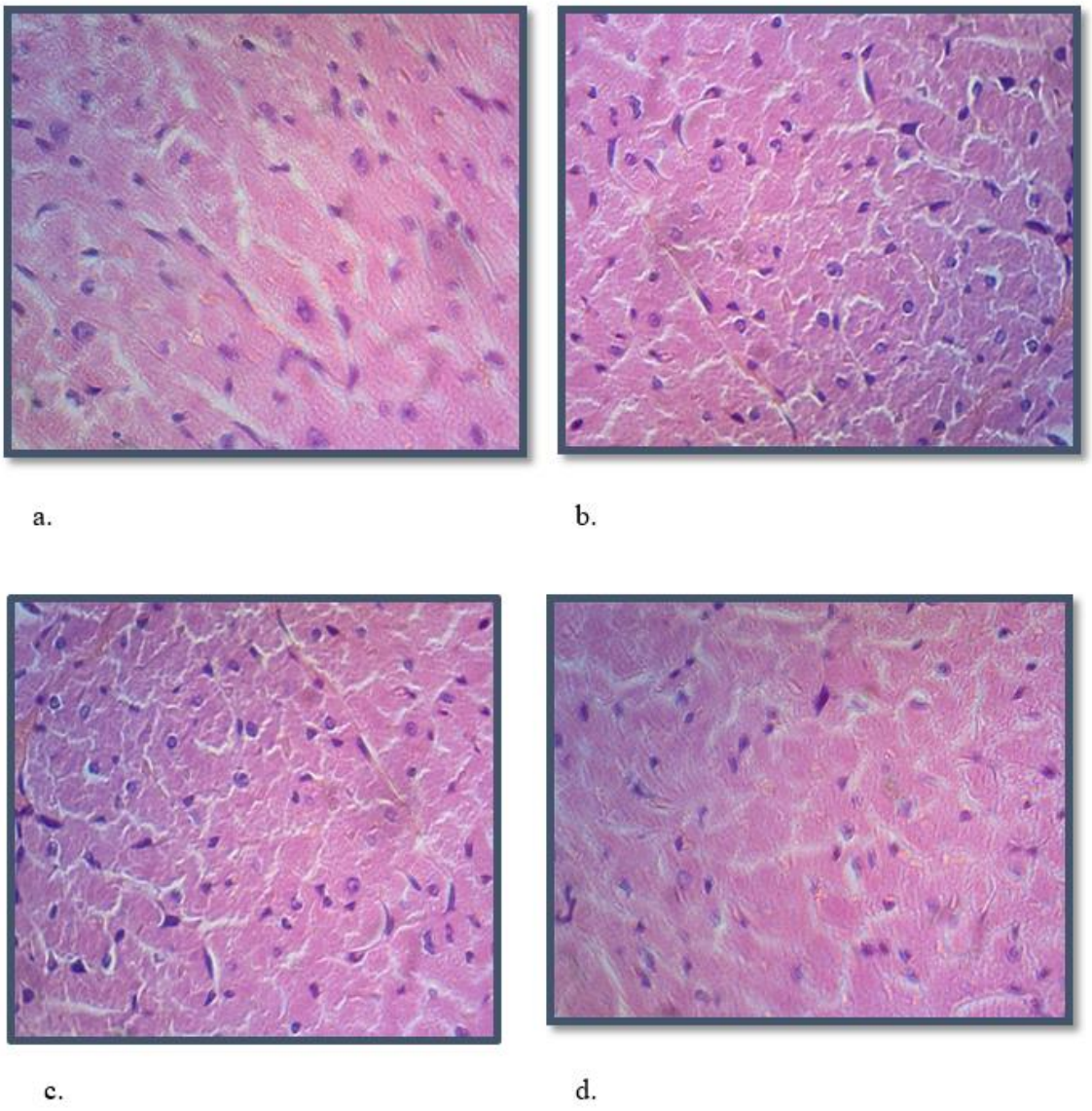

Figure 38 (a) Heart cells of a normal mouse (b) Heart cells of a mouse treated with vitexin only, (c) Heart cells of a mouse treated with 250 µg dosage of vitexin liposomes, (d) Heart cells of a mouse treated with 500 µg dosage of vitexin liposomes

Kidneys

Figure 39 shows histological images of control and the 3 dosages of vitexin show that vitexin did not show any cytotoxic effect on kidney cells even after repeated exposure of 14 days. Capillaries, proximal tubules, bowen capsule, all are intact over the four groups. Mesengial cells also has well intact cell membrane. Podocytes are also presents.

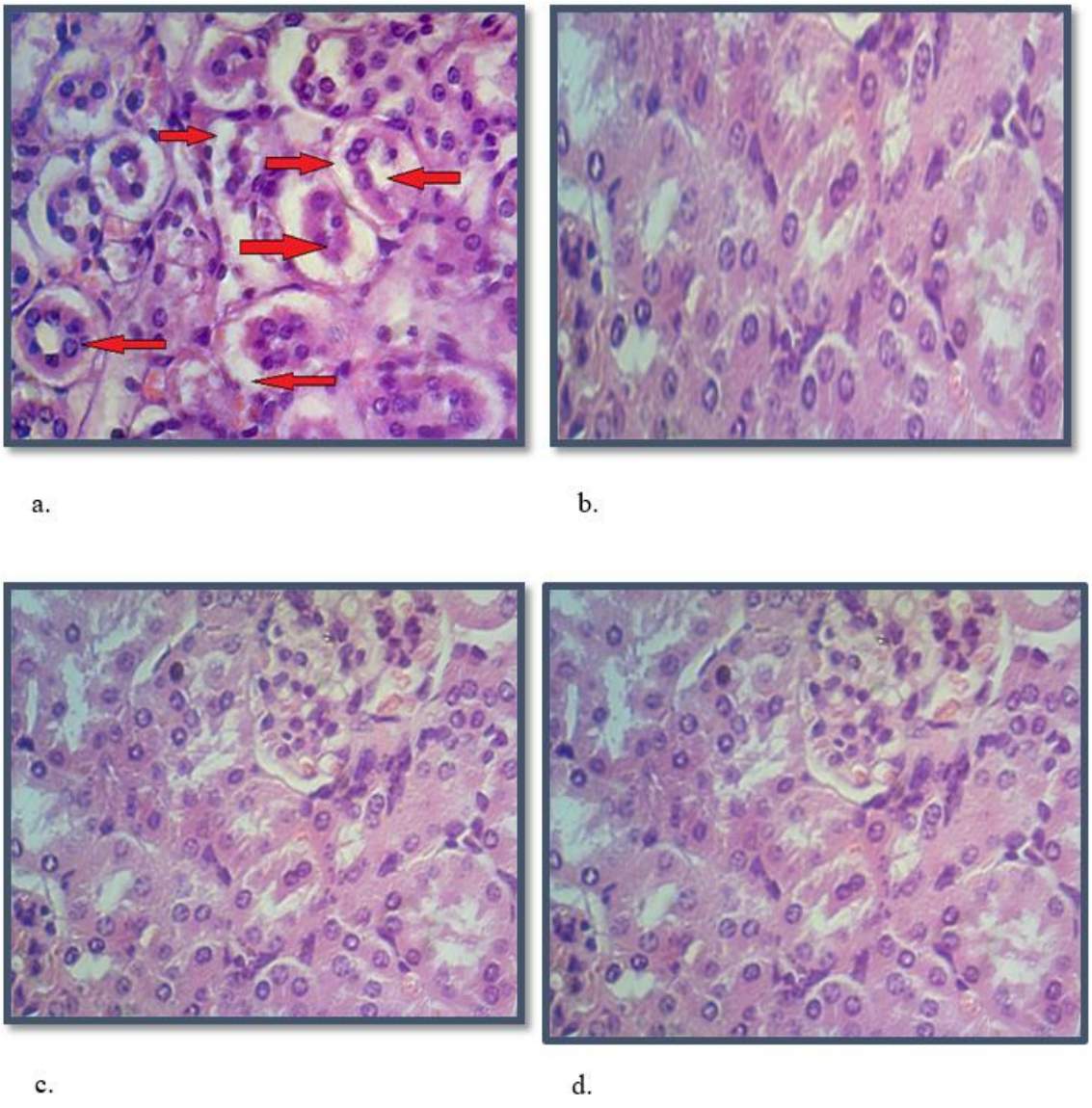

Figure 39 (a) Kidney cells of a normal mouse (b) Kidney cells of a mouse treated with vitexin only, (c) Kidney cells of a mouse treated with 250 µg dosage of vitexin liposomes, (d) Kidney cells of a mouse treated with 500 µg dosage of vitexin liposomes

Spleen

Figure 40 shows histological images of control and the 3 dosages of vitexin show that vitexin did not show any cytotoxic effect on the cells of the spleen even after repeated exposure of 14 days. Red and white pulp is evenly distributed. Sheathed capillaries are present. Splenic cords are a meshwork of erythrocytes, macrophages, plasma cells and granulocytes.

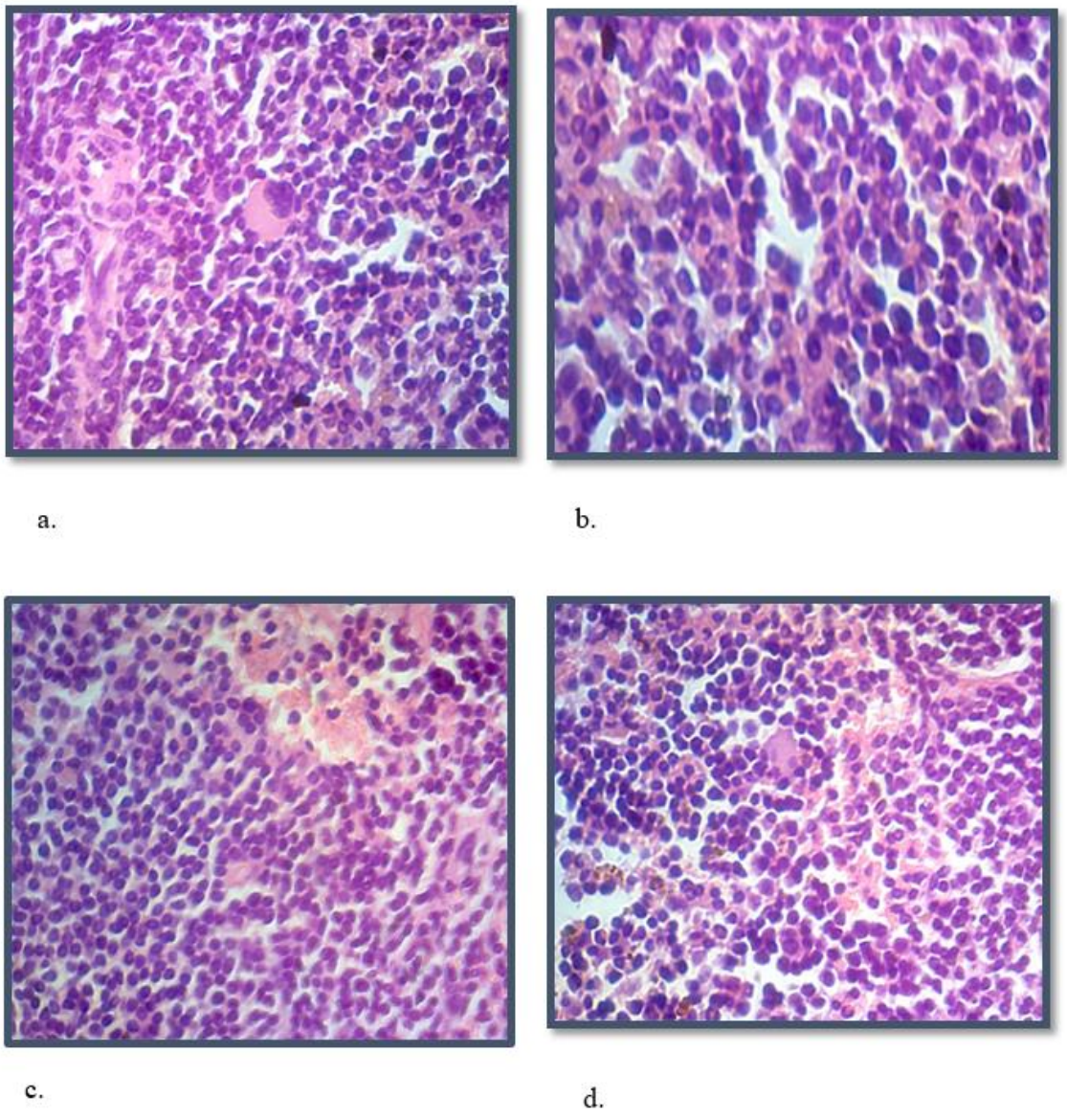

Figure 40 (a) Spleen cells of a normal mouse (b) Spleen cells of a mouse treated with vitexin only, (c) Spleen cells of a mouse treated with 250 µg dosage of vitexin liposomes, (d) Spleen cells of a mouse treated with 500 µg dosage of vitexin liposomes

Chapter 5

Discussion

This study was done to assess the impacts of liposomal vitexin on the CNS by first inducing depression in mice using the UCMS protocol and then utilizing behavioral tests, after induction of depression and after treatment. Two weeks of UCMS protocol which was subjected on mice showed traits of depressive-like behavior which were apparent even before the behavioral tests were conducted, like lethargy, social withdrawal, excessive grooming, tail biting, etc. Then tests were done to confirm this visual analysis to give quantified results. Following depression two weeks of chronic treatment was given of simple vitexin, vitexin liposomes in 250 µg and 500 µg. Forced swim tests, open field tests and tail suspension tests were done to check probable antidepressant-like impacts of vitexin.

The FSTs and TSTs are widely recognized animal models utilized for antidepressant drug screening. Both these tests depend on the perception that mice, after preliminary escape attempts, build up a stationary stance when set in a temporary stressful inescapable situation. This immobility, alluded to as social misery in mice, replicates a case of depression in humans. Subsequently, a decrease in the complete span of immobile status demonstrates an antidepressant-like impact. In this study, when evaluated in TST, vitexin (at 10mg/kg) diminished the immobility time of mice contrasted to those in the control group, demonstrating a stimulant like impact. The FST results affirmed these discoveries and gave extra data identified with a conceivable system for this antidepressant action. The diminished immobility time and swimming time combined with an expansion in the climbing time showed that the antidepressant-like impact of vitexin might be identified with catecholaminergic, instead of serotonergic, mechanisms in the CNS.

It was also observed that when mice were given a dose of simple vitexin, they developed a slightly higher body temperature compared to the mice that were given the liposomal dosages, demonstrating that a direct dosage of vitexin does cause some adverse effects. There was also a change in urine color, with it being a more murky brown than the slight yellow of other mice, indicating that large amounts of vitexin, which is of a brownish color, wasn't being adequately absorbed by the body, strengthening the fact that liposomes increase bioavailability of the drug by increasing the stay time and half-life of the drug. The mice with simple vitexin were slightly lethargic compared to their counterparts who were given vitexin liposomes, these mice showed slight improvements in their behavior within 2 days of the start of therapy, and two weeks of therapy made sure that any lingering effects of stressor protocol were completely eliminated.

Taking everything into account, this study demonstrates that vitexin liposomes shows a stimulant like activity superior to the dosage of simple vitexin, when the TST and FST were performed, which are broadly utilized animal behavior models for studies of antidepressant drug screening (Ö. D. Can et al., 2013). With dependable safety and diverse biological activities, vitexin liposomes may be legitimate possible therapeutic contenders for some maladies and disorders.

Chapter 6

Conclusion

Liposomes were synthesized successfully. Behavioral tests demonstrated favorable symptoms of depression and anxiety. Behavioral tests after treatment with vitexin nanoparticles gave encouraging results to the positive effects of vitexin nanoparticles on depression. 500 µg of lnps gave the best result compared to a dosage of 250 µg of lnps and simple vitexin. One-way ANOVA and post hoc Tuckey's test demonstrated the significance of the results. Vitexin and Vitexin liposomes did not demonstrate any cytotoxic effects after sub-chronic dosages.

Future perspectives

Work can be done to find out the pathways that vitexin liposomes take to further understand their antidepressant-like behavior. Further histological and blood test can also be performed to further analyze their effect on different parts of the body over a period of time longer than 14 days, this may also bring about further behavioral indices. Effect on the gut flora can also be observed.

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