Annexing Blood-Brain Barrier for Targeted Drug Delivery Designed for Brain Instigated Pathologies; An Application of Solid Lipid Nanoparticles

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Dedicated

To my late grandfather Engr. Jehandad Khan, whose strong attitude inspired me to be a hard working person.

To my parents, who have been there to support me and let me achieve my goals. They have been my ultimate inspiration.

To my best friend, who has always picked me up during difficult times and encouraged me morally and practically. Whose firm support, helped me in achieving this goal.

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LIST OF ACRONYMS

BBB	Blood Brain Barrier
SLNP	Solid Lipid Nanoparticle
SEM	Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
XRD	X-ray Diffraction
HPLC	High Performance Liquid Chromatography
Con-A	Concanavalin A
P-80	Polysorbate 80
NVU	Neurovascular Unit
TJ	Tight Junction
AJ	Adherent Junction
ANG1	Angiopoetin-1
bFGF	Basic Fibroblast Growth Factor
GDNF	Glial Derived Neurotrophic Factor
TGF-β	Transforming Growth Factor- β

MAGUK	Membrane Associated Guanylate Kinase
ZO-protein	Zonula Occluden protein
TEER	Trans-Endothelial Electrical Resistance
CSF	Cerebrospinal Fluid
PNS	Peripheral Nervous System

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ABSTRACT

In terms of brain pathologies, the major obstacle for treatment is due to insufficient transport of drugs across the blood brain barrier. This barrier is basically the junctions between endothelial cells and neurons of brain. Drug transport is halted due to the tight junctions present between the endothelium cells which are not permeable for most of the entities. This has beneficial role in preventing pathogens and toxic materials to reach brain but have disadvantage during treatment when most of the drug molecules are prevented to cross the blood to brain barrier. Viewing this factor, it is very important to devise a system that is capable and efficient enough to transport therapeutic drug to brain. Emergence of nanotechnology has paved way to overcome such obstacle through formulation of solid lipid nanoparticle as a carrier. Just like a vehicle, SLNPs have the ability to encapsulate transporting entity within its core and transport it to that area. Over the decade SLNP has proved to deliver drugs for other pathologies. In this case test drug diosgenin has been in focus due to its anti-cancer and anti-inflammatory properties. As it's a natural extract of plant it showed higher effectivity and low toxicity in many studies.

In this study, polysorbate-80 coated stearic acid SLNP were formulated with and without drug encapsulation. These SLNP were characterized through SEM, FTIR, XRD and HPLC. This formulation was then tested in-vitro and in-vivo. U87 MG cell lines were used in order to check the anti-cancer effect (MTT assay) by using blank SLNP, diosgenin only and diosgenin SLNP. Cell scratch assay was used with the IC-50 of the above formulations in order to check its anti-metastatic effect on glioma cells. Then sickness model were formed by injecting Concanavalin-A into mice and treating them with PBS, Fluoxetine, Diosgenin,

blank SLNP and diosgenin SLNP. These groups were then undergone behavioral analysis to assess its behavior and effectivity of treatment. Open field test, elevated maze plus, social interaction, grooming test, tail suspension test and forced swim test were performed for analysis of their behavior. Histological sectioning and staining were performed on mice brains for anti-inflammatory response analysis in different groups of mice. Drug levels were analyzed in different organs and blood of mice through spectrophotometry.

The characterization SLNP showed desired size with anionic charge which has encapsulated diosgenin in its core. The in-vitro assays showed the anti-proliferative activity of SLNP with and without drug and diosgenin exhibited high cytotoxicity level than diosgenin SLNP and blank SLNP. This suggests that diosgenin is quite effective at low concentrations. The in-vivo tests showed that the treatment which were given to mice had healing effect in sickened mice. The group diosgenin and diosgenin with SLNP showed higher level of social interaction and exploratory behavior than others. Which were further proved by the histology that the inflammation was controlled in these treated mice than the Con-A group. The blood tests proved that diosgenin is not toxic to cells. These tests and assays showed that diosgenin as a drug has potential for controlling glioma proliferation and showed quite high anti-inflammatory responses in mice.

Chapter 1

Introduction

In human physiology, brain is one of the highly developed organ on which we as specie have pride on. Comparing to other specie on the basis of size, structure, association with other systems and intelligence; our brain is highly unique and complicated. Blood-brain barrier is basically a barricade for diffusion which hinders the influx process of most of the chemical agents that tends to move from blood to brain (Ballabh, Braun, & Nedergaard, 2004). It has great importance in neuro-therapeutics but despite of its importance this has been neglected so far in industrial, pharmaceutics and therapeutic programs (Pardridge, 2005). Blood brain barrier permeability has been attributed as a major milestone in development of brain targeted therapies. From neurodegenerative diseases to malignancies; blood brain barrier permeability is a common factor which must be considered in drug development. The advancement of new medications for the brain has not kept pace with advancement in the neuro and therapeutic-sciences, because most of the drugs that are designed for neurological disorders are not capable enough to cross the blood-brain barrier (BBB). Albeit 100% of drugs (large molecules) do not cross the BBB and on other side this issue exists for small molecule drugs — approximately more than 98% of these drugs do not cross the BBB (Ghose, Viswanadhan, & Wendoloski, 1999). In spite of the above study, no therapeutic and pharmaceutical organizations have been focusing on developing or designing strategies to deliver drugs in an efficient way from blood to brain. However strategies can be developed that will have an insight on the endogenous system at molecular level to get a clear picture on how we can efficiently transport drug without losing the

integrity of that chemical agent (Pardridge, 2005). Designing and developing a new drug for neurological disorder has been unnerving challenge and majority of the disorders have no proper treatment due to the complications that brain impose. But if these complications as such one is the blood brain barrier are overcome then there are greater chances to treat the disorders effectively. For such obstacle to overcome there is a therapeutic drug delivery system that can be implicated in this case. Nano-particle has become one of the important strategies in drug delivery system that has been utilized to transport conventional drugs, vaccines, proteins, recombinant nucleotides, etc. Nanoparticle drug-delivery system has been successful in modification of the distribution and kinetics of the drug that has to be delivered (Parveen, Misra, & Sahoo, 2012). Various types of Nano-particulate systems have been designed which are small in size but have larger surface area which have the capability to improve the therapeutics and drugs safety profiles. And one of the major advantage by using this system is that they can be administered orally, dermally, pulmonary, nasal, parenteral, etc. (Ramasamy, Khandasami, Ruttala, & Shanmugam, 2012). Lipid Nano-particles are efficient when it comes to crossing cell membrane. Due to its lipidic nature it is readily taken up by brain and it is less toxic due to its bio-acceptable and biodegradable nature (Kaur, Bhandari, Bhandari, & Kakkar, 2008). Due to their small size, prolong circulation in blood vessels, high loading drug capacity, large scale production from small amount and absence of abrupt high effect makes them suitable candidate for drug delivery through blood-brain barrier (Blasi, Giovagnoli, Schoubben, Ricci, & Rossi, 2007).The use of lipid Nano-particles of stearic acid is effective because it does not impose any serious health issues because in body it is converted into oleic acid which has no effect on cholesterol level (Kris-Etherton et al., 2005). And its transport is

rapid across the blood brain barrier and uptake into the brain is high with no observable toxicity. In studies it showed efficient uptake by tumor cells (Xie, Du, Yuan, & Hu, 2012). The desired drug which has to be delivered and transported through blood-brain barrier is Diosgenin. Basically it's a natural steroidal sapogenin which is extracted from fenugreek (the Dioscorea specie). In experimental studies of tumor cells, results showed that this drug was able to down regulate the peroxidation reaction and has enhanced the body's own antioxidant defense mechanism. It is suggested that due to its strong anti-oxidant nature which has down regulated the enzymatic activity of tumor cells. Due to which it was concluded that Diosgenin poses anticancer and apoptotic activity via elevation of internal anti-oxidant defense mechanism (Jagadeesan, Nandakumar, Rengarajan, & Balasubramanian, 2012) (Selim & Al Jaouni, 2015). By encapsulating Diosgenin in lipid Nano-particles can improve the drug delivery through blood-brain barrier for neurological disorders like gliomas and psychological disorders like depression. Till now this drug and this system with stearic acid Nano-particles have not been experimentally tested on gliomas and depression. And through this we can analyze this drug is capable enough to show anti-cancer activity on gliomas or not.

Chapter 2

LITERATURE REVIEW

2.1 Central Nervous System

The complexity and integrity of nervous system depends on two main systems that make up this complicated biological network of nerve cells. Nervous system comprise of central nervous system (CNS) and peripheral nervous system (PNS) that are involved in the regulation of body's reaction and response to all kind of stimulus that can be internal or external (Abbott et al., 2010). Nerves which are originating from brain and spinal cord are a part of central nervous system which is protected in the skull and vertebral canal. And rests of the body originating nerves are part of the peripheral nervous system. It is through these nerve cells our body communicates or show response to the stimulus and also many other systems of the body are controlled and regulated via this extensive network of cells (Kappers, Huber, & Crosby, 1936).

The central nervous system (CNS), as the name indicates its position and role consist of brain and spinal cord which makes it highly complex yet delicate enough that any causality can lead to paralysis and even death. Due to which this requires a micro-environment that is regulated precisely enough to process every bit of information it receives. As the brain is only 2% of the body mass so it requires around 20% of the body's energy which is available through blood circulation. This requires continuous blood supply and in case the circulation slows down the brain senses it because it does not have any other energy reservoir to compensate for that (Abbott et al., 2010). Ions, neurotransmitters and proteins

level must be maintained and regulated properly in order to prevent neurotoxicity or any kind of disturbance in the normal functioning of CNS (Iversen & Fonnum, 1994). There are varieties of metabolites and other substances that are the by-product of digestion or from pharmacological interventions might be not that toxic to PNS and its organs but could be quite toxic to CNS.

Central nervous system homeostasis can be fluctuated due to any entity that is circulating in the blood. So it is very important to maintain the homeostasis of CNS from the blood-borne entities due to which it has developed a number of barriers which have evolved greatly at cerebral micro-vasculature level. These barriers are different at permeability levels which consist of the blood-brain barrier (BBB), blood-cerebrospinal fluid (CSF), blood-spinal cord barrier and blood-retinal barrier (Cipolla, 2009). Among all of the barriers the most exclusive and selected one is on the basis of its cellular architecture and role; is the blood-brain barrier (BBB) (Saraiva et al., 2016). BBB main role is in the blocking and prevention of circulatory entities in the blood from entering CNS and allows entry of those substances that are vital to CNS regulatory functions (Banks, 2009). This barrier act as a bridge between CNS and blood that allows exchange of necessary molecules that are required for homeostatic equilibrium, nutritive or immunological purposes (Quan & Banks, 2007).

2.2 Historical Perspective of BBB

In 1885, the German neuro-anatomist Paul Ehrlich observed that most of the water soluble acidic dyes when injected into peripheral system were capable of dying tissues outside the CNS but failed to stain the brain and cerebrospinal fluid (CSF) (Ehrlich, 1885). Due to which he attributed this significant characteristic to the fact that the nervous tissues lack

certain kind of binding affinities for these dyes (Lewandowsky, 1900) (Liddelow, 2011). In 1900, another investigator Lewandowsky observed that when potassium Ferro cyanide was injected via intravenous route that didn't induce any pharmacological actions but when it was injected directly into the central nervous system it stained the brain and CSF but didn't stain the tissues of peripheral system (Edwin E Goldmann, 1909) (Saunders et al., 2014). These observations led Lewandowsky to coin a term "blood brain barrier (BBB)" and named it "bluthirnschranke" for its unique and well controlled milieu of CNS which imposed a barrier for circulating entities between blood and brain. Later on after the discovery of BBB, Edwin Goldman (1909, 1913) student of Paul Ehrlich further demonstrated the concept of BBB through intravenous (i.v.) administration of trypan blue an acidic dye which stained all tissues except the brain and CSF. But when the same dye was injected directly into the subarachnoid area, the choroid plexus were stained which clarified that brain cells do have binding affinity with the dye but when injected directly (Edwin Ellen Goldmann, 1913) (Saunders et al., 2014). This experimentation also suggested that there is a barrier between CNS and peripheral system (Krogh, 1946).

After the elucidation of BBB, several years later investigators like Friedman (1942) used high lipid soluble basic dyes that were efficient enough to cross the cerebral microvasculature and highly stain the brain cells. This suggests that the solubility of lipid plays a significant role in the transport of substances across the BBB (Long, McAfee, & Winkelman, 1963) (Saunders et al., 2014). Friedemann and Broman (1942) both observed that the BBB and blood-CSF barrier are two different and distinct systems. The BBB is localized to the cerebral vasculature in the brain and blood-CSF barrier (BCSFB) is localized to the choroid plexus. This debate that whether the BBB functions through

capillary endothelial cells or through astrocyte end-feet was sorted out with the cytochemical study through the advent of electron microscopy (Pardridge, 2012) (Reese & Karnovsky, 1967). These studies were carried out in late 60's by various investigators which were Karnovsky and Reese in 1967 and then in 1970 by Brightman.

For cytochemical study of BBB, investigator Karnovsky and Reese used horseradish peroxidase that was administered into the CSF via intra-cerebroventricular injection. That stained the extracellular fluid only which showed that the horseradish peroxidase diffused only in the astrocyte end-feet and basement membrane but didn't diffuse when it reached the endothelial cell layer junctions in the brain. This study elucidated that how BBB decreases the vascular permeability is mainly due to the presence of these tight junctions that are present in between cerebral endothelial cells which prevent the diffusion or movement of molecules across the barrier. Horseradish peroxidase somehow fails to cross the junctions because these junctions are fused with the outer layer membrane of the adjacent endothelial cell in the brain. Vesicles for transporting entities are also rare in the brain which is supposed to be involved in the transport of materials from luminal side to perivascular area of endothelial cell. This reduction in transport between the luminal and perivascular spaces also led to the reduced permeability of the barrier (Brightman & Reese, 1969) (Stewart & Wiley, 1981).

2.3 The concept of Neurovascular Unit: An Insight to the barrier properties of neuronal micro vessels

Discovery and the cytochemistry of BBB lead to a proven fact that the micro vessels of brain and that of other organs are phenotypically different from each other which then made researchers to find out how brain micro vessels acquired their highly selective vascular permeability or barrier properties. Two scientists named Stewart and Wiley in 1981 underwent an experiment in which they have examined the role of neural tissue micro-environment in the differentiation process of BBB. In this experiment they have transplanted a small fragment of embryonic brain into the coelomic cavity with non-neural environment exposure. And in the same way they have transplanted a portion of mesoderm into the brain with neural environment exposure where they can be vascularized. It was observed that the BBB functions and properties were acquired by the abdominal vessels during the vascularization of neural fragments but on other side BBB properties were not absent in the mesoderm fragment during its vascularization by the brain vessels. This study suggests that BBB differentiation is basically due to the role played by the factors within neural environment of the brain (Zloković, Begley, Djuričić, & Mitrovic, 1986).

From past few decades, brain endothelial cells has been considered to be the functional part of one unit and is involved in the communication with other types of cells in the CNS. In neurovascular unit (NVU), BBB is one of the main functional component. Basically the NVU comprise of brain vessels which lie in the close vicinity of other parts of NVU that are pericytes, astrocyte end-feet, microglia and neuronal cells. For accurate and proper neuronal activity to occur it is must the intercellular signaling should be regulated properly. Endothelium plays a vital role as a connecting bridge between blood and brain, and it also

helps in transporting important nutrients to the cells while confining the entry of toxic entities. Almost every neuron has its own capillary system (Guillemin & Brew, 2004).

2.3.1 Pericytes: are mesodermal in origin and basically contained in the basement membrane which is present on the endothelial layer. It is thought that pericytes migrated to the brain during the late phases of vascularization (Hamilton, Attwell, & Hall, 2010). Mainly pericytes resides over the tight endothelial junctions (TJ) and they also extend their cytoplasmic projections to other microvessels. They plays role in vascular contractility; during metabolic needs the brain vasculature basically dilates due to neuronal firing in which response pericytes have contractile protein that helps in vascular contractility (Fisher, 2009). Pericytes are also involved in the synthesis of components that are important part of basement membrane such as laminin, type IV collagen and glycosaminoglycans (Armulik et al., 2010). Pericytes have been observed in the polarization of astrocyte end-foot which undergoes the sheathing process of vasculature and also they act as neural stem cells (Dore-Duffy, 2008) (Tait, Saadoun, Bell, & Papadopoulos, 2008).

2.3.2 Astrocyte: signaling between astrocyte and endothelium is involved in the maintenance of BBB integrity. In NVU, astrocyte is in such a position that it interacts with both neuronal (gets neuronal input) and endothelial cells (intercellular communication). Astrocyte end-feet wraps around endothelial in such a way that it helps in water regulation through aquaporin-4 expression (Abbott, Rönnbäck, & Hansson, 2006). It is involved in the regulation of transport proteins expression and localization on the endothelium and also involved in the secretion of many mediators that helps in promoting the BBB phenotype. These mediators are angiopoetin-1 (ANG1), basic fibroblast growth factor (bFGF), glial

derived neurotrophic factor (GDNF) and transforming growth factor β (TGF-β) (Ramsauer, Krause, & Dermietzel, 2002). Astrocyte are necessary in the proper organization of neural microvasculature (Estrada, Bready, Berliner, Pardridge, & Cancilla, 1990) and studies suggests that endothelial secretes such compounds that required for differentiation and growth of astrocytes which highlight the importance of astrocyte and endothelial cell signaling (Mi, Haeberle, & Barres, 2001).

2.3.3 Microglia: are responsible for the immune responses in the CNS either that be innate or adaptive immune response. Microglia are in resting stage when the physiological conditions are normal, the shape of microglia is ramified with small circular bodies and projections processing out that interact with endothelium as a sensor. But when there is trauma or conditions are not normal then the microglia activates and retracts back their projections and the cell bodies enlarge (Han & Suk, 2005). They secrete cytokines, mediators and this state is referred to as CNS pathology (Bell & Zlokovic, 2009). During immune response these activated microglia become reactive microglia which have phagocytic activity and act as an antigen presenting cell in the CNS (Nelson, Soma, & Lavi, 2002). It is observed that during normal physiological condition the microglia have role in development and maintenance of BBB, the same microglia under pathological conditions have destructive role on BBB.

2.3.4 Neurons: are known to be involved in the regulation of different aspects of BBB functioning. According to study neurons induces BBB related enzymes that helps in the uptake of neutral amino acids (Tontsch & Bauer, 1991).

This above makes it very clear that to maintain brain homeostasis it is must that all cell and components work in confluence and conjunction.

2.4 Molecular Makeup of the Blood-Brain Barrier

BBB restricts most of the molecules transport from blood to brain (passive diffusion) except few gas molecules like oxygen and carbon dioxide and lipophilic molecules that are <200nm and <400 Da. BBB's trans-endothelial electrical resistance (TEER) is basically higher than peripheral endothelium and that is 1500-2000 $\Omega \cdot cm^2$ compared to 3-30 $\Omega \cdot cm^2$ of peripheral endothelium which makes BBB very less permeable to the molecules and this is due to the tight junction (TJ) complexes that makes the plasma membrane of adjacent cells to fuse together tightly (Butt, Jones, & Abbott, 1990) (Crone & Olesen, 1982). Proteins which basically constitute the major portion of TJ are the claudins, occludins and ZO protein.

2.4.1 Occludin: the first ever highly expressed integral TJ membrane protein that was discovered in the liver of chick (Furuse et al., 1993). Occludin is a polypeptide of 522 amino acid with a molecular mass of 60-65kDa. It's a trans-membrane protein with four domain whose carboxyl (C-) and amino (N-) terminals are oriented in the cytoplasmic region and two outer loops that is in the intercellular cleft/region (Furuse et al., 1993). Occludin's second extracellular loop is involved in mediating high electrical resistance in TJ (Wong & Gumbiner, 1997). Serine and threonine residue in occludins have multiple phosphorylation sites and this property of occludin phosphorylation helps in regulating the association of occludins with the cell membrane (Sakakibara, Furuse, Saitou, Ando-Akatsuka, & Tsukita, 1997) (Wachtel et al., 1999) (Clarke, Soler, & Mullin, 2000) (Kale,

Naren, Sheth, & Rao, 2003). Many *in vitro* studies have elucidated the role of mutated or truncated occludin in the permeability property of BBB. That is when the N-terminus of occludin was deleted the barrier property of TJ was decreased to an extent. Truncated C-terminus shows less paracellular permeability to low molecular weight molecules (Balda et al., 1996). The C-terminus also enhances the barrier property of occludin and it also oligomerizes the occludin by the formation of disulfide bond in redox sensitive manner (Medina, Rahner, Mitic, Anderson, & Van Itallie, 2000) (Wong & Gumbiner, 1997). About C-terminus, it has been observed that it helps in the regulation of intracellular trafficking of the occludins (Matter & Balda, 1998) and has been involved in the interaction with other proteins like zonula occludens (ZO) which has regulatory properties helps occludin in anchoring within the actin cytoskeleton of cytoplasm (Feldman, Mullin, & Ryan, 2005). Occludin also has serine, threonine and tyrosine residues which when undergo kinases or phosphorylation have regulatory function. Increase in phosphorylation of tyrosine residues leads to an increase in vasculature permeability (Haorah et al., 2007) while on other side phosphorylation of serine and threonine relates to the occludin localization in the membrane (Balda et al., 1996) (Takenaga, Takagi, Murotomi, Tanonaka, & Takeo, 2009).

2.4.2 Claudin: have almost same cell membrane topography but sequence homology is not same as that of the occludins and they are also expressed on the endothelial and epithelial cells TJs. They are of 20-27 kDa by molecular weight proteins with four trans-membrane domain with their C- and N-terminus in the cytoplasmic region and two extracellular loops. Claudin have specific binding motifs (PSD-95/Discs-large/ZO-1 (PDZ)) in their carboxyl terminus which let them bind with ZO proteins (Itoh, Morita, & Tsukita, 1999). Various claudins are expressed in a tissue specific manner which basically forms the TJ stretch.

Isoform of claudin showed specific interaction between or within TJ stretch (Furuse, Sasaki, & Tsukita, 1999) (Lippoldt et al., 2000) (Morita, Furuse, Fujimoto, & Tsukita, 1999) (Nitta et al., 2003). Claudin-5 is one of the highly expressed claudin in the BBB by endothelial and epithelial cells. In an experiment in which mice were deficient of claudin-5 showed loosening of BBB and its function to reject large molecules was reduced. This suggested that claudin-5 is important in maintain the BBB size selectiveness during transport of molecules and allow only small molecules.

2.4.3 Zonula Occluden: present at adherent junctions (AJ), TJs or at the gap junctions and contain ZO-1, ZO-2 and ZO-3 (Furuse et al., 1994). This protein basically belongs to the membrane associated guanylate kinase like protein family (MAGUK). This protein is known as scaffolding proteins which helps the occludin and claudin to affix in the actin cytoskeleton in cytoplasm (Itoh et al., 1999). Apart from their scaffolding nature they are also observed to be involved in signal transduction pathways that have cell behavior or genetic expression role (Bauer, Zweimueller-Mayer, Steinbacher, Lametschwandtner, & Bauer, 2010).

In order to deliver essential nutrients to the brain from the blood that would not normally cross the BBB, a number of transporters are expressed at the luminal surface of the BBB to import these substances. These include transporters and/or receptors for glucose, amino acids, monocarboxylic acids, nucleosides, purines, amines, vitamins, ions, and peptides and proteins such as insulin, leptin, and transferring (Zlokovic, Begley, & Chain-Eliash, 1985).

2.5 Solid Lipid Nanoparticles: New Aspect of Nanotechnology

The worldwide market for drugs for CNS is highly underpenetrated and would need to develop by more than 500% just to be equivalent to the worldwide market for cardiovascular drugs (Nadkar & Lokhande, 2010). The sole reason behind this is that most of the drugs do not cross BBB, in vivo. There is a small class of drugs with atomic mass ranging from <400 to 500Dalton and high lipid solubility crosses the BBB (Loxley, 2009). And only few diseases reacts to such category of small sized drugs.

Over the past few decades significant efforts have been made towards the drug development, enabling the drugs to cross the blood brain barrier. Such advancements include direct injection into the brain, drug structural modifications, chemical drug delivery systems, osmotic opening of the tight junctions, and the use of nanoparticles as carriers, such as liposomes (Bodor & Buchwald, 1999) (Huwyler, Wu, & Pardridge, 1996) solid lipid nanoparticles, or polymeric nanoparticles (Blasi et al., 2007) (Tosi, Costantino, Ruozi, Forni, & Vandelli, 2008). Among these strategies, the most promising results are yielded by solid lipid nanoparticles as the drug delivery vector through central nervous system.

Solid lipid nanoparticles are also known as liposphere are known to be efficient and safe form of drug delivery vehicle. Introduced in 1991, they proved to be a preference over traditionally utilized carrier systems like liposomes, emulsions, micro and nano-particles (Mukherjee, Ray, & Thakur, 2009).SLNP which are made up from solid lipid are highly considered as a colloidal drug carrier. They range in nano size and are formed from physiological solid lipids which are dispersed in surfactant solution (aqueous form) (Garud, Singh, & Garud, 2012). During the formation of SLNPs, the liquid oil phase has been replaced by solid lipid phase which makes the SLNP matrix to be in solid phase at both

room and body temperature (Pardeike, Hommoss, & Müller, 2009).The drug load is encapsulated inside its solid core. These particles have many advantages e.g., smaller in size, higher surface area, higher drug loading capability, ensure the safe delivery of drug, preventing degradation as well as increasing the drug concentration at the targeted site (Ekambaram, Sathali, & Priyanka, 2012). Also, the modified helps avoiding uptake by the mononuclear phagocyte system of the body (Gref et al., 1994).

The increase in use of lipid carrier system for drug delivery are due to the following main reasons:

- Enhancement in bioavailability and reduction in the variation of plasma profile.
- Site specific drug delivery.
- Better and efficient characterization.
- High bio-compatibility and safe for biological use.
- Less to no toxicity shown by the carrier system.
- High surface area provide high drug load.
- Controlled drug release can be achieved.
- Can be produced at large scale.

SLNPs have small structure which is basically a small spherical lipid particles which ranges in nanometer having a hydrophobic solid lipid core with monolayer of phospholipid with or without coating. The drug which is loaded either is in absorbed form or dispersed in the solid matrix. And the hydrophobic phospholipids are basically entrenched in the matrix of SLNPs. SLNPs are the particles which have the combination of advantageous properties

of emulsions, liposomes and nanoparticles. Schematic representation of these particles are shown in the figure.

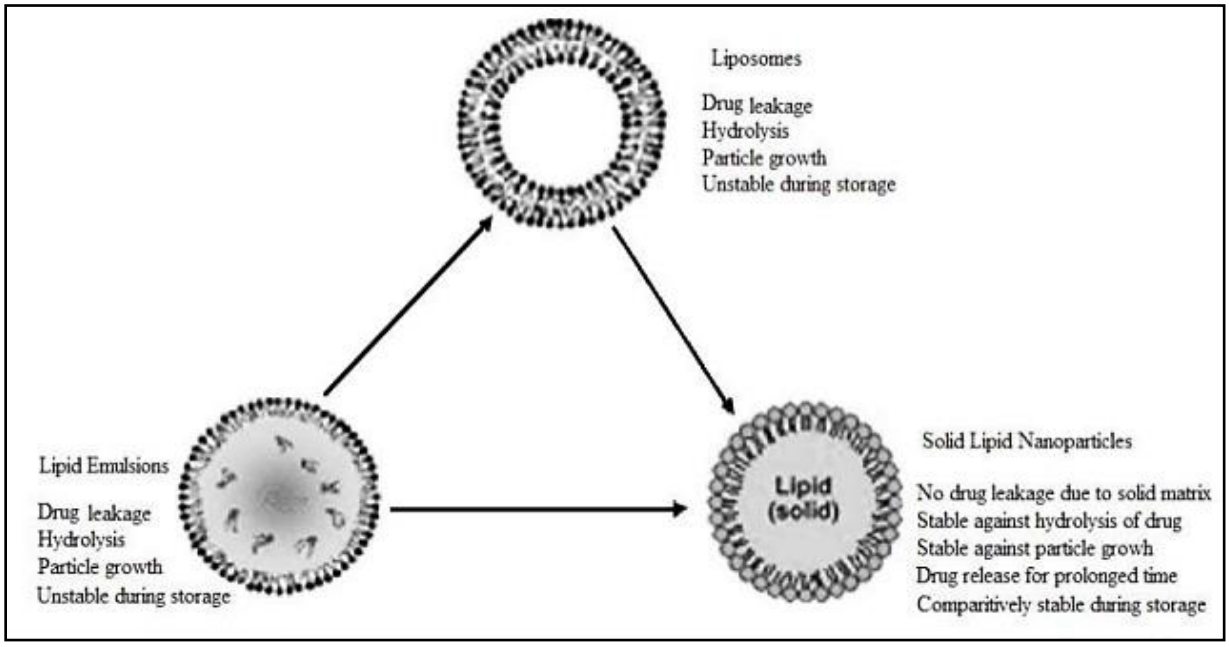

Figure2.1: Diagrammatic representation of properties SLNP, emulsion and liposome(Abbott, Patabendige, Dolman, Yusof, & Begley, 2010)

The main principles behind the release of drug from SLNPS are as follows:

- Lipid carrier's crystallinity behavior and higher rate of drug mobility leads to efficient and faster release of drug from carrier.
- Due to small size of particle in nanometer have larger surface area which also helps in efficient release of drug.
- When drug is homogenized with the lipid core matrix the drug release gets slow. This mainly depends on the entrapment and type of drug entrapment SLNP (Radhika & Sriram, ., 2016).

2.6 Stability of SLNP as a Nano-Carrier

The small size and appropriate surface functioning of SLNPs have led to effective transporting of various drugs across the blood–brain barrier. But for this purpose stability of SLNPs are must in order to avoid its degradation once it is injected into the body. Emulsifiers or surfactants are highly utilized in the formulations of SLNPs (Beyth et al., 2008) (Rawat, Jain, & Singh, 2011). Surfactants which are highly utilized for their stabilizing property are Tweens, Spans, Poloxamer 407 and Poloxamer 188.One such example is a non-ionic hydrophilic surfactant polysorbate-80 which coats lipids (Table 2.1) that can transport therapeutic agents in significant quantities across the blood–brain barrier. The transported drugs can be one of the many anticancer agents, analgesics, antagonists, antibiotics, peptides, N-methyl-D-aspartate receptor and nonsteroidal anti-inflammatory drugs (Miyazaki, Takahashi, Kubo, Bachynsky, & Löbenberg, 2003) (Alyautdin et al., 1997). Tween-80 helps in the reduction of overall SLNP size, higher absorption and specific but effective delivery of SLNPs to brain (Prabhakar, Afzal, Surender, & Kishan, 2013) (Göppert & Müller, 2005).

Lipids	Surfactants
Triacylglycerols:	***Phospholipids:***
Tricaprin	Soy lecithin
Trilaurin	Egg lecithin
Trimyristin	Phosphatidylcholine
Tripalmitin	
Tristearin	
Acylglycerols:	***Ethylene oxide/propylene oxide***
Glyceryl monostearate (ImwitorÒ900)	***copolymers:***
Glyceryl distearate(Precirol)	Poloxamer 188
Glyceryl monooleate(Peceol)	Poloxamer 182
Glyceryl behenate (CompritolÒ 888 ATO)	Poloxamer 407
Glyceryl palmitostearate (PrecirolÒ ATO 5)	Poloxamine 908
Fatty acids:	***Sorbitan ethylene oxide/propylene***
Stearic acid	***oxide copolymers:***
Palmitic acid	Polysorbate 20
Decanoic acid	Polysorbate 60
Behenic acid	Polysorbate 80
Acidan N12	
Waxes:	***Alkylaryl polyether alcohol***
Cetyl palmitate	***polymers:***
Cetyl palmitate	Tyloxapol
Cyclic	***Bile salts:***
complexes:	Sodium cholate
Cyclodextrin	Sodium glycocholate
	Sodium taurocholate
	Sodium taurodeoxycholate
Hard fat types:	***Alcohols:***
Witepsol W 35	Ethanol
Witepsol H 35	Butanol
WitepsolÒ H 45	Butyric acid
WitepsolÒ E 85	Dioctyl sodium sulfosuccinate
	Monooctylphosphoric acid sodium

Table 2.1: Different types of Lipids and Surfactant for the synthesis and stabilization of SLNPs (Abbott et al., 2010)

2.7 Diosgenin: Natural sapogenin with potential therapeutic properties

Pharmaceuticals and drug industries have been focusing on the use of natural compounds as well as natural steroids from plant extracts that act as a main drug for many pathologies (Jiang et al., 2016) (Salvador et al., 2013). An example relating to this is that few years ago natural steroids and sapogenins have been extracted which showed anticancer properties as well as low toxicity in the body (Podolak, Galanty, & Sobolewska, 2010) (Yan et al., 2015). Diosgenin is a steroid which was extracted through hydrolysis of saponin dioscin that can be acquired from Dioscorea, Smilax and Trigonella Costus species. This compound has been under high consideration and topic of interest for industrial production of many sex hormones and corticosteroids from such natural and especially from diosgenin (Dong, Lei, Lu, & Wang, 2015) (Al Jasem, Khan, Taha, & Thiemann, 2014). Over the past decades various experiments and studies has been conducted in order to understand the mechanism of diosgenin in different pathologies including cancers and inflammatory diseases. This has been find out that diosgenin have anti-inflammatory, antioxidant and anti-cancerous activity which can be a useful therapeutic drug for brain and blood pathologies, diabetes, obesity, cancers, etc (Chen, Shih, Huang, & Cheng, 2011) (Kim, Jeon, Lee, Woo, & Mun, 2012).

This steroid has been emerging as a potential anti-cancerous agent and it has been an attractive option for pharmaceuticals and drug industries. This has shown its chemo preventive activity in many cancers which makes it an effective anti-tumor agent against many organ tumor. The development of cancer therapeutics from steroidal compounds has been an attractive choice for medicinal chemists and many active molecules have emerged (Huang et al., 2012). In this context, several preclinical studies investigated the effects of

the diosgenin as a chemo preventive/therapeutic agent against cancers of several organs, and this has demonstrated the high interest of this molecule as a potential antitumor agent (Yan et al., 2015) (Raju & Mehta, 2008). The anticancer effect of diosgenin has been studied on various cell lines which were cancerous in nature showed its activity on them which was dependent on the concentration of diosgenin and type of cell used. Cancer cell lines on which it showed its anti-tumor effect are erythro-leukemia (HELcells), colon carcinoma (HCT-116 and HT-29) (Lepage et al., 2011), prostate cancer(PC-3and DU-145) (Chen et al., 2011), squamous carcinoma(A431,Hep2,andRPMI2650) (Das et al., 2012), hepatocellular carcinoma(HepG2andHCC) (Kim et al., 2012), lung cancer (A549) (Mohammad, Somayyeh, Gholamreza, Majid, & Yousef, 2013), human chronic myeloid leukemia (CML) (K562) (Jiang et al., 2016), gastric cancer (BGC-823) and breast cancer (MCF-7). It has been suggested that the mechanism on which diosgenin works is the modification of certain signaling pathways which are involved in the cell proliferation, differentiation, transition migration of epithelial mesenchyme, angiogenesis and oncogenesis. Diosgenin basically induces apoptosis of tumorigenic cells. In previous studies it has been observed that diosgenin activity is mainly mediated through different factors for example: cell cycle arrest, p53 activation, modification and modulation of caspase-3 activity, immunomodulation and activation of STAT3 pathway. Diosgenin has also showed anti-metastatic activity in human breast cancer cell lines (MDA-MB-231) by suppression of Vav proteins (Jesus, Martins, Gallardo, & Silvestre, 2016).

The anti-inflammatory effect of diosgenin has been reported and keen interest had been shown for this property but still its proper mechanism is not clearly understood. Jung et al has observed reduction in the inflammatory contents like IL-1, IL-6 and NO mediators in

murine macrophages which were treated with diosgenin (Jung et al., 2010). The superoxide inhibitory activity of diosgenin elucidated that diosgenin inhibits superoxide anion production in a potent and dose dependent manner. This was also observed that the inhibitory effect of diosgenin was due to inhibition of certain pathways that are PAK, MAPK, cAMP, PKA, etc (Lin et al., 2014). Recent study has provided an insight that the diosgenin has provided protection against hypertension in rats which were induced by the monocrotaline and preserved changes in hemodynamics of rat blood. Diosgenin has also been investigated which showed immunomodulatory and anti-inflammatory effects in osteoarthritis by inhibition of IL-1$^{\beta}$ inducing expressions different types of inflammatory mediators. This shows that it can be a drug of interest for many other pathologies (Wang et al., 2015).

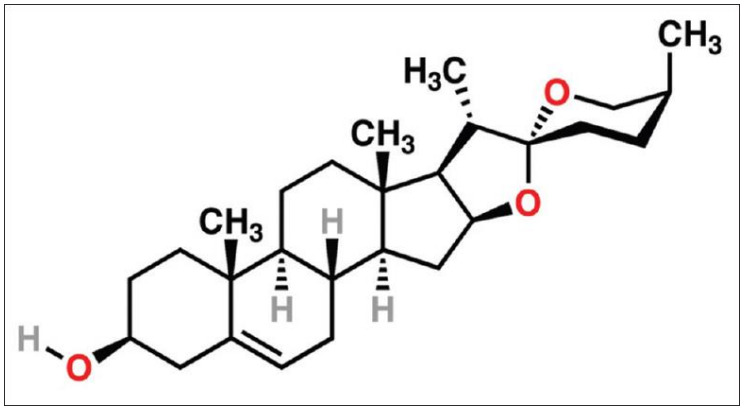

Figure 2.2: Structure of Diosgenin (Jayachandran, Vasanthi, & Gurusamy, 2016)

Hypothesis

Encapsulation of Diosgenin in stearic acid Solid Lipid Nano-particles to check its anti-cancer and anti-inflammatory activity in cell line and mouse model for brain incited pathologies.

Aims and Objectives

- To prepare stearic acid solid lipid nanoparticles coated with polysorbate-80 to check its drug encapsulation and transport efficiency through blood-brain barrier via different techniques.
- Characterization of solid lipid nanoparticles through scanning electron microscopy, Fourier transform infrared spectroscopy , ZETA potential, x-ray diffraction and high performance liquid chromatography.
- Evaluation of anti-glioblastoma and anti-metastatic potential of Diosgenin and Diosgenin solid lipid nanoparticles.
- To create a sickness behavior model with Con-A in mice in order to evaluate the anti-inflammatory activity of Diosgenin and Diosgenin solid lipid nanoparticles. And to evaluate its toxicity in mice.

Chapter 3

MATERIALS AND METHODS

Materials

Lecithin, reagent grade stearic acid (95%) from SIGMA-ALDRICH, polysorbate-80 from SIGMA-ALDRICH, isopropanol, phosphate-buffered saline (PBS), HPLC grade acetonitrile, Diosgenin (93% purity) from SIGMA, Formaldehyde solution 37% from Riedel-de Haen, extra pure Chloroform from Riedel-de Haen, Paraformaldehyde from DAEJUNG, DMEM (1X) + GlutaMAX (1g/L d-Glucose, 110mg/L Sodium Pyruvate) from Gibco, 10% Fetal Bovine Serum from Gibco, Penstrep from Gibco, 0.4% trypan blue from Gibco.

Methods

3.1 Preparation of SLNPs

Blank SLNPs: were prepared by using Hot Injection method (Upadhyay, Patel, Patel, & Saluja, 2012). For organic phase, 380mg of stearic acid with 200mg of lecithin were dissolved in 20mL of isopropanol. The mixture of organic phase was continuously stirred with a medium sized magnet in it on a hot plate at 700rpm with heating of 75^{o}C for 60 minutes. Aqueous phase was prepared by mixing 1g of polysorbate-80 in 60mL of PBS, continuously stirred on a hot plate at 700rpm with heating of 75^{o}C for 40 minutes.

Phase Transfer: 6mL of prepared organic was rapidly transferred into the stirred aqueous phase via 25mm injection needle. The final mixture was stirred for 2.5 hours at 1000rpm

with heating of 75°C. The dispersion was then passed through 0.2µm filter fitted on injection and transferred into 2mL microtubes.

Pellet Formation: Microtubes were centrifuged in refrigerated centrifuge for 1 hour at 20,000rpm with temperature set at 4°C. The supernatant was discarded and pellet was air dried. The final product was then kept in desiccator containing silica gel for overnight drying of the moisture in the pellet.

Drug Incorporated SLNPs: were prepared by using the same solvent injection method. During organic phase, 300mg of powdered Diosgenin drug was added in the mixture of stearic acid and lecithin (polymerization step). After 1 hour of organic phase polymerization step, 6ml of organic phase (with drug added) was injected into the aqueous phase which is continuously being stirred on hotplate with a medium sized magnet. After 2.5 hours of mixing the dispersion was transferred into Microtubes passing via 0.2µm filter and centrifuged at 20,000 for an hour at 4°C. The acquired pellet was separated from supernatant and dried just like that of blank SLNP.

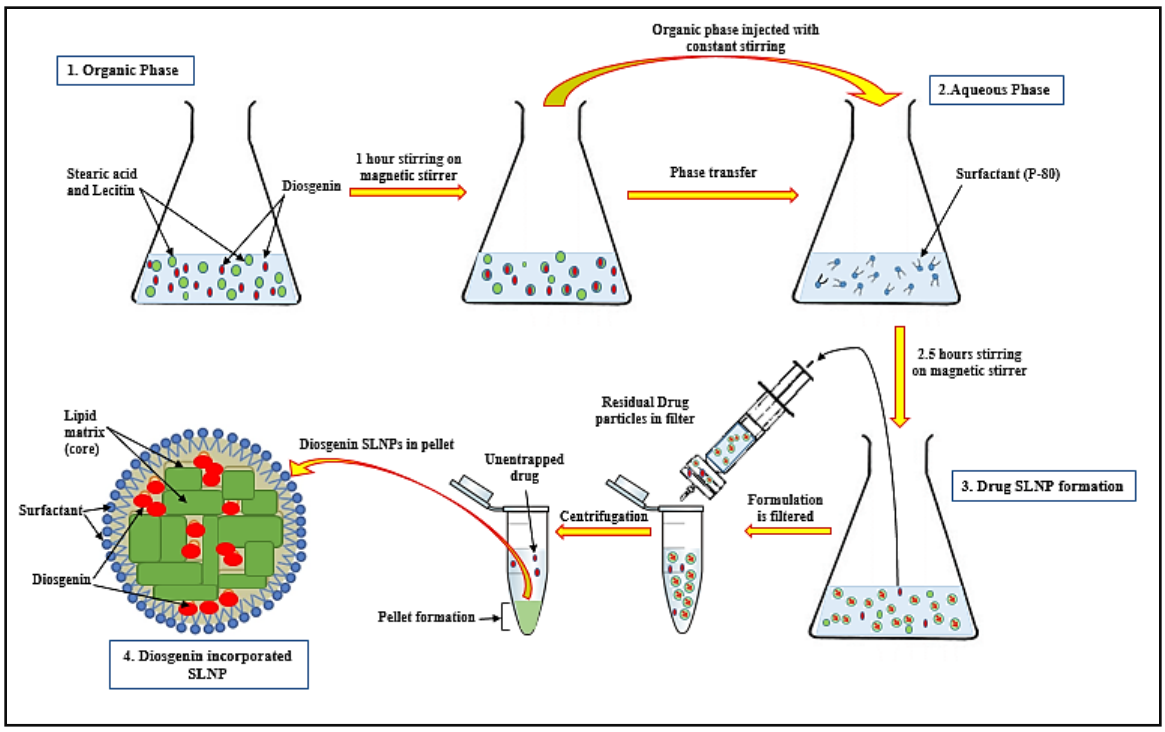

Figure 3.1: Diagrammatic representation of Diosgenin SLNPs formation through Hot Injection method. 1. Organic Phase, 2. Aqueous Phase, 3. Drug SLNP formation, 4. Diosgenin SLNP structure. Modification did in the procedure mentioned by (Kunasekaran & Krishnamoorthy, 2015)

3.2 PHASE I (Physical Characterization of SLNPs)

3.2.1 Scanning Electron Microscope (SEM)

1mg of Blank and drug incorporated SLNP were weighed and then dissolved in 2ml of PBS solution. The solution was kept in incubator at 37°C for 15 minutes. After that the solution was kept in ice and sonicated well till the solution gets clear. A drop of solution was placed on square cut glass slide and the droplet was air dried. With an ion sputter, the samples were coated with gold particles before SEM analysis. In analysis the samples were observed at different resolutions and photographs were taken with size (in nm) mentioned on them (Law, Huang, & Chiang, 2000).

For evaluation of elements concentration and drug incorporated in the SLNP Energy-dispersive X-ray spectroscopy (EDS) was also performed which was coupled with SEM apparatus. (Hădărugăa et al., 2010).

3.2.2 Fourier Transformed Infrared Spectroscopy (FTIR)

FT-IR studies were performed using a Nicolet Magna-IR 560 optical bench (Madison, WI, USA). The FTIR spectra of SLNP and drug incorporated SLNP were recorded in order to determine the interaction of SLNP components and functional groups. As SLNP were dissolved in PBS so they were recorded by KBr disc method in which the disc was prepared by pressing KBr at 5 Kpsi pressure. A droplet of samples was place on the disc and dried at room temperature after which they were analyzed in FTIR system at wave number of 4000cm^{-1} to 400cm^{-1} (Sriamornsak et al., 2008).

3.2.3 X-ray Diffraction Analysis (XRD)

The samples were in paste form so they were mixed well enough to make a fine non-granular paste. The paste was then transferred on glass slide that is fixed on an aluminum disc (sample holder). The samples were left to set and dry for a while. Then the disc was placed in the XRD disc holder. The samples were exposed to a monochromatic, collimated X-ray beam emitted from a Rigaku-Denki RU3 rotating copper anode generator and brought to a line focus by a diffraction camera containing a single vertical Franks' mirror to enhance the monochromaticity of the beam (Herbette et al., 1977). Diffraction patterns were recorded for approximately 1 h with a one-dimensional quartz wire detector. The graphs were attained with miniflex software (Miller, Herbette, & White, 1996).

3.2.4 Zeta Potential and Zeta Size Measurement

Size and zeta potential of blank and drug incorporated SLNP were analyzed by a Nano-ZS zeta sizer (Malvern Instruments, Malvern, UK) at 25 ◦C. The analyses were carried out on both types of SLNP within few days after their preparation. The SLNP were diluted with PBS solution prior to analysis and graphs were obtained for their size and potential charge (Li et al., 2009).

3.2.5 High Performance Liquid Chromatography (HPLC)

Stock Solution Preparation: 10mg of Diosgenin drug was weighed and dissolved in 1ml of acetonitrile through sonication for 10 minutes. The solution was diluted up to 10ml with acetonitrile in order to get final concentration of 1mg/1ml. Further stocks were made from this initial stock in order to get different concentrations (10mg, 5mg, and 1mg) to get a standard curve for diosgenin drug. 20µl of stock solution was automatically injected and chromatographic separation was carried out on universal C18 column with flow rate of 3min/1ml. Detection was carried out with UV range of 210nm and mobile phase was HPLC grade acetonitrile/water at a ratio of 90:10 v/v. The retention time for diosgenin was 6.01 minutes this was set according to the flow rate.

3.2.6 Drug Release Efficiency of SLNP

Drug release efficiency of SLNPs was observed through HPLC comparing with the calibration curve that was obtained through readings of standard diosgenin. Different concentration of blank and diosgenin encapsulated SLNPs were weighed (10mg, 5mg and 1mg) and dissolved in 1ml of acetonitrile through sonication and was kept at room temperature for an hour. Then the suspension was spun in a mini centrifuge for 30 sec so

the SLNPs settle down and released drug was suspended in the supernatant. 20ul of that supernatant was automatically injected and according to the above set parameters absorbance was observed for the released drug. Efficiency of released drug from SLNPs was calculated by applying Beer's Law concept and using slope-intercept formula with respect to the standard curve generated from drug diosgenin absorbance.

- $y = mx + c$

3.2.7 Drug Entrapment/Encapsulation Efficiency of SLNPs

To determine encapsulation ability, SLNPs final suspension was centrifuged for 30 minutes at 15,000 rpm so to get pellet. The supernatant was separated and passed through 0.2µm syringe filter. 100µl of the supernatant was distributed in 900µl of acetonitrile and un-entrapped drug absorbance was observed through HPLC at 210nm with above set parameters. The entrapped drug was then determined through subtraction.

The entrapment efficiency of SLNPs was calculated as follows:

$$\text{Entrapment Efficiency (\%)} = \frac{total\ amount\ of\ drug - free\ unentrapped\ drug\ in\ suspension}{total\ amount\ of\ drug} \times 100$$

All the above measurements were done in triplicates (Dora, Singh, Kumar, Datusalia, & Deep, 2010).

3.2.8 Percent Yield of Diosgenin SLNPs

The yield of diosgenin SLNPs were calculated through weighing the dried up drug SLNPs that was recovered through centrifugation and dividing it with the total amount of drug and lipids added initially (Arora, Khattar, Parashar, & Garg, 2012).

$$\text{Percentage Yield} = \frac{weight\ of\ dried\ up\ drug\ SLNPs}{total\ weight\ of\ drug+lipids} \times 100$$

3.3 PHASE II (In Vitro)

3.3.1 Culturing of U87-MG Cell line

Cell cytotoxicity experimentations were performed on adherent cell line, U87-MG. The cells were cultured in T-25 flasks in pre-warmed complete RPMI DMEM (Gibco by Life Technologies, catalogue # 31800-022). The media was supplemented with 10% Fetal Bovine Serum (Gibco by Life Technologies, catalogue # 16050) and Sodium bicarbonate (Riedel de-Haën, catalogue # 13433) to obtain a pH of 7.4 for the medium. Also, 1% of Penstrep (Gibco by Life Technologies, catalogue # 00580) was added to cell culture medium to prevent contamination of micro-organisms.

The culture flasks were kept in a humidified water-jacketed incubator at 37˚C temperature and 5% CO_2 to allow for U87-MG cell growth. The cells were maintained at a density of less than 1.0 x 10^5/ml (75%) in order to avoid contact inhibition.

3.3.2 Drug and SLNPs Treatment

Assays were performed upon Diosgenin drug, blank SLNPs and Diosgenin SLNPs exposure of 24 hours after which the assay procedures were done. Various concentrations of Diosgenin drug, blank SLNPs and Diosgenin SLNPs were prepared by dissolving the required amount in double distilled, filtered PBS through thorough sonication. These

different solutions with different concentrations were given to U87 cells with a density of 1.0 x 10^3 cells/ml for 24 hours.

3.3.3 Trypan Blue Exclusion Assay

This assay was performed before MTT assay to determine the number of cells present in the medium per ml of the medium. This was done to seed the right amount of cells viable for MTT assay, to obtain accurate results.

A dilution of 1:1v/v of the cell suspension was prepared with 0.4% trypan blue staining solution (Gibco by Life Technologies). 10µl of stained cell dilution was loaded into the disposable counting chamber of cell counting chamber slide (Countess, Invitrogen). Non-viable cells stained blue. Calculations of viable, non-viable cells and total cell density were determined by the automated cell counter (Countess, Invitrogen) in which the slide was installed.

3.3.4 MTT Assay (Anti-cancer Assay)

This assay was used to observe the anti-cancer property of drug and SLNPs through percent cell viability. This happens due to the cleaving of the redox indicator that is tetrazolium, component of the yellow MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] to blue insoluble crystals of Formazan by the viable cells. This reduction reaction is catalyzed by the active mitochondrial dehydrogenase enzymes and is therefore an indicator of cell viability (Riss & Moravec, 2004).

Day 1: U87 cells (100µl/well) were plated at a density of 1.0 x 10^3 cells/ml into 96 well plates and were incubated in CO_2 incubator for 24 hours to adhere.

Day 2: When cells were seeded then 100µl of diosgenin drug, blank SLNPs and diosgenin SLNPs dilutions at different concentrations were added to the adhered cells in triplicates. These were allowed to produce its effect on the cells for another 24 hours in an incubated environment.

Day 3: After completion of 24 hours duration, 50µl of filter sterilized MTT (5 mg/ml in PBS) was added to the microtiter wells. The plates were again incubated for 3 hours for the reduction reaction to take place.

After a 3 hour incubation period with MTT, the wells were carefully emptied through pipetting. 100µl of sterile DMSO was added to the wells and were given 10 minutes incubation at 37^{o}C to precipitate the purple colored insoluble crystals of formazan that were entrapped in the viable cells. After this, the absorbance of formazan was measured at 405 nm with a spectrophotometric micro plate reader.

3.3.5 Cell Scratch Assay

Count the cells in order to have 0.5-1.0 x 10^5 cells/ml in a cell suspension. Seed cells (3ml/well) in a 6 well plate and incubate it for 24 hours in a CO_2 incubator and grew to confluency of 75-80%. Then the wells with monolayer cells were scratched with 1ml sterile tip to get a denuded region with no adherent cells. Then the cells were washed with PBS to remove debris from well and add fresh media. IC50 concentrations of drug and SLNPs were added after 24 hours. After incubation for 24 hours the plate was observed under light microscope. The wound region was measured and the cells were counted that crossed or were present in the scratched region

3.4 PHASE III (In Vivo)

3.4.1 Ethical Statement

All mice were housed in Atta-ur-Rahman School of Applied Biosciences animal house (LAH) under controlled condition and environment. All experimentations which were performed and protocols which were followed were approbated by the members of Internal Review Board (IRB), ASAB, National University of Sciences and Technology (NUST).

3.4.2 Animals and housing

A total of 80 male Balb/c mice were used for this study. They were provided by National Institute of Health, Pakistan. All of them were 5-6 weeks old with weight ranging from 25-35 grams. Animals were housed in polycarbonate cages (475x350x200mm) in groups of 5 mice in each cage. They were kept under standard conditions with light-dark schedule maintained at 12-hours, room temperature maintained at $23^{o}C \pm 2^{o}C$, with access to feed and water ad libitum. The cages were provided with bedding of wooden shavings which were changed after two days. Mice were not been on experimentation before so all of them were naïve experimentally. This study was conducted under the rules and regulations of NIH, USA (Guide for the Care and Use of Laboratory Animals: Eight Edition, 2011).

3.4.3 Grouping of Animals

Mice were divided into six groups, Control (PBS), Con-A, Con-A + Fluoxetine, Con-A + Diosgenin, Con-A + Blank SLNP and Con-A + Diosgenin SLNP. Each group contained 8 male mice with normal behavior. All the mice were of same age during the commencement of experimentation and all were given same feed and conditions.

Groups	Route of administration	Condition	Treatment
Control	Intra-peritoneal (IP)	PBS	Nil
Concanavalin A (Con-A)	Intra-peritoneal (IP)	Con-A	PBS
Con-A + Fluoxetine	Intra-peritoneal (IP)	Con-A	Fluoxetine
Con-A + Diosgenin	Intra-peritoneal (IP)	Con-A	Diosgenin drug
Con-A + Blank SLNP	Intra-peritoneal (IP)	Con-A	Blank SLNP
Con-A + Diosgenin SLNP	Intra-peritoneal (IP)	Con-A	Diosgenin encapsulated SLNP

Table 2: Shows different groups of mice with condition induced and treatment given IP.

Groups	Compounds dissolved in PBS	Concentrations mg/ml (w/v)	Weight range (grams)
Control	PBS	10ml	25.3g-38.6g
Concanavalin A (Con-A)	Con-A/PBS	56mg/35ml	27.9g-37.8g
Con-A + Fluoxetine	Fluoxetine/PBS	6.4mg/4ml	26.9g-39.9g
Con-A + Diosgenin	Diosgenin/PBS	32mg/4ml	24.6g-40.3g
Con-A + Blank SLNP	Blank SLNP/ PBS	32mg/4ml	23.8g-31.3g
Con-A + Diosgenin SLNP	Diosgenin SLNP/ PBS	32mg/4ml	24.2g-36.2g

Table 3: Shows concentrations of compounds dissolved in PBS on the basis of their body weight.

3.4.4 Inflammation Induced Sickness Behavior Model Development

Except from control group, all the other five groups mice were given intra-peritoneal injections of Concanavalin-A (Con-A). The concentration of injected Con-A depended on their weight. Con-A induces sickness behavior within 5-10 minutes after IP injection. And this can be observed through their behavior under controlled conditions. After 30 minutes of Con-A injections these groups were injected with different testing compounds and their injection volume also depended on the weight of every mouse. Universally, a mouse of 40g is given 500µl of IP injection and keeping this in view the volume was calculated with respect to every mouse that was being injected.

Behavior Analysis

Behavior tests were performed when the mice were 8 weeks old. All behavior tests were performed in a separate room for behaviors were all equipment were placed. Mice were allowed to habituate in that room for a while before starting the tests. And after each test about 20 minutes break was given to each mouse before starting a new test.

3.4.5 Open Field Test

Open field test is used asses locomotory and exploratory activity as well as depression and anxiety level in mice (Gould, Dao, & Kovacsics, 2009). Parameters that tells their behavior are when they spend more time in center arena or rearing in the sides of walls depicts exploratory behavior while grooming behavior shows how much anxious they are. The more anxious they are the more grooming they will do (Komorowska & Pisula, 2003) (Negishi et al., 2005). Mice were placed one by one in the square shaped 40x 40x 40cm

arena and recorded their behavior for 5 minutes in a camera. After every mouse trial the apparatus was cleaned with 70% ethanol so the behavior of other mouse was not affected. Parameters like total time spent in arena and the number of times mice crossed the line of arena were recorded and observed.

3.4.6 Social Interaction Test

Crawley's social interaction test has been designed in order to observe social behavior, interaction and memory. This test took place in a three chambered rectangular glass box with two divisions in it with opening for mice to enter. This tests have two sessions; Session1 in which mice social preference are checked and after break is Session 2 in which mice social memory is observed. These sessions are recorded in camera and each session is of 10 minutes with break in between these session is of 20 minutes. First five minutes are given to mouse to habituate in the box. After each session the box was cleaned thoroughly.

Session 1 is basically to observe mice social affiliation and preferences with other mouse or the object while session 2 is mostly for mice's memory and novelty observation. The basis of this test performance is based on the Kaidanovich-Beilin's description (Kaidanovich-Beilin, Lipina, Vukobradovic, Roder, & Woodgett, 2011).

Habituation

Before starting the recording the mouse was kept in the box in order to habituate and acclimatize according the environment of the box. In this way mouse explore the box and it is for 5 minutes.

Session 1 (Social Preference Test)

In this session it is for 10 minutes and the test mouse is placed in the center chamber. Two wired cages S1 and S2 are placed in opposite chambers in such a way that one is empty and the second one contains a strange mouse in it of same sex. Strange 1 mouse is basically has never been in contact with the test mouse is of same age and weight. The test mouse was freely allowed to move around, touch or interact with S1 and empty cage in all the three chambers. And this was recorded in camera. Test mouse's exploratory, touching or sniffing with S1 or empty cage was considered as interaction behavior.

Session 2 (Social memory/novelty Test)

This session starts after 20 minutes break from session 1. This session is also for 10 minutes. In this the test mouse is again placed in the center chamber and both cages were placed in opposite chamber like in session 1. But in this case S1 is the same mouse like that in session 1 and cage 2 or S2 contains new mouse with which test mouse has never been interacted with. Movement, interaction and exploration of test mouse was recorded in camera for 10 minutes in all the chambers and with S1 and S2. Sniffing and touching the cages or mice were observed as interaction of the test mouse. Test mouse has free choice whether with which mice it will interact more either with familiar S1 or the new unfamiliar S2 or through this its social novelty and memory is elucidated.

3.4.7 Elevated Maze Plus Test

This test is a modification of what Liser had developed for NIH swiss mice. This apparatus is basically an elevated maze having a 'Plus' shape with two open arms extending at opposite directions and two covered or dark arms extending in other directions. This tests

basically elucidates the exploratory behavior either it stays in dark arms due to stress or depression or is anxious enough to get into open arms.

The test was performed for 5 minutes and recorded in a camera. The test mouse placed in the central platform of the maze plus facing an open arm and let it freely move into any arm for 5 minutes. Parameter like how many entries into open and closed arms and the time spent in open arms were observed. The maze was thoroughly cleaned for every mouse to obtain better results of their behavior (Rodgers & Johnson, 1995).

3.4.8 Grooming/Splash Test

This test is done to evaluate test mice grooming behavior that either it's in stress or anxiety. In this test a clean glass rectangular box was used. This test was given 7 minutes. A test mouse was sprayed thrice with sugar solution on its coat and placed in the center of the box. The box didn't have any other object in order to not deviate the test mouse from the performance. The mouse was freely allowed to move around in the box. Parameter for this test was cleaning and licking its coat and paws were observed as recorded to evaluate its grooming behavior. For every mouse the box was cleaned thorough (Orvoen, Pla, Gardier, Saudou, & David, 2012).

3.4.9 Tail Suspension Test

This test is based on the fact that when test subjects develop an immobile position/posture when they are hung or placed in a stressful in-escapable position like hanging them by their tail. This involves hemodynamic stress that is developed when hung by their tail. This test is basically to observe its involvement in escaping that position. The test mouse was hung by its tail on a firm wooden support with the help of a paper tape. And it was allowed to

freely twist or move its body in any direction to escape that position. The test mouse was not disturbed during this period and it was given 6 minutes. The parameters that were observed were its twisting movement, the mobile and immobile phase during hanging. This was done to observe how much effort that mouse is putting to escape and to evaluate the condition of that mouse (Cryan, Mombereau, & Vassout, 2005).

3.4.10 Forced Swim Test

This paradigm is basically performed to check the effort a test subject applies in order to escape the un-escapable situation that will elucidate the condition of that test subject either it's in stress or anxiety. The test mouse were place in a cylinder that was 35 cm tall with a diameter of 30 cm and filled up to 21.5 cm ±1.5 with water having temperature of about 24±0.5 ◦C. The test mouse was allowed to swim around the cylinder in order to avoid drowning. The test was conducted for 6 minutes for every mouse. Were recorded was immobility when the mouse didn't put effort to swim or escape, the climbing in which it forcefully moved its paws to escape the cylinder and the diving. Total time was calculated for this. This elucidates the condition of mice when they were in this stressful scenario (Sáenz, Villagra, & Trías, 2006).

3.5 Histological Analysis

3.5.1 Brain Perfusion

Brains of three mice were fixed by heart perfusion method as described by Gregory J. Gage (Gage, Kipke, & Shain, 2012)Animals were anesthetized via IP injection of cocktail of Ketamine + Xylazine (87.5mg/kg + 12.5mg/kg) and that is 300µl/50g by body weight. Mice were place ventrally on dissection board and appendages were fixed on the board.

Abdominal incision was made and carefully heart was exposed. A butterfly needle was inserted into the left ventricle of beating heart about 5mm deep. Around 80-90ml of cold saline solution (0.9% NaCl solution) was injected slowly at the rate of 5ml/min through the vascular system and simultaneously right atrium was incised so all the blood was drained out with this. After that 4% Paraformaldehyde was injected around 90-100ml for perfusion and hardening of tissues. After that brain was harvested through dissection and kept in 4%PFA solution for 24 hours at 4^{o}C.

3.5.2 Brain Fixation

Harvested brains were then dehydrated through different concentrations of ethanol (organic solvent). These were immersed in the solvents for 60 minutes in the following order:

70% Ethanol > 80% Ethanol > 85% Ethanol > 90% Ethanol > 100% Ethanol

Then the brains were further hardened in Xylene for 30 minutes.

3.5.3 Paraffin Embedding

Dehydrated and hardened brains were then placed in molten paraffin wax and formed blocks of it in molds in such a way that the brains were completely covered in wax. These were then air dried first and then kept in 4^{o}C for 20 minutes for further solidification. These were prepared for microtome sectioning.

3.5.4 Tissue Sectioning

Embedded brains were sliced on SLEE Mainz (CUT6062) MICROTOME and the thickness of slices were kept 3µm to get cortex and hippocampus in the slices. The slices were transferred on glass slides and placed on hot plate at 60^{o}C until the wax was melted. They were deparaffinized in xylene for 10 minutes followed by rehydration for 5 minutes

in the following order:

95% Ethanol > 80% Ethanol > 70% Ethanol > 50% Ethanol

3.5.5 Hematoxylin and Eosin Staining (H & E)

The deparaffinized brain tissue slices were then exposed to H and E staining in order to stain the tissues. Once they were stained the slide were ready to be observed under microscope at different magnification.

3.5.6 Microscopic Examination

The prepared slides were then observed under light microscope. They were observed under 4X, 10X and 40X lens and pictures were taken for further observation of changes in cell density, morphology and observe inflammation.

3.6 Evaluation of Drug Concentration in Blood and Organs

4 weeks old mice were IP injected with Diosgenin drug, Blank SLNP and Diosgenin SLNP dissolved in sterile PBS (5mg/ml) and the injection volume was set according to the body weight.

3.6.1 Blood and Organs Removal (Dissection)

After 30 minutes of the injection the mice were anesthetized with Ketamine + Xylazine (87.5mg/kg + 12.5mg/kg) cocktail via IP injection. The abdomen was incised and heart was exposed. A 1ml insulin syringe was inserted into the heart and blood was injected out slowly which after that was collected in EDTA tubes placed in ice. After that mice were sacrificed and organs were removed from mice which includes brain, heart, liver, spleen and kidneys. Organs were washed with PBS solution, dried with filter paper, weighed and kept in ice for extraction.

3.6.2 Extraction of Diosgenin from Blood

Blood was pooled into 2ml Microtubes and centrifuged for 2 minutes in order to get plasma and serum separated. The serum layer was collected and methanol was added at a ratio of 2:1 of methanol to serum. This sample was then vortexed for 20 seconds for complete extraction and precipitation of proteins. This mixture was kept at room temperature for an hour. After 1 hour, the mixture was then centrifuged for 12 minutes at 16,000g. The supernatant acquired from centrifugation was separated in another microtube for extracted drug analysis (Shadkchan, Zaslavsky, & Segal, 2003).

3.6.3 Extraction of Diosgenin from Organs

Folch's method was followed for drug extraction from organs (Folch et al., 1957). The tissues were homogenized with mixture of chloroform/methanol (2:1) to a final volume that is 20 times to that of tissue sample (1g in 20ml). The whole mixture was agitated in a cell disruptor at room temperature. The homogenate was then transferred to 2ml microtubes and centrifuged at 1000rpm for 5 minutes to get the liquid phase. The solvent was washed with 0.9% NaCl solution with thorough vortexing for 10 seconds and then centrifuged it at 2000rpm for 1 minute to get two separate phases. The upper phase is removed containing polar organic molecules and the lower chloroform phase was separated in other tube for extracted drug analysis (Folch, Lees, & Sloane-Stanley, 1957).

3.6.4 UV Spectrophotometry of Standard Diosgenin Drug

The concentration of drug released from SLNP in blood and organs were assessed via spectrophotometry. Different concentration of diosgenin drug (10mg, 5mg and 1mg) were dissolved in 10ml of acetonitrile and sonicated well. Further five dilutions were made from original stocks. The sample were then placed in cuvette and analyzed at 410nm for

absorbance. The acquired absorbance was used to make a calibration curve and the graph was used further to analyze drug concentration in blood and organs.

3.6.5 UV Spectrophotometry of Analytes from Blood and Organs

The level of diosgenin was analyzed through UV spectrophotometry at 410nm of wavelength according to protocol. The analyst were prepared by mixing the separated phase in 1ml of acetonitrile. In order to get accurate reading the plasma solution and acetonitrile was blanked before taking reading for analytes. Absorbance was recorded for every sample in triplicates. Concentrations of diosgenin in all samples were calculated by interposing with the calibration curve of standard diosgenin and using slope-intercept formula on the basis of Beer's law.

3.7 Blood CP for Evaluation of Toxicity

To check the toxicity level, blood cp (complete picture) test was done. 6 weeks old 9 mice were IP injected with 100µl of diosgenin drug, blank SLNP and diosgenin SLNP (3mg/ml). And there blood was collected after two weeks of injections through the heart. The blood was collected in EDTA tubes and was sent for Blood CP testing. Total leukocyte, lymphocyte and other cells were performed. [61]

3.8 Statistical Analysis

Statistical analysis was done by using the Graphpad Prism 5.01 software. One-way ANOVA was applied on those data that had two variable and two-way ANOVA on more than two variables. P-values were considered significant which less than 0.05 was. Data was represented in the form of ± SEM with up to two significant figures with confidence interval of 95%.

Chapter 4

RESULTS

4.1 PHASE I

4.1.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopies of blank SLNP and SLNP encapsulating the drug Diosgenin in the below figures shows morphology and size which were air dried. It can be observed from the SEM images that SLNPs are present and are of various sizes ranging from micro-meter to nano-meter. SLNPs ranging from 10nm to 200nm which is the desired range of size to cross BBB were observed in larger quantity. Images were taken at 20,000X, 40,000X, 50,000X and 80,000 X magnifications with spatial resolution ranging from 2µm to 0.5µm shows that blank SLNP and SLNP encapsulating the Diosgenin are of spherical structure with desired sizes to cross the BBB.

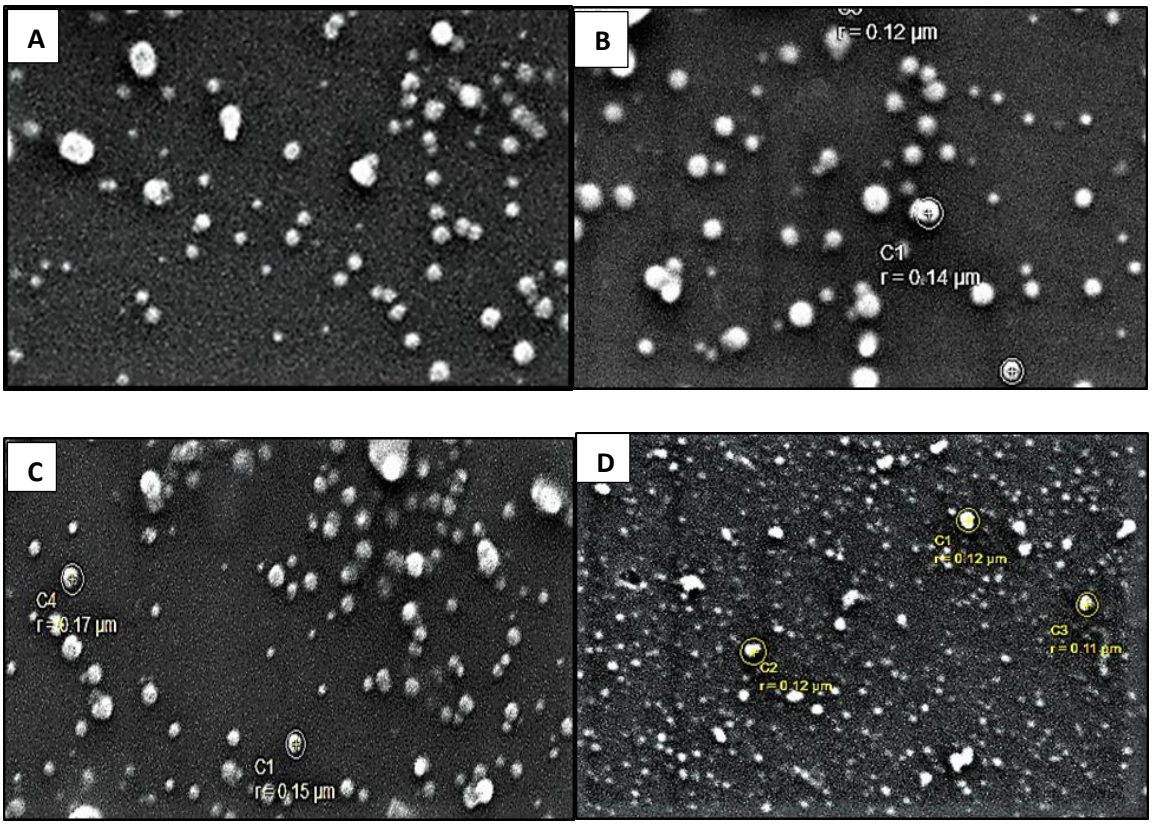

Figure 4.1*:* SEM images of blank and diosgenin SLNPs. Images were taken at 20,000x and 40,000x magnifications with size ranging from 20nm to 150nm. **(A)(B)** Blank SLNP. **(C)(D)** Diosgenin SLNP

4.1.2 Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR has been done for both blank SLNP and diosgenin encapsulating SLNP. FTIR spectra of both SLNPs are presented below in fig. which shows that there is not much difference in the characteristic peaks in both graphs. This preservation of characteristic absorption bands of P-80 in the diosgenin-loaded formulation interprets no significant interruption by the drug on functional groups of P-80 coated SLNP. However spectra show slight intensification of the peak at 2918 cm^{-1} and 2850 cm^{-1} which shows alkane C-H bond stretch which suggests H bond interaction between drug and SLNP components.

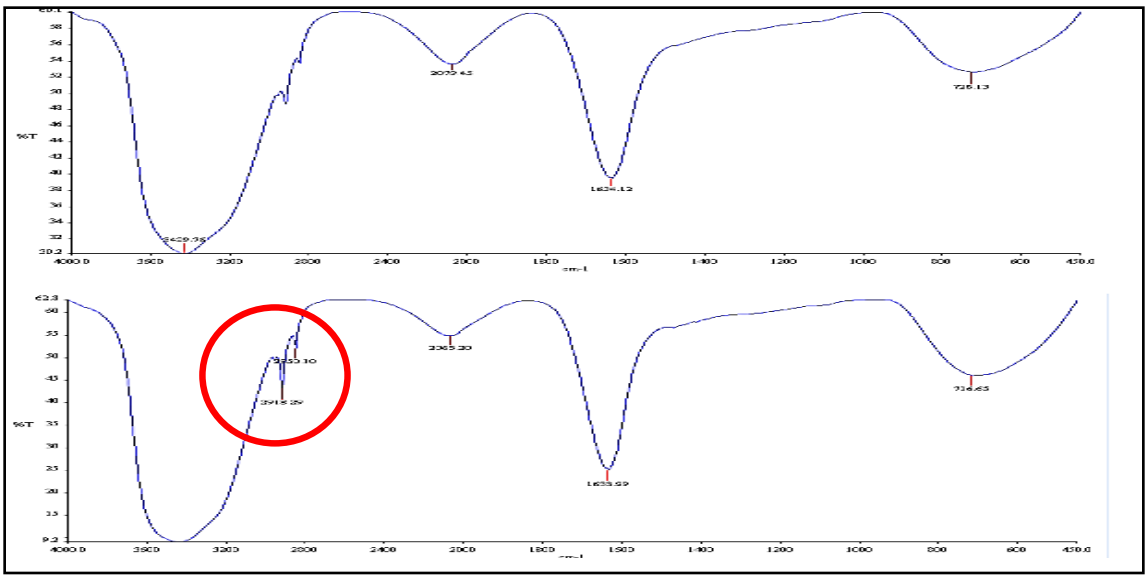

Figure 4.2: FTIR Peaks showing the P-80 coating through stretching of C-H and C-C bonds.

4.1.3 X-ray Diffraction Analysis (XRD)

XRD was performed to identify the physical state of diosgenin which was encapsulated in SLNP and different patterns of diosgenin drug, blank SLNP and diosgenin SLNP are shown in the following figures. The data was collected at room temperature in the 2ɵ range 5.0° to 69.99°. In this XRD pattern (fig) the diosgenin drug shows characteristic peaks at different range and this is attributed to its crystalline structure in pure drug form. The patterns of blank SLNP and diosgenin SLNP are quite different from each other but do have characteristic peaks of diosgenin in diosgenin encapsulated SLNP. This shows that the SLNP are formed with diosgenin encapsulated in its core due to which characteristic peaks range of drug, drug SLNP and blank SLNP are a bit different with respect to height and range position.

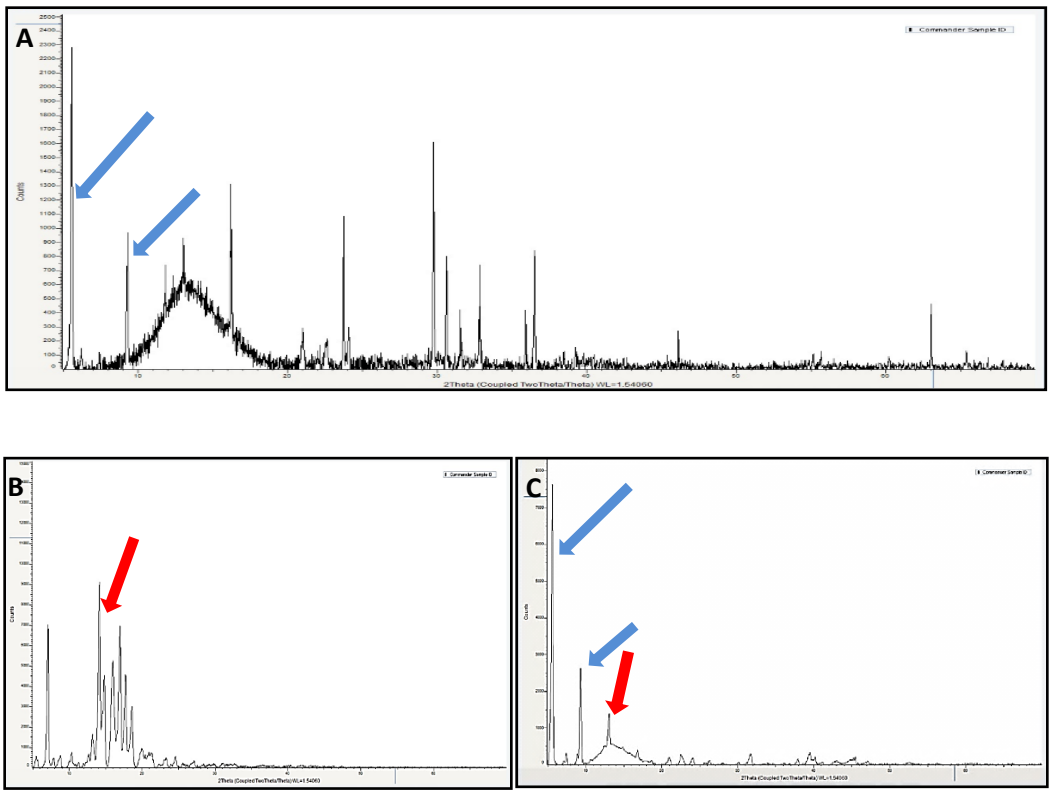

Figure 4.3: XRD patterns of SLNP. **(A)** Shows peak pattern of P-80 coated stearic acid SLNP. **(B)** Shows peaks for Diosgenin only. **(C)** Shows peaks for diosgenin that is encapsulated in SLNP. Red arrow indicates for diosgenin peak and blue for SLNP.

4.1.4 Particle Size and Zeta Potential Measurement

Graphical patterns shown in the figure below shows zeta potential of SLNPs which is an important factor for its stability. The pattern of zeta potential shows that the blank SLNP and diosgenin SLNP average ZP is -14.1mV and -26.0Mv which shows that they are anionic in nature and stable enough and won't flocculate in the solution.

Zeta size graphs shows that the formulation with which SLNPs were prepare got us different size of particles that are in desirable range. The pattern showed different peaks and shows that average size of blank and diosgenin SLNP to be 436.5d.nm and 15.92d.nm. This suggests that SLNPs are quite suitable size for BBB crossing.

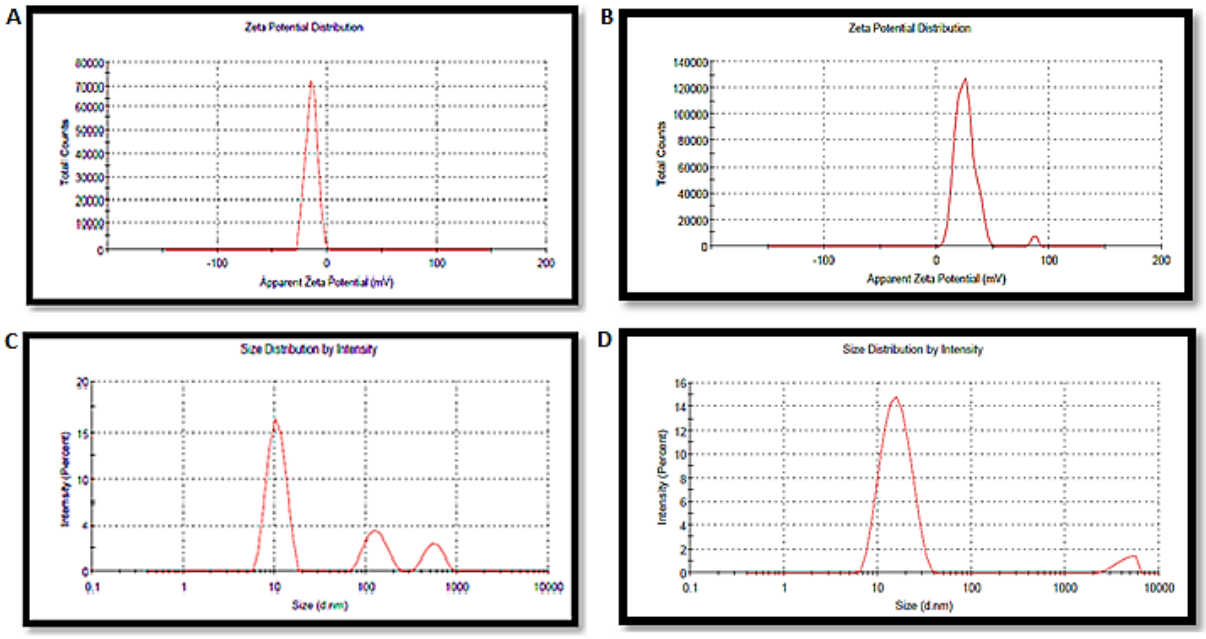

Figure 4.4*:* Zeta potential and zeta size of SLNP. **(A)** Zeta potential of stearic acid SLNP. **(B)** Zeta potential of diosgenin incorporated SLNP. **(C)** Zeta size of stearic acid SLNP. **(D)** Zeta size of diosgenin incorporated SLNP.

4.1.5 High performance Liquid Chromatography (HPLC)

As the objective of doing HPLC was to find out the drug release and entrapment efficiency of SLNP so for that the initial step was to form calibration curve for the standard drug diosgenin that was dissolved in 10ml of acetonitrile. The standard curve for different concentrations of diosgenin on the basis of absorbance is shown in the following figure:

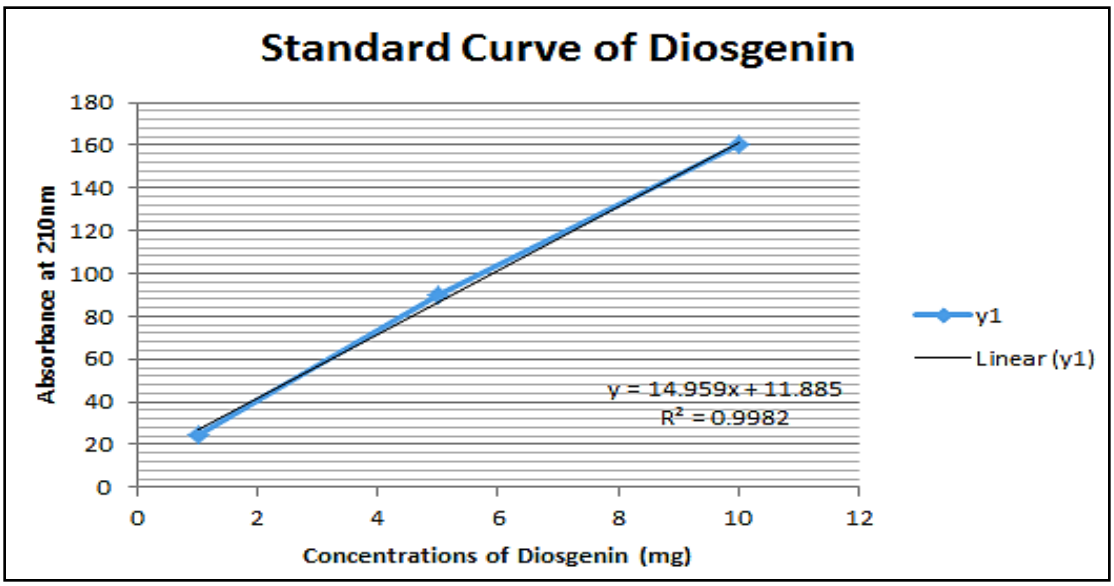

Figure 4.5: Standard curve of diosgenin drug with different concentrations which gave absorbance given at 210 nm with retention time of 6.1s.

This calibration curve gave values for slope and intercept for diosgenin on the basis of which concentrations of diosgenin (encapsulated in SLNP) were calculated from the absorbance that are acquired for drug release and drug entrapment efficiency of SLNP.

4.1.6 Drug Release Efficiency of SLNP

SLNPs capability to release the drug when in solvent environment was elucidated with HPLC under specific parameters with respect to standard curve. The following graph elucidate the release efficiency of Diosgenin from SLNP is almost half of the concentration of standard drug. The release of Diosgenin turns out to be significantly different from the standard drug concentration with *p* value less than 0.001.

It is shown in the figure below that the release of Diosgenin from SLNP was less in 1mg SLNP and its release from SLNP increased as the concentration of SLNP was increased. So this shows a direct proportional relationship between drug release concentration and

initial concentration of SLNP taken. And it also suggests that SLNP is quite stable enough to retain the drug inside its core and release a significant amount when in solvent.

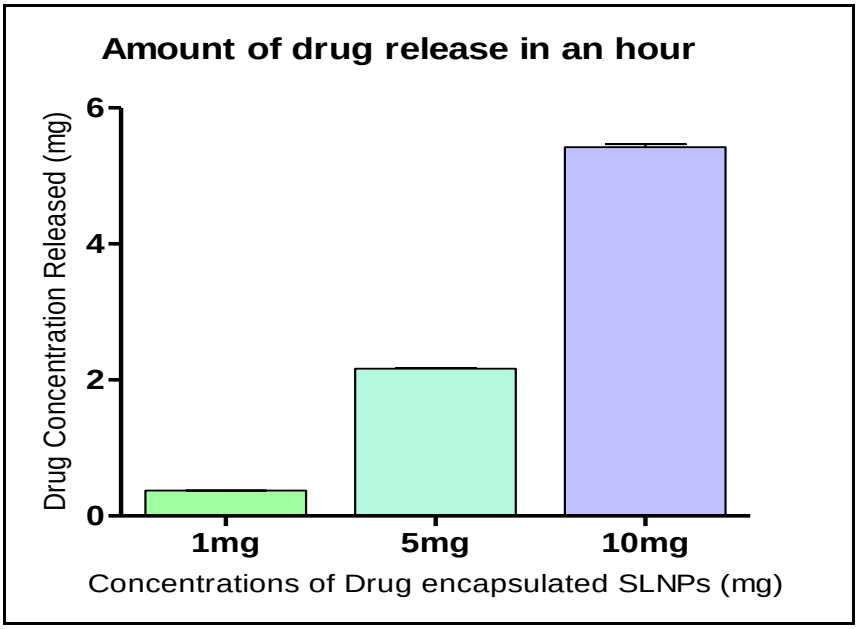

Figure 4.6: HPLC histogram for drug release from SLNPs after 1 hour. Shows concentration dependent drug release.

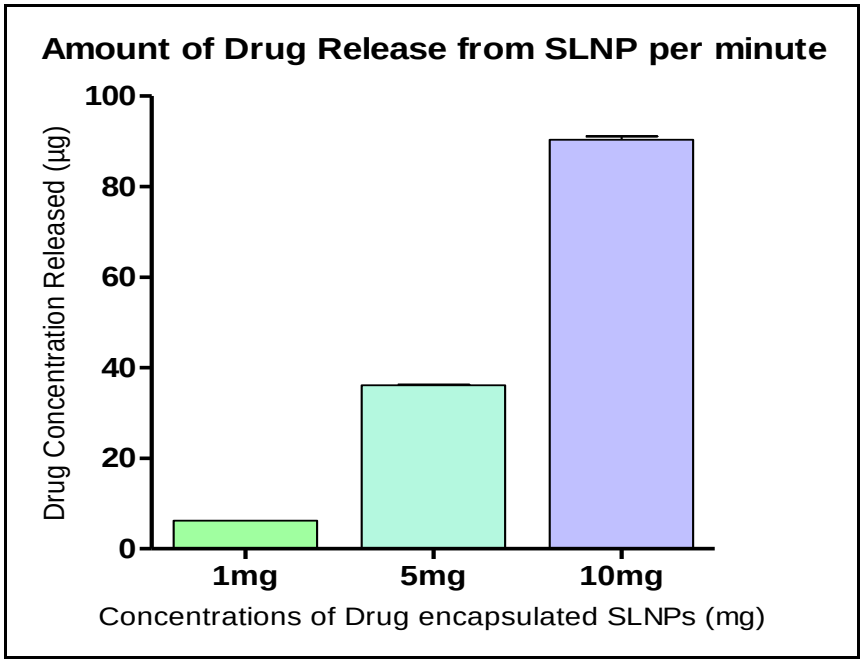

Figure 4.7: Histogram showing release of drug per minute from SLNPs.

4.1.7 Drug Entrapment/Encapsulation Efficiency

The drug entrapment ability of SLNP was checked from the free unentrapped drug that was present in the supernatant after centrifugation of the final mixture. The HPLC absorbance when was converted and calculated through the percent efficiency formula it turns to be that the amount of drug that was initially added to the aqueous phase was almost all entrapped. On the basis of absorbance, percentage was taken out for all three concentrations and it shows high entrapment efficiency of the SLNPs. Total entrapment ranged between 97%-99% on the basis of different concentrations of drug SLNPs. Statistical analysis shows that 1mg drug entrapment efficiency of SLNP is significantly different from 5mg and 10mg drug SLNPs. While there is no significant difference between the entrapment efficiency of 5mg and 10mg. The figure below shows the comparison among different concentration of drug SLNPs in order to evaluate the percentage of entrapped drug in SLNPs.

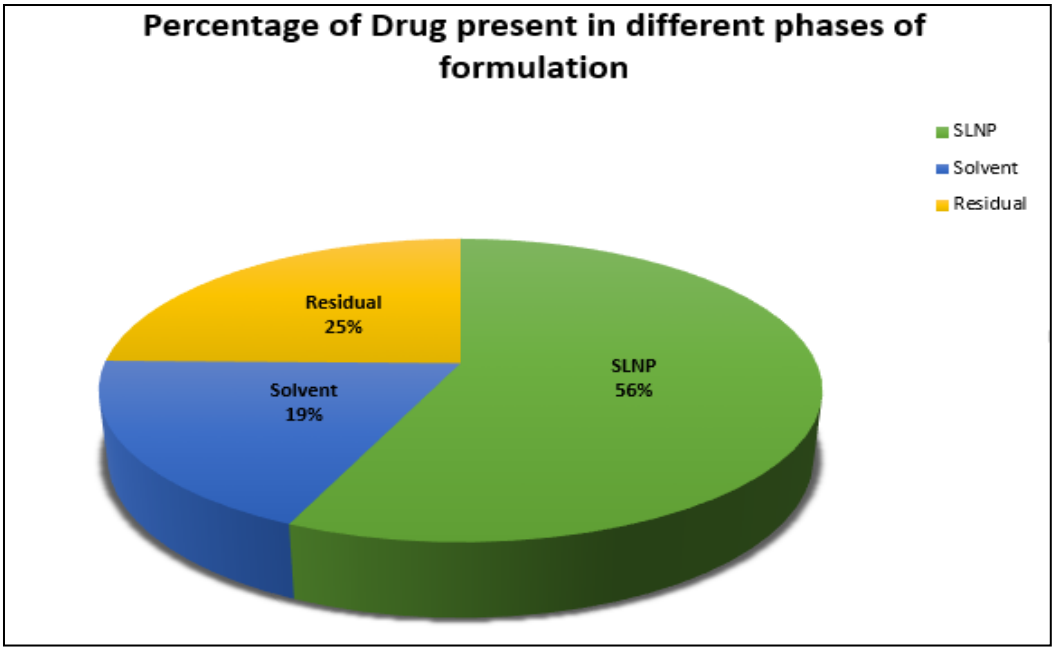

Figure 4.8: Pie chart representation of Drug presence in SLNP, formulation solvent (un-entrapped) and residue in syringe filter.

The percent yield of formulation was to be 51.70% and this shows that the yield of formulation increases with the increase in the concentration of Diosgenin as they are directly proportional to each other.

4.2 PHASE II: In Vitro (Anti-cancer Effect of Diosgenin and Diosgenin SLNP)

4.2.1 MTT Assay

MTT assay was done on U87 cell lines (glioma) with Diosgenin drug alone in order to check the cytotoxicity level on cell viability. It turns out that the cytotoxicity of Diosgenin is dose dependent on U87 cells. As the dose was increased cell viability reduced gradually to a greater extent. And there was a point when cells showed constant and high viability at 0.0001-0.000001µM. This shows that the cytotoxicity level depends on the concentration of drug.

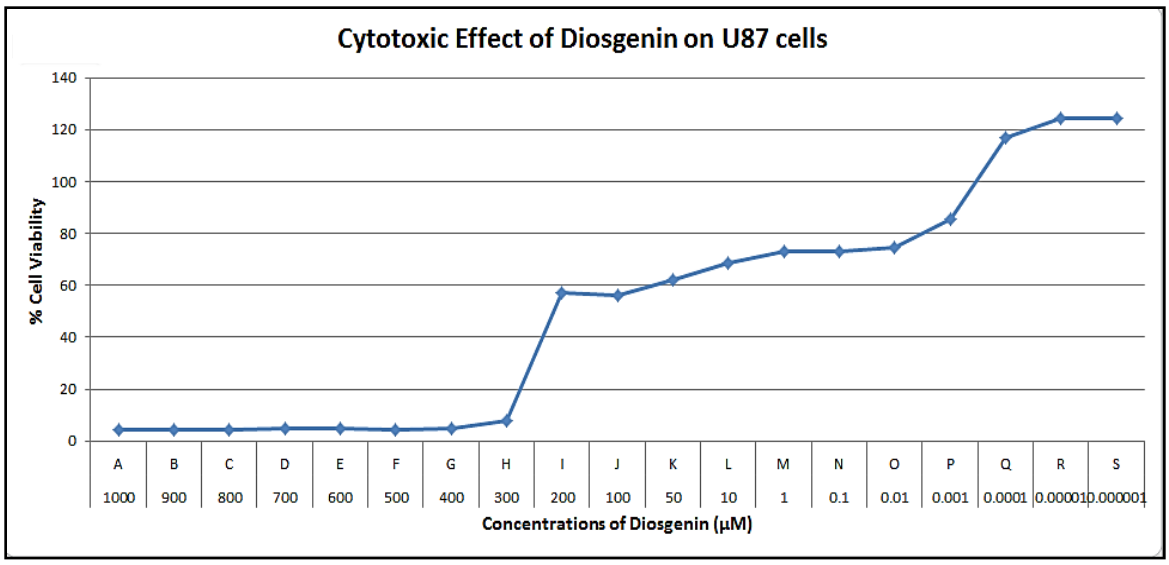

Figure 4.9: Graphical representation of U87 viability at different concentrations of Diosgenin which suggests a dose dependent relation.

To elucidate the cytotoxic effect (anti-cancer effect) of Diosgenin SLNP, MTT assay was carried out with similar concentrations of diosgenin drug, blank SLNP and diosgenin SLNP on U87. The percent cell viability was calculated from the absorbance which was acquired from microplate reader. Bar graph was plotted as a comparison among the viability percent of these three. In the fig below, the bar height shows that the cell viability increases as the concentrations drops down for all the three components. There is quite large difference between the viability percent of diosgenin and blank SLNP. It shows significant difference ($p<0.001$ *and* $p<0.01$) for all concentrations between diosgenin and blank SLNP. Which suggests that diosgenin alone at 2mg concentration shows high cytotoxicity than blank SLNP. Observing the bars for diosgenin and diosgenin SLNPs, it showed no significant difference except from 0.002mg which showed $p<0.5$ difference. That might be due to the less amount of drug entrapped in 0.002mg SLNP. Significance difference is shown by first three concentrations between diosgenin SLNP and blank

SLNPs which is *p<0.5.* Which suggests that the concentration of entrapped diosgenin at first three concentration is more in SLNPs than the rest of the concentrations. Once again this graph represents the dose dependent cytotoxicity of the components on U87. And diosgenin alone is more cytotoxic and showed anticancer effect on U87 than the other two. While diosgenin SLNP showed cytotoxicity quite near to the diosgenin alone than blank SLNPs.

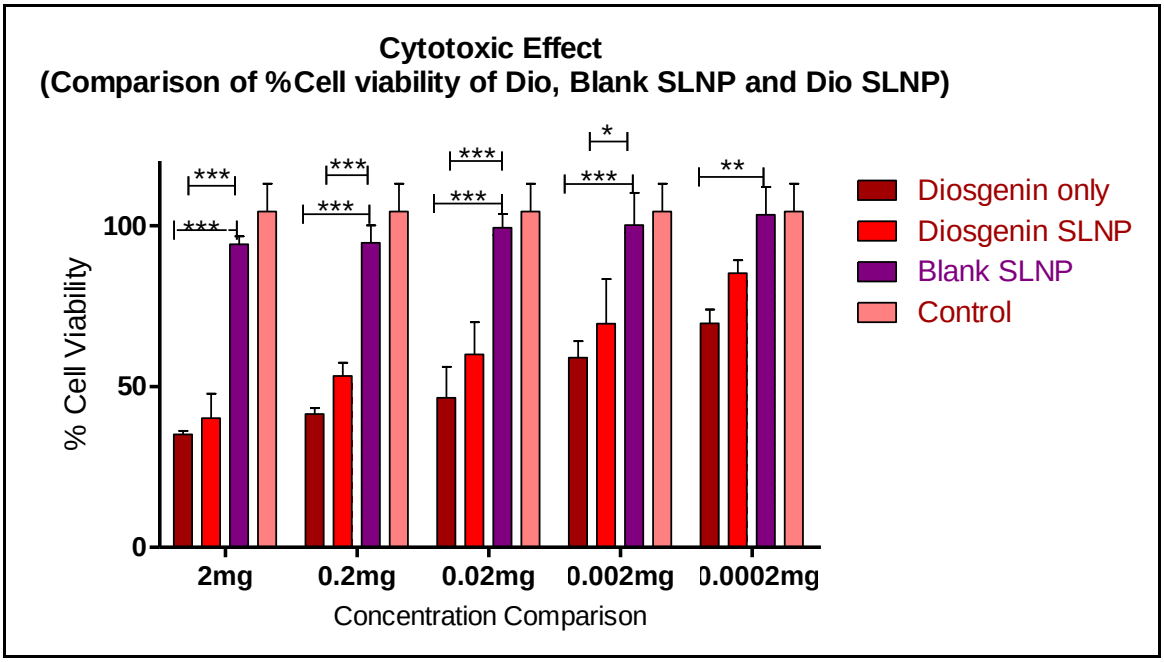

Figure 4.10: Bar graphs represents the comparison of cell viability among Diosgenin, blank SLNP and Diosgenin SLNP at different concentrations with comparison to control (PBS).

4.2.2 Cell Scratch Assay

For this assay when cells were incubated for 24 hours and a scratch was applied with a tip then after further incubation the well showed extreme cell death with the concentrations. Due to which the assay was not completed and it failed to show any results for U87 metastasis in the presence of drug or SLNPs.

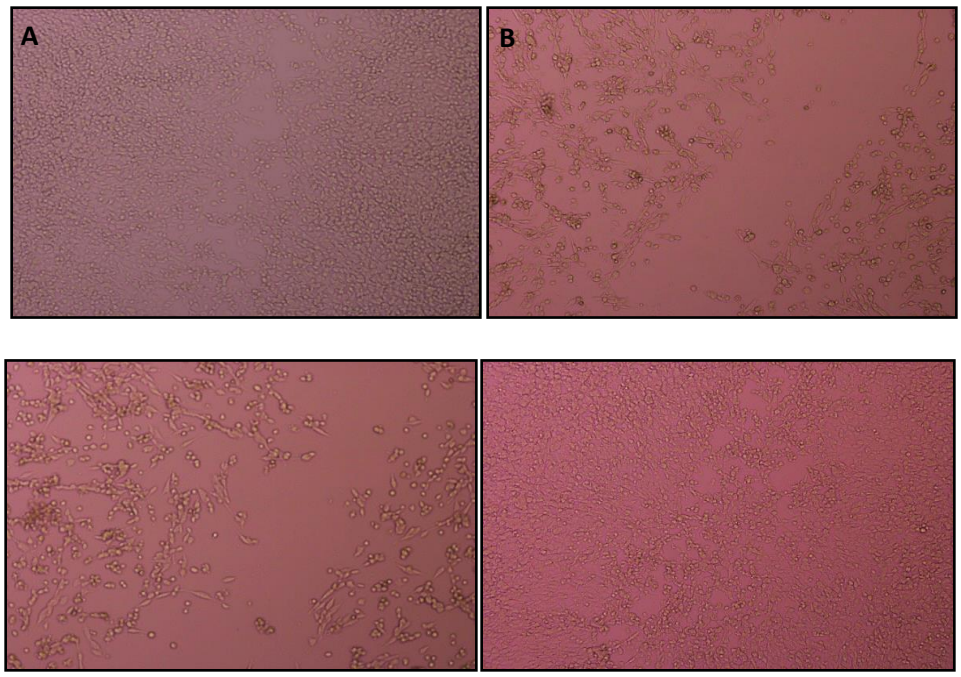

Figure 4.11: Above pictures shows U87 cells in 6-well plate. **(A)** Confluency of U87 after 24 hours incubation. **(B)** Shows 0.2µm scratch area.

4.3 PHASE III: *In Vivo Analysis*

Confirmation of Model via Behavioral Tests

4.3.1 Open Field Test

This test was performed in order to determine the overall activity, anxious, exploratory and stressed behavior of mice for all the groups and compare them. In this test parameter of focus was time (in seconds) spent in the center square.

The figure shows bar graph comparison of all groups of mice in which it is observed that all groups spent time that is less than the control group. Control group showed normal behavior while its exploratory behavior to be more than the rest of groups. Con-A showed significantly low exploratory behavior and displayed a significant difference to Con-A+Flo ($p< 0.05$), Con-A+Dio ($p<0.01$) and Con-A+Dio.SLNP ($p< 0.01$) groups. Groups Con-

A+Dio and Con-A+Dio.SLNP showed quite significantly more time spent in center and in exploring than Con-A+Flo. Group Con-A+B.SLNP also showed stressed behavior and less time spent in the square just like group Con-A.

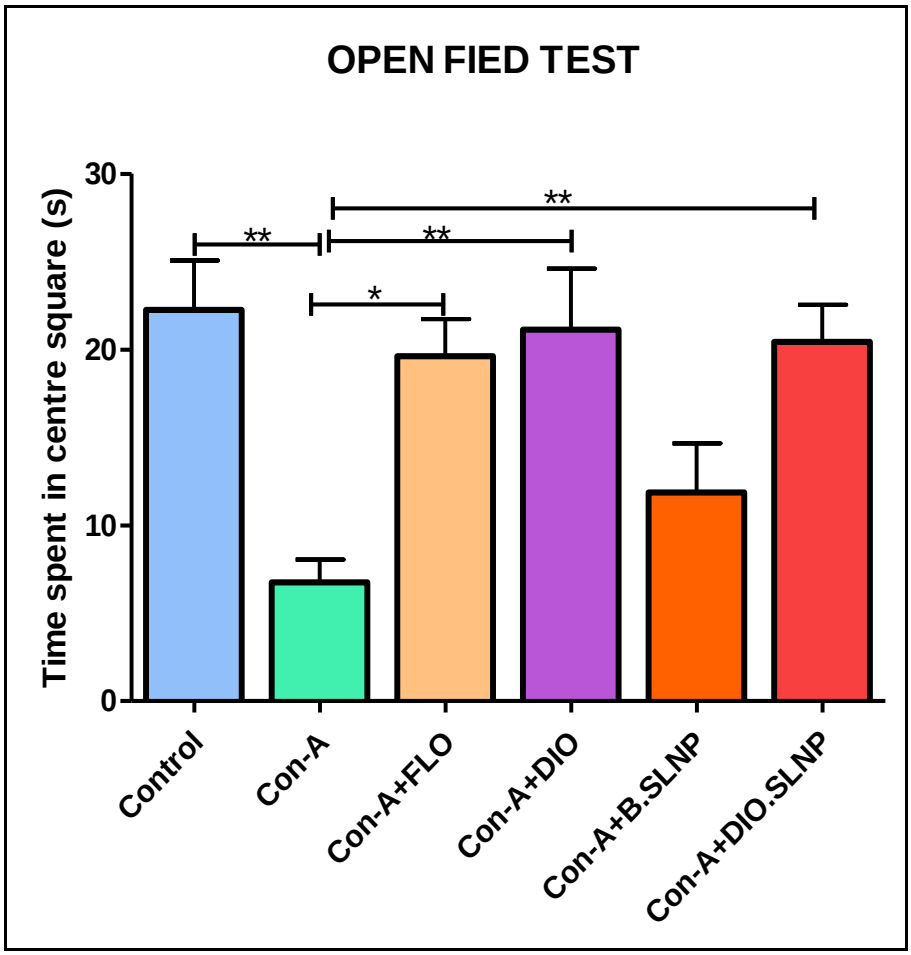

Figure 4.12: Effect of drug and SLNPs on mice with sickness behavior in Open Field test. The figure depicts; Histogram which shows total time spent in the center square of arena for Control (PBS), Con-A, Con-A+Flo, Con-A+Diosgenin, Con-A+Blank SLNP and Con-A+Diosgenin SLNP groups. Error bars represent mean ± SEM for one-way ANOVA followed by Bonferroni's multiple comparison analysis. Significant values: * = $p<0.05$, ** = $p<0.01$

4.3.2 Social Interaction Test

This test is used to determine the sociability, social preference and social novelty of mice. Time spent by mouse with S1 and S2 mice were recorded and evaluated for behavior among different groups.

Mice of all groups exhibited less or more sociability with the S and S2. Control group showed more interaction than other groups and are the active ones. Groups showed preference to S2 more than S1. Except Control group, Groups like Con-A+Dio and Con-A+Dio.SLNP from other groups showed higher preference to S2 than S1 which shows social preference to novel mice. Control group is significantly have spent more time with S2 than Con-A, Con-A+Flo and Con-A+B.SLNP groups.

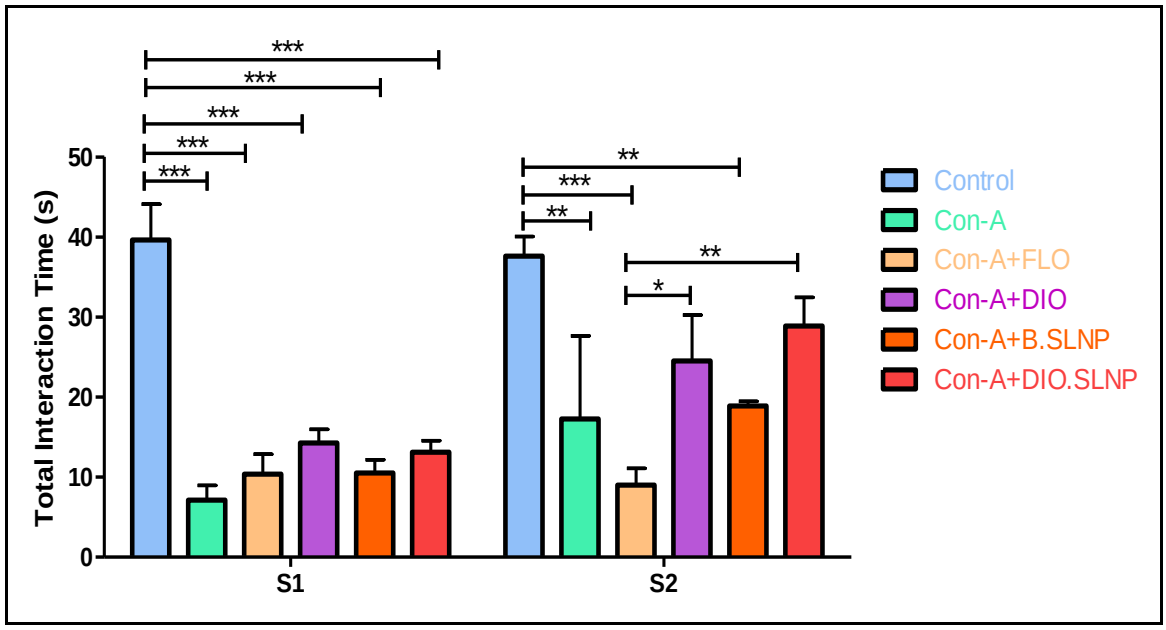

Figure 4.13: Effect of drug and SLNPs on mice with sickness behavior for social preference and novelty. The histogram represents total time spent in seconds with S1 and S2 for Control (PBS), Con-A, Con-A+Flo, Con-A+Diosgenin, Con-A+Blank SLNP and Con-A+Diosgenin SLNP groups. Error bars represent mean ± SEM for one-way ANOVA followed by Bonferroni's multiple comparison analysis. Significant values: ** = $p<0.01$, *** = $p<0.001$.

4.3.3 Grooming/Splash Test

This test is used to illustrate the grooming behavior of mice once sprayed with sugar solution on their coat. The time spent by mouse in licking its coat and paws are recorded and evaluated in the form of histogram. The histogram showed normal grooming behavior of Control group among other groups and time spent in cleaning or licking itself is more than other groups. Control groups showed significance with Con-A and Con-A+Flo groups ($p< 0.01$) due to their least grooming behavior (least time spent in grooming). Con-A+Dio.SLNP showed to be more active after Control group with second high time spent in self grooming than the other groups. This group showed significant difference to Con-A and Con-A+Flo due to their timings. Other two groups Con-A+Dio and Con-A+B.SLNP also spent time in cleaning itself but less than the control and Con-A+Dio.SLNP groups.

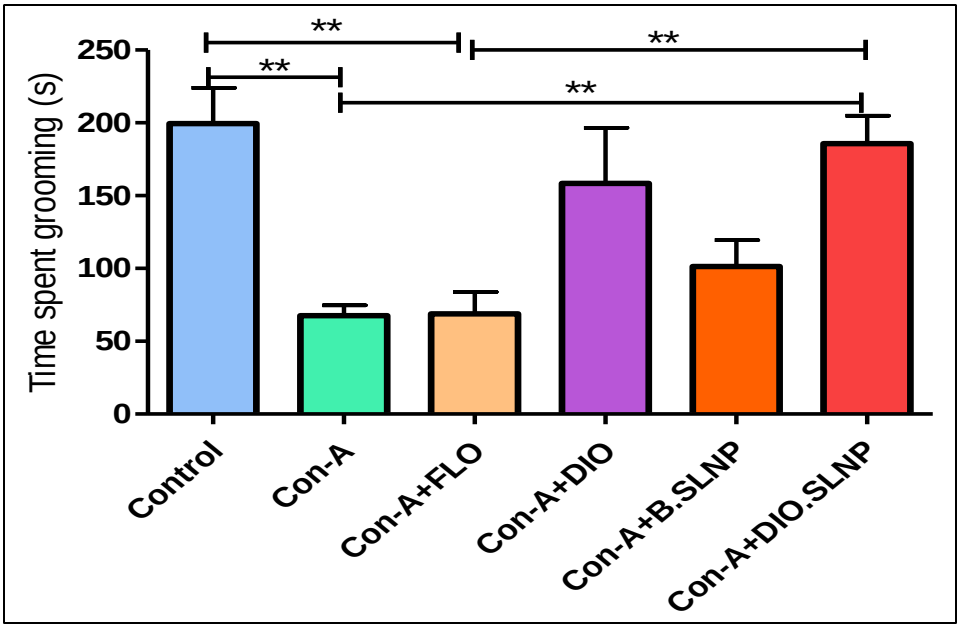

Figure 4.14: Effect of drug and SLNPs on mice with sickness behavior in Grooming test. The figure depicts; Histogram which shows total time spent in cleaning and grooming for Control (PBS), Con-A, Con-A+Flo, Con-A+Diosgenin, Con-A+Blank SLNP and Con-A+Diosgenin SLNP groups. Error bars represent mean ± SEM for one-way ANOVA followed by Bonferroni's multiple comparison analysis. Significant values: ** = $p<0.01$

4.3.4 Tail Suspension Test

This test was done to observe the effort mice put in escaping from a position that was un-escapable and makes them immobilize. The parameter which was focused was the forcefully twisting with rashly moving its limbs to escape the position. Among all groups Control mice were the active ones with high significance to Con-A+Dio, Con-A+B.SLNP and Con-A+Dio.SLNP groups ($p < 0.001$). Histogram elucidates that the next to Control, Con-A mice were actively putting effort in escape with second highest time spent in the twisting. While other groups somehow show almost alike time spent in the effort to escape the position and were quite immobile than control. This parameter spent time was counted in seconds.

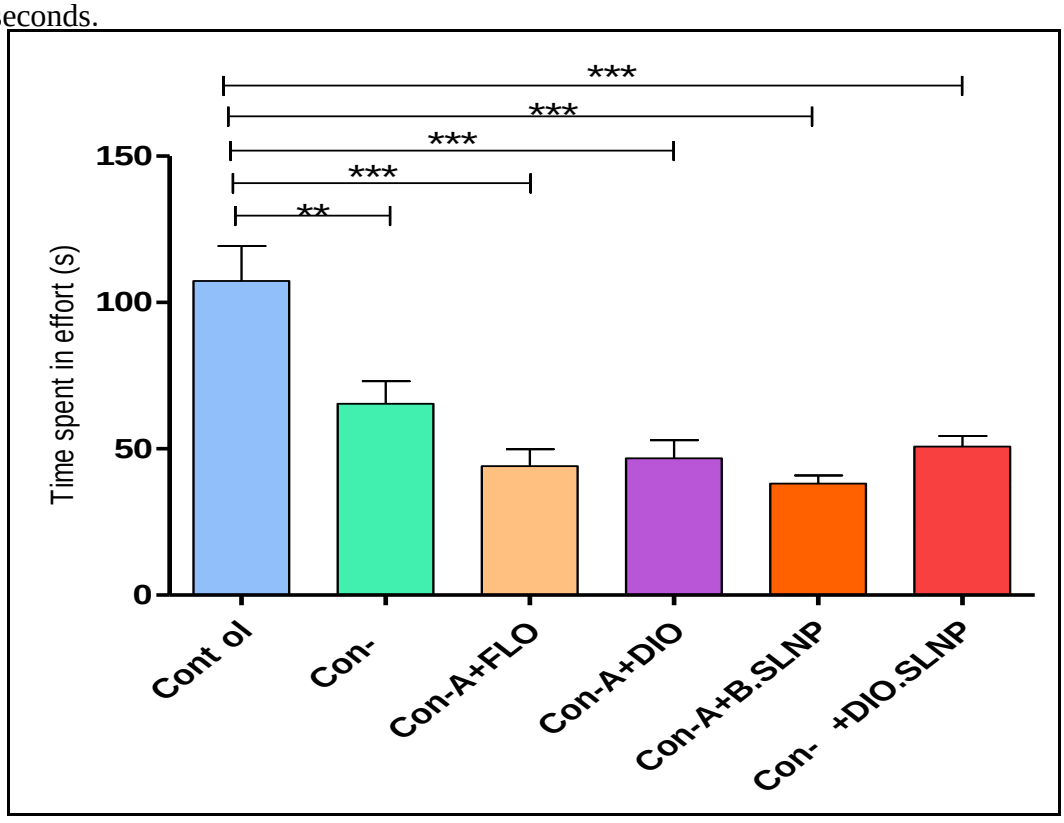

Figure 4.15: Effect of drug and SLNPs on mice with sickness behavior in Tail Suspension test**.** Histogram which shows total time spent in twisting and moving their limbs for Control (PBS), Con-A, Con-A+Flo, Con-A+Diosgenin, Con-A+Blank SLNP and Con-A+Diosgenin SLNP groups. Error bars represent mean ± SEM for one-way ANOVA followed by Bonferroni's multiple comparison analysis. Significant values: *** = $p<0.001$

4.3.5 Forced Swim Test

In this test, the group which showed more time spent in swimming by forceful limb movements was Con-A+Dio. While the group with least time spent was Con-A which shows significance difference with Con-A+Flo. In the histogram it elucidates that Control is significantly lower in time spent with groups Con-A+Dio, Con-A+B.SLNP and Con-A+Dio.SLNP ($p<0.001$). Groups Con-A+Flo and Con-A+Dio.SLNP show almost alike time spent in the swimming to escape.

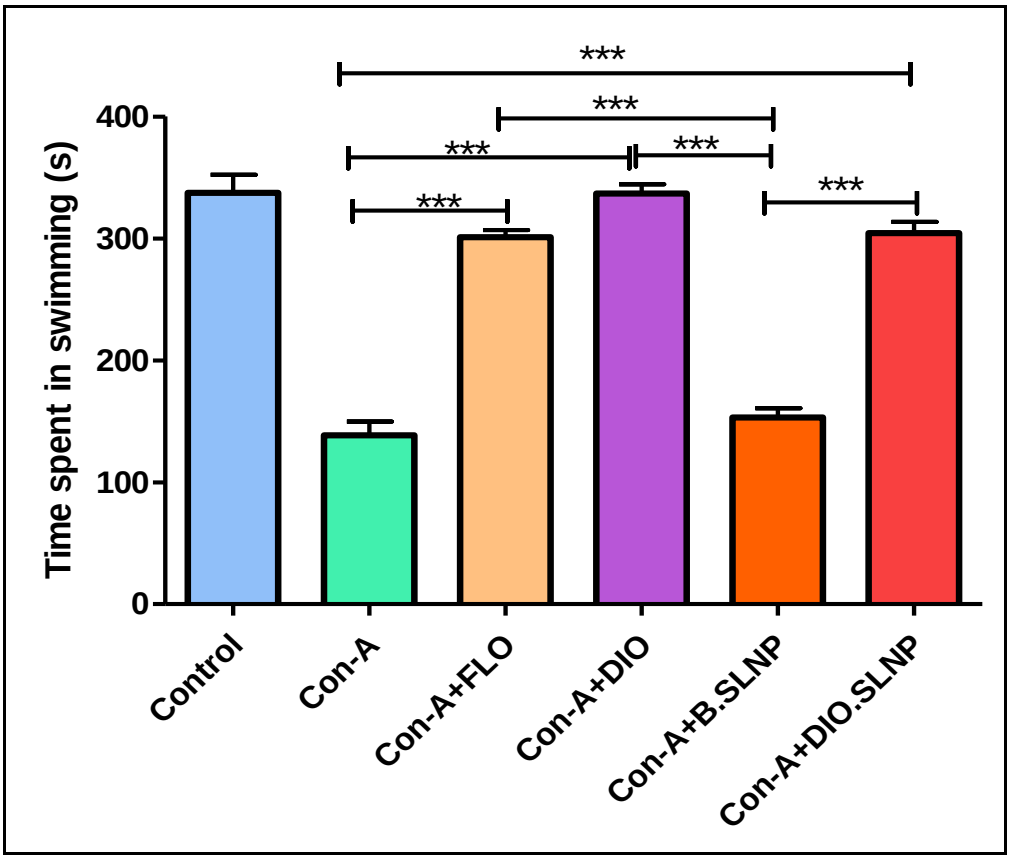

Figure 4.16: Effect of drug and SLNPs on mice with sickness behavior in Forced swim test. The figure depicts; Histogram which shows total time spent in swimming and rashly moving their limbs for Control (PBS), Con-A, Con-A+Flo, Con-A+Diosgenin, Con-A+Blank SLNP and Con-A+Diosgenin SLNP groups. Error bars represent mean ± SEM for one-way ANOVA followed by Bonferroni's multiple comparison analysis. Significant values: *** = $p<0.001$

4.3.6 Histological Analysis

Qualitative analysis of histological slides were performed on the cortical and hippocampus sections of brain which were stained with H & E. Micrographic represents the brain sections from Control, Con-A, Con-A+Dio, Con-A+B.SLNP and Con-A+Dio.SLNP groups were taken at 4X, 10X and 40x resolutions.

The H & E staining of brain slices were done to observe the presence of pyknosis, neuro-inflammation, and vacuolization in the brain. Histopathological changes in the regions of brain (cortex and hippocampus regions) for all groups are shown in the figure 4.16. Control group brain section showed normal glial and neuronal cells in the hippocampal and cortex region. No vacuolization has been observed. Mice of group Con-A exposure showed neuro-inflammation with pyknosis and inflammatory infiltrates were in abundance. Edema and vacuolization has been observed as well. Images of hippocampus and cortex showed state of inflammation. While the brain sections of Con-A + Dio.SLNP showed moderate level of inflammatory cell with less pyknosis and gliosis as compared to the Con-A ones.

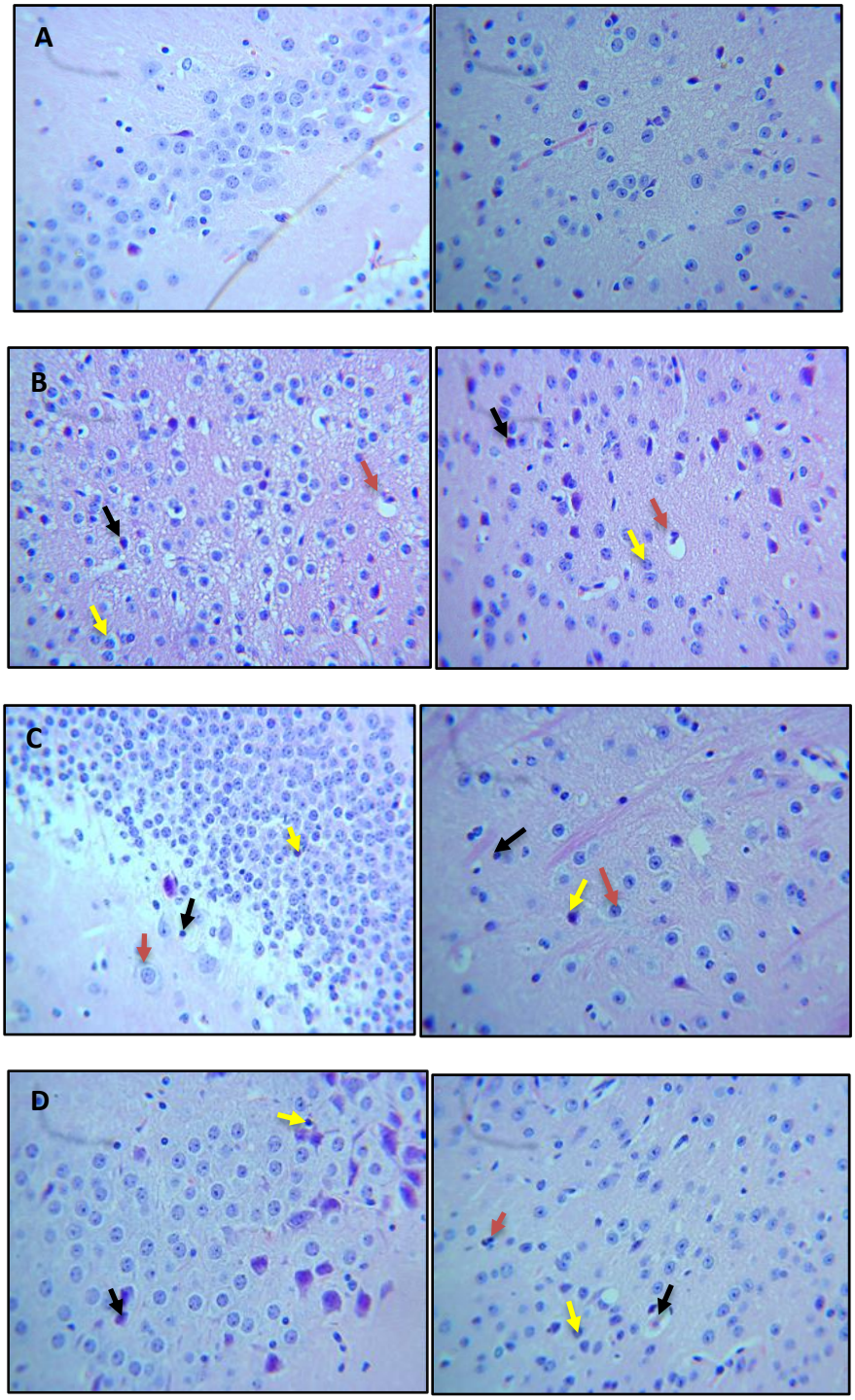

Figure 4.17: Micrographs showing Hematoxylin and eosin staining of brain section. This figure represents histological images of A: Control, B: Con-A, C: Con-A+Dio, D: Con-A+B.SLNP and Con-A+Dio.SLNP groups. Red arrow: vacuolization, black arrow: pyknosis and yellow arrow: infiltration of inflammatory cells.

4.4 UV Spectrophotometry of Drug Extracted from Blood and Organs

Spectrophotometric analysis was carried out for the concentration of diosgenin present in the extracts acquired from mice organs; brain, heart, liver, spleen and kidneys when IP injected with diosgenin and diosgenin SLNPs. These measurements were taken at the same time as those for blood.

Brain showed the highest level of drug either that be injected diosgenin only or diosgenin SLNP. These values were taken for 100µl of the extract solution and the values showed that the concentration of diosgenin was high in mice which were injected diosgenin SLNP than those which were injected diosgenin only. There was 50% increase in uptake of diosgenin by the brain with SLNP then diosgenin alone. Liver extract of group diosgenin SLNP showed high level of diosgenin with SLNP than the diosgenin alone. And there was a high difference between the concentrations of diosgenin in both groups. Which shows vulnerability of liver towards SLNP. The extract which shows least drug concentrations was of heart and kidneys somehow had equal amount of drugs for both of the group with no difference. Extracts from spleen showed higher level if drug for group which got diosgenin injected only. And it showed low level of drug in diosgenin SLNP injected mice extracts. There was a quite significant difference between the drug levels in the extracts. Heart, liver and spleen extracts for both groups showed a significant difference of $p<0.001$.

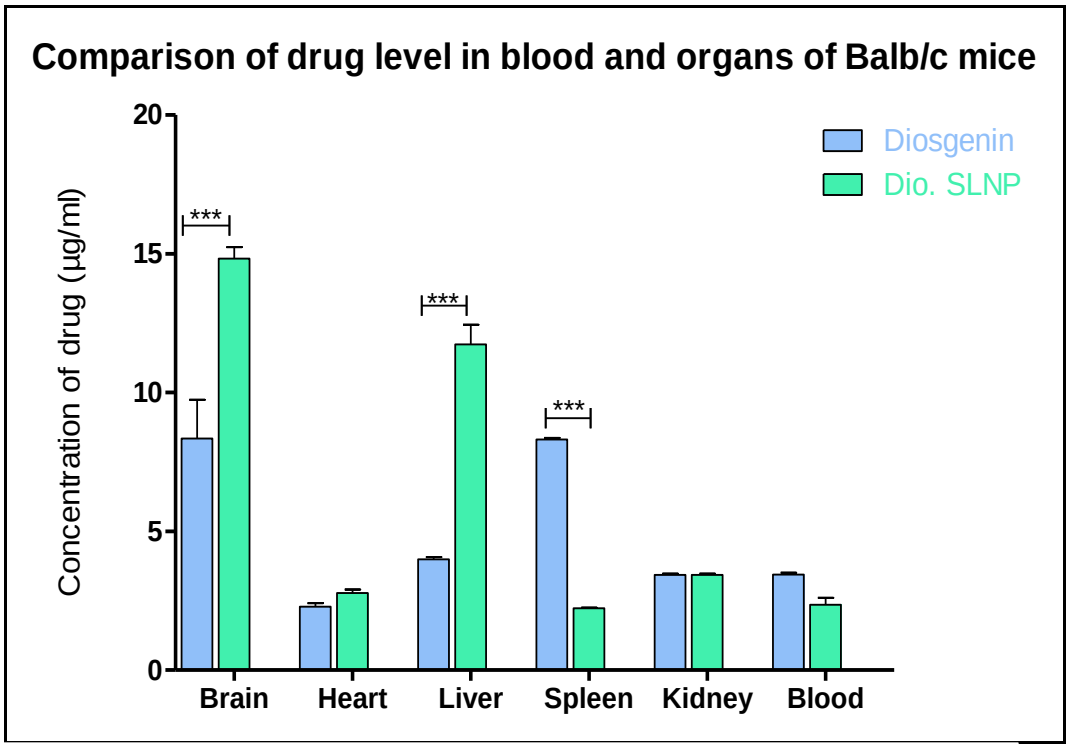

Figure 4.18: Comparative histogram shows concentrations of diosgenin level in brain, heart, liver, spleen, kidney and blood.

When the measurements of drug was evaluated for blood extracted drug it showed that the concentration of diosgenin for group diosgenin SLNP was low in blood then the diosgenin alone given. There is a significance difference of $p<0.05$ group injected with diosgenin only and diosgenin SLNP.

4.5 Blood CP for Evaluation of Toxicity

Complete blood profile or picture was done for mice of all groups to elucidate the toxicity level through lymphocyte, leukocyte and neutrophils number. The following table shows levels of neutrophils, leukocytes and erythrocytes in the blood with respect to control.

GROUPS	MOUSE	LEUKOCYTE ($\times10^9$/L)	LYMPHOCYTE (%)	NEUTROPHIL (%)	ERYTHROCYTE ($\times10^{12}$/L)
CONTROL	FEMALE,3	4.5-10	--	60-80	5.1-7.5
DIOSGENIN ONLY	M1	2.9	--	49.1	7.0
	M2	2.2	--	70.6	7.6
	M7	3.2	--	77.8	9.4
BLANK SLNP	M3	5.9	--	92.6	8.4
	M8	6.9	--	81.4	8.6
	M9	5.7	--	80.5	8.3
DIOSGENIN SLNP	M4	4.1	--	78	4.2
	M5	4.8	--	80	9.6
	M6	8.9	--	69	6.8

Table 4: Representing levels of leukocyte, neutrophil and erythrocyte in mice blood.

The blood cp of all group mice showed different level of cell count after injecting them IP with these treatments. Blank SLNP group mice showed slightly high level of neutrophils with leukocytes which lies in normal range so they are in reverse proportion. Mice which were injected with Diosgenin SLNP, showed no significant rise in levels of neutrophils but somehow there leukocyte level that lies in normal range as well. Group Diosgenin blood cp showed normal range of neutrophils but level of leukocyte is somehow low than the normal range. In this blood cp somehow lymphocyte level didn't give values.

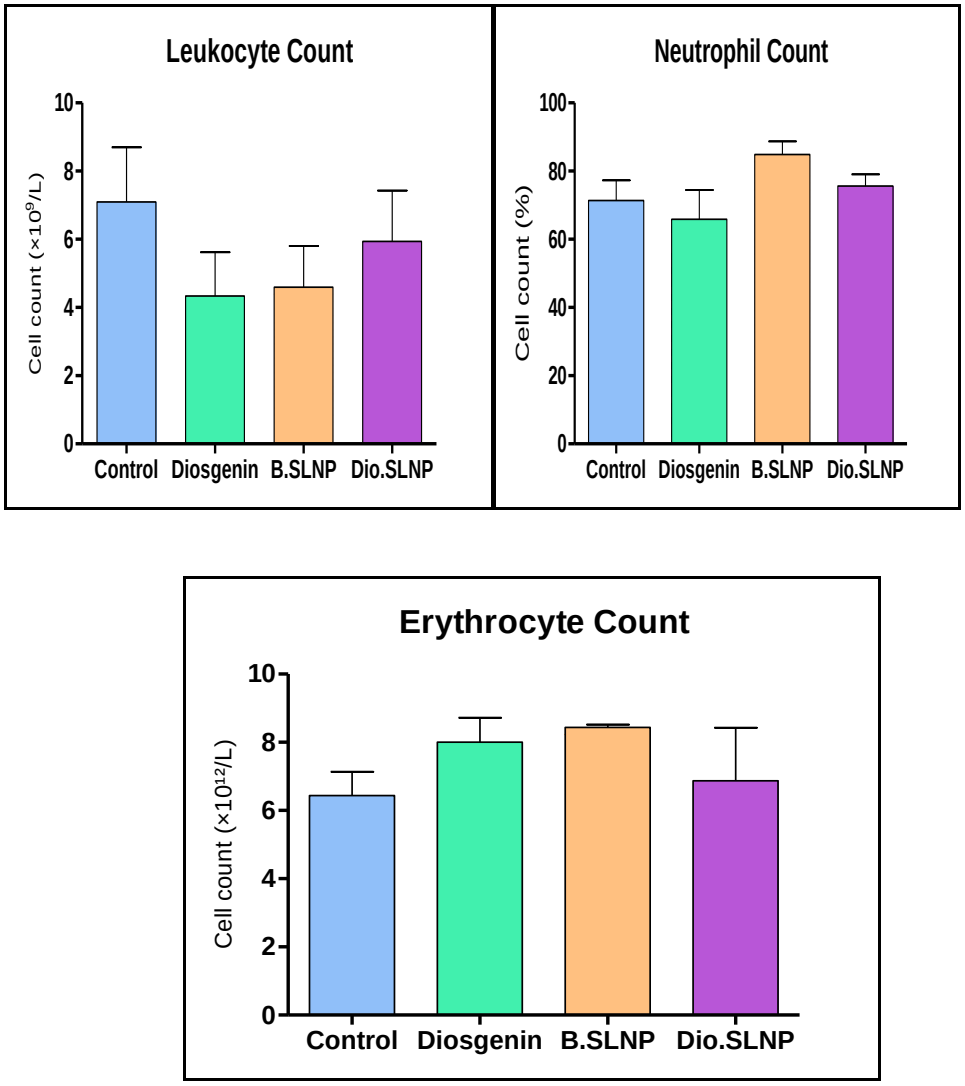

Figure 4.19: Blood complete profile of heart blood acquired from different groups of mice. Graphs shows the levels of Leukocytes, neutrophils and erythrocytes in blood for different groups.

Chapter 5

DISCUSSION

The BBB is very important for maintenance of CNS homeostasis. Alterations or disruption of any kind and extent leads to disease state that can be of any level (Bell & Zlokovic, 2009). BBB serve more as a blocking barrier to most of the components than any other barrier. In order to overcome this obstacle during delivery of potential therapeutic drugs, an efficient delivery system is required. For this purpose to efficiently carry a potential drug to cross the BBB, solid lipid nanoparticles are designed as a vehicle that is small but efficient enough to transport the drug. This study elucidated the formation of stearic acid P-80 coated SLNPs which encapsulated the targeted drug diosgenin. And explored its effects at different levels of *in-vitro and in-vivo* experimentation.

In the first phase of experimentations, the desired SLNP with and without drug encapsulation has been through different characterization analysis. The SEM results showed that the SLNPs in both blank and diosgenin encapsulated, have the desired size ranging from 10nm to 200nm that is basically required by the particle to get access to cross the barrier (Neves et al., 2015). Zeta size have also showed the average size of drug SLNP to be in range and it can easily cross the barrier. Zeta potential of the SLNP were found out to be negative in charge so they are anionic in nature over all. This characteristic of SLNP shows that the negative charge makes them more prone for cellular uptake as compared to the positive charged particles. The higher the negative charge the more stable particle is and in our result it turns out that SLNP which were formulated are stable, no flocculation and high cellular uptake of the drug through SLNP will result (Bernfield et al., 1999). The

previous studies shows that due to large negative domains of cell membrane it is possible that these negative charged vehicles get repelled. Which made scientist to experiment furthermore that made them to come to a conclusion in which it was elucidated that there are cationic sites (positively charged) in the membrane that allows the adsorption or entry of such negative charged vehicles (Patila et al., 2007) (Verma & Stellacci, 2010). This suggests that the entry of such negative vehicles or drugs through specific cationic sites can leads toward site specific internalization of drug in the membrane (Win KY and Feng SS., 2005). The patterns of SLNPs which were acquired from XRD shows intense peaks for diosgenin drug alone but when diosgenin SLNP were analyzed the pattern showed less intense peaks for drug. This suggests that drug itself is crystalline in nature but when encapsulated in SLNP the intensity of its crystallinity lower down to an extent. Which in this case is positive for a reason that low order crystallinity provides successful inclusion of drug in the SLNP as compared to the high order crystallinity of lipids. This increases the drug accommodation in its core and expulsion of drug is limited. The reduction in peaks of diosgenin indicated the decrease in its crystal nature when encapsulated in SLNP (Venkateswarlu and Manjunath., 2004) (Kumar et al., 2007). The FTIR peaks showed that the SLNP were coated with P-80 as the peaks showed intensity for certain bonds.

The drug release and entrapment efficiency of SLNP has been evaluated which showed better release pace of drug from SLNP and the amount of released drug was almost 50% to that of the standard drug concentrations. This might be due to the fact that drug concentrations used for standard curve formation were 10mg, 5mg and 1mg of drug alone and on other side same concentrations were taken for drug encapsulated SLNPs due to which there was a difference in the concentration of drug release. The higher surface area

of small sized SLNP could be a reason for better/faster release of drug (Venishetty et al., 2013). The entrapment efficiency of SLNP proves to be highly efficient. According to the calculated results it showed that the entrapment capability of P-80 coated SLNPs is more than 95% at different concentrations. Which means that drug concentration did not affect the entrapment efficiency of SLNP and due to small size it provides larger surface area for drug to be entrapped according to the previous studies (Komuravelli et al., 2013).

In-vitro study were analyzed in which MTT assay were performed first to check the extent of its cytotoxicity or anti-proliferative activity on U87 glioma cell lines (to check anti-cancer activity). Results represented that the diosgenin still showed cytotoxicity at 0.001µM concentration and showed highly constant anti-proliferative nature at the concentration ranging from 1Mm-0.3mM. Which suggested its anti-cancerous nature on U87 cell line and showed it is quite cytotoxic to cancerous cell lines by inhibiting its proliferation and inducing apoptosis in it. Diosgenin showed this activity in a time and dose-dependent manner according to the previous study (Kim et al., 2012). Cytotoxicity analysis was also done with the blank SLNP and diosgenin SLNP with comparison to diosgenin drug only. Results showed that the diosgenin SLNP were quite efficient to kill more than 50% of the cells which is more than the blank SLNP. This also showed that the killing of the cells with same concentration of drug were more than the same concentration of diosgenin SLNPs. This might be due to the fact that the drug encapsulated in that amount of SLNP might be moderately less than the actual drug concentration. But the difference was not that much between both of them. This study proved that diosgenin still showed high anti-proliferative activity on glioma cell lines when encapsulated in the SLNPs and this didn’t decreased its anti-cancerous activity on U87 glioma cell lines. Whereas blank

SLNP showed mild killing of the cells. Previous studies showed diosgenin dose-dependent cytotoxic effect on k562 human leukemia cells and osteosarcoma 1547 cell lines of human (David et al., 2004) (Moalic et al., 2001) and (Mirunalini et al., 2011).

In order to validate further the activity of diosgenin alone and diosgenin with the SLNPs, in-vivo studies had been also conducted. This study was basically to create sickness behavior model of Balb/c mice in order to check the anti-inflammatory activity of diosgenin. As it has been reported before that diosgenin imposed a high puissance of anti-inflammatory activity in diseases (Punitha et al., 2013). The model was created by injecting mice with Concanavalin-A which induced inflammatory response by activation of immune system in which lymphocytes are beget to release wide range of lymphokines (Tiegs et al., 1992). These mice were given treatments with commercially available drug and test drug with SLNPs and behavioral analysis were done on them. These behavior analysis showed that the diosgenin with SLNP and diosgenin alone had a potential therapeutic effect on the sickened mice with respect to other groups. The behavior analysis in open field test showed that the mice of group that was given diosgenin only as a treatment had high exploratory rate after control group. The mice were actively crossing the center square and spent time in exploring the arena more Difference between diosgenin and diosgenin SLNP were not drastic as with the sickened mice. This showed that the effectivity of drug encapsulated in SLNP had not been diminished rather it was quite active in displaying its therapeutic effect just like that of diosgenin alone. In social interaction tests, mice of group diosgenin SLNP showed high preference for socializing with novel mouse than with the known one. Which indicated that mice natural behavior is too prefer social novelty thus they spent more time with the Stranger 2 mouse than Stranger 1. This

study has been in line with the reported review literature (File and Seth, 2003, Robinson et al., 2005). This suggests that diosgenin have revived the normal sociable behavior of mice than fluoxetine.

Mice behavior in grooming test showed that mice with diosgenin SLNP treatment showed more grooming behavior than the rest of the treated groups. This group showed significant difference between the sickened mice of Con-A group. This suggested that the drug was efficient enough to restore back normal self-cleaning and grooming behavior in mice. These mice showed more time consumed in the cleaning and licking its coat and paws with sugar solution sprayed on it. This active nature showed the effectivity of drug SLNPs more than the commercially available drug and blank SLNPs. Tail suspension test of mice showed hyperactivity of mice in Con-A group which was sickened group with no treatment. This group showed more effort to escape the position than other groups. Which somehow indicates the hyperactivity of mice during sickness which according to some literature relates to anxiety level. But after this group, diosgenin SLNP showed more time spent in effort of escaping which provides an insight to drug efficacy with SLNP. The forced swimming test is an indication of continuous effort of survival in the water in which this study suggested that the group which was treated with diosgenin only showed more time spent in vigorous swimming for survival which overall indicated the effectivity of diosgenin as an anti-inflammatory drug. In all these experiments, results indicated that the effect of blank SLNPs are not that much when injected alone. When they are injected as a drug vehicle, they as a combination showed drastic therapeutic effect as an anti-inflammatory entity. They had lowered the inflammation which was caused by the Concanavalin-A and these above test proved its effectivity on sickness model.

Further to validate the anti-inflammatory effect of diosgenin and SLNPs, histological analysis was done on the brain of these mice. They were compared to control mice which have no inflammatory responses going on in the CNS vasculature. Group which were injected with Con-A only, there histology showed higher level of inflammatory infiltrates in the hippocampal and cortex region. The slides showed pyknosis at higher rate with vacuolization and high number of neutrophils and eosinophils in the region. When compared to that of diosgenin an diosgenin SLNP there were slight inflammation but the level of infiltrates were low than that in Con-A group (Kerschensteiner et al., 1999). The slides for blank SLNP showed pyknosis higher than the drug treated groups which somehow suggested that the blank SLNP are not that effective for therapeutic purpose the way drug has effected the inflammation. Which again provide an insight to the anti-inflammatory role of diosgenin with no other observable side effects. This examination showed high performance by the drug SLNP and poor effectivity performance by the blank SLNPs (Tohda et al., 2012).

Drug extraction performed on different organs and on blood which elucidated that the drug concentration were high in brain and then in liver than the rest of organs. Which showed capability of SLNP to deliver the drug more than that of drug alone when injected. It has also proved that the specificity of SLNP is more toward brain due to specific sites for entrance and the P-80 coating make it site specific as well. The level of drug in blood was low for that of diosgenin SLNPs which showed that the uptake of drug through SLNP was more than the drug alone (Tian et al., 2011). There was no value for blank SLNP as this was not detected with the diosgenin parameters. This study was done after one hour of injection which elucidated the greater uptake of drug by the brain in high quantity through

SLNPs which show significant difference level with the diosgenin alone. Blood cp was performed for the mice with blank SLNP, diosgenin drug and diosgenin SLNP. The blood was taken after 2 weeks of IP administration. Level of leukocytes, erythrocytes and neutrophils shows that there is no significant toxicity shown in the blood. Most of the cell count lies within the normal range which were assumed from the normal control mice blood. This experiment represent that the diosgenin alone and with SLNPs doesn't have greater extent of toxicity and do not show elevated cell count in the light of the previous studies done on toxicity of diosgenin (Qin et al., 2009) (Patel et al., 2012).

This study concludes that the BBB has been an obstacle for many potential therapeutics which are efficient enough to treat the CNS diseases. To overcome such obstacle solid lipid nanoparticle has been a great vehicle to carry a potential drug to the site and treat it effectively. Which in this study showed positive results for the polysorbate-80 coated stearic acid SLNP which transported test drug that was diosgenin to the BBB and treated the inflammation induced by Con-A. This study has also highlighted the anti-cancer and anti-inflammatory activity of diosgenin on gliomas and in sickness model which turns out to be quite effective enough. It has showed high anti-proliferative activity on glioma cell lines which indicated its anti-cancer activity at such low dose and showed to be not highly toxic to the normal cells. Its anti-inflammatory activity gave an insight as a potential drug for inflammation that is caused in many diseases. This study does not show that it will completely eradicate the problem but suggested that it can be a potential drug for many diseases with less side effects.

Yet this study has paved a way to for this type of carriers with a drug that has not been tested on gliomas yet. Further experimentations needed in order to find out the exact

molecular mechanism involved in the anti-cancer property of such drug. Further investigation are required to be done on BBB with SLNP incorporated with diosgenin to get an insight of the effect it has on cellular components of CNS. And exactly how these drugs cross the barrier through which specific channel.

APPENDIX

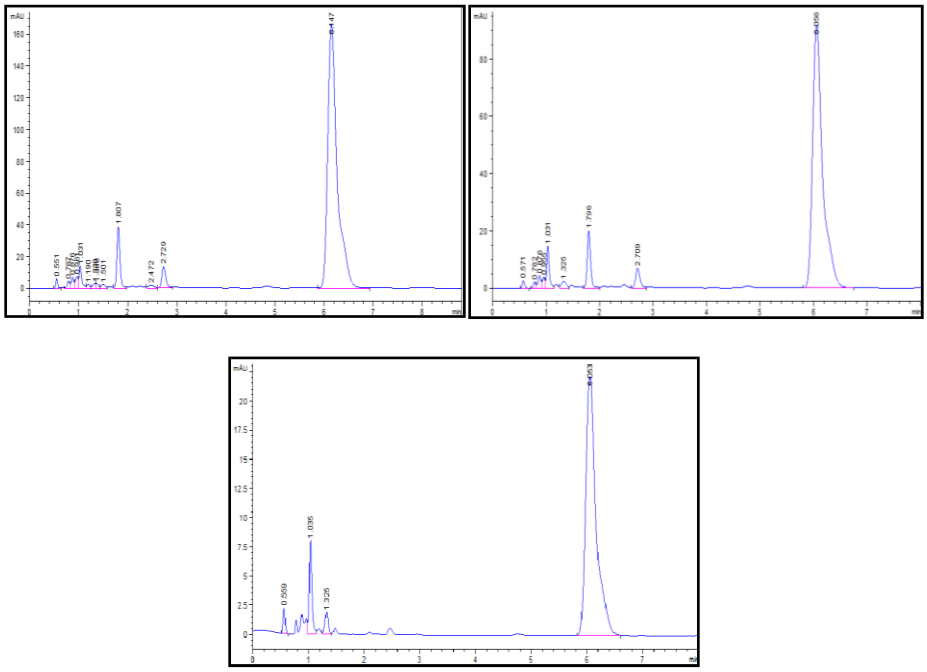

Figure 1: Shows retention time peak of diosgenin drug at different concentrations.

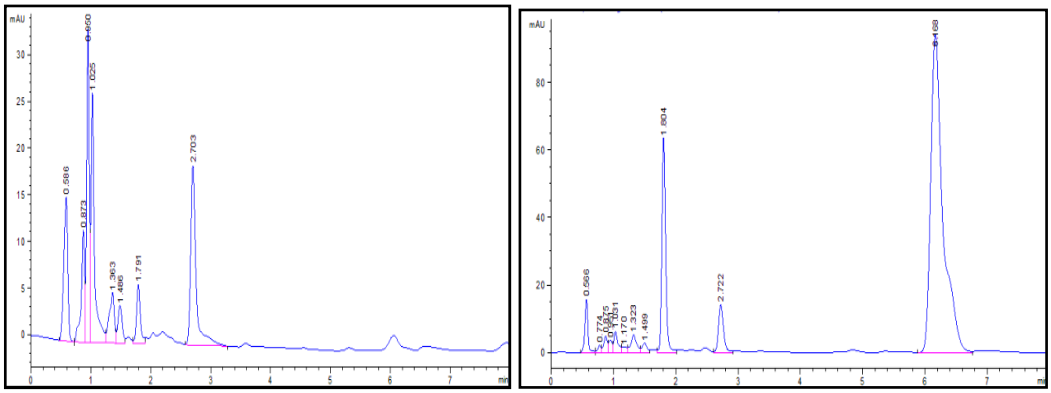

Figure 2: Representing graphs comparison of peaks between stearic acid SLNP and diosgenin incorporated SLNP. (A) Shows peaks for stearic acid SLNP. (B) Shows characteristic peak for diosgenin incorporated SLNP at 6.1 min.

Chapter 6

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