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Development of Nano-Niosomal Formulation of Alkaloids from *Tylophora Indica* for Improving Bioavailability through Oral Delivery

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LIST OF ABBREVIATIONS

nm	-	Nano metre
mm	-	Milli metre
µg	-	Micro gram

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ABSTRACT

Phytochemicals, the natural biochemical substances produced by plants possess a range of medicinal values. These phytochemicals get into our system through food and bring different physiological benefits. However, many of these phytochemicals lack essential physicochemical properties that can provide them effective drug-likeness properties. Tylophora indica alkaloids are known for their therapeutic values especially in the cancer treatment domain. However, the physiochemical properties of these molecules limit their bioavailability greatly. This current research aimed at improving the bioavailability of Tylophora indicaalkaloids through the nanovector system, niosomes. Tylophora indica alkaloids were extracted, purified, screened and quantified. Niosomal constructs of the alkaloids were made, characterized through scanning electron microscope and ex- vivo studies were carried out using goat intestine. Results shows that niosomal formulation can increase bioavailability of Tylophora indica alkaloidstwo folds compared to the native alkaloid extract.

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CHAPTER 1

INTRODUCTION

Pharmaceutical denotes any substance or compound that provide medicinal or health benefits. It is majorly used for curing a wide range of diseases. It is called as a multidisciplinary field which gained numerous dimensional roles in curing the disease. It deals with understanding the depth of molecular level for designing the drug. Traditional medicine serves as the lifesaver when compared with the modern medicine [1]. Plants and its therapeutical compound possess to be effective in preventing the disease and one of the advantages over the traditional medicine is it doesn't accelerate the disease for ex: deleterious effect of the drug. Tylophara is a genus of the family Asclepiadaceae consisting of about 60 species being widely distributed throughout the world. Tylophora species are slender perennial climber and commonly known to occur in Africa, Asia, Australia and Oceanic Islands [2]. The plant name "Tylophora" is made up of Two ancient Greek words wherein "Tylos" stands for "knot" while "phoros" stands for "bearing". Tylophora indica commonly known as "antmool" is one of the most medicinally important species of the Tylophora genus. Tylophora indica is well distributed in the plains, forest and hilly tracks of southern and eastern India, occurring up to an altitude of 900 m. The climber being indigenous to Inida inhabits sub-Himalayan tract up to an elevation fo 1260 m extending from Uttar pradesh to Meghalaya. It is an endangered perennial woody medicinal climber. It possesses long, fleshy, and knotty roots and long and twinning stem that grows up to 1.5 m [3].

A. Medicinal applications of Tylophora indica

The various medicinal applications of *Tylophora indica* include the cure against hypotension and cancer [4,5]. The plant is even used as a sedative and tranquilizing agent in animal hunting and other purposes. The other medicinal uses include for the treatment of elevated levels of blood glucose, loss of memory, bleeding gums etc. The list of its medicinal application of this plant is ever expanding and promising. The attempts to deliver the enhanced bio-available alkaloids to the target site are a novel one and the results of the attempts are promising too. The novel drug delivery mechanism involves delivering the alkaloids obtained from the plant to a targeted site using nano carriers. Currently many drug delivery mechanisms are in row and are in use. But using nano carriers is one of the finest and novice mechanisms to deliver the alkaloids from the plant [6,7]. The added advantage of this drug delivery system is controlled release of the drug, targeted site of action and safe to mankind. This attempt will help in better availability and better targeted sites of action against deadliest diseases like cancer and diabetes [8,9].

B. Problems associated with plant therapeuticals

Phytopharmacological compounds like alkaloids are highly soluble in aqueous layer and have low absorption, because they are unable to penetrate the lipid bilayer of the cells, which results in loss of bioavailability. Due to this obstacle the phytochemicals is formulated using nanostructured system which enables to potentiate the action of the phytochemicals, reducing the side effects and improving the therapeutic activity [10]. Pharmaceutical industries henceforth focus on the plant compounds and their improvement through nanotechnology.

C. Pharmaceutical nanotechnology

Pharmaceutical nanotechnology represents the revolutionary opportunities to fights against threat full disease like cancer, diabetes mellitus, and neurodegenerative diseases etc., [11]. The activities of active compound in plants are being researched to understand its complexity for in further developing them into therapeutic formulations. Phyto-therapeutics requires a

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delivery system to improve the bioavailability and sustained release in the system and reduce the effect of frequent administration [12]. Delivery of drug through nanovector system of phytocompounds has gained a potential future for the effective treatment and to overcome the drug side effects, to reduce the toxicity and to minimize the degradation of the compounds [13]. The advantage ofusing nano vector are improving the solubility of the drug, promote the sustain release of the active constituents and reaches the target place for the action [14]. Currently nano technology is developing innovative delivery systems like nanovesicles using bio-degradable and biocompatible substances for effective drug delivery in the system.

D. Nanovesicles

There are numerous drug delivery and drug molecule targeting systems, such as nano polymers, nano vesicles such as liposomes, micelles are currently utilized with the aim to minimize the drug degradations upon administration and to prevent from undesirable side-effects due to over/under load of active molecules in the cell and increase drug bioavailability etc. Nanovesicles are one type of nano delivery agents.

A nanovesicle is a lipid bilayer rolled up into a spherical shell which is enclosing a small amount of liquid and separating it from the external environment (which is usually aqueous) and usually ranges in the size of1 to 100 nanometres.

Nanovesicles like other vesicles are formed based on the molecular self-assembly process. They can be developed based on both the top- down (larger to smaller) and bottom-up (smaller to larger) approaches.

Different type of nanovesicles are artificially synthesised in a controlled environment and has been widely used across different fields. An artificial vesicle is any lipid bilayer that is formed in a spherical shape using the molecular self-assembly processes. These vesicles are usually made up of lipids which are natural or synthetic in origin. These artificial vesicles are made from single type of lipid compounds or group of compounds, which may be of different origin and properties. These artificial vesicles are easy to produce and hence they have beenextensively studied, characterised and being applied.

The size of these artificial vesicles ranges highly from the macroscopic, microscopic to nanoscopic level. Some of the artificial vesicles are liposomes, transferosomes, bilosomes. ethosomes, colloidasomes and niosomes. In this study, inorder to increase the bioavailability of the chosen drug, niosomes were synthesized to encapsulate the drug so that it enhances the sustained release of thedrug [15].

E. History of Nanovesicles

Nanovesicles were developed as a result of the expectation to produce novel lipid vesicle based carriers to deliver a range of pharmaceutical compounds and other compounds. Liposomes were the first type of nanovesicles that were first described in the mid of 1960s. The first patented nanovesicles were “niosomes” in the year 1970 to 1980. Transferosomes are the first generation of elastic nanovesicles introducedby Cevc et al. in the later 90s [16].

F. Nature of nanovesicles

Nanovesicles are chemically stable. They possess both hydrophilic and hydrophobic regions with the internal environments structure; henceforth, most of the hydrophilic and lipophilic active molecules are being entrapped in the nanovesicles. Nanovesicles can be prepared using relatively simple methodologies. Nanovesicles do not require high method protocol to maintain the activeness of the vesicle. Nanovesicles enhance the absorption of active ingredients; therefore, increase the bioavailability. The outer limiting layer of the nanovesicles which is

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usually made up of lipid base is similar to that of the biological cell membranes. This resemblance expands the usage of the nanovesicles greatly. Apart from lipids, nanovesicles can also be made from the compounds which have lipid like properties, such as modified peptides. The nanovesicles are usually non-toxic, biocompatible and biodegradable in nature; hence, making their wide usage in medicinal and food applications preferably. Transport, delivery, targeted site-specific delivery, localised delivery, protection, continuous delivery, etc. are the major application nature of the nanovesicles.

It has been well established that nanovesicles made of different molecular compounds must have size-dependent physiochemical properties. Most of the nanovesicles exist in the range of 1–100 nm. The size, shape and composition of the nanovesicles determine their properties and thereby their applications. It has been well established that nanovesicles made of different molecular compounds must have size-dependent physiochemical properties. Most of the nanovesicles existin the range of 1–100 nm. The size, shape and composition of the nanovesicles determine their properties and thereby their applications. The nanovesicles usually contain an aqueous layer and a bilayer membrane made up of amphiphilic molecules. The amphiphilic molecules that make the nanovesicles are usually lipids, especially phospholipids. The charge, degree of saturation and the length of the fatty acid chains of the lipids that are present in the bilayer have more influence over the physical properties of the vesicles, which include curvature, stability and permeability.

The thermodynamic system is a vital part of the nanovesicular development. The study of the surroundings like heat and melting points are the major parameters involved in the nanovesicle developmentprocess [17].

G. Factors affecting the nanovesicles

In order to achieve the mission functionality, nanovesicles should possess two important functionalities namely stability and entrapment ability. These two important properties are highly influenced by the various external and internal factors of the nanovesicles which include:

- Methodology of preparation
- Permeability of the bilayer
- Fluidity of the bilayer
- Size of the liposomes
- Storage conditions, etc.

The stability and entrapment ability are highly disturbed due to the damage of the nanovesicles. Nanovesicles undergo physical or chemical damage during preparation as well as storage. High temperatures during preparation and storage usually damage the nanovesicles. Oxidation of the lipids (fatty acids) is an important factor that leads to the damage of the vesicles including permeability of bilayers. The quality of lipids used in the vesicle preparation also influences the quality of vesicles. Nanovesicles with anti-oxidants or phospholipids with more saturated fatty acids can resist the damage through the process of oxidation. The entrapment efficiency of the nanovesicle is influenced majorly by the size and the lamellarity (the number of bilayers, uni or multi). A proper mechanical stress during the dispersion process of preparation step can be able to influence the size and lamellarity. A simple mechanical stress through agitation can lead to the production of multilamellar vesicles (MLV); whereas, the high level mechanical stress may lead to small or unilamellar nanovesicles. Multilamellar vesicles have been found to entrap more hydrophilic and hydrophobic compounds. However, the stability of such MLV is lesser than that of unilamellar vesicles [18].

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H. Self-assembly mechanism in nanovesicle synthesis

Self-assembly is a ubiquitous process by which the objects autonomously assemble into complexes. Nanovesicles are formed through the molecular self-assembly process. Many examples exist in nature and some of them are given below

- Atoms react to form molecules
- Molecules react to form crystals
- Molecules react to form supramolecules

Molecular self-assembly is a spontaneous organisation of molecules under thermodynamic equilibrium conditions into a structurally well-defined and rather stable arrangement through a number of non-covalentinteractions.

Formation of several non-covalent weak chemical bonds between molecules, such as hydrogen bonds, ionic bonds, van der Waals interactions, etc., are collectively called as the *non-covalent* interactions. These interactions are reversible in nature. The self-association process leads the molecules to form stable hierarchical macroscopic structures. Though non-covalent bonds are weak, their collective interaction often results in very stable assemblies. Self-assembled entities may be either discrete constructions or extended assemblies, these assemblies include

- dimensional polymolecular chains and fibres
- dimensional layers and membranes
- dimensional solids

Nanovesicles are of the kind of second type of assemblies.

I. Process of self-assembly

There are three basic steps that define a process of molecular self- assembly namely molecular recognition, growth and termination. Elementary molecules selectively bind to others which are commonly termed as molecular recognition. These elementary molecules or intermediate assemblies are the building blocks that bind to each other following a sequential or hierarchical assembly which is often termed as growth. Assemblies can potentially grow infinitely but their growth is interrupted by physical and/or environmental constraints, following withthe process of self-assembly undergoes termination.

In the nanovesicles formation, the process of self-assembly contains two major parts.

- The first part is the formation of a bi-layer.
- The second part is the closing of the bilayer to form a vesicle.

Nanovesicles which are characterised by the presence of lipids and amphiphilic compounds usually become a self-assembled structure as a result of a balance of attractive and repulsive forces. The amphiphilic surfactants compounds at low concentration form the micelles. The lipidsform lamellar bilayer structures which in turn form the vesicular structure on dilution. Usually the size of the micelles and vesicles vary depending on the relative geometry of hydrophobic and hydrophilic moieties and relative hydrophobicity. In the part of vesicle development process, the polymer (the tecton) (e.g. Lipid) prefers a parallel molecular arrangement which results in the formation of sheet like structure. At lower concentration, the sheet like structures of tectons are large; the energy loss due to surface tension is high, and the elasticity is less andthis tends to fold to form the vesicular structure. Vesicle formation requires two types of energy namely line energy and bending energy.

J. Theory of lipid assembly

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Assembly of lipid class of molecules into organised structure like bilayer, micelles depends on three major factors:

- Interactive free energy of the molecule
- Geometry of the molecules
- Thermodynamics

K. Mechanism of assembly

Lipids that organise into micelles or bilayer in oil-water complex is based on the forces between the hydrophobic tail and hydrophilic head of lipid molecules. Hydrocarbon tail tries to reduce water interaction; whereas, the hydrophilic head increases the water interaction. These two opposing forces initiate lipid assembly. At certain point, each head group reaches its optimal surface area where the total interactive free energy is minimum which further may organise into either monolayer/bilayer vesicles or micelles depending on the geometry of the molecules. Now geometry of bilayer/vesicles is hinge on optimal surface area and concentration of hydrophobic chain. **E.g.** Single carbon chain lipids like "phosphatidylcholine" will form micelles but lipids with two or more carbon chains like "diacyl phosphatidylcholine" that will form bilayer cannot form micelles and vice versa. Concept of self-assembly of lipids is the combined effect of entropy, geometry and interactive free energies to form organised structures.

L. Features of self-assembly

Co-operativity and non-linear behaviour often characterise molecular self-assembly. Molecular self-assembly is a highly parallel and time- dependent process.The process of self-assembly can be influenced by the physical and chemical conditions, such as pH, temperature and concentrations.

M. Principles of characterisation of nanovesicles

Nanosized particles and vesicles play a major role in various applications, for example, in pharmacology, food science, agriculture and environment, etc. Analysis of nano dimension is vital before proceeding into applications of nanovesicels. Structural analysis involves physical, chemical and biological properties. The fundamental principle to analyse these properties is through "CHARACTERISATION" of nano-entities. Apart from nanoparticle synthesis, characterisation of these nano sized materials is also an emerging field which delegates various techniques to study the morphology, property and dimension.

The various parameters checked for a nanoparticle are:

- Size
- Shape
- Charge
- Entrapment
- Drug release
- Stability
- Storage

N. The Size

As the size of the particles reduces to nanoscale, the properties also change.

Based on the size, the nanoparticles are classified as:

- Ultrafine particles (1 to 100 nm)
- Fine particles (100 to 2500 nm)
- Coarse particles (2500 to 10000 nm)

The various properties of the particle that depends on size are:

- Optical property
- Thermal property
- Mechanical property
- Chemical property

The colour of gold nanoparticles varies with the size. Particles with small size (<100 nm) have red colour, while larger nanoparticles have bluish or purple colour. Also, silver nanoparticles are yellow. This change is attributed to extinction spectra, i.e. the total of absorption and scattering. The extinction spectra depend on the size of the spherical particles. Nanoparticles have the ability to scatter phonons. Phonons are a quantum of energy associated with sound or vibration of crystal lattice.With reduction in diametre, the thermal conductivity also reduces. The phonon influenced thermal conductivity of nanoparticles is size- dependent.

The mechanical property of nanoparticles is different from that of the bulk. For example, let us take the case of organic or inorganic nanoparticles containing polymer nanocomposites.Compared to the bulk materials, the nanoparticles have increased surface to volume ratio. When the unbounded polymers are close to the exposed surface of nanoparticles, interaction occurs better than that in the bulk.The polymer will tightly wrap around the nanoparticle; thereby, changing its properties. 50% of the atoms are surface atoms in nanoparticles. Therefore, the electrical conductivity properties are directly related to the chemistry of these nanoparticles. Due to large number of surface atoms, the atoms have higher energy compared to the bulk state. The nanoparticle interaction depends on the surface chemistry. The increased surface area might also attract impurities on its surface. The surface properties/interactions can be changed by using molecular monolayers. The melting points of nanoparticles are lower than that for the bulk.

O. Shape

Nanoparticles can be synthesised in different shapes like spheres and rods. The shape of the particles can be influenced by two parameters:

- Thermodynamic stability
- Kinetic stability

The shapes obtained from thermodynamic stability can also be kinetically stable. However, the converse is not true.

The shape of the nanoparticles also influences their cellular uptake. Spherical nanoparticles are readily taken up by the cell, irrespective of their angle of projection. On the other hand, rod shaped particles can be taken up by the cell only when their major axis is perpendicular to thecell membrane.

P. Surface Charge

The surface charge of the particles influences their interaction and cell penetration. The positively charged gold nanoparticles can penetrate deep into the negatively charged cell wall. The negatively charged particles in contrast are repelled by the cell wall. The positively charged particles also have been observed to have higher cytotoxicity, better imaging efficiency, drug delivery and gene transfer. Recent findings suggest that, the delivery of drugs to the brain can be accomplished by using neutral or low anionic nanoparticles. The cationic particles have immediate toxic effect to the blood-brain barrier [19].

Q. Entrapment Efficacy

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The amount of entrapped drug can be calculated by estimating the amount of unentrapped drug and subtracting it from the total drug added. The entrapment efficiency depends on variety of factors including physical entrapment, precipitation, covalent bonding, surface absorption etc.

R. Drug Release

The ideal drug carrier should release the drug only in the target site, in a controlled way. The drug carrier should not be subjected to rapid clearance or the blood barrier. The nanoparticles can be manipulated to release the drug rapidly or in a controlled constant way. The use of antigens or proteins or molecules on the surface of the nanoparticle enables delivery of the drug only into the target site. Nanoparticles that are in the diametre of 5 nm are rapidly cleared from the body. Therefore, appropriately sized particles can be designed for effectively delivering the drugs. The drug is released from the nanoparticle by simple diffusion, influence of pH, exchange of ions with the environment, degradation or even by enzyme activity.

S. Stability

Stability is enhanced at nanoscale than in the bulk. Stability of nanoparticles has also been observed inside the body. Quantum dots are retained in the body for more than 100 days along with their fluorescenceability. The reactivity and stability depend on the surface chemistry and the surface charge of the particles.

The stability can be controlled by:

- pH of the solution
- Ionic strength
- Type of ion, either monovalent or multivalent

T. Storage

Storage of nanoparticles at lower temperatures prolongs the shelf-life. The particles should not be frozen but stored at 4 to 25°C. Improper storage may cause aggregation and loss of the particles. This can be characterised by colour change in the solution [19].

U. Preparation of nanovesicles

The method of preparation of nanovesicles depends on the functions of biovesicles. The properties, such as size, bilayers, distribution of the drug molecules, entrapment efficiency and membrane permeability play a vital role in choosing the preparation methods. Following are some of the methods used to synthesise nanocarriers for effective drug delivery at targeted place 20.

V. Ether injection method

This method is effective in preparing vesicles based on the conditions with the range of 50 to 1000 nm in diametre. The vesicles can be prepared by introducing a solution of surfactant dissolved in diethyl ether into water which is warm (i.e. water maintained at 60°C). The surfactant mixture in the ether solution is often injected through 14 gauge needle into an aqueous solution of vesicle material. The ether vapourisation leads to single layered vesicle formation.

W. Thin film hydration technique

The key materials used for the synthesis of nanovesicles like surfactant and cholesterol are dissolved in a volatile organic solvent, such as chloroform, methanol and diethyl either in a round-bottomed flask in rotatory evaporator instruments. Due to pressure, the organic solvent is removed at room temperature conditions; this produces a thin layer of vesicles deposited on the

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wall of the round-bottomed flask. The surfactant film gets rehydrated with the aqueous solution at 0-60°C with gentle agitation.

X. Sonication method

This method is found to be a typical approach to produce nanovesicles by sonication method. In this method, the active molecules (drugs) in buffer solution are added to the surfactant/cholesterol mixture. This mixture is probe sonicated at 60°C for 5 min using a sonicator instrument attached with a titanium probe to yield better nanovesicles, for example, noisomes.

Y. Microfluidisation method

Microfluidisation method is an advanced technique to prepare unilamellar vesicles with defined size distribution. This method is based on the submerged jet principle which consists of two fluids that interact with ultra-high velocities in precise micro channels with the interaction chambers. The impingement of thin liquid sheet along with a front with energy supplied to the system pretends to be in the area of niosomes formation.[3] The resulted niosomes found to possess uniformity, miniature size and better reproducibility of vesicles, for example, niosomes.

Z. Multiple membrane extrusion method

The anionic material of the surfactant and lipid composition like cholesterol and dicetyl phosphate in chloroform solution is made into thin film by evaporation method. The formed film is dissolved with aqueous drug polycarbonate membrane solution and the resultant suspension is extruded through a series of 8 passages. This method is found to be an apt method for controlling the vesicle size.

AA. Reverse phase evaporation technique

The phospholipid and surfactant in the ratio 1:1 are dissolved in the organic solvents, such as ether and chloroform. An aqueous phase contacting the drug molecules is added to this and the resulting two phases are sonicated for getting unilamellar vesicles at 4-5°C for 3 minutes.[4] The gel formed is further sonicated for controlling the vesicle size and maintaining proper pH after the addition of small amount of phosphate buffered saline solution. The organic phase is removed at 40°C under low pressure conditions. The resulted solution of vesicles suspension is further diluted with buffer solutions and heated over a water bath at 60°C for 10 min to yield nanovesicles 20.

BB. Nanovesicle in Pharmacology

The conventional drugs are those that are consumed orally or injected intravenously. These drugs enter the blood circulation and are distributed throughout the body. Relatively a small portion of the drug reaches the target site. The conventional drugs that are available have many limitations such as need of frequent administration, small therapeutic index and reduced bioavailability.

The common problem of reduced bioavailability associated with the conventional delivery of pharmaceutically active ingredients is due to

- Poor permeability through skin and membrane linings of organs
- Insolubility in physiological fluids

Nanotechnology can be used to overcome these drawbacks. Mainly attributed to their small size, nanomaterials have been effective in delivering drugs. One such widely used carrier is the nanovesicles.

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Nanovesicles are lipid bilayer spherical particles of 10-100nm size. The increased surface area of the nanovesicles enables the vesicles to have maximum interaction with the drug. Both hydrophobic and hydrophilic drugs can be encapsulated into these nanovesicles. The amphiphilic nature of nanovesicles makes it suitable for interacting with the cell or biological membranes and thereby increase the drug permeation. The usage of various nanovesicle for delivering drugs effectively have been explored extensively [20].

CC. Advantages of nano vesicles

The advantages of utilizing nanovesicles are as follows:

- Nanovesicles are chemically stable
- They possess both hydrophilic and hydrophobic regions with the internal environments structure, henceforth most of the hydrophilic and lipophilic active molecules are being entrapped inthe nanovesicles
- Nanovesicles do not require high method protocol to maintain the activeness of the vesicle, most preferably high temperatures are not recommended for storage purposes.
- Nanovesicles enhances the absorption of active ingredients therefore increases the bioavailability.
- Due to the high advantages of using nanovesicles it is mostly preferable for the drug delivery processes.

DD. Pharmacological applications of nanovesicles

Nanovesicles greatly help in the drug delivery processes. Nanovesicles can be employed in different delivery processes such as Transdermal delivery, Intravenous delivery, Ocular delivery, Pulmonary delivery and Oral delivery.

EE. Transdermal delivery through nanovesicles

Transdermal delivery involves application of the drug formulation on to the skin. The skin being the largest organ in our body, has wide surface area suitable for maximum absorption of the drug. The skin delivery can be used to avoid rapid clearance of the drug, GI track (gastro-intestinal) irritation and first pass mechanism. The nanovesicles help in penetrating the drug through the skin layers and in reaching the target site. Some vesicles are formulated into gels for easy application and enhanced skin absorption. The various nanovesicles in transdermal application include niosomes, transferosomes, ethosomes and liposomes. The various drugs Such as anti-inflammatory drugs, local Anaesthetics, genetic materials, hormones, immunisations, hair loss medicines, Non- steroidal Anti-Inflammatory Drugs, anti-histamines etc., can be delivered in transdermal way using nanovesicles.

FF. Intravenous delivery

In intravenous delivery, the drug is directly injected into the vein This method of delivery is chosen to induce immediate activity of the drug. Some of the liposomal drugs delivered intravenously are Doxil, AmBisomeetc.

GG. Ocular drug delivery

Ocular drug delivery is the process of delivering drugs to the eyes, mostly through eye drops. The conventional drug formulations are not efficient in ocular delivery because of the reasons such as short residence time impermeability to corneal epithelia, tear flow, blinking reflux etc. The epithelial layers prevent drug penetration into deeper layers of cornea and aqueous humour. To effectively deliver the drugs through ocular route, nanovesicle formulation is preferred. Nanovesicles have better localizing and drug activity maintaining ability. The nanovesicles can easily pass through the barriers and extend the rate and degree of drug absorption. Liposomes are the most frequently used formulation for ocular delivery. An example is idoxuridine liposomal formulation. Idoxuridine is an anti-herpes simplex anti-viral drug.

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Liposomal formulation of idoxuridine was tested on herpes simplex infected corneal lesions of rabbits. Precorneal retention times of the drug were enhanced, even in presence of mucoadhesives. Liposomes had migrated to conjunctival sac with very little activity in the corneal surface [21].

HH. Pulmonary drug delivery

Pulmonary drug delivery is delivers therapeutic molecules into systemic circulation through alveolar absorption. Use of drug delivery system for treatment of pulmonary diseases is gaining expertise as potential for localized topical therapy in lungs, prophylactic agents such as peptides and proteins can be targeted, due to large surface area for absorption, low metabolic activity as there is no first pass mechanism etc. Nanovesicles in pulmonary drug delivery, can be used for the treatment of cystic fibrosis, asthma, pulmonary infections and lung cancer. Cytotoxic agents, bronchodilators, anti-asthma drugs, antimicrobial, antiviral agents and drugs for systemic action: insulin and proteins are being investigated for pulmonary delivery [22].

II. Oral Drug Delivery

The most commonly used route for delivery of pharmaceutical compounds is through mouth. The drugs are released into the gastro intestinal tract (mouth, stomach, small and large intestine). The commonly used nanovesicles for oral delivery are niosomes and liposomes. Delivery of anti-cancer drugs and targeted delivery of drugs are explained as examples of oral drug delivery system [23]. Drug delivery for cancer therapy

Cancer is a disease in which cells grow uncontrollably and destroy body tissue. Anti-cancer drugs have low therapeutic index i.e. the dosage level required to have the necessary effect on cancer cells is toxic to normal cells. This is observed due to low concentrations of drug at the target site. Liposome formulations are being investigated as it can be used for selective targeting of the drug. Liposomes are chosen as drug carriers because of enhanced drug circulation lifetime, higher concentration in the infected tissue, protection from metabolic degradation of the drug, altered tissue distribution of the drug etc.

Enhanced uptake in organs rich in mononuclear phagocytic cells (liver, spleen and bone marrow) and reduced uptake in kidney, myocardium and brain. Anthracyclines, a potent class of cytotoxic drugs that chelates DNA is used for cancer treatment. However, it is commonly found in hair, gastrointestinal mucosa, and blood cells. Liposomal formulations showed reduced toxicity to normal cells compared to cancer cells. Moreover, the formulations showed reduced cardiotoxicity and dermal toxicity. Methotrexate loaded niosomes have also been used for oral administration. Higher levels of the drug were observed in serum, liver and brain. This indicates that there is enhanced drug absorption in niosomal formulation compared to conventional consumption [22].

JJ. Niosomes

Niosomes is one such nanovesicle that can be used for entrapping the active phytochemical compounds and delivering them effectively through different drug delivery systems such as oral, topical, intravenous etc. Niosomes are artificially synthesized vesicles that are used for targeted delivery in the body. This is a novel technique used for targeted drug delivery in human body involving the medication to be encapsulated inside the vesicle which is then orally administered in the humans to reach specific target tissues or organs.

Niosomes composed of non-ionic surface active agents (surfactants) and hence named niosomes. They can be either unilamellar or multilamellar based on their method of preparation. They are structurally similar to liposomes but the bilayer in niosomes is made of

non-ionic surface active agents. Most surface agents yield micellar structure but some surfactants give a bilayer vesicle that is called Niosome.

Niosomes have hydrophilic ends that lie on the outward side and hydrophobic end on the inside so they hold hydrophobic drugs on the aqueous side and the hydrophilic drugs within the bilayer. A typical Niosome vesicle would consist of vesicle with surfactant such as SPAN-60 to which a stabilizing agent such as cholesterol is added and also a non-ionic surfactant such as diacetyl phosphate is added for stability.

KK. Preparation of niosomes

Niosome preparation involves mixing of cholesterol and surfactant in a specified ratio (usually 1:1) and then dissolving in a medium of organic solvents (usually ether, chloroform etc.). The mixture is subjected to rotary vacuum evaporation for the removal of organic phase and it result in formation of thin layer cholesterol and surfactants. Drugs in aqueous phase are added to the medium which usually results in the formation of vescicular structures in the size range of nano to micro. Further the size of the vesicles can be reduced to nano usually through sonication process. The unentrapped is further removed from the vesicles through the processes such as dialysis, centrifugation or Gel filtration [19].

LL. Applications of niosomes

Niosomes are applied in the therapeutical sector greatly. Niosomes helps in carrying and delivering wide range of drugs. Sustained drug delivery nature of Niosomes helps in incorporating them in different disease treatments. Niosomes helps in delivering peptides and proteins greatly and hence it finds better place in vaccine delivery. Niosomes function as gene delivery vector due to its multi beneficial nature than the viral vectors. Niosomes helps in DNA vaccination. Niosomes with necessary coating can also be used targeted drug delivery. Niosomes usedin anti-neoplastic treatment and helps greatly in cancer management [24].

MM. Niosome as a drug carrier

Drug carrier is a substance that improves the delivery and the effectiveness of drug. Drug carriers are used in drug delivery system such as controlled release technique, prolong invivo drug action, decrease drug metabolism and reduce drug toxicity. The concept of drug carriers is thatto deliver the drugs to target organs and modify the drug disposition [25].

There has been keen interest in the development of a novel drug delivery system and the aim is to deliver the drug at a rate directed by the needs of the body during the period of treatment [26].

Some of the drug carriers which are used to deliver the drugs are immunoglobulins, plasma protein, liposomes, niosomes. The slow drug release may reduce the toxicity of drug and hence these carriers play an important role in drug delivery [27].

Many drugs those currently available in the market have poor aqueous solubility that result in decrease bioavailabilities. So to improve the bioavailability of drug, drug carrier is used [28].

NN. Current research focus

In this research work we have engaged in developing niosomal nano formulations of Tylophora indica alkaloids. Tylophora indica alkaloids are series of secondary of metabolites that are isolated from the medicinal plant Tylophora indica rosea. These alkaloids were reported to possess a range of medicinal activities that includes anti-cancer activity, anti-diabetic activity, treating hypertension etc. Even though the therapeutic application profile of these alkaloids is high, there are many challenges in reaching these alkaloids in destination for treatment.

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This may be due to their physio chemical properties which lead to poor bioavailability and possible side effects. Therefore Tylophora indica alkaloids could be one effective target compound group among the range of phytochemicals to be experimented for developing a nanoformulation. This research work deals with extraction of alkaloids from the Tylophora indica leaves, developing niosomal formulation of Tylophora indica alkaloids, characterising the nanovesicle formulation and studying the ex-vivo application of the nanoformulation and to determine the improved deliverability of Tylophora indica alkaloids through niosomes.

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CHAPTER 2

LITERATURE SURVEY

Niosomes are typical vesicular bodies which can be synthesized in nano scale sizes. These niosomes can be used to carry different small sized compounds for variety of purposes including pharmaceutical applications, cosmetic applications, enhanced nutrition delivery of food supplements etc. Over the period of two to three decades several researches has been conducted in the nanovesicles part. In the category of nanovesicles liposomes are kind of ancestors of niosomes.

A. Improvements in niosomes over years

Bangham et al developed liposomes in the year of 1961. Since then liposomes were encountering variety of development and the application scope of this type of nanovesicles are expanding [22].

Even though the liposome was a wide researched nanovesicle it contains several limiting factors because of its own physio chemical nature. The stability and leaky nature of the liposomes are two of the major disadvantages of this lipid based nanovesicle. This leads to the demand of developing further improved stable nanovesicle formulations [29].

Niosome was first developed by L'Oréal in the 1980's and it was patented. Based on the developer's perspective, the niosomes were originally developed for cosmetic applications with factors such as good penetration of skin barrier, improved bioavailability and high stability [30].

Later the focuses on niosomes were further expanded in dimensional ways. Researchers found more effective nature of niosomes in the delivery of pharmaceutical compounds. Several research work reported that niosomes can be been used to encapsulate different kind of drugs such as natural and synthetic.

Carafa et al in 2002, analysed the efficiency of improved deliverability of Lidocaine through niosomes. Lidocaine is an amino amide chemical compound used of anesthetic purposes. This research focused on developing novel formulations of lidocaine loaded niosomal vesicles and studies the efficacy under in-vitro release conditions. The lidocain niosomal formulations were found to be effective in crossing the Silastic and mouse abdominal skin in-vitro [31].

affeine is one of the common ingredients found in widely used beverages, such as coffee, tea, etc. It acts as a mental stimulant and also possesses a range of pharmacological activities, which make its application wider. A research work attempting transdermal delivery of caffeine was carried out by Payam Khazeli et al. in 2006.

Niosomal formulation of caffeine was made and experimented with for release studies through Franz diffusion cell in vitro. The researchers tried altering the outer charge of the niosomes and studied the efficiency in the release of caffeine. Positively charged niosomes entrapped less caffeine than the neutral one; however, they were able to deliver more caffeine comparatively [32].

Oryza sativa (rice) is one of the most highly consumed staple foods across countries. Rice contains a hard outer layer called bran, which is usually removed during processing. The

chemical constituents of the rice bran, such as oryzanol, ferulic acid, etc. possess therapeutic values and find usage in cosmetic products.

Degradation over a short period is one of the key challenges in using the antioxidants in cosmetic applications and hence Aranya Manosrai et al. developed gel and cream containing niosomal formulations of rice bran extracts. The niosomal formulations were experimented in vitro, ex vivo and in vivo and were found to be more effective in producing good antioxidant activity and high skin hydration ability [33].

In 2008, Ghada Abdelbary and Nashwa El-gendy performed an analytical study of controlled ophthalmic delivery of an antibiotic called Gentamycin. The researchers have analysed various compositions of niosomes and achieved a better delivery rate of gentamicin under in-vitro conditions. They also found that nano formulations of Gentamycin do not cause any irritations on albino rates [34].

Shatalebi et al in 2010 has worked in N-acetyl glucosamine niosomes and directed it towards topical applications. N-acetyl glucosamine whichis been reported for treating disorders of hyperpigmentation was well delivered through the niosomal formulation the research claims. Niosomes containing the formulation based on span – 40 produced higher movement of the N-acetyl glucosamine across the skin barrier [35].

Gymnema sylvestre is an herb used widely in traditional medicine for its antidiabetic and antidiuretic properties. Gymnemic acid is one of the important components of the *Gymnema sylvestre* which is pharmacologically active. However, the drug-likeness property of this gymnemic acid is poor. The solubility and instability in gastric conditions and affinity towards cholesterol make it less preferable for therapeutical purposes.

Niosomes can be a better option for improved delivery of these gymnemic acids. Bhagyashree Kamble et al. in 2013 developed niosomal constructs entrapping alcoholic extract of *Gymnema sylvestre* and tested their deliverability efficiency under in vitro and in vivo conditions. Gymnema-niosomal formulation exhibited a higher percentage of blood glucose level reduction comparatively (Kamble et., 2013). .

Living fossil, *Ginkgo biloba* is a very old plant species that possess excellent medicinal properties including antioxidant and anticancer abilities. The phytochemical components of this plant were known to induce a neuroprotective effect. It is also a better candidate to treat diseases, such as Alzheimer's. However, the bioavailability of the phytocompounds is poor.

To overcome the same, Ye Jin et al. in the year 2013 reported niosomal formulation development of *Ginkgo biloba* extract and it's in vivo evaluation experiments in the rats. The niosomal formulations were found to cross the blood-brain barrier (BBB), which demonstrated niosomes as a potent vehicle for improving the bioavailability of therapeutical molecules across BBB [36]·

A research work in 2015 carried out by Karim M. Raafat & Sally A. El- Zahaby involved development of niosomes by entrapping active phytomolecules of a medicinal plant called Fumaria officinalis. The study involves in-vivo analysis of the niosome construct to determine its efficiency in enhancement of antineuropathic and anti-inflammatory potential. The niosomes were found to entrap two alkaloids from the plant namely Stylopine, and Sanguinarine in greater proportions which are found to be anti-diabetic. The researchers can able to develop the alkaloid entrapped niosomes successfully in the average size of 96 nm.

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The researchers concluded the formulation to be efficient and novel for practical oral applications [37].

Asthana et al in 2016 developed niosomal gel containing Etodolac, a pain relieving drug, for topical applications. This nanogel was proposed to be a pain relieving ailment. The researchers could able to successfully entrap 96.72% of drug into the niosomal formulations. The experiments were carried out in both exvivo as well as invivo. The results shows sustainable and prolonged delivery of the etodolac through the niosomal gel [38]. Curcumin is one of the major components of the widely used *Curcuma longa*. It is well known for its medicinal properties across the world. But still, the solubility and stability issues of the compound makeit less preferable for clinical applications.

Xu et al. in the year 2016 developed a novel niosomal formulation of curcumin using chemical compositions, such as Span 80, Tween 80, and Poloxamer 188. The niosome construct was able to retain more than 92% of the loaded curcumin and proved to be a bioavailability enhancer (1.40 folds) in the antitumor cell line study performed in comparison to the crude extract [39].

Withania somnifera is a medicinal plant well known in ayurvedic medicines. It is commonly known as aswagandha. It is being used for its variety of medicinal properties, such as antibacterial, antidiabetic, antihypertensive, antiaging, anticancer, etc. Tawona N Chinembiri et al. developed niosomes entrapping crude extract of *Withania somnifera* and characterized the same. The complex was aimed for topical applications and the penetration of the phytoconstituents through the skin. In vitro and ex vivo studies have been performed for analyzing drug release efficiency of the niosomal construct. Niosomes have successfully takenthe phytochemicals across the stratum corneum barrier [40].

Myrtus communis is a common flowering plant known for its traditional medicinal activities including wound and burn healing, curing ulcers, bleeding of nose, etc. It is a very good source of antibacterial compounds. Niosomal formulation of *Myrtus* extract was developed by Mahboobeh Raeiszadeh et al. and tested against different pathogenic microorganisms. The niosomal formulation tested has shown up to 93.4% entrapment efficiency and has shown consistent and steady release of the phytochemicals under in vitro conditions. The formulation was proposed by the researchers for oral drug delivery purposes [41].

Niosomes can able to help us in improving the bioavailability of bio compounds which are of poor soluble in nature. Mahmood barani and his team of researchers in 2018 researched on improving the bioavailablity, stability and permeability of Lawsone, a plant compound through niosomes.

The research was able to produce niosomes with 70% entrapment of lawsone which is directed for anti-tumor applications. The researchers can able to store the niosomes for 2 months in a stable manner [42]. Apart from aiding in improving the bioavailability and quality of oral formulations, niosomes can also be used to improve the topical applications through skin. Many research works have been reported in this phenomenon [43].

Kassem et al in 2017 have conducted a research in developing niosomes loaded with imatinib mesylate. The niosomes have been tested for efficacy over the cancer cell lines such as HepG2, MCF7 and HCT-116 in-vitro. The formulation developed showed improved efficacy and selectivity of the drug toward cancer cells [44]. In 2018, Raeiszadeh et al worked on constructing niosomes with an active medicinal extract of *Myrtus communis.*

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The plant has been reported to be used for treatment of mouth ulcer, nosebleed, burn, wound etc. However, the plant compounds have poor pharma efficiency due to low solubility and low permeability. The researchers successfully tried to improve the efficiency of this plant drug through niosomal formulation. They have developed and experimented different composition of Span and Tween and Cholesterol to find out the optimal composition for *Myrtus communis* extract. It was found that 3:3:4 ratios of Span, Tween and Cholesterol were optimal for better performance of the formulation [41].

Lawsone is of the phytochemical compounds present in the plants, such as henna and water hyacinth. It is a dye compound that renders orange stain to hair and skin through binding with the keratin. Apart from staining, medicinal values of lawsone have also been reported[10]. However, wide application of lawsone was limited as a result of its poor solubility, which in turn affects its stability, permeability and bioavailability. An attempt to improve the bioavailability of lawsone through niosomes was carried out by M. Barani et al. in 2018. The researchers have developed nano size (~250 nm) niosomes of henna extract using cholesterol and non-ionic surfactants as nanovesicles forming composition. The efficacy study of niosomal construct was carried out in MCF-7 cell line, which has shown increased anticancer activity thus proving the improved stability and bioavailability of the formulation [45].

Annona squamosa is one of the plant members known for its fruit called sugar apple and also for medicinal properties of its different plant parts. Different research analyzing its efficacy in evaluating antioxidants, antibacterial, anticancerous and antidiabetic potential has been performed so far. For improving the bioavailability, stability and prolonged release E.A. Mohammed et al [44]. have developed niosomal formulations of the leaves extract of *Annona* squamosa. They have tested the usefulness of the niosomal form through in vitro experiments and also ex vivo experiments using the abdominal skin of the rat.

The in vitro results have shown that, the niosomes help in the reduction of the rapid release of the extracts and increase the consistent release in a prolonged way. The skin-based penetration studies have shown the active penetration of the niosomal formulation across the skin barrier. The authors claim the usage of niosomal formulation for better transdermal delivery of the phyto extracts [42]. Curcumin is an active bio compound from the plant Curcuma longa. It is having wide medicinal and dietary supplement value. However the compound is poorly soluble and unstable which leads to less common application in different treatment procedures. Ghadi et al in 2019 involved in curcumin loaded niosomal research. They have incorporated hyaluronan in their niosomal formulation and they could able to produce more stable composition which could able to produce higher anti-inflammatory effect comparatively [46].

A novel study on treating wounds through methylene blue loaded niosomes was experimented in 2020 by Farmoudeh et al. This research group involved in developing niosomes entrapping methylene blue through the preparatory technique called ultra-sonication. They can achieve 63.27 percentage of entrapment of the dye in the niosomes and used the same to treat wounded rats under experimental conditions. Compared to other controls, niosomal formulations found to be very effective in treatment [47].

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CHAPTER 3

METHODOLOGY

A. Experimental Methods

All the chemicals utilised in the study were obtained from Merck Chemicals, India. Glasswares and other lab wares utilised in the study were obtained from Borosil and Labmate, India.

B. Plant specimens

*Tylophora indica*plant leaves used in the study were collected from Ayanavaram, Chennai.

C. Sterilisation

Glass wares were soaked in chromic acid solution (10% potassium dichromate in 25% Sulphuric acid) for few hours and washed thoroughly using detergent solution and dried in hot air oven at 160°C for 20 min.Media and other utilities used in the research work were sterilised in an autoclave at 121°C with 15 lb pressure for 20 min.

D. Extraction of Tylophora indicaalkaloids

4 g of fresh leaves of *Tylophora indica*was taken and rinsed with sterile distilled water, and air-dried. Leaves were then crushed with 0.1M HCl solution using a mortar and pestle. It was then kept for stirring in a magnetic stirrer for 2 h and centrifuged. The supernatant was then added with petroleum ether and shaken well in a separating funnel. The acid fraction was carefully removed and transferred to a separating funnel containing an equal volume of 0.1 N NaOH solution and shaken well. The funnel was kept undisturbed for 30 min. To that, diethyl ether (1:1) was added and mixed by inverting gently and the internal pressure was released slowly. The mixing action was repeated thrice and observed for two layers of separation. The upper organic layer containing salts of alkaloids was separated evaporated and stored for further experiments [48].

E. Purification of Tylophora indicaalkaloids using column chromatography

Column chromatography was performed on a glass column (20 × 2 cm) packed with 90 g of alumina. The fresh *Tylophora indica* alkaloid extract (5 ml) was applied to the column by using a pipette and the column was eluted with methanol. Five fractions were collected and each fraction was tested for the presence of alkaloids using Dragendorff's reagent. The fractions were then evaporated and dissolved in 5 ml of methanol and refrigerated for further analysis [49].

F. Identification of Tylophora indicaalkaloids by thin layer chromatography (TLC)

Silica gel-coated aluminium plates (obtained from Merck, India) were taken in the size of 2 x 9 cm. The *Tylophora indica* alkaloids sample in methanolic solution was loaded at the base of the TLC plate using an applicator stick. The solvent system containing 80% ethanol and 1 N HCl (in the ratio 25:1) was taken in the TLC chamber and a thin layer chromatogram was developed. The plate was then allowed for drying and Dragendorff's reagent was sprinkled over the plate and observed for yellow spots [50].

G. Separation of Tylophora indicaalkaloids by high-performance thin layer chromatography (HPTLC)

HPTLC was performed on 10 x 20 cm aluminum-backed silica gel F254 HPTLC plates from Merck, India. In order to avoid possible interference of manufacturing-based impurities on silica plates, the plates were prewashed with methanol, dried and activated for 30 min at 110°C. *Tylophora indica* alkaloids dissolved in methanolic solution was applied on plates as

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6 mm bands, by 6 mm apart and 1 cm from the edge of theplate, by means of automatic sample applicator, fitted with a syringe. Vinblastine sulphate was used as standard and was applied to the parallel track. The mobile phase used in the analysis was hexane: ethylacetate: glacial acetic acid (3:1:0.1). The plate was then moved with the mobile phase and developed to a distance of 90 mm and then removed from the chamber, dried and scanned at 210 nm using scanner densitometer [51]

H. Estimation of Tylophora indicaalkaloids using Bromocresol Green (BCG) method

Tylophora indica alkaloids dried in rotary vacuum evaporator was taken and dissolved in 2 N HCl and mixed well. The solution was then filtered. 1 mlof this solution was transferred to a separating funnel and washed with 10 ml of chloroform (3 times) followed by pH adjustments to neutral using 0.1 N NaOH. Then, 5 ml of BCG solution and 5 ml of phosphate buffer were added to this solution and mixed well. The complex thus formed was then extracted with chloroform (1, 2, 3 and 4 ml) by continuous and vigorous shaking. The absorbance of the complex in the chloroform was measured at 470 nm. Vinblastin sulphate obtained from Sigma chemicals was used as the standard [52].

I. Synthesis of Tylophora indicaalkaloids niosomal formulation

Tylophora indica alkaloids niosomal formulation was prepared by thin-film hydration technique. Niosomes were prepared using Span-60 and cholesterol in the ratio 1:1 and 1:2 respectively. For each ratio, non- ionic surfactant (Span-60) and cholesterol was weighed accurately anddissolved in 15 ml of diethyl ether. The contents of the above ratio weretaken in a round-bottomed flask and left in a rotary shaker evaporator for 24 h. The surfactant/lipid thin film was formed by the evaporation of chloroform. Purified *Tylophora indica* alkaloids dissolved in the methanolic solution was then taken in a syringe and injected slowly through a 16 gauge needle into the beaker containing vesicles maintained at 60- 65^{O}C and agitated slowly. The solution thus obtained was transferred to the centrifuge tube and centrifuged at 4500 rpm for 30 min. The supernatant which consists of extra unentrapped *Tylophora indica* alkaloids was carefully removed [53].

J. Entrapment efficiency of Tylophora indicaalkaloids niosomal formulation

Tylophora indica alkaloids niosomal formulation was centrifuged at 4500 rpm for 30 min and the supernatant obtained was separated from the sediment which forms the nanoformulations. The separated niosomal suspension (1 ml) was disrupted using 3 ml of 50% propanol for 5 min, which was then analyzed spectrophotometrically for alkaloid concentration at λ_{max} 210 nm to calculate the amount of entrapped *Tylophora indica* alkaloids against 50% propanol as blank.

The percentage of entrapped *Tylophora indica* alkaloids was calculated by the following equation:

$$\% \text{ Entrapment} = A_e x 100 / A_i$$

Where, A_e is the amount of entrapped *Tylophora indica* alkaloids and A_i is the initial amount of *Tylophora indica* alkaloids in the lipid phase [39].

K. SEM analysis of Tylophora indicaalkaloids niosomes

Samples of *Tylophora indica* alkaloids niosomal formulations were mounted on the cover glass fixed on the specimen stub using adhesive and coated with gold to a thickness of about 100Å. Coated samples were viewed in SEM operated at 15 kV with different magnifications and photographed [54].

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L. Ex vivo drug release analysis of Tylophora indicaalkaloids niosomal formulation

Phosphate-buffered saline was prepared and its pH was adjusted to 7.4. The buffer has been used as a donor solution to dissolve *Tylophora indica* alkaloids niosomal formulation. The surface area of the receiver cell opening was 2 sq. cm and the cell volume was 50 ml. Isotonic phosphate buffer with a pH of 7.4 was prepared and used as the receptor solution. Domestic goat intestine was obtained from the local market, cleaned well using deionized water and with phosphate-buffered saline. The intestine was then dissected longitudinally and rimmed (2.3 x 2.3 cm^2) and used in the drug release study. 5 mg of *Tylophora indica* alkaloids niosomal formulation was weighed and dissolved in 10 ml of fresh phosphate-buffered saline (pH 7.4) and vortex mixed. The receptor cell was filled with a receptor solution (fresh phosphate-buffered saline - pH 7.4). The intestine (2.3 x 2.

cm^2) was mounted on the receptor opening and the donor cell was placed above and clamped carefully. The donor cell was filled with 5 ml of *Tylophora indica* alkaloids niosomal formulation. The receptor compartment was agitated uniformly using a teflon-coated magnetic stir bar. 3 ml of samples from the reservoir compartment were collected through thesample collection port at every 60 min for a period of 12 h. All the samples were subjected to UV-spectrophotometrical absorbance analysis at the wavelength of 210 nm for quantification of released *Tylophora indica* alkaloids. Purified total *Tylophora indica* alkaloids were taken as control, and a release study was performed. Readings were tabulated and compared [55].

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CHAPTER 4

RESULTS AND DISCUSSION

The alkaloids of *Tylophora indica*are one of the most important and widely used antineoplastic agents. The *Tylophora indica* alkaloids arrest cell growth during metaphase and exhibit strong cytotoxic activity [56–58]. These *Tylophora indica*alkaloids are more specific to the stages of the cell cycle in order to exhibit their potential therapeutical activity. Hence, it is highly required for the compounds to get exposed more to the site of tumor cells in orderto maximize their efficacy. Various delivery systems have been under research to increase the bioavailability of these *Tylophora indica* alkaloids. This current research is also focused on developing such a novel drug delivery system to increase the bioavailability of *Tylophora indica* alkaloids using nanosized particulate bodies called niosomes [59–61].

A. Extraction of Tylophora indicaalkaloids by acid-base extraction methodology

*Tylophora indica*contains different compounds, namely vindoline, catharanthine, vinblastine, vincristine, etc. under the class of alkaloids and hence the study is focused on extracting these alkaloids in total from the fresh leaves of *Tylophora indica*and furthering nano research with them. Fresh leaves of the herb *Tylophora indica*were collected (Fig. 1.1.) and washed with distilled water, and further subjected to alkaloid extraction methodology (Fig. 1.2.). The hydrochloric acid reduces the pH of the extraction solution and alkaloids which are basically amines are converted into salts at this pH. The salt remains soluble in the acidic solution. The solution is then treated with the organic solvent (petroleum ether) in order to remove the non-polar constituents present in the solution. The acidic portion was again treated with alkaline solution which helped in increasing ed in the diethyl ether layer and applied in further experiments [48,62].

Fig. 1.1. *Tylophora indica*leaves

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Fig. 1.2. Acid base extraction

B. Purification of Tylophora indicaalkaloids using column chromatography

The total alkaloid extract obtained by the end of extraction methodology was further continued with the purification procedure which involved the alumina-based column chromatography. The chromatographic procedure was preceded following the Jóźwiak and Hajnos work [49]. Since alumina (aluminium oxide) is a better versatile sorbent to produce the best results in wide varying pH ranges in practice, it was used here for purification of *Tylophora indica* alkaloids which was obtained by acid-base extraction procedure. Alumina possesses both Lewis acid and basic sites and is found to be more excellent at adsorbing plant alkaloids, possibly through strained Al-O bonds. The sample was eluted with methanol, and 5 different fractions were collected. All the fractions were qualitatively tested for the presence of alkaloids using Dragendorff's reagent test method. Of the five fractions collected, fraction five has shown maximum turbidity which helped us to sense the presence of alkaloids in the fraction. To strengthen the screening, further the fraction sample was subjected to TLC and HPTLC [63,64].

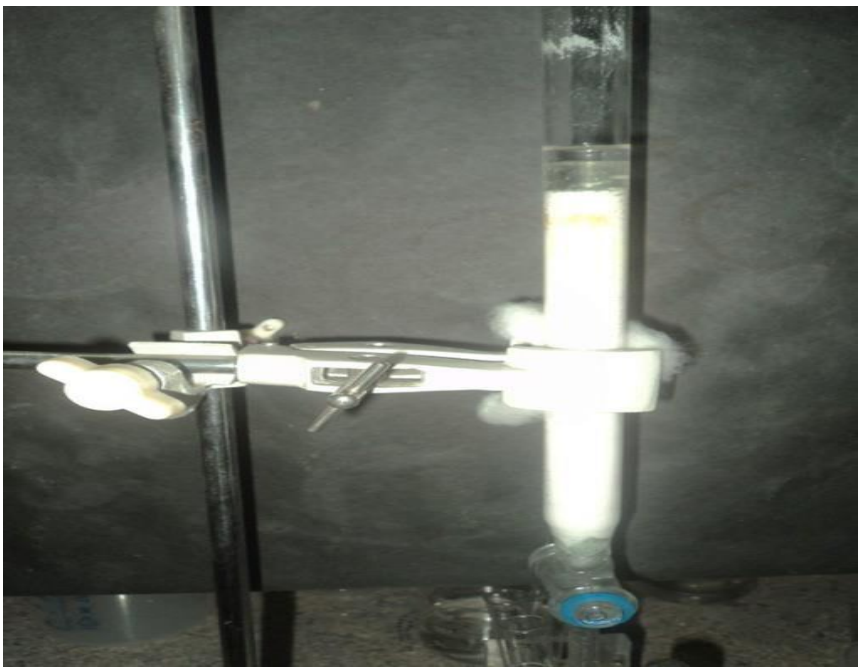

Fig. 2: Purification of *Tylophora indica* alkaloids using Column chromatography.

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C. Screening of Tylophora indicaalkaloids by TLC

TLC was performed following Wu and Sharp procedure**.** The silica plate was then developed with Dragendorff's reagent which formed yellow spots (Fig. 2) and confirmed the presence of the alkaloids [50,65].

Fig. 3.1. Thin layer chromatographic screening for *Tylophora indica* alkaloids.

D. Screening of Tylophora indicaalkaloids by HPTLC

TLC was followed with HPTLC, which produced a more standard screening result that confirms the presence of alkaloids in the fifth fraction of alumina column chromatography. The HPTLC procedure was performed following Hamrapurkar et al [65]. The results are shown in Fig. 3. HPTLC chromatogram of both the vinblastine sulfate (standard) and all the *Tylophora indica* alkaloids samples was compared, and closely similar peaks were found on the following Rf values, such as 0.78, 0.91, 0.98, and 1.04. This confirms the presence of alkaloid-like compounds in the chromatographic fraction of the extract [66].

Peak No	Start Position	Start Height	Max position	Max height	Max %	End Position	End Height	Area	Area %	Assigned substances
1	0.08 Rf	0.1 AU	0.11 Rf	15.4 AU	2.79%	0.11 Rf	12.5 AU	202.5 AU	1.34%	unknown
2	0.13 Rf	9.5 AU	0.14 Rf	16.5 AU	2.99%	0.16 Rf	4.6 AU	233.8 AU	1.55%	unknown
3	0.27 Rf	2.2 AU	0.29 Rf	15.5 AU	2.81%	0.32 Rf	1.0 AU	349.1 AU	2.32%	unknown
4	0.59 Rf	4.1 AU	0.63 Rf	12.7 AU	2.31%	0.64 Rf	11.0 AU	349.8 AU	2.32%	unknown
5	0.71 Rf	6.8 AU	0.75 Rf	46.9 AU	8.53%	0.78 Rf	19.0 AU	1545.7 AU	10.26%	unknown
6	0.78 Rf	20.2 AU	0.85 Rf	104.7 AU	19.04%	0.86 Rf	103.2 AU	4224.7 AU	28.03%	unknown
7	0.88 Rf	103.3 AU	0.89 Rf	106.0 AU	19.27%	0.92 Rf	88.9 AU	2862.8 AU	19.00%	unknown
8	0.96 Rf	100.1 AU	0.96 Rf	100.6 AU	18.30%	1.02 Rf	49.7 AU	4044.4 AU	26.84%	unknown
9	1.03 Rf	25.1 AU	1.04 Rf	131.7 AU	23.95%	1.06 Rf	2.9 AU	1257.9 AU	8.35%	unknown

Table 2: Standard (Vinblastin sulfate)

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Peak No	Start Position	Start Height	Max position	Max height	Max %	End Position	End Height	Area	Area %	Assigned substances
1	0.78 Rf	23.2 AU	0.83 Rf	109.0 AU	27.52%	0.84 Rf	108.6 AU	3048.7 AU	29.60 %	unknown
2	0.91 Rf	100.9 AU	0.92 Rf	104.1 AU	26.30%	0.96 Rf	86.8 AU	3192.4 AU	30.99 %	unknown
3	0.98 Rf	89.4 AU	0.99 Rf	92.5 AU	23.36%	1.03 Rf	4.5 AU	3189.7 AU	30.97 %	unknown
4	1.04 Rf	5.1 AU	1.05 Rf	90.4 AU	22.83%	1.06 Rf	1.1 AU	869.1 AU	8.44%	unknown

Table 3: Test sample

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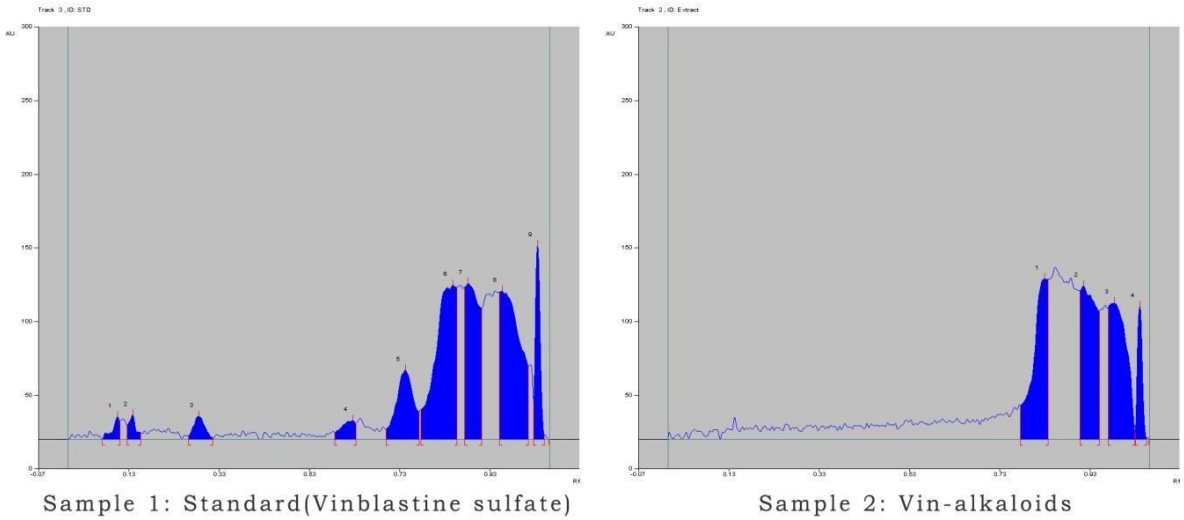
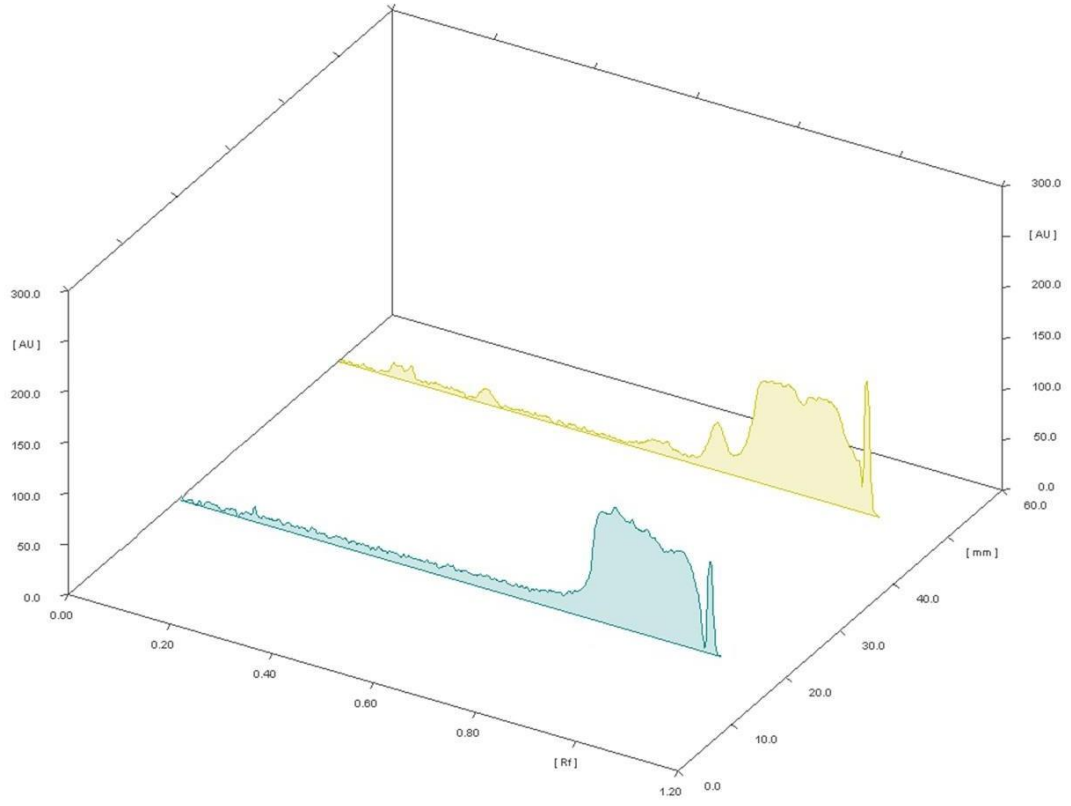


Sample 1: Standard(Vinblastine sulfate) Sample 2: Vin-alkaloids



Comparative Spectral analysis

Fig. 3.2 HPTLC – Densitometric scanning analysis of samples vinblastinesulphate (standard) and *Tylophora indica* alkaloids

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E. Estimation of Tylophora indicaalkaloids using BCG method

Usually a variety of methods including high-performance liquid chromatography (HPLC), fluorimetry, ion chromatography, colorimeter, gas chromatography, and electrochromatography were involved for the determination of alkaloids along with the simple spectrophotometrical methods. Here, the spectroscopical method which is simple, sensitive, and rapid was employed for the determination of total alkaloids in the extract. The estimation procedure was performed based on the principle of BCG reaction with alkaloids, which produced a yellow colored complex. The total alkaloids present in 100 g of *Tylophora indica* leaves material were foundto be 58 mg [52].

F. Synthesis of Tylophora indicaalkaloids niosomal formulation

Niosomes are nano-shaped vesicular bodies formed by the process of self-assembly of non-ionic amphiphilic molecules in aqueous media, which results in closed bilayer structures that entrap both hydrophilic and lipophilic therapeutical agents irrespective of their chemical origin, namely synthetic or natural [67]. Niosomes show more chemical stability, lower toxicity, less requirement of handling care, biodegradability and biocompatibility. Niosomes possess more ability to improve the performance of therapeutical agents by increasing their bioavailability and controlled delivery [31,68]. Hence, niosomes were chosen to enhance the retention properties of these *Tylophora indica* alkaloids. Niosomal formulation of extracted *Tylophora indica* alkaloids was prepared using thin-film hydration technique [69]. The technique was found to be more simple and effectivein terms of synthesizing niosomes for therapeutical research purposes.

G. Entrapment efficiency of Tylophora indica rosea alkaloids niosomal formulation

Various factors are involved in determining the entrapment efficiency of the niosomal formulations, which include the nature of the surfactant used in synthesizing niosomal formulation, cholesterol ratio, etc [34]**.** Thetotal amount of *Tylophora indica* alkaloids entrapped in the niosomal vesicles are determined by the propanolysis method [70] and was found to be 74.02%.

H. SEM analysis of Tylophora indicaalkaloids niosomal formulation

SEM is an important tool, capable of producing high-resolution images of the sample surface. The scanning electron microscopical photograph of the *Tylophora indica* alkaloids niosomal formulation was observed to have spherical and uniform morphology. The size of the niosomes was found to be in the range of 400 to 800 nm (Fig. 4).

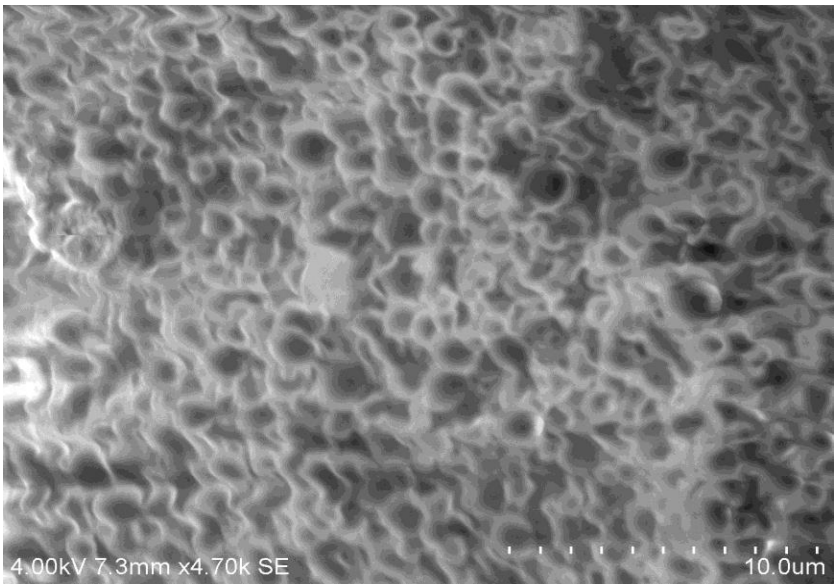

Fig. 4. SEM image of *Tylophora indica* alkaloids niosomal formulation.

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I. Ex vivo drug release analysis of Tylophora indicaalkaloids niosomalformulation

Niosomal formulation of *Tylophora indica* alkaloids was synthesized and tested for its improving efficacy in terms of bioavailability. Broadly, bioavailability refers to the absorption of the administered dosage of a therapeutical compound that reaches the systemic circulation. Usually, bioavailability decreases due to incomplete absorption and first-pass metabolism. This current study has focused to improve the bioavailability of *Tylophora indica* alkaloids using niosomal suspensions. Intestinal skin of domestic goat was used for the ex vivo bioavailability study and the bioavailability experiment was performed in the modified Franz diffusioncell chamber.

The purified total *Tylophora indica* alkaloid extract and the niosomal formulations of *Tylophora indica* alkaloids were tested through ex vivo release studies and the results have shown that the bioavailability has been increased to two folds in terms of niosomal formulations than the total extract (Fig. 5). Apart from increasing the bioavailability, the niosomal formulations aidin the consistent release of the *Tylophora indica* alkaloids over the tested period (Fig.6). The niosomal formulations and their oral delivery could be a wise route for the steady release of the active alkaloid molecules of the wonder plant *Tylophora indica rosea*.

Fig. 5.1. Domestic goat intestinal skin

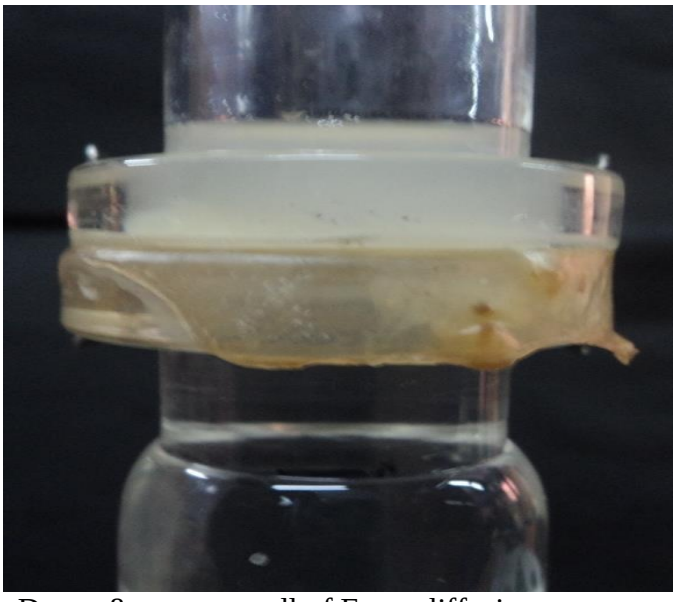

Fig. 5.2. Donor & receptor cell of Franz diffusion apparatus

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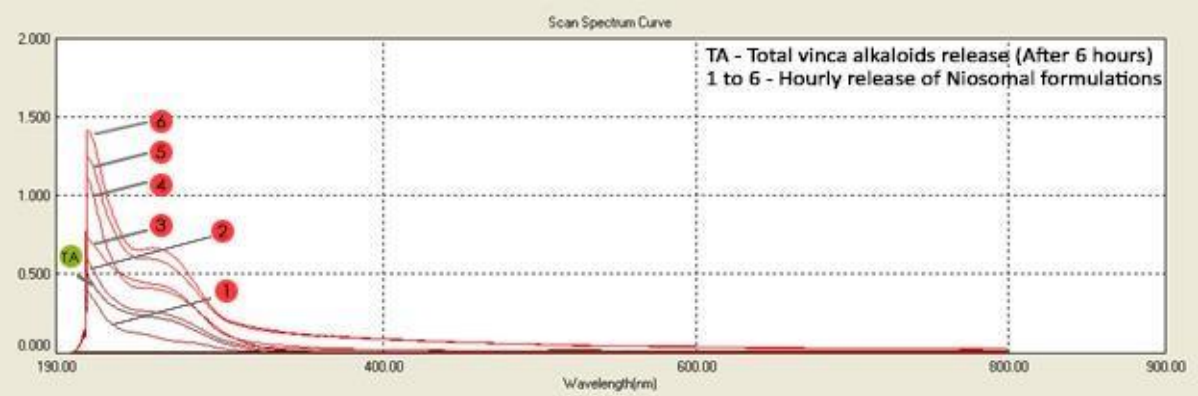

Fig. 5.3. UV spectrum of *ex vivo* release of total alkaloids and niosomalformulations.

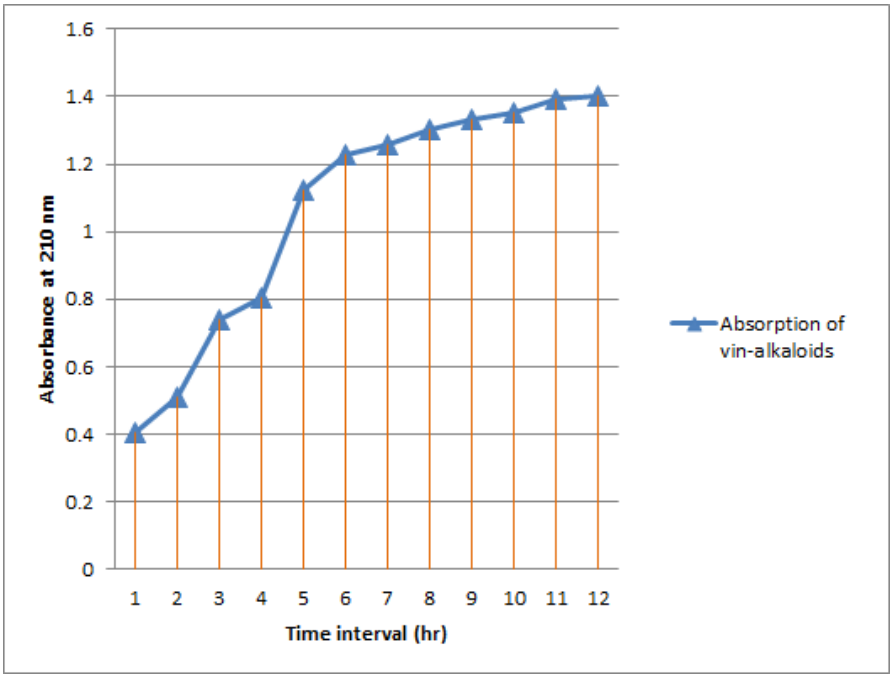

Fig. 5.4. Steady release of *Tylophora indica* alkaloids through niosomes in *ex vivo* experiments.

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CHAPTER 5

SUMMARY & CONCLUSION

Plants are elixir of our life through their consistent support since fromthe development of human race. Plants are the fundamental components of the food chain. Apart from the food supply plants also helps mankind by supplying essential compounds that can cure a wide variety of diseases. There are variety of such essential compounds present in plants and are collectively called as phyto chemicals. These phytochemicals are produced as a part of the protective mechanism of plants against microbial, animal and environmental stress attack.

Our traditional medicine explores this greatly and uses the phytochemicals in treatment across almost all countries in the world. Even though the phytochemicals can treat wide range of diseases including cancer, the efficacy of these compounds greatly and they are less competitive when compared to the synthetic drug molecules. Many of the phytocompounds produce extra ordinary results in the lab conditions however there performance in-vivo is less. There are varieties of factors involved in the same. One important parameter is the bioavailability. Most of the phytochemicals possess less bioavailability, which makes the practioners to increase the dosage which in turn develop toxicity and other side effects. This makes limitation to their wide usage.

Tylophora indicais one such wonderful plant which acts as reservoir of some of the excellent alkaloids known in the phyto-medical history. However, the bioavailabilities of these Tylophora indica alkaloids are less. The present research was carried out to develop a novel drug delivery system to increase the bioavailability of the active compound (Tylophora indica alkaloids) present in the plant *Tylophora indica*using the nano-sized vesicles called niosomes.

Tylophora indica alkaloids were extracted, purified, screened and quantified. Niosomal constructs of the alkaloids were made, characterized through scanning electron microscope and ex-vivo studies were carried out using goat intestine.

From the SEM analysis, it was observed that it has spherical and uniform morphology. The size of the niosomes was found to be in the range of 400 to 800 nm. Through ex vivo release studies the results shows that the bioavailability has been increased to two folds in terms of niosomal formulations than the total extract. The current study with its positive results, demonstrated the possibility to improve the bioavailability of the compound using a nanotechnological approach.

Further optimization of the niosomes should be carried out in order to bring the formulation to the market. Like niosomes, nanotechnology is providing varied tools that can be used to improve the drug delivering efficiency of different phyto chemicals and can able to bring effective treatment results at low dosages.

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