DEVELOPMENT OF FORMULATION AND EVALUATION OF NIOSOMAL DRUG DELIVERY SYSTEM FOR IMPROVED ANTI-CANCER ACTIVITY

THESIS

Submitted to

The Tamilnadu Dr. M. G. R. Medical University, Chennai

in partial fulfillment for the award of degree of

DOCTOR OF PHILOSOPHY

in

FACULTY OF PHARMACY

Submitted by

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January 2017

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my guide **Prof. Dr. K. S. G. Arulkumaran, M.Pharm., Ph.D.,** Vice –Principal & Head, Dept. of Pharmaceutics, KMCH College of Pharmacy, Coimbatore for his continuous support of my Ph.D., study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better guide and mentor for my Ph.D., study.

I extend my heartful thanks and respectful regards to our beloved Chairman **Dr. Nalla G. Palaniswamy MD,** and Madam Trustee **Dr. Thavamani D Palanisami, MD**., Kovai Medical Centre Research and Educational Trust, Coimbatore, for all the facilities which have been provided to me at the institution, enabling me to do a work of this magnitude.

I would like to extend my gratitude to **Prof. Dr. A. Rajasekaran, M.Pharm., Ph.D.,** Principal, KMCH College of Pharmacy, Coimbatore, providing me with necessary facilities to work successfully.

I express my sincere respect and gratitude **to Dr. C. Sankar, M. Pharm, Ph.D.,** Head, Department of Pharmacy practice for his insightful comments and encouragement in the completion of my thesis.

My sincere thanks also goes to **Dr.A.Meena, M.Pharm., Ph.D.,** Principal, K.K. College of Pharmacy for her encouragement and support towards my research work.

I am grateful to my friends **Mr. E.Krishna Kumar, M.Pharm., B.Anbarasan, M.Pharm., and Mr. C.Dhandapani, M.Pharm.,** for their stimulative discussions, valuable advices, throughout my research. Without their precious support, it would not be possible to complete this research.

My heartfelt thanks also goes to my **friend Dr. R. Prakash, M. Pharm., Ph.D., Head, Dept. of Pharmacology,** K.K. College of Pharmacy, whose positives, informed and encouraging nature was an inspiration throughout.

I submit my sincere thanks to **O. T. Buvaneswaran, Ph.D., CEO, Dr. Natesan. Ph.D., KMCRET,** Coimbatore, for their kind support.

I extend my heartfelt thanks to all my colleagues for their encouragement and support. I take this occasion to owe my indebted gratitude towards all those who have continued to support me accomplish this work. This thesis is the result of unbounded and immense contribution and support by many people.

Last but not the least, I would like to thank my family: my parents and to my wife for supporting me spiritually throughout writing this thesis.

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CERTIFICATE

This is to certify that the Ph.D., dissertation entitled **"DEVELOPMENT OF FORMULATION AND EVALUATION OF NIOSOMAL DRUG DELIVERY SYSTEM FOR IMPROVED ANTI-CANCER ACTIVITY"** being submitted to The TN Dr.M.G.R. Medical University, Chennai for the award of **Doctor of Philosophy** in **Faculty of Pharmacy** was carried out by Mr**. K. KARTHICK** at KMCH College of Pharmacy, Coimbatore under my direct supervision and guidance to my fullest satisfaction. The content of this thesis in full or in parts have not been submitted to any other institute or university for the award of any degree or diploma.

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DECLARATION

I hereby declare that the Ph.D., dissertation entitled **"DEVELOPMENT OF FORMULATION AND EVALUATION OF NIOSOMAL DRUG DELIVERY SYSTEM FOR IMPROVED ANTI-CANCER ACTIVITY"** being submitted to The TN Dr. M.G. R. Medical University, Chennai for the award of **Doctor of Philosophy** in **Faculty of Pharmacy** was carried out under the direct supervision and guidance of **Dr. K. S. G. Arulkumaran,** Vice-Principal**,** KMCH College of Pharmacy, Coimbatore during the academic year **2012 –2016.** The content of this thesis in full or in parts have not been submitted to any other institute or university for the award of any degree or diploma.

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LIST OF ABBREVIATIONS

5-FU	-	5-Fluorouracil
ADP	-	Adenosine diphosphate
AFP	-	Alfa- Fetoprotein
ALP	-	Alkaline phosphatase
ATP	-	Adenosine triphosphate
CAT	-	Catalase
CDNB	-	1-chloro-2, 4-dinitrobenzene
CEA	-	Carcinoembryonic antigen
COPD	-	Chronic obstructive pulmonary disease.
COX	-	Cyclooxygenase
CPP	-	Critical Packing Parameters
DMH	-	1,2-dimethyl hydrazine
DNPH	-	2,4-dinitro phenyl hydrazine
DSC	-	Differential scanning Colorimetry
DTNB	-	5,5'-dithiobis (2-nitro benzoic acid)
EDTA	-	Ethylene diamine tetra acetic acid
GPx	-	Glutathione peroxidase
GSH	-	Reduced glutathione
GST	-	Glutathione-S-transferase
HLB	-	Hydrophilic Lipophilic Balance
HPLC	-	High-performance liquid chromatography.
HPMA	-	Hydroxypropyl methacrylamide
LPO	-	Lipid Peroxidation

LUV	-	Large unilamellar vesicles
LV	-	Leucovorin
MDA	-	Malondialdehyde
MUV	-	Multi-lamellar vesicles
NLC	-	Nanostructured lipid carriers
NMR	-	Nuclear magnetic resonance
PEG	-	Polyethylene glycols
PL	-	Phospholipids
PSA	-	Particle Size Analysis
RBC	-	Red blood corpuscles
RES	-	Reticulo endothelial system
SCID	-	Severe combined Immuno deficiency
SDS	-	Sodium dodecyl sulphate
SEM	-	Scanning electron microscopy
SGOT	-	Serum glutamate oxaloacetate transaminases
SGPT	-	Serum glutamate pyruvate transaminases
SLN	-	Solid lipid nanoparticles
SOD	-	Superoxide dismutase
SUV	-	Small Unilamellar Vesicles
TBA	-	Thiobarbituric acid
TCA	-	Trichloro acetic acid
TEM	-	Transmission electron microscopy
TEP	-	1, 1, 3, 3-tetra ethoxypropane
TG	-	Triglycerides
WBC	-	White blood corpuscles

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1. INTRODUCTION

1.1. Background

The delivery of drug molecules to the human body for treatment purpose is an important fact in the medical sciences[1].Since the ancient era, progresses in the pharmaceutical field have also been made in the research and development of diverse types of effective drug delivery dosage forms. During past few decades, there has been an enhanced demand in the pharmaceutical field still for more patient-friendly and compliant effective drug delivery dosage forms[2]. The drug properties such as drug solubility, drug absorption, bioavailability and carrier properties (*e.g*., residence time at the particular target-site) are the chief constraints for effective drug delivery by any dosage forms. The novel drug delivery dosage forms able to manage the drawbacks of the conventional dosage forms through delivering the drug molecules over longer period, enhancing drug solubility, maintaining drug activity, availing target-specificity, reducing chances of side-effects,*etc*[3]. The goals of any ideal novel drug delivery systems include delivering of the drug molecules to desired target sites to limit side-effects, supplying drug molecules through the specific areas of the body without any chances of drug degradations, maintaining therapeutic plasma-drug level for prolonged time periods, predicting controlled drug releasing profiles, reducing dosing intervals and thereby, increasing patient compliances[4]. Globally, several researches are performing on for the development of effective targeted drug delivery dosage forms.

1.2. Targeted drug delivery

In 1909, Paul Ehrlich first initiated the era of the development for targeted delivery of molecules, when he predicted the drug delivery mechanism that would target to diseased cells, directly [3, 5, 6]. Paul Ehrlich proposed drug delivery to be a 'magic bullet'. According to him, targeted drug delivery as an event, where a drug releasing carrier conjugate/complex delivers drug molecules exclusively to the preidentified target cells in a specific manner. Gregoriadis described drug targeting using novel drug delivery systems as 'old design in new cloths' [6].

The 'drug targeting' can be defined as the capability to direct the drugmolecules, specifically to the desired site of action with little or absence ofany interaction with the non-target tissues[6, 7].The perception of the 'targeted drug delivery' is intendedfor endeavoring to concentrate the desired drug molecules in the tissues ofinterest at the desired site,while dropping the relative drug concentrations in the other tissues of the body, and thus, the targeted drug molecules are localizedatthe desired targeted site[8]. Therefore, the remaining tissues are notaffected by the targeted drug molecules due to the maintenance lower concentrations of the free drug molecules in the blood stream[9]. Additionally, the loss of drugs doesnothappen due to localization of drug molecules, leading to achieve utmost effectiveness of the medication[5, 6].Unwanted side-effects can be diminished by delivering the maximal portion of the applied dose accomplishment of the desired site through the targeted drug delivery systems, which can be partially attempted by the use of novel carriers[10, 11].Various important pharmacokinetic issues like tissue distributions, plasma clearance kinetics, drug metabolisms as well as cellular interactions of drugs can be controlled through the site-specific delivery systems[5-7]. The drug targeting to the

diseased organs or sites in the body can be attained by linking particulate systems or to cell specific ligands (*e.g.,*glycoproteins, or immunoglobulins), or macromolecular carriers to monoclonal antibodies, or by modifications in the surface characteristics, so that these are not identified by the reticulo endothelial systems (RES) [7].The targeted drug deliveries to the desired site of the diseased organs are the most challenging research fields in the drug delivery and medical sciences[12].The target specific drug delivery may be occurred at cellular, molecular or sub-molecular levels[2]. The high concentration at the target site is a result of relative cellular uptake of the drug carrier, release of drug molecules at the target site of desired area of the body[13]. Drug targeting is signified if the target part is distinguished from other parts, where toxicity may arise, and also if the active drug molecules could be placed predominantly in the proximity of the target site. The restricted distribution of the parent drug molecules to the non-targeting sites with effective accessibility to the target sites could maximize the advantages of the targeted drug therapy[3]. It isvery difficult and multifaceted for any drug molecule to reach at the target site in the complex cellular network of the diseased area[2]. Researches and investigations associated to the development of targeted drug delivery system is currently aday is highly preferred and facilitating the field of drug delivery and medicine world[3]. The current research on the targeted drug delivery systems is burgeoning with the newer ways to expand the effective drug delivery technologies and methodologies. Currently, a numbers of drug delivery carriers were investigated to deliver drug molecules at the target organs/tissues of the diseased body, which include synthetic polymers, proteins, serum, immunoglobulins, microspheres, erythrocytes, liposomes, niosomes, *etc*[3, 7]. The targeted drug deliveryis especially important in cancer chemotherapy and enzyme replacement treatment[13, 14].

1.3. Targeted drug delivery carriers

Carriers are the important entities, which is required for the successful deliveries of the loaded drugs to the target organs or sites. These elute or deliver the drugs within or in vicinity of the target. Drug loaded carriers can perform these either through an inherent properties or acquired (by structural modifications), to interact specifically with the biological targets, or otherwise, these are engineered to deliver the loaded drug molecules in the proximity of preidentified target cell lines demanding the optimal therapeutics. An ideal drug delivery carrier as a selective targetable device should have the following characteristics[3]:

1. The targeted drug deliverycarrier should be biodegradable, non-toxic and non-immunogenic.
2. It should be capable of optimal drug loading and drug releasing properties, according to the therapeutic need.
3. After recognition and internalization, the targeted drug deliverycarrier system should release the drug molecules at proximity of preidentified target organs, tissues or cells, demanding the optimal therapeutics.
4. The targeted drug delivery carrier system should be recognized specifically by the target organs, tissues or cells and must maintain the activity.
5. The carrier system should be able to cross the anatomical barriers and in case of tumor chemotherapy tumor vasculature.
6. The biomodules utilized for the carrier investigation and site recognition must not be ubiquitous, otherwise it can cross over the sites, defeating the targeting concept.

Some examples of targeted drug delivery carrier systems appended as pilot molecules to deliver the drug molecules selectively to the intended organs, tissues or cells have been presented below [32]

1. Colloidal carrier systems
    a) Vesicular systems: Liposomes, Immunoliposomes, Niosomes, Virosomes, Pharmacosomes, *etc*.
    b) Particulate systems: Microparticles, Nanoparticles, Nanocapsules, Solid lipid nanoparticles, Magnetic microparticles, *etc*.

2. Cellular carrier systems

    Resealed erythrocytes, Antibodies, Leukocytes, Platelets, Serum albumin, *etc*.

3. Polymer-based carrier systems

    Mucoadhesive, Signal sensitive, *etc*.

4. Supramolecular carrier systems

    Micelles, Mixed micelles, Reverse micelles, Polymeric micelles, Lipoproteins (Low density lipoproteins, High density lipoproteins, Chylomicron), Synthetic LDL mimicking particles (supramolecule biovector system)

5. Macromolecular carrier systems
    a) Proteins, Glycoproteins, Neoglycoproteins, Artificial viral envelops (AVE), *etc*.
    b) Lectins and Polysaccharides
    c) Glycosylated water soluble polymers
    d) Monoclonal antibodies (Mabs), Immunological Fab fragments, Bispecific Abs, Antibody enzyme complex
    e) Toxins, Immunotoxins, rCD4 toxin conjugates

Based on the nature of the origin of these above mentioned targeted drug delivery carrier systems, these are also classified as[3]: (a) Endogenous: *e.g*., Resealed erythrocytes, Serum albumin, Low density lipoproteins, High density lipoproteins, Chylomicron, *etc*., and (b) Exogenous: *e.g*., Microparticles, Nanocapsules, *etc*.Recent years, a wide variety of effective site-specific as well as targeted drug delivery carrier systems have been investigated and developed by the drug delivery and medical researchers. Amongst the various site-specific and targeted drug delivery carriers, fewer are at the stage of clinical trials, where colloidal vesicular carrier systems prove their strong potential for the effective drug delivery to the target-site of the diseased organs, tissues or cells of the body.

Recent research directed that the colloidal vesicular carrier systems appear to resolve the troubles of oral bioavailability of drugs[31]. Among various colloidal vesicular drug carriers, lipid-based vesicular systems like liposomes and niosomes are gaining remarkable status due to their biocompatibility as these aredevoid of any antigenic, allergic or pyrogenic reactions[33]. These carrier systems can perform as drug reservoirs as well as provide controlled release of the loaded drug molecules. Additionally, modifications of their compositions or surfaces can allow the targeting of the diseased organs, tissues or cells of the body. Even, these are widely investigated for the effective use in cancer targeting therapeutics, recently[14].

1.4.Cancer and cancer targeting

Currently, cancer is a most leading reason of death worldwide. In 2012, about 55 million people died worldwide, out of which more than 8 million people died from various kinds of cancers[15]. Cancer is a complex fatal disease, which is associated to genetics, age, gender, life-style, geographical region, *etc*[16]. Cancer is occurring as a

result of progressive accumulation of genetic or epigenetic alterations [17]. The important attribute of cancer is the rapid progressive uncontrolledgrowth of abnormal cells and the abnormal growth can invade the adjacent parts of the body (invasion) and spreads to the other organsin the body via lymph or blood (metastasis)[33]. During the development of cancers, the transformation of normal somatic cells into malignant tumor cellshappens via a complex multi-stage course of actions involving various biochemical changes as well as genetic changes[16].The entire process of cancerous development comprises 3 distinct steps.In the first step, the normal cells get converted into neoplastic cells (*i.e*., initiation)followed by transformation into overt neoplasm (*i.e*., promotion) thatfinally lead to the final progression stage[17].

Currently, the major challenges in cancer chemotherapy are associated to toxicity on healthy proliferating cells and multi-drug resistance against anticancer drugs[18]. The life-threatening side-effects caused by non-specific tissue distribution of anticancer drugs have constrained the systemic high dose approach [19]. The resistance instigated by anticancer agents extends the cross-resistance to variety of anticancer drugs having diverse chemical structures and cellular targets [20,21]. Once the drug resistance develops, the systemic administration of anticancer drugs becomes ineffective and the drug resistance is further stimulated. Accordingly, there is an emergency in cancer therapy. The success of cancer therapy is understood by its ability to reduce and eliminate tumors without damaging healthy tissues in the infected organs. These facts put the accent on the requirement for new generation, more effective and safe therapies for the treatment of cancers[21]. A diverse species of cancerous cells have specific characteristics that are trying to exploit through the applications of nanotechnology to target cancerous cells and treat cancers [22].

Recently, the progress of nanotechnology offers wonderful promise in cancer therapy, prevention and diagnosis [23]. The combination of two disciplines- 'nanotechnology' and 'oncology (cancer science)' has given rise the new field, "nano-oncology" providing the cancer researchers and scientists with various innovative ways to diagnose and treat various types of cancers [18, 24]. The anticancer agents used in the cancer therapy mainly can kill target cells, but also normal cells in the body resulting undesired side-effects[25]. For achieving better therapeutic application, nanocarriers are considered for the target-specific drug delivery to various cancer-infected sites in the body in order to improve the therapeutic efficiency, while minimizing undesirable side-effects[26, 27]. Improvements in target to non-target concentration ratios, increased drug residence at the target site, are some of the major reasons for greater emphasis on the use of nanoparticulate anticancer drug delivery systems[26]. Nanotechnology also presents the capacity diseases in initial stages, like finding hidden colonies often observed in patients diagnosed with lungs, breast, colon, prostate and ovarian cancers [28]. It is apparent that almost all types of cancers, which can be treated by cytotoxic drugs not only demand for controlled drug releasing but also target specific drug releasing patterns at the quantitative levels [14]. The rationale of using nanotechnological applications for cancer targeting is based on these following issues[2, 26]:

[1] For cancer targeting applications, nanoparticles should be able to deliver concentrate dosing of drugs in the surrounding area of thetumor targets via the improved permeability and retention effects or active targeting through theligands on the surface of nanoparticles;

[2] Nanoparticles should decrease the drug exposure ofhealth tissues through limiting the drug distribution to the target organs, tissues or cells.

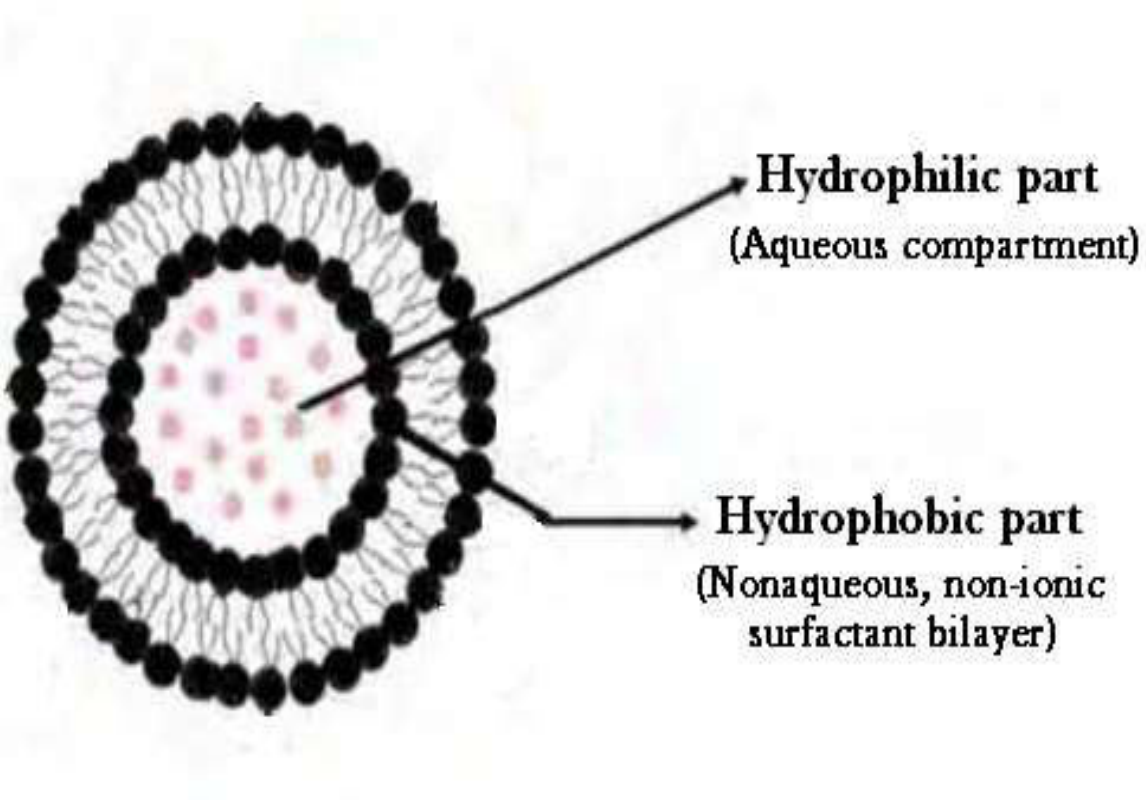
Efforts in the field of medicinal chemistry, combinatorial chemistry, drug delivery and nanotechnology continue to give rise to wider range of discovery and effective delivery of various kinds of anticancer drugs with enormous therapeutic prospective[29]. However, many of the anticancer drugsencompasswith some of the important physicochemical issues such as solubility and stability or biological issues like toxicity,which arise as barrier for their developmentinto viable treatment strategies in the cancer therapy through the incorporation of these within the nanoparticulate devices. In numerous cases, the formulation technologies of nanoparticulate devicesfor the effective delivery of anticancer drugs areinvestigated as a means to take benefits of the therapeutic potential of these anticancer drugs. Since the clinical applications of various nanoparticulate carriers as drug deliverysystems began in cancer therapeutics, a series of chemotherapeutic strategies have been developed as well asmodified to improve the therapeutic efficacy of anticancer drugs incorporated within the nanoparticulate carrier systems[14].The conception of incorporating the anticancer drugs into different types of nanoparticulate carrier systems for abetter targeting of the anticancer drugs at appropriate preidentified cancerous organs, tissues or sellsas destination is extensively acceptedby the medical researchers and academicians[26]. Moreover,nanoparticulate carrier systems represent promising drug delivery modules and hence, these can represent alternative vesicular systems with respectto other delivery systems because of their capability to encapsulate different type of anticancer drugs within their multienvironmental structure[27].A wide variety of nanoparticle-based systems for drug delivery in cancer treatment like biodegradable polymeric nanoparticles, polymeric micelles, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), nanoliposomes, niosomes, inorganic nanoparticles, magnetic nanoparticles,

dendrimers, *etc* are available [26, 27, 29,30]. Among these nanoparticulate carrier systems, colloidal vesicular carrier systems mainly lipid-based systems like liposomes and niosomes are gaining remarkable status for the effective use in cancer targeted drug delivery therapeutics[31].

1.5. Niosomes as nanocarriers

Niosomes are the lipid-based carriers for the use as promising vehicles for drug delivery applications[34]. In 1975, first niosomal formulations were formulated and patented by L'Oreal[35].These are mainly non-ionic surfactant vesicles having microscopic bilayer lamellar structure obtained on the proper mixtures of non-ionic surfactantslike spans or tweens and cholesterol with the subsequent hydration in the aqueous media[36].In the bilayer lamellar structure, the hydrophobic parts are oriented away from the aqueous solvents, whereas the hydrophilic heads stay behind in contact with the aqueous solvents. The common structure of niosome is presented in **Fig. 1.1**. However, these may also be prepared with various ionic amphiphiles like dicetylphosphate, stearylamine, *etc*., in order to attain a stable suspension. Similar to liposomes, niosomes are formed on the hydration on non-ionic surfactant films, which hydrate imbibing or encapsulating the hydrating aqueous solutions [36]. Basically, niosomal vesicles do not form spontaneously[37]. Thermodynamically stable niosomal vesicles form only in the presence of proper mixtures of non-ionic surfactants as well as charge inducing agents[36].The size of the niosomes lies in nanometric scale usually in the range of 100 – 300 nm[37]. The characteristics of the niosomes can be altered through varying the composition, surface charge, lamellarity, tapped volume, *etc*[38]. A variety of forces work inside the vesicular structure of niosomes and are accountable for maintaining the vesicular structure of these, *e.g*., the van der Waals forces amongstthe surfactant molecules, the entropic repulsive forces of head groups of the

surfactant molecules, the repulsive forces rising from electrostatic interactions between charged species of surfactant molecules, the shortacting repulsive forces, *etc*. However, the stability of niosomes are influenced by types of the surfactants, characteristics of the encapsulated drug molecules, storage temperature, the use of membrane-spanning lipids, the inclusion of charged molecules, the interfacial



polymerization of the surfactant monomers *in situ*,*etc*[38].

Fig. 1.1: Structure of niosome

Niosomes present a structural similarity to liposome and hence, these are able to represent as alternative kinds of vesicular systems with respect to liposomes. These are believed to be better carriers for drug delivery as compared to liposomes due to various issues like cost, stability at pH variations, sterilization,*etc*[39]. The drug encapsulation efficiency of these systems increases with increment in the concentration as well as lipophilicity of surfactant[40]. Niosomes canperform as drug

depots and these are able to release the drugs in a controlled manner[3]. These also behave like liposomes*in vivo* and to prolong the circulation of encapsulated drugs,varying the organ distribution of the carrier system as well as the metabolic stability[36]. Niosomes are able to modify the plasma clearance kinetics, tissue distribution, drug metabolism and cellular interaction of the drug molecules[41, 42].These may be employed as vehicles for poorly absorbable drug molecules to develop the novel drug delivery system[4].Delivery of various kinds of drug molecules can be possible using niosomal systems like oral, topical, parentral, ophthalmic, targeting, *etc*[39]. Niosomes are able to improve the bioavailability of the encapsulated drug molecules through crossing the barrier of gastrointestinal tract (GIT) by transcytosis of 'M cells' of Peyer's patches in the intestinal lymphatic tissues[43].Several drugs also encapsulated within the niosomes for the delivery through the transdermal route to enhance the drug permeation and the therapeutic efficacy [44].The niosomes are also taken up by the reticulo endothelial system (RES). Such kind of localized drug accumulation strategy is employed in treatment of various diseases like cancer, leishmania, *etc* [36, 45]. Also, some non-RES like immunoglobulins recognize the lipid surfaces of niosomal delivery systems [38]. The encapsulation of various kinds of anticancer agents with in niosomal carriers has reduced the drug induced toxic adverse effects while improving cancer targeting and the anticancer efficacies [46]. Since niosomes are non–ionic in nature, these are less toxic and increasethe therapeutic index of drug moleculesthrough restricting their action to the target cells[45].The drug targeting through the use of linkages to niosome via antibody is presented in **Fig. 1.2**.

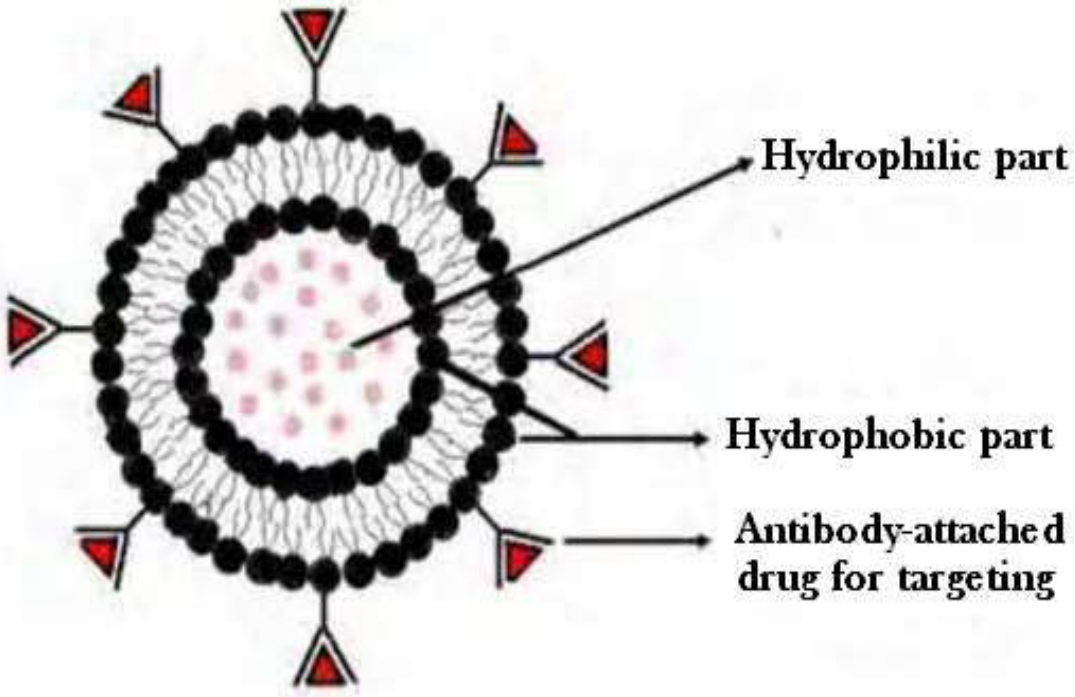


Fig. 1.2: Drug targeting through the use of linkages to niosome via antibody

1.6. Classes of niosomes:

On the basis of vesicle sizes, niosomes can be classified into three categories[3]:

a) Small Unilamellar Vesicles (SUV, vesicle size: 0.025-0.05 μm)

b) Multi-lamellar Vesicles (MLV, vesicle size: > 0.05 μm)

c) Large Unilamellar Vesicles (LUV, vesicle size: >0.10 μm).

1.7. Advantages of niosomes

The potential advantages of niosomes are presented below[37,3,39]:

[1] Simple methods are required for the formulation of niosomes. Even large–scale production can be possible.

[2] The excipients,apparatus and equipments used for the formulation of niosomes are economical.Therefore, niosome formulation process is not expensive.

[3] Since the niosomal structure presents position to accommodate hydrophilic, lipophilic and amphiphilic drug moieties, these can be employed for a variety of drug molecules.

[4] Niosomes displays the flexibility in their structural features (*i.e.*, composition, size and fluidity) and can be intended in relation to the preferred situation.

[5] Niosomesenhance the therapeutic potential of the drug molecules by protecting from the biological environment as well as restricting influences to the target cells, thuslowering the clearance of the drugs.

[6] Niosomes perform as a depot for releasing the drug molecules slowly and present controlled release of drug molecules.

[7] Niosomes enhance the stability of the encapsulated drugs at various pH.

[8] Niosomes enhance the oral bioavailability of various drugs.

[9] Niosomes are osmotically active as well as stable at various pH environments.

[10] Niosomes can improve the skin penetration of drug molecules through topical administration.

[11] Niosomes are able to reach the site of action through oral, parenteral and topical routes.

[12] Niosomal carriers are biocompatible, biodegradable and non-immunogenic in nature.

[13] Niosomal dispersions in an aqueous phase can be emulsified easily in a non-aqueous phase. This is helpful to manage the release rate of the drugs and administer normal vesicles in the external non-aqueous phase.

[14] The niosomal vesicle dispersions offer better patient compliances.

[15] Niosomal formulations possess longer shelf-lives.

[16] Contrasting liposomes, niosomes are stable even at room temperature and less susceptible to light.

1.8. Compositionof Niosomes

The composition of niosomes includes surfactants (mainly non-ionic), cholesterol and charge inducing substances[3, 39,47].

❖ ***Surfactants:***

A wide variety of surfactants and their blends in different molar ratios have been employed to encapsulate many kinds of drugs in the niosomes [48].In the preparation of niosomes, non-ionic surfactants are the commonly employed surfactants. The non-ionic surfactants are employed for this purpose because of some potential advantages associated with the stability and compatibility compared with other types of surfactants [49]. Non-ionic surfactants contains both polar (hydrophilic) and non-polar (hydrophobic) parts and possess high interfacial activity. The non-polar tail contains one or two alkyl or perfluoro alkyl groups or a single steroidal group [50]. The non-ionic surfactants aregenerally non-toxic, less irritating and less haemolytic to the biological cellular surfaces. These are also helpful to maintain their surface active property near the physiological pH in formulations. These also exert several important functions including acting as stabilizer, solubilizer, emulsifier, wetting agents, permeability enhancers, *etc*[49]. The non-ionic surfactant types which are mostly employed in the preparation of niosomes are ether linked surfactants, ester linked surfactants, sorbitan esters, alkyl amides, fatty acids and amino acid compounds[50], [47].

❖ ***Ether-linked surfactants:***

Ether-linked surfactantsare mainly polyoxyethylene alkyl ethers, which encompass hydrophilic as well as hydrophobic moieties, and both are linked with the ether linkage[47]. The common formula of thesetypes of surfactant surfactants is C_nEO_m, where n should be 12-18 and m should be 3-7. Ether-linked surfactants with

polyhydroxyl headand ethylene oxide parts are also reported to be employed in the niosomepreparation[47]. The single alkyl chain surfactants, C-16 monoalkyl glycerol ethers with average of three glycerol parts is one of the examples of theether linked surfactants employed for the niosome preparation. Polyoxyethylene 4-lauryl ether (Brij30) has a'Hydrophilic Lipophilic Balance' (HLB) value of 9.7 and phase-transitiontemperature of more than 10°C[47]. Contrasting with other alkyl ether derivatives, these reduce the vesicle formation in the presence of cholesterol; Brij 30 forms LUV, when combined with 30 mM/Literof cholesterol. It cannot be employed to formulate some drugs, iodides, salicylates, sulfonamides, phenolic substances, mercury salts, tannins, *etc*., as it cause oxidation leading to the discoloration of the product. Polyoxyethylene stearyl ethers (Brij72and76) and Polyoxy ethylene cetyl ethers (Brij 58) are also employed in preparation of niosomes [36, 49].

- **Ester-linked surfactants:**

Ester-linked surfactants have ester linkage between hydrophilic and hydrophobic groups[47]. These surfactants have been investigated for their use inthe preparation and delivery of sodium stibogluconate to experimental marine visceral leishmaniasis[46].

- **Sorbitan esters:**

Sorbitan esters orpolyoxy ethylene esters are the most extensivelyemployed ester-linked surfactants in the food industry in particular [47]. These esters are often used in the cosmetic industry to make soluble the essential oils in water based products [49].Commercially available sorbitan esters are the mixtures of the partial esters of sorbital and its mono as well as di-anhydrides with the oleic acid. Sorbitan esters have been employed to encapsulate a wide range of drugs including anticancer drugs (*e.g.,* doxorubicin)[51].

❖ **Alkyl amides:**

Alkyl amides are the alkyl galactosides and glucosides, which have ability to incorporateamino acid spacers[47]. The alkyl groups are fully and/or partially saturated C-12 to C-22 hydrocarbons as well as some novel amide compounds have fluorocarbon chains.

❖ **Fatty acids and amino acid compounds:**

These are amino acids, which are prepared amphiphilic through the addition of hydrophobic alkyl side-chains and long-chain fattyacids[47].These form "Ufasomes" vesicles.Ufasomes are formed from fatty acid bilayers[47].

❖ ***Cholesterol:***

Cholesterol, a waxy steroid-based metabolite material is an important component to manufacture vesicular systems, which lead tothe changes in the fluidity as well as permeability of the bilayer structures[47]. It is generally added to the non-ionic surfactants to afford thefluidity, rigidity, permeability andorientational order to the bilayer structures of the vesicular systems.Cholesterol does not able form the bilayer itself. It can be incorporated in a larger molar ratio like 1: 1 in most of the vesicular formulations to prevent the vesicle aggregation through the inclusion of molecules, which stabilize the vesicular systems against the formation of aggregates by the repulsive steric/electrostatic influences[52].This directs to the transition from the gel-state toliquid phase in the niosomal systems. As a result, niosomal systems become less leaky in nature.Being anamphiphilic molecule, cholesterol adjusts its hydroxyl group towards the aqueous phase and aliphatic chain, also towards the hydrocarbon chain of the surfactant used in the preparation of vesicular structures including niosomes[47]. The rigidization of the prepared vesicles is offeredthrough the

alternative positioning of rigid steroidal skeleton with the surfactantmolecules in the bilayer structures by restricting the movement of the carbons of hydrocarbon[47]. Cholesterol is also recognizedfor preventing the leakage through abolishing gel to the liquid-phase transition [52]. Cholesterol imparts the rigidity to the niosomal vesicles, which is very vital under in severe stress conditions [53]. The interaction of cholesterol with Span 60 in bilayer of niosomes is because of hydrogen bonding [49] (**Fig. 1.3**).

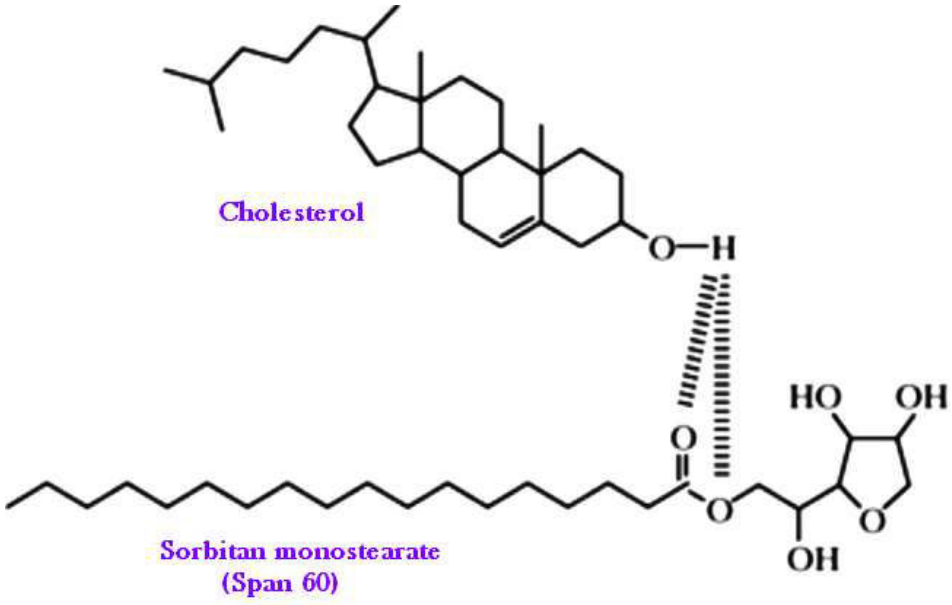


Fig. 1.3: Structural interaction between Span 60 and cholesterol

❖ ***Charge inducers:***

Charge inducers enhance the stability of the vesicular structures including niosomesthrough the induction of charge on the vesicular surface[47]. These performthrough the preventing fusion of vesicles because of the repulsive forces of the same charge, which offers higher values of zetapotential to the vesicular structure. The frequentlyemployed negative charge inducers are dihexadecyl phosphate, dicetyl phosphate, lipoamineacid.The frequently employed positive charge inducers are sterylamine, cetyl pyridinium chloride, *etc*[54,55].

❖ ***Hydration medium:***

Hydration medium is considered as an important excipient issue in the preparation of niosomes. Phosphate buffers of various pHs are among the commonly used hydration medium for preparation of niosomes. The pH of the hydration medium is the important factor in this case. The pH of the hydration medium depends on the solubility of the drugs being encapsulated within the niosomes [50]. Accordingly, pH 5.5 phosphate buffer was employed in the preparation of ketoconazole niosomes[56]; whereas pH 7.4 phosphate buffer was employed in the preparation of meloxicam niosomes[57].

1.9. Preparation methods of niosomes

Niosomes are prepared using non-ionic surfactants through different methodologies.The desired vesicle sizes, number of double layers in their structure, their distributions, drug encapsulation efficiency of the aqueous phase used and permeability of vesicles are potentially influenced the way of niosome preparation [3]. All these constraints should be taken into consideration while making selecting the optimum methodology for the formulation of niosomes of desired property[37]. For the preparation of various types of niosomes, various preparation methodologies are adopted. Most of the niosome preparation methodologies comprise the hydration of a mixture of surfactants or lipids at the elevated temperature subsequently optional size reduction to achievedesired qualities of colloidal dispersions containing niosomes. Afterward, the un-entrapped drugs are separated from the niosomes containing entrapped drug through the centrifugation, dialysis or gel filtration[50]. Preparation methods of various types of niosomes are described below [3, 37, 39].

1.9.1. Preparation of small unilamellar vesicles (SUV)

❖ ***Sonication:***

These SUV type niosomes (diameter: >100 nm) are larger than the SUV type liposomes (diameter: <100 nm)[3]. Basically in the sonication method, the aqueous phase containing drugs to be encapsulated is added to the mixture of surfactants and cholesterol in the scintillation vials [36]. Then, the mixture is homogenized using a sonication probe for 3 minutes at 60°Cto produce uniform sized SUVtypes of niosomes. It is also possible to get SUV types of niosomes through the sonication of MLV types of niosomes. For the preparation of SUV types of niosomes, probe type sonicator is employed [36].

❖ ***Microfluidization:***

Among different preparation techniques of niosomes, microfluidization is a recently adopted technique to prepare the SUV types of niosomes[36].The SUV niosomes prepared from microfluidization technique of defined size distribution[50]. This microfluidization techniquefor the preparation of the SUV types of niosomes is based on submerged jet principle,where two fluidized streams interrelateat ultra-high velocities in precisely defined micro channels within the interaction chambers.The impingement of the thin liquid sheet along a common front is assembled in away that the energy is supplied to the system stays within the niosomal formation area. As a result,SUV types of niosomes are formed with a greater uniformity, smaller vesicularsized and better reproducibility [34].

1.9.2.Preparation of multi-lamellarvesicles (MUV)

- **Hand shaking method:**

The hand shaking method is also called as 'Thin film hydration technique', which is used to prepareMUV types of niosomes [36]. In this methodology, surfactants and cholesterol are dissolved in the suitable volatile organic solvents (such as, chloroform,diethyl ether, or methanol) in round bottom flasks to prepare a mixture of surfactants and cholesterol. At room temperature (24°C), the volatile organic solvent is removed using a rotary evaporator, which leaves a thin layer of solid mixture deposition on the wall of the round bottom flasks used in the preparation. Then, the dried surfactant film is required to be hydrated with the aqueous phase containing drugs with the application of gentle agitation at 50-60°C. This process forms typical MUV niosomes[36].

- **Trans-membrane pH gradient (inside acidic) drug uptake process (remote loading):**

In the method of trans-membrane pH gradient (inside acidic) drug uptake process (remote loading), MUV types of niosomes can be produced [36]. Required surfactants and cholesterol are dissolved in chloroform to prepare surfactants-cholesterol mixture solution and it is then evaporated under the application of reduced pressure to obtain a thin-film on the wall of the round-bottom flasks used in the preparation. The developed thin-film is hydrated using 300 mM of citric acid (pH 4.0) solutions through vortex mixing. The multi-lamellar vesicles are then frozen and thawed for three times.The thawed multi-lamellar vesicles later sonicated using a bath type sonicator to prepare a niosomal suspension.To these niosomal suspensions, aqueous solutions containing drugsare added and then, vortexed. The pH of these niosomal dispersions is then increased up to 7.0-7.2 with 1M disodium phosphate.

These dispersion mixtures arethen heated for 10 minutes at 60°C to manufacture the desired MUV types of niosomes.

1.9.3. Preparation of large unilamellarvesicles (LUV)

❖ **Reverse phase evaporation technique:**

Reverse phase evaporation technique is being employed to formulate different vesicular carrier systems like liposomes, nanoliposomes, niosomes, archaeosomes, *etc*[31]. In the reverse phase evaporation technique, surfactants and cholesterol are dissolved in mixtures of ether and chloroform[36, 58]. An aqueous phase containing drugs is added to these mixtures. The resulting mixtures containing two phases are sonicated at 4-5°C to form a clear gel.Then, clear gel is hydrated by the 0.25 volume of the phosphate buffer saline. Alternatively, hydrogenated or non-hydragenated egg phosphatidyl choline is dissolved in chloroform and phosphate buffer saline. The formed clear gel is further sonicated after the addition of a small amount of phosphate buffersaline, pH. Under low pressure, the organic phase is removed at 40°C. The resulting viscous niosomal suspensionsarethen diluted with phosphate buffer saline, pH and then, heated in a water-bath at 60°C for 10 minutes to manufacturelarge unilamellar niosomes.Free drugsto be encapsulated (unentrapped drugs) areusually removed through the dialysis and/or centrifugation[36]. Protamine is added prior to the centrifugation procedure to attainthe phase separation[50].

❖ ***Ether injection technique:***

The ether injection technique is basically based on the slow injection of the niosomal ingredients in diethyl ether by a 14gauge needle at approximately 0.25 ml/minutes rate into a pre-heated aqueous phase, which should be maintained at 60°C[36, 59].The possiblecause behind the development of LUV is that the slow

vaporization of the solvents results in an ether gradient, which extends towards the non-aqueous interfaces. The former may be accountable for the development of the bilayer structures. Depending upon the parameters employed the diameter of the niosomal vesicles range from 50 to 1000 nm.The drawbacks of the ether injection technique are that a small quantity of ether is recurrently present in the niosomal suspensions and it is very difficult to remove.

1.9.4.Miscellaneous

❖ **Multiple membrane extrusion technique:**

In this technique, a mixture of surfactants, cholesterol and diacetyl phosphate in chloroform is prepared into thin film through the evaporation[36,50]. The formed thin film is hydrated with aqueous solution of drugs to be encapsulated.The resultant vesicular suspensions extruded through the polycarbonate membranes, which are placed in a sequence for up to 8 channels. This technique is recognized as anexcellenttechnique for controlling the niosomal vesicle size[34].

❖ **Emulsion method:**

In the emulsion method, the oil-in-water (o/w) emulsionsareformed from the organic solutions of surfactants, cholesterol and aqueous solution of drugs to be encapsulated [50, 60].Then, the organic solvent phase is evaporated from the mixture dispersion, forming niosomes dispersed in the aqueous phase of the system.

❖ ***Lipid injection technique:***

Lipid injection technique does not need any expensive organic phase for the preparation of niosomes[36]. In this technique, the mixture of lipids and surfactants is first melted.Then,the melted mixture is injected into heated and a highly agitated

aqueous phase containing the dissolved drugs. A homogenous lamellar film is formed by shaking. The mixture is ultra-centrifuged and agitated at a controlled temperature.

❖ ***Bubble method:***

The bubble method is a novel technique for the one-step preparation of liposomes as well as niosomes without utilizingthe organic solvents [36]. The bubbling-unit comprises roundbottom flask with the three necks positioned in a water-bath to control the temperature of the system. The watercooled reflux and the thermometer are positioned in the first neck and second neck, respectively. The nitrogen supply was performed through the third neck of the round bottom flask. Surfactants and cholesterol are dispersed together in the phosphate buffer at 70°C.The dispersion mixture is mixed for 20 seconds with the use of high shear homogenizer and afterwards, immediately bubbled at 70°C using nitrogen gas[61]. As the organic solvents are not required, this technique is recognized as one of the economical technique of niosome preparation [36].

❖ ***Heating method***

Heating method is a scalable, non-toxic and one-step preparation of niosomes[35]. The mixtures of non-ionic surfactants, cholesterol and/or charge inducing molecules are introduced to the aqueous medium (*e.g.*, buffer, distilled H_2O, *etc.*) in the presence of a polyols, such as glycerol. The mixture is then heated with stirring at low shear forces until the niosomal vesicles are formed [35].

1.9.5. Niosome preparation using polyoxyethylene alkyl ether

The sizes and number of bilayer in vesicles comprising of cholesterol and polyoxyethylene alkyl ether can be altered using an alternative method[62]. The temperature increase above 60°C temperature transforms SUV to LUV (less than 1 μm), while the vigorous shaking at the room temperature demonstrates the opposite effect (*i.e.,* transformation of multilamellar vesicles into unilamellar ones). The transformation from the unilamellar vesicles into unilamellar ones at the higher temperaturesmay be the typicalattribute for polyoxyethylene alkyl ether surfactant, since it is recognized that polyethylene glycols (PEGs) and water remix at higher temperaturesbecause ofthe breakdown of hydrogen bonds between the water and the PEG moieties[50]. Usually, the free drugs are removed from the encapsulated drugsthrough the gel permeation chromatography method, dialysis method or centrifugation method or ultracentrifugation method [3].Often, the density differences between the niosomes and the external phase are much smaller than that of the liposomes.This makesthe separation through centrifugation very difficult. The addition of protamine to the vesicle suspension facilitates phase separation during the centrifugation[50].

1.9.6. Niosome preparation using Micelles

Niosomes can also be prepared from mixed micellar solutionsthrough the use of enzymes. Mixed micellar solution of C-16 G2, dicalcium hydrogen phosphate and polyoxyethylene cholesteryl sebacetate diester converts to niosomal dispersions when incubated with the enzyme, esterases. Polyoxy ethylene cholesteryl sebacetate diester is cleaved through the esterases to yield polyoxyethylene, sebacic acid and

cholesterol. Then, cholesterol in combination with C-16 G2 and dicalcium hydrogen phosphate yields the C-16 G2 niosomes.

1.9.7. Formation of niosomes from proniosomes

Another alternative method of preparing niosomes is to coat a water-soluble carrier like sorbitol with surfactants[63, 64]. The result of the coating process is dry formulations, in which each water-soluble particle is covered with a thin-film of dry surfactants. These dry preparations are termed as "Proniosomes".Hu and Rhodes have reported that proniosomes are dry formulations of surfactant-coated carriers, which can be rehydrated by brief agitation in hot water[63]. The schematic presentation of preparation of niosomes from proniosomes is shown in **Fig. 1.4**.

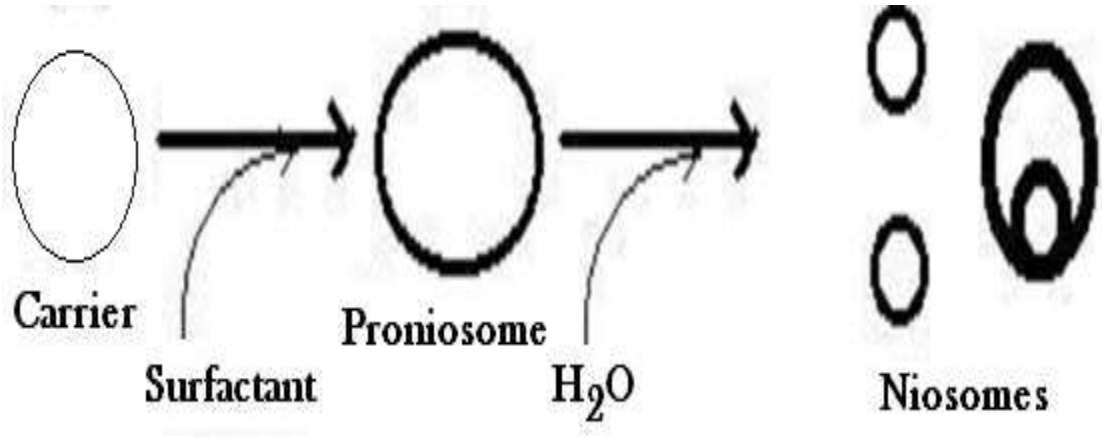


Fig.1.4: The schematic presentation of preparation of niosomes from proniosomes

Proniosomes are found as dry powder and thus, can be dispensed in capsule form [64]. These proniosomal carriers possess the capability to diminish the problems associated with niosome such as (i) physical instability; (ii) aggregation; (iii) fusion; (iv) leaking of the encapsulated drugs; and (v) hydrolysis of the encapsulated drugs, which also limits the shelf life of the dispersion [64]. Niosomes, which are derived from proniosomes are much better than the conventional niosomes, which provide optimal flexibility, easy processing, unit-dosing facility and packaging [3]. In the stability

viewpoint, the dry proniosomes is supposed to be more stable than the pre-manufactured niosomal formulations. Size distributions and the release performance of niosomes derived from proniosomes is also the superior to conventional niosomes [61, 65]. Blazek-Walsh *et al* [61] have reported the formulation of niosomes from maltodextrin-based proniosomes. This affords a rapid reconstitution of niosomes with the minimal residual carrier. The slurry of maltodextrin and surfactants was dried to form a free-flowing dry powder, which could be rehydrated through the addition of warm water [61].

1.10. Separation of unencapsulated drugs

As in most cases 100 % of the drugs to be encapsulated cannot be encapsulated within the niosomal vesicles [66]. The unencapsulated drugs should be separated from the entrapped ones. The separation of unencapsulatedsolute from theformulated vesicular dispersion can be carried outthrough various techniques, which includes[3]:

- ***Dialysis:***

The prepared aqueous niosomal dispersionsare dialyzed to separate the unencapsulateddrugsusing dialysis tubing against phosphate buffers or normal saline or glucose solution[66].

- ***Gel filtration:***

The unencapsulateddrugs is separated from the prepared aqueous niosomal dispersions through the gel filtration using a Sephadex-G-50 column and elution with phosphate buffered saline or normal saline[61,67].

❖ ***Centrifugationor ultracentrifugation:***

The prepared niosomal suspensions are centrifuged (7000 × *g* for 30 minutes for the niosomal suspensions prepared through handshaking method and ether injection method) and ultracentrifuged (150000 × *g* for 1.5 hours).Then, the supernatants areseparated. The pellets are washed and then resuspended to attain niosomal suspensions free from unencapsulateddrugs [63, 68].

1.11. Postpreparation processes in the manufacturing of niosomes

The most important postpreparation processes in the manufacture of niosomes are downsizing the prepared niosomes as well as separation of unencapsulateddrugs. After preparation, the size reduction of niosomes is attained using one of thesetechniques given below:

i. For downsizing of the niosomes, the probe sonication is preferred over the bath sonicator. The use of probe sonication results in the manufacturing of the niosomes of 100–140 nm size range. For the preparation of SUV types of niosomes, probe type sonicator is employed [36].

ii. Extrusion of the prepared niosomal dispersions through the filters of defined pore sizes.

iii. The combination of sonication and filtration has also been employed to attain niosomes of 200-250 nm diameter size ranges (*e.g.,* doxorubicin niosomes)[3].

iv. The microfluidization technique can be employed to yield niosomes of less than 50 nm in diameter sizes[36, 50].

v. High-pressure homogenisation technique also can be employed to yield niosomes of below 100 nm in diameter sizes.

1.12.Factors affecting physico-chemical properties of niosomes

Various factors that affect the physico-chemical properties of the niosomes are discussed below:

1.12.1. Nature of Surfactants

Surfactantsemployed for preparation of niosomal formulationshould have a hydrophilic head and hydrophobic tail[3]. The hydrophobic tail consists of one or two alkyl or perfluoroalkyl groups.In some cases, a single steroidal group is present within these[50]. The ether-type surfactant groups with a single-chain alkyl as hydrophobic tail is considered as much more toxic than the corresponding dialkyl ether-chains[46]. The ester-type surfactants are generally chemically less stable than the ether-type surfactants. Also, the former is comparatively less toxic than the latter one due to the chances of degradation of ester-linked surfactants by the enzymes like esterases to triglycerides as well as fatty acids,*in vivo*[46].The surfactants with alkyl chain length from C-12 to C-18 are considered as suitable for the formulation of niosomal vesicles[69, 70]. Non-ionic surfactants like C-16EO5 (polyoxyethylene cetyl ether) and/or C-18EO5 (polyoxyethylene steryl ether) are usually employed for preparation of polyhedral niosomal vesicles[71]. Non-ionic surfactants of Span series having the HLB number between, 4 and 8 are able to form vesicles[72].Examples of various types of non-ionic surfactants used in the manufacturing of niosomes are summarized in **Table 1.1**

Table 1.1: Varioustypes of non-ionic surfactants used in the manufacturing of niosomes

Non-ionic surfactants types	Examples
Fatty alcohol	Steryl alcohol, Cetyl alcohol, Cetosteryl alcohol, Oleyl alcohol, *etc*.
Ethers	Brij, Decyl glucoside, Lauryl glucoside, Octyl glucoside, Nonoxynol-9, Triton X-100,
Esters	Spans, Polysorbates (Tweens), Glyceryl laurate, *etc*.
Block copolymers	Poloxamers

1.12.2. Structure of surfactants

The structural geometry of the vesicular formulations to be prepared from surfactants is basically affected by the structure of surfactants used in the manufacturing procedure[3].The structure of surfactants is recognized to be related to the critical packing parameters, which is able to influence the structural geometry of the vesicles. Based on the critical packing parameters of surfactants used in the manufacturing procedure of niosomes, structural geometry of the formed niosomes can be predicted.

The critical packing parameters of surfactantsused in the manufacturing procedure of niosomes can be calculated using following equation[3]:

Critical Packing Parameters (CPP) = v/lc ×a0

v = non-polar group volume,

lc = critical non-polar group length,

a_0= area of polar head group.

From the critical packing parameter value of surfactants used in the manufacturing procedure of niosomes,the types of micelle structure formed can be determined as given below[3]:

1) The critical packing parameters are less than ½ (½ < CPP) means the indication of the formation of spherical micelles.
2) The critical packing parameters are more than ½ and less than 1 (½ < CPP < 1) means the indication of the formation of bilayer micelles.
3) The critical packing parameters are more than 1(CPP > 1) means the indication of the formation of inverted micelles.

1.12.3. Amounts and types of surfactants

The mean vesicle size of the niosomes enhances proportionally with the incrementof the HLB values of non-ionic surfactants such as Span 85 (HLB value, 1.8) to Span 20 (HLB value, 8.6) since the surface free energy reduces with an increase in the hydrophobicity of surfactants used[72].The bilayers of theseniosomal vesicles are either in the so-called liquid-state or in the gel-state depending on temperature of the formulation systems, the types of lipidsand/or surfactantsas well asalso the presence of other components like cholesterol. In the gel-state, the alkyl chains are present in a well-ordered structureas well asin the liquid-state[72]. The lipids andsurfactants are also characterized by the gel-liquid phase transition temperature[73].

Phase-transition temperature of surfactant also influences the encapsulation efficiency of the niosomes,*i.e.,* Span 60 having the higher transition temperature, which supplies better encapsulation to the niosomal vesicles.

1.12.4. Membrane composition

The stable niosomal vesicles can be formulated with the addition of different chemical additives along with the surfactants as well as drugs. The formulated niosomes encompassseveral morphologies, their permeability and stability characteristics.These can be changedthroughthe manipulation of membrane characteristics by the different additives. In case of polyhedral niosomes formed from C-16G2, the shape and morphology of these niosomal vesicles remains unchangedthrough the addition of low amount of solulan C-24 (cholesteryl poly-24-oxyethylene ether), which checks the aggregation because of the development of steric hindrance[74]. The average vesicle sizes of the niosomes is affected by the membrane compositions,likepolyhedral niosomes formed by C-16G2: solulan C-24 in ratio of 91:9, having a comparatively bigger sizes than the spherical or tubular niosomes formed by C-16G2: cholesterol: solulan C-24 in ratio of 49:49:2[74]. The addition of cholesterol to the niosomal system also offers the rigidity to the membrane and decreases the leakage of the drugs from niosome[75]. The inclusion of cholesterol in the niosomal system enhances the hydrodynamic diameter and also the encapsulation efficiency[72]. Generally, the action of cholesterol is of two folds:on one hand, cholesterol enhances the chain order of liquid-state bilayers of the niosomal system and on the other hand, cholesterol reduces the chain order of gel-state bilayers of the niosomal system. At a high concentration of cholesterol, the gel-state is usually transformed to the liquid-ordered phase of the system[68].An increment in the cholesterol content of the bilayers of the niosomal system resulted in a reduction in

the release-rate of encapsulated drug material and therefore, an incrementof the rigidity of the bilayers of the niosomal system obtained[68,76].The presence of the charge in the niosomal system tends to amplify the inter-lamellar distance between the successive bilayers in the MLV structures and also leads to the greater overall encapsulated volume.

A. Nature of the encapsulated drugs

The physico-chemical properties of the encapsulated drugs influence both the charge and the rigidity of the niosome bilayer. The drugmolecules interact with the surfactant-head groups and produce the charge so as to generatea mutualrepulsion between the surfactant bilayers and hence, thisenhances the vesiclesizes of the niosomal system[77].The aggregation of the vesicles is prevented because of the chargedevelopment on the bilayers of the system. In case of polyoxyethylene glycol (PEG) coated vesicles, some drugsare encapsulated in the long PEG-chains, and thus, this reduces the tendency to improve the vesicle sizes of the niosomes[63]. The HLB value of the drugs affects the degree of encapsulation of the drugs within the niosomal system. Effect of the nature of drugs on the formation of niosomes is summarized in **Table 1.2.**

Table 1.2: Effect of the nature of drugs on the formation of niosomes

Nature of the drugs	Leakage from the vesicles	Stability	Other properties
Hydrophobic drugs	Reduced	Improved	Improved transdermal delivery of drugs
Hydrophiliic Drugs	Improved	Reduced	-
Amphiphilic drugs	Reduced	-	Increased drug encapsulation, Altered elecrophoretic mobility
Macromolecules	Reduced	Improved	-

B. Temperature of hydration

The hydration temperature of the manufacturing system influences the size and shape geometry of the niosomal vesicles. For ideal condition, this should be above the gel to liquid-phase transition temperature of the manufacturing system. The temperature alteration of the niosomal system affects the assembly of surfactants into vesicles and also, induces vesicle-shape transformation [50, 74]. Arunothayanun*et al.*[74] have reported that a polyhedral vesicle formed by C-16G2 : solulan C-24 in a ratio of 91 : 9 at 25°C [74]. On heating, this transformed into the spherical vesicles at 48°C, but on cooling from 55°C, the spherical vesicles fabricated a cluster of smaller sized spherical niosomes at 49°C before varying to the polyhedral structures at 35°C.In contrast, the vesicles formed by C-16G2 : cholesterol : solulan C-24 in a ratio of 49 : 49 : 2 showed no shape-transformation on cooling or heating (temperature change) [78]. Along with the above mentioned issues, the volume of hydration medium and the time of hydration of niosomes are also critical issues. Inappropriate selection of these issues can result in the formation of fragile niosomes or the formation of the drug leakage problems.

C. Effect of pH of the hydration medium

The encapsulation efficiency of niosomes is significantly influenced by the pH of the hydration medium employed for the formulation of niosomes[79]. At pH 5.5, the high encapsulation of flurbiprofen was reported with a maximum encapsulation efficiency of 94.60 %. The fraction of flurbiprofen encapsulated within niosomes enhanced to about 1.5 times as the pH reduced from 8 to 5.5 and decreased considerably at pH less than 6.8. At pH 7.4 and 8, the lowest encapsulation of flurbiprofen occurred with the absence of significant differences between them. The enhancement in the encapsulation efficiency of flurbiprofen at the lower pH was most

probably because of its ionizable carboxylic acid-group.Atthe lower pH, proportionsofunionizedflurbiprofenfound enhancedand the partitions morereadilyintothelipid-bilayerthantheionized species[79]. At the lowerpH, the niosomal formulations were examined by the opticalmicroscopy method forthepresenceofdrug precipitates bothbeforeandaftercentrifugationandwashing. This facilitatesto measure theconcentrationof the drugs inthe hydration medium employed giving the optimumencapsulationinniosomes.

D. Preparation methodology

The hand shaking method formulates the vesicles with greater diameter of 0.35-13 nm compared to the vesicles formulated by the ether injection method (50-1000 nm)[34]. Small-sized niosomes can be formulated by the reverse-phase evaporation method[58, 80]. Microfluidization method provides a greater uniformity and small-size vesicles[34]. Niosomes formulated throughthe trans-membrane pH gradient (inside acidic) drugs uptake process demonstrated greater drug encapsulation efficiency as well as better retention of drugs[80].

E. Resistance to osmotic stress

The addition of hypertonic salt solutions to a niosomal suspension transports about the reduction in diameter. In the hypotonic salt solutions, there is apreliminary slow release with a slight swelling of the vesicles possiblybecause ofthe inhibition of eluting fluid from the vesicles, followed by the faster release, which can be because of mechanical loosening of the vesicles structure under the osmotic stress[34; 37].

1.13. Stability of niosomes

Niosome vesicles are stabilized on the basis of formation of 4 different forces[35]:

- ✓ Van der Waals forces among the surfactant molecules used in the preparation of niosomes.
- ✓ Repulsive forces emerging from the electrostatic-interactions among the chargedgroups of the surfactant molecules used in the preparation of niosomes.
- ✓ Entropic repulsive forces of the head groups of surfactants used in the preparation of niosomes.
- ✓ Short-acting repulsive forces.

The electrostatic repulsive forces are generated among the vesicles upon addition of the chargedsurfactants to the double-layer, increasing the stability of the niosomal vesicle systems[35]. The biological stability of the niosomes formulated with alkyl glycosides was exploredby Kiwada et al[80, 81]. In these reports, Kiwada et al.[81, 82]have reported that the formulated niosomes by them were not stableenough in the plasma, whichcan be because ofthe single–chain alkyl surfactants used in the preparation of these niosomes. In these researches, the formulated SUVs werefound more stable enough.Niosomes in the form of liquid crystals and gelsareremaining stable enough at both the roomtemperature (25°C) and 4°C for 2 months. Any significant dissimilarity hasnot been examinedbetween the stability of these 2 types of niosomes regarding leakage. Although, any correlation between the storage temperature and the stability has not been observed,it is advocated that niosomes should be stored at 4°C. Preferably, these niosomal vesicular systems required to be stored as dry for the reconstitution by the nursing staffs or by the patients and also, whenrehydrated niosomal systems should displaythe dispersion characteristics that are

analogous to the originaldispersion.The simulation studies performed to investigate the physical stability of these niosomesfor the duration of transportation to the purchaser revealed that the mechanical forces didn't have anyeffect on the physical stability of the niosomal systems. It is believed that the causefor the stability ofniosomes can be due to the aggregation prevention of vesicles caused by the steric-interactionsamong the large polar head groups of surfactants used for the preparation.The issues which may affect the stability of niosomes are[35]:

a. Types of surfactants used in the preparation of niosomes;
b. Nature of encapsulated drugs within niosomes;
c. Storage temperatures;
d. Detergents;
e. The use of membrane spanning lipid materials;
f. The interfacial polymerization of surfactant monomers *in situ*;
g. Inclusion of charged molecules.

1.14. Characterization of niosomes:

❖ ***Determination of drug encapsulation efficiency:***

After the preparation of niosomal dispersion, unencapsulated drugs are separated by dialysis [66, 82], centrifugation[82], or gel-filtration [72,83].The drugs remained ncapsulated within niosomes is determined throughthe complete vesicle disruption using 50% n-propanol or 0.1% Triton X-100 and analyzing the resultant solutionsthrough appropriate assay method for the drugs [40].

$$\text{Drug encapsulation efficiency (\%)} = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug}} \times 100$$

❖ ***Determination of vesicle size and vesicle size distribution analysis:***

The drug permeability is dependent on the vesicle sizes of niosomes. Therefore, the vesicle sizes and vesicle size distribution ofniosomes are essential.To determine the mean vesicle sizes and vesicle size distribution, instrumental methodsemployed mainly are as follows[34,37]

a. Malveran Mastersizer
b. Optical microscopy
c. Laser diffraction particle size analyzer
d. Coulter submicron size analyzer
e. Photon correlation microscopy
f. Molecular sieve chromatography, and
g. Freeze fracture electron microscopy

Recent years, freeze fracture electron microscopy is widely employed in the determination of vesicle size and vesicle size distribution analysis. The freeze thawing condition of niosomes (keeping the niosomal vesicular suspensions at - 20°C for 24 hours and then, heating to an ambient temperature) increases the vesicle sizes that may be attributed to the fusion of vesicles during the cycle [34].

❖ ***Determination of vesicle shape and vesiclesurface characterization:***

To determine vesicle shape and vesicle surface characterization, important instrumental methods employed are as follows [34,37]

a. Optical microscopy
b. Transmission electron microscopy (TEM)
c. Scanning electron microscopy (SEM)
d. Field emission scanning electron microscopy (FESEM)

❖ ***Determination of Zeta potential:***

To determine the colloidal properties of niosomalformulations, zeta potential value is essential.Zeta potential can be determined using Malveran Zetasizer.

❖ ***Determination of vesicular surface charge:***

Niosomes are usually prepared through the inclusion of charged molecules in the lipid bilayers to avoid the aggregation ofvesicles. A decrease in the aggregate formation was seen, when the charged molecules like dicetyl phosphate wasincorporated within the vesicles. The surface charge on the vesicles is expressed in terms of zeta potential and it is calculated using the Henry's equation, shown below[84]

$$£ = \mu E \pi \eta / \Sigma$$

where,£ = Zeta potential; μE = Electrophoretic mobility; η = Viscosity of the medium; and Σ = Dielectric constant

❖ ***Determination of number of lamellae:***

The number of lamellaeis determined by using these following instrumental methods[85]

a. Nuclear magnetic resonance (NMR) spectroscopy,
b. Small angle X-ray scattering and
c. Electron microscopy

❖ ***Measurement of membrane rigidity:***

The membrane rigidity can be measured through mobility of fluorescence probe as the function of temperature [79].

❖ ***Measurement of bilayer formation:***

The assembly of non-ionic surfactants to form the bilayer vesicles is characterized through X-cross formation under the lightpolarization microscopy [85].

❖ ***In vitro drug release study:***

A method of *in vitro*drug release rate study methodology with the help of dialysis tubing was reported by Yoshioka *et al.* [72].According to this methodology reported, a dialysis sac is washed and then, is soaked in distilled water. The vesicle suspensionsare pipetted into a bag made of the tubing and sealed. The dialysis bag containing the niosomal vesicles is then positioned in the 200 ml of buffer solution in a 250 ml beaker with application of constant shaking at 37 ± 0.5°C. At various predetermined time intervals, the buffer solutions are collected. The collected buffer solutions are analyzed for the estimation of drug contentsthrough an appropriate assay method.

A different type of *in vitro* drug release rate study methodology of niosomes is reported by Karki *et al.*[86]. According to Karki *et al.*[86]1 ml of prepared niosome suspensionsare placed first on the top of the column and the elution isperformed using normal saline as *in vitro* drug release medium. Niosomes are filled within dialysis tubing to which a dialysis sac is attached to the one end. The dialysis tube is suspended in the phosphate buffer (pH, 7.4) at 37 ± 0.5°C with continuous stirring using a mechanical stirrer. At various predetermined time intervals, the buffer samples are withdrawn. The collected buffer samples are analyzed for the estimation of drug contents through an appropriate assay method using high-performance liquid chromatography (HPLC).

❖ ***In vivo studies:***

For *in vivo* studies of the niosomal formulations rats and rabbits are used as experimental animal. These experimental animals are subdivided with groups as control and test. Niosomal formulations to be tested *in vivo* are administered to the experimental animals through appropriate route. At various predetermined time intervals, appropriate samples as well as changes in physical condition are recorded for pharmacokinetic and pharmacodynamic responses, respectively.

❖ ***Stability study:***

The stability study is performed to ensure that the absence ofany degradation of active ingredients or drugs encapsulated in the niosomes and the niosome formulations does not exhibit any physical or chemical interactions between active ingredients or drugs and excipients. The stability study test is also conducted to ensure that the activity of the preparation is not lost. Therefore, the formulated niosomes are subjected for stability studies for a period of three months. The formulated niosomes are divided into 3 portions. First portion is kept at refrigeration temperature (4°C ±1°C). Second portion at room temperature and third portion is kept at 40°C± 2°C.

1.15. Drug delivery applications of niosomes

Niosomes are potentially applicable to encapsulate many pharmacological agents and their delivery for their action against various diseases[35,47]. The administration of niosomes through various routes has been also reported[49]. Some of their drug delivery applications are discussed below[35,49,47].

❖ ***Oral drug delivery:***

The oral route is usually chosen as a leading popular route of drug administration. An enhanced oral bioavailability of griseofulvin through the oral

administration of griseofulvin loaded niosomes was also observed[43]. An *in vitro–in vivo* correlation demonstrated that niosomes were the efficient way to enhance the oral bioavailability as well as sustained delivery of griseofulvin through the oral administration. Span60-based niosomal formulations containing fluconazole (an antifungal drug) for oral use withanencapsulation efficiencyof greater than 91% demonstrated sustaine drug release pattern by zero-order followed by first-orderkinetics[87]. In a report, orally administered niosomes loaded with rifampicin and gatifloxacin were found effective against thetuberculosis causing bacteria, *Mycobacterium tuberculii* for a prolonged period and also, were observed better results than the conventional dosage formsdue to the reduced dosingas well as greater patient compliances[88]. Yet,the major difficulties to oral delivery of niosomal formulations are the hepaticfirst-passmetabolismandgastrointestinal irritation.These limitations can be conquered via other routes of drug administration.For the oralimmunization,a type of mannosylatednon-ionic surfactant-basedniosomal vesicles have been formulated for efficient delivery of plasmid DNA encoding small lsub-unit proteins of hepatitisBvirus[49].

- ***Parenteral drug delivery:***

Niosomes of sub-micron sized vesicles are usually employed for the parenteral administrations of several drugs. Usually, niosomalvesicles up to 10 μm vesicle-size are administered via intraperitoneal or intramuscular route.[R]-^{59}Fe-deferroxamine trioxyethylene cholesterol niosomal vesicles were developed for intravenous administration[89]. It was observed that the distribution of such niosomal vesicles depended upon the vesicle size as apparentfrom the result indicating the greater distribution in liver as well as spleen.The results indicated that the encapsulation of doxorubicin within the niosomes decreases the cardiactoxicity upon intravenous

administration.Uchegbu *et al.*[51, 90]have investigated the influence of the dose on the plasma-drugconcentration through theintraperitoneal administration of doxorubicin-containing niosomes inmouse.

❖ ***Pulmonary drug delivery:***

Niosomal vesicles are employed in the treatment of asthmatic patients also. Terzano*et al.* [91] havedevelopedpolysorbate 20-based niosomes containing beclomethasone dipropionate for the pulmonary delivery to the patients suffering from chronic obstructive pulmonary disease (COPD). They have reported that theseniosomes containing beclomethasone dipropionatepresented targeted and sustained delivery of encapsulated beclomethasone dipropionate. Along with this, these niosomes exhibitedenhanced mucus permeation and improved therapeutic effect. Theseniosomal vesicles exhibit an increased permeation rate of beclomethasone dipropionate through the model mucosal-barrier and therefore, presenting anenhanced targeting of beclomethasone dipropionate in the treatment of COPD. In another investigation, Moazeni *et al.,*[92] have also investigated the formulation and *in vitro* analysis of niosomes containing ciprofloxacin for pulmonary delivery.

❖ ***Drug targeting:***

a) Drug targeting to reticulo-endothelial system (RES): The cells of RES preferentially uptake the vesicles[47]. The uptake of niosomesby the cells of RES is also through the circulating serum factors known as opsonins, which is recognized to mark them for the clearance. However, such types of localized drug accumulation have been developed in the treatment of tumors commonly known to metastasize to both the spleen and liver.

b) Drug targeting to organs other than reticulo-endothelial system (RES): The drug targeting to the organs other than RES is also employed by the various researchers. This has been advocated that the carrier systems can be directed to the specific-sites in the body through use of antibodies. Various kinds of immunoglobulins areinvestigated to bind quite readily to the lipid-surface of the vesicles.This offers a convenient and effective meansfor the targeting of drug carriers. Numerous cells have the intrinsic capability to identify and also to bind particular carbohydrate-determinants.This can be developed to direct the carriers system to target any particular cells [93].

❖ ***Transdermal drug delivery:***

The slow penetration of drug molecules through the skin barrier is the main disadvantage of transdermal drug delivery. An enhancement in penetration rate for the drug molecules has been attained through the transdermal delivery of drugs incorporated in the niosomal formulations. Jayraman *et al.*[94] *has* researched the transdermal delivery of erythromycin from various types of formulations including niosomes on the hairless mouse. From results of these studies, it was observed that these non-ionic niosomal vesicles are found effective to target the pilosebaceousglands.

❖ ***Ocular drug delivery:***

Bioadhesivecoated niosomal formulationscontaining acetazolamide was formulated usingSpan 60, dicetyl phosphate or cholesterol stearylamine showed more affinity for the reduction of intra-ocular pressure as compared to the marketed formulation of acetazolamide, Dorzolamide[95]. The chitosancoated niosomal formulation containing timolol maleate (0.25%) displayed more effectivenessfor the

reduction of intra-ocular pressure as compared to the marketed formulation of timolol maleate (0.25 %) with lesser chances of cardiovascular side-effects[96].

❖ ***Delivery of peptide drugs:***

In an investigation, Yoshida*et al.*[72] have investigated the oral delivery of 9-desglycinamide and 8-arginine vasopressin encapsulated in the niosomes in the *in vitro* intestinal-loop model. They have reported that the stability of these encapsulated peptides found increased significantly.

2. AIMS AND OBJECTIVES

2.1. AIM

Niosomes are used to prolong the circulation of the drugs, to alter the distribution of drugs and they also have many advantages. Niosomes favor selective drug delivery and enhances the therapeutic efficacy and decreases the severity of side effects. The need for present study is to encapsulate the drug in the niosomes vesicles for effective colon drug delivery for a prolonged period of time. 5-Fluorouracil is one of the most effective drugs in the treatment of colon cancer. Our objective of this study is to treat colon cancer by formulating niosomes containing 5-fluorouracil and leucovorin. Leucovorin is used in the formulation to increase the activity of the drug fluorouracil. Since, niosomes prolong the circulation of many drugs and alters the distribution of drugs, we aimed at formulating 5-Fluorouracil and leucovorin niosomal drug delivery, thereby dose can be minimized and also to achieve sustained release for a prolonged period of time and to compare the anticancer activity of 5-Fluorouracil and leucovorin niosomes with a marketed formulation at different concentrations of the drug. The reason for selecting niosomal drug delivery is that niosomes can entrap both hydrophilic and lipophilic drugs and also have better stability than liposomes.

2.2. OBJECTIVES

2.2.1. Reason for selecting 5–fluorouracil and leucovorin

5–Fluorouracil is the most continuously used drug in treating colon cancer [97, 98]. It belongs to the category anti-metabolites [99]. It inhibits with the production of proteins which is required for cell growth and reproduction [100.] It is used in combination with many other drugs now-a-days. Leucovorin is not actually

chemotherapeutic agent, but it potentiates the activity of the drug 5-Fluorouracil to a great extent [101]. Also, it does not possess any serious side effects. Leucovorin is a vitamin–B derivative. Conventional dosage forms are available with 5-fluorouracil and leucovorin is given in the adjuvant therapy. So both these drugs were selected in combination to be formulated as niosomes to treat colon cancer effectively.

2.2.2. Reason for choosing niosomes

Though many carriers are available under targeted drug delivery systems, niosomes was selected in this study because these host many advantages over other carriers especially over liposomes, which are structurally similar to niosomes [85]. Niosomes are osmotically active and more stable compared to liposomes [102]. Since these can encapsulate both hydrophilic and lipophilic drugs, a wide range of solubility of drugs can be accommodated [84]. The non-ionic surfactants employed in the formulation are biodegradable, bio compatible and non–immunogenic [49, 47]. It is known that most of the anti-cancer drugs possess severe side effects. But, niosomes have the ability to alter the metabolism of a drug[34]; these can also prolong the circulation and half–life of a drug so that the side effects of the drug can be decreased[37].

2.3. Plan of Work

The following experimental protocol was envisaged for a systemic approach to the study.

- Purchase of drugs 5- Fluorouracil, Leucovorin and other excipients used in the study.
- Literature review of the drugs and niosomes.
- Preformulation studies:

a. Identification of drugs, excipients and its mixture using:
   - Infrared spectroscopy (FTIR Technique)
   - Melting point determination
   - Differential scanning colorimetry

b. Preparation of calibration curve of drugs in DMSO and buffer

c. Formulation of niosomes containing 5 –Fluorouracil and combination of 5-Fluorouracil and leucovorin niosomes with different surfactants by Lipid Hydration technique using Rotary Flash Evaporator.

d. Selection of best formulation for characterization

e. Characterization of formulated niosomes:
   - Particle size distribution
   - Vesicle shape and surface characterization by Scanning Electron Microscopy(SEM) and Transmission Electron Microscopy (TEM)
   - Percentage yield
   - Drug Entrapment Efficiency
   - In –vitro release study
   - Sterility test of niosomes
   - Stability studies using ICH guidelines
   - Comparison of anti –cancer effect of the prepared niosomal formulations with the marketed formulation.

3. REVIEW OF LITERATURES

Recent years, various colloidal vesicular carrier systems are gaining remarkable status for the effective use in various kinds of drug delivery therapeutics [31]. Among these various colloidal vesicular carrier systems, niosomes are the lipid-based vesicular systems for the use as promising vehicles for drug delivery applications [34]. In 1975, L'Orealformulated and patented the first niosomal formulations [35]. Niosomes are the microscopic bilayer lamellar vesicles formed on the hydration on non-ionic surfactant films, which hydrate imbibing or encapsulating the hydrating aqueous solutions [36]. The size of the niosomes lies in nanometric scale usually in the range of 100 – 300 nm [37]. During last few decades' considerable research efforts have been directed towards the research and development of various niosomal formulations for the use in potential drug delivery applications through different routes [47,49, 51, 84, 102].

Wang *et al.*[99] have prepared and characterized 5-fluorouracil loaded pH-sensitive niosomes made of a non-ionic surfactant mixed with cholesteryl hemisuccinate (a derivative of cholesterol as a pH-sensitive agent) and succinic acid. These 5-fluorouracil loaded pH-sensitive niosomes were prepared through the film hydration-probe ultrasound technique. Both the normal and pH-sensitive niosomes exhibited spherical morphology structure under the transmission electron microscopy (TEM) with average vesicle sizes of 172.00 ± 6.20 nm and 153.00 ± 4.70 nm, respectively. The thermotropic status, structure changes and interaction of 5-fluorouracil with other excipients were also characterized by the differential scanning calorimetry (DSC) and the disappearance of the melting peak of 5-fluorouracil revealed that the fact of 5-fluorouracil encapsulation within these niosomes. The structure of 5-fluorouracil loaded pH-sensitive niosomes was analyzed

by the mass spectrometry and proton nuclear magnetic resonance (^{1}H NMR) spectrum. The pattern of 5-fluorouracilreleases from normal niosomes and pH-sensitive niosomes was tested using bulk equilibrium reverse dialysis method. The 5-fluorouracil releases from normal niosomes and pH-sensitive niosomes in different pH medium exhibited a pH-sensitive behavior from the niosomes made of cholesteryl hemisuccinate. The *in vivo* tumor targeting efficacy of these 5-fluorouracil loaded pH-sensitive niosomes demonstrated a remarkable high concentration of 5-fluorouracil in the tumor-site of the mice transplanted with the tumor cell.

Namdeo and Jain, (1999)[100] have formulated niosomes containing 5-fluorouracil using a series of Spans, i.e. Span 20, 40, 60 and 80. They have prepared these Span-based niosomes containing 5-fluorouracil through the hand shaking method, reverse phase evaporation method and ether injection method. The niosomes containing 5-fluorouracil prepared by hand shaking method were found less permeable, when niosomal vesicles were studied for the effect of various process variables such as types of Spans, composition of the lipid and total lipid concentration on drug entrapment efficiency and also the drug release rate. The niosomes containing 5-fluorouracil prepared using Span 40 and 60 were found to release 5-fluorouracil of 40.90 % and 37.10 % within 6 hours; while niosomes prepared using Span 20 and 80 exhibited 52.20 % and 57.10 % of 5-fluorouracil releases, respectively within the same time period. Niosomes prepared using Span 40 showed a mean vesicle size of 8.1 μm, drug entrapment efficiency of 15.30 ± 1.30 % and released 78.60 % of 5-fluorouracil within 6 hours. The incorporation of cholesterol within the niosomal formulations found to be reduced the vesicle size to 4.8 μm, drug entrapment efficiency to 12.30 ± 0.90 % and released 50.50 % of 5-fluorouracil within 6 hours; while the incorporation of dicetyl phosphate within the niosomal

formulations further decreased the vesicle size to 3.87 μm, drug entrapment efficiency to 10.90 ± 1.10 % and reduced of 5-fluorouracil to 40.90 % within 6 hours. The increment in the amounts of lipids used for the niosomes containing 5-fluorouracil was found to be translated into an almost linear enhancement in the drug entrapment efficiency. The bio-distribution of 5-fluorouracil in the rats was found modified on the encapsulation within the nisomal formulations. The concentrations of niosomal 5-fluorouracil in the liver, lung and kidney were found increased; while it was found to be decreased in the intestine as compared to the free drug solution following intravenous administration of niosomes containing 5-fluorouracil. These niosomes also exhibited a higher and sustained plasma-drug level shape as compared to the free drug solution. The pharmacokinetic parameters revealed an improvement in the half-life, area under the curve and a decrease in the volume of distribution of 5-fluorouracil on encapsulation within the niosomes.

Cosco *et al.,* (2009)[103] have formulated niosomes composed of α, ω-hexadecyl-bis- (1-aza-18-crown-6) (bola), Span 80 and cholesterol in a molar ratio of 2:5:2. They have encapsulated 5-fluorouracil (an anti-tumoral drug, which is mostly used in the breast cancer treatment) within these bola-niosomes. After the sonication procedure these 5-fluorouracil loaded bola-niosomes demonstrated mean vesicle sizes of ~ 200 nm and a drug loading capacity of ~ 40 %. Similar results were also attained in case of polyethylene glycol (PEG)-coated bola-niosomes (bola, Span 80, cholesterol, and DSPE-mPEG2000, in a molar ratio of 2:5:2:0.1, respectively). 5-fluorouracil loaded PEG-coated and uncoated bola-niosomes were evaluated for their activity on MCF-7 and T47D cells. Both the types of 5-fluorouracil loaded bola-niosome formulations (PEG-coated and uncoated) presented an enhancement in the cytotoxic effects on MCF-7 and T47D cells with respect to the free 5-fluorouracil.

The confocal-laser scanning microscopy analyses were performed to evaluate both the extent as well as the time-dependent bola-niosome/cell interactions. *In vivo* analysis on MCF-7 xeno-graft tumor SCID-mice models demonstrated a more effective anti-tumoral activity of the PEG-coated 5-fluorouracil loaded bola-niosomes at a concentration, which was 10 times lower than that of the free drug solution of 5-fluorouracil after a treatment of 30 days period.

The same research group, in another study has investigated the same niosomal formulation [composed of α, ω-hexadecyl-bis- (1-aza-18-crown-6) (bola), Span 80 and cholesterol in a molar ratio of 2:5:2] for the use in the topical delivery of 5-fluorouracil for the treatment of skin cancer[104]. These 5-fluorouracil loaded bola-niosomes demonstrated a mean vesicle size of approximately 400 nm. These niosomal vesicles were found to be decreased to around 200 nm through a sonication method of niosomepreparation with 0.1 of polydispersion index value. These niosomes exhibited 5-fluorouracil loading of about 40 % regarding the amount of 5-fluorouracil added the preparation systems. These 5-fluorouracil-loaded bola-niosomes were evaluated on HaCaT (non-melanoma skin cancer with specific mutations in the p53 tumor suppressor gene) and SKMEL-28 (human melanoma) to evaluate the cytotoxic action regarding the free 5-fluorouracil. These 5-fluorouracil-loaded bola-niosomes illustrated an enhancement of the cytotoxic action regarding the free 5-fluorouracil. The confocal-laser scanning microscopy analyses were performed to evaluate both the extent as well as the time-dependent bola-niosome/cell interactions. The percutaneous permeation of 5-fluorouracil-loaded bola-niosomes was assessed by the using of human stratum corneum as well as epidermis membranes. These 5-fluorouracil-loaded bola-niosomes presented an enhancement of the drug penetration through the human stratum corneum and the epidermis membranes of 8- and 4- folds, respectively,

regarding an aqueous solution of 5-fluorouracil and a mixture of empty bola-niosomes with an aqueous solution of 5-fluorouracil.

Parikh *et al.,* (1998)[97] have reported the formulation and *in vitro* evaluation of niosomes containing 5-fluorouracil using a non-ionic surfactant series of Spans through thin-film hydration method. They have prepared niosomes using Span 40, 60, 80 and 85. The higher drug entrapment efficiencies of 5-fluorouracil within these niosomes made of Span 40 and 60 were seen as compared to the niosomes made of Span 80 and 85. It was also seen that incorporation of increased amounts of cholesterol increased the entrapment of 5-fluorouracil into these niosomal formulations. The leakage of 5-fluorouracil in these niosomes was found reduced on the freeze drying.

Hao *et al.,* (2002)[60] have prepared niosomes of high encapsulation efficiency for a soluble drug. They have employed Span 60 as non-ionic surfactant and cholesterol to prepare these niosome through the evaporation-sonication technique. These niosomes exhibited good stability for at least 40 days. In these niosomes, colchicine was encapsulated as a model drug. To get higher colchicine encapsulation efficiency, several processing factors such as the structure of the non-ionic surfactant used, level of lipid, colchicine content and cholesterol amount were analyzed and optimized. From the obtained results, it was clearly evident that the Span 60 was the most ideal surfactant among four kinds of Spans used for the preparation of these niosomes containing colchicine. Moreover, colchicines release in the *in vitro* analysis from these niosomes displayed prolonged colchicine release profile over 24 hours. The results of this investigation suggested that these optimized niosomes containing colchicines not only contained high encapsulation efficiency, but also expected less

chance of side-effects. These types of niosomes can be prepared for the encapsulation of other soluble drugs.

Desai and Finlay (2002)[105] have investigated the potential of the encapsulation of all-trans-retinoic acid through the niosomal formulations and delivering it as an inhaled aerosol. Various types of non-ionic surfactants like Span and Tweens of various grades were employed to achieve the optimal encapsulation and nebulization efficiencies. The optimal formulations were obtained with combinations of Span 20-Tween 80 blend and Span 60-Tween 80 blend using an all-trans-retinoic acid concentration of 1 mg/ml.

Balasubramaniam *et al.,* (2002) [106] developed niosomes for the encapsulation of daunorubicin hydrochloride through the reverse evaporation process. These developed niosomes containing daunorubicin hydrochloride exhibited entrapment efficiency of 20 %, which were the higher than the theoretically possible by the reverse evaporation process. These niosomes containing daunorubicin hydrochloride were found very stable at a storage temperature of 4°C for 3 months period. Even, the drug leakage from these formulations was found to be restricted to just only 10 %. The *in vivo* drug release advocated a prolonged daunorubicin hydrochloride release over a period of 20 hours. The niosomes containing daunorubicin hydrochloride displayed an improved anti-tumor efficiency, when compared to free daunorubicin hydrochloride. The niosomes containing daunorubicin hydrochloride was found capable to damage the Dalton's ascitic lymphoma cells in the peritoneum within the 3rd day of treatment; while free daunorubicin hydrochloride took around 6 days and the process was found incomplete. The haematological analysis also confirmed that the treatment using niosomes containing daunorubicin hydrochloride were superior as

compared to the treatment using free daunorubicin hydrochloride. An improved mean survival-time was attained by the developed niosomes containing daunorubicin hydrochloride, which finally confirmed the overall effectiveness of these niosomal formulations. The results of this study recommended that these multilamellar developed niosomes containing daunorubicin hydrochloride resisted the immediate lysis in the Kupffer cells, whereby a prolonged daunorubicin hydrochloride concentration was attained, which improved the cell-lysis. However, the key issue accountable for the rapid onset of action could be the improved permeability of these niosomes into cell membranes and cytoplasm of the Dalton's ascitic lymphoma cells.

Uchegbu *et al.,* (1995)[107] have investigated that PK1 was an *N*- (2-hydroxypropyl) methacrylamide (HPMA) copolymer-doxorubicin conjugate. The niosome encapsulation was planned a means to enhance the PK1 blood residence-time, which could potentially promote the tumour uptake and produce a slower-sustained release of the niosome encapsulated drug. The factors effecting the drug encapsulation efficiency and vesicle size of PK1-niosomal formulations were evaluated. 5 non-ionic surfactants were employed to formulate the PK1-niosomes, namely, hexadecyl poly-5-oxyethylene ether (C16EO5), octadecyl poly-5-oxyethylene ether (C18EO5), hexadecyl diglycerol ether (C16G2), sorbitan monostearate (Span 60) and sorbitan monopalmitate (Span 40). Each surfactants were mixed in the equimolar ratio with cholesterol with varying amounts (9–39 mol %) of Solulan C24 (a cholesteryl poly-24-oxy ethylene ether). Dicetylphosphate (2 mol %) was also added to each mixtures. The passive association of PK1 with the preformed C16G2 and Span 60 niosomal vesicles was found low (only 3–4 %), while the subsequent dehydration (*i.e.,* freeze drying) afterward rehydration of the niosomal formulation enhanced the drug entrapment up to 61 % in the C16G2 niosomal

formulation. Transmission electron microscopy (TEM) observation revealed that these formulated niosomes possessed an electron dense core, which was an evidence of intravesicular concentration of the encapsulated PK1. The increment of Solulan C24 content within these niosomal formulations resulted in reduced PK1 entrapment within niosomes after the freeze drying and the vesicle sizes were found to be decreased. The incorporation of SolulanC24 (39 mol %) caused the marked vesicle aggregation on the freeze drying, while at the lower levels (9 mol %), PK1 emerged to work as a cryoprotectant. The mean vesicle size of C16G2 niosomes was found 235 nm. The C16G2 niosomes were unable to induce the lysis of red blood cells at the planned dose for the *in vivo* application. The preliminary *in vivo* bio-distribution analyses demonstrated PK1-C16G2 niosomes were mainly taken up by the spleen and liver.

Chandraprakash *et al.,* (1990)[40] have prepared unilamellar niosomes for the encapsulation of an anti-cancer drug, methotrexate. The drug entrapment efficacy within these niosomes was found to be enhanced with the increment in lipophilicity of the non-ionic surfactants used for the preparations. The niosomes were prepared using span 60 as non-ionic surfactant demonstrated maximum drug entrapment and its pharmacokinetic profile in the mice transplanted with S-180 tumor was noticeably different as compared with the unentrapped free methotrexate.

The same research group (Chandraprakash *et al.,* 1993)[108] has evaluated the effect of niosome encapsulation of methotrexate (prepared by the reverse phase evaporation technique using Span 60 as non-ionic surfactant), macrophage activation on the tissue distribution of methotrexate and tumor size after the administration of intravenous infusion into the mice. After the intravenous infusion of the prepared

niosomes containing methotrexate into the mice, the methotrexate concentration was measured as higher in all tissues of the treated mice except the kidney as compared with the free methotrexate solution treatment. The niosomal encapsulation of methotrexate and macrophage activation improved the methotrexate delivery to the tumor and also found to increase the anti-tumor activity.

Udupa *et al.,* (1993)[109] have formulated niosomes using various grades of Tweens and Spans as non-ionic surfactants. They have planned to encapsulate the anti-cancer agent, methotrexate. The influences of vesicle size distribution, methotrexate encapsulation efficiency, and pharmacokinetic parameters on the tumour remission of the mice transplanted with S-180 Sarcoma cells were evaluated. Niosomes containing methotrexate prepared with Span 60 have shown the promising results.

In an investigation, Parthasarathi *et al.,* (1994)[80] have formulated niosomes for the encapsulation of anticancer drug, vincristine sulfate to evaluate the effectiveness of these formulated niosomes as promising drug carrier. These niosomes encapsulated vincristine sulfate were prepared through the trans-membrane pH-gradient drug uptake process (*i.e.,* the so-called remote loading method). These formulated niosomes containing vincristine sulfate were evaluated for the toxicity and anti-tumour activity after administration of these niosomes to the tumour bearing mice. The toxicity of vincristine sulfate was found decreased after the administration of niosome containing vincristine sulfate. The *in vivo* evaluation in the tumour bearing mice indicated an enhanced anticancer activity with the reduced toxicity in mice. These niosomes can be employed to attain a better delivery of vincristine sulfate at the tumour site.

Ruckmani et al., (2000)[110] developed niosomes containing cytarabine hydrochloride and evaluated their effectiveness for the treatment of leukemias. These niosomes containing cytarabine hydrochloride were prepared through a lipid hydration technique which excluded dicetyl phosphate. The vesicle sizes of these niosomes measured were ranged, 600 to 1000 nm. The release of cytarabine hydrochloride from these formulated niosomes displayed prolonged cytarabine hydrochloride release pattern over 16 hours of period. The drug entrapment efficiency of these niosomes containing cytarabine hydrochloride was measured about 80 % for the niosomes prepared using Tween 80, Span 60 and Tween 20 as non-ionic surfactant. For the niosomes prepared using Span 80, the drug entrapment efficiency was measured as 67.50 %. The physical stability of these niosomes containing cytarabine hydrochloride was found good over 4 weeks of stability period.

Mokhtar*et al.,* (2008)[79] have investigated the formulation of niosomes containing a non-steroidal anti-inflammatory drug, flurbiprofen. They have found that the flurbiprofen encapsulation efficiency of niosomes was found significantly influenced by the pH of the hydration medium employed the formulation of niosomes. At pH 5.5, the high encapsulation of flurbiprofen was reported with a maximum encapsulation efficiency of 94.60 %. The fraction of flurbiprofen encapsulated within niosomes enhanced to about 1.5 times as the pH reduced from 8 to 5.5 and decreased considerably at pH less than 6.8. At pH 7.4 and 8, the lowest encapsulation of flurbiprofen occurred with the absence of significant differences between them. The enhancement in the encapsulation efficiency of flurbiprofen at the lower pH was most probably because of its ionizable carboxylic acid-group. At the lower pH, proportions of unionized flurbiprofen enhanced and the partitions more readily into the lipid-bilayer than the ionized species. At the lower pH, the niosomal formulations were

examined by the optical microscopy method for the presence of drug precipitates both before and after centrifugation and washing.

Bhaskaran and Panigrahi, (2002)[111]have formulated niosomes loaded with salbutamol sulphate using various types of non-ionic surfactants like Tweens (20, 40, 60 and 80) Spans (20, 40, 60 and 80) and Brij 35 through the transmembrane pH-gradient technique. The salbutamol sulphate encapsulation efficiencies in these niosomes were found varied from 28 % to 79 %. These niosomal vesicles were characterized by infrared (IR) spectroscopy. *In vitro* salbutamol sulphate release evaluations were performed using dialysis bag. Phosphate buffer, pH 7.4 was employed as dissolution medium. The *in vitro* salbutamol sulphate release evaluations were carried out for a period of 24 hours. These niosomes loaded with salbutamol sulphate displayed retarded release of salbutamol sulphate for 24 hours. Span 60 was observed as the most satisfactory surfactant among others, which released 78.40 % of salbutamol sulphate within 24 hours. The vesicle-size distribution analyses were performed by the optical microscopy. Most of these niosomes loaded with salbutamol sulphate were found spherical in shape. The thermal stability analyses were performed at 4°, 25° and 50° for one month. The niosomal product was lyophilized. The tissue distribution analyses were performed on the rabbits and the maximum concentration was measured in their lungs.

Manconi *et al.,* (2002)[112] have prepared tretinoin loaded niosomes from the polyoxy ethylene (4) lauryl ether, sorbitan esters and a mixture of octyl/decyl poly glucosides, in the presence of cholesterol and dicetyl phosphate. Liposomes prepared from non-hydrogenated and hydrogenated phosphatidyl choline were prepared as comparison references to analyze the formulated tretinoin loaded niosomes. An investigation was planned to analyze the influence of vesicle-compositions and the

preparation method on the vesicle structure (MLV, LUV, or SUV), vesicle size distribution, drug entrapment efficiency and *in-vitro* release of incorporated tretinoin from these niosomes. Results obtained demonstrated that in the presence of cholesterol, all the amphiphiles employed were found capable to form stable vesicular dispersions with or without tretinoin. The vesicle sizes were found dependent on the preparation technique, the bilayer composition and the drug-load. The multilamellar (MLV) vesicles were observed as larger than the extruded (LUV) and the sonicated (SUV) vesicles; while the tretinoin-loaded vesicles were found generally smaller than the empty ones. The tretinoin entrapment efficiencies were always observed as very high particularly for the multilamellar (91–99 %) and extruded (88–98 %) vesicles. The *in vitro* tretinoin release from the formulated vesicular formulations was evaluated using the vertical Franz-diffusion cells. The *in vitro* tretinoin release rates through the Silastic membrane from liposomal and niosomal tretinoin dispersion were found faster than from a solution containing tretinoin. The *in vitro* tretinoin release data demonstrated that the tretinoin delivery through the niosomal formulations was found mainly affected by the vesicular structures and that tretinoin delivery was also observed increased from the MLVs to the LUVs to the SUVs.

In an investigation, Himanshu *et al.,* (2007)[113] have designed and evaluated verapamil hydrochloride loaded niosomes. The verapamil hydrochloride loaded niosomes were prepared by thin film hydration technique using Span 40 and Span 60 as non-ionic surfactants and cholesterol as lipids, in different ratios. The polymer to drug ratios was varied and the formulation variables were optimized to attain such verapamil hydrochloride release kinetics with a view to attain release in a controlled manner, improve bioavailability and decreased dosing frequency. Among these non-

ionic surfactants, Span 60 showed an ecstatic result with high verapamil hydrochloride entrapment efficiency and *in vitro* verapamil hydrochloride release.

Jain and Vyas (1995)[114] have formulated niosomes containing rifampicin (an anti-tubercular agent) us Span as non-ionic surfactant and cholesterol in a ratio of 50 : 50 mole fraction. The rifampicin-entrapped niosomal vesicles were characterized for their vesicle-shapes, sizes, drug entrapment efficiencies and *in vitro* release rates. The rifampicin-entrapped niosomes showing maximum rifampicin entrapment and minimum rifampicin release rate were chosen for the *in vivo* studies. The cumulative percent doses of rifampicin were recovered in the thoracic lymph following the intravenous as well as intraperitoneal administrations of rifampicin-entrapped niosomes and free rifampicin solution were compared. The results of this study displayed that the effective compartmentalization of rifampicin occurred in lymphatic system following the intraperitoneal administration of rifampicin-entrapped niosomes. Thus, rifampicin-entrapped niosomes could be employed for the tuberculosis treatment.

Mullaicharam and Murthy (2004)[115] also developed and optimized rifampicin (a first-line anti-tuberculosis drug) loaded niosomes. The formulation procedure along with the rifampicin entrapment efficiency of these niosomes was optimized using factorial design. The influences of changes of various formulation variables such as volume of solvents, volume of hydrating medium, hydration-time and sonication time were analyzed. The prepared rifampicin loaded niosomes were characterized for vesicle size, vesicle shape and lamellarity. The stability of these prepared rifampicin loaded niosomes was measured in terms of retention of the drug (rifampicin) at the ambient temperature (25-35°C) as well as refrigerated temperature (5°C) for 60 days.

The developed rifampicin loaded niosomes can be practical in the treatment of pulmonary tuberculosis.

Rangasamy *et al.,* (2008)[116] have formulated acyclovir encapsulated niosomes through hand shaking technique and ether injection technique using various ratios (1:1, 1:2 and 1:3) of cholesterol (lipid) to Span 80 (Non-ionic surfactant). The vesicle size range was measured as 0.5-5 μm in the case of acyclovir encapsulated niosomes prepared by hand shaking technique and 0.5-2.5 μm in the case of acyclovir encapsulated niosomes prepared by ether injection technique. The acyclovir encapsulation efficiency was found increases, when the concentration of Span 80 was increased during preparation. *In-vivo* acyclovir release study of acyclovir encapsulated niosomes indicated that 76.64 % acyclovir release was measured for the niosomal formulation prepared with cholesterol to Span 80 ratio, 1:1. These niosomes indicated an extended acyclovir releasing period of 1 day.

Ahuja *et al.,* (2008)[117] have formulated and evaluated lansoprazole (widely used as antiulcer agents and antacid) loaded niosomes. They have formulated these lansoprazole loaded niosomes through a modified reverse phase evaporation technique using Span 60 as non-ionic surfactant and cholesterol in a molar ratio of 1:1. They dissolved Span 60 and cholesterol within a mixture of diethyl ether. Aqueous phase (5 ml) containing lasoprazole was added the above said mixture and the resulting 2 phases was homogenized using a homogenizer for 3 minutes at 4-5°C and 800 rpm. The formed clear gel was homogenized using a homogenizer once again for 2 minutes with the addition of phosphate buffer saline. The prepared suspension was heated using a water-bath for 10 minutes at 60°C.

In a study, Terzano *et al.,* (2005)[91] havedeveloped polysorbate 20-based niosomes containing beclomethasone dipropionate for the pulmonary delivery. They have formulated these niosomal formulations for the patients suffering from chronic obstructive pulmonary disease (COPD). They also have reported that these niosomes containing beclomethasone dipropionate presented targeted and sustained delivery of encapsulated beclomethasone dipropionate. Along with this, these niosomes exhibited enhanced mucus permeation and improved therapeutic effect. These niosomal vesicles exhibit an increased permeation rate of beclomethasone dipropionate through the model mucosal-barrier and therefore, presenting an enhanced targeting of beclomethasone dipropionate in the treatment of COPD.

Pardakhty *et al.,* (2006)[118] have studied the encapsulation of insulin within the niosomes prepared polyoxy ethylene alkyl ethers (Brij 35, Brij 58 and Brij 92) by the film hydration technique. They have found that both Brij 35 and Brij 58 were unable to form niosomes without cholesterol owing to comparatively larger polar-head groups with reference to their alkyl chains. The vesicle sizes of these niosomes were depended on the cholesterol content as well as the incorporation of charges or the hydrophilicity of surfactants used in the preparation of niosomes. The encapsulation of insulin within the bilayer structure of niosomes guarded it against the proteolytic activity of α-chymotrypsin, pepsin and trypsin *in vitro*. The maximum protection activity against the proteolytic activity of α-chymotrypsin, pepsin and trypsin *in vitro* was observed when Brij 92 to cholesterol was employed in a molar ratio of 7:3. These niosomes containing insulin exhibited only 26.30 ± 3.98 % of encapsulated insulin was found to be released during 24 hours in the simulated intestinal fluid. The drug release kinetics for most of the niosomal formulations were best described by the Baker-Lonsdale equation indicating the diffusion-based drug delivery mechanism.

These results of this investigation indicated that niosomes of insulin can be developed for sustained releasing delivery of peptides and proteins such as insulin through oral route.

Fang *et al.,* (2001)[119] have evaluated the skin permeation and partitioning of an antibacterial agent of fluoroquinolone group, enoxacin within liposomes and niosomes. They assessed the effectiveness of these vesicle formulations of enoxacin after the topical application. The *in vitro* percutaneous enoxacin absorption through the nude mouse skin was estimated using Franz-diffusion cells. The effects of these enoxacin loaded liposomes and niosomes on the physicochemical properties and stabilities were evaluated. The improved enoxacin delivery across the nude mouse skin from these enoxacin loaded liposomes and niosomes was observed after choosing the appropriate vesicular formulations. The optimized formulation of enoxacin was found able to reserve a larger amount of enoxacin in the nude mouse skin. A significant correlation between the skin permeation and the cumulative amount of enoxacin in the nude mouse skin was seen. Both the effects of permeation enhancer as well as the direct vesicle fusions with the stratum corneum were found to contribute to the skin permeation of enoxacin across nude mouse skin. These enoxacin loaded niosomes have revealed a higher stability after 48 hours of incubation as compared to enoxacin loaded liposomes. The addition of cholesterol enhanced the stability of enoxacin loaded liposomes consistent with the results from the enoxacin encapsulation and the turbidity. However, the addition of negative charges decreased the stability of the enoxacin loaded niosomes. The capability of the liposomes and niosomes to modulate the drug delivery without any significant toxicity, which indicated that these two vesicles (enoxacin loaded liposomes and niosomes) as useful for topical delivery.

Jin *et al.,* (2013)[120] have developed novel niosomal formulations for the oral delivery of *Ginkgo biloba* extract. They formulated these niosomes containing *Ginkgo biloba* extract through the film dispersion homogenization technique. The formulated niosomes containing *Ginkgo biloba* extract was spray-dried or freeze dried to enhance the stability of the niosomal formulations. These niosomes were characterized for their vesicle morphologies, vesicle sizes, zeta potentials, entrapment efficiencies, angle of reposes and thermal stability. The vesicle sizes of optimized niosomes containing *Ginkgo biloba* extract, prepared using Span 80, Tween 80 and cholesterol was measured about 141 nm. A significant difference ($p< 0.05$) was observed in the drug entrapment efficiency between the spray-drying (77.50 %) and the freeze-drying (50.10 %). The stability evaluation of the optimized niosomes containing *Ginkgo biloba* extract revealed that there was absence of any significant changes in the drug entrapment efficiency for these niosomes at 4°C as well as at 25°C after 3 months period. The *in vitro* release analyses suggested that these optimized niosomes containing *Ginkgo biloba* extract were able to prolong the release of *Ginkgo biloba* extract in the phosphate buffer solution (pH 6.8) over 48 hours. The *in vivo* evaluation of these optimized niosomes containing *Ginkgo biloba* extract demonstrated in rats that the *Ginkgo biloba* flavonoid glycoside content in the lung, heart, kidney, brain and blood of rats treated with the niosomes was found greater than that in the rats treated with the oral *Ginkgo biloba* extract tablet ($p< 0.01$). Any trace of the *Ginkgo biloba* flavonoid glycoside content was detected in the brain tissue of rats treated with the oral *Ginkgo biloba* tablets. However, the *Ginkgo biloba* flavonoid glycoside content was detected in the brain tissue of rats, when rats are treated with the optimized niosomes containing *Ginkgo biloba* extract. Thus, these niosomes containing *Ginkgo biloba* extract had shown a promise for the oral delivery of *Ginkgo*

biloba extract to the brain with enhanced oral bioavailability and to substitute the conventional *Ginkgo biloba* extract tablets.

Azeem *et al.,* (2008)[121] have formulated and evaluate the niosomal vesicles containing frusemide in order to enhance transdermal permeation of frusemide. The formulation development of these niosomes containing frusemide was performed to analyze the process variables on the preparation and properties of these niosomal formulations. The process variables investigated such as type of Spans, Span to cholesterol ratio, cholesterol to dicetyl phosphate ratio, concentration of frusemide, solvent type, hydration media and hydration time were found to affect the preparation and properties of these niosomal vesicles containing frusemide. The maximum frusemide entrapment efficiency of 77.73 ± 2.36 % was observed with the niosomes formulated from Span 60 ∶ cholesterol ∶ dicetyl phosphate (47.5 ∶ 47.5 ∶ 5) and chloroform : methanol (4 ∶ 1) as the solvent system at the 1 hour of hydration time. The stability of the niosomes containing frusemide in terms of drug holding ability was evaluated for 30 days on storage. A direct correlation was seen between the percentage leaching of frusemide out of the niosomal vesicles and temperature. The higher transdermal flux of frusemide was attained with the frusemide niosomal gel (9.20 ± 0.50 μg/cm^2/hour) as compared to the conventional frusemide gel (6.40 ± 0.30 μg/cm^2/hour).

Pillai *et al.,* (1999)[122] have incorporated indomethacin within niosomes. In this investigation, the effect of indomethacin encapsulated niosomal formulations on the platelet functions like the inhibition of aggregation and ATP release induced through a range of agonists such as adenosine diphosphate (ADP), epinephrine, arachidonic acid, and ristocetine were investigated. The feasibility of these indomethacin encapsulated niosomal formulations based carrier-mediated drug delivery to the

platelets were also explored by this investigation. These indomethacin encapsulated niosomes were of MLV, which were prepared from Tween 60 through the lipid hydration technique. The freshly prepared human platelet rich plasma was employed for the aggregation/inhibition analyses. The extent of aggregation/inhibition was measured as an alteration in the light transmission, which was measured through the Chronolog Aggregometer. The percentage inhibition of the aggregation induced through the agonist ADP, ranged from 28.21 ± 0.28 % at the 2.0 μM level to 92.60 ± 1.20 % at 12.7 μM of the encapsulated indomethacin; while the same concentrations of the indomethacin found to inhibit the aggregation only to the extent of 13.75 ± 0.13 % and 36.82 ± 0.57 %, respectively. 100 % of aggregation inhibition induced by the arachidonic acid was attained by the action of indomethacin encapsulated niosomes; while the inhibition by the free indomethacin was found 41.90 % at the equimolar concentrations. The ATP release study demonstrated that the 100 % inhibition was attained by 8 μM of the encapsulated indomethacin; while the inhibition by the free indomethacin was 40.00 ±1.82 %. Thus, at the equimolar doses, the indomethacin encapsulated niosomal formulation demonstrated a more proficient activity in inhibiting the platelet aggregation than the free indomethacin, probably because of the greater quantity of the indomethacin reaching the specific-site of inhibition within the interior of the platelets as well as performing directly on the cyclooxygenase (COX) system to avert the formation of thromboxane.

Carter *et al*., (1989)[67] have reported that the multiple dosing with sodium stibogluconate-loaded niosomal formulations was found to be effectual against the parasite causing leishmaniasis. They have also investigated the characteristics and capability of sodium stibogluconate-loaded niosomal formulations to clear parasites from the spleen, liver and bone marrow in the *Leishmania donovani* infected BALB/c

mice. These sodium stibogluconate-loaded niosomal formulations were found to be effectual against the *Leishmania donovani* in the spleen, liver and bone marrow as compared to the simple solution of sodium stibogluconate. Therefore, these sodium stibogluconate-loaded niosomal formulations can be employed in the treatment of visceral leishmaniasis.

Jadon *et al.,* (2009)[43] have investigated the formulation of griseofulvin loaded niosomes for the use in oral administration. They have observed an enhanced oral bioavailability of griseofulvin through the oral administration of griseofulvin loaded niosomes. An *in vitro–in vivo* correlation demonstrated that niosomes were the efficient way to enhance the oral bioavailability as well as sustained delivery of griseofulvin through the oral administration.

In an investigation, Sharma *et al.,* (2009)[87] have formulated Span 60-based niosomal formulations containing fluconazole (an antifungal drug) for oral use. They have found the formulated Span 60-based niosomal formulations containing fluconazole contained an encapsulation efficiency of greater than 91 %. These niosomal formulations demonstrated sustained drug release pattern by the zero-order followed by first-order kinetics.

Pavala Rani *et al.,* (2010) [88] have developed niosomes loaded with rifampicin and gatifloxacin to investigate the effectiveness of the developed formulations against tuberculosis. They have observed that the orally administered niosomes loaded with rifampicin and gatifloxacin were found effective against the tuberculosis causing bacteria, *Mycobacterium tuberculii* for a prolonged period. It was also seen that these niosomes exerts better results than the conventional dosage forms due to the reduced dosing as well as greater patient compliances.

Raja Naresh *et al*. (1994)[58] have reported the anti-inflammatory activity of niosome encapsulated diclofenac sodium in the arthritic rats. They have formulated these niosomes using Tween 85 and cholesterol. They have also found that the niosomal formulation prepared through employing a 1 : 1 combination of Tween 85 and cholesterol exhibited an improved consistent in the anti-inflammatory activity for more than 72 hours after the administration of single-dose.

Florence and Cable, (1993) [89]have reported the development of [59]Fe-deferroxamine trioxyethylene cholesterol niosomal vesicles containing doxorubicin for the use in intravenous administration. They observed that the distribution of such niosomal vesicles was depended upon the vesicle-size as apparent from the result indicating the greater distribution in liver as well as spleen. The results indicated that the encapsulation of doxorubicin within the niosomes decreases the cardiac toxicity upon intravenous administration.

Aggarwal *et al.,* (2007)[95] have prepared and evaluated bioadhesive coated niosomal formulations containing acetazolamide using Span 60, dicetyl phosphate or cholesterol stearylamine. These bioadhesive coated niosomal formulations containing acetazolamide demonstrated more affinity for the reduction of intra-ocular pressure as compared to the marketed formulation of acetazolamide, Dorzolamide.

In another investigation, the same group (Aggarwal and Kaur, 2005)[96] have formulated chitosan coated niosomal formulations containing timolol maleate (0.25 %). The chitosan coated niosomal formulations containing timolol maleate (0.25 %) displayed more effectiveness for the reduction of intra-ocular pressure intraocular

pressure as compared to the marketed formulation of timolol maleate (0.25 %) with lesser chances of cardiovascular side-effects.

Blazek-Walsh *et al.,* (2001)[61]have reported the formulation of niosomes from maltodextrin-based proniosomes. This affords a rapid reconstitution of niosomes with the minimal residual carrier. The slurry of maltodextrin and surfactants was dried to form a free-flowing dry powder, which could be rehydrated through the addition of warm water.

Yoshida*et al.,* (1992)[76] have investigated the oral delivery of 9-desglycinamide and 8-arginine vasopressin encapsulated in the niosomes in the *in vitro* intestinal-loop model. They have reported that the stability of these encapsulated peptides found increased significantly.

Rogerson *et al.,* (1987)[75] have developed adriamycin-loaded niosomes. The efficiency of entrapment of aqueous solutions of adriamycin was found actually dependant on neither the niosomal vesicle composition nor the method of preparation and evidence of a degree of surfactant/adriamycin association. The light induced adriamycin degradation was found decreased through the encapsulation of adriamycin within the niosomes. The efflux of encapsulated adriamycin was found reduced through the incorporation of cholesterol into the these niosomal vesicles to that reported for the liposome preparations.

4. MATERIALS AND METHODS

4.1. Preformulation Studies

Preformulation is defined as the stage of research and development process where physical, and chemical characteristics of a new drug entity are characterized alone and when combined with excipients in order to develop stable, safe and effective dosage form.

A thorough understanding of physicochemical properties may eventually provide a rationale for formulation design or carry, the need for molecular modification or purely confirms that there are no significant hindrances to the development of a substance. Therefore, preformulation studies were performed on the obtained sample of drug for solubility analysis, identification and compatibility studies.

4.1. 1. Solubility analysis

Preformulation solubility analysis was done, which include the selection of a suitable solvent to dissolve 5-Fluorouracil (5-FU) and Leucovorin (LV) as well as various excipients.

4.1. 2. Compatibility studies

Compatibility of drug and lipids which are used to prepare niosomes was established by infrared absorption spectra and DSC analysis.

4.1. 3. FTIR Spectral analysis

FTIR spectral analysis of pure drug and excipients was carried out and observation was made whether changes in the chemical constitution of drug after combining it with the excipients occurred. The samples were crushed with KBr to get pellets by applying pressure on 600 Kg/cm^2 and scanned with the IR instrument (Shimadzu, 8400 Series, Tokyo, Japan) from 400-4000cm^{-1}.

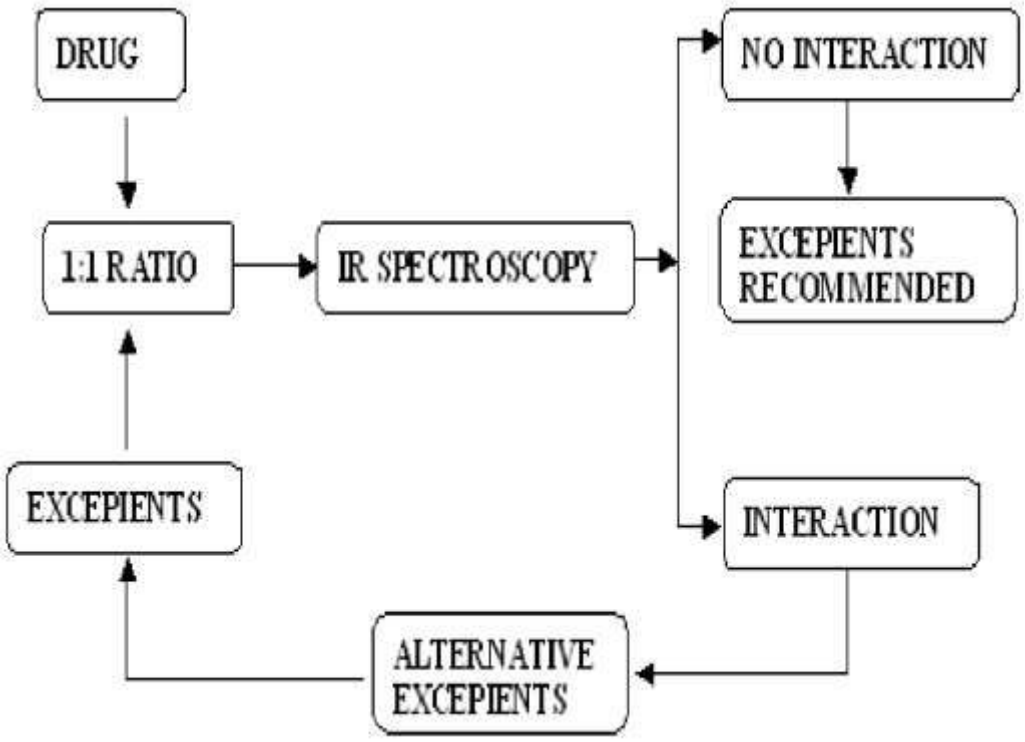


Fig 4.1. Schematic representation of compatibility studies

Infra Red spectroscopy is one of the most powerful analytical techniques to recognize functional groups of a drug.

4.1. 4. DIFFERENTIAL SCANNING COLORIMETRY (DSC)

In Differential Scanning Colorimetry, the samples were heated from 25°C to 100°C at a constant temperature increment of 10° C/min and purged with nitrogen gas at 40 ml/min.

- **Calibration curve**

The calibration curve for 5-Fluorouracil and Leucovorin (LV) was prepared by using phosphate buffer (pH 7.4).

- **Phosphate buffer pH 7.4 -** Phosphate buffer was prepared by placing 25ml of 0.2M potassium dihydrogen orthophosphate solution and 19.55ml of 0.2N sodium hydroxide solution in a 100ml volumetric flask and volume was made up to the mark with distilled water.

❖ **Primary stock solution for 5-Fluorouracil and Leucovorin (LV)with phosphate buffer (pH 7.4)**

Accurately weighed 100mg of 5-Fluorouracil and Leucovorin (LV) was dissolved in 10ml of ethanol in a 100 ml of volumetric flask and volume was made up to the mark with pH 7.4 buffer to get a concentration of 1000µg/ml.

❖ **Secondary stock solution for phosphate buffer (pH 7.4)**

From primary stock solutions of phosphate buffer 10ml was pipetted out in a 100ml of volumetric flask and volume was made up to the mark with pH 7.4 buffer to obtain a concentration of 100µg/ml.

From secondary stock solutions 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1.0ml were pipetted out and diluted to 10ml with respect buffers to get a concentration range from 5µg/ml to 50µg/ml. The samples were analyzed spectrometrically at wavelength of 266 nm for 5-FU and 288 nm for LV.

The calibration curve was plotted with the absorbance Vs concentration (µg/ml) and r^2 and slope were calculated.

4.2. Preparation of Niosomes

Niosomes were prepared by a thin film hydration method using a surfactant mixture consisting of (span 40 and 60, tween 60 and 80) and cholesterol, at various ratios, at different specified ratios as given in Table. Surfactant and cholesterol was dissolved in 8 ml of diethyl ether and the drugs were dissolved in 2 ml of ethanol. Then the mixture was poured to a round bottom flask, and the solvent was evaporated under reduced pressure at a temperature 20-25º, using a rotary flash evaporator until a thin film of lipid layer was formed. The formed film was hydrated with 10 ml of

phosphate buffer saline pH 7.4. The hydration was continued for 1 h, while the flask was continued to rotate at 55-65°. The niosomes which were hydrated were sonicated for 20 min using a bath sonicator to obtain niosomal dispersion.

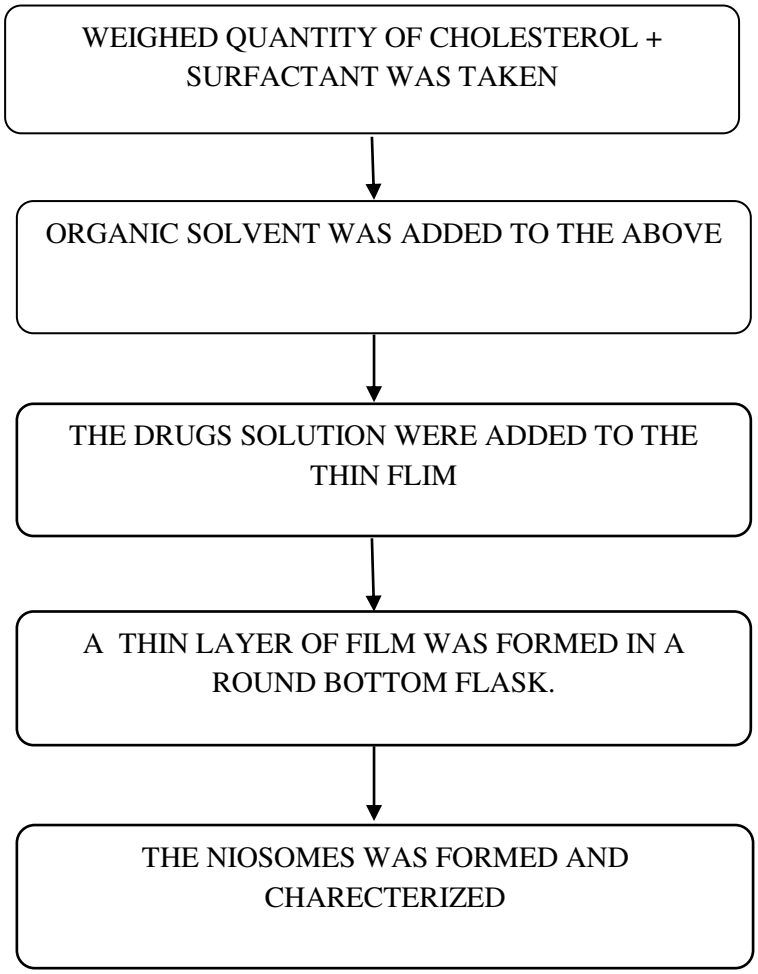


Fig 4. 2: Flow Chart for Preparation of Niosomes

Table 4.1: Formulation composition of Niosomes

Type of formulation	F1	F2	F3	F4	F5	F6	F7	F8
Drug	10	10	10	10	10	10	10	10
Cholesterol	10	20	10	20	10	20	10	20
Span 40	10	10	-	-	-	-	-	-
Span 60	-	-	10	10	-	-	-	-
Tween 40	-	-	-	-	10	10	-	-
Tween 60	-	-	-	-	-	-	10	10
Drug :cholesterol: SA	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1

4.3. Evaluation of Niosomes

4.3.1. Entrapment efficiency for the Niosomes

The niosomal dispersion is a homogeneous milky white suspension which is centrifuged at 12000 rpm for 15 min and entrapment efficiency of niosomes were evaluated by measuring the ultraviolet absorption of the supernatant. The corresponding calibration curves were made by testing the supernatant of blank niosomes. Each sample was measured in triplicate. 5-FU was measured at 266 nm where an intense characteristic peak was displayed while LV showed a characteristic peak at 288 nm. The percentage of the entrapment of the drug can be obtained for the niosomal formulations.

4.3.2. Particle Size Analysis (PSA)

The size division of the niosomes was determined using the particle size analyzer (Beckman Coulter, Delsa nano C, Brea, USA) prepared with a dry accessory system. Sample was diluted with water and temperature maintained at 25°C.

4.3.3.Zeta Potential Analysis

The zeta potential was measured using the appropriate instrument (Beckman Coulter Delsa Nano C, Brea, USA). The sample was made to dilute with distilled water and tranferred in the cuvettes and temperature maintained at 25°C.

Zeta potential is the potential difference between the continuous phase and the stationary layer of the liquid attached to the particle dispersed. Stability of colloidal dispersions (dispersed systems, multi particulate, multi charge systems) can be predicted by Zeta potential value.

Zeta potential indicates the degree of repulsion between adjacent, similarly charged particles (likely charged particles) in dispersion. A high Zeta potential value will indicate stability for molecules and particles that are small enough (i.e. the solution or dispersion will resist aggregation/ do not flocculate).

Colloids having higher Zeta potential are made to stabilize electrically while colloids with Low Zeta potential tends to coagulate or flocculate.

Table 4.2: Zeta Potential Range

Zeta Potential (mV)	Stability behaviour of colloid
From 0 to ±5 range	Rapid coagulation or Flocculation
From ±10 to ±30 range	Insufficient instability
From ±30 to ±40 range	Medium stability
From ±40 to ±60 range	Good stability
More than ±61 range	Excellent stability

4.3.4. Morphological analysis of niosomes

Transmission electron microscopy (TEM) was used in the morphological analysis of niosomes. Samples were prepared by placing one drop of freshly prepared nano-suspension on a copper grid with carbon coated film support and the excess fluid was removed by using filter paper. Samples were viewed directly with TEM (JEM-100, JEOL, Tokyo, Japan) once they were made to dry under room temperature without any further staining.

The Scanning Electron Microcopy (SEM) is used to identify the particle size and the surface morphology of the nioosomes. For niosomes, the drop of the sample is placed on the covered glass slide and then dried by applying vacuum, later it was coated with gold to a thickness of 100Å using VEGAS TESCAN Vacuum evaporator and the image was captured for the niosomal formulation.

Vesicle dispersions were characterized by photo microscopy for vesicle formation and morphology. The size and shape of vesicles in niosomes formulations were observed by optical microscopy using a calibrated eyepiece micrometer, and photographs were taken at × 400 magnifications with a digital camera (Olympus, 8.1 megapixel, Japan).

4.3.5. *In-vitro* release studies for the niosomes

In vitro release of 5-FU and LV niosomes was conducted by a dialysis membrane having a pore size of 2.4 mm (LA-395-5Mt Himedia Pvt. Ltd, Mumbai, India) with 75 ml of pH 7.4 phosphate buffer at 37°C. Briefly in a 100 ml beaker 75ml of pH 7.4 phosphate buffer was placed. 2 ml of niosomal formulation was transferred into a dialysis bag and made to dip into the buffer solution. The dialysis membrane was activated earlier using by soaking in 1% w/v NaOH over night. The flask was placed on a magnetic stirrer. Stirring was continued to maintain at 250 rpm and temperature of the buffer was maintained at 37°C. Sampling was done by withdrawing 1 ml of aliquots from a beaker. Immediately 1 ml of new buffer was added to keep the sink condition. Samples were analyzed after sufficiently diluting with buffer by using a UV-Visible Spectrophotometer (UV/VIS-Double beam Spectrophotometer, V-530, Jasco, Tokyo, Japan) at a wavelength of 266 nm for 5-FU and 288 nm for LV.

Figure 4.3: *In vitro* release study of niosomes

4.3.6. *In vitro* release Kinetic study

The release data obtained were fixed into various mathematical models like zero order, First Order, Higuchi and Korsmeyer-Peppas to know which mathematical model was best fitting the obtained release profile.

In order to analyze the release mechanism, several release models were experiencing such as

Zero order $Q_t = Q_0 + K_0 t$ (1)

Where, Q_t is the amount of drug released at time t,

K_0 is the apparent dissolution rate constant or zero order release constant and

Q_0 is the initial concentration of the drug in the solution resulting from a burst effect; in this case the drug release runs at a constant rate.

It describes the systems where the drug release rate is autonomous of its concentration of the dissolved substance.

A graph was plotted against time taken on x-axis and the cumulative percentage of drug release on y-axis and it yields a straight line.

Application: This relationship can be used to describe the drug dissolution of various types of modified release dosage forms, as in the case of some transdermal systems, as well as matrix particles with low soluble drugs in coated forms, osmotic systems, etc.

The release of the drug that obeys first order kinetics may be given by the equation:

$$dc/dt = -Kc$$

Where K is first order rate constant expressed in units of $time^{-1}$.

Equation can be expressed as:

$$\log C = \log C_0 - Kt / 2.303$$

Where C_0 is the initial concentration of drug, k is the first order reaction rate constant, and t is the time taken. The data obtained are plotted as log cumulative % of the remaining drug against time, which will give a straight line with a slope of -K/2.303.

Application: This relationship can be used to explain the drug dissolution in pharmaceutical dosage forms such as those containing water-soluble drugs in permeable matrices.

The Higuchi release equation is

$$\mathbf{Q = K_H t^{1/2}}$$

Where

Q is the cumulative amount of drug release at time "t"

K_H is Higuchi constant

T is time in hours

The Higuchi equation suggests that drug release by diffusion.

A graph is plotted between the square root of time taken on x-axis and the cumulative percentage of drug release on y-axis and it gives a straight line.

Application: This relationship can be used to illustrate the drug dissolution from several types of modified release pharmaceutical dosage forms, as in the case of some transdermal systems and matrix particles with water soluble drugs.

The korsmeyer-peppas equation was given as

$$\mathbf{F = (M_t/M) = K_m t^n}$$

Where

F is fraction of drug released at time‘t’

Mt is amount of drug release at time‘t’

M is total amount of drug in dosage form

K_m is kinetic constant

n is diffusion or release exponent

t is time in hours

n is estimated from linear regression of log (Mt/M) versus log t

If n = 0.45 it indicates fickian diffusion

$0.45<n<0.89$ it indicates anomalous diffusion or non-fickian diffusion

Anomalous diffusion or non-fiction diffusion refers to a combination of both diffusion and erosion controlled rate rules.

A graph was plotted between the log time release on x-axis and the log cumulative percentage of drug release on y-axis and it gives a straight line.

Interpretation of drug release mechanism is given in following Table 4.3.

Table 4.3 Interpretation of Drug Release Mechanism

Release exponent (n)	Drug release mechanism	Rate as a function of time
0.5	Fickian diffusion	$t^{-0..5}$
0.5 < n <1	Anomalous transport	t^{n-1}
1	Case-II transport	Zero order release
> 1	Super case II transport	t^{n-1}

4.4. Sterility Test for Niosomes

After sterilization of 5-FU and LV loaded niosomes, the formulations were subjected to sterility test. The test for sterility is intended for detecting the presence of viable forms of bacteria, fungi and yeast in preparations. The tests were carried out under aseptic conditions designed to avoid accidental contamination of the product during the test.

Culture Media

A. For aerobic Bacteria and Fungi: Fluid thioglycollate medium:

This medium can be used for the detection of aerobic bacteria and fungi. Preparation of fluid thioglycollate medium Trypticase 15g, L-cystine 0.5 g, Dextrose 5g, Yeast extract 5g, Sodium chloride 2.5 g Sodium Thioglycollate 0.5 g, Reazurin 0.001 g, Agar 0.75 g .The above ingredients were dissolved completely in 1000ml of distilled water and the medium was boiled for 10 minutes. The pH was adjusted to 7.2 ± 0.2. Media was distributed into 4 ½ size test tubes and sterilized by autoclaving at a pressure of 15 lbs/in 2 and at a temperature of 121°C for 15 minutes. Autoclaved medium was kept in room temperature.

For Anaerobic Bacteria Chopped meat (CM) medium: This medium can be used for the detection of anaerobic bacteria.

Preparation of chopped meat medium

Lean ground beef 500 g, Distilled water 1000 ml, Sodium hydroxide (In Solution) 25 ml b. Trypticase 30 g, Yeast extract 5 g, Dipotassim hydrogen phosphate 5 g L-cystine 0.5 g, Hemin (1% solution) 0.5 g, Vitamin K (1% alcoholic solution) 0.1 ml Procedure 1. 500g meat was mixed with 1000ml distilled water and 25ml sodium hydroxide.It was heated to boiling while stirring. After mixture has cooled,it was refrigerated overnight at 4°C. Mixture was filtered through two layers of gauze. Meat particles were retained and liquid was filtered. Enough distilled water was added to filtrate to give final volume of 1000ml. All ingredients were added into above two liquids except L-cystine. It was heated until ingredients dissolved completely, cooled to less than 50°C and L-cystine was added and mixed to dissolve it completely.The pH of broth was adjusted to 7.4. Meat particles were washed several times with distilled water to remove excess sodium hydroxide and spreaded thinly on clean towel to partially dry. About 0.5g meat particles was dispensed with small scoop into 15X90mm screw cap tubes. 7ml enriched broth filtrate was added to each tube. The tubes were autoclaved at 1210°C for 15 minutes. Tubes were cooled and with caps loosened, they were passed into anaerobic glove box to cover the surface of the media with white petroleum jelly, so that atmosphere of approximately 85% nitrogen (N2), 10% hydrogen (H2) 5%carbon dioxide (CO2) replaces air in tubes. After caps are tightened securely, tubes were removed from glove box. Chopped meat (CM) medium tubes were stored in refrigerator at 4°C at ambient temperature.

Procedure

The niosomal formulation (F4) was added to the fluid thioglycollate medium and incubated at 20-25°C for not less than seven days. Chopped meat medium is incubated for not less than fifteen days. At intervals during the incubation period, the

media were examined visually for microbial growth. In order to support the above performed test, a positive control and negative control tests were also carried out. Positive control test was carried out in order to confirm that the media and the environment provided for incubation were suitable for the growth of micro organisms. α - haemolytic Streptococci were inoculated into the fluid thioglycollate medium and Clostridium tetani was inoculated into the chopped meat medium and the growth promotion was observed.

4.5. Stability Study of Niosomes

Stability studies for the optimized niosomes (F4) were carried out at refrigeration temperature, room temperature and elevated temperature (4 ± 2°C, 25 ± 2°C/ 60 % RH ± 5 % RH and 37 ± 2°C/ 65 % RH ± 5 % RH) for a period of three months. Samples from each batch were withdrawn at definite time intervals and evaluated using following parameters like appearance, size, assay, and dissolution.

4.6. Preclinical Anti Colon Cancer Evaluation of Niosomes

4.6.1. Chemicals:

The 1, 2-dimethyl hydrazine (DMH) was obtained from Sigma Chemical Company, Mumbai, India and all other chemicals and reagents used were of analytical grade.

4.6.2. Animals

Male Sprague-Dawley rats (300-400gm body weight) were procured from KMCH college of Pharmacy, Coimbatore, Tamilnadu, India. All the animals were kept at room temperature of 22°C under 12 hr light/12 hr dark cycle in the animal house. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. All animal procedures were performed in accordance with the recommendation of CPCSEA the proper care and use of laboratory animals the

proposal of the present study was approved by IAEC of KMCH college of Pharmacy, Coimbatore, Tamilnadu, India

4.6.3. Preparation of DMH solution

DMH was dissolved in 1 mm EDTA just prior to use and the pH was adjusted to 6.5 with 1 mm sodium bicarbonate to ensure the stability of the chemical.

4.6.4. Induction of Colon Cancer

Animals were given a weekly subcutaneous (s.c.) injection of DMH in the groin area at a dose of 20 mg/kg body weight for 15 weeks.

4.6.5. Study design

The animals were divided into five groups of six animals each as follows,

Group I: Control, received 1 ml of Normal saline. p.o everyday for 15weeks

Group II: Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c)

Group III: Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c) + 5 fluorouracil (20mg/kg) i.p

Group IV: Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c) + 5-FU and Leucovorin market formulation.

Group V: Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c) + 5-FU and Leucovorin Niosomes formulation.

4.6.6. Blood Collection

After end of treatment period, the animals were anaesthetized with ketamine 2mg/kg (i.p route), blood was collected by Retro orbital puncture , with EDTA and 7

without EDTA for the enumeration of blood cell (i.e. RBC, WBC,),estimation of Hemoglobin and for estimation of various biochemical parameters respectively.

Separation of Serum

For estimating the biochemical parameters such as SGOT, SGPT, ALP and total protein the serum was separated from blood by centrifuging at 10,000 rpm for 10 minutes. The separated serum were collected and used for the parameter estimation.

Separation of Plasma

For the estimation of tumour markers such as Alpha-feto-protein (AFP), Carcinoembroyonic antigen (CEA), the blood was collected with EDTA, and centrifuged at 10,000 rpm for 5 min and the separated plasma was used for the parameter estimation.

4.6.7. Estimation of Haematological Parameters[123]

4.6.7.1. Estimation of RBC

Procedure

The RBC pipette was filled with blood up to the mark 0.5, immediately RBC diluting fluid (Hayem's fluid) was filled up to the mark 101. Pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept aside for a while. The counting chamber was placed and the RBC squares were focused under low power first, when markings were identified then turn to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again, the counting chamber was charged with the mixed blood. After charging mount the slide, allow the fluid to settle then using a 45X lens the RBC

4.6.7.2. Estimation of WBC

Procedure

The WBC pipette was filled with blood up to the mark 0.5, immediately RBC diluting fluid (Hayem's fluid) was filled up to the mark 11.Pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept aside for a while. The counting chamber was placed and the WBC squares were focused under low power first, when markings were identified then turn to high power .The first 3-4 drops of blood mixture was discarded and it was mixed once again, the counting chamber was charged with the mixed blood. After charging mount the slide, allow the fluid to settle then using a 10X lens the WBC were counted uniformly in corner squares. The number of cells was expressed as $10^{[9]}$ Cmm.

4.6.7.3. Estimation of Haemoglobin

Procedure

The heamoglobinometer tube was filled with N/ 10 HCl up to the marking 10, to this 20µl of blood was added with the help of pipette. The superficial ac1d was sucked and it rinsed repeatedly till all the blood in the pipette washed out in mud. The contents in the tube were mixed by stirring, and allowed to stand for 10 minutes. A clear brown colour solution was formed due to the formation of acid hematin. Then distilled water was added drop by drop to dilute. The colour of diluted fluid was compared with the standard; dilution was continued until the colour of the flu1d exactly matches the standard. The lower meniscus of the fluid was noted and reading was noted directly 31 ~ from the graduated tube g/ percentage of haemoglobin.

4.6.7.4. Estimation of Serum glutamate oxalo acetate transaminase (SGOT)

The enzyme catalyzes the reaction,

L-aspartate + 2-oxoglutarate → oxaloacetate + L-gluatamate

The enzyme activity was assayed by the method of Reitman and Frankel [124]

Reagents:

- ✓ Substrate:1.33 g of L-aspartic acid and 15 mg of 2-oxo glutaric acid were dissolved in 20.5 ml of buffer and 1 N sodium hydroxide to adjust the pH to 7.4 and made up to 100 ml with the phosphate buffer.
- ✓ 0.1N Sodium hydroxide
- ✓ 2, 4-dinitro phenyl hydrazine (DNPH) (0.2% in 1 N HCl).
- ✓ Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains 1 μmole of pyruvate/ml

Procedure:

1 ml of buffered substrate was incubated at 37°C for 10 minutes. Then 0.2 ml of serum /tissue homogenate was added in the test tubes and incubated at 37°C for 30 minutes. The reaction was arrested by adding 1 ml of DNPH reagent and tubes were kept at room temperature for 20 minutes. Then 10 ml of 0.4N sodium hydroxide solution was added. A set of pyruvic acid was also treated in a similar manner for the standard. The colour developed was read at 520 nm against the reagent blank. The activity of the enzyme was expressed as IU/L / μmoles of pyruvate liberated/min/mg protein.

4.6.7.5. Estimation of Serum glutamate pyruvate transaminase (SGPT)

This enzyme catalyzes the reaction,

L-alanine + 2-oxoglutarate → oxaloacetate + L-gluatamate

The enzyme activity was assayed by the method of Reitman and Frankel [124]

Reagents:

- ✓ Phosphate buffer: 0.1 M; pH 7.4.
- ✓ Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxo glutaric acid weredissolved in 20 ml of buffer. About 0.5 ml of 1 N sodium hydroxide was added and made up to 100 ml with buffer.
- ✓ 0.1N Sodium hydroxide.
- ✓ 2, 4-dinitro phenyl hydrazine (DNPH): 0.2% in 1 N HCl.
- ✓ Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 μmole of pyruvate/ml.

Procedure:

1 ml of buffered substrate was incubated at 37°C for 10 minutes. Then 0.2ml of serum/tissue homogenate was added in the test tubes and incubated at37°C for 30 minutes. The reaction was arrested by adding 1 ml of DNPH reagent and tubes were kept at room temperature for 20 minutes. Then 10 ml of0.4N sodium hydroxide solution was added. A set of pyruvic acid was also treated in a similar manner for the standard. The colour developed was read at 520 nm against the reagent blank. The activity of the enzyme was expressed as IU/L / μmoles of pyruvate liberated/min/mg protein.

4.6.7.6. Estimation of Lipid parameters

Extraction of serum lipids

Serum lipids were extracted according to the method of Folch et al[125].

Reagents

- ✓ Alcohol and ether as a mixture (3: 1, v/v)
- ✓ Chloroform-methanol mixture (1 : I , v/v)

Procedure

1ml of serum sample was extracted with 3: 1 v/v alcohol ether mixture into 15ml test tubes and kept at 65°C in a water bath for 2 hours. Cooled and centrifuged for 30 mins. Then another 6 ml of 3: l alcohol-ether mixture was added to the residue and heated for 2hrs at 65°C.Centrifuged and decanted the supernatant to the same tube containing previously decanted filtrate. Then 6 ml of 1: 1 chloroform methanol rnixture was added to the residue and heated at- 65°C for 1 hr. Centrifuged and decanted the supernatant to the same tube containing the filtrate previously decanted. The total volume was made to 25ml with chloroform methanol mixture. This was used for the estimation of total cholesterol, phospholipids (PL) and triglycerides (TG).

4.6.7.6.1. Estimation of Cholesterol

Total cholesterol was estimated by the method of Zlatkis et al[126].

Reagents

- ✓ Ferric chloride (0.05 per cent solution of $FeC1_3.6H_2O$ in acetic acid).
- ✓ H_2S0_4
- ✓ Stock cholesterol standard, 100 mg in 100 ml acetic acid.
- ✓ Working standard. The stock standard was diluted 1 to 25 with ferric chloride-acetic acid reagent.
- ✓ Acetone-ethanol reagent (1 : 1)

Procedure

0.1 ml of serum was added to 10 ml of the ferric chloride acetic-acid reagent in a stoppered centrifuge tube. Mixed and allowed to stand for 10- 15 min for the proteins to flocculate. Then 5 ml of the clear supernatant fluid was transferred to a stoppered centrifuge tube after centrifugation. For standard, 0.1 ml of physiological saline was mixed with 10 ml of the cholesterol standard and 5 ml was transferred to a second stoppered centrifuge tube. As blank 5 ml. of the ferric chloride -acetic acid reagent was taken. 3 ml. of H_2S0_4 was added to all three tubes stoppered and mixed. Allowed to stand for 20 to 30 min. The unknown and standard were read against the blank at 560 nm in a spectrophotometer. The values were expressed as mg/dl.

4. 6.7.6.2. Estimation of Triglycerides

Triglycerides were measured by the method of Foster and Dunn [127].

Reagents

- ✓ Alumina (neutral-chromatography grade) -Wash the alumina with water until all the fine particles are removed. Dry it in an oven at 100 – 110 ℃ overnight, cool and store in a desicator. Stable for six months.
- ✓ Saponification reagent – 5 g of KOH was dissolved in 60 ml distilled water and to this 40 ml Isopropanol was added.
- ✓ Sodium metaperiodate reagent - 77 g of anhydrous ammonium acetate was dissolved in 700 ml distilled water and to this 60 ml glacial acetic acid and 650 mg of sodium metaperiodate were added. The contents were mixed and made up to 1000ml distilled water.

- ✓ Acetyl acetone reagent – To the 0.75 ml of acetyl acetone 20 ml of isopropanol was added and the contents were mixed and to this Add 80 ml of distilled was added.
- ✓ Stock standard solution -4 mg/ml of triolein.

Procedure:

0.1ml of serum was taken and to this 0.6 ml of saponification reagent was added and incubated at 60 - 70º C for 15 minutes. The mixture was allowed to cool and to this 1 ml of sodium metaperiodate solution was added and mixed well followed by 0.5 ml acetyl acetone reagent and mixed. The final contents were incubated at 50 º C for 30 minutes. After cooling the color was read at 405 nm in a spectrophotometer. The estimated triglyceride was expressed as mg/dl.

4.6.7.7. Analysis of Tumor Markers

4.6.7.7.1. Estimation of Carcinoembryonic antigen (CEA)

CEA is a cell-surface 200-kd glycoprotein. Elevated plasma levels are related to the stage and extent of the disease, extent of the differentiation in the tumour, area of metastasis. CEA is also found in normal tissue.

The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to CEAThen the samples were added to the suitable microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for CEA. Next, Avidin conjugated to Horseradish Peroxidase was placed to all the microplate wells and incubated. After that a TMB substrate solution was placed to all wells. Only those wells that contain CEA, biotin-conjugated antibody and enzyme-conjugated Avidin may show a change in colour. The reaction between substrate and enzyme was terminated by the addition of a sulphuric acid solution

and the color change is measured spectrophotometrically at a wavelength of 450nm. Then the amount of CEA in the samples was assessed by comparing the O.D. of the samples to the standard curve.

Assay procedure

- ✓ Wells for diluted standard, blank and sample were determined. Seven wells for standard and 1 well for blank were prepared. 100 µl each of dilutions of standard, blank and samples was added into the appropriate wells. Covered with the plate sealer and incubate for 2 hours at 37 °C.
- ✓ The liquid was removed from each well.
- ✓ 100 µl of detection reagent A working solution was added to each well and incubated for 1 hour at 37 °C after covering it with the Plate sealer.
- ✓ The solution was aspirated and washed with 400 µl of 1x wash solution to each well using a squirt bottle, multichannel pipette, manifold dispenser or auto washer, and let it sit for 1~2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. Repeated for three times. After the last wash, remaining wash buffer was removed by aspirating or decanting. Plate was inverted and bloted with absorbent paper.
- ✓ 100 µl of detection reagent B working solution was added to each well. Incubated for 30 minutes at 37 °C after covering it with the plate sealer.
- ✓ The aspiration/wash process was repeated for five times as in step 4.
- ✓ 90 µl of substrate solution was added to each well. Covered with a new plate sealer and incubated for 15 -25 minutes at 37 °C. It was protected from light. The liquid turned blue by the ad dition of substrate solution.

- ✓ 50 µl of stock solution was added to each well. The liquid turned yellow by the addition of stock solution. The liquid was mixed by tapping the side of the plate. If color change does not appeared uniform, the plate was gently taped to ensure thorough mixing.
- ✓ The drop of water was removed and fingerprinted on the bottom of the plate and it was confirmed there was no bubble on the surface of the liquid. Then,the microplate reader was run and measurement was conducted at 450 nm immediately.

Calculation

- ✓ The standard curve was plotted by using the average absorbance for all standards on the x axis against the concentration on the y-axis and the best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the CEA concentrations versus the log of the O.D. and the best fit line was determined by regression analysis.

Detection range

- ✓ 0.156-10 ng/ml. The standard curve concentrations used for the ELISA's were 10 ng/ml, 5 ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml, 0.156 ng/ml

4.6.7.7.2. Estimation of Alfa- Fetoprotein (AFP)

AFP is a glycoprotein with a molecular weight of between 65,000 and 70,000 daltons including 4% of carbohydrate. During fetal development, AFP maintains high levels in the serum and drops to lesser levels throughout the rest of life. AFP values are higher in the malignant diseases of colon, hepatocellular, testicular nonseminomatous origin, and occasionally of other entodermal origin. AFP

measurement is widely accepted as tumor marker and for monitoring the therapeutic effectiveness of colon cancer and nonseminomatous testicular cancer.

Principle

The microtiter plate present along with this kit was already coated with an antibody which is specific to AFP. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated preparation which is specific for AFP. Then, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Specific wells that contain AFP, biotin-conjugated antibody, conjugated enzyme Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a solution of sulphuric acid and the change in colour was determined spectrophotometrically at a wavelength of 450 nm±2 nm. Then the amount of AFP in the samples was assessed by comparing the O.D. of the samples to the standard curve.

Sample collection and storage

- ✓ Serum– A serum separator tube was used and samples were allowed to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000x g. Freshly prepared serum was assayed immediately or store samples in aliquot at -20 or -80°Cfor later use.
- ✓ Plasma –Plasma was collected using EDTA or heparin as an anticoagulant. Samples were centrifuge for 15 minutes at 1000x g at 2 –8 °C within 30 minutes of collection. Serum was removed and assayed immediately or store samples in aliquot at -20 or -80°C for later use.

Reagent preparation

- ✓ All kit components and samples were kept atroom temperature (18-25 °C) before use.
- ✓ **Standard** : Standard was reconstituted with 1 ml of standard diluent, kept for 10 minutes at room temperature, shaked gently (not to foam). The concentration of the standard in the stock solution is 80 ng/ml. stock solutiondiluted to 20 ng/ml and the diluted standard serves as the high standard (20 ng/ml). Diluted standard was used to produce a dilution series. Each tube was thoroughly mixed before the next transfer. Seven points of diluted standard such as 20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml were setup, and the last EP tubes with standard diluent is the blank as 0 ng/ml
- ✓ **Assay Diluent A and Assay Diluent B:** 6mlof assay diluent A or B were diluted, concentrated with 6ml of deionized or distilled water to prepared 12 ml of assay diluents A or B.
- ✓ **Detection Reagent A and Detection Reagent B:** The stock detection A and B were centrifuged before use. The working concentration with working assay diluent A or B were diluted, respectively (1:100)
- ✓ **Wash Solution:**600 ml of wash solution was prepared as per instruction in the protocol
- ✓ **TMB substrate**: The needed dose of the solution was aspirated with sterilized tips and the residual solution was not dumped into the vial again.

Assay procedure

- ✓ W ells for diluted standard, blank and sample were determined. Seven wells for standard and 1 well for blank were prepared. 100 µl each of dilutions of standard, blank and samples was added into the appropriate wells. Covered with the plate sealer and incubate for 2 hours at 37 °C.
- ✓ The liquid was removed from each well.
- ✓ 100 µl of detection reagent A working solution was added to each well and incubatedfor 1 hour at 37 °C after covering it with the Plate sealer.
- ✓ The solution was aspirated and washed with 400 µl of 1xwash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or auto washer, and let it sit for 1~2 minutes. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. Repeated for3 times. After the last wash, remaining wash buffer was removed by aspirating or decanting. Plate was inverted and bloted with absorbent paper.
- ✓ 100 µl of detection reagent B working solution was added to each well. Incubatedfor 30 minutes at 37°C after covering it with the plate sealer.
- ✓ The aspiration/wash process was repeated for five times as in step 4.
- ✓ 90 µl of substrate solution was added to each well. Covered with a new plate sealer and incubated for 15 -25 minutes at 37°C. It was protected from light. The liquid turned blue by the addition of substrate solution.
- ✓ 50 µl of stock solution was added to each well. The liquid turned yellow by the addition of stock solution. The liquid was mixed by tapping the side of the

plate. If color change does not appeared uniform, the plate was gently taped to ensure thorough mixing.

- ✓ The drop of water was removed and fingerprinted on the bottom of the plate and it was confirmed there was no bubble on the surface of the liquid. Then, the microplate reader was run and measurement was conducted at 450nm immediately.

Calculation

- ✓ The standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and the best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of the AFP concentrations versus the log of the O.D. and the best fit line was determined by regression analysis

Detection range

- ✓ 0.312-20 ng/ml. The standard curve concentrations used for the ELISA's were20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml.

4.6.7.8. Evaluation of Enzymic Hepatic Antioxidants

4.6.7.8. 1.Estimation Superoxide dismutase (SOD)

This enzyme catalyzes the dismutation of superoxide anion ($O2^{-\cdot}$) to hydrogen peroxide and molecular oxygen in the following manner

$$H_2O + 2O_2^{-\cdot} + 2H^+ \rightarrow 2H_2O_2 + O_2$$

The enzyme activity was assayed by the method of Misra and Fridovich, [128].

Reagents

- ✓ 0.1 M Carbonate-bicarbonate buffer; pH 10.2.
- ✓ 0.6 mM EDTA solution
- ✓ 1.8 mM Epinephrine (prepared in situ)
- ✓ Absolute ethanol.
- ✓ Chloroform

Procedure

0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (cooled with ice) and then it was centrifuged. To the 0.5 ml of supernatant liquid, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. Then the reaction was proceeded by adding of 0.5 ml of 1.8 mM epinephrine (freshly prepared) and the increase in absorbance at 480 nm was measured.One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation.

4.6.7.8. 2.Estimation of Catalase (CAT, E.C. 1.11.1.6)

This enzyme catalyzes conversion of hydrogen peroxide into water and molecular oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The enzyme activity was assayed by the method of Sinha,[129].

Reagents

- ✓ Dichromate-acetic acid reagent: 5% potassium dichromate in water was mixed with glacial acetic acid in the ratio of 1:3 (v/v).
- ✓ 0.01 M Phosphate buffer; pH 7.0.
- ✓ 0.2M Hydrogen peroxide

Procedure

0.1 ml of the tissue homogenate was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0) pre-warmed to 37°C, 0.4 ml of distilled water and the mixture was incubated at 37°C. The reaction was initiated by the addition of 0.5 ml of 0.2 M hydrogen peroxide and the reaction mixture was incubated at 37°C for one minute. The reaction was terminated by the addition of 2 ml of dichromate-acetic acid reagent after 15, 30, 45, and 60 seconds. Standard hydrogen peroxide in the range of 4-20 μmoles were taken and treated in the same manner. All the tubes were heated in a boiling water bath for 10 minutes, cooled and the green colour that developed was read at 590 nm against blank containing all components except the enzyme. Catalase activity was expressed in terms of μmoles of H_2O_2 consumed/min/mg protein.

4.6.7.8. 3.Estimation of Glutathione peroxidase (GPx)

This enzyme catalyzes the reduction of H_2O_2 using glutathione as substrate.

$$\mathbf{2GSH + H_2O_2 \rightarrow GSSG + 2H_2O}$$

The enzyme activity was assayed by the method of Rotruck et al.[130].

Reagents

- ✓ 0.32 M Sodium phosphate buffer; pH 7.0.
- ✓ 0.8 mM EDTA
- ✓ 10 mM Sodium azide.
- ✓ 4mM reduced glutathione.
- ✓ 2.5 mM Hydrogen peroxide.
- ✓ 10%Trichloro acetic acid (TCA).
- ✓ 0.3M Disodium hydrogen phosphate.
- ✓ 0.04% 5,5' - dithiobis (2-nitro benzoic acid) (DTNB); 40 mg of DTNB in1% sodium citrate.
- ✓ 10 mM Standard reduced glutathione.

Procedure

The assay mixture containing 0.5 ml sodium phosphate buffer, 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, and 0.5 ml of 1:10 tissue homogenate was taken and the total volume was made up to 2.0 ml with distilled water. The tubes were incubated at 37 8C for 3 min and the reaction was terminated by the addition of 0.5 ml of 10% TCA. To determine the residual glutathione content, the supernatant was removed after centrifugation, and to this 4.0 ml disodium hydrogen phosphate (0.3 M) solution and 1 ml DTNB reagent were added. The colour that developed was read at 412 nm against a reagent blank containing containing only phosphate solution and DTNB reagent using a spectrophotometer Aliquot portions of the standard were treated similarly. The enzyme activity is expressed in terms of μg of GSH utilized/min/mg protein.

4.6.7.8. 4.Estimation of Glutathione-*S*-transferase (GST)

This detoxifying enzyme catalyzes the conjugation of xenobiotics/ electrophiles (E) with the reduced glutathione (GSH), which acts as an electrophile.

E + GSH → EH + GSSG

The enzyme activity was assayed by the method of Habig et al.[131]

Reagents

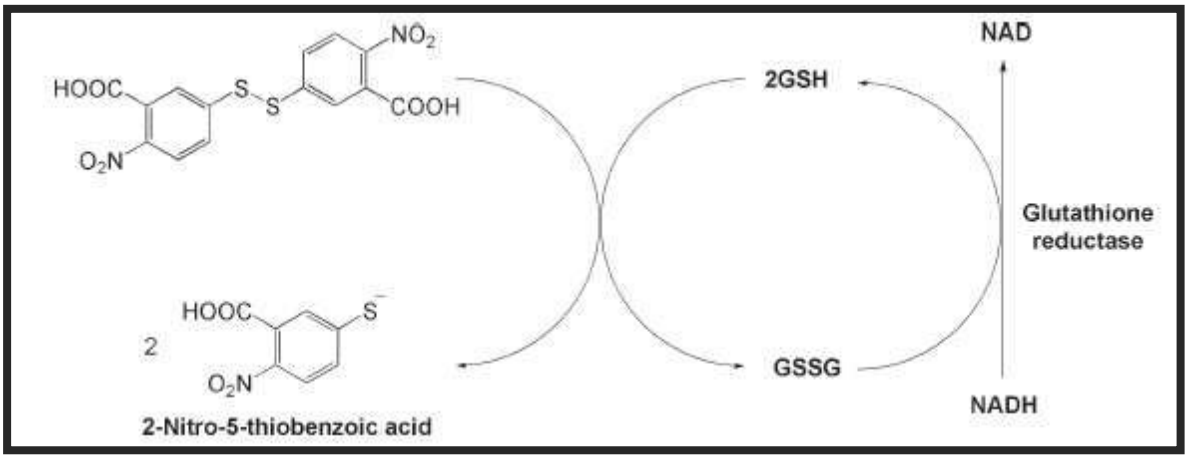
- ✓ 0.3 M Phosphate buffer; pH 6.5.
- ✓ 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) in 95% ethanol.
- ✓ 30 mM reduced glutathione.
- ✓ Double distilled water

Procedure

The reaction mixture contained 1.0 ml of 0.3 M phosphate buffer, 0.1 ml of 30 mM CDNB in 95% ethanol and 1.7 ml of distilled water. After completing the pre-incubation of the reaction mixture at 37°C for 5 mins, the reaction was started by the addition of 0.1 ml of tissue homogenate and 0.1 ml of 30 mM glutathione as substrate. Then, the total reaction mixture (3 ml) was mixed well and the absorbance was read at 340 nm for 3 min at an interval of 30 sec. Reaction mixture without the enzyme was used as the blank solution. The activity of GST is expressed as μmoles of GSH-CDNB conjugate formed/min/mg protein.

4.6.7.8. 5.Estimation of reduced glutathione (GSH)

The total reduced glutathione was determined according to the method of Ellman, [132]. The assay procedure is based on the oxidation of Ellman´s reagent [5, 5´-dithio bis (2- nitrobenzoic acid)] (DTNB) by SH groups of glutathione to form 2-nitro-S-mercaptobenzoic acid per mole of glutathione. The product is measured spectrophotometically at 412 nm.



Reagents

- ✓ 0.2 M Phosphate buffer; pH 8.0.
- ✓ 0.6 mM DTNB reagent.
- ✓ 5%TCA

Procedure

0.1 ml of tissue homogenate was made to precipitate using 5% TCA. Then the contents were mixed well for complete precipitation of proteins and centrifuged. To 0.1 ml of supernatant, 2 ml of 0.6 mM DTNB reagent and 0.2 M phosphate buffer (pH 8.0) were added to make up to a final volume of 4 ml. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards were also treated in the same manner to determine the amount of glutathione. The amount of glutathione was expressed as nmoles/g tissue.

4.6.7.9. Measurement of Lipid peroxidation

Estimation of Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al.[133] in which the malondialdehyde (MDA) released served as the index of LPO. The extent of LPO in the hepatic tissue was assayed by measuring one of the end products of this process, the thiobarbituric acid-reactive substances (TBARS). As 99% TBARS is malondialdehyde (MDA), thus this assay is based on the reaction of 1 molecule of MDA with 2 molecules of TBARS at low pH (2- 3) and at a temperature of 95°C for 60 min. The resultant pink chromogen can be detected spectrophotometrically at 532 nm.

Reagents

- ✓ Standard: 1, 1, 3, 3-tetra ethoxypropane (TEP).
- ✓ 8.1% Sodium dodecyl sulphate (SDS)
- ✓ 20%Acetic acid
- ✓ 0.8%Thiobarbituric acid (TBA)
- ✓ 15:1 v/v n-butanol: pyridine mixture

Procedure

To 0.2 ml of tissue homogenate, following were added - 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA. Then the mixture was made up to 4 ml with water and heated in a water bath at 95.8ºC for 60 min using glass ball as a condenser. After cooling, 1 ml of water and 5 ml of n-butanol: pyridine (15:1 v/v) mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance was observed at 532 nm. The concentration of lipid peroxides was denoted as nmoles of MDA formed per mg of protein.

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4.6.7.10. Histopathological Studies

Hematoxylin, a basic bye is oxidized to hematein with a mordant, a metallic ion such as the salts of aluminium. The positively charged aluminium-hematein complex combines with the negatively charged phosphate groups of the nucleic acids (DNA and RNA) forming blue/purple colour, which is characteristic of hematoxylin stains. Eosin is an acidic dye, which is consideredto have a selective affinity for the basic parts of the cell, i.e., the cytoplasm. Thus, the hematoxylin and eosin (H & E) stain is used to demonstrate different structures of the tissue.

The various steps involved in the preparation of tissues for histological studies are as follows:

- **Fixation**

In order to avoid tissue by the lysosomal enzymes and to preserve its physicaland chemical structure, a bit of tissue from all the organs was cut and placed in bouin's fluid immediately once it is removed from the animal body. The tissues were fixed in bouin's fluid for about 24 hours. The tissues were then taken and washed in glass distilled water for a day to remove excess of picric acid.

- **Dehydration**

The tissues were kept in the following solutions for an hour each; 30%, 50%, 70% and 100% alcohol. Inadequately dehydrated tissues cannot be successfully infiltered using paraffin. Also too much of dehydration resultswhich would be difficult for sectioning. So, careful precautions shoule be followed whilw performing the dehydration process.

❖ **Clearing**

Dealcoholization or replacement of alcohol from the tissues with a clearing agent is called as clearing. Xylene was used as the clearing agent for a period of one to two hours and two or three times. Because the clearing agent is miscible with both dehydration and embedding agents, it permits paraffin to infilterate the tissues. So, the clearing was performed next after dehydration to allow tissue spaces to be filled with paraffin. The tissues were kept in the clearing agent till they become transparent and impregnated with xylene.

❖ **Impregnation**

In this process the clearing agent xylene was placed by paraffin wax. The tissues were taken out of xylene and were kept in paraffin embedding bath which was molten. It comprises of of metal pots which is filled with molten wax maintained. The tissues were given three changes in the molten wax at half an hour intervals.

❖ **Embedding**

The paraffin wax which is used for embedding shall be fresh and heated to the optimum melting point at about 56–58^{o}C. A clear glass plate was smeared with glycerine. L-shaped mould was placed on it to from a rectangular cavity. The molten paraffin wax was discarded and the entrapped air bubbles were withdrawn by using a hot needle. Then the tissue was placed in the paraffin and oriented with the surface to be sectioned. Then the tissue was pressed slowly on the glass plate so that it settles uniformly with a metal pressing rod and allowed the wax to settle and solidity room temperature. The paraffin was kept in cold water for cooling

❖ **Section Cutting**

Section cutting was done using a rotating microtome. The excess of paraffin around the tissue was removed by trimming, leaving ½ cm around the tissue. The

block was then fixed to the warm holder. Extra support was given by some additional wax, which was applied on the sides of the block. Before sectioning, all set screws used for holding the object holder and knife were carefully tightened by using hands to avoid vibration. To make uniform sections, the microtome knife was placed to the appropriate angle in the knife holder so that the cutting edge alone is coming in contact with the paraffin block. The tissue was cut in the thickness range of about 7µm.

❖ **Flattening and Mounting of Sections**

The procedure was carried out using tissue flotation warm water bath. The sections were made to fall on a warm water bath after they were detached from the knife using a hair brush. Clean slides which are free from dust were coated with egg albumin over the whole surface. Required sections were made to spread on clean slide and maintained at room temperature.

Staining of Tissue Sections

The sections were stained as follows; deparaffinization with xylene two timeseach for five minutes

Dehydration through descending grades of ethyl alcohol

- ✓ 100% alcohol - 2 mins
- ✓ 90% alcohol - 1min
- ✓ 50% alcohol - 1 min

Staining with Ehrlich's Haemaoxylin was done for 15-20 minutes. Then the sectioned tissues were thoroughly washed in tap water for 10 minutes. Rinsed with distilled water and stained with Eosin. Dehydration again with ascending grades of alcohol.

- ✓ 70% alcohol - 2 minute
- ✓ 90% alcohol - 2 minute

- ✓ 100%alcohol - 1 minute

Finally the tissues were cleared with xylene two times, each for about 3 minutes interval.

❖ **Mounting**

On the stained slide, DPX mountant was applied uniformly and microglass cover slides were spread. The slides were observed in Nikon microscope and microphotographs were taken.

5. RESULTS AND ANALYSIS

5.1. Preformulation Studies

5.1.2. Organoleptic Characteristics

The color, odor, and appearance of the drug were characterized and recorded using descriptive terminology; the results were shown in the Table 5.1

Table 5.1: Organoleptic Charecteristics of 5-Fluorouracil and Leucovorin

Properties	5-Fluorouracil	Leucovorin
Odour	Odourless	Odourless
Colour	White to off-white	Pale White
Form	Crystalline	Crystalline

5.1.3. Melting point

The melting point of the 5-Fluorouracil and Leucovorin was reported in Table 5. 2.

Table 5.2: Melting point of 5-Fluorouracil and Leucovorin

Sample	Reported	Observed
5-Fluorouracil	282-283^{0}C	287.15^{0}C
Leucovorin	240-250^{0}C	241.02^{0}C

5.1.4. Solubility analysis

The available literature on solubility profile of 5-Fluorouracil and Leucovorin indicated that the drug is soluble in water, alcohol and methanol and insoluble in chloroform, ether and benzene.

5-Fluorouracil and Leucovorin was found to be soluble in water, alcohol and methanol and insoluble in chloroform, ether and benzene.

5.1.5. Calibration curve

Table 5.3: Observations for Standard graph of 5-Fluorouracil in pH 7.4 at 266nm

Concentration (µg/ml)	Absorbance (266nm) in pH 7.4
10	0.206
20	0.394
30	0.586
40	0.766
50	0.913

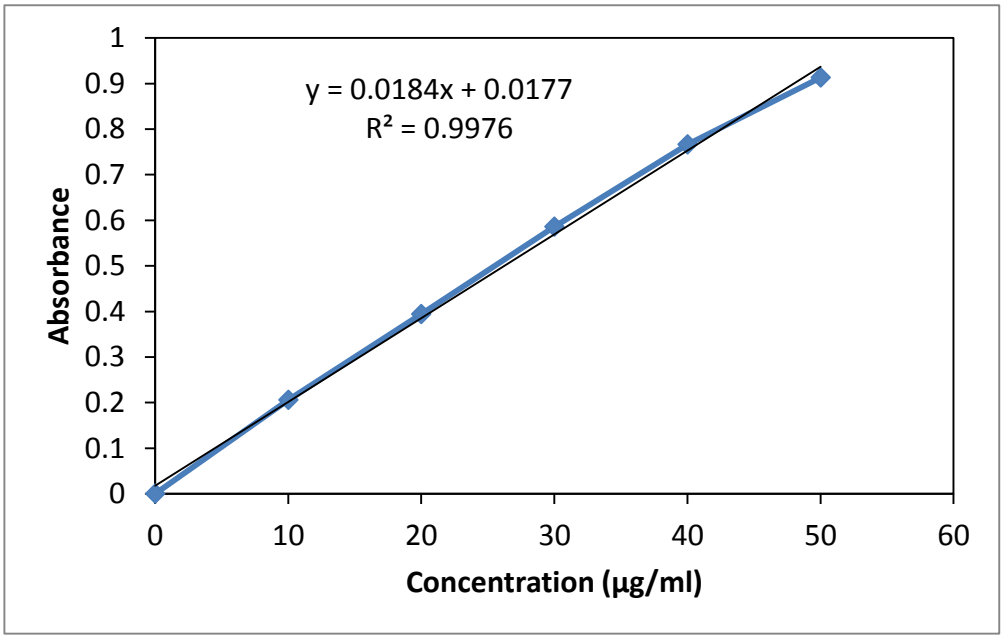


Fig 5.1:Calibration curve of 5-Fluorouracil in pH 7.4 at 266nm

Table 5.4: Observations for Standard graph of Leucovorin in pH 7.4 at 288 nm

Concentration (µg/ml)	Absorbance (288nm) in pH 7.4
10	0.109
20	0.323
30	0.475
40	0.697
50	0.86

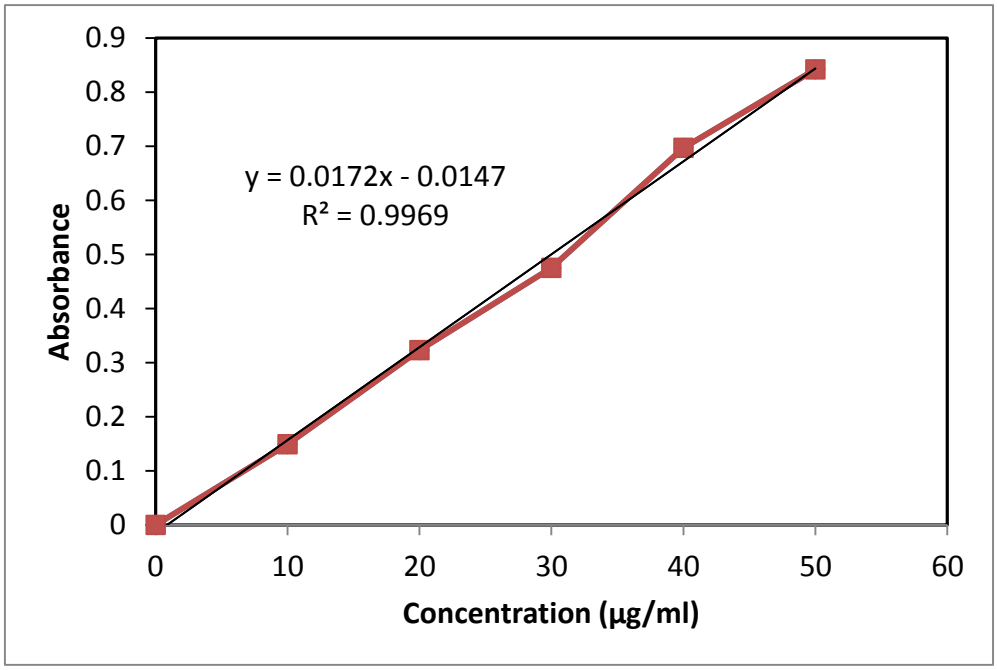


Fig 5.2: Calibration curve of Leucovorin in pH 7.4 at 288nm

The data are plotted and the graph is shown in the Figure 5. 1 &5.2. The r^2 value was obtained by using the linear curve and it was found to be nearly to 1.The slope the curve (5-FU and LV) was found 0.0184 and 0.0172 respectively.

5.1.6. FT-IR Spectral Analysis

The development of a successful formulation depends only on a suitable selection of excipients. Hence the physical state of the drug 5-Fluorouracil and leucovorin and the combination of drug and cholesterol and surfactant used for niosomes preparation were studied by FTIR (Fourier transform infrared spectroscopy) to know the drug – excipients compatibility. The physicochemical compatibility of the drugs and the excipients was obtained by FTIR studies with the interpretation values of the FTIR were mentioned in **Fig No (5.3A, 5.3.B and 5.3.C).**

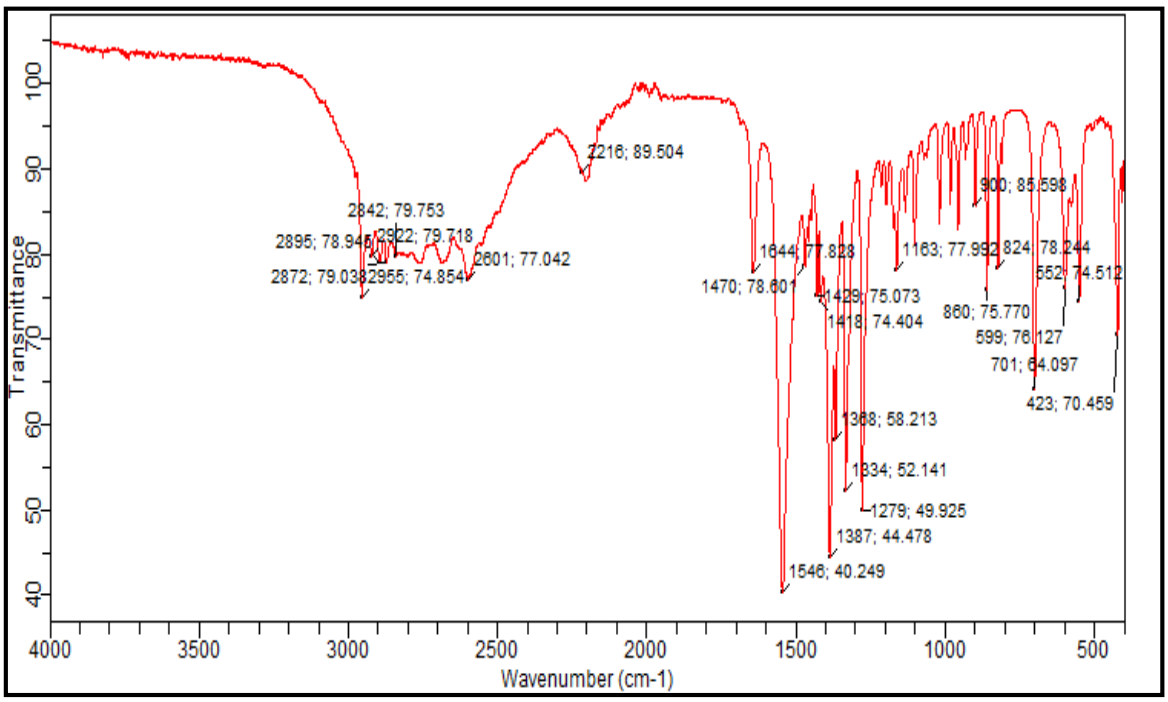


Fig 5.3.A: FTIR spectrum of 5-fluorouracil

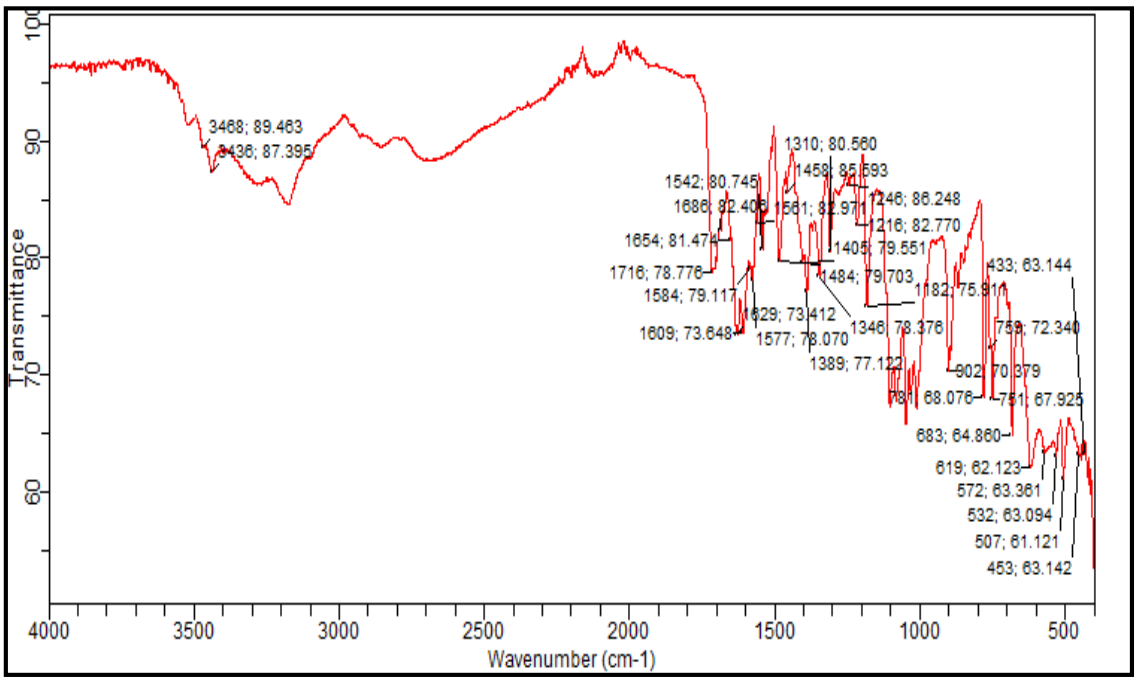


Fig 5.3.B: FTIR spectrum of Leucovorin

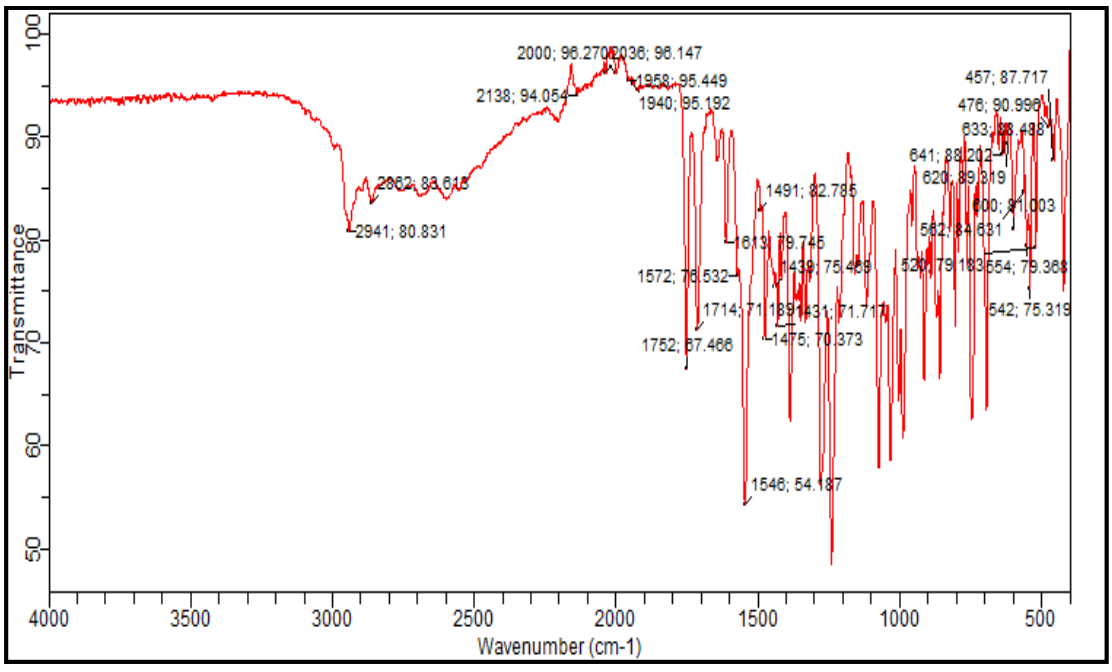


Fig 5.3.C: FTIR spectrum of 5-FU + LV Niosomes

5.2. Optimization of Niosomes

The optimization of the niosomes was made by altering the cholesterol ratio and evaluating the niosomes by using *in vitro* drug release (Table 5.5).

Table 5.5: Optimization of niosomal formulation compositions

Type of formulation	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
Drug	10	10	10	10	10	10	10	10
Cholesterol	10	20	10	20	10	20	10	20
Span 40	10	10	-	-	-	-	-	-
Span 60	-	-	10	10	-	-	-	-
Tween 40	-	-	-	-	10	10	-	-
Tween 60	-	-	-	-	-	-	10	10
Drug :Cholesterol: SA	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1

5.3. Evaluation of Niosomes

5.3.1. Particle Size Analysis

The particle size of the niosomes was analyzed by using the particle size analyzer (Malvern) and it is shown in the Figure 5.4.The particles of the drug loaded niosomes are in the size of 0.1 to 1 μ range. More number of the particles is in the 0.1μ to 0.8 μ approximately and then larger particle size distribution is also seen.

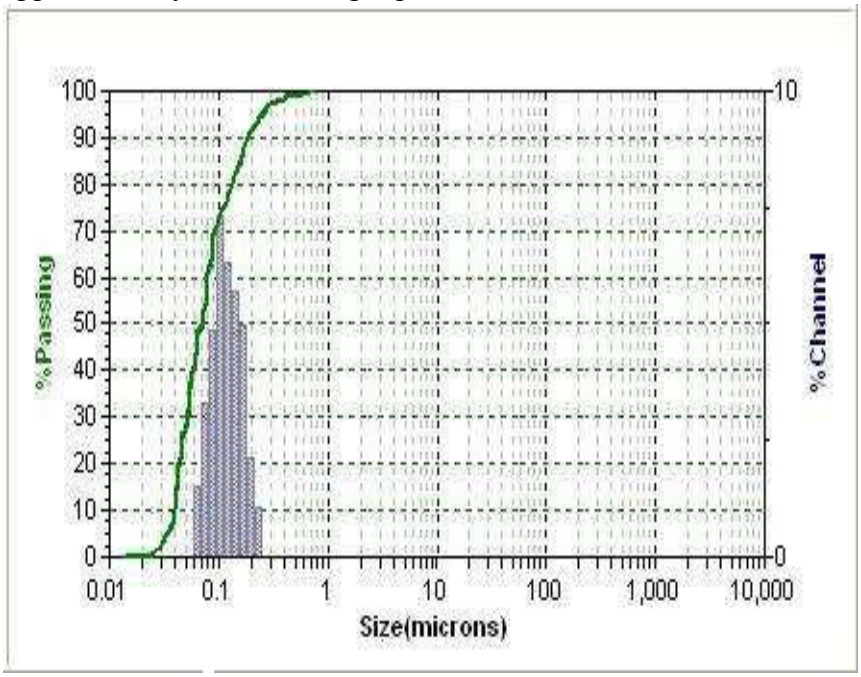


Figure 5.4: Particle size analysis of 5-FU + LV Niosomes

5.3.2. Optical Microscopy

The formulated niosomes were viewed through optical microscope (Fig 5.5)

Fig 5.5: Optical microscopy images of 5-FU + LV Niosomes

5.3.3. Morphological studies:

High-Resolution Transmission Electron Microscopy helps to find the internal morphology of the sample. For this the sample is diluted and it is placed on the TEM grid, approximately two or three drops is sufficient. Then it is air dried until the water molecule is removed from it. Further it is taken for the analysis. The images of the 5-FU and LV loaded niosomes are shown in the Figure 5.6. The TEM image shows the discrete formation of the structure and the internal morphology.

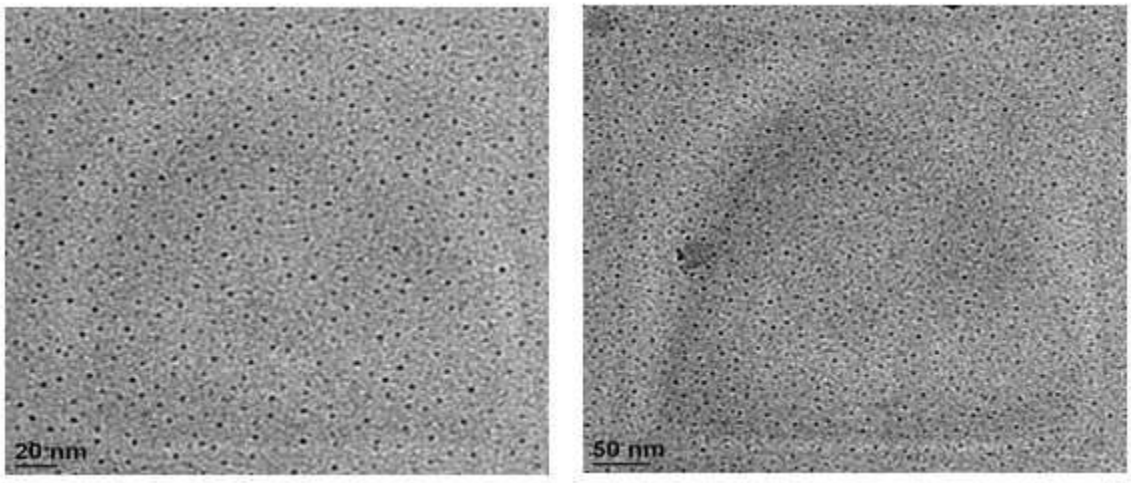


Fig 5.6: TEM image of 5-FU + LV Niosomes

5.3.4. Scanning Electron Microscopy

Niosomes were characterized by SEM (JEOL). Niosomes containing 5-FU + LV was taken in a cover glass and transferred on a specimen stub. Dried samples were coated using a platinum alloy upto a thickness of 100 Å by a sputter coater. After coating, scanning was done to examine the shape and size (Fig 5.7).

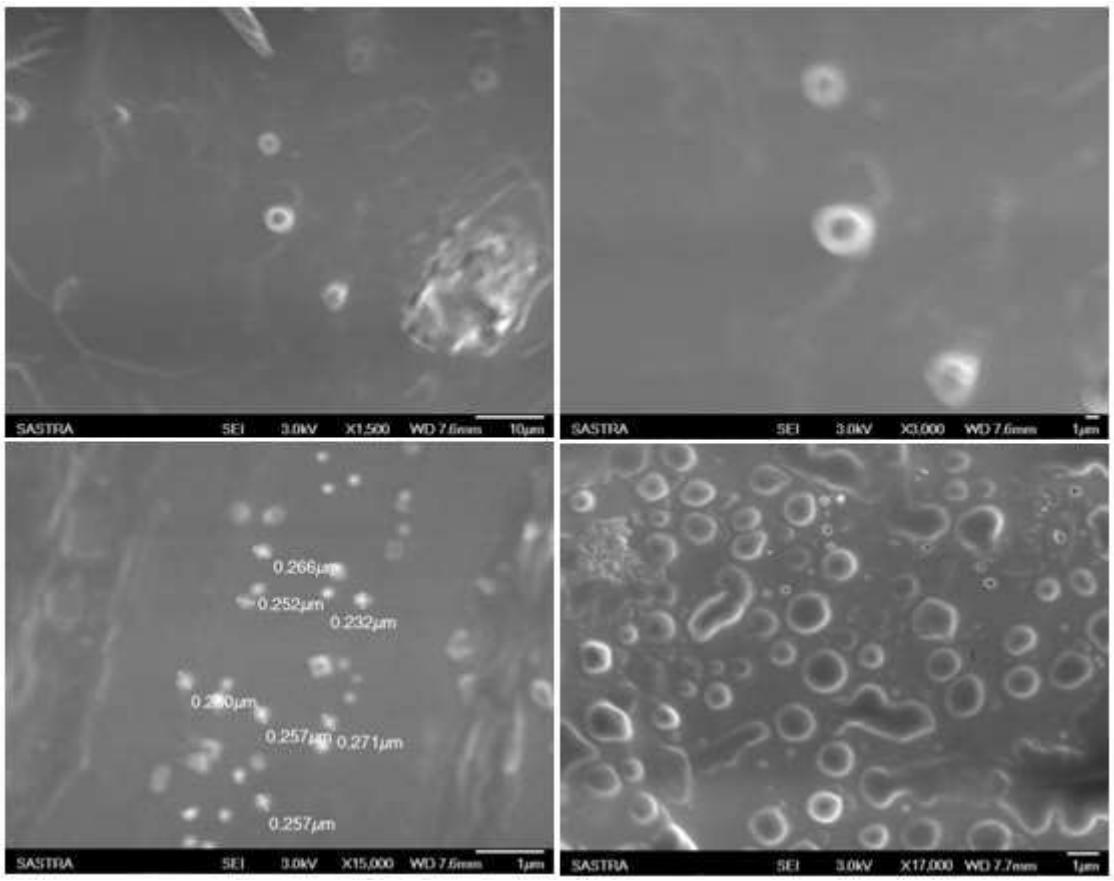


Fig5.7: SEM image of 5-FU + LV Niosomes

5.3.5.Differential scanning Colorimetry

The drugs 5-Fluorouracil, Leucovorin, cholesterol, span 40, span 60, span 80 and mixture of drug and excipients were subjected to DSC studies for testing the compatibility of the drug with the excipients used in the formulation (Fig 5.8).

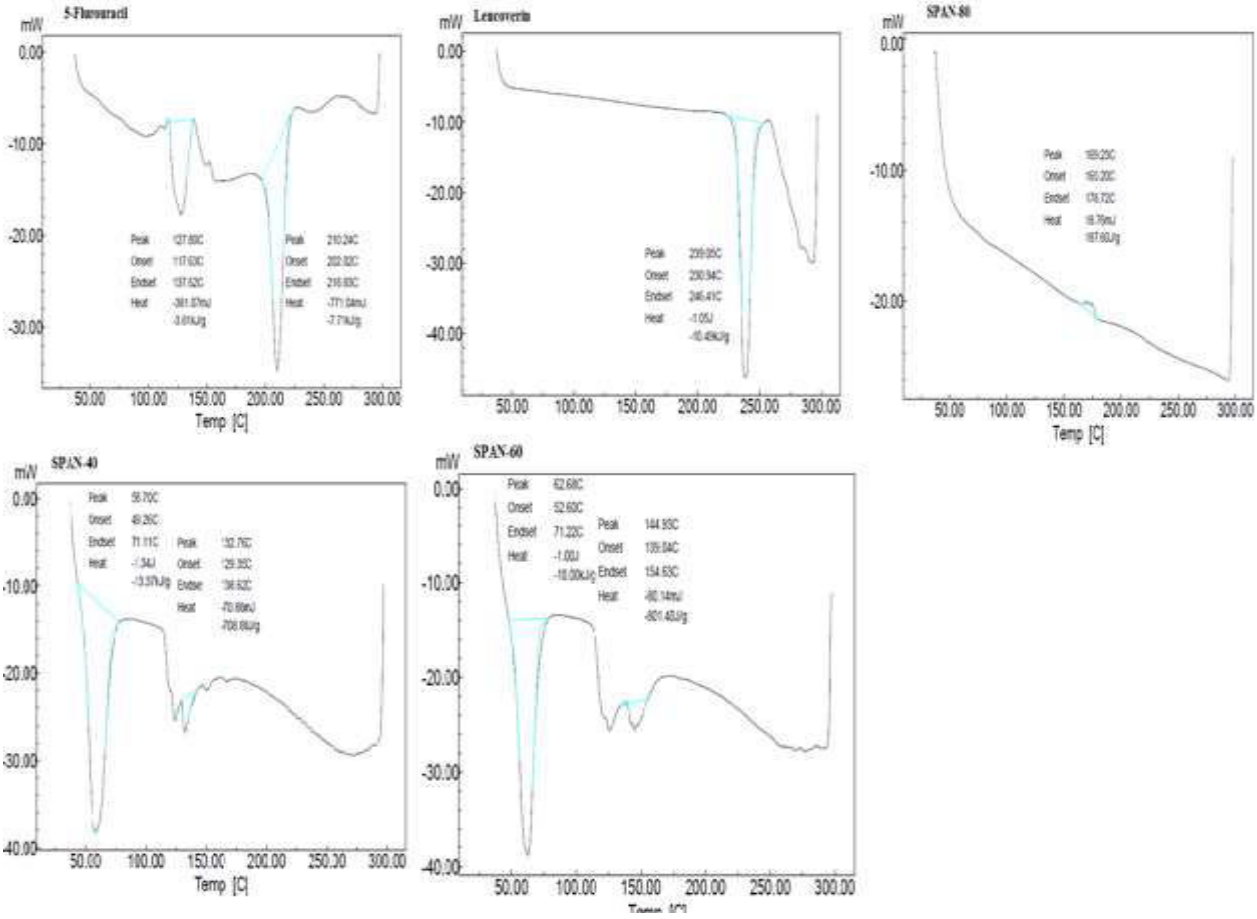


Fig 5.8: DSC studies of 5-FU + LV niosomes

5.3.6. Zeta Potential

The surface charge of 5-Fluorouracil and leucovorin niosomes was measured using Zetapotentiometer. The zeta potential graph was shown in Figure 5.9.

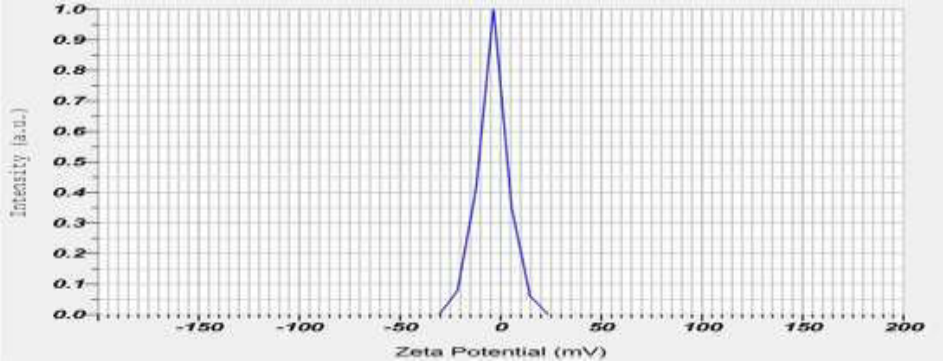


Figure 5.9.: Zeta potential of Niosomes

5.3.7. Drug entrapment efficiency:

The Entrapment Efficiency of the formulations was calculated and Tabulated in the Table 5.6. The entrapment efficiency of drug containing span 60 was found to be 72.21% which showed maximum percent drug entrapment where as those containing span 40, span 60, tween 40, tween 60 were found to encapsulate 66.35, 72.21, 50.44, and 52.24. These results indicates that span 60 is the more suitable surfactant along with higher concentration of cholesterol for enhancing maximum entrapment for the drugs 5-Fluorouracil and leucovorin.

Table 5.6: Entrapment Efficiency of the 5-FU + LV niosome formulations

Formulations Code	5-FU Entrapment (%)	LV Entrapment (%)
F1	62.44	63.28
F2	66.35	65.02
F3	68.30	64.86
F4	72.21	69.45
F5	47.05	54.76
F6	50.44	57.92
F7	50.92	59.55
F8	52.24	60.27

5.3.8. *In Vitro* Drug Release

The formulated niosomes were subjected to *in vitro* drug release. The amount of 5-Fluorouracil and leucovorin diffused was estimated spectrophotometrically at 266 nm and 288 nm respectively and the results were shown in Table 5.7 &5.8.

Niosomes containing 5-Fluorouracil prepared with span 60 showed 99.65 % of drug release upto 12 hours (Figure 5.10). 5-Fluorouracil release from the formulation containing span 40, Tween 40 and Tween 60 was observed almost 100% drug release within 10 hours. These results showed that niosomal formulation containing 5-Fluorouracil prepared with span 60 has sustained release upto 12 hours. Leucovorin release profile from niosomal formulation was shown in Figure 5.11. Niosomes containing leucovorin prepared with span 60 showed 99.91 % of drug release upto 12 hours. Leucovorin release from the formulation containing span 40, Tween 40 and Tween 60 was observed almost 100% drug release within 10 hours. These results showed that niosomal formulation containing leucovorin prepared with span 60 has sustained release upto 12 hours.

Table 5.7: *In-vitro* release study of the 5-FU niosomes

Time in Hours	% Drug Released							
	F1	F2	F3	F4	F5	F6	F7	F8
1	25.12	20.78	23.43	19.23	28.34	23.02	24.72	21.66
2	42.04	39.02	40.12	30.22	40.22	33.22	35.15	32.17
4	51.06	48.9	52.22	46.89	61.27	48.82	49.42	50.92
6	69.35	70.33	68.79	65.88	72.52	67.94	70.21	66.25
8	90.23	83.45	88.16	79.43	84.67	82.64	82.56	81.92
10	99.34	95.74	95.34	89.58	99.96	99.52	98.9	95.99
12	99.9	99.13	100.02	99.65	100	99.99	100	100

Table 5.8:*In-vitro* release study of the 5-FU + LV Niosomes

Time in Hours	% Drug Released							
	F1	F2	F3	F4	F5	F6	F7	F8
1	38.96	36.72	35.07	30.01	40.04	39.02	39.27	38.12
2	50.22	50.38	48.39	45.22	52.78	51.35	50.55	49.55
4	65.15	62.12	62.07	59.12	66.9	66.17	65.22	62.49
6	79.6	76.2	72.98	70.36	84.56	72.56	79.21	78.01
8	92.1	90.08	90.14	85.43	95.9	88.54	90.56	88.32
10	99.34	98.94	99	93.12	99	98.18	99.82	100
12	99.9	100	100	99.91	99.98	99	100	100

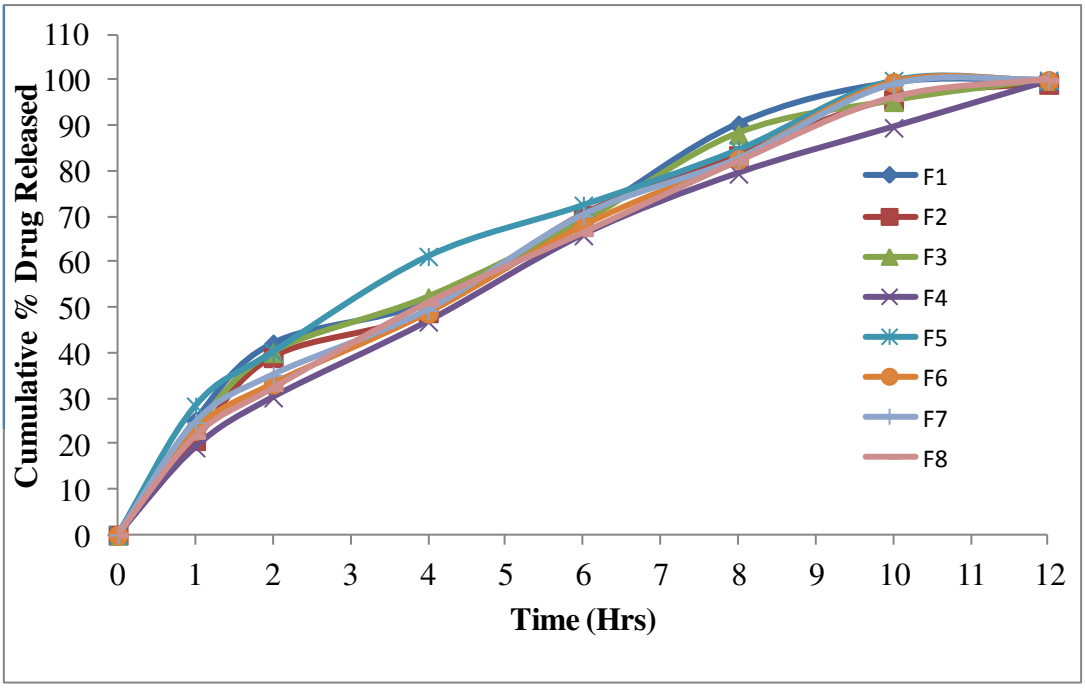


Fig5.10: In-vitro release study of the 5 – FU from Niosomes

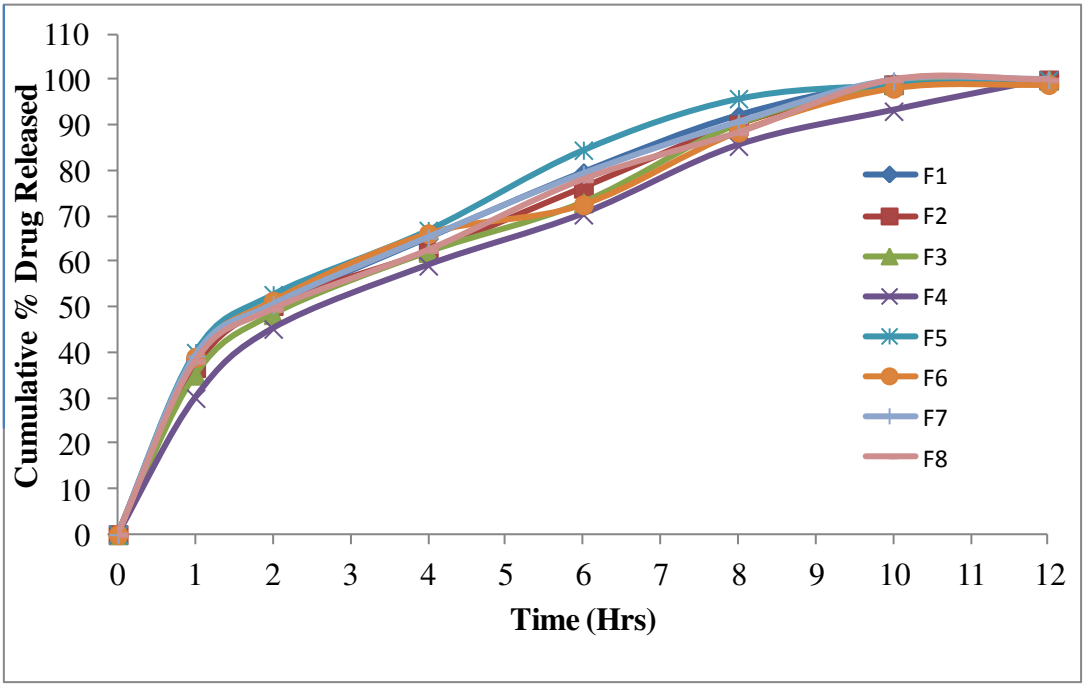


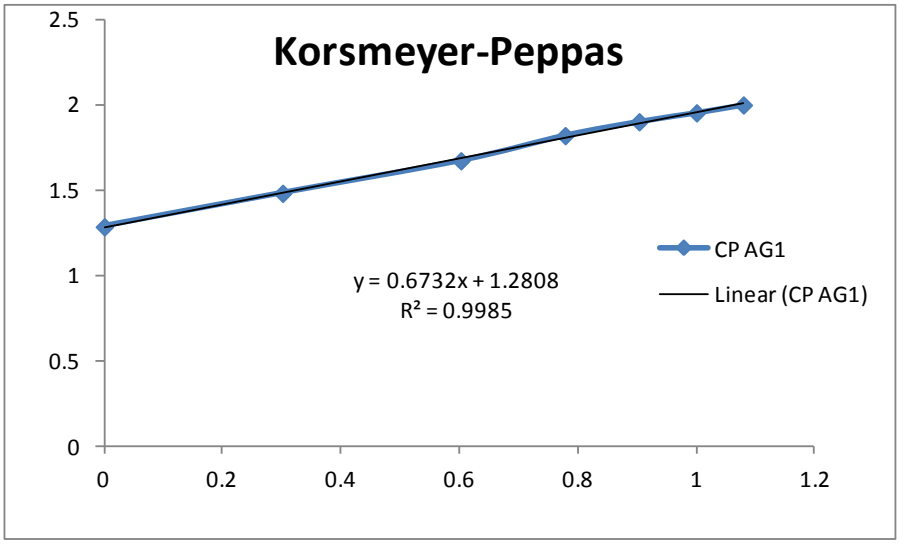
Fig5.11. *In-vitro* release study of the 5-FU + LV Niosomes

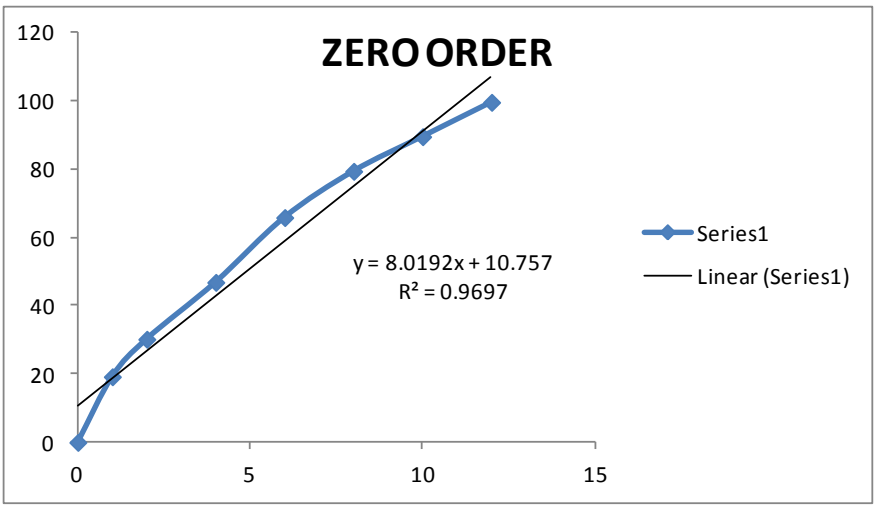
5.3.9. *In vitro* release kinetics:

In vitro release kinetics data was sown in Table 5.9. The results shown that formulation F4, Span 60 with cholesterol follows zero-order kinetics. Calculation of Higuchi's correlation coefficient confirms that drug release was proportional to the square root of time indicating that drug release from niosomes was diffusion controlled (Figure 5.12 and 5.12.A). The *n* value from the Korsmeyer-Peppas model for release 5-FU from niosomal formulation was 0.67 which confirms the Non Fickian type diffusion, whereas release LV from niosomal formulations follow an Fickian diffusion mechanism ($n > 0.47$).

Table 5.9: *In vitro* release kinetics of 5-FU + LV niosomes

Formulation code	Higuchi R^2	Korsmeyer-Peppas		Zero order		First order		Release mechanism
		R^2	*N*	R^2	K_0 (%mg/h)	R^2	K_1 (h^{-1})	
F4 (5- FU)	0.996	0.998	0.67	0.969	8.01	0.770	0.161	Non Fickian
F4 (LV)	0.996	0.995	0.47	0.907	7.44	0.745	0.194	Fickian





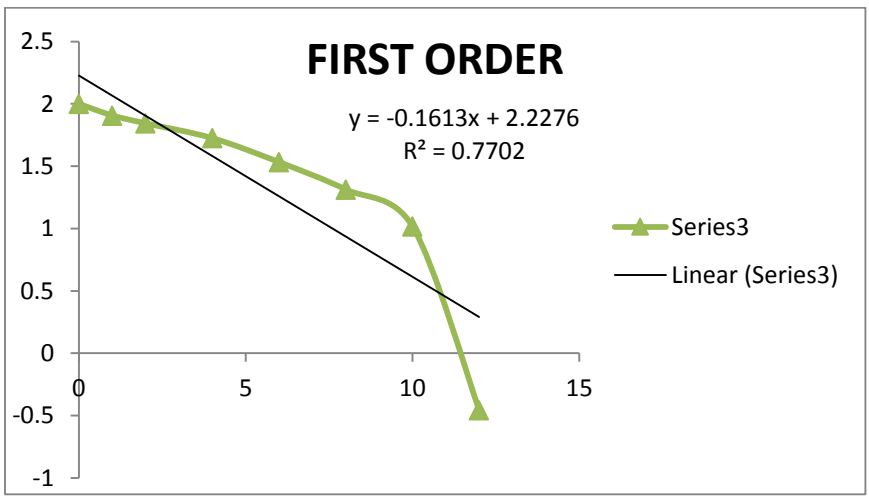


Figure 5.12 In vitro release kinetics of 5-FU + LV niosomes (F4)

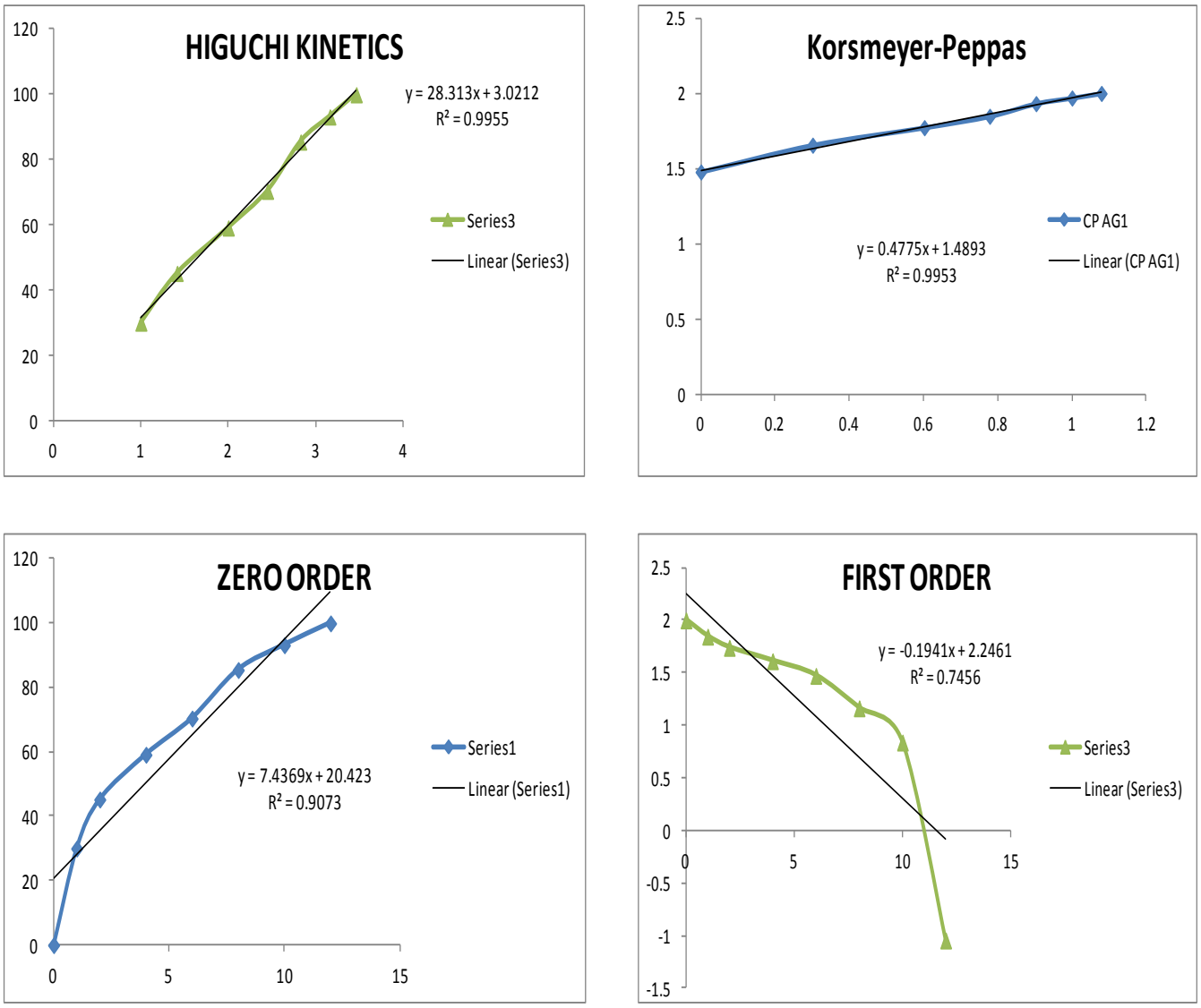


Figure 5.12 .A: *In vitro* release kinetics of 5-FU + LV niosomes (F4)

5.4. Preclinical evaluation of Niosomes

5.4.1. Effect of 5-FU + LV niosomes (F4) against 1, 2 Dimethyl hydrazine (DMH) induced colon cancer

5.4.1.1. Effect of 5-FU + LV niosomes (F4) on Hematological parameters on DMH induced colon cancer

In this study, haematological parameters were significantly altered in the DMH treated as a carcinogenesis and altered immune function. The level of RBC and haemoglobin were significantly ($p<0.05$) reduced in DMH groups, whilst the WBC level was significantly ($p<0.05$) increased as that of the control rats. Meanwhile, treatment with 5-FU+ LV niosomes significantly ($p<0.05$) restored the altered blood parameters level to normalcy (Table 5.10 and Fig 5.13).

Table 5.10: Effect of 5-FU + LV niosomes (F4) onHematological parameters on DMH induced colon cancer

Groups	RBC	WBC	Hb
Control	7.53 ± 0.17	2.53±0.11	14.48±0.26
DMH	3.21± 0.14[a*]	8.42±0.16 [a*]	8.97±0.16 [a*]
DMH+5-FU	4.40±0.13 [b*]	5.64±0.17 [b*]	11.23± 0.21 [b*]
DMH + (5-FU +LV) Market formulation	5.87±0.16[c*]	4.32±0.12 [c*]	12.24±0.23 [c*]
DMH + (5-FU +LV) Niosomes	6.40±0.18 [d*]	3.15±0.09 [d*]	13.89±0.18 [d*]

Units: RBC: millions/µl of blood; WBC: thousands/µl of blood; Hb: g/dl. The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes. * denotes statistically significant $p< 0.05$.

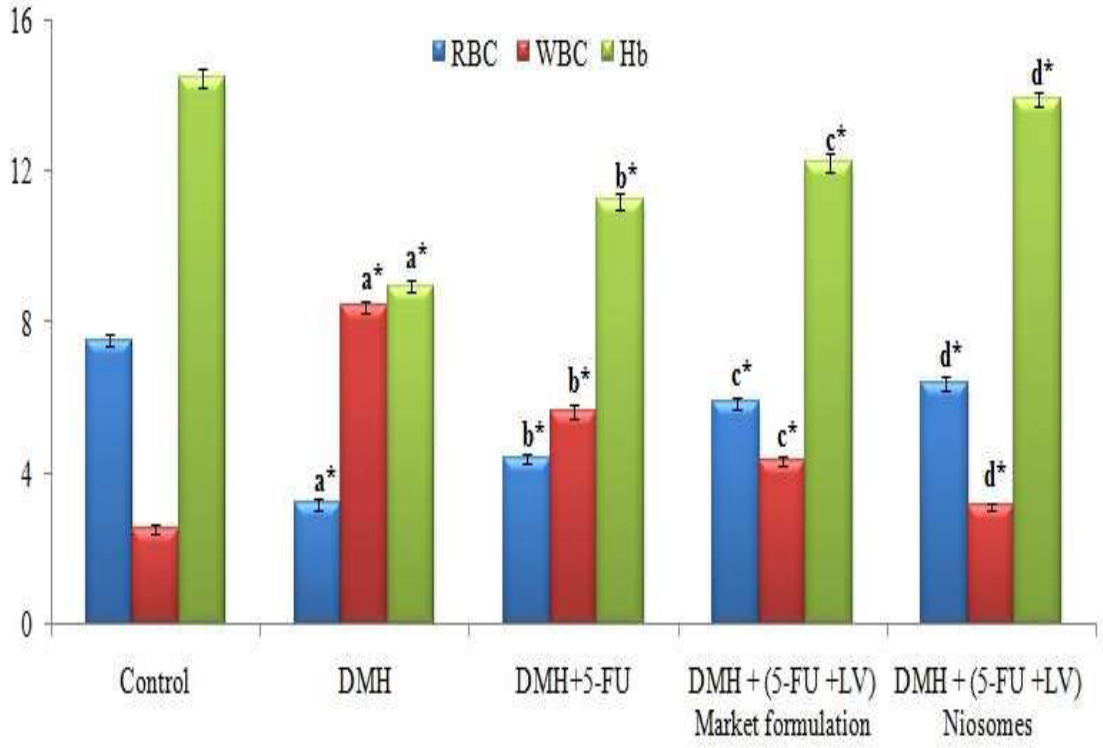


Fig 5.13: Effect of 5-FU + LV niosomes (F4) on Hematological parameters on DMH induced colon cancer

5.4.1.2. Effect of 5-FU + LV niosomes (F4) on antioxidants and MDA level in DMH induced colon cancer

The present result show that, the colonic tissue level of the LPO (Table 5.11& Fig 5.13) increased significantly ($p<0.05$) and GSH (Table 5.11 and Fig 5.14), SOD, CAT, GPx and GST level (Table 5.12 and Fig 5.15) were decreased significantly ($p<0.05$) in DMH alone group compared with normal control group. Whilst, treatment with 5-FU+ LV niosomes significantly ($p<0.05$) decreased the level of MDA in colonic tissue of DMH treated rats, as well as significantly ($p<0.05$) increased the level of the antioxidants defense enzymatic system of SOD, CAT, GPx and GSH level.

Table 5.11: Effect of 5-FU + LV niosomes on GSH and MDA level in DMH induced colon cancer

Groups	GSH	MDA
Control	4.98±0.54	1.4±0.12
DMH	1.52±0.15 [a*]	4.24±0.35 [a*]
DMH+5-FU	2.67±0.21 [b*]	3.47±0.26 [b*]
DMH + (5-FU +LV) Market formulation	3.12±0.29 [c*]	2.42±0.18 [c*]
DMH + (5-FU +LV) Niosomes	3.92±0.42 [d*]	1.86±0.16 [d*]

GSH: Reduced Glutathione (nmole/mg of tissue) ; MDA: Malondialdehyde (nmole/mg of tissue). The values are expressed as mean ±S.E.M, n=6. The statistical analysis was performed by one way ANOVA and Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes. * denotes statistically significant $p< 0.05$.

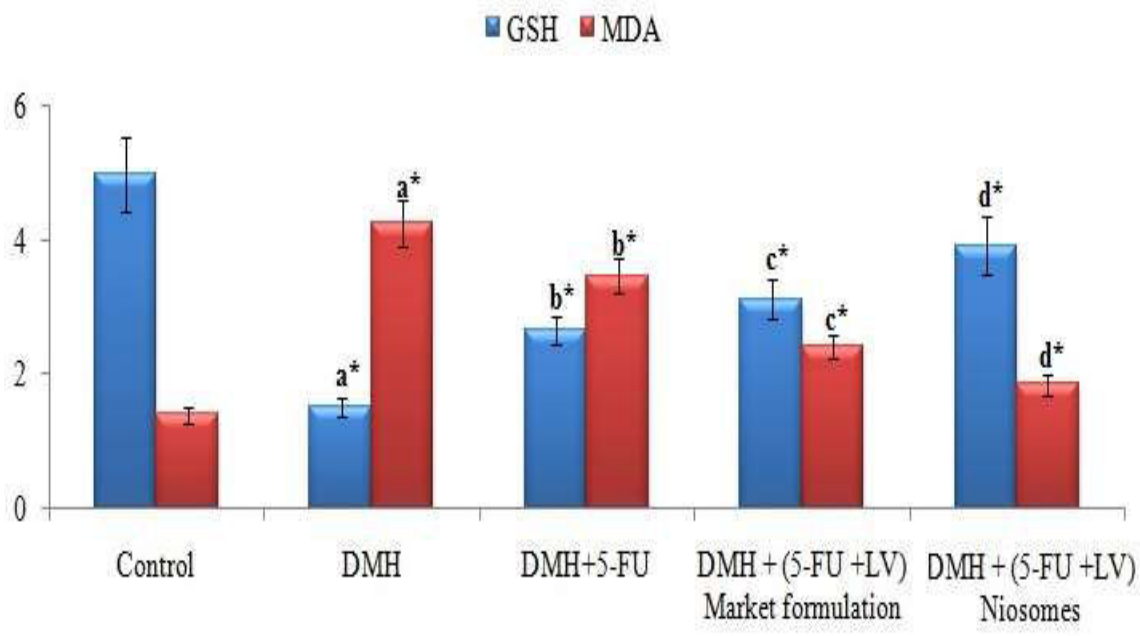


Fig 5.14: Effect of 5-FU + LV niosomes on GSH and MDA level in DMH induced colon cancer

Table 5.12: Effect of 5-FU + LV niosomes on colon antioxidant enzymes in DMH induced colon cancer

Groups	SOD	CAT	GPx	GST
Control	7.84±0.75	7.14±0.68	2.34±0.05	0.75±0.006
DMH	2.89±0.29 a*	2.63±0.25 a*	0.57±0.01 a*	0.25±0.003 a*
DMH+5-FU	4.87±0.35 b*	4.32±0.29 b*	1.6±0.03 b*	0.48±0.002 b*
DMH + (5-FU +LV) Market formulation	5.92±0.37 c*	5.75±0.42 c*	1.98±0.03 c*	0.65±0.005 c*
DMH + (5-FU +LV) Niosomes	7.12±0.67 d*	6.87±0.54 d*	2.12±0.04 d*	0.71±0.006 d*

SOD: Superoxide dismutase (U/mg protein); CAT: Catalase (nmoles of H2O2 utilized/min/mg/protein); GPx: Glutathione peroxidase (nmoles of GSH utilized/min/mg protein); GST: Glutathione -S- Transferase (nmoles of CDNB conjugate formed/min/mg/protein).The values are expressed as mean ±S.E.M, n=6. The statistical analysis was performed by one way ANOVA and Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes. * denotes statistically significant p< 0.05.

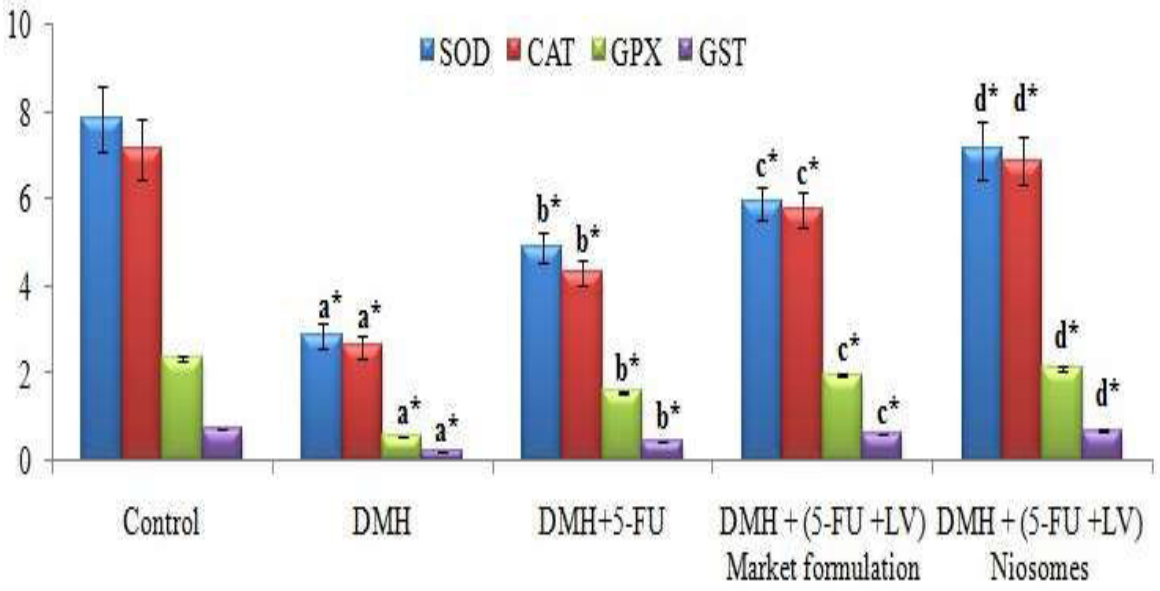


Fig 5.15: Effect of 5-FU + LV niosomes on colon antioxidant enzymes in DMH induced colon cancer

5.4.1.3. Effect of 5-FU + LV niosomes (F4) on serum lipid profiles in DMH induced colon cancer

In this study, serum level of cholesterol and triglycerides were significantly ($p<0.05$) elevated in DMH induced group may be due to higher cholesterogenesis induced by DMH. Whilst, treatment with 5-FU+ LV niosomes in DMH induced rats significantly ($p<0.05$) reduced the serum level of total cholesterol and triglycerides (Table 5.13 and Fig 5.16).

Table 5.13: Effect of 5-FU + LV niosomes on serum lipid profiles in DMH induced colon cancer

Groups	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control	76.94±2.46	122.6±3.33
DMH	171.4±7.65 [a*]	164.4±2.68 [a*]
DMH+5-FU	110.7±2.05 [b*]	147.1±3.7 [b*]
DMH + (5-FU +LV) Market formulation	90.44±2.47 [c*]	138.28±1.88 [c*]
DMH + (5-FU +LV) Niosomes	81.17±1.08 [d*]	125.5±5.23 [d*]

The values are expressed as mean ±S.E.M, n=6. The statistical analysis was performed by one way ANOVA and Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes. * denotes statistically significant $p< 0.05$.

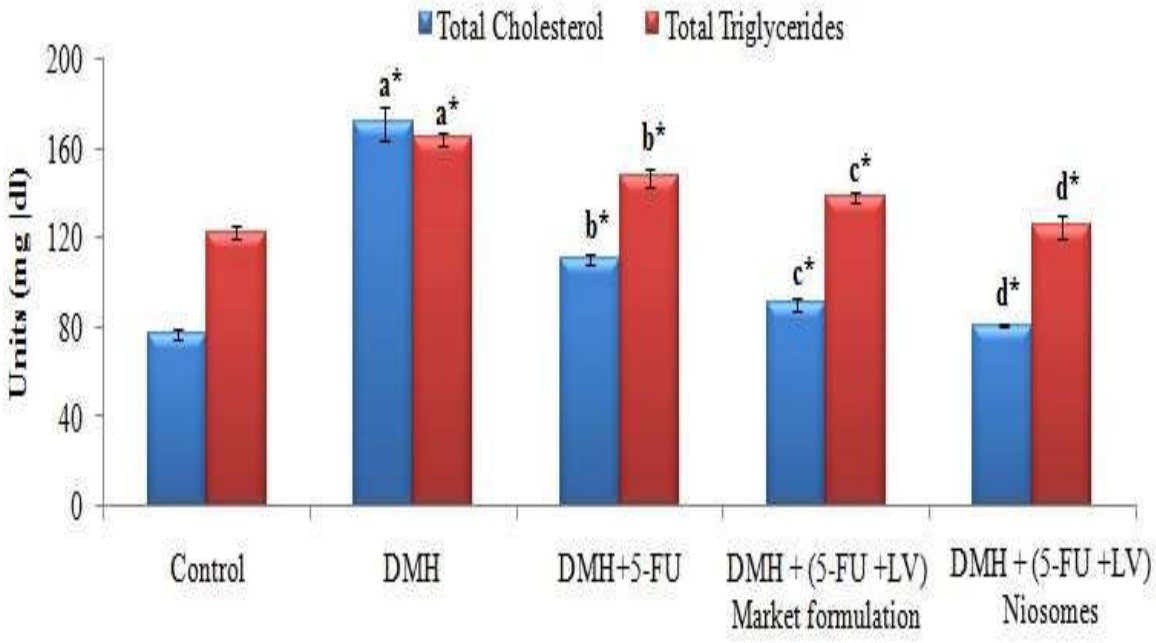


Fig 5.16: Effect of 5-FU + LV niosomes on serum lipid profiles in DMH induced colon cancer

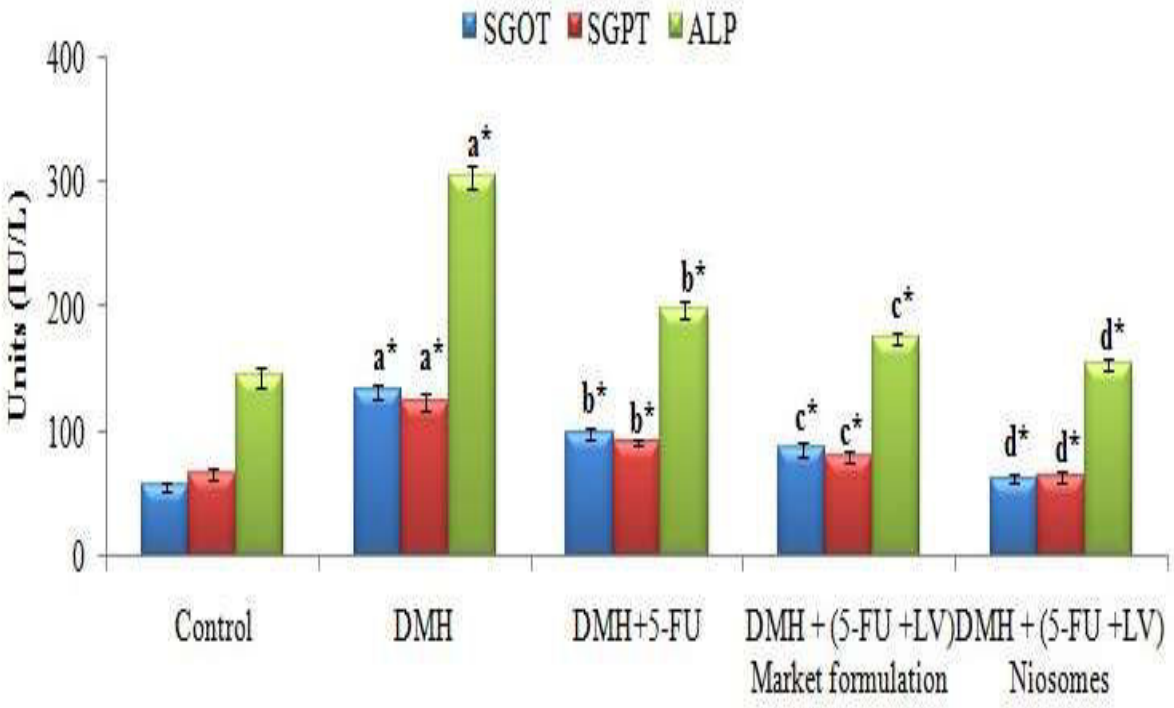
5.4.1.4. Effect of 5-FU + LV niosomes (F4) on serum hepatic markers enzyme in DMH induced colon cancer

In this study, DMH intoxicated rats displayed significantly ($p<0.05$) elevated level of hepatic marker enzymes (SGOT, SGPT and ALP) in serum and treatment with 5-FU+ LV niosomes significantly ($p<0.05$) decreased the hepatic markers enzyme level and restores the hepatocellular membrane damage elicited by DMH (Table 5.14 and Fig 5.17).

Table 5.14: Effect of 5-FU + LV niosomes (F4) on serum hepatic markers enzyme in DMH induced colon cancer

Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
Control	56.87±3.23	66.45±3.76	144.34±7.98
DMH	132.65±5.76 [a*]	124.32±6.84 [a*]	304.65±9.65 [a*]
DMH+5-FU	98.76±4.25 [b*]	92.12±3.25 [b*]	197.65±6.87 [b*]
DMH + (5-FU +LV) Market formulation	86.87±5.65 [c*]	79.67±4.82 [c*]	175.42±4.56 [c*]
DMH + (5-FU +LV) Niosomes	62.76±3.29 [d*]	64.65±3.98 [d*]	153.89±5.25 [d*]

The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes. * denotes statistically significant $p< 0.05$.



Fig**g 5.17: Effect of 5-FU + LV niosomes (F4) on serum hepatic markers enzyme in DMH induced colon cancer**

5.4.1.5. Effect of 5-FU + LV niosomes (F4) on tumour markers in DMH induced colon cancer

In this study, serum level of CEA and AFP were significantly ($p<0.05$) elevated and 5-FU+ LV niosomes treatment significantly ($p<0.05$) reduced the level of tumor markers (CEA and AFP) to normalcy (Table 5.15 and Fig 5.18).

Table 5.15: Effect of 5-FU + LV niosomes (F4) on tumour markers in DMH induced colon cancer

Groups	CEA (ng/dl)	AFP (ng/dl)
Control	0.21±0.02	0.45±0.01
DMH	1.24±0.07 a*	1.62±0.05 a*
DMH+5-FU	0.56± 0.06 b*	0.89±0.04 b*
DMH + (5-FU +LV) Market formulation	0.45±0.03 c*	0.72±0.03 c*
DMH + (5-FU +LV) Niosomes	0.29±0.05 d*	0.55±0.04 d*

CEA: Carcinoembryonic Antigen; AFP-Alpha-Feto-Protein. The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes. * denotes statistically significant $p< 0.05$.

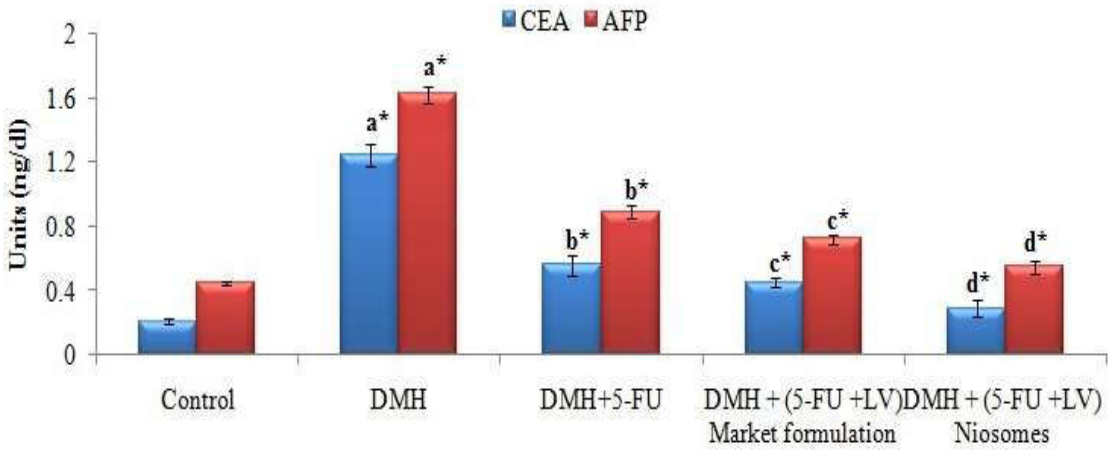


Fig 5.18: Effect of 5-FU + LV niosomes (F4) on tumour markers in DMH induced colon cancer

5.4.1.6. Effect of 5-FU + LV niosomes on colon histology in DMH induced colon cancer

The histopathological analysis revealed the thickened mucosa with densely packed inflammatory cell infiltration and a higher degree of hyperplasia in DMH intoxicated rats and treatment with 5-FU+ LV niosomes showed normal appearing glands with normal mucosa of colon (Fig5.19).

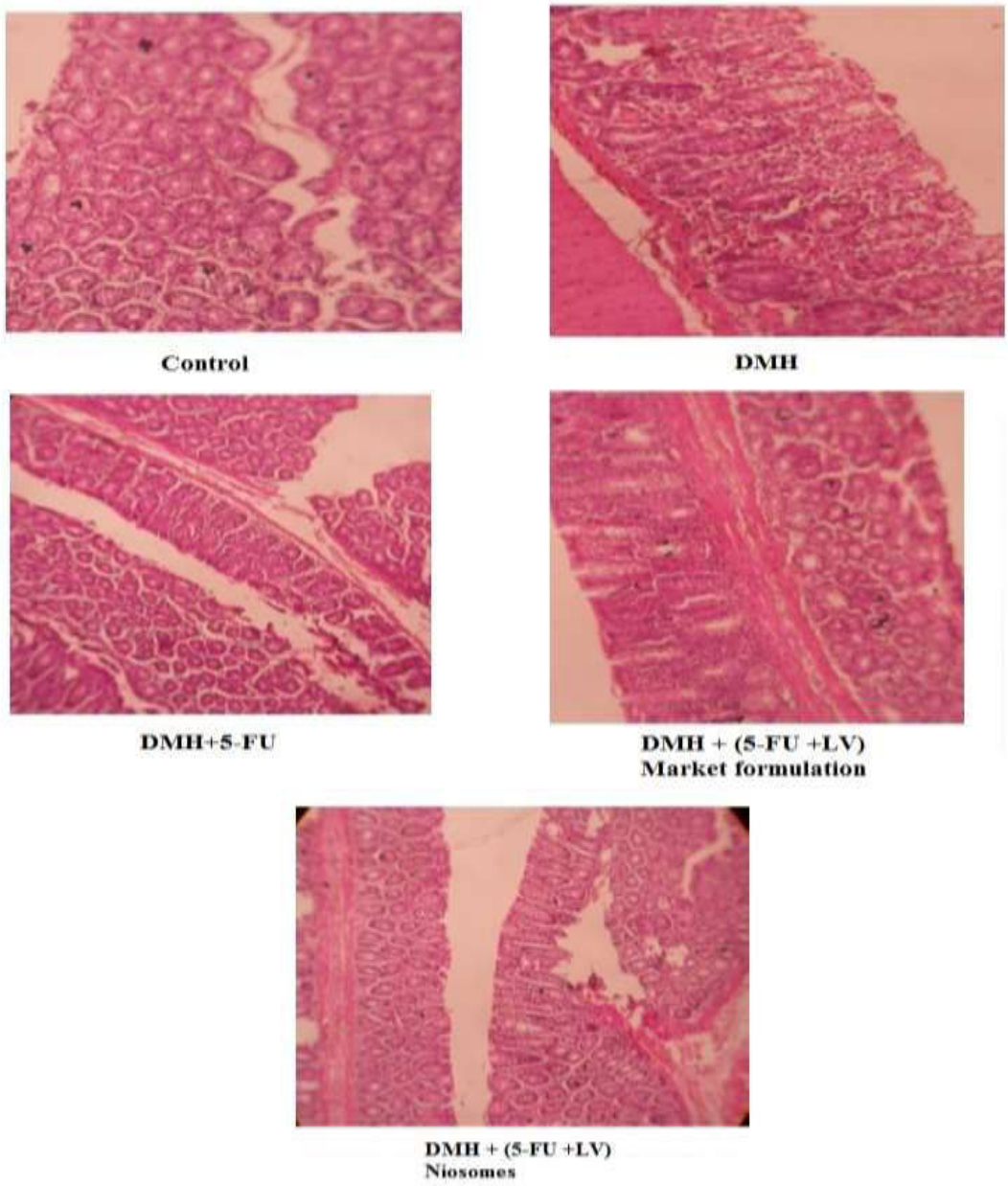


Fig 5.19: Effect of 5-FU + LV niosomes on colon histology in DMH induced colon cancer

6. DISCUSSION

6.1. Preformulation Studies

Preformulation study is the primary step in the rational development of dosage forms of a new drug entity. It is the investigation of physical and chemical properties of a drug substance alone and when combined with excipients. The overall objective of preformulation testing is to provide information related to the formulation in developing stable and bioavailable dosage forms that can be mass-produced. The type of information needed will depend on the dosage form to be developed.

The first step in any formulation design activity is careful consideration of the Preformulation data. It is important that the formulation have a complete physicochemical profile of the active ingredients available prior to initiating formulation development activity.

6.1.2. FT-IR Spectral Analysis

Based on the results, intense characteristic peaks at 3160, 1727, 1662, 1426, 1247, 811.7 and 547cm^{-1} are detected due to the vibration of imide stretch (amide II and amide III) and aromatic ring in the structure of 5-FU . In the case of the FTIR spectra of LV, some absorption bands are observed at 1609, 1324, 1190, and 763cm^{-1} corresponding to the vibration of NH stretch, aromatic ring, C-O, C–H groups. The characteristic peaks of 5-Fluorouracil and leucovorin appeared in spectra without any remarkable change position in that of prepared niosomes. The results revealed that no chemical interaction between drugs and excipients.

6.2. Optimization of Niosomes

The optimization of the niosomes was made by altering the cholesterol ratio and evaluating the niosomes by using *in vitro* drug release. The uniformity of the particle size and the formation of the particles are considerably noted. Following which the sonication process was carried out. The larger the cholesterol content the formation of the flakes in the solution takes place and the formation of the thin film in the round bottom flask is increasing timely. Optimum amount is by taking the equal proportion of the cholesterol and the surfactant (Span 40, Span 60, Tween 40 and Tween 60) gives a better formulation.

From composition of all the formulations, the best formulation is obtained from the as we increase the cholesterol concentration and keeping the surfactant concentration constant the formation of the niosomes is uniform and the visibility of the cholesterol flakes in the formulation is nil. But the time taken for the organic solvents to get evaporated is increasing. The film which is formed on the round bottom flask is kept overnight for the complete removal and drying of the organic solvents from the film. The best formulation is taken from the further evaluation.

Large volume of organic solution of lipids is most easily dried in a rotary evaporator fitted with a coil which is cool and a thermostatically controlled water bath. Rapid evaporation of solvent is carried out by gentle warming (20° - 40°C) under reduced pressure (400 - 700 mmHg). Rapid rotation of the solvent containing flask increases the surface area for evaporation. In case, where sufficient vacuum is not attainable or if the concentration of lipids is particularly high, it may be difficult to remove the last traces of chloroform from the lipid. Therefore, it is recommended as a matter of routine that after rotary evaporation some further means is provided so that

the residue is completely dried. Attachment of the flask to the manifold of lyophilizer and overnight exposure to high vacuum is a good method.

Increasing the sonication time resulted into reduction in percent drug entrapment the decrease drug entrapment is due to leakage of the drug during sonication. Cholesterol provides endurance against mechanical strain during sonication and centrifugation. Sonication brings about size reduction by breaking large niosomes to smaller ones and in doing so, leakage of small quantities of drug from the niosomes occur. Hence the sonication time was made to optimize for 15 minutes, and further size reduction by increasing the time of sonication was not attempted.

6.3. Evaluation of Niosomes

6.3.1. Particle Size Analysis

The particles of the drug loaded niosomes are in the size of 0.1 to 1 μ range. More number of the particles is in the 0.1μ to 0.8 μ approximately and then larger particle size distribution is also seen.

6.3.2. Optical Microscopy

The microscopic methods include the use of Bright field, phase contrast microscope and fluorescent microscope and are useful in evaluating the vesicle size of large vesicles (>1μm) particularly the upper end of the size distribution for miltilamellar vesicles. Vesicular dispersion appropriately diluted are wet mounted on a haemocytometer and photographed by using phase contrast microscope. Then the negatives can be observed on a piece of calibrated paper using a photographic enlarger at X 1250 diameters of approximately 500 vesicles are determined and

hence this method is tedious and coupled with the limitation of resolution, hence electron microscopic methods with greater resolutions are preferred.

6.3.3. Morphological studies

The TEM image shows the discrete formation of the structure and the internal morphology. The mean size of the niosome increased with progressive increase in the HLB value because surface free energy decreases on increasing hydrophobicity of surfactant. Surfactants with longer alkyl chains generally give larger vesicles. These result reasonable with that of higher entrapment efficiencies with span 60 niosomes.

6.3.4. Zeta Potential

Niosomes has a Negative Zeta Potential which indicates that it has excellent stability due weak electrostatic repulsive force exists in the niosomal bilayer. The niosome formulations have more tendencies to be in suspended condition/ dispersed condition for longer duration. Particles with zeta potential close to zero are less able to be phagocytosed than charged particles

6.3.5. Drug entrapment efficiency:

The quantity of the drug entrapped in the niosomes is very essential to know before analysing the behaviours of the drug entrapped in physical or biological system. The process and formulation variables like cholesterol was altered and optimized to obtain the niosomes with maximum drug entrapment. 5-Fluorouracil and leucovorin entrapped niosomes were subjected to percentage drug entrapment.

With increase in the cholesterol concentration more number of niosomes per ml of the niosomal dispersion was formed, though the optimum concentration resulting into an increased percent drug entrapment. The solvent mixture composition used was chloroform / methanol, is an attempt to enhance percent drug entrapment.

The solvent mixture composition was optimized to 1:1 (5ml v/v). The presence of large volume of hydration medium helps in faster and efficient hydration of the niosomes. Therefore, the volume of the hydration medium was optimized to 10ml.

Incorporation of cholesterol into niosomes at ratios up to 1:2 increased the encapsulation efficiency of drugs. Inclusion of cholesterol increases the viscosity of the formulation indicating more rigidity of the bilayer membrane. Moreover, drug partitioning will occur rapidly in highly ordered systems containing both surfactant and cholesterol. The ability of the lamellar surfactant phase to accommodate drug, depends upon the structure of the surfactant phase. The entrapment efficiency of Span 60 was higher than Span 40. The higher entrapment may be due to the solid nature, hydrophobicity, and high-phase transition temperature of the surfactant.

6.3.6. *In Vitro* Drug Release

The slower release of 5-Fluorouracil and leucovorin from the lipid layer may be due to slow partitioning and diffusion of 5-Fluorouracil and leucovorin from the lipid layer to the surrounding aqueous layer. Increasing cholesterol markedly reduces the efflux of the drug. Inclusion of cholesterol fills the pores in vesicular bilayers and abolishes the gel-liquid phase transition of liposomal and niosomal systems resulting in niosomes that are less leaky. This confirms that cholesterol in the formulation acts as a membrane stabilizing agent that helps to sustain drug release. Differences in the *in vitro* release profiles may be due to vesicle size, lamellarity, and membrane fluidity as a function of chain length of surfactant and cholesterol content. *In vitro* release data of niosomal formulation containing 5-Fluorouracil and leucovorin prepared with span 60 was selected for kinetics study analysis.

6.4. Preclinical evaluation of Niosomes

6.4.1. Effect of 5-FU + LV niosomes (F4) against 1, 2 Dimethyl hydrazine (DMH) induced colon cancer

Colon carcinogenesis multistage process which involves initial, secondary, and progression phases. Hence, the multistage sequence of events has many phases for prevention and intervention. Chronic inflammation is a well-recognized risk factor for development of human cancer. DMH is a chemical carcinogen known to cause colon cancer with a reproducible experimental in vivo system for studying "sporadic" (nonfamilial) forms of colon carcinoma. Metabolism of DMH leads to the formation of AOM, MAM, and methyl diazonium ions which involves alkylation of colonic mucosal DNA. The primary metabolite of DMH, namely AOM, w is responsible for the methylation at the O-6 position of guanine, occurs within 6 to 12 h of DMH injection[134].

6.4.2. Effect of 5-FU + LV niosomes (F4) on antioxidants and MDA level in DMH induced colon cancer.

Studies suggest that rapid cell proliferation involved in colon cell. Hence, cancer cell have certain characteristics that promote proliferation and tend to faster cell proliferation[135]. DMH itself can generate H_2O_2 in the presence of copper ions. In the presence of metal ions such as Fe^{2+} and Cu^{2+}, H_2O_2 can react with O_2 to convert it into the more reactive OH radical. If sufficient amounts of CAT or GPx are not available to decompose H_2O_2, the generated OH radicals are capable of attacking DNA basement. GSH an important non-protein thiol in conjunction with GPx and GST plays a significant role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species[136]. The present study, correlates

with the decline in circulatory antioxidants such as SOD, CAT, GPx and GSH. This may be due to their overusage to scavenge the products of lipid peroxidation and sequestration by tumor cells. GPx uses H_2O_2 to catalyse the oxidation of GSH to GSSG, thereby nullifying the deleterious effects of H_2O_2[137]. Diminished GPx activity indicates cellular accumulation of the lipid hydroperoxides, which can potentially turn on a chain reaction, wherein more polyunsaturated fatty acid become targets for further peroxidative tissue injury. In the present study, GPx level was reduced in colonic tissue, which could be due to the elevated levels of hydroperoxides. However, 5-FU + LV niosomes treatment effectively reduced the lipid peroxidation and improved the antioxidant status.

6.4.3. Effect of 5-FU + LV niosomes (F4) on serum lipid profiles in DMH induced colon cancer

Previous studies elicits that xenobiotic like DMH causes an increase in nonspecific microsomal enzyme activities like liver microsomal HMG-CoA reductase which is involved in the synthesis of cholesterol. Also, DMH induce liver damage and consequently results in loss of feedback control of cholesterol synthesis in liver, thereby increasing the concentration of bile acids and cholesterol in the bowel and serum [138,139]. In our study, serum level of cholesterol and triglycerides were significantly elevated in DMH induced group may be due to higher cholesterogenesis induced by DMH. Whilst, treatment with 5-FU+ LV niosomes in DMH induced rats markedly reduced the serum level of total cholesterol and triglycerides.

6.4.4. Effect of 5-FU + LV niosomes (F4) on serum hepatic markers enzyme in DMH induced colon cancer

The hepatic cell membrane damage releases the enzymes SGOT, SGPT, ALP into circulation, which can be measured in serum. High levels of SGOT indicate liver damage. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Hence, SGPT is highly specific to the liver, and therefore a better parameter for detecting liver injury. Previous studies indicate the heaptotoxicity role of DMH during the event of colon cancer [140]. In this study, DMH intoxicated rats displayed markedly elevated the level of hepatic marker enzymes (SGOT, SGPT and ALP) in serum and treatment with 5-FU+ LV niosomes effectively decreased the hepatic markers enzyme level and restores the hepatocellular membrane damage elicited by DMH.

6.4.5. Effect of 5-FU + LV niosomes (F4) on tumour markers in DMH induced colon cancer

CEA is the best marker in colorectal cancer patients and also most thoroughly characterized tumour-associated antigens, in both biochemical and clinical aspects[141]. AFP is rarely reported until now in colorectal cancer compared to other cancers but it can also be an indicative of colorectal cancer[142]. In this study, serum level of CEA and AFP were significantly elevated and 5-FU+ LV niosomes treatment effectively reduced the level of tumor markers (CEA and AFP) to normalcy.

6.4.6. **Effect of 5-FU + LV niosomes (F4) on colon histology in DMH induced colon cancer**

Histopathological observations of the tissue sections of different experimental group of animals showed that the administration of 5-FU+LV niosmes inhibited the histological changes induced by DMH in rats and effectively preserved colonic epithelium from carcinogenic effect of DMH.

7. SUMMARY AND CONCLUSION

Niosomal formulations containing 5- Fluorouracil and leucovorin were successfully prepared with different surfactants like Span 40, Span 60, Tween 40, and Tween 60 by thin film hydration technique. The evaluation parameters revealed that 5-Fluorouracil and leucovorin niosomes shows reduced particle size distribution with better entrapment efficiency. Niosomes has negative surface charges which indicate excellent stability. *In vitro* release of 5-Fluorouracil and Leucovorin from niosomes was carried out. Formulation F4 was shown better sustained release among other formulations. *In vitro* release kinetics study was done for formulation F4 to find out the release mechanism. The release of 5-FU from niosomal formulation follow Non Fickian type diffusion, whereas release LV from niosomal formulations follows Fickian diffusion mechanism. The result suggests that niosomal formulation can provide consistent and prolonged release of the entrapped drug molecules. Niosomal delivery system can provide sustained action of the entrapped drug and reduce the side effects associated with frequent administration of the drug and potentiate the therapeutic effects of the drug.

Further, preclinical evaluation of prepared 5-FU+ LV niosomes displayed efficient anticancer potential in DMH induced colon cancer by restoration of altered biochemical levels. Furthermore, 5-FU+ LV niosomes displayed marked anticancer efficacy as that of the 5-FU+ LV market formulation and 5-FU alone

8. IMPACT OF THE STUDY

Attempt has been made to incorporate 5-Fluorouracil and Leucovorin niosomes in blend of surfactants to provide sustained release of the entrapped drug for colon delivery system. The niosomes designed and prepared in the current study elicits reduced particle size distribution with marked entrapment efficiency. Further, the prepared niosomes displayed better sustained release as that of the marketed formulation containing 5-FU+LV and 5-FU alone.

The highlights of the study, was the marked anti-colon cancer activity exhibited by the 5-FU+LV niosomes in a preclinical model as that of the market formulation 5-FU+LV and 5-FU alone.

Further trials could be attempted in future on these 5-FU+LV niosomes,

- ✓ The anti colon cancer activity of 5-FU+LV niosomes could be tested in cell lines.
- ✓ Attempt towards further preclinical studies for 5-FU+LV niosomes could be carried out based on the pharmacokinetic evaluation.

KMCH College of Pharmacy, Coimbatore, Tamil Nadu, India.

Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA)
Institutional Animal Ethics committee (IAEC).

CERTIFICATE

Title of the Project	:	Development of formulation and evaluation of niosomal drug delivery system for improved anti-cancer activity
Proposal Number	:	KMCRET/Ph.D/02/2012-13
Date received after modification (if any)	:	-NA-
Date received after second modification	:	-NA-
Approval date	:	23-06-2012
Animals	:	Wistar rats / Mice / Rabbit / Guinea pig
No. of animals sanctioned:	:	Male/Female 48 NOS
Expiry date (Termination of the Project)	:	23-07-2013
Name of IAEC chairperson	:	**Dr. A. Rajasekaran**
		Signature of IAEC Chairperson A Rajasekaran
		Date:

Vivek
(Shri. Vinayak Kulkarni)
CPCSEA Inspector & Nominee

(Dr.P. Chinnaswamy)
Biological Scientist

(Shri. S. Sengottuvelu)
Biological Scientist

TR Muneera 23/6/12
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Research Article **CODEN: IJRPJK** **ISSN: 2319 – 9563**

International Journal of Research in Pharmaceutical and Nano Sciences

Journal homepage: www.ijrpns.com

FORMULATION AND EVALUATION OF NIOSOMES CO-LOADED WITH 5-FLUOROURACIL AND LEUCOVORIN: CHARACTERIZATION AND *IN VITRO* RELEASE STUDY

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ABSTRACT

In the present study, niosomes co-loaded with 5-Fluorouracil and Leucovorin was prepared and evaluated for their characterization and in vitro drug release. Formulation of niosomes was optimized for highest percentage of drug entrapment. Microscopic observation confirmed that all particles were uniform in size and shape. The entrapment efficiency was optimized using different concentration of cholesterol and non-ionic surfactants. The in vitro release studies of drug from niosomes exhibited a prolonged drug release as observed over a period of 12 h. The negative values of zeta potential indicated that the 5-Fluorouracil and Leucovorin loaded niosomes were stabilized by electrostatic repulsive forces. Results from stability study have shown that the drug leakage from the vesicles was least at 4°C followed by 25°C and 37°C. The mechanism of release of 5-FU and LV was found Non-Fickian and Fickian diffusion respectively. Niosomes can be formulated by optimised process parameters to enhance 5-FU and LV entrapment efficiency and sustainability of release. These improvements in 5-Fluorouracil and Leucovorin niosomal formulation may be useful in developing a more effective combination for cancer therapy.

KEYWORDS

Niosomes, Multiple drug loading, Leucovorin, 5-Flurouracil and Colon cancer.

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INTRODUCTION

In recent years, niosomes as a drug carrier has been received much attention in pharmaceutical academia and industrial research. Generally niosomes have a bilayer structure and are formed by self-association of nonionic surfactants and cholesterol in an aqueous phase[1]. It has many advantages like biodegradable, biocompatible, and nonimmunogenic, long shelf life, exhibit high stability and achieves the delivery of drug at target

site in a controlled and/or sustained manner. Also niosomes may alleviate the disadvantages associated with liposomes such as chemical instability, variable purity of phospholipids and high cost[2]. Encapsulation of a large number of drugs with a wide range of solubility in niosomes using various types of nonionic surfactants has been extensively studied[3].

Combination therapy with drugs of different therapeutic effects shows an improving efficacy in the treatment of various diseases. Particularly, cancer treatment requires simultaneous administration of different combination of drugs due to the molecular complexity of cancer diseases[4,5]. Niosomes show potential in combination of drug delivery and targeting combine transdermal and tumor targeting ability in cancer therapy[6,7]. 5-Fluorouracil (5-FU) has been in clinical use as an anticancer drug for more than 30 years. Although 5-FU has a broad spectrum of anticancer activity including common solid tumors present in the gastrointestinal system. But only a minority of patients treated with 5-FU experience an objective response to therapy[8]. Leucovorin is an active metabolite of folic acid and an essential coenzyme for nucleic acid synthesis. Leucovorin may significantly increase both the clinical efficacy and the clinical toxicity of 5-FU in cancer patients. From literature we found that 5-Flurouracil in combination with leucovorin is well acknowledged in the treatment of cancer[9-11]. But only a few approaches consider the developing new drug delivery systems co-loaded with 5-FU and Leucovorin has been investigated.

The purpose of this study was to evaluate the process parameters that critically affect the formulation of niosomes with respect to entrapment and release of 5-FU and Leucovorin. There is little information in the literature on optimizing the different processing variables that are important in the formulation of 5-FU and Leucovorin niosomes for development of an improved drug delivery system. In this investigation, we optimized concentration of cholesterol and non ionic surfactants (Span 40, Span 60, Tween 40 and Tween 60) used in the niosomal formulations. The niosomes was characterized such as entrapment efficiency, particle size analysis, surface morphology (SEM and TEM) and zeta potential. Measurements of in vitro drug release of 5-FU and leucovorin were done to assess the effectiveness of the drug delivery system.

MATERIAL AND METHODS

Drugs and Chemicals

5-Fluorouracil and Leucovorin was procured from Sigma Aldrich, India. Cholesterol, polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and dicetyl phosphate (DCP) were procured from Merck, India. All materials used in the study were of analytical grade.

FTIR Spectral analysis

FTIR spectral analysis of pure drug and excipients was carried out and observation was done to analyze any changes in the chemical nature of the drug after combining it with the excipients occurred. The drug samples were mixed with KBR and the pressure (600 Kg/cm^2) were applied to get pellets and scanned with the IR instrument (Shimadzu, 8400 Series, Tokyo, Japan) from 400-4000cm^{-1}.

Differential Scanning Colorimetry (DSC)

In differential Scanning Colorimetry, the samples were heated from 25°C to 100°C at a constant temperature increment of 10° C/min and purged with nitrogen gas at 40 ml/min.

Preparation of the niosomes

Niosomes were prepared by a thin film hydration technique using a mixture of surfactants encompassing (span 40, span 60, tween 60 and tween 40) and cholesterol, at different specified ratios as given in Table No.1. Surfactant and cholesterol was dissolved in 8 ml of diethyl ether and the drugs were dissolved in 2 ml of ethanol. The mixture was then transferred to a round bottom flask, and the solvent was evaporated under reduced pressure at a temperature 20-25º, using a rotary flash evaporator until the formation of a thin lipid film. The resultant film was made wet with 10 ml of phosphate buffer saline pH 7.4. The hydration was

continued for 1 h, while the flask was kept rotating at 55-65°. The hydrated niosomes were sonicated for 20 min using a bath sonicator to obtain niosomal dispersion.

Entrapment efficiency for the Niosomes

The niosomal dispersion is a homogeneous milky white suspension which is centrifuged at 12000 rpm for 15 min and entrapment efficiency of niosomes were evaluated by measuring the UV absorption of the supernatant. The corresponding calibration curves were made by testing the supernatant of blank niosomes. Each sample was measured in triplicate. 5-FU was measured at 266nm and elicited an intense characteristic peak whilst LV displayed a characteristic peak at 288 nm. The percentage of the entrapment of the drug can be obtained for the niosomal formulations.

Particle Size Analysis (PSA)

The size division of the niosomes was analyzed using the Beckman particle size analyzer (Beckman Coulter, Delsa nano C, Brea, USA) prepared with a dry accessory system. Samples were mixed with water and maintained at a temperature of 25°C.

Zeta Potential Analysis

The zeta potential was measured using a Beckman Coulter (Beckman Coulter Delsa Nano C, Brea, USA). The sample was diluted with double distilled water and transferred to cuvettes and maintained at a temperature of 25°C.

MORPHOLOGICAL ANALYSIS OF NIOSOMES

Optical microscopy

Characterizations of vesicle dispersions were done by photo microscopy for vesicle formation and morphology. The size and shape of vesicles in niosomes formulations were observed by optical microscopy using a calibrated eyepiece micrometer, and images were captured at resolution of 400 X with a digital camera (Olympus, 8.1 megapixel, Japan).

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used in the morphological analysis of niosomes. Samples were processed by spreading a drop of freshly prepared nano-suspension onto copper grid with carbon film support and excess solution was cleared using a filter paper. Samples were allowed to dry at room temperature and observed directly with TEM (JEM-100, JEOL, Tokyo, Japan) without further staining.

Scanning Electron Microscopy (SEM)

The SEM is used to identify the particle size and the surface morphology of the niosomes. For niosomes, the drop of the sample is placed on the covered glass slide and then dried by applying vacuum, later it was coated with gold to a thickness of 100A using VEGAS TESCAN Vacuum evaporator and the image was captured for the niosomal formulation.

***In-vitro* release studies for the niosomes**

In vitro release of 5-FU and leucovorin niosomes was conducted by a dialysis membrane having a pore size of 2.4 mm (LA-395-5Mt Himedia Pvt. Ltd, Mumbai, India) with 75 ml of pH 7.4 phosphate buffer at 37°C. Briefly in a 100 ml beaker 75ml of pH 7.4 phosphate buffer was taken. A 2 ml of formulation was taken into a dialysis bag and immersed into the buffer solution. The dialysis membrane was activated earlier using by soaking in 1% w/v NaOH over night. Then the flask was placed on a magnetic stirrer. The stirring was done at 250 rpm and the temperature of the buffer was maintained at 37°C. Sampling was done by withdrawing 1 ml of aliquots from a beaker. Immediately 1 ml of new buffer was added to keep the sink condition. Samples were analyzed after sufficiently diluting with buffer by using a UV-Visible Spectrophotometer (UV/VIS-Double beam Spectrophotometer, V-530, Jasco, Tokyo, Japan) at a wavelength of 266 nm for 5-FU and 288 nm for LV [15].

***In vitro* release kinetics study**

The release data obtained via the above procedure was derived using Ritger and Peppas model to devise its release mechanism. The initial 60% cumulative release data were used to estimate the diffusion exponent 'n' by using following equation.

$$M_t / M_\infty = Kt^n$$

Where M_t is the amount of drug released at time t, $M\infty$ the nominal total amount of drug released, K the kinetic constant, and n the diffusion exponent

that is used to characterize the release mechanism. For spheres, a value of $n \leq 0.43$ indicates Fickian release and 'n' value between 0.43 and 0.85 is an indication of non-Fickian release (both diffusion-controlled and swelling-controlled drug release). An 'n' value ≥0.85 indicates case-II transport that involves polymer dissolution and polymeric chain enlargement or relaxation.

Stability studies

Stability studies for the optimized niosomes (F4) were carried out at refrigeration temperature, room temperature and elevated temperature (4 ± 2°C, 25 ± 2°C/ 60 % RH ± 5 % RH and 37 ± 2°C/ 65 % RH ± 5 % RH) for a period of three months. At definite time intervals, the samples from each batch were taken and evaluated using following parameters like appearance, size, assay, and dissolution.

RESULTS AND DISCUSSION

FT-IR spectral analysis

The development of a successful formulation depends only on a suitable selection of excipients. Hence the physical state of the drug 5-Flurouracil and leucovorin and the combination of drug and chloestrol and surfactant used for niosomes preparation were studied by FTIR to study the drug and excipients compatibility. The physicochemical compatibility of the drugs and the excipients was obtained by FTIR studies with the interpretation values of the FTIR were mentioned in Figure No.1.

The spectra were recorded for pure drugs, surfactants, cholesterol and optimized formulations using FTIR spectrophotometer. The spectra of pure 5-FU, LV and niosomes loaded with 5-FU and LV was shown the intense characteristic peaks at 3160, 1727, 1662, 1426, 1247, 811.7 and 547cm^{-1} are detected due to the vibration of imide stretch (amide II and amide III) and aromatic ring in the structure of 5-FU . In the case of the FTIR spectra of LV, some absorption bands are observed at 1609, 1324, 1190, and 763cm^{-1} corresponding to the vibration of NH stretch, aromatic ring, C O, C–H groups. The characteristic peaks of 5-Fluorouracil and leucovorin appeared in spectra without any remarkable change position in that of prepared niosomes. The results revealed that no chemical interaction between drugs and excipients.

Optimization of niosomes

The optimization of the niosomes was made by altering the cholesterol ratio and evaluating the niosomes by using in vitro drug release. The uniformity of the particle size and the formation of the particles are considerably noted. Following which the sonication process was carried out. The larger the cholesterol content the formation of the flakes in the solution takes place and the formation of the thin film in the round bottom flask is increasing timely. Optimum amount is by taking the equal proportion of the cholesterol and the surfactant (Span 40, Span 60, Tween 40 and Tween 60) gives a better formulation.

From composition of all the formulations (Table No.1), the best formulation is obtained from the as we increase the cholesterol concentration and keeping the surfactant concentration constant the formation of the niosomes is uniform and the visibility of the cholesterol flakes in the formulation is nil. But the time taken for the organic solvents to get evaporated is increasing. The film which is formed on the round bottom flask is kept overnight for the complete removal and drying of the organic solvents from the film. The best formulation is taken from the further evaluation.

Large volume of organic solution of lipids is most easily dried in a rotary evaporator fitted with a cooling coil and a thermostatically controlled water bath. Rapid evaporation of solvent is carried out by gentle warming (20° - 40°C) under reduced pressure (400 - 700 mmHg). Rapid rotation of the solvent containing flask increases the surface area for evaporation. In case, where sufficient vacuum is not attainable or if the concentration of lipids is particularly high, it may be difficult to remove the last traces of chloroform from the lipid. Therefore, it is recommended as a matter of routine that after rotary evaporation some further means is employed to bring the residue to complete dryness. Attachment of the flask to the manifold of lyophilizer and overnight exposure to high vacuum is a good method.

Increasing the sonication time resulted into reduction in percent drug entrapment the decrease drug entrapment is due to leakage of the drug during sonication. Cholesterol provides endurance against mechanical strain during sonication and centrifugation. Sonication brings about size reduction by breaking large niosomes to smaller ones and in doing so, leakage of small quantities of drug from the niosomes occur. Hence the sonication time was optimized to 15 minutes, and further reduction in the size by increasing sonication time was not attempted.

Particle Size Analysis

The particle size of the niosomes was analyzed by using the particle size analyzer (Malvern) and it is shown in the Figure No.2. The particles of the drug loaded niosomes are in the size of 0.1 to 1 µ range. More number of the particles was in the 0.1µ to 0.8 µ approximately and also found some larger particle size distribution.

Optical microscopy

The formulated niosomes were viewed through optical microscope and image was shown in Figure No.3. The microscopic methods include the use of Bright field, phase contrast microscope and fluorescent microscope and are useful in evaluating the vesicle size of large vesicles (>1µm) particularly the upper end of the size distribution for miltilamellar vesicles. Vesicular dispersion appropriately diluted is wet mounted on a haemocytometer and photographed with a phase contrast microscope. The negatives then can be projected on a piece of calibrated paper using a photographic enlarger at X 1250 (Vyas and Katar, 1991), diameters of approximately 500 vesicles are measured and thus this method is tedious and coupled with the limitation of resolution, hence electron microscopic methods with greater resolutions are preferred.

Morphological studies

The TEM and SEM images of the 5-FU and LV loaded niosomes are shown in the Figure No. 4 and 5**.** The images are shown the discrete formation of the structure and the internal morphology. The mean size of the niosome increased with progressive increase in the HLB value because surface free energy decreases on increasing hydrophobicity of surfactant[12]. Surfactants with longer alkyl chains generally give larger vesicles. These result reasonable with that of higher entrapment efficiencies with span 60 niosomes.

Differential scanning Colorimetry

The drugs 5-Flurouracil, Leucovorin, cholesterol, span 40, span 60, span 80 and mixture of drug and excipients were subjected to DSC studies for testing the compatibility of the drug with the excipients used in the formulation. DSC thermo gram was shown in Figure 6. The results clearly indicates that there is no interaction between drug and excipients.

The surface charge of 5-Fluorouracil and leucovorin niosomes was measured using Zeta potentiometer. The zeta potential graph was shown in Figure No.7. Zeta value of niosomes was found to be – 10 mV. Niosomes has a Negative Zeta Potential which indicates that it has excellent stability due weak electrostatic repulsive force exists in the niosomal bilayer. The noisome formulations have more tendencies to be in suspended condition/ dispersed condition for longer duration[13]. Particles with zeta potential close to zero are less able to be phagocytosed than charged particles.

Drug entrapment efficiency

The quantity of the drug entrapped in the niosomes is very essential to know before studying the behaviours of this entrapped drug in physical or biological system. The process and formulation variables like cholesterol was altered and optimized to obtain the niosomes with maximum drug entrapment. 5-Fluorouracil and leucovorin entrapped niosomes were subjected to percentage drug entrapment. The entrapment efficiency of drug containing span 60 was found to be 72.21% which showed maximum percent drug entrapment where as those containing span 40, span 60, tween 40, tween 60 were found to encapsulate 66.35, 72.21, 50.44, and 52.24. This result indicates that span 60 is the more suitable surfactant along with higher concentration of cholesterol for enhancing maximum entrapment for the drugs 5-Fluorouracil and leucovorin.

With increase in the cholesterol concentration more number of niosomes per ml of the niosomal

dispersion was formed, though the optimum concentration resulting into an increased percent drug entrapment. The solvent mixture composition used was chloroform / methanol, is an attempt to enhance percent drug entrapment. The solvent mixture composition was optimized to 1:1 (5ml v/v). The presence of large volume of hydration medium helps in faster and efficient hydration of the niosomes. Therefore, the volume of the hydration medium was optimized to 10ml. The Entrapment Efficiency of the formulations was calculated and Tabulated in the Table No.2.

Incorporation of cholesterol into niosomes at ratios up to 1:2 increased the encapsulation efficiency of drugs. Inclusion of cholesterol increases the viscosity of the formulation indicating more rigidity of the bilayer membrane. Moreover, drug partitioning will occur more easily in highly ordered systems of surfactant and cholesterol. The ability of the lamellar surfactant phase to accommodate drug, depends upon the structure of the surfactant phase. The entrapment efficiency of Span 60 was higher than Span 40. The higher entrapment may be due to the solid nature, hydrophobicity, and high-phase transition temperature of the surfactant[12].

***In vitro* drug release**

The formulated niosomes were subjected to *in vitro* drug release. The amount of 5-Fluorouracil and leucovorin diffused was estimated spectrophotometrically at nm 266 and 288 nm respectively[14,15]. The *in vitro* release profile of drugs was shown in Table No.3 and 4. Niosomes containing 5-Fluorouracil prepared with span 60 showed 99.65 % of drug release up to 12 hours shown in Figure No.8. 5-Fluorouracil release from the formulation containing span 40, Tween 40 and Tween 60 was observed almost 100% drug release within 10 hours. These results showed that niosomal formulation containing 5-Fluorouracil prepared with span 60 has sustained release up to 12 hours. Leucovorin release profile from niosomal formulation was shown in Figure No.9. Niosomes containing leucovorin prepared with span 60 showed 99.91 % of drug release up to 12 hours. Leucovorin release from the formulation containing span 40, Tween 40 and Tween 60 was observed almost 100% drug release within 10 hours. These results showed that niosomal formulation containing leucovorin prepared with span 60 has sustained release up to 12 hours.

The slower release of 5-Fluorouracil and leucovorin from the lipid layer may be due to slow partitioning and diffusion of 5-Fluorouracil and leucovorin from the lipid layer to the surrounding aqueous layer. Increasing cholesterol markedly reduces the efflux of the drug. Inclusion of cholesterol fills the pores in vesicular bilayers and abolishes the gel-liquid phase transition of liposomal and niosomal systems resulting in niosomes that are less leaky. This confirms that cholesterol in the formulation acts as a membrane stabilizing agent that helps to sustain drug release. Differences in the *in vitro* release profiles may be due to vesicle size, lamellarity, and membrane fluidity as a function of chain length of surfactant and cholesterol content. *In vitro* release data of niosomal formulation containing 5-Fluorouracil and leucovorin prepared with span 60 was selected for kinetics study analysis.

***In vitro* release kinetics**

In vitro release kinetics data was sown in Table No.5. The results shown that formulation F4, Span 60 with cholesterol follows zero-order kinetics. Calculation of Higuchi's correlation coefficient confirms that drug release was proportional to the square root of time indicating that drug release from niosomes was diffusion controlled. The n value from the Korsmeyer-Peppas model for release 5-FU from niosomal formulation was 0.67 which confirms the Non Fickian type diffusion, whereas release LV from niosomal formulations follow an Fickian diffusion mechanism ($n > 0.47$).

Stability study

The stability study result indicates that stability studies for the optimized niosomes (F4) were carried out at refrigeration temperature, room temperature and elevated temperature (4 ± 2°C, 25 ± 2°C/ 60 % RH ± 5 % RH and 37 ± 2°C/ 65 % RH ± 5 % RH) for a period of three months. Niosomes were evaluated for physical appearance, size, assay and dissolution studies. 5 – FU and LV loaded niosomes have not shown any significant change in storage condition 4°C ± 2°C. But drug leakage was

found in conditions 25 ± 2°C/ 60 % RH ± 5 % RH and 37 ± 2°C/ 65 % RH ± 5 % RH. This may be attributed to phase transition of surfactant and lipid causing vesicles leakage at higher temperature during storage[13]. Hence, it is concluded from the obtained data that the optimum storage condition for niosomes was found to be 4°C.

Table No.1: Formulation composition of Niosomes

S.No	Type of formulation	F1	F2	F3	F4	F5	F6	F7	F8
1	Drug	10	10	10	10	10	10	10	10
2	Cholesterol	10	20	10	20	10	20	10	20
3	Span 40	10	10	-	-	-	-	-	-
4	Span 60	-	-	10	10	-	-	-	-
5	Tween 40	-	-	-	-	10	10	-	-
6	Tween 60	-	-	-	-	-	-	10	10
7	Drug :cholesterol: SA	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1

Table No.2: Entrapment Efficiency of the Niosomal formulations

S.No	Formulations Code	5-FU (%)	LV (%)
1	F1	62.44	63.28
2	F2	66.35	65.02
3	F3	68.30	64.86
4	F4	72.21	69.45
5	F5	47.05	54.76
6	F6	50.44	57.92
7	F7	50.92	59.55
8	F8	52.24	60.27

Table No.3: *In-vitro* release study of the 5 – FU from Niosomes

S.No	Time(hr)	% Drug Released							
		F1	F2	F3	F4	F5	F6	F7	F8
1	1	25.12	20.78	23.43	19.23	28.34	23.02	24.72	21.66
2	2	42.04	39.02	40.12	30.22	40.22	33.22	35.15	32.17
3	4	51.06	48.9	52.22	46.89	61.27	48.82	49.42	50.92
4	6	69.35	70.33	68.79	65.88	72.52	67.94	70.21	66.25
5	8	90.23	83.45	88.16	79.43	84.67	82.64	82.56	81.92
6	10	99.34	95.74	95.34	89.58	99.96	99.52	98.9	95.99
7	12	99.9	99.13	100.02	99.65	100	99.99	100	100

Table No.4: *In-vitro* release study of the Leucovorin from Niosomes

S.No	Time (hr)	% Drug Released							
		F1	F2	F3	F4	F5	F6	F7	F8
1	1	38.96	36.72	35.07	30.01	40.04	39.02	39.27	38.12
2	2	50.22	50.38	48.39	45.22	52.78	51.35	50.55	49.55
3	4	65.15	62.12	62.07	59.12	66.9	66.17	65.22	62.49
4	6	79.6	76.2	72.98	70.36	84.56	72.56	79.21	78.01
5	8	92.1	90.08	90.14	85.43	95.9	88.54	90.56	88.32
6	10	99.34	98.94	99	93.12	99	98.18	99.82	100
7	12	99.9	100	100	99.91	99.98	99	100	100

Table No.5: *In vitro* release kinetics of 5-FU and LV from niosomes

S.No	Formulation code	Higuchi R^2	Korsmeyer-Peppas		Zero order		First order		Release mechanism
			R^2	*N*	R^2	K_0 (%mg/h)	R^2	K_1 (h^{-1})	
1	F4 (5- FU)	0.996	0.998	0.67	0.969	8.01	0.770	0.161	Non Fickian
2	F4 (LV)	0.996	0.995	0.47	0.907	7.44	0.745	0.194	Fickian

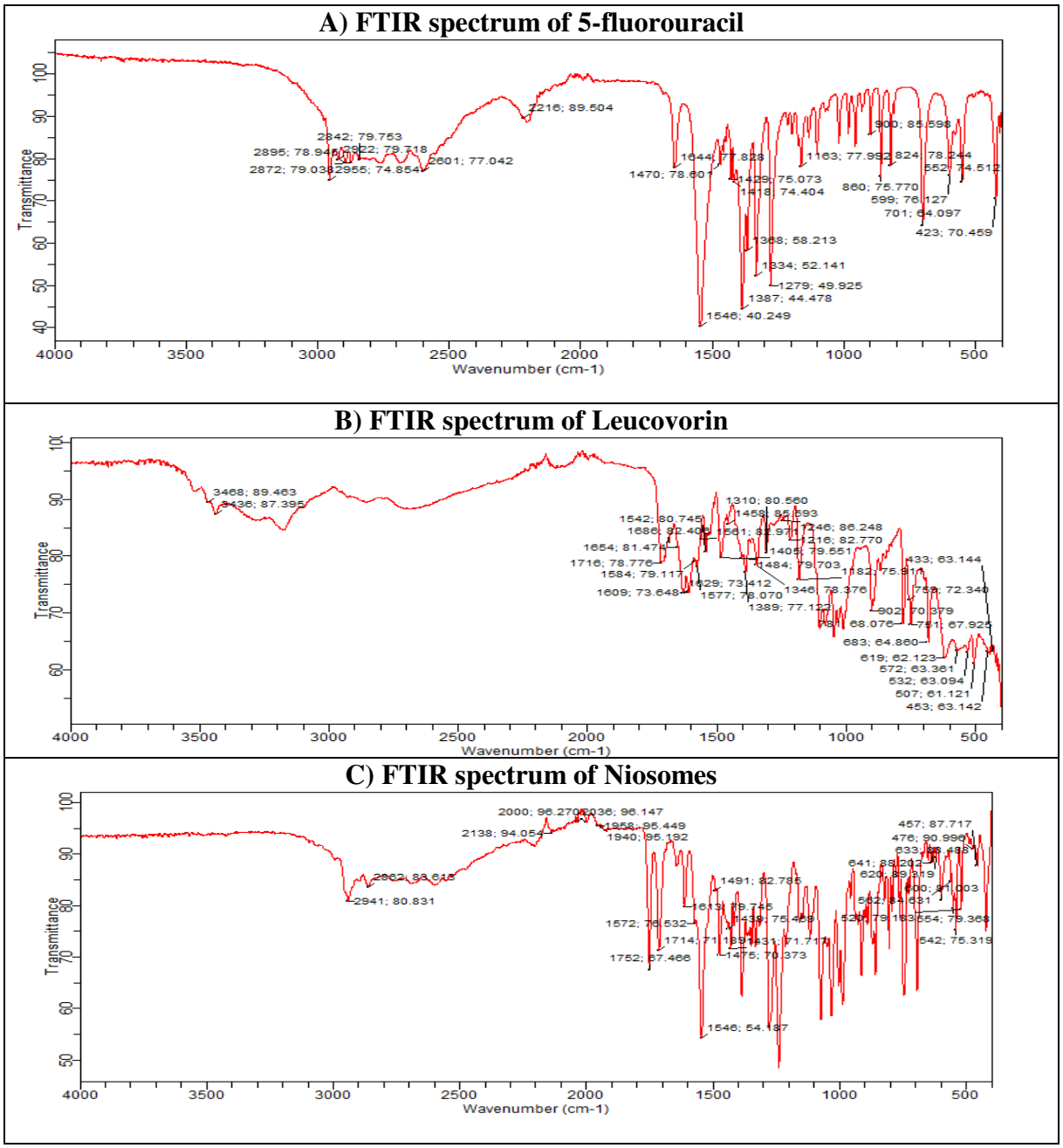

Figure No.1: FTIR spectrum

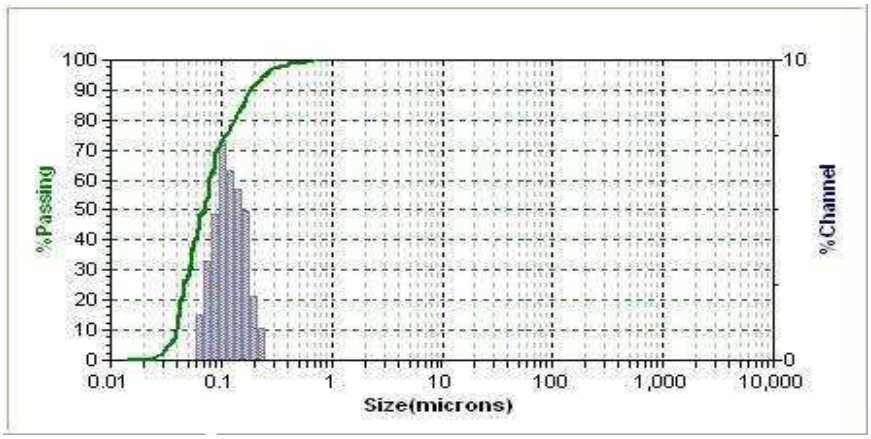

Figure No.2: Particle size analysis of niosomes

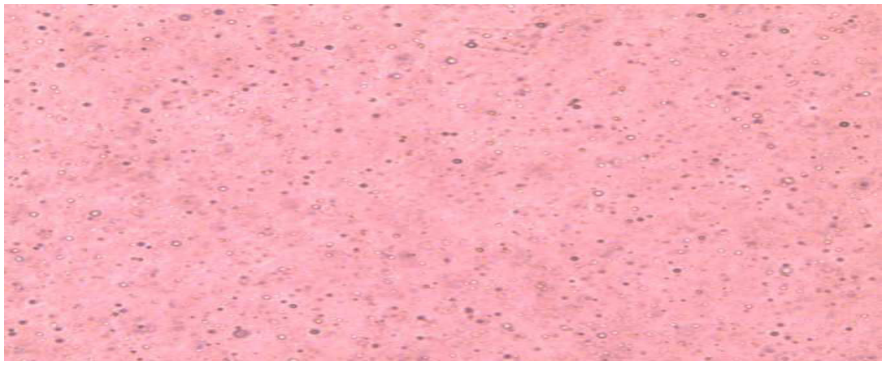

Figure No.3: Optical microscopy images of Niosomes

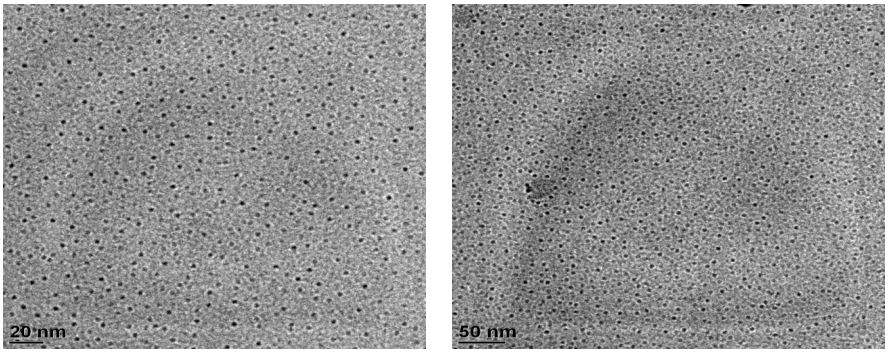

Figure No.4: TEM image of Niosomes

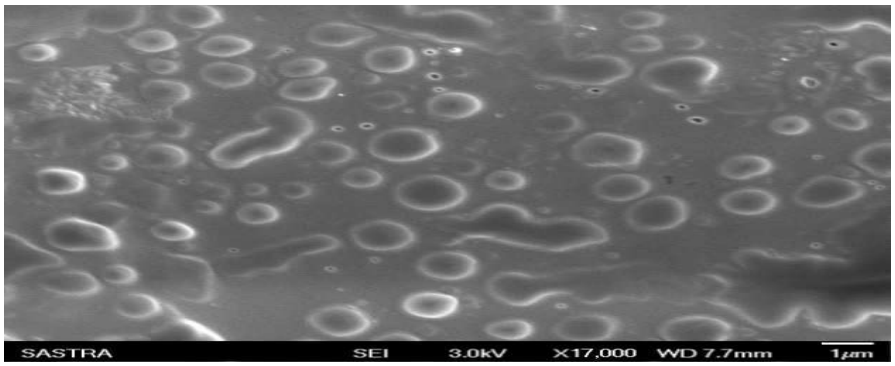

Figure No.5: SEM image of Niosomes

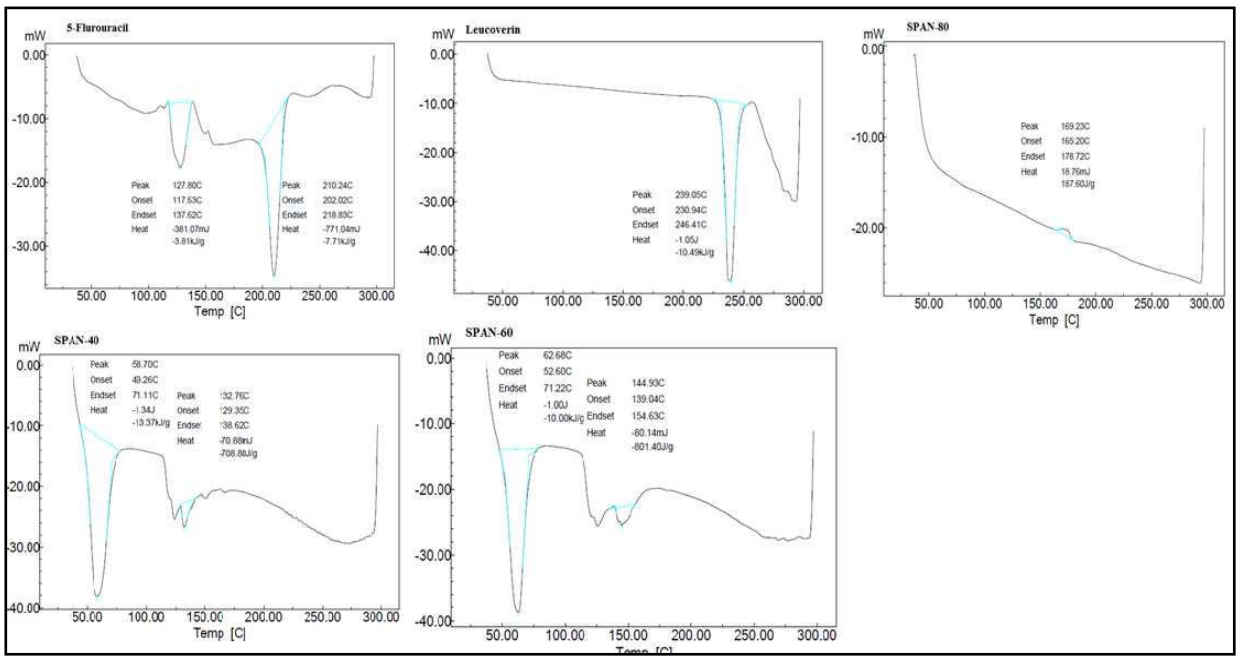

Figure No.6: DSC Studies of niosomes Zeta potential

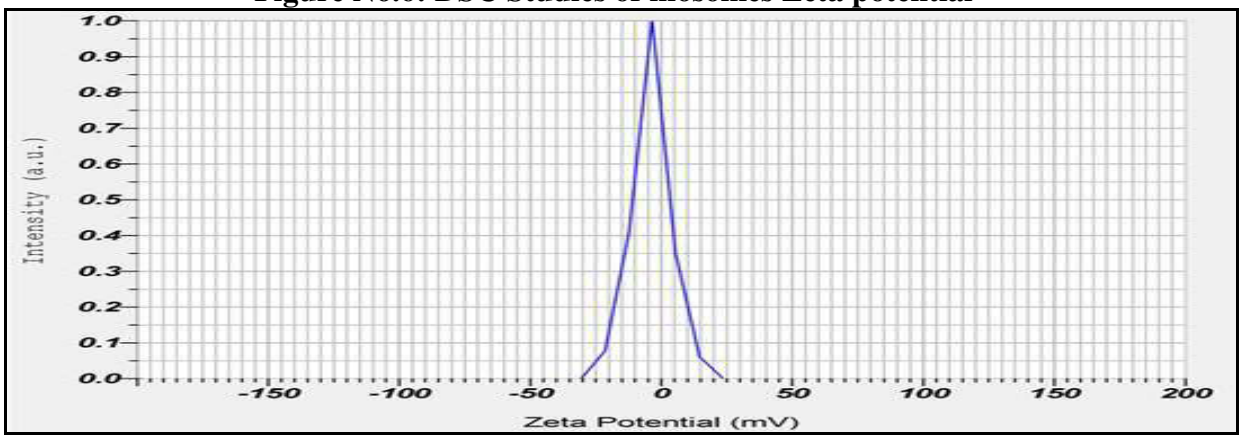

Figure No.7: Zeta potential of Niosomes

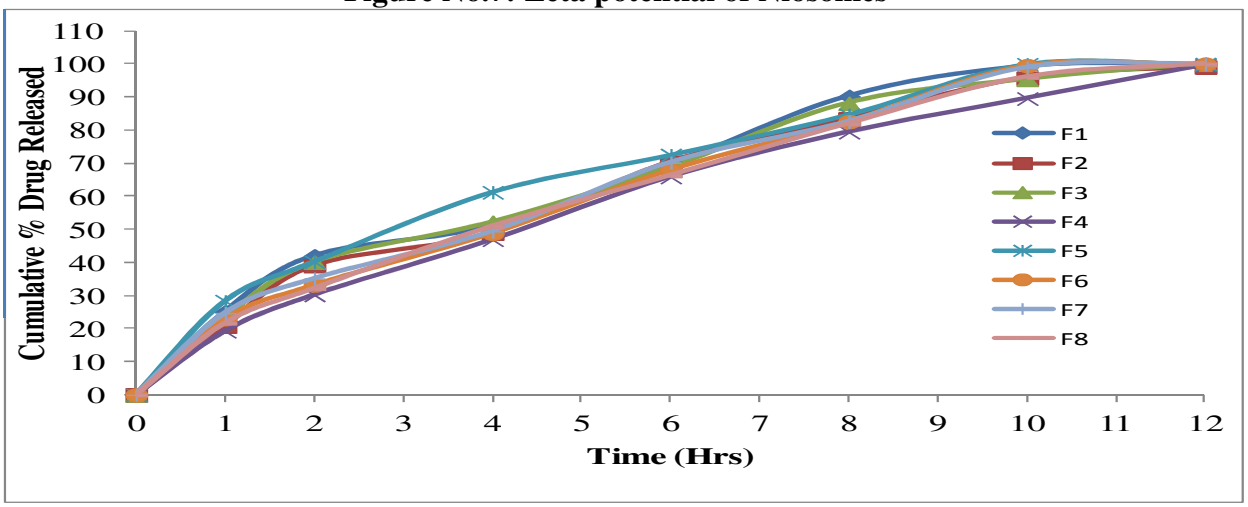

Figure No.8: *In-vitro* release study of the 5 – FU from Niosomes

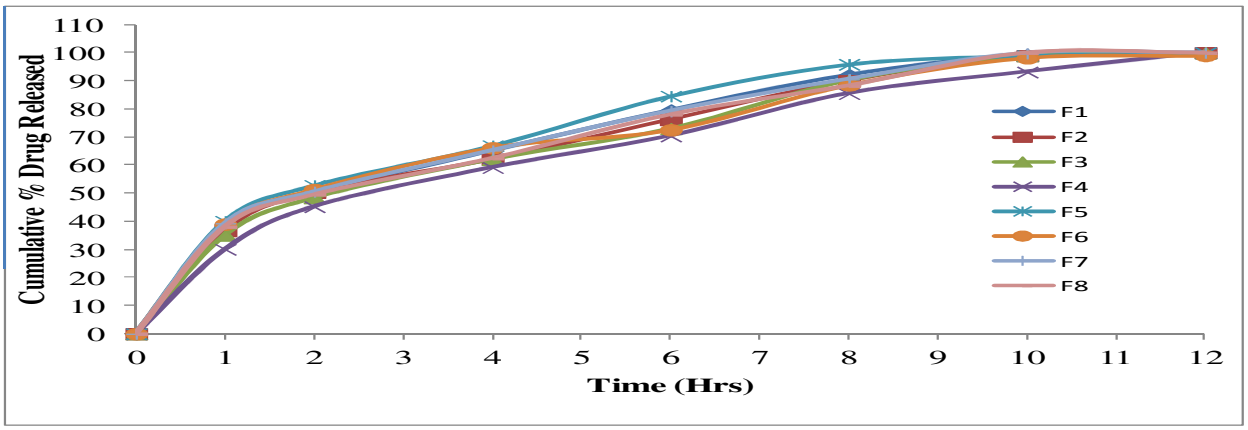

Figure No.9: *In-vitro* release study of the Leucovorin from Niosomes

CONCLUSION

Niosomal formulations containing 5- Fluorouracil and leucovorin were successfully prepared with different surfactants like Span 40, Span 60, Tween 40, and Tween 60 by thin film hydration technique. The evaluation parameters revealed that 5-Fluorouracil and leucovorin niosomes shows reduced particle size distribution with better entrapment efficiency. Niosomes has negative surface charges which indicate excellent stability. In vitro release of 5-Flurouracil and Leucovorin from niosomes was carried out. Formulation F4 was shown better sustained release among other formulations. In vitro release kinetics study was done for formulation F4 to find out the release mechanism. The release of 5-FU from niosomal formulation follow Non Fickian type diffusion, whereas release LV from niosomal formulations follows Fickian diffusion mechanism. The result suggests that niosomal formulation can provide consistent and prolonged release of the entrapped drug molecules. Niosomal delivery system can provide sustained action of the entrapped drug and reduce the side effects associated with frequent administration of the drug and potentiate the therapeutic effects of the drug.

ACKNOWLEDGEMENT

The authors would like to thank Dr.A.Meena, Principal and management of K.K.College of Pharmacy, Chennai and management of KMCH college of Pharmacy, Coimbatore for their support towards my research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: Karthick K and Arulkumaran K S G. Formulation and evaluation of niosomes co-loaded with 5-fluorouracil and leucovorin: characterization and *in vitro* release study, *International Journal* of *Research in Pharmaceutical and Nano Sciences,* 5(5), 2016, 239-250.

Research Article **CODEN: IJRPJK** **ISSN: 2319 – 9563**

International Journal of Research in Pharmaceutical and Nano Sciences

Journal homepage: **www.ijrpns.com**

FORMULATION AND PRECLINICAL EVALUATION OF NIOSOMES CO-LOADED WITH 5-FLUOROURACIL AND LEUCOVORIN

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ABSTRACT

In the present study, niosomes co-loaded with 5-Fluorouracil and Leucovorin was prepared and evaluated for *in vivo* anticancer efficacy in Dimethyl hydrazine (DMH) induced colon cancer. In the present study, colon cancer was induced by s.c injection of DMH (20 mg/kg b.wt) for 15 weeks. The animals were divided into five groups as follows control, DMH alone, DMH and 5-FU, DMH and 5-FU + LV market formulation and DMH and 5-FU + LV niosomes formulation and the treatment was carried out for 15 weeks. At the end of the study period the blood was withdrawn and serum was separated for haematological, biochemical analysis and tumor markers. Further, the colonic tissue was removed for the estimation of antioxidants and histopathological analysis. The results of the study displays that DMH intoxication elicits altered haematological parameters (RBC, WBC and Hb), elevated lipid peroxidation and decreased antioxidants level (SOD, CAT, GPX, GST and GSH), elevated lipid profiles (cholesterol and triglycerides), tumor markers (CEA and AFP) and altered colonic tissue histology. Mean while, treatment with 5-FU + LV niosomes significantly restored the altered biochemicals parameters in DMH induced colon cancer mediated by its anticancer efficacy. Further, 5-FU + LV niosomes showed marked efficacy as that of the 5-FU + LV market formulation and 5-FU-alone.

KEYWORDS

Niosomes, Multiple drug loading, Leucovorin, 5-Flurouracil, Colon cancer, Dimethyl hydrazine, Antioxidant and Lipid peroxidation.

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INTRODUCTION

In recent years, niosomes as a drug carrier has been received much attention in pharmaceutical academia and industrial research. Generally niosomes have a bilayer structure and are formed by self-association of nonionic surfactants and cholesterol in an aqueous phase[1]. It has many advantages like biodegradable, biocompatible, and nonimmunogenic, long shelf life, exhibit high

stability and achieves the delivery of drug at target site in a controlled and/or sustained manner. Also niosomes may alleviate the disadvantages associated with liposomes such as chemical instability, variable purity of phospholipids and high cost[2]. Encapsulation of a large number of drugs with a wide range of solubility in niosomes using various types of nonionic surfactants has been extensively studied[3].

Combination therapy with drugs of different therapeutic effects shows an improving efficacy in the treatment of various diseases. Particularly, cancer treatment requires simultaneous administration of different combination of drugs due to the molecular complexity of cancer diseases[4,5]. Niosomes show potential in combination of drug delivery and targeting combine transdermal and tumor targeting ability in cancer therapy[6,7]. 5-Fluorouracil (5-FU) has been in clinical use as an anticancer drug for more than 30 years. Although 5-FUhas a broad spectrum of anticancer activity including common solid tumors present in the gastrointestinal system. But only a minority of patients treated with 5-FU experience an objective response to therapy[8]. Leucovorin is an active metabolite of folic acid and an essential coenzyme for nucleic acid synthesis. Leucovorin may significantly increase both the clinical efficacy and the clinical toxicity of 5-FU in cancer patients. From literature we found that 5-Flurouracil in combination with leucovorin is well acknowledged in the treatment of cancer[9-11]. But only a few approaches consider the developing new drug delivery systems co-loaded with 5-FU and Leucovorin has been investigated.

The purpose of this study was to prepare 5-FU and Leucovorin niosomes formulation and evaluate its anticancer efficacy in Dimethyl hydrazine (DMH) induced colon cancer in a animal model.

MATERIAL AND METHODS

Drugs and Chemicals

5-Fluorouracil and Leucovorin was procured from Sigma Aldrich, India. Cholesterol, polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60) and dicetyl phosphate (DCP) were procured from Merck, India. All materials used in the study were of analytical grade.

Preparation of 5-FU and Leucovorin Niosomes

Niosomes were prepared by a thin film hydration technique using a mixture of surfactants encompassing (span 40, span 60, tween 60 and tween 40) and cholesterol, at different specified ratios as given in Table No.1. Surfactant and cholesterol was dissolved in 8 ml of diethyl ether and the drugs were dissolved in 2 ml of ethanol. The mixture was then transferred to a round bottom flask, and the solvent was evaporated under reduced pressure at a temperature 20-25º, using a rotary flash evaporator until the formation of a thin lipid film. The resultant film was made wet with 10 ml of phosphate buffer saline pH 7.4. The hydration was continued for 1 h, while the flask was kept rotating at 55-65°. The hydrated niosomes were sonicated for 20 min using a bath sonicator to obtain niosomal dispersion.

Evaluation of 5-FU and Leucovorin Niosomes

The evaluation of niosomes was carried in our laboratory as described in the previously published paper[12].

Preclinical anti colon cancer evaluation of 5-FU and Leucovorin Niosomes

Chemicals

The 1, 2-dimethyl hydrazine (DMH) was obtained from Sigma Aldrich, India and all other chemicals and reagents used were of analytical grade.

Animals

Male Sprague-Dawley rats (300-400gm body weight) were procured from KMCH college of Pharmacy, Coimbatore, Tamilnadu, India. All the animals were kept at room temperature of 22°C under 12 hr light/12 hr dark cycle in the animal house. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. All animal procedures were performed in accordance with the recommendation of CPCSEA the proper care and use of laboratory animals the proposal of the present study was approved by IAEC of KMCH college of Pharmacy, Coimbatore, Tamilnadu, India.

Preparation of DMH solution

DMH was dissolved in 1 mm EDTA just prior to use and the pH was adjusted to 6.5 with 1mm sodium bicarbonate to ensure the stability of the chemical.

Induction of colon cancer

Animals were given a weekly subcutaneous (s.c.) injection of DMH in the groin area at a dose of 20 mg/kg body weight for 15 weeks.

Study design

The animals were divided into five groups of six animals each as follows,

Group I - Control, received 1 ml of Normal saline. p.o everyday for 15weeks.

Group II - Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c)

Group III - Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c) +5 fluorouracil (20mg/kg) i.p.

Group IV - Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c) +5-FU and Leucovorin market formulation.

Group V - Received DMH (20mg/kg body weight once in a week for 15 weeks, s.c) +5-FU and Leucovorin Niosomes formulation.

Blood Collection

After end of treatment period, the animals were anaesthetized with ketamine 2mg/kg (i.proute), blood was collected by Retro orbital puncture, with EDTA and 7 without EDTA for the enumeration of blood cell (i.e. RBC, WBC,) estimation of Hemoglobin and for estimation of various biochemical parameters respectively.

Separation of Serum

For estimating the biochemical parameters such as SGOT, SGPT, ALP and total protein the serum was separated from blood by centrifuging at 10,000 rpm for 10 minutes. The separated serum were collected and used for the parameter estimation.

Separation of Plasma

For the estimation of tumour markers such as Alpha – feto - protein (AFP), Carcinoembroyonicantigen (CEA), the blood was collected with EDTA, and centrifuged at 10,000 rpm for 5 min and the separated plasma was used for the parameter estimation

Estimation of Haematological parameters

Total Leukocyte count (TLC), total erythrocyte count (TEC) and hemoglobin (Hb) content were determined by auto analyzer according to methods described by Dacie and Lewis[13].

Assay of serum hepatic markers

The level of serum glutamate oxalo acetate transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) was determined by the method of Reitman and Frankael[14]. The level of alkaline phosphatase (ALP) was estimated by the method of Plummer[15] and the protein was measured by the Lowry et al method[16].

Estimation of Lipid profiles

The extraction of serum lipids was done according to the procedure of Folch *et al*[17]. The estimation of total cholesterol was carried out by the method of Zlatkis *et al*[18] and triglycerides by the method of Foster and Dunn[19].

Analysis of Serum tumor markers

The serum hepatic tumor markers - α-feto protein (AFP) and carcinoembryonic antigen (CEA) were measured using ELISA assay kits from USCN LIFE science and technology (Wuhan, China).

Estimation of oxidative stress parameters

The colonic tissue level of superoxide dismutase (SOD) was assayed by the method of Misra and Fridovich[20]. Catalase (CAT) level was estimated by the method described by Sinha[21]. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*[22]. Glutathione S-transferase (GST) was assayed by the method of Habig *et al*[23]. Reduced glutathione (GSH) was determined by the method of Ellman[24].

Estimation of Lipid peroxidation:

The colonic tissue lipid peroxide level was determined as MDA by the method of Ohkawa *et al*[25]. The absorbance was measured photometrically at 532 nm and the concentrations were expressed as nmol malondialdehyde (MDA) min/mg/protein.

Histopathology Studies

The colon was fixed for 24 h in 10 % buffered formalin solution for histological study. Then a 2 × 2 mm dissected tissue was then sectioned (5 μm thickness), embedded and stained with haematoxylin and eosin (H and E).

Statistical Analysis

Values were expressed as mean ± SEM. The variation between groups was estimated by the one-way ANOVA followed by Dunnet's multiple comparison test using SPSS version 20.0 (SPSS Inc. Chicago, IL, USA).

RESULTS AND DISCUSSION

In our study, the formulation F4of 5-FU and Leucovorin noisome was found to satisfactory based upon the various parameters done for the evaluation of niosomes[12].

Colon carcinogenesis is a multistage process which involves initiation, promotion, and progression phases. Thus, the multistage sequence of events has many phases for prevention and intervention. Long term inflammation, is a hallmark risk factor for the progression of development of cancer in humans. DMH is a chemical carcinogen known to cause colon cancer with a reproducible preclinical *in vivo* system for evaluating "sporadic" (nonfamilial) forms of colon carcinoma[26]. Metabolism of DMH leads to the formation of AOM, MAM, and methyl diazonium ions which involves colonic mucosal DNA alkylation process. The metabolite, AOM, is a cardinal moiety involved in the methylation at the O-6 position of guanine, occurs within 6 to 12 h of DMH injection.

In our study, haematological parameters were significantly altered in the DMH treated as a carcinogenesis and altered immune function. The level of RBC and haemoglobin were significantly ($p<0.05$) reduced in DMH groups, whilst the WBC level was significantly ($p<0.05$) increased as that of the control rats. Meanwhile, treatment with 5-FU+ LV niosomes significantly ($p<0.05$) restored the altered blood parameters level to normalcy (Table No.2). The present result show that, the colonic tissue level of the LPO (Table No.3) increased significantly ($p<0.05$) and GSH (Table No.2), SOD, CAT, GPx and GST level (Table No.4) were decreased significantly ($p<0.05$) in DMH alone group compared with normal control group. Suggest that rapid cell proliferation involved in colon cell. Hence, cancer cell have certain characteristics that promote proliferation and tend to faster cell proliferation[27]. DMH itself can generate H_2O_2 in the presence of copper ions. In the presence of metal ions such as Fe^{2+} and Cu^{2+}, H_2O_2 can react with O_2 to convert it into the more reactive OH radical. If sufficient amounts of CAT or GPx are not available to decompose H_2O_2, the generated OH radicals are capable of attacking DNA basement. GSH an important non-protein thiol in conjunction with GPx and GST plays a significant role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species[28]. The present study, correlates with the decline in circulatory antioxidants such as SOD, CAT, GPx and GSH, and. This may be due to their accelerated synthesis to inhibit the lipid peroxidation end products the products of lipid peroxidation as well as sequestration by tumor cells. GPx uses H_2O_2 to catalyse the oxidation of GSH to GSSG, thereby nullifying the deleterious effects of H_2O_2[29]. Diminished GPx activity indicates cellular accumulation of the lipid hydro peroxides, which can potentially turn on a chain reaction, wherein more polyunsaturated fatty acid become targets for further per oxidative tissue injury. In the present study, GPx level was reduced in colonic tissue, which could be due to the elevated levels of hydro peroxides. Whilst, treatment with 5-FU+ LV niosomes significantly ($p<0.05$) decreased the level of MDA in colonic tissue of DMH treated rats, as well as significantly ($p<0.05$) increased the level of the antioxidants defense enzymatic system of SOD, CAT, GPx and GSH level.

Previous studies elicits that xenobiotic like DMH causes an increase in nonspecific microsomal enzyme activities of hepatic microsomal HMG -Co A reductase which mediates the cholesterol synthesis process. DMH also provoke hepatic damage and consequently results in loss of feedback control of cholesterol synthesis in liver, thereby elevating the level of bile acids and cholesterol in the bowel and serum[30,31]. In our study, serum level of cholesterol and triglycerides were significantly ($p<0.05$) elevated in DMH induced group may be due to higher cholesterogenes is induced by DMH. Whilst, treatment with 5-FU+ LV niosomes in DMH induced rats significantly ($p<0.05$) reduced

the serum level of total cholesterol and triglycerides (Table No.5).

The hepatic cell membrane damage releases the enzymes SGOT, SGPT, ALP into circulation, which can be measured in serum. High levels of SGOT indicate liver damage. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury. Previous studies indicate the hepatotoxicity role of DMH during the event of colon cancer[32]. In our study, DMH intoxicated rats displayed significantly ($p<0.05$) elevated level of hepatic marker enzymes (SGOT, SGPT and ALP) in serum and treatment with 5-FU+ LV niosomes significantly ($p<0.05$) decreased the hepatic markers enzyme level and restores the hepato cellular membrane damage elicited by DMH (Table No.6).

CEA is the best marker in colorectal cancer patients and also most thoroughly characterized tumour-associated antigens, in both biochemical and clinical aspects[33]. AFP is rarely reported until now in colorectal cancer compared to other cancers but it can also be an indicative of colorectal cancer[34]. In our study, serum level of CEA and AFP were significantly ($p<0.05$) elevated and 5-FU+ LV niosomes treatment significantly ($p<0.05$) reduced the level of tumor markers (CEA and AFP) to normalcy (Table No.7).

Further, the histopathological analysis revealed the thick enedmucosa with densely packed inflammatory cell in filtration and a higher degree of hyperplasia in DMH intoxicated rats and treatment with 5-FU+ LV niosomes showed normal appearing glands with normal mucosa of colon (Figure No.1).

Units

RBC

Millions /µl of blood; WBC: thousands/µl of blood; Hb: g/dl. The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes* denotes statistically significant $p< 0.05$.

GSH

Reduced Glutathione (nmole/mg of tissue); MDA: Malondialdehyde (nmole/mg of tissue). The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes* denotes statistically significant $p< 0.05$.

SOD

Superoxide dismutase (U/mg protein); CAT: Catalase (nmoles of H_2O_2 utilized/ min/ mg/ protein); GPx: Glutathione peroxidase (nmoles of GSH utilized/ min/ mg protein); GST: Glutathione - S- Transferase (nmoles of CDNB conjugate formed/ min/ mg/ protein). The values are expressed as mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU+LV) Niosomes. *denotes statistically significant $p< 0.05$.

The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes* denotes statistically significant $p< 0.05$.

The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test. The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes* denotes statistically significant $p< 0.05$.

CEA

Carcinoembryonic Antigen; AFP - Alpha – Feto - Protein. The values are expressed as mean ±S.E.M, n=6. The statistical analysis was carried out using one way ANOVA followed by Dunnet's multiple comparison test.

The comparison were made between a- Control vs DMH; b - DMH vs DMH+5-FU; c- DMH vs DMH + (5-FU +LV) Market formulation; d- DMH vs DMH + (5-FU +LV) Niosomes denotes statistically significant $p< 0.05$.

Table No.1: Formulation composition of 5-FU and Leucovorin Niosomes

S.No	Type of formulation	F1	F2	F3	F4	F5	F6	F7	F8
1	Drug	10	10	10	10	10	10	10	10
2	Cholesterol	10	20	10	20	10	20	10	20
3	Span 40	10	10	-	-	-	-	-	-
4	Span 60	-	-	10	10	-	-	-	-
5	Tween 40	-	-	-	-	10	10	-	-
6	Tween 60	-	-	-	-	-	-	10	10
7	Drug: cholesterol: SA	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1	1:1:1	1:2:1

Table No.2: Effect of 5-FU + LV niosomes on Haematological parameters on DMH induced colon

S.No	Groups	RBC	WBC	Hb
1	Control	7.53±0.17	2.53±0.11	14.48±0.26
2	DMH	3.21±0.14^{a*}	8.42±0.16 ^{a*}	8.97±0.16 ^{a*}
3	DMH+5-FU	4.40±0.13^{b*}	5.64±0.17 ^{b*}	11.23± 0.21 ^{b*}
4	DMH + (5-FU +LV) Market formulation	5.87±0.16^{c*}	4.32±0.12 ^{c*}	12.24±0.23 ^{c*}
5	DMH + (5-FU +LV) Niosomes	6.40±0.18^{d*}	3.15±0.09 ^{d*}	13.89±0.18 ^{d*}

Table No.3: Effect of 5-FU + LV niosomes on GSH and MDA level in DMH induced colon cancer

S.No	Groups	GSH	MDA
1	Control	4.98±0.54	1.4±0.12
2	DMH	1.52±0.15 ^{a*}	4.24±0.35 ^{a*}
3	DMH+5-FU	2.67±0.21^{b*}	3.47±0.26 ^{b*}
4	DMH + (5-FU +LV) Market formulation	3.12±0.29^{c*}	2.42±0.18 ^{c*}
5	DMH + (5-FU +LV) Niosomes	3.92±0.42^{d*}	1.86±0.16 ^{d*}

Table No.4: Effect of 5-FU + LV niosomes on colon antioxidant enzymes in DMH induced

S.No	Groups	SOD	CAT	GPx	GST
1	Control	7.84±0.75	7.14±0.68	2.34±0.05	0.75±0.006
2	DMH	2.89±0.29 ^{a*}	2.63±0.25 ^{a*}	0.57±0.01 ^{a*}	0.25±0.003 ^{a*}
3	DMH+5-FU	4.87±0.35 ^{b*}	4.32±0.29 ^{b*}	1.6±0.03 ^{b*}	0.48±0.002 ^{b*}
4	DMH + (5-FU +LV) Market formulation	5.92±0.37 ^{c*}	5.75±0.42 ^{c*}	1.98±0.03 ^{c*}	0.65±0.005 ^{c*}
5	DMH + (5-FU +LV) Niosomes	7.12±0.67 ^{d*}	6.87±0.54 ^{d*}	2.12±0.04 ^{d*}	0.71±0.06 ^{d*}

Table No.5: Effect of 5-FU + LV niosomes on serum lipid profiles in DMH induced colon cancer

S.No	Groups	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
1	Control	76.94±2.46	122.6±3.33
2	DMH	171.4±7.65 [a*]	164.4±2.68 [a*]
3	DMH+5-FU	110.7±2.05 [b*]	147.1±3.7 [b*]
4	DMH + (5-FU +LV) Market formulation	90.44±2.47 [c*]	138.28±1.88 [c*]
5	DMH + (5-FU +LV) Niosomes	81.17±1.08[d*]	125.5±5.23[d*]

Table No.6: Effect of 5-FU + LV niosomes on serum hepatic markers enzyme and total protein level in DMH induced colon cancer

S.No	Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Tot. Protein (mg/dl)
1	Control	56.87±3.23	66.45±3.76	144.34±7.98	8.22±0.50
2	DMH	132.65±5.76 [a*]	124.32±6.84 [a*]	304.65±9.65[a*]	5.21±0.31 [a*]
3	DMH+5-FU	98.76±4.25 [b*]	92.12±3.25 [b*]	197.65±6.87[b*]	6.76±0.35[b*]
4	DMH + (5-FU+LV) Market formulation	86.87±5.65 [c*]	79.67±4.82 [c*]	175.42±4.56[c*]	7.02±0.25[c*]
5	DMH + (5-FU +LV) Niosomes	62.76±3.29 [d*]	64.65±3.98 [d*]	153.89±5.25[d*]	8.02±0.50 [d*]

Table No.7: Effect of 5-FU + LV niosomes on tumour markers in DMH induced colon cancer

S.No	Groups	CEA (ng/dl)	AFP (ng/dl)
1	Control	0.21±0.002	0.45±0.01
2	DMH	1.24±0.007 [a*]	1.62±0.05 [a*]
3	DMH+5-FU	0.56± 0.006 [b*]	0.89±0.04 [b*]
4	DMH + (5-FU +LV) Market formulation	0.45±0.003 [c*]	0.72±0.03 [c*]
5	DMH + (5-FU +LV) Niosomes	0.29±0.005 [d*]	0.55±0.04 [d*]

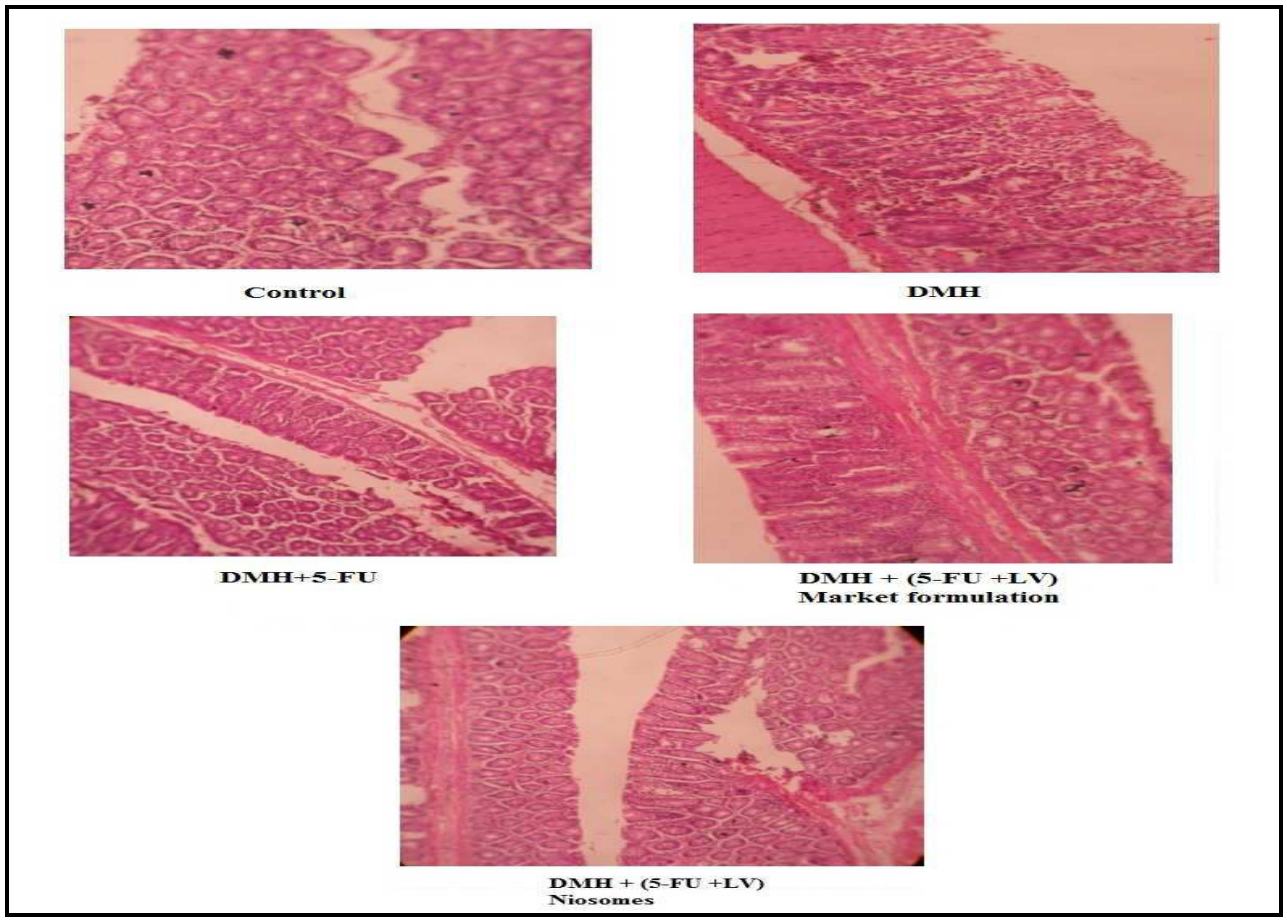

Figure No.1: Effect of 5-FU + LV niosomes on colon histology in DMH induced colon cancer

CONCLUSION

Niosomal formulations containing 5- Fluorouracil and leucovorin were successfully prepared with different surfactants like Span 40, Span 60, Tween 40, and Tween 60 by thin film hydration technique. Further, preclinical evaluation of prepared 5-FU+ LV niosomes displayed efficient anticancer potential in DMH induced colon cancer by restoration of altered biochemical levels. Furthermore, the in our study 5-FU+ LV niosomes displayed marked anticancer efficacy as that of the 5-FU+ LV market formulation and 5-FU alone.

ACKNOWLEDGEMENT

The authors would like to thank Dr. A. Meena, Principal and management of K. K. College of Pharmacy, Chennai and management of KMCH college of Pharmacy, Coimbatore for their support towards our research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: K. Karthick and K. S. G. Arul Kumaran. Formulation and preclinical evaluation of niosomes co-loaded with 5-fluorouracil and leucovorin, *International Journal* of *Research in Pharmaceutical and Nano Sciences,* 5(5), 2016, 283-292.