



Original Research Article

Solid nanostructured lipid carriers loaded with silymarin for oral delivery: Formulation development and evaluation

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ARTICLE INFO

Article history:

Received 24-10-2021

Accepted 02-11-2021

Available online 16-11-2021

Keywords:

Nanostructured lipid carrier

Silymarin

Adsorbent

Lymphatic uptake

Bioavailability

ABSTRACT

Aims: The present study was aimed at preparing stable dry adsorbed nanoparticles (DANs) of silymarin loaded nanostructured lipid carriers (NLCs). The prepared silymarin loaded NLCs and DANs were characterized for various quality parameters.

Methods and Material: Silymarin loaded NLCs were prepared by a modified hot melt emulsification ultrasonication method using glyceryl monostearate (GMS), capmul MCM C8 EP (CAP) and gelucire 50/13 (G50/13) as solid lipid, liquid lipid and surfactant respectively. For better stability, NLC dispersion was converted into DANs by adsorbing them onto some suitable carriers. NLCs and DANs were characterized for particle size, polydispersity index, zeta potential, entrapment efficiency, drug loading, assay, thermal behavior, crystallinity and morphological study.

Results: The optimized NLCs have a mean particle size of 206.1 ± 0.125 nm (size distribution of 0.249 ± 0.058), a zeta potential of -32.5 ± 1.2 mV with high entrapment of $95.60 \pm 0.45\%$ and drug loading of $1.90 \pm 0.08\%$. The X-ray diffraction and endothermic peaks confirmed the maximum encapsulation of active in lipid matrices. The particles were spherical with smooth surface morphology. In-vitro release studies showed sustained drug release for up to 24 h. Ex-vivo permeation in the presence and absence of lymphatic blocker indicates the uptake of silymarin loaded NLCs by the lymphatic route.

Conclusions: Silymarin loaded NLCs prepared had a nanosize distribution with high entrapment efficiency. The ex-vivo permeation study for optimized NLC formulation exhibited the lymphatic uptake of active. Dispersion stability was increased by preparing the DANs. The solid dry powder is used for oral reconstitution and can be further converted into tablets or filled into capsules.

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1. Introduction

Nanotechnology based formulations have important prospects in pharmaceutics and can generally be divided into two groups: polymer- and lipid-based systems. Commercial use of polymer systems is restricted due to their toxicity, lack of suitable production scale, minimal number of biopolymers and use of organic solvents. As most bioactive molecules are lipophilic, colloidal dispersions are often required in aqueous environments. The lipid

system facilitates bioactive molecule absorption in the small intestine because of the formation of micelles that solubilize and transport lipophilic molecules. Important lipid based systems include nano- and microemulsions, liposomes, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). SLNs are modified into NLCs by combining solid lipid (fat) with liquid lipid (oil). The NLCs contain partially crystallized and less ordered crystalline structure lipid droplets.¹ The NLCs produce particles where liquid lipid is blended with the solid lipid core. Thus, more amount of drug dissolves in the liquid lipid and simultaneous encapsulation in the solid lipid. This

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lipidic blend exhibits a slower polymeric transition and a low crystallinity index. The liquid lipid in the core aids the spherical morphology of the particles.²

Silymarin, milk thistle extract (*Silybum marianum*) has been a natural remedy for liver related disorders and hepatic protection from various toxins. Silymarin also protects the liver cells from ischemic injury, radiation, iron toxicity and viral hepatitis.³ The hepatoprotective nature of silymarin includes enhanced detoxification, anti-lipid peroxidation and antioxidant. It reduces the formation of leukotrienes from polyunsaturated fatty acids in the liver by inhibiting the enzyme lipoxygenase. It was also reported that silymarin increased the synthesis of hepatocyte protein, reducing the tumor promoter's activity, mast cell stabilization and modulating immune functions.^{4,5} The 20-50% of human oral bioavailability of silymarin is due to poor aqueous solubility.⁶ High phase II degradation and rapid elimination of silymarin in bile and urine lead to poor therapeutic efficiency. The polyphenols are large multiple ring compounds that are not absorbed by simple diffusion.⁷ In animals and humans, maximum concentrations in plasma are observed at 4-6 h and the elimination half-life is 6-8 h. Silymarin continues enterohepatic circulation with absorption in the intestine, liver conjugation, elimination through bile, hydrolysis by intestinal flora and further intestinal reuptake.⁸ It was reported that silymarin has a half maximal cytotoxic concentration (CC50) of 160.20 $\mu\text{g/mL}$ in rhabdomyosarcoma cells and a half maximal inhibitory concentration (IC50) of $15.20 \pm 3.53 \mu\text{g/mL}$ against enterovirus 71 subgenotype B4 strain 41. The bioavailability and liver targeting of silymarin through NLCs can be enhanced by lymphatic uptake and their ability to bypass the first pass metabolism. Thus, more drugs will reach the site of action to show their therapeutic activity. Also, sustained drug release is obtained due to the entrapment of the drug in the lipid matrix, which retards drug release, reduces its dosing frequency and improves patient compliance.⁹

Silymarin NLCs were prepared by the emulsification and ultrasonication method with a particle size of $223.73 \pm 43.39 \text{ nm}$ with an entrapment of $78.65 \pm 2.2\%$. Lymphatic absorption of silymarin NLCs was observed in-vivo and the study was confirmed by the presence of chylomicron inhibitor in blood plasma. The serum concentration of silymarin NLCs was twice that of conventional silymarin suspension.¹⁰ The freeze dried product of silymarin loaded NLCs was by the emulsion-evaporation-solidifying method and had an encapsulation of 92%. The formulated NLCs exhibited enhanced permeation of silymarin in-vitro and significant lowering of blood glucose and triglycerides in-vivo.^{11,12} A comparative bioavailability study of silymarin SLNs and NLCs dispersion was performed in dogs. Both SLNs and NLCs were prepared by the conventional hot homogenization method using suitable excipients.

Pharmacokinetic analysis in dogs showed a decrease in bioavailability of 74.86% and 59.09% for lipolysates compared to integral NLCs and SLNs, respectively.¹³ The freeze dried silymarin NLCs were converted into gel form for dermatokinetic study. The kinetic study showed an increase in $C_{\text{skin max}}$ in treated skin with NLCs gel as compared with traditional gel formulation. Epidermal drug deposition enhancement was observed due to lipid bilayer fluidization.¹⁴ The silymarin NLCs were prepared by high pressure homogenization and the oral bioavailability was compared with silymarin solid dispersion pellets in beagle dogs. The NLCs dispersion had a mean particle size of 78.87 nm and an entrapment efficiency of 87.55%. The relative oral bioavailability of silymarin NLCs was 2.54 and 3.10 fold that of marketed formulations and solid dispersion pellets.¹⁵ Silymarin NLCs were prepared by a hot high pressure homogenization process and further converted into topical gel form for anti-proliferative activity. Silymarin NLC possessed activities against progression and proliferation, which were associated with enhanced solubility and stability of silymarin and greater permeation into the affected cells.¹⁶ The previously reported studies highlighted the freeze drying technique for solidification of NLCs to improve stability. The present study mainly focused on the development of silymarin loaded NLCs and the solidification of the dispersion system by adsorption technique for better oral delivery and to improve the bioavailability of silymarin.

2. Materials and Methods

Silymarin (70%) was obtained as a gift sample from Bio-gen Extracts Pvt. Ltd. India. Compritol ATO 888 (glyceryl dibehenate), precinol ATO 5 (glyceryl distearate), capryol PGMC (propylene glycol monocaprylate), labrafac lipophile WL 1349 (medium chain triglycerides of caprylic and capric acids), plulrol CC oleique 497 (polyglyceryl-3 dioleate), ricebran oil, gelucire 50/13 (stearoyl polyoxyl-32 glycerides), transcutol HP (diethylene glycol monoethylether) were gift samples from Gattefosse Pvt. Ltd. India. Dynasan 114 (trimyristin), dynasan 116 (tripalmitin) and dynasan 118 (tristearin) were obtained as gift samples from Cremer oleo GmbH&Co, Germany. Capmul MCM C8 (glyceryl caprylate) was obtained from Abitec Corporation, USA. Kolliphor ELP (polyoxyl castor oil), solutol HS 15 (polyoxyethylene glycol (15)-hydroxystearate), cremophor RH 40 (PEG-40 hydrogenated castor oil), tween 20, tween 40, tween 60, tween 80, kollidon CLSF (crospovidone, water insoluble) were obtained as gift samples from BASF, India. Glyceryl monostearate, stearic acid, palmitic acid, oleic acid, ethyl oleate, span 20 (sorbitan monolaurate) were obtained from Loba Chemie, India. Sodium carboxymethylcellulose (blanose, molecular weight 90,500) was obtained from Ashland, India. Lactose monohydrate (supertab 11SD) was a gift sample from

DFE Pharma Ltd., India. The mannitol (pearlitol 100SD) was a gift sample from SPI Pharma Ltd., India. Aerosil 200 (silicon dioxide) and aerosil R 972 (hydrophobic fumed silica) were obtained from Evonik Industries Ltd., India. Avicel CL 611 (microcrystalline cellulose and carboxymethyl cellulose sodium) was obtained as a gift sample from FMC Biopolymer, USA. Syloid 244 FP (silica) was obtained as a gift sample from Grace GmbH & Co KG, Germany. Neusilin US 2, neusilin UFL 2 (magnesium aluminometasilicate) and fujicalin (dibasic calcium phosphate anhydrous) were obtained as gift samples from Fuji Chemical Industry Ltd., India. Analytical grade solvents and reagents were used for the study.

2.1. Formulation development of silymarin loaded NLCs

2.1.1. Screening of formulation components: solid lipids, liquid lipids and surfactants

All the formulation components were screened for solubility of silymarin. This is performed by a semi-quantitative method in which the lipids are initially melted 5°C higher than their melting point and the silymarin is added to them and then placed on a vortex mixer (Remi CM 101 Plus; Remi Labs.) for uniform mixing and determining the maximum amount of silymarin dissolved in each lipid. Further, these solutions were checked for the presence or absence of silymarin visually and the process was continued till the lipid got saturated with the silymarin.¹⁷ The lipid in which comparatively more amount of silymarin was dissolved was chosen for further studies. The above method was also used for screening other components such as liquid lipids and surfactants without heating. The ratio of solid lipid to liquid lipid is also important for stable formulation development. Hence, various ratios, including 50:50 to 90:10 were screened.

2.1.2. Preparation of silymarin loaded NLCs

Silymarin loaded NLCs were prepared by the method mentioned by Qianwen.¹⁸ In the modified hot melt emulsification ultra-sonication method, solid lipid, liquid lipid and emulsifier were weighed in a test tube and melted in a water bath using a heating mantle. The temperature was maintained at a 5-10°C higher than the melting point of the lipid. Then to this molten mixture, an accurately weighed silymarin was added and mixed on a vortex mixer. The water phase and the lipid phase were maintained at the same temperature separately. After complete solubilization of the silymarin, the water phase was added to the molten mixture. It was mixed well in the vortex (Remi labs, India) for sufficient time. This primary emulsion was stirred for 15 min at 1200 rpm on a magnetic stirrer (Remi Labs, India) under heated conditions to form a fine; stable emulsion. It was further subjected to probe sonication (Oscar Ultrasonic Co. Ltd., India) with a 3 mm horn, 30-40% variance and

90-watt power for 10 min.

2.1.3. Preparation of silymarin suspension

The conventional silymarin suspension was prepared by using sodium carboxymethylcellulose as a suspending agent. Briefly, the blanose (0.5% w/v) was slowly dissolved in purified water under stirring for 10 min at 1200 rpm on a magnetic stirrer (Remi Labs, India). The silymarin (10 mg/10 ml) was slowly suspended in blanose media under stirring for 15 min at a 1200 rpm magnetic stirrer (Remi Labs, India).

2.1.4. Formulation design: preliminary screening of formulation and process related parameters

Various factors affect the formulation. These include formulation related and process related parameters. The formulation related parameters like solid lipid, liquid lipid, surfactant concentration, and process related important parameters like sonication time were taken into consideration and accordingly, the studies were carried out as mentioned in Table 1. After the screening of each parameter, the optimum value of that parameter was kept constant for the next parameter screening. Total lipid concentration, the volume of the dispersion medium and silymarin concentration were kept constant throughout the process. The effect of each parameter was observed and was analyzed based on physical stability, particle size, size distribution and entrapment efficiency.

2.2. Formulation development of dry adsorbed nanoparticles (DANs) of silymarin loaded NLCs

2.2.1. Preparation of DANs of silymarin loaded NLCs

It is difficult to maintain NLC dispersion stable for a longer period of time. There may be the chances of an increase in particle size and the formation of aggregates during stability. The physical and chemical stability of the NLCs formulation was improved by preparing the DANs using the granulation evaporative drying method with some modifications.¹⁹ The DANs can be used as dry powder for reconstitution. Briefly, 5 ml of NLCs dispersion was added to the adsorbent and kneaded. After kneading, the granules were dried overnight at room temperature to form a dry powder. The granules were sized through mesh no #40 to form a free flowing powder.

2.2.2. Optimization of DANs of silymarin loaded NLCs

Adsorbents were screened and selected considering various factors like % desorption, particle size and size distribution and flow properties of DANs.

2.2.2.1. Redispersion of NLCs. Redispersion was performed by the method as mentioned by Hywel et al.²⁰ Briefly, the DANs equivalent to one dose of silymarin were reconstituted in 250 ml of water and stirred for 15

Table 1: Formulation design for preparation of silymarin loaded NLCs

| Variable | Formulation code | Solid lipid (3% w/v) | Liquid lipid (2% w/v) | Surfactant | SC (% w/v) | ST (min) |
|-------------|------------------|-------------------------|--------------------------|------------|------------|----------|
| Solid lipid | F1 | GMS | CAP | G50/13 | 2.0 | 10 |
| | F2 | CMPR | CAP | G50/13 | 2.0 | 10 |
| | F3 | PRE | CAP | G50/13 | 2.0 | 10 |
| | F4 | GMS+CMPR | CAP | G50/13 | 2.0 | 10 |
| | F5 | GMS+PRE | CAP | G50/13 | 2.0 | 10 |
| Surfactant | F6 | GMS | CAP | T80 | 2.0 | 10 |
| | F7 | GMS | CAP | THP | 2.0 | 10 |
| | F8 | GMS | CAP | G50/13+T80 | 2.0 | 10 |
| | F9 | GMS | CAP | G50/13+THP | 2.0 | 10 |
| SC | F10 | GMS | CAP | G50/13 | 1.5 | 10 |
| | F11 | GMS | CAP | G50/13 | 2.5 | 10 |
| ST | F12 | GMS | CAP | G50/13 | 2.0 | 5 |
| | F13 | GMS | CAP | G50/13 | 2.0 | 15 |

The silymarin concentration was 10mg/10ml in all formulations. GMS- Glyceryl monostearate, CMPR- Compritol ATO 888, PRE- Precirol ATO 5, CAP- Capmul MCM C8, G5013- Gelucire 50/13, THP-Transcutol, HP, T80-Tween 80, SC- Surfactant concentration, ST- Sonication time.

min on a magnetic stirrer (Remi labs, India) to allow the desorption of NLCs. The reconstituted DANs were then filtered through whatman filter paper 1 (pore size around 11 μ m) and the residue on the filter paper was poured into a volumetric flask and diluted with methanol. Sonication was done for about 1 h to aid the complete extraction of the silymarin. The contents were again filtered and the filtrate was analyzed for silymarin content by a UV- visible spectrophotometer (Shimadzu 1800) against methanol as a blank at 287nm. This gave the amount of silymarin in the NLCs that was not desorbed by the adsorbent. The percent of NLCs desorbed was calculated as follows:

$$\%NLC\ desorbed = \frac{W(\text{total drug}) - W(\text{undesorbed drug})}{W(\text{total drug})} \times 100$$

Where, W (total drug): the amount of drug present in the DANs. W (undesorbed drug): the amount of drug which did not desorb from DANs upon reconstitution.

2.2.2.2. Particle size and size distribution of desorbed NLCs. DANs are solid carriers that contain adsorbed NLCs on their surfaces. It is important to check the particle size of NLCs after redispersion for their nano range particle size. The DANs batch which was giving less particle size and better PDI was selected for further trials.

2.2.2.3. Micromeritic properties. The DANs batch having good flow properties (angle of repose, compressibility index and hausners ratio) was selected as an optimized batch of DANs.²¹

2.3. Ex-vivo intestinal permeability and lymphatic uptake study of silymarin loaded NLCs

Ex-vivo intestinal permeability was performed on chicken intestinal sacs (non-everted chicken jejunum).²² The tissue selected was chicken jejunum because the jejunum has

about 6 scattered payer's patches where lymphatic uptake takes place. Also, it consists of p-glycoprotein which actively effluxes toxic molecules including many drugs from the cell. The non-everted model of the chicken jejunum was used. The medium used was phosphate buffer, pH 7.4 maintained at $37 \pm 0.5^\circ\text{C}$ with agitation of 100 rpm with proper oxygen supply to the tissue. Suitable aliquots were withdrawn at regular intervals for up to 3 h and replaced with the same buffer. The aliquots were filtered and analyzed using a UV-visible spectrophotometer (Shimadzu 1800) against pH 7.4 as a blank at 287 nm to know the amount of silymarin permeated from the silymarin suspension and NLC formulation.

For the lymphatic uptake study, the tissue was incubated in a 20 μ g/ml solution of a lymphatic uptake blocker (pluronic F-68) for 1 h with proper aeration. After 1 h the NLCs formulation was added to the tissue and the rest of the procedure was followed as mentioned above. There is a possibility of permeation of intact NLCs through the tissue or only the silymarin permeating from the NLCs due to the breakdown of lipid by the enzymes present in the enterocyte. Therefore, the study was also done with blank NLCs so as to nullify any interference shown by the lipids or surfactants in the formulation at the time of analysis. Intestinal permeability of plain silymarin from suspension and formulation was compared. The permeability of formulation with or without lymphatic uptake blocker was also compared so as to get a thought about whether the silymarin loaded NLCs can be absorbed through the lymphatic route.²³

2.4. Characterization and evaluation of silymarin loaded NLCs and DANs

2.4.1. Particle size, size distribution and zeta potential

Particle size and the extent of the size distribution of the silymarin loaded NLCs were measured using a Malvern zeta sizer ZS90 at a 90 degree scattering angle using dynamic light scattering (DLS). Zeta potential was also determined by Malvern zetasizer ZS90.²⁴ For light scattering measurements, the samples were redispersed in a suitable amount of double distilled water to maintain a scattering intensity of between 100-500 kilopoise, stirred on a magnetic stirrer for about 10 min and then filtered. The filtrate was measured at a fixed angle of 90° at 25° C. The whole measurement was carried out in duplicate. The DANs were redispersed as mentioned earlier. The redispersed NLCs were evaluated for particle size, size distribution and zeta potential.

2.4.2. Entrapment efficiency and drug loading

The entrapment efficiency of silymarin loaded NLCs was determined by an indirect method (aqueous phase manipulation by addition of a saturated solution of sodium chloride) wherein, the amount of unincorporated (untrapped) silymarin in the aqueous phase of NLCs was determined.²⁵ 1 ml of NLCs formulation and 1 ml of saturated sodium chloride solution were taken in an eppendorf tube and centrifuged at 15000 rpm for 15 min. The saturated sodium chloride solution helps in the separation of the formulation into two separate layers of lipid and dispersion medium. The lower layer was separated and diluted with methanol. The absorbance was recorded at 287 nm using a UV-visible spectrophotometer (Shimadzu 1800) against methanol as a blank.

$$\% \text{ Entrapment efficiency (EE)} = \frac{W_t - W_f}{W_t} \times 100$$

$$\% \text{ Drug loading (DL)} = \frac{W_t - W_f}{W_L + (W_t - W_f)} \times 100$$

Where, W_t stands for the total amount of drug used in the formulation. W_f stands for the amount of free drug quantified by the indirect method. W_L stands for the total amount of lipid used in the formulation.

2.4.3. Drug content/Assay

The silymarin loaded NLCs formulation was diluted with methanol, sonicated for a sufficient period of time and filtered. The filtrate was diluted with methanol and its absorbance was taken on a UV-visible spectrophotometer (Shimadzu 1800) against methanol as a blank at 287 nm. The same procedure was followed with a blank formulation and its absorbance was subtracted from that of the silymarin loaded formulation to avoid any interference by the lipid.

DANs equivalent to 1mg of silymarin were analyzed in a similar way to that of NLCs.

$$\% \text{ Drug content} = \frac{\text{Amount of silymarin recovered}}{\text{Amount of silymarin added}} \times 100$$

2.4.4. Thermal behavior

The crystallization and thermal behavior were studied by differential scanning calorimetry (DSC).²⁶ A sample weight of 10 mg was heated at 10°C/min between 30–250°C temperature ranges in an open solid pan. The calibration of the temperature and energy scale of the DSC apparatus was performed using alumina as the standard reference material. Nitrogen gas was purged at the rate of 50 ml/min to maintain an inert atmosphere. A thermal behavior study was carried out for plain silymarin, silymarin loaded NLCs and placebo NLCs.

2.4.5. Crystallinity

Lipid molecules can arrange themselves into different patterns, therefore exhibiting different crystallographic structures. X-ray diffraction (XRD) helps in the determination of crystalline nature.²⁷ XRD of plain silymarin, adsorbent and DANs were performed using PAN analytical X'Pert PRO MPD. The samples were loaded onto an X-ray diffractometer and then the spectrum range of 0-5000 intensity was observed at 2 θ° . The analysis was performed in a similar way to that of silymarin. XRD of the adsorbent and DAN were performed using PAN analytical X'Pert PRO MPD. The analysis was performed in a similar way to that of NLCs.

2.4.6. Surface morphology

The external surface morphology of silymarin loaded NLCs formulation was recorded using scanning electron microscopy (SEM) (Philips XL30 FEG) at 20 kV as an accelerating voltage. A small amount of sample was mounted and scattered on an aluminum stub. A thin layer of gold was sputter coated on the stub with the sample to make the sample conductive. The sample was then subjected to analysis under different magnification levels. The surface morphology of the adsorbent and DANs were analyzed using SEM (Philips XL30 FEG). A SEM study was carried out to check if any morphological changes occurred after adsorption.

2.4.7. In-vitro drug release

In-vitro release studies were carried out by the dialysis method.²⁸ The dialysis membrane (molecular weight cut-off between 12,000 and 16,000) was soaked in double distilled water for 12 h before being used for the study. The appropriate amounts of the silymarin loaded NLCs formulation (5 ml), silymarin suspension (5 ml) and DANs (redispersed in water, 5ml) were filled separately in dialysis bags. The medium which was used for the drug release

study was phosphate buffer, pH 7.5 + 2 % sodium lauryl sulphate (SLS), (100 ml). The solution was maintained at 37 ± 0.5 °C and agitated at 50 rpm with magnetic stirring bars (Remi Labs, India) during the experiment. At fixed time intervals, 5 ml of the sample was withdrawn and replaced with the fresh dissolution medium to maintain the sink conditions. Solutions were analyzed by a UV spectrophotometer (Shimadzu 1800) at 287 nm. Similarly, for DANs, the DANs equivalent to 5 mg of silymarin were redispersed in 5 ml of water and poured into the dialysis bag and tied to a magnetic bead. Further procedures were performed in a similar way to that of NLCs.

3. Results

3.1. Formulation development of silymarin loaded NLCs

3.1.1. Screening of formulation components: Solid lipids, liquid lipids and surfactants

The solubility of silymarin in different formulation components is mentioned in Table 2.

The melting point of solid components used in solubility screening is below 100°C. Further, the silymarin was thermally stable up to 100°C. The published literature showed that the thermal decomposition was not observed till 140°C for silymarin components.²⁹ Similarly, another study revealed the thermal stability of silymarin content up to 250°C.³⁰ Thus, the thermostable nature of silymarin retains its effectiveness at the temperature used for screening of components and formulation development. Silymarin showed maximum solubility in GMS, followed by CMPR and PRE. Also, in the case of liquid lipids, maximum solubility was observed in CAP followed by CPLYL and PLU. GMS and CAP showed the maximum solubility for the silymarin, so the screening of these excipients was carried out to find the best ratio of solid to liquid lipid. It was found that the maximum amount of silymarin was soluble in a 50:50 ratio followed by 60:40. There are fair chances of silymarin leakage from NLCs containing a high concentration of liquid lipid and, hence a ratio of 60:40 was selected over 50:50.

F1 to F5 represent variations in solid lipids. Amongst the various solid lipids utilized, NLCs prepared using GMS gave the smallest particle size of $206.1 \pm 0.12.5$ nm and 0.249 ± 0.058 PDI. Thus, GMS was selected as a solid lipid for further studies. Surfactant variations are represented by Formulations F6 to F9. Formulations prepared with THP or in combination with G50/13 gave higher particle size and resulted in gelling during storage. Formulations containing T80 resulted in smaller particle size but drug leakage was observed. The minimum particle size and PDI were obtained when G50/13 was used alone with the formulation being physically stable for a period of a minimum of 3 days. Formulation F10 and F11 represent variations

in surfactant concentration. It was observed that, as the surfactant concentration increased, particle size decreased. An increase in surfactant concentration above 200 mg did not affect the particle size and PDI substantially and hence concentration was selected as 200 mg. Formulations F12 and F13 represent variations in sonication time. Particle size and PDI generally decrease with increasing sonication time. An increase in particle size is seen at high sonication time with a broad distribution of the particles. This may be due to physical instability (aggregation was observed). Thus, by varying process-related parameters, an optimized formulation was obtained (Figure 1 A). All the results are mentioned in Table 3.

3.2. Formulation development of DANs of silymarin loaded NLCs

3.2.1. Preparation and optimization of DANs of silymarin loaded NLCs

Various hydrophilic and hydrophobic adsorbents were screened for the development of DANs. The conversion of NLCs into dry adsorbed nanoparticles by a drying technique using suitable carriers represents a simple and cost effective technique. The DANs were prepared by adsorbing the NLCs onto adsorbents like mannitol, lactose, aerosil 200, aerosil R 972, kollidon CL SF, syloid 244 FP, avicel CL 611, neusilin US 2, neusilin UFL 2 and fujigalin. It is very important that NLCs desorb completely upon reconstitution in purified water. If the adsorbent is not able to desorb NLCs properly, then it would remain as a microparticle rather than a nanoparticle. The adsorbents were finalized on the basis of the amount required of adsorbent and the % desorption are mentioned in Table 4. It was observed that lactose, kollidon CL SF, neusilin US 2 showed redispersion of more than 96%. The avicel CL 611 showed a redispersion of $99.10 \pm 0.78\%$ with the powder having a sticky nature. Hence, it was not further selected for optimization. Further, the combination of adsorbents was also tried for the development of DANs to improve the flow properties of DANs.

It was observed that less quantity of these adsorbents were required when used in combination to adsorb NLCs. Thus, a combination of water soluble and water insoluble adsorbents was chosen because the water soluble carrier helps to dissolve easily with the release of NLCs when dispersed in water, and because insoluble carriers have a large surface area, fewer adsorbents are required for adsorption. Lactose being water soluble showed the highest % desorption but required a large quantity for the formation of a thick paste. Kollidon CL SF, crosslinked polyvinyl pyrrolidone being insoluble, was required in less quantity but due to its super-disintegrating ability, showed good desorption. Neusilin US 2 (a granular form of magnesium aluminosilicate) has good flow and compressibility properties. It is highly porous and has a high specific

Table 2: Solubility of silymarin in various formulation components

| Solid lipid (200 mg) | Solubility of silymarin (mg) | Liquid lipid (200 mg) | Solubility of silymarin (mg) | Surfactant (200 mg) | Solubility of silymarin (mg) |
|----------------------|------------------------------|-----------------------|------------------------------|---------------------|------------------------------|
| GMS | 5.00±0.50 | CAP | 5.50±0.50 | G50/13 | 10.00±2.00 |
| CMPR | 3.00±0.75 | CPRYL | 3.00±0.50 | T80 | 6.00±1.50 |
| PRE | 3.00±1.00 | PLU | 3.00±1.00 | THP | 6.00±1.00 |
| Dynasan 114 | 2.00±1.00 | LAF | 2.50±0.50 | S20 | 5.00±1.00 |
| Dynasan 116 | 2.00±1.00 | oleic acid | 2.50±0.50 | T60 | 4.00±0.50 |
| Dynasan 118 | 2.00±0.50 | IPM | 1.00±0.50 | KHS15 | 4.00±0.50 |
| Stearic acid | 2.00±0.75 | Ethyl oleate | 1.00±0.50 | KELP | 4.00±1.00 |
| | | Ricebran oil | 0.75±0.50 | KRH40 | 4.00±1.00 |
| Palmitic acid | 1.00±0.50 | Sunflower oil | 0.75±0.50 | T40 | 2.00±0.50 |
| | | | | T20 | 2.00±0.50 |

Values expressed in mean ± SD, n=3. GMS- Glyceryl monostearate, CMPR- Compritol ATO 888, PRE- Precirol ATO 5, CAP- Capmul MCM C8, CPRYL- Capryol PGMC, PLU- Plurol CC olique 497, LAF- Labrafac lipophile w1 1349, IPM- Isopropyl myristate, G50/13- Gelucire 50/13, T80- Tween 80, THP- Transcutol HP, S20-Span 20, T60-Tween 60, KHS15- Kolliphore HS 15, KELP- Kolliphore ELP, KRH40- Kolliphore RH 40, T40- Tween 40, T20-Tween 20.

Table 3: Physicochemical properties of silymarin loaded NLCs

| Formulation Code | Physical stability | Particle size (nm) | Size distribution (PDI) | Entrapment efficiency (%) |
|------------------|--------------------|--------------------|-------------------------|---------------------------|
| F1 | Stable | 206.1±012.5 | 0.249±0.058 | 95.60±0.45 |
| F2 | Stable | 554.1±115.6 | 0.880±0.120 | 92.26±0.34 |
| F3 | Stable | 735.6±156.2 | 1.000±0.256 | 91.56±0.74 |
| F4 | Stable | 568.7±102.2 | 0.890±0.172 | 93.75±0.83 |
| F5 | Stable | 291.5±078.2 | 0.420±0.080 | 94.25±0.22 |
| F6 | Drug leakage | 217.4±064.5 | 0.470±0.064 | 90.14±2.85 |
| F7 | Gelling | 317.7±101.4 | 0.580±0.114 | 89.12±1.20 |
| F8 | Drug leakage | 293.0±043.2 | 0.430±0.075 | 85.21±2.45 |
| F9 | Gelling | 371.1±018.5 | 0.220±0.046 | 94.12±0.15 |
| F10 | Stable | 271.7±014.5 | 0.340±0.042 | 79.47±1.85 |
| F11 | Stable | 194.0±018.4 | 0.240±0.056 | 93.12±0.46 |
| F12 | Aggregation | 280.2±126.5 | 0.300±0.126 | 88.64±0.54 |
| F13 | Aggregation | 214.7±118.5 | 0.350±0.145 | 90.15±0.15 |

Values expressed in mean ± SD, n=3

Table 4: Redispersion properties of various adsorbents used in the development of DANs of silymarin loaded NLCs

| Adsorbents | Quantity of adsorbent used for adsorption of 5 ml of NLCs dispersion (g) | Redispersion (%) |
|--------------------------------------|--|------------------|
| Pearlitol 100SD | 1.50±0.05 | 92.20±0.21 |
| SuperTab 11 SD | 1.60±0.06 | 99.60±0.33 |
| Kollidon CLSF | 0.70±0.12 | 96.68±0.19 |
| Aerosil 200 | 0.35±0.07 | 82.20±0.08 |
| Aerosil R 972 | 0.23±0.04 | 65.40±0.45 |
| Avicel CL 611 | 0.15±0.02 | 99.10±0.78 |
| Syloid 244 FP | 0.80±0.05 | 35.20±0.54 |
| Neusilin US 2 | 0.50±0.04 | 96.78±0.89 |
| Neusilin UFL 2 | 0.35±0.03 | 72.10±0.16 |
| Fujicalin | 0.35±0.01 | 95.20±0.13 |
| Supertab 11 SD + Neusilin US 2 (1:1) | 0.40±0.04 | 97.62±0.44 |
| Kollidon CLSF + Neusilin US 2 (1:1) | 0.68±0.05 | 96.60±0.25 |
| Kollidon CLSF + SuperTab 11 SD (1:1) | 0.40±0.09 | 95.50±0.14 |

Values expressed in mean ± SD, n=3.

surface area of 300 m²/g for adsorption. It was required in a small quantity, showing good desorption. A combination of kollidon CL SF and neusilin US 2 was required in a comparatively larger amount, which may be attributed to the hygroscopic nature of kollidon CL SF. The same quantity of combination of lactose with both neusilin US 2 and kollidon CL SF was required for DAN formation with a minor increase in %desorption in the case of neusilin US 2, which may be due to its greater surface area (Table 4). The optimization results showed that the combination of lactose with neusilin US 2 gave reasonable particle size, redispersion properties and better flow properties (Figure 1B) (Table 5).

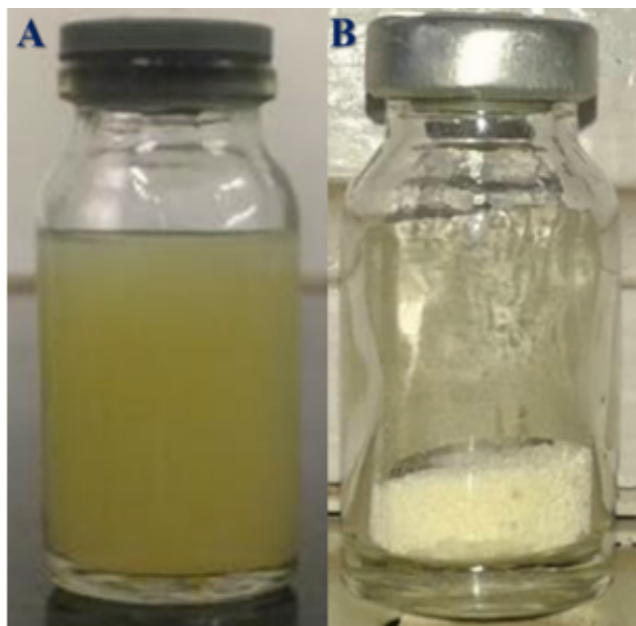


Fig. 1: Optimized formulations of A. silymarin loaded NLCs, B. DANs of silymarin loaded NLCs

3.3. Ex-vivo intestinal permeability and lymphatic uptake study of silymarin loaded NLCs

Drug permeation from the formulation was compared with silymarin suspension. From Figure 2, it was observed that the permeation of NLCs through untreated jejunum (without pluronic F-68) was 36 % and that of plain silymarin suspension was about 30% within 3 h, while the permeation of NLCs through treated jejunum with pluronic F-68 was found to be 20%. The permeation of silymarin from NLCs through the tissue treated with pluronic F-68 solution was found to be less compared to the untreated tissue. This gives an idea that absorption of NLCs was through lymph along with blood capillaries and a decrease in the permeation of silymarin was observed due to blockage of lymphatic uptake of NLCs by pluronic F-68.

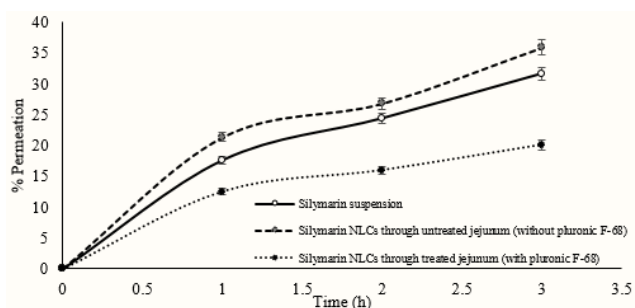


Fig. 2: Ex-vivo intestinal permeability of silymarin suspension, silymarin NLCs through untreated jejunum (without pluronic F-68) and silymarin NLCs through treated jejunum (with pluronic F-68)

3.4. Characterization and evaluation of silymarin loaded NLCs and DANs

3.4.1. Particle size, size distribution and zeta potential

Measurement of particle size was done to assure the production range. The range of particle size of NLCs is 10-1000nm. PDI gives an indication of the width of the distribution. PDI 0.1-0.3 shows a narrow size distribution. The results of the various experiments indicated that particle size was significantly influenced by most of the formulation and process variables. The particle size of the silymarin NLCs dispersion was found to be 206.1±012.5 nm with a PDI of 0.249±0.058. Silymarin NLCs have a zeta potential of -32.5±1.2 mV confirming their stability. The zeta potential shows the degree of repulsion between the particles which are charged in the dispersion. The higher negative zeta potential will prevent the aggregation of the particles due to electric repulsion, which is good for the stabilization of a nanodispersion. Based on the optimization of DANs, a combination of neusilin US 2 with lactose was finalized for DANs. The particle size of NLCs after reconstitution from DANs was found to be 347.2±042.5 nm with a PDI of 0.521±0.021. This particle size is more than that of NLCs, but not very significant.

3.4.2. Entrapment efficiency and drug loading

Higher EE would result in improved bioavailability as the drug remains entrapped in the formulation. Entrapment efficiency depends on the excipients used in the formulation and method of preparation. Higher entrapment efficiency offers protection of drugs against degradation. The entrapment efficiency and drug loading of NLCs were found to be 95.60±0.45 % and 1.90±0.08% indicating no drug leakage from the fine lipid matrix.

3.4.3. Drug content/assay

An assay of the silymarin was done to verify the content of the drug actually present in the formulation. By calculating the percentage recovery, it can be concluded that the

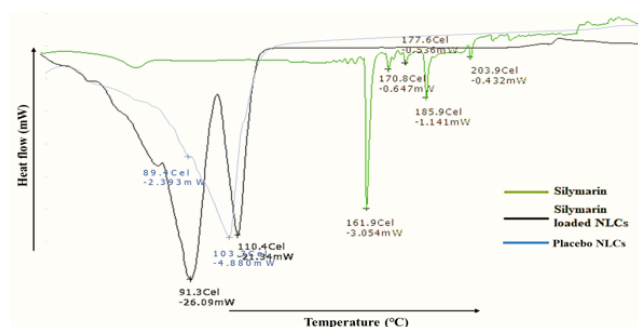
Table 5: Results of optimization of DANs of silymarin loaded NLCs

| Adsorbents | Particle size (nm) | PDI | Angle of repose (°) | Flow properties Compressibility index (%) | Hausners ratio |
|-----------------------------------|--------------------|-------------|---------------------|--|----------------|
| SuperTab 11 SD | 305.2±062.1 | 0.401±0.125 | 13.82±1.45 | 23.20±1.15 | 1.30±0.16 |
| Kollidon CL SF | 499.7±126.2 | 0.541±0.085 | 22.04±2.14 | 16.77±1.47 | 1.20±0.07 |
| Neusilin US 2 | 325.7±108.5 | 0.616±0.042 | 14.89±4.12 | 19.04±0.75 | 1.23±0.04 |
| SuperTab 11 SD + Neusilin US 2 | 347.2±042.5 | 0.521±0.021 | 10.42±2.47 | 7.69±1.12 | 1.08±0.06 |
| SuperTab 11 SD + Kollidon CLSF | 417.1±088.5 | 0.570±0.134 | 30.83±3.14 | 15.93±2.47 | 1.18±0.12 |
| Kollidon CLSF+ Neusilin US 2 | 463.3±156.2 | 0.851±0.064 | 14.03±1.54 | 19.87±4.15 | 1.24±0.25 |

formulation contained a specified quantity of the drug. The assay of silymarin loaded NLCs was found to be $100.32 \pm 1.91\%$. Similarly, the assay of optimized DANs was found to be $98.60 \pm 0.98\%$.

3.4.4. Thermal behavior

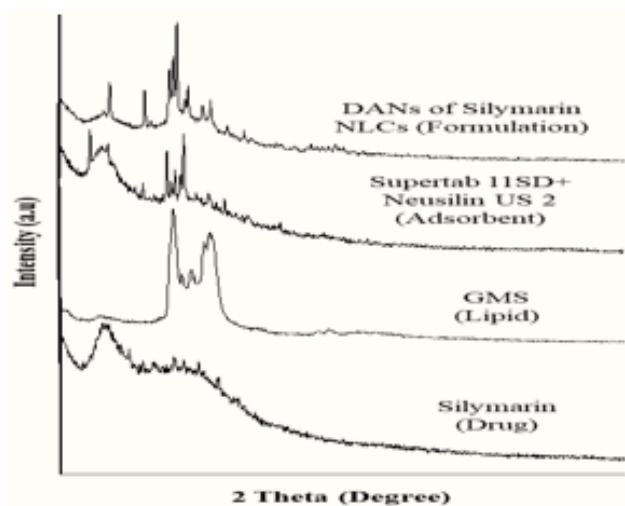
The DSC of plain silymarin and silymarin loaded NLCs formulation is shown in Figure 3. The DSC curve of the silymarin showed a sharp, intense endothermic peak at its melting point of 161°C indicating its crystallinity, but the thermogram of silymarin loaded NLCs did not show a peak at the same temperature. The peaks were observed at 91°C and 110°C which were slightly broader. The disappearance of the sharp endothermic peak of the silymarin in placebo and silymarin NLCs indicates that the silymarin is solubilized in a lipid matrix. The appearance of new peaks may be attributed to the matrix of solid lipid, liquid lipid and surfactant mixture.

**Fig. 3:** DSC thermogram of silymarin, silymarin loaded NLC formulation and placebo NLC formulation

3.4.5. Crystallinity

The XRD spectra of silymarin (drug), GMS (lipid), supertab 11SD+neusilin US 2) (adsorbent) and DANs of silymarin loaded NLCs are depicted in Figure 4. The XRD of the silymarin shows a partly crystalline nature, which can be attributed to the fact that silymarin is a mixture of four different flavonolignans, including silybin, isosilybin,

silychristin and silydianin. Thus, it can be concluded that it has both an amorphous and a crystalline nature. XRD of DANs does not show characteristics corresponding to amorphous nature. It shows the combination of peaks as obtained in the adsorbent and other components. It can be thus concluded that the silymarin does not undergo complete amorphization during processing.

**Fig. 4:** XRD spectra of silymarin (drug), GMS (lipid), supertab 11SD+neusilin US 2) (adsorbent) and DANs of silymarin loaded NLCs

3.4.6. Surface morphology

SEM analysis was performed on the surface morphology of particles. Figure 5 depicts the SEM image of silymarin loaded NLCs, adsorbent and DANs. It was observed that the NLCs were spherical in shape with smooth surface morphology. Some aggregated particles were also observed. The adsorbent was found to be irregular in shape with aggregated masses. SEM of DANs showed adsorption of spherical NLCs on the surface of adsorbents.

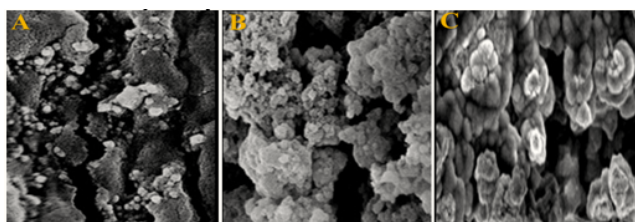


Fig. 5: SEM images of A. silymarin loaded NLCs formulation, B. adsorbents and C. DANs of silymarin loaded NLCs

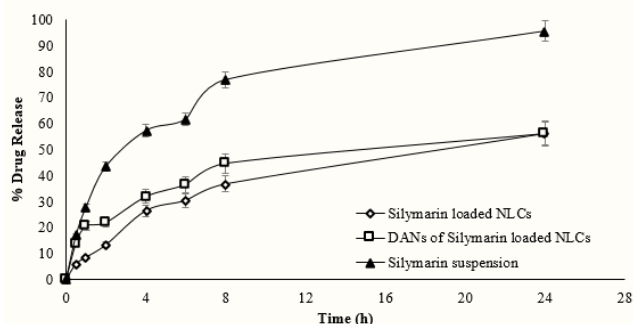


Fig. 6: In-vitro drug release profile of silymarin suspension, silymarin loaded NLCs and DANs of silymarin loaded NLCs

3.4.7. In-vitro drug release

An in-vitro release study was performed in phosphate buffer pH 7.5+2% SLS for 24 h. The % cumulative release at predetermined time intervals was calculated and plotted against time as shown in fig. 6. Drug release from silymarin loaded NLCs formulation and DANs of silymarin loaded NLCs were compared with silymarin suspension in water. It was observed that drug release was sustained as compared with silymarin suspension. Sustained drug release from the formulation was observed, which may be due to the entrapment of the drug in the lipid matrix. The conversion of NLCs into DANs did not affect the release pattern of the drug.

4. Discussion

GMS is a monoglyceride, whereas CMPR and PRE contain a mixture of fatty acid esters. The possibilities of conversion of crystalline to different polymorphic forms of these lipids are less and thus they can incorporate a higher amount of drug. The lesser incorporation of the drug in dynasan 114, 116, 118, which are triglycerides of myristic, palmitic, and stearic acid due to their crystalline nature and symmetrical in structure. The effect of solid lipids on lipid based systems was reported by Sarker et al. The crystal structure of the lipid depends on the changes in temperature and cooling speed. Further, aggregation may occur because of polymeric transitions of the crystalline NLCs. The stability of the SLN/NLCs system is maintained

by the presence of surfactants which compactly pack the structures to avoid aggregation of the particles. The stability of the colloidal systems is improved by achieving and maintaining a uniform particle size distribution. The smaller the particle size, the more stable the formulation.³¹ The use of surfactants increases the stability of NLC dispersion. During the development of NLCs, G50/13 showed better stability. While formulations with some other surfactants showed a gelling effect, drug leakage, etc. Sanming li et al studied the effect of surfactants on lipid based systems. In addition to suitable surfactants in the colloidal system, the electrostatic repulsion among the particles will be increased, which prevents the aggregation of the particles and leads to enhanced stability of the system. The particles without the protection of surfactant will flocculate under the effect of intermolecular van der Waals forces. This will create the formation of a gel by gravitational potential energy due to a change in surface state.³² Minimum particle size and PDI were obtained when G50/13 was used alone with the formulation being physically stable for a period of a minimum of 3 days. Another advantage of using G50/13 in the preparation of NLCs is that it inhibits the p-glycoprotein efflux pump and thus increases absorption of the drug after oral administration.³³ The decrease in particle size was observed with an increase in surfactant concentration. Higher surfactant concentration reduces the surface tension of the molten lipid droplets, causing the further breakdown of the lipid drops into a smaller size. An optimum concentration of surfactant will act as a protective layer to prevent aggregation of particles and form a stable emulsion.³⁴ Particle size and PDI generally decrease with increasing sonication time. Acoustic energy generated from the probe tip forces the fluid to an extremely high pressure and this leads to a physical phenomenon called cavitation. Cavitation causes sudden formation and collapse of high-pressure bubbles within the fluid and results in particle size reduction. Larger particles are formed during the emulsification step, which breakdown into smaller particles by sonication. Hence, sonication significantly affects the particle size. It was found that a significant decrease in particle size occurred at a medium level of sonication time. The excessive collisions between particles and the formation of aggregates were observed due to high sonication time, which ultimately increased the particle size of the system.³⁵ An ex-vivo study was carried out to check intestinal permeability and lymphatic uptake of the drug. It was evident from Figure 2, that the decreased permeation of silymarin in the presence of pluronic F-68 treated sac as compared to that of the increased permeation in the absence of pluronic F-68 (non treated sac). This could be attributed to pluronic F-68 blockage of intestinal lymphatic transport. It is evident from the literature that the transport of lipidic particles to the lymphatic circulation via the intestinal lymph seems to gain access to the

lymphatic system via three potential pathways: via the paracellular route with the aid of absorption enhancers, through M cells and gut associated lymphoid tissue (GALT), and via a transcellular route in association with the triglyceride lipoprotein core of the chylomicrons.^{36–38} The results reported in this study were similar and comparable to the lymphatic blockage study of pluronic F-68 reported.²³ The increased permeability/diffusion of silymarin loaded NLCs is attributed to several factors, like high permeation of drug from NLCs due to nano range of particle size, reduction of interfacial tension of formulation due to presence of surfactant, lipidic excipients used in the formulation, permeation through the chylomicron production or permeation through payer's patch, enhanced membrane fluidity, the opening of tight cellular junctions, inhibiting p-glycoprotein efflux pump by surfactants. Improved diffusion of the drug from NLCs may be because of the inhibitory action of G50/13 on the p-glycoprotein efflux pump and hence an increase in absorption after oral administration.³³

5. Conclusion

Stable and optimized silymarin loaded NLCs prepared by a modified hot melt emulsification ultrasonication method with high entrapment efficiency, drug loading, acceptable particle size distribution, and zeta potential. It was formulated for oral administration to increase its solubility and bioavailability. For better stability of NLCs, the DANs were prepared by an adsorption technique using suitable carriers. A combination of water soluble and water insoluble carriers made free flowing NLC granules. This can be used as a dry powder for oral reconstitution. Several formulations and process related parameters were varied at different levels to get an optimum silymarin NLC formulation on the basis of particle size and PDI. From the observations, the GMS, CAP and G50/13 exhibited good candidates for the preparation of NLCs. The ex-vivo permeation study in the presence and absence of lymphatic uptake blocker exhibited uptake of silymarin loaded NLCs by the lymphatic route. Thus, developed formulations may lead to increased bioavailability due to lymphatic targeting.

6. Acknowledgement

The authors are also thankful to Biogen extract Pvt. Ltd. for providing a gift sample of silymarin. The authors are thankful to Tata Institute of Fundamental Research (TIFR) for providing the facility for analysis of XRD samples.

7. Source of Funding

None

8. Conflict of Interest

None

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Cite this article: Hirlekar R, Patil E, Bhairy S. Solid nanostructured lipid carriers loaded with silymarin for oral delivery: Formulation development and evaluation. *Curr Trends Pharm Pharm Chem* 2021;3(4):56-67.