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Research Article

Spray-dried nanoemulsion for improved oral delivery of silymarin against hepatic cancer cells

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Abstract

Silymarin recognized for numerous activities, but the use is limited due to poor aqueous solubility, inefficient intestinal permeability, and low-erratic bioavailability. The aim of the current research was formulation of spray-dried nanoemulsion to enhance the solubility of silymarin. The nanoemulsion was prepared by aqueous titration method, spray dried and characterized for thermal analysis by diffraction scanning calorimetry, crystallography analysis by x-ray diffraction, surface morphology by scanning electron microscopy. The reconstitution properties were determining for droplet size, polydispersity index and microscopic structure. Optimized nanoemulsion composed of 15% v/v of oil, 33% v/v of Smix and 52% v/v of distilled water demonstrated lowest droplet size (52.4 ± 1.63 nm) and polydispersity index (0.112), optimum viscosity (23.37 ± 2.36 cps), maximum % transmittance (94.55), optimum cloud point (88°C) and cumulative % drug release (98.43%). The microscopic structure of spray-dried nanoemulsion after reconstitution in distilled water revealed spherical shape free from any aggregation. Spray-dried nanoemulsion demonstrated amorphous state of silymarin after fabrication into solid state. The cumulative % release of silymarin was significantly higher than marketed conventional suspension (Limarin). The developed spray-dried nanoemulsion was robust and stable for a period of 3 months that could be recommended for oral administration of silymarin after further study. Solids are preferred over liquid dosage form; the formulation may offer better patient compliance over liquid nanoemulsion. In addition, the in-vitro cytotoxicity study revealed more cytotoxicity of SD-NE than plain silymarin against HepG2 cell line after 48 h of incubation. Moreover, the HepG2 cellular uptake silymarin was found to be substantially higher from NE when compared to the plain silymarin. Further, silymarin loaded SD-NE could be potential approach against hepatic cancer.

Keywords: Silymarin, spray-dried nanoemulsion, dextran, phase diagram, solubility, stability

INTRODUCTION

Cancer is a type of malignant disease that is the world's second-leading cause of death, after coronary artery disease. The most common kinds of liver cancer are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). HCC is the third most prevalent cause of cancer-related death worldwide, accounting for 75–85 percent of all primary liver malignancies and affecting predominantly men. CCA, on the other hand, is the second most common hepatic malignancy, accounting for 10%–15% of all cases ¹.

Silymarin, a potential phytochemical compound is isolated from *Silybum marianum*, popularly known as milk thistle plant. Silymarin demonstrates numerous activities including anti-oxidant, anti-inflammatory, anti-cancer and anti-viral activities, as well as potential usefulness in the treatment of several liver disorders, such as chronic liver diseases, cirrhosis and hepatocellular carcinoma ²⁻⁵. In the past, it has been demonstrated that silymarin treatment inhibited the proliferation and induced apoptosis in the human HCC cell line (HepG2) ⁶. Despite of numerous therapeutic activities, the limiting factors restricting its use are low permeability across intestinal epithelial cells, extensive metabolism in phase II, gastrointestinal degradation, and rapid biliary-urinary excretion ^{7,8}. The aforementioned problems are mainly due to lipophilicity ($\log p$ 1.41) and poor aqueous solubility (50-430 µg/mL) of drug lead to poor bioavailability (23-47%) and requirement of high oral dose (280–1000 mg).

Recent trends in drug development had led to fabricate a robust and suitable formulation to augment its solubility, bioavailability and to address the major drawbacks concerned ⁹. Numerous nanotization approaches have been adopted in the past including liposomes ¹⁰, solid-dispersion ¹¹, solid lipid nanocarrier ¹², micro- nanoemulsion ^{13,14}, and self-emulsifying formulation ¹⁵. Among all, micro or nanoemulsion emerged as a promising nanocarrier to improve its solubility and oral bioavailability. Higher solubilization capacity, rapid onset of action, decreased inter-and intra-subject variability and the possibility of large scale production by high pressure homogenization are the main benefits offered by nano-sized emulsion over other nanocarrier system ¹⁶.

Solids are preferred over liquid dosage form due to many reasons including ease to patient and accuracy of dose as well as robust nature for adequate physical stability. The direct filling of nanoemulsion into capsule causes stability issue with the gelatin shell due presence of surfactant. Hence, transformation of liquid-lipid formulation/nanoemulsion into spray-dried form by adopting suitable fabrication technique could offer the benefits of both lipid formulation as well as solid dosage form ^{17,18}. The current study aims to develop a robust spray-dried nanoemulsion (SD-NE) and investigate its therapeutic efficacy using liver cancer cell line (HepG2).

MATERIAL AND METHODS

Materials

Silymarin was generously provided as a gift sample from

Ranbaxy, Gurgaon, India. Maisine™ 35-1 (oil), capryol 90 (co-surfactant) were obtained as a gift sample from Gattefosse Corp., France. Tween 80 (surfactant) was purchased from Merck India Ltd. Dextran 40 was supplied as a gift sample from Shanghai Huamao Pharmaceutical Company, Shanghai, China. Water was procured from Milli-Q water purification system (Millipore, Billerica, MA). The liver cancer cells (HepG2) were procured from American Type Culture Collection, USA. All the components used in the formulation meet the requirements for the suitability of oral administration.

Nanoemulsion excipients selection

Lipid components for nanoemulsion formulation are usually oils, surfactant and co-surfactant. The assessment of solubility of drug in various components and identifying the area of nanoemulsion region in the phase diagram are among the important task in formulation development. To select suitable excipients, these components were examined by solubility study to ascertain the maximum solubility of silymarin. Briefly, an excess amount of silymarin was added into the excipients previously kept in a glass vial of 5 mL capacity and fixed on a biological shaker for 72 h with temperature control of 25 °C followed by centrifugation at rotational speed of 5000 rpm. After 10 min, the clear upper layer was removed and filtered (0.45 µm filter). The absorbance of the sample was measurement spectrophotometrically at 288 nm, and the drug concentration was calculated using standard plot ¹⁹.

Construction of ternary phase diagram

Surfactant (tween 80) and co-surfactant (capryol 90) in six volume ratios, Smix-1:0, 1:1, 1:2, 1:3, 2:1, and 3:1 were prepared in a glass volumetric flask. From each group of Smix ratio, 100 mL of stock solution was made for further use. Oil (Maisine™ 35-1) and Smix ratio ranging from 9:1 to 1:9 were made for each phase diagram to achieve maximum ratios and to delineate the boundary. Aqueous titration using double distilled water was adopted for each oil/Smix ratios prepared above and after every 5% addition of water, visual observation was made. The nanoemulsion components (% v/v) were selected for ternary phase diagram construction. The corners of ternary phase diagram representing three axes; one axis water, other axis oil and third axis Smix.

Nanoemulsion selection from phase diagram

From the phase diagram constructed, the formulation was selected in such a way that the concentration of oil could easily dissolve 140 mg of silymarin. In brief; four concentration of oil ranging from 10-25% at a difference of 5%, and minimum surfactant concentration was selected from each phase diagram.

Thermodynamic stability

Three stability stress tests were performed by exposing the formulation selected from each phase diagram. Heating cooling cycle; exposing at 45 °C and 25 °C temperature alternately for not <24 h (six cycles at each temperature), centrifugation; 5000 rpm for a time period of 30 min and freeze-thaw cycle; exposing at -20 °C and 25 °C temperature alternately for not <24 h. Finally, the formulation was detected for any aqueous and oily phase separation, creaming or cracking. The most stable formulation towards each stress tests were selected for further study.

Dispersibility tests

The purpose of dispersibility test was to assess the self-emulsification efficiency of the passed formulations in thermodynamic stress tests. Dissolution apparatus USP II was filled with 500 mL distilled water, 0.1N HCl, and phosphate

buffer (PB) as a dispersion media and the temperature was controlled at 37 ± 0.5 °C. 1 mL formulation was added into the glass jar and paddle speed was fixed at 75 rpm provided gentle agitation to the formulation. Then, the formulation was assessed visually for its *in vitro* performance and compared with the reported grading system ²⁰. Grade A: formation of nanoemulsion within 1 min with clear transparent or slightly bluish in appearance; Grade B: formation of nanoemulsion rapidly but slightly less clear and bluish white appearance; Grade C: formation of emulsion in 1-2 min with fine milky appearance; Grade D: formation of emulsion in more than 2 min with dull, grayish white and oily appearance; Grade E: formation of emulsion with large oil droplets clearly visible on the surface.

Silymarin loaded nanoemulsion

Only those formulation was incorporated with drug that passed the stress tests and demonstrated self-emulsification ability to form nanoemulsion in Grade A and B. Briefly, silymarin (20 mg) was dissolve in each concentration of oil ranging from 10-25% with respective Smix ratio. The whole content was vortex mixed with addition of water dropwise until a clear solution was obtained. The drug loaded nanoemulsion formulation was subjected for interim study by keeping at 40 ± 2 °C/65 ± 5% RH for 48 h and observed for any phase separation or precipitation.

Physicochemical characterization

Droplet size and size distribution

Size of nanoemulsion droplets and its distribution (polydispersity index, PDI) was determined by dynamic light scattering technique using a Zetasizer ZS 90 (Malvern, Worcestershire, UK). 1 mL sample was diluted with 100 mL double distilled water and filtered using 0.22 µm membrane filter to avoid multiscattering phenomena and experimental error. Light scattering was recorded at temperature of 25 °C and measurement angle of 90°.

Viscosity measurement

The sample without dilution was used for viscosity measurement. The instrument used was Brookfield DV III ultra V6.0 RV cone with plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA, USA) fitted with spindle # C 50-1. Other parameters were set to spindle speed: 30 rpm, data interval: 1.0, Loop start: 1, Wait time: 30 min, and share rate maintained to 60 s⁻¹. The software used was RHEO3000.

Transmittance and cloud point

Shimadzu UV spectrophotometer (Shimadzu, Japan) was used to measure the transmittance (%) and cloud point (°C). Dilution of the sample (1:100) was made using double distilled water and transmittance was measured at 288 nm wavelength. For cloud point measurement, 5 mL sample was taken in a glass test tube, placed into a water bath and temperature was increased slowly. The drop in % transmittance was measured at the same wavelength at 288 nm ²¹.

Preparation of spray-dried nanoemulsion

A single stage pilot-scale spray dryer (Anhydro F1 Lab, Copenhagen, Denmark) equipped with a two-fluid nozzle atomization system (Type 1/8 JAC 316ss) and counter-flow drying was used to achieve spray drying of nanoemulsion. Drying mixtures were prepared by dissolving dextran (5 g), a water soluble solid carrier, in 100 mL distilled water and agitated using magnetic stirrer followed by addition of 5 g nanoemulsion formulation with continuous stirring until a homogenous mixture was obtained. Other process parameters

optimized were: inlet temperature: 145 °C, outlet temperature: 80 °C, water evaporation rate: 10 kg/h, aspiration: 85%, rate of air flow: 600 nL/h, and feeding rate: 5 mL/min. The assay of the spray-dried nanoemulsion (SD-NE) obtained after drying of nanoemulsion was 20.6 % w/w.

Thermal analysis

Differential scanning calorimeter, DSC 131EVO (Setaram, Caluire, France) was used for thermal analysis of silymarin, dextran, and SD-NE to investigate the solid state of each ingredient alone as well as in spray-dried nanoemulsion. 5 mg sample was placed separately in an aluminium crucible. The operation was optimized at; heating rate: 5 °C /min; temperature: ranges from 10-200 °C, nitrogen purging rate: 20 mL/min, and temperature scale calibration using Indium.

Crystallography analysis

The crystallography structure of silymarin, dextran and SD-NE were examined using Philips X'Pert PRO diffractometer (PANalytical, the Netherlands). The measurements were carried out using operation voltage of 40 kV and supplied current of 40 mA. The sample was exposed to a Cu-K α radiation, over an angular range of 5-60° (2 θ), at scanning speed of 5°/min, and sampling interval of 0.02° 2 θ .

Morphological evaluation

A FEI Sirion-200 scanning electron microscope (FEI, the Netherlands) was used to investigate the outer morphological structure of SD-NE. The sample was fixed on a SEM-stub using double-sided adhesive tape and then coated with a thin layer of gold. The operation was run at 10 kV.

Reconstitution Properties

Droplet size and microstructure of nanoemulsion

The droplet size, PDI and microstructure of nanoemulsion was determined to investigate the reconstitution properties of SD-NE. In brief, 50 mg SD-NE was reconstituted in 10 mL distilled water followed by sonication for 10 min and filtration using 0.22 μ m membrane filter. The droplet size and PDI were measured by the same instrument used above. For determining the microstructure of reconstituted nanoemulsion, the sample was diluted (1:100) with distilled water and applied on carbon coated grid with 2% phosphotungstic acid. The coated grid was placed on the slide, covered with a cover slip and examined for transmission electron microscopy (TEM A TOPCON 002B) operated at 200 kV.

In-vitro release of silymarin

The dialysis bag ((MWCO: 1200 g/mole) method was used to determine the release of silymarin from nanoemulsion, spray-dried nanoemulsion and marketed conventional suspension (Limarin). 900 mL of simulated intestinal fluid was filled into the jar of the dissolution apparatus USP II with temperature control at 37 \pm 0.5 °C and speed of the paddle set to 50 rpm. The dialysis bag was kept into the dissolution media and left overnight for pre-treatment. Each formulation equivalent to 140 mg silymarin was placed into the pre-treated dialysis bag. Time to withdraw the sample was pre-determined at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h. 1 mL of the sample was withdrawn from the jar and replaced with same volume of fresh dissolution media to maintain the sink condition. Amount of drug in each sample was analyzed spectrophotometrically at 288 nm.

In-vitro cytotoxicity study

Dulbecco's modified eagle's medium (DMEM) supplemented

with 10% Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 μ g/mL) was used for culture of cells. Cells were grown in humidified incubator in the atmosphere of 5% CO₂/37°C/95% RH. Each well of 96 well-plates were seeded with 5 \times 10⁴ cells and allowed to adhere and grow. Then the plates were incubated overnight in humidified atmosphere of 5% CO₂/37°C/95% RH. After that, the supernatant DMEM was replaced with 100 μ L of serially diluted test solutions followed by incubation for 48 h (at 37°C/5% CO₂/95% RH). After 48 h of incubation, the test solutions were replaced with 100 μ L of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT: 0.6 mg/mL) in phosphate buffer saline and further incubated for 4 h. Finally, the supernatant was removed and 100 μ L dimethyl-sulphoxide was added to solubilize the formazan crystals formed in cells. The absorbance was measured at 570 nm wavelength using a microplate. The IC₅₀ values were then calculated using dose-response curves.

In-vitro cellular uptake

HepG2 cells were used to study the cellular uptake of plain silymarin and SD-NE. Briefly, 3 mL of 5 \times 10⁴ cells/well were placed into 24-well plates and incubated overnight at 37° C. The cells were then treated with plain silymarin and SD-NE (20 μ g/mL) and incubated for 4 h. The cells were lysed with triton X-100 (1% v/v; 0.5 mL/well) after being rinsed twice with phosphate buffer saline. Then, 0.5 mL of acetonitrile was added to each well to extract silymarin and precipitate proteins. Finally, the supernatant was filtered using 0.45 μ m filter, and silymarin content was determined using an in-house HPLC method.

HPLC for analysis for estimation of silymarin

The drug content was determined by reported method with slight modification ²³. The instrument for analysis was Shimadzu LC-10AT VP equipped with C18 column (25 cm x 4.6 mm, 5 mm particle size) and UV detector (Shimadzu, Kyoto, Japan). Mobile phase, water and acetonitrile in 50:50, v/v was pumped at a flow rate of 1 mL/min and column temperature set to 40°C. The sample was analyzed at detection wavelength of 288 nm. The software used was Class VP, version 5.032.

Stability of spray-dried nanoemulsion

The spray dried nanoemulsion was subjected to stability studies at different temperature and relative humidity (25 \pm 2 °C/60 \pm 5% RH, 40 \pm 2 °C/65 \pm 5% RH.). Briefly, the formulation was placed into amber colored glass container and charged for stability with humidity and temperature control. The samples were taken out at specified time intervals of 0, 15, 30, 60, and 90 d and evaluated for drug content, reconstituted droplet size and polydispersity index.

RESULTS AND DISCUSSION

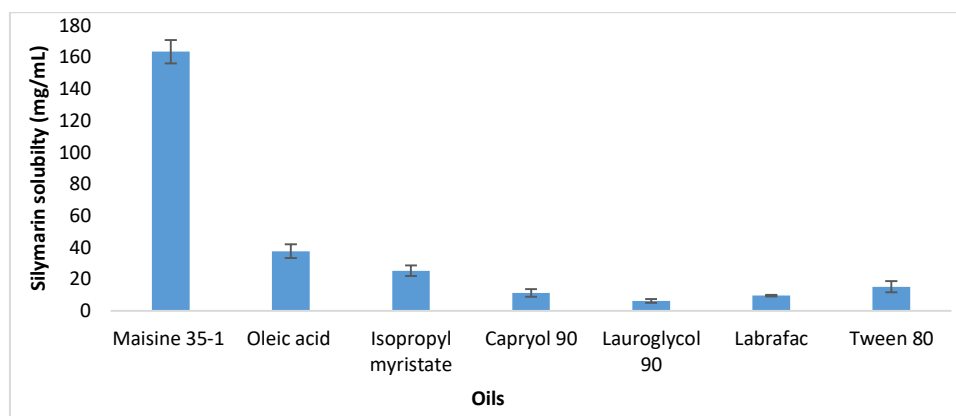
Nanoemulsion components

Prior to development of nanoemulsion formulation, it becomes important to identify suitable oil that can solubilize required amount of drug. Moreover, higher solubilization of drug in oil is vital for nanoemulsion to maintain the drug in solubilized state *in vivo*. The solubility of silymarin in Maisine™ 35-1 was highest (163.5 mg/mL) followed by oleic acid (37.6 mg/mL) and isopropyl myristate (25.3 mg/ml). Fig. 1 represents the solubility of silymarin in different excipients. Tween 80 and capryol 90 were selected as surfactant and co-surfactant, respectively. Surfactant let down the interfacial tension, aid in dispersibility, while the co-surfactants provide flexibility to the interfacial film for nanoemulsion formation ²⁴. Finally, the excipients selected were in the GRAS (Generally Regarded as Safe) category.

Table 1: Nanoemulsion formulation selected at a difference of 5% v/v of oil.

Smix ratio (S:CoS)	Nanoemulsion components (% v/v)			Thermodynamic stability test			Dispersibility test			Inference
	Oil	Smix	Water	H/T	Centr.	F/T	Water	HCl	PB	
1:0 (Fig. 3a)	10	33	57	X	-	-	-	-	-	Failed
	10	40	60	√	X	-	-	-	-	Failed
1:1 (Fig. 3b)	10	36	54	X	-	-	-	-	-	Failed
	10	40	50	√	X	-	A	C	-	Failed
	10	42	48	√	X	-	-	-	-	Failed
	15	31	54	√	X	-	-	-	-	Failed
	15	38	47	√	√	√	B	B/C	B	Passed
	15	43	42	√	√	√	A	A	A	Passed*
	20	30	50	√	√	X	-	-	-	Failed
	20	35	45	√	√	√	A	A	A	Passed*
	20	40	40	√	√	√	A	B/C	B	Passed
	25	36	39	√	√	X	-	-	-	Failed
2:1 (Fig. 3c)	10	28	62	X	-	-	-	-	-	Failed
	10	38	52	√	√	X	-	-	-	Failed
	10	40	50	√	√	√	A	A	B	Passed
	15	31	54	√	√	X	-	-	-	Failed
	15	33	52	√	√	√	A	A	A	Passed*
	15	38	47	√	√	√	A	A/B	A	Passed
	20	40	40	√	√	√	A	A	A	Passed*
	20	42	38	√	√	√	A	A	B	Passed
	20	44	36	√	√	√	B	C	-	Failed
	25	36	39	√	√	√	A	A	A	Passed*
3:1 (Fig. 3d)	10	28	62	√	√	A	A	A	A	Passed*
	10	32	58	√	√	√	A	B	C	Failed
	10	37	53	√	√	√	B	C	-	Failed
	15	38	47	√	√	X	-	-	-	Failed
1:2 (Fig. 3e)	10	37	33	√	√	√	E	-	-	Failed
	10	40	60	√	X	-	D	-	-	Failed

(Heating cooling cycle: H/C, Centrifugation: Centr, Freeze thaw cycle: F/T, *Formulation selected)

**Figure 1:** Bar diagram showing the solubility of silymarin in different oils, surfactant and co-surfactant.

Construction of ternary phase diagram

Aqueous titration method was adopted for constructing ternary phase diagram. Mixtures of oil and Smix were slowly titrated with double distilled water to produce water concentration ranging from 5-95% of total volume at a difference of around 5%. The ternary phase diagram was constructed using percentage of oil, surfactant and co-surfactant. Phase diagrams were constructed separately for each Smix ratio (Fig. 2a-e). The three corners of the phase diagrams comprise of Maisine™ 35-1, Smix ratio (tween 80: capryol 90) and water. At Smix ratio of 1:0 (Fig. 2a), when surfactant was used alone without co-surfactant, 40% v/v of surfactant was required to solubilize 10% v/v of oil i.e., a very low concentration of oil could be solubilized by a high concentration of surfactant. When the concentration of surfactant was further increased, solubilization of oil was less than 10% v/v. At Smix ratio of 1:1 (Fig. 2b), when equal

concentration of surfactant and co-surfactant were used, there was decline in nanoemulsion area compared to Smix ratio of 2:1 (Fig. 2c). At Smix ratio of 2:1, the nanoemulsion area was more than Smix ratio of 1:1 and only 36% v/v Smix was required to solubilize a high concentration (25% v/v) of oil. When surfactant concentration was further increased (Smix ratio-3:1, Fig. 2d), there was decrease in nanoemulsion area compared to Smix ratio-2:1. And only up to 15% v/v oil could be solubilized with a surfactant concentration of 38% v/v. At Smix ratio of 1:2 (Fig. 2e), only up to 10% v/v of oil could be solubilized at higher concentration of Smix (40% v/v). At Smix ratio of 1:3 (data not shown), there was significant decline in nanoemulsion region and phase separation after 12 h of storage. Hence, the construction of phase diagram delivers valued info on the role of each component in different concentrations in nanoemulsion formulation. Results suggested that Smix ratio of 2:1 demonstrated better result compared to Smix ratio of 1:0, 1:1, 1:2, 1:3 and 3:1.

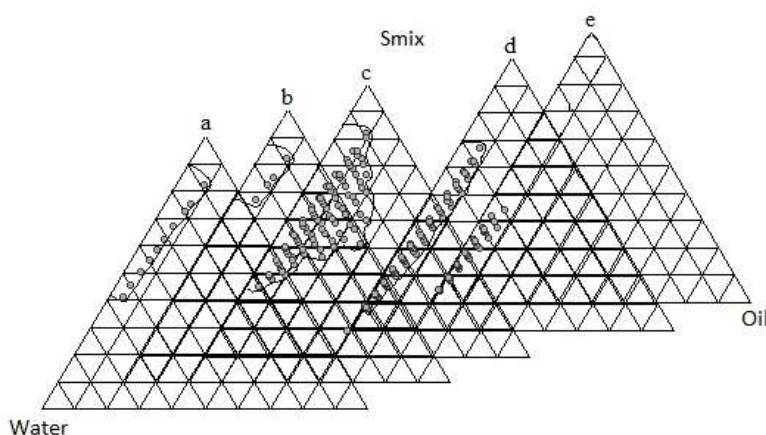


Figure 2: Pseudo-ternary phase diagrams for Smix ratio; 1:0 (a), 1:1 (b), 2:1 (c), 3:1 (d), 1:2 (e).

Test for thermodynamic stability

In order to ensure the stability of nanoemulsion, selected formulation from each phase diagrams were passed through thermodynamic stability test. The formulations remain to be stable after heating/cooling cycle, centrifugation, and freeze-

thaw cycles (observations recorded in Table 2). The formulations passed the thermodynamic test were subjected to dispersibility test. Formulations demonstrating the self-emulsification ability to form nanoemulsion in Grade A and B was selected for further study.

Table 2: Droplet size, PI, viscosity, % transmittance and cloud point of nanoemulsion.

Code	Smix ratio	Oil	Smix	Water	Interim Stability	Droplet Size	PDI	Viscosity	T**	Cloud Point
		% v/v								
NE-1	1:1	15	43	42	Unstable	-	-	-	-	-
NE-2	1:1	20	35	45	Stable	77.5 ± 1.28	0.244	38.26 ± 1.52	72.46	76
NE-3	2:1	15	33	52	Stable	52.4 ± 1.63	0.112	23.37 ± 2.36	94.55	88
NE-4	2:1	20	40	40	Stable	66.3 ± 1.12	0.166	42.24 ± 1.51	86.28	74
NE-5	3:1	25	36	39	Unstable	-	-	-	-	-

Physicochemical characterization

The droplet size measurement is important to differentiate micro- and nanoemulsion and PDI is a measure of uniformity of droplet size distribution. The nanoemulsion formulation NE-3 showed smallest particle size and PDI (52.4 ± 0.291 nm, 0.112) followed by NE-4 (66.3 ± 1.12 nm, 0.166), and NE-2 (77.5 ± 1.28, 0.244). The results demonstrated that the droplet size increased with increase in oil concentration. The lowest

PDI of NE-3 suggested narrow size distribution of droplets in the formulation. The viscosity of NE-3 was minimum (23.37 ± 2.36 cps) followed by NE-2 (38.26 ± 1.52), and NE-4 (42.24 ± 1.51). The formulation NE-3 showed lowest viscosity revealed direct relationship between the concentration of oil and viscosity of the formulation i.e. with increase in concentration of oil phase viscosity increases. The formulation NE-3 composed of lowest concentration of oil (15% v/v) and hence

demonstrated lowest viscosity (23.37 cps). The transmittance was highest for NE-3 (94.55%) followed by NE-4 (86.24%) and NE-2 (72.46%). Moreover, the cloud point for NE-3, NE-2 and NE-4 was 88 °C, 76 °C, and 74 °C, respectively. The transmittance closer to 100% means the droplet size in nanometer range and cloud point demonstrate its stability in GI fluid. Overall, selection of suitable oil, surfactant, and co-surfactant ratios were crucial for obtaining smaller particle size and PDI, optimum viscosity, higher % transmittance and cloud point for a stable nanoemulsion.

Differential thermal and x-ray diffraction analysis

In the past, many water-soluble solid carriers were used and reported including sucrose, maltodextrine, hydroxypropyl methylcellulose and dextran 40²⁵⁻²⁷. Among all the materials, maltodextrine and dextran 40 demonstrated good results to reduce the degree of agglomeration between spray-dried nanoparticles. In this study, dextran 40 was used as a solid carrier. The physical state of silymarin in SD-NE would have an important role on the release characteristic of silymarin

both *in vitro* and *in vivo*. Hence, the internal physical state of the drug was confirmed using differential scanning calorimetry (DSC) (Fig. 3a) and X-ray diffraction (XRD) chromatogram (Fig. 3b). Silymarin demonstrated a wide endothermic peak at about 90 °C and glass transition temperature of 70 °C indicated its crystalline nature (3a). Dextran produced a sharp endothermic peak at 33.75 °C and glass transition temperature of 30.7 °C. The indicative peaks for silymarin and dextran disappeared with a slight decrease around 75 °C and an increase around 150 °C in the DSC thermogram of SD-NE. This suggested that the drug and dextran was well solubilized in the formulation and silymarin was encapsulated in an amorphous or molecularly dispersed state within the spray-dried nanoemulsion. XRD chromatogram (Fig. 3b) exhibited sharp peaks in a 2θ angle ranging from 10 to 30, indicating a typical crystalline structure for both silymarin and dextran. No such peaks signifying crystals of silymarin were seen for the SD-NE revealing the transformation of crystalline structure of silymarin to amorphous state in fabricated spray-dried formulation.

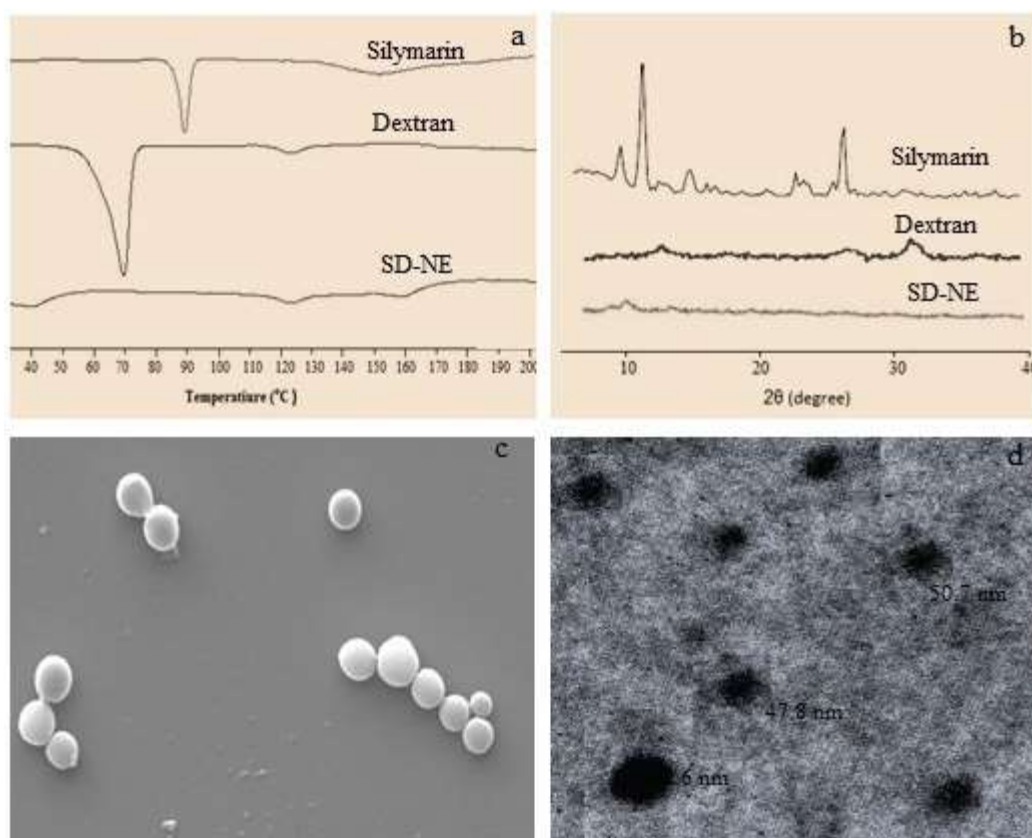


Figure 3: (a) Differential scanning calorimetry (DSC) thermogram of silymarin, dextran and SD-NE. (b) X-ray diffraction (XRD) patterns of silymarin, dextran, and SD-NE (c) SEM images of SD-NE, and (d) TEM images of reconstituted SD-NE.

Reconstitution properties

The droplet size and polydispersity index of the reconstituted nanoemulsion were 54.6 ± 1.44 and 0.124, respectively. Both the values increased slightly compared to the NE-3 but were statistically non-significant ($p > 0.05$). The SEM image (Fig. 3c) of SD-NE demonstrated well-separated particles with regular spherical shape without any aggregation. TEM images (Fig. 3d) of reconstituted nanoemulsion demonstrated spherical droplets. Incorporation of nanoemulsion in dextran 40 and spray drying did not lead to any significant effect on droplet size.

In-vitro release of silymarin

The nanoemulsion (NE-3), spray-dried nanoemulsion (SD-NE) were examined for *in vitro* release study, and compared with the marketed conventional suspension of silymarin (Limarin). The graph between percent cumulative release and time is shown in Fig. 4, demonstrating release profile of silymarin. The % drug release was highest (98.43%) for the formulation NE-3 followed by SD-NE (95.29%) and marketed conventional suspension (26.07%). Approximately 75% of the initial drug was released from SD-NE at the end of 2h. This burst effect may be due to the availability of drug particles distributed on the surface which tends to release immediately into the dissolution media. Both NE-3 and SD-NE exhibited enhanced drug release profile compared to conventional suspension. The NE-2, NE-3 and NE-4 were also compared for release of

silymarin utilizing same dissolution media and process parameters. The formulation NE-3 showed highest release (98.43%) in 24 h and hence selected for spray drying. NE-3 also demonstrated smallest droplet size (52.4 nm) and PDI (0.112), optimum viscosity (23.37 cps), highest % transmittance (94.55) and cloud point (88 °C).

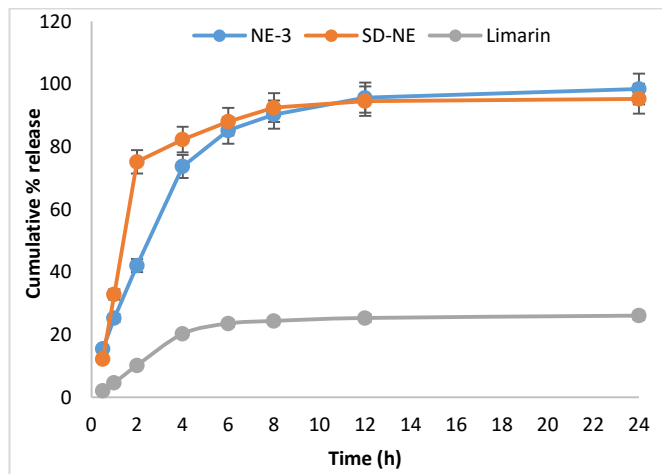


Figure 4: Comparative *in vitro* release profile of different formulations of silymarin (NE-3, SD-NE and marketed conventional suspension).

In-vitro cytotoxicity study

The cytotoxic potential of plain silymarin and SD-NE was studied by MTT assay against hepatic cell line (HepG2). The silymarin-loaded SD-NE demonstrated IC₅₀ of 18 µM displayed significantly (*p* < 0.01) more cytotoxicity than plain silymarin (IC₅₀: 32 µM) against HepG2 cell line after 48 h of incubation.

In-vitro cellular uptake

The purpose of cellular uptake study was to investigate the effect of SD-NE formulation on augmenting uptake of silymarin into HepG2 cells. The uptake of plain silymarin was found to be 145.7 ng/µg. The SD-NE showed about 3.6-fold increased cellular uptake of silymarin (524.3 ng/µg). These results could be correlated to the ability of SD-NE to improve cellular uptake of loaded drugs owing to the presence of lipids.

Stability of spray-dried nanoemulsion

The stability study of spray-dried formulation at different temperature and relative humidity for a period of 90 days demonstrated stable and robust nature of nanoemulsion in dried form. As mentioned in Table 3, all the evaluated parameters were slightly changed with respect to time but statistically not significant (*P* > 0.05) in terms of drug content, droplet size and PDI.

Table 3: Stability results after 3 months of storage.

Days	Storage condition: 25 ± 2 °C/60 ± 5% RH			Storage condition: 40 ± 2 °C/65 ± 5% RH		
	Drug content (%)	Droplet size (nm)	PI	Drug content (%)	Droplet size (nm)	PI
0	100.0	54.6 ± 1.63	0.124	100.0	54.6 ± 1.63	0.124
15	99.83	54.9 ± 1.44	0.127	99.53	55.2 ± 2.07	0.129
30	99.34	55.7 ± 1.53	0.133	99.25	56.8 ± 1.15	0.136
60	98.44	57.3 ± 2.05	0.141	99.04	58.3 ± 1.98	0.144
90	97.36	60.8 ± 1.39	0.146	96.42	61.8 ± 2.41	0.148

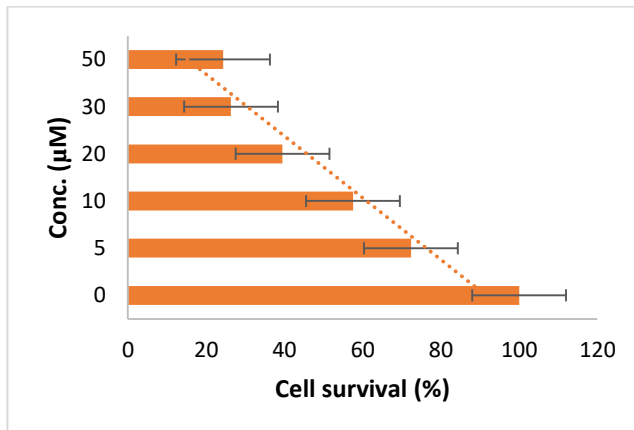
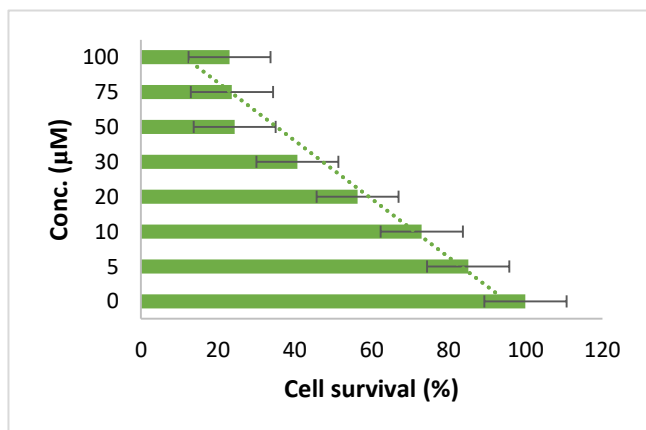


Figure 5:

CONCLUSION

In the research envisaged, the nanoemulsion was prepared by aqueous titration of oil and Smix, and spray-dried using dextran as solid carrier. The spray-dried nanoemulsion demonstrated particles with smooth surface without any

aggregation. Reconstitution of spray-dried nanoemulsion produced nanodroplets with all the required properties of nanoemulsion. Solid state characterization revealed the amorphous state of silymarin in fabricated spray-dried nanoemulsion. *In vitro* release of silymarin both from nanoemulsion and spray dried nanoemulsion were faster

compared to marketed conventional suspension. Results obtained from the stability studies demonstrated that the formulation remain stable over the storage period of 90 days as there was no significant ($p > 0.05$) change in drug content, droplet size and polydispersity index. Hence, fabricated spray-dried nanoemulsion may offer a useful oral solid dosage form for silymarin and other poorly water soluble drug as well. The in-vitro cell line study approve the use of silymarin for treatment of liver cancer.

Conflict of interest

The authors confirmed no conflicts of interest.

REFERENCES

- Bruix J, Han K, Gores G, JM, Llovet JM, Mazzaferro V. Liver cancer: approaching a personalized care. *J Hepatol* 2015; 62(1):S144-S156. <https://doi.org/10.1016/j.jhep.2015.02.007>
- Bijak M. Silybin, a major bioactive component of Milk Thistle (*Silybum marianum* L. Gaernt.)-chemistry, bioavailability, and metabolism. *Molecules* 2017; 22:942. <https://doi.org/10.3390/molecules22111942>
- Esmail N, Anaraki SB, Gharagozloo M, Moayedi B. Silymarin impacts on immune system as an immunomodulator: One key for many locks. *Int Immunopharmacol.* 2017; 50:194-201. <https://doi.org/10.1016/j.intimp.2017.06.030>
- Mastron JK, Siveen KS, Sethi G, Bishayee A. Silymarin and hepatocellular carcinoma: A systematic, comprehensive, and critical review. *Anticancer Drugs.* 2015; 26:475-486. <https://doi.org/10.1097/CAD.0000000000000211>
- Polachi N, Bai G, Li T, Chu Y, Wang X, Li S, Gu N, Wu J, Li W, Zhang Y., Zhou S, Sun H, Liu C. Modulatory effects of silibinin in various cell signaling pathways against liver disorders and cancer-A comprehensive review. *Eur J Med Chem* 2016; 123:577-595. <https://doi.org/10.1016/j.ejmech.2016.07.070>
- Ramakrishnan G, Lo Muzio L, Elinos-Báez CM, Jagan S, Augustine TA, Kamaraj S, Anandakumar P, Devaki T. Silymarin inhibited proliferation and induced apoptosis in hepatic cancer cells. *Cell Proliferation* 2009; 42:229-240. <https://doi.org/10.1111/j.1365-2184.2008.00581.x>
- Giacomelli S, Gallo D, Apollonio P, Ferlini C, Distefano M, Morazzoni P, Riva A, Bombardelli E, Mancuso S, Scamvia G. Silybin and its bioavailable phospholipid complex (IdB1016) potentiate in-vitro and in-vivo activity of cisplatin. *Life Sci* 2002; 70:1447-1459. [https://doi.org/10.1016/S0024-3205\(01\)01511-9](https://doi.org/10.1016/S0024-3205(01)01511-9)
- Javed S, Kohli K, Ali M. Reassessing bioavailability of silymarin. *Altern Med Rev* 2011; 16:239-249.
- Ahmad U, Faiyazuddin M, Hussain MT, Ahmad S, Alshammari TM, Shakeel F. Silymarin: an insight to its formulation and analytical prospects. *Acta Physiol Plant* 2015; 37:253:1-7. <https://doi.org/10.1007/s11738-015-2008-3>
- Schwendener RA, Schott H Lipophilic 1-beta-D-arabinofuranosyl cytosine derivatives in liposomal formulations for oral and parenteral antileukemic therapy in the murine L1210 leukemia model. *J Cancer Res Clin Oncol* 1996; 122:723-726. <https://doi.org/10.1007/BF01209119>
- Chen W, Xia H, Wu W. Optimized preparation of silymarin dripping pills by a central composite design-response surface method. *Chin Trad Herb Drug* 2005; 36:679-683.
- He J, Hou S, Lu W, Zhu L, Feng J. Preparation, pharmacokinetics and body distribution of silymarin-loaded solid lipid nanoparticles after oral administration. *J Biomed Nanotech.* 2007; 3:195-202. <https://doi.org/10.1166/jbn.2007.024>
- Jadhav KR, Shaikh IM, Ambade KW, Kadam VJ. Applications of microemulsion based drug delivery system. *Curr Drug Deliv* 2006; 3:267-273. <https://doi.org/10.2174/15672010677731118>
- Akhtar J, Siddiqui HH, Fareed S, Badruddin, Khalid M, Aqil M. Nanoemulsion: for improved oral delivery of repaglinide. *Drug Deliv* 2016; 23:2026-2034. <https://doi.org/10.3109/10717544.2015.1077290>
- Woo JS, Kim TS, Park JH, Chi SC Formulation and biopharmaceutical evaluation of silymarin using SMEDDS. *Arch Pharm Res* 2007; 30:82-89. <https://doi.org/10.1007/BF02977782>
- Mehnert W, Mader K. Solid lipid nanoparticles: production, characterization and applications. *Adv Drug Deliv Rev* 2001; 47:165-196. [https://doi.org/10.1016/S0169-409X\(01\)00105-3](https://doi.org/10.1016/S0169-409X(01)00105-3)
- Abbaspour M, Makhmalzadeh BS. Development and evaluation of a solid self-nanoemulsifying drug delivery system for loratadin by extrusion-spheronization. *Adv Pharm Bull* 2014; 4(2):113-119.
- Rashid R, Kim DW, Yousaf AM, Mustapha O, Fakhar-ud-Din, Park JH, Yong CS, Oh Y, Youn YS, KJ Oh, Choi Comparative study on solid self-nanoemulsifying drug delivery and solid dispersion system for enhanced solubility and bioavailability of ezetimibe. *Int J Nanomedicine* 2015; 10:6147-6159. <https://doi.org/10.2147/IJN.S91216>
- Wei Wu, Wang Y, Li Qu. European journal of pharmaceutics Enhanced bioavailability of silymarin by self-micro emulsifying drug delivery system. 2006; 63(3):288-94. <https://doi.org/10.1016/j.ejpb.2005.12.005>
- Parveen R, Baboota S, Ali J, Ahuja A, Vasudev SS, Ahmad S. Oil-based nanocarrier for improved oral delivery of silymarin: In-vitro and in-vivo studies. *Int J Pharm* 2011; 413:245-53. <https://doi.org/10.1016/j.ijpharm.2011.04.041>
- Shafiq S, Shakeel F, Talegaonkar S, Ali J, Baboota S, Ahuja A, Khar RK, Ali M Formulation development and optimization using nanoemulsion technique: a technical note. *AAPS Pharm Sci Tech* 2007b; 8(2):E1-E6. <https://doi.org/10.1208/pt0802028>
- Rahman MA, Iqbal Z, Hussain A. Formulation optimization and in vitro characterization of sertraline loaded self-nanoemulsifying drug delivery system (SNEDDS) for oral administration. *J Pharm Investig* 2012; 42:191-202. <https://doi.org/10.1007/s40005-012-0029-0>
- Rahman MA, Mujahid M. Development of self-nanoemulsifying tablet (SNET) for bioavailability enhancement of sertraline. *Braz J Pharm Sci* 2018; 54(1):e17232. <https://doi.org/10.1590/s2175-97902018000117232>
- Gosh PK, Murthy RSR. Microemulsions: a potential drug delivery system. *Curr Drug Deliv* 2006; 3:167-180. <https://doi.org/10.2174/156720106776359168>
- Christensen KL, Pedersen GP, Kristensen HG. Preparation of redispersible dry emulsions by spray drying. *Int J Pharm* 2001; 212:187-194 [https://doi.org/10.1016/S0378-5173\(00\)00596-2](https://doi.org/10.1016/S0378-5173(00)00596-2)
- Christensen KL, Pedersen GP, Kristensen HG. Physical stability of redispersible dry emulsions containing amorphous sucrose. *Eur J Pharm Biopharm* 2002; 53:147-153 [https://doi.org/10.1016/S0939-6411\(01\)00232-6](https://doi.org/10.1016/S0939-6411(01)00232-6)
- Dollo G, Corre PL, Guerin A, Chevanne F, Burgot JL, Leverage R. Spray dried redispersible oil-in-water emulsion to improve oral bioavailability of poorly soluble drugs. *Eur J Pharm Sci* 2003; 19:273-280. [https://doi.org/10.1016/S0928-0987\(03\)00134-9](https://doi.org/10.1016/S0928-0987(03)00134-9)