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Improved Efficacy and Stability of Silymarin Loaded Nanocochleates Over Liposomes for the Treatment of Skin Diseases

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Authors' contributions

This work was carried out in collaboration among all authors. Author NM contributed to the study conception and design. Material preparation, data collection and analysis were performed by all the authors. Author AW performed thorough literature review. The first draft of the manuscript was written by authors NM and RR. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Silymarin, a complex polyphenolic component mixture with anti-oxidant, anti-inflammatory, and membrane-stabilizing property is being investigated in several dermatological conditions. Present research aims to evaluate potential of silymarin loaded nanocochleates and liposomal topical application for treating chronic skin diseases.
Study Design: Silymarin loaded liposomes and nanocochleates were formulated and optimized using Design Expert software. Different invitro and exvivo tests were performed to compare their performance.
Place and Duration of Study: The study was conducted in Smt. Kashibai Navale College of Pharmacy, Pune, India, between January 2019 till February 2020.
Methodology: Liposomes were prepared using ethanol injection method and further treated with

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calcium chloride to form nanocochleates by trapping method. Design of experiments (3^2 Factorial Design) was used for optimization of nanocochleates. Cell line studies (HaCaT cell lines) and short term stability studies were performed to compare the efficacy and stability respectively.
Results: Particle size, entrapment efficiency and drug deposition in Wistar Rat Skin was found to be statistically significant for nanocochleates over liposomes proving superiority of cochleates. Both the carriers sustained release of silymarin for 24h. Antimicrobial efficacy of nanocochleates against *E.coli* and *S.aureus* was significant. Inhibition of hyper proliferation of HaCaT cell lines (key mechanism by which most of the antipsoriatic drugs act) demonstrated the superiority of nanocochleates over liposomes.The nanocochleates also displayed better stability compared to liposomes due to decreased entrapment efficacy and leakage of drug.
Conclusion: Silymarin loaded Nanocochleates could prove as a promising topical drug delivery system for the treatment of chronic skin diseases like psoriasis.

Keywords: Liposomes; Nanocochleates; Silymarin; Design of Experiments; HaCaT cell lines.

1. INTRODUCTION

The topical route for drug administration has advantages over other pathways as it avoids hepatic first pass effect, provides continuous drug delivery, has fewer side effects and improves patient compliance [1]. especially while treating immune mediated skin disorders like dermatitis, urticaria, angioedema, psoriasis, etc that are chronic, inflammatory and proliferative in nature [2,3].

The stratum corneum (SC) presents main barrier against drug transport and SC intercellular lipids help to regulate penetration. This lipid matrix, composed of ceramides, free fatty acids, cholesterol (CHOL), and minor lipids, plays a major role to overcome the barrier function [4]. Despite the advantages of topical drug delivery, low SC permeability may limit its usefulness. To lower SC barrier properties and increase permeability, chemical and physical approaches have been examined like lipid based vesicular systems such as liposomes niosomes, ethosomes and have proven advantageous [5,6].

For aiming drug delivery via topical administration it is necessary that sufficient quantity of drug penetrates the Stratum corneum and reaches the target layers of the skin to impart desired therapeutic effects. Skin infections cause clinical manifestations such as scaling, fissures, skin maceration, hyperkeratosis and vesiculation. A typical change occurs in the skin thickness, further increasing the barrier effect of the skin and challenging the penetration of drugs. In such circumstances lipid based systems like liposomes would be advantageous [6].

Liposomes at physiological temperature are comprised of fluid bilayer membrane with aqueous space contained within compartments bounded by the lipid bilayers. The liposomal bilayer is susceptible to harsh environmental conditions like extremes of pH or enzymes that digest lipid. Also they tend to aggregate or become leaky upon prolong storage [7,8]. To overcome these limitations, modification of liposomes to form nanocochleates was carried out in the present investigation. Since cochleates are liposome based structures, it can be hypothesized that they too might have prospective for topical delivery of drugs in more efficient manner.

Nanocochleates are spiral, elongated, tubular and multi lamellar stable structures devoid of aqueous space. The lipid bilayers are associated with each other via positively charged calcium which interacts with negative head groups on the opposing lipid bilayers. The interactions further stabilize the structure and remove the water of hydration. Hence these carriers can also be useful for delivery of drugs that undergo oxidation or hydrolytic degradation [6].

The intrinsic properties of cochleates have led to advantages in the important areas of safety, stability, efficacy, immune response targeting, combining vaccines to multiple infectious agents. They can also be useful for intradermal drug delivery for various skin conditions [7].

Nanocochleate delivery of Amphotericin B, adopted and patented by BioDelivery Sciences Inc. was a huge success [9]. They find application in topical delivery of important drugs like triamcinolone acetonide, acyclovir, lidocaine, econazole etc [10-12]. Acidic phospholipids that may be useful in preparing cochleates are phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, and, phosphatidylserine [7]. It

has been reported that Ca++ forms a more tightly packed, highly ordered and less hydrated structure than does Mg++ with phospholipids. It is well documented that Ca++ plays a vital role in natural membrane fusion phenomena and is most compatible with the body. Thus calcium is the most suitable divalent cation reported for preparing cochleates and so, used in the present work.

At present, silymarin is being used for various skin disorders such as melasma, anti-aging, sunscreen, acne, rosacea, psoriasis, skin cancer, photo protection, cosmeceuticals [13]. It strongly prevents photo carcinogenesis, and significantly prevent melanin production [14].

There are many studies that claim the photo protection ability of silymarin in literature. In a study on epidermal cells by Katiyar and co-workers, it has been shown that the apoptosis and DNA damage caused by UV radiation was markedly decreased by silymarin via the nucleotide excision repair mechanism which makes silymarin an excellent option for prophylaxis of skin cancers [15].

In another study, Vaid M et al. showed silymarin to be effective in preventing not only skin cancers but also melanoma cell migration via the B-catenin signaling pathway [16]. According to Choo et al. Silymarin inhibited L-dihydroxyphenyla- lanine (L-DOPA) oxidation activity of tyrosinase, the rate-limiting melanogenic enzyme, in cell based-systems [17]. Further, western blot analysis done by Altaei T, indicated that silymarin decreased the expression of tyrosinase protein, thus explaining its effect in melasma [18]. Eszter Firdrus and co-workers found that silymarin pre-treatment reduced Reactive oxygen species production of skin keratinocytes after high-dose (20 J/cm^2) of UVA irradiation in a dose-dependent manner and predicted it to be due to a strong antioxidant potential of Silymarin [19].

Thus present research aims at comparative study of silymarin loaded liposomes and nanocochleates as an effective carrier for topical drug delivery.

2. MATERIALS AND METHODS

Dimyristoylphosphatidylglycerol (sodium salt, molecular weight 688.85 g/mol) [DMPG] was a generous gift from Lipoid (Germany). Silymarin was purchased from Yucca Enterprises, Mumbai (India). Cholesterol was purchased from Research-Lab Fine Chem Industries, Mumbai (India). Chloroform, Sodium acetate, Disodium EDTA, Sodium hydroxide and other reagents and solvents were of analytical grade. Double distilled water was used wherever required.

2.1 Preparation of Liposomes

Silymarin loaded liposomes were formulated using ethanol injection metho [20-22]. After preliminary trials, specific amount of DMPG (60mg), cholesterol (10mg) and drug (10mg) were dissolved in 2 mL ethanol. This solution was heated up to phase transition temperature (30°C) of DMPG for 5 minutes and was rapidly injected into 10 mL of the aqueous phase (distilled water) under constant stirring at 500 rpm using magnetic stirrer. Spontaneous formation of vesicles was observed as soon as ethanolic solution was dropped into aqueous phase. It was further stirred magnetically at 500 rpm at room temperature for 1 h for complete removal of ethanol. Water was added to adjust the volume of final lipid vesicles suspension to 10 mL. Finally, the vesicles were purified by passing through a 0.45 µm membrane filter to obtain purified, small liposomes.

2.2 Preparation of Nanocochleates

Nanocochleates were prepared by trapping method [22,23]. Calcium chloride (50 micro litre solution of concentration 0.1 M) was added drop-wise into the prepared silymarin-loaded liposomes under vortex. The vesicle phase immediately turned turbid because of nanocochleate formation. Precipitated nanocochleates were refrigerated at 2-8°C. These nanocochleates were optimized using 3^2 factorial design using Design Expert Software (Version 11).

2.3 Optimization of Silymarin loaded Nanocochleates by using 3^2 Factorial design

Silymarin loaded nanocochleates (SN) were optimized to study the effect of independent variables like concentration of DMPG and drug on dependent variables viz. particle size, entrapment efficiency and drug deposition in the rat skin. Experimental trials were performed at 3 possible combinations higher, lower and middle. The resulting data were fitted into Design Expert 11 software and analysed statistically using

analysis of variance (ANOVA).The data were also subjected to 3-D response surface methodology to determine the influence of DMPG and silymarin on dependent variables. The probable formulations (coded and actual levels) using 3^2 factorial design and their responses are shown in Table 1.

2.4 Evaluation of Silymarin Loaded Liposomes (SL) and Nanocochleates (SN)

2.4.1 Particle size/ Vesicle size determination

The mean particle size and particle size distribution of SL and SN were studied using optical microscope (Motic) and same for the optimized batch (SN5) was obtained by particle size analyzer (Sympatec- Nanophox NX0088). The instrument measures the particle size and particle size distribution based on dynamic light scattering theory. The apparatus consists of a He-Ne laser beam of 632.8 nm focused with a minimum power of 10 mw using scattering angle 90°C to the sample cell of 10×10 mm and calculate particle size by 3D cross correlation technique [22].

2.4.2 Determination of Entrapment Efficiency (EE) of Liposomes and Nanocochleates

EE of liposomes loaded with silymarin (SL1-SL5) was determined by separating unencapsulated silymarin by centrifugation of liposomal dispersion at 12,000 rpm for 2 h at 4°C. The sedimented pellet was disrupted with absolute ethanol to release the entrapped drug. This dispersion was filtered through Whatmann filter paper (45μ) and then was suitably diluted with ethanol and the absorbance measured at 287 nm using UV Spectrophotometer. The percent EE was calculated using Equatio [20,22].

EE (%) = Amount of drug entrapped in the vesicle / Total amount of drug present × 100 [1]

One hundred micro litres of nanocochleates (SN1-SN9) was added into centrifugation tubes. The tube was centrifuged at 6000 rpm for 20 min at 4°C and supernatant and pellets were separated. To each tube 60 μL pH 9.5 EDTA and 1mL of ethanol were added while vortexing. The resulting solution is clear and colourless. The samples were suitably diluted and absorbance determined at 287 nm to calculate entrapment efficiency as per equation.

EE(%) = Amount of drug entrapped in the cochleates / Total amount of drug present × 100 [2]

2.4.3 Differential Scanning Calorimetry

The thermograms of drug, DMPG, cholesterol, physical mixture of drug and excipients and freeze dried nanocochleates were obtained using DSC – Differential Scanning Calorimeter with thermal analyzer (Perkin Elmer 4000). Accurately weighed samples (about 1 mg) were placed in sealed aluminium pans, under nitrogen flow (20 mL/min) at the scanning rate of 10°C per min from 30 to 300°C using PYRIS Manager Software.

2.4.4 Zeta Potential Measurements

Charge on the surface of drug loaded liposomes and nanocochleates was determined by Beckman Coulter Delsa Nano. Analysis time was kept for 1 min at pH 6.5 and average zeta potential and charge on the liposomes and nanocochleates in distilled water was determined. Temperature was kept 25°C at and 3 runs were carried out.

2.4.5 In Vitro Release of Silymarin from Liposomes and Nanocochleates

The in vitro release of silymarin from liposomes and nanocochleates was carried out in phosphate buffer saline (PBS, pH 7.4) using dialysis bag diffusion technique. Formulations(SL and SN equivalent to 1 mg of silymarin) and 1 mg silymarin solution (1 mg/mL in 30% w/w mixture of polyethylene glycol (PEG) 400 and water) as control was introduced into a dialysis bag (cellulose membrane, molecular weight cut off 12,0000 Da), hermetically sealed and immersed into 100 mL of release medium. The entire system was kept at 37 ± 0.5°C with continuous magnetic stirring at 100 rpm/min. At selected time intervals of 0, 1, 2, 5, 7, 9, 12 and 24 h sample (5mL) was removed and replaced with equal volume of fresh medium in order to maintain sink conditions.The absorbance of silymarin in the solution was determined using the double beam UV-Vis Spectrophotometer.

2.4.6 In Vitro Drug deposition and Permeation Studies through Skin Barrier

In vitro permeation of silymarin from liposomes and nanocochleates was evaluated by using full thickness abdominal skin, which was excised

from adult Wistar rats weighing 160–200 g. Visceral side of the freshly excised skin was cleaned by removing adhering subcutaneous tissue. Epidermal hair was removed and skin was hydrated for 24 h in phosphate buffer solution (PBS) of pH 7.4. The skin samples were mounted on Franz diffusion cell with receptor volume of 14.5 mL. The surface area of skin mounted between donar and receptor compartment was 3.14 cm^2. The receptor compartment contained phosphate buffer (pH 7.4), and its temperature maintained at 37 ± 0.5°C and stirred continuously using a magnetic stirrer at 100rpm. On the epidermal side of skin (donar compartment), 1mL of the nanocochleates were spread evenly. At set intervals of 0, 1, 2, 5, 7, 9, 12 and 24 h, respectively, 5.0 mL of the receptor phase was removed and immediately replaced by an equal volume of PBS (pH 7.4) solution. Samples were analysed spectrophotometrically at 287 nm, in triplicate, for determination of the content of silymarin. At the end of 24 h, excised rat abdominal skin was minced and the drug deposited in the skin (% drug deposition) was analyzed spectrophotometrically.

2.4.7 Antibacterial activity

The antibacterial activity of silymarin solution in ethanol (1mg/mL), silymarin loaded liposomes and nanocochleates were examined against bacterial strains *S.aureus* (ATCC 29213) *and E.coli* (ATCC 35218) cultures maintained on sterile nutrient agar. The antimicrobial activity was evaluated using the cup plate technique using an official method in IP and antimicrobial activity was determined by using zone of inhibition (mm) [24]. The overnight grown subcultures of organisms were inoculated on the surface of the sterile nutrient agar solidified in sterile glass petriplates, which were incubated at 37°C in an incubator for 24h to form lawn culture. The sterile borer was used to prepare cups of 8 mm diameter, in the medium of each petri-plate. Solutions (formulations) were placed in different cavities. All the plates were kept at room temperature for effective diffusion of the drug from solutions/formulations. The plates were incubated at 37º C over a period of 24 h. Zone of inhibition (mm) of silymarin loaded liposomes and nanocochleates were measured after 24 h.

2.4.8 Anti-proliferative activity on HaCat Cell Lines using MTT assay

Anti proliferative activity (cell inhibition%) on HaCaT cell lines which is proven model for invitro antipsoriatic activity was evaluated for SL and SN using MTT Assay [25]. The monolayer cell culture was trypsinized and the cell count was adjusted to 5.0 x 10^5 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 24h in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

2.4.9 Stability studies

Short term stability studies of freshly prepared silymarin loaded liposomal and nanocochleates suspension was studied at 5°C ± 3°C up to three months and the effect on various parameters was studied. Also, lyophilized formulations of liposome 0.2 mg were filled into amber colour glass vials. Sealed and stored at the above mentioned condition. Storage conditions for liposomes and nanocochleates or any lipid based drug delivery is refrigeration [26]. Hence in the present investigation this temperature condition was selected.

3. RESULTS AND DISCUSSION

The present investigation was focused on comparative study of silymarin loaded liposomes and nanocochleates as carrier on topical application of silymarin. Nanocochleates were prepared from preformed liposomes (SL), which showed entrapment efficacy of 60.98%.This formulation was used for comparison with prepared nanocochleates. These liposomes were treated with calcium chloride and by virtue of folding action of Ca^{++} they were converted to cylinder shaped cochleates. In the preliminary

study, 1M concentration of calcium chloride was found optimum to convert liposomes into cochleates and was kept constant throughout the study. These nanocochleates were optimized to study the effect of concentration of lipids and drug on particle size, entrapment efficiency and drug deposition in skin using Design Expert Software Version 11. Results are mentioned in Table 1.

3.1 Optimization of Silymarin loaded Nanocochleates

The implementation of a 3^2 factorial design aided the understanding of the roles and extent to which the negatively charged phospholipid and the drug affected the properties and performance of nanocochleates.

3.1.1 Evaluation of particle size/Vesicle size

Particle size of nanocochleates is greatly influenced by the concentration of lipids as they are the main constituents of membrane of nanocochleates. Amount of drug also has effect on particle size. Particle size determination is an important parameter in case of nanocochleates as it affects the drug entrapment, release and permeation. In the present study, the observed particle sizes for all batches were in range of 417 to 937 nm.

- **Final Equation in Terms of Coded Factors**

Particle size= +555.95 + 145.00A+ 94.06 + 97.93AB [3]

- **Final Equation in Terms of Actual Factors**

Particle size = +554.95333 + 144.99833DMPG+ 97.93500DMPG*Silymarin [4]

As seen from equation 3 and 4, a positive correlation is seen for the effect of independent variables i.e. concentration of lipid and drug on particle size. As the concentration of lipid is increased, thickness of the lipid surface also increases resulting in increase in particle size [21]. Silymarin being lipophilic in nature will reside in the lipidic layers, thereby augmenting the thickness of the phospholipid bilayer and in turn increasing the particle size [27].

Very small particle size results in decreased entrapment efficacy of drug and also nanocochleates may cross the skin layer and cause transdermal delivery of silymarin resulting in its systemic absorption which is not desirable in the present investigation. Present research work aims at maximizing the deposition of drug in the skin layer for local action and not systemic action. Hence, Batch SN5 with particle size of 536.01±2 was considered as optimized batch as, further increase in the size may result in decreased drug deposition in the skin layers.

Data was analyzed statistically by one-way analysis of variance (ANOVA) as seen in Table 2. Sum of squares is Type III – Partial. The Model F-value of 13.54 implies the model is significant. There is only a 0.78% chance that an F-value this large could occur due to noise. *P*-values less than 0.05 indicate model terms are significant. In this case A, B, AB are significant model terms.

3.1.2 Evaluation of entrapment efficiency (E.E)

Entrapment efficiency (EE) the nanocochleates was influenced by concentration of lipid and amount of drug. EE of the prepared nanocochleates was found to be in the range of 56.36**±** 0.23to 88.34± 3.21%

- **Final Equation in Terms of Coded Factors**

Entrapment efficiency= +83.33+7.46A- 1.98B - 0.8525AB -1.80A²- 18.49 B² [5]

- **Final Equation in Terms of Actual Factors**

Entrapment efficiency= +83.32889 + 7.45667 DMPG - 1.98500 silymarin - 0.852500 DMPG * silymarin - 1.80333 DMPG²-18.48833 silymarin² [6]

As seen from the equations 5 and 6, as the concentration of lipid increases, entrapment efficiency goes on increasing due to more lipid domains are available for binding the drug. Silymarin is a hydrophobic drug, so it has the tendency to get incorporated in the lipid bilayer which already has cholesterol embedded in it for improved stability of nanocochleates. Hence, upon increasing the amount of drug beyond particular limit, due to presence of cholesterol in the lipid bilayer, more drug cannot be accommodated. Batch SN5 was selected as optimized batch though Batch SN6 showed more E.E (difference is not very significant), because it

had more particle size, which may lead to decreased skin deposition of drug.

Data were analyzed statistically by one-way analysis of variance (ANOVA) as shown in Table 3. Sum of squares is Type III – Partial. The Model F-value of 14.39 implies the model is significant. There is only a 2.62% chance that an F-value this large could occur due to noise. *P*-values less than 0.05 indicate model terms are significant.

3.1.3 Drug Deposition Study

The formulations in the present study were prepared with the objective of penetrating the drug in the skin through keratinized layer, but at the same time to retain the drug in the skin without transferring it into systemic circulation. This study was performed using skin of Wistar Rat mounted on Franz diffusion cells. It was observed that the drug was not detected in the receiver compartment of Franz diffusion cell (which simulates systemic circulation) form liposomes as well as nanocochleates even after 24 h of study. After 24 h the tissue was minced and the drug content was analysed which is said to be drug deposited in the skin. The in-vitro drug deposition from cochleates varied from 49.17 to 75.36 %

- **Final Equation in Terms of Coded Factors**

Drug deposition= +76.38+ 8.21A+2.65B-3.05AB- 4.03 A^2- 12.83 B^2 [7]

- **Final Equation in Terms of Actual Factors**

Drug deposition= +76.37556 +8.21000 DMPG+ 2.65000 silymarin-3.04500 DMPG* Silymarin-4.03333 $DMPG^2$-12.83333 $silymarin^2$ [8]

As seen from the above equations 7 and 8, the concentration of lipid and drug had significant effect on drug deposition. This probably could be correlated to the size of cochleates. Overall, it could be said that higher concentration of drug lead to cochleates of bigger size which simply formed a film over the stratum corneum and did not penetrate to cause deposition [26]. As compared to liposomes (64.98%), higher drug deposition was demonstrated by cochleate formulation. This may be attributed to the shape of nanocochleates which is cylindrical or rod shaped, so it can easily pass through pores on the skin vertically, also due to presence of calcium as one of the components of nanocochleates, which might have caused perturbations of the cells and eventually, more increased uptake and deposition in the skin. Data were analyzed statistically by one-way analysis of variance (ANOVA) using software and the results are as seen in Table 4. Sum of squares is Type III – Partial. The Model F-value of 4.07 implies the model is significant. There is only a 0.0.138% chance that an F-value this large could occur due to noise. *P*-values less than 0.05 indicate model terms are significant. In this case A, B, AB , A^2B^2 are significant model terms.

The response surface graphs for optimization of silymarin loaded nanocochleates for Particle size, Entrapment Efficiency and Drug deposition are shown in the Fig. 1.

3.1.4 Desirability for feasibility of model for silymarin nanocochleates

Table 5 shows model fit summary for silymarin nanocochleates. It can be seen that Predicted R^2 for Particle size, Entrapment Efficiency and Drug deposition of optimized batch is close to the Adjusted R^2. The difference is not more than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable which indicates an adequate signal. Hence, this model can be used to navigate the design space.

Observed responses were fitted to Design Expert software version 11, the dependant variables demonstrated that the model was significant for all the response variables (EE, PS and DD). Comparison between the experimental and predicted values for the most probable optimal formulation SN5 showed the desirability of 0.975. This demonstrates the feasibility of the model in the development of Silymarin loaded nanocochleates formulation.

3.2 Evaluation of Liposomes and Nanocochleates

3.2.1 Particle size analysis and surface morphology

As seen in Fig. 2, liposomes were smaller in size than nanocochleates as they are rod shaped. Large size of nanocochleates can incorporate more amount of drug as seen from increased entrapment efficiency of SN as compared to SL. Cylindrical shape of nanocochleates (batch SN3) and spherical shape of liposomes (batch SL3) was also confirmed using SEM studies.

Table 1. Formulation and Evaluation of Silymarin loaded Nanocochletes using 3^2 factorial design

Batch*	Coded Level**		Response 1 ***PS (nm)	Response 2 ***EE (%)	Response 3 ***DD (%)
	X_1	X_2			
SN1	-1	-1	417.84 ± 3	58.24 ± 1.23	57.29
SN2	0	-1	450.32 ± 2	65.17 ± 2.11	62.89
SN3	1	-1	493.3 ± 5	73.46 ± 1.25	67.43
SN4	-1	0	421.99 ± 4	70.63 ± 5.01	65.27
SN5	0	0	536.01 ± 2	87.41 ± 2.14	75.36
SN6	1	0	749.32 ± 6	88.34 ± 3.21	70.43
SN7	-1	1	470.48 ± 3	56.36 ± 0.23	49.17
SN8	0	1	517.64 ± 4	60.43 ± 4.32	54.21
SN9	1	1	937.68 ± 2	68.17 ± 1.25	67.13

**Each formulation batch (SN1-SN9) contains 10mg cholesterol*
***Actual values [X1(DMPG): -1=60mg, 0=70mg , 1=80mg; X2 (Silymarin): -1=5mg, 0=10mg,1=15mg)]*
**** PS=Particle Size; EE=Entrapment Efficiency ; DD=Drug Deposition in Rat skin*

Table 2. Statistical analysis by one-way analysis of variance (ANOVA) for Particle size of Silymarin Nanocochleates

Source	Sum of Squares	Df	Mean Square	F-value	*P* value	
Model	2.176E+05	3	72530.70	13.54	0.007	Significant
A-DMPG	1.261E+05	1	1.261E+05	23.55	0.004	
B-silymarin	53079.94	1	53079.94	9.91	0.02	
AB	38365.06	1	38365.06	7.16	0.04	
Residual	26781.47	5	5356.29			
Cor Total	2.444E+05	8				

Table 3. Statistical analysis by one-way analysis of variance (ANOVA) for Entrapment Efficiency of Silymarin Nanocochleates

Source	Sum of Squares	Df	Mean Square	F-value	*P-value*	
Model	1050.30	5	210.06	14.39	0.02	Significant
A-DMPG	333.61	1	333.61	22.86	0.01	
B-silymarin	23.64	1	23.64	1.62	0.02	
AB	2.91	1	2.91	0.1992	0.06	
A^2	6.50	1	6.50	0.4456	0.05	
B^2	683.64	1	683.64	46.84	0.006	
Residual	43.79	3	14.60			
Cor Total	1094.09	8				

Table 4. Statistical analysis by one-way analysis of variance (ANOVA) for Drug Deposition(%) of Silymarin Nanocochleates

Source	Sum of Squares	Df	Mean Square	F-value	*P*-value	
Model	479.69	5	95.94	4.07	0.01	significant
A-DMPG	184.37	1	184.37	7.82	0.04	
B-silymarin	48.73	1	48.73	2.07	0.02	
AB	15.29	1	15.29	0.6483	0.04	
A^2	3.74	1	3.74	0.1584	0.04	
B^2	227.56	1	227.56	9.65	0.05	
Residual	70.74	3	23.58			
Cor Total	550.43	8				

Table 5. Model Fit Summary for Silymarin loaded Nanocochleates

	PS	EE	DD		PS	EE	DD
Std. Dev.	73.19	3.82	3.54	**R^2**	0.8904	0.9600	0.9574
Mean	554.95	69.80	65.13	**Adjusted R^2**	0.8247	0.8933	0.8864
C.V. %	13.19	5.47	5.44	**Predicted R^2**	0.7580	0.7749	0.7887
				Adequate Precision	9.9581	11.0711	12.0885

PS=Particle Size; EE=Entrapment Efficiency; DD=Drug Deposition in Rat skin

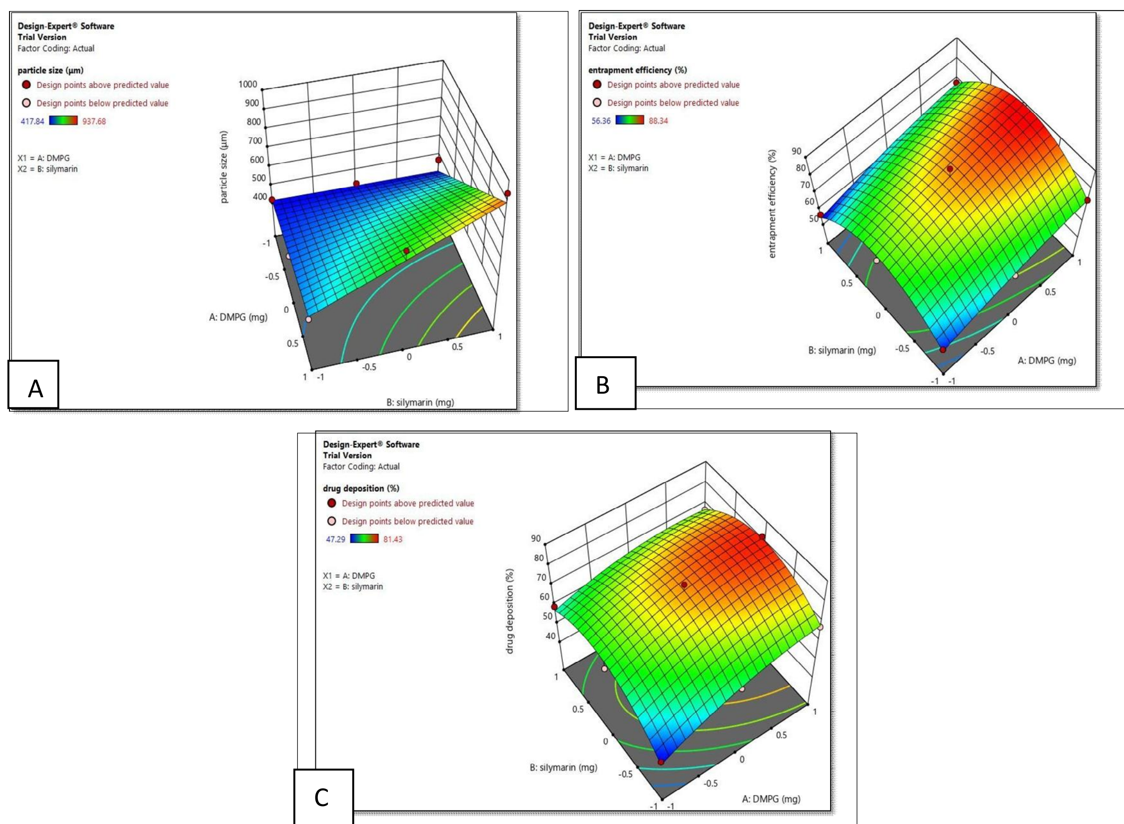

Fig. 1. Response surface graphs for optimization of silymarin loaded nanocochleates for Particle size (A), Entrapment Efficiency (B) and Drug deposition (C)

3.2.2 Zeta Potential Measurements

Zeta potential measurement can be used to determine the stability of a colloidal system. The measurements indicate the overall surface charge of a particle and therefore information as to whether the system may remain stable or consequently undergo aggregation or flocculation. The values of Zeta Potential of optimized batch of Liposomes batch SL3 was found to be -45.3 mV while that of nanocochleates batch SN3 was found to be -22.8 mV as shown in the Fig. 3.

It indicates prepared liposomes and nanocochleates have sufficient charge to avoid aggregation of vesicle. The negative zeta potential is probable due to the anionic nature of the employed lipid (DMPG). The addition of calcium ions to nanoliposome induces fusion of lipid membrane and the formulation of planer sheets, which eventually coil around and initial point of folding to form rods shape cochleates. Zeta potential of nanocochleates have greater charge due to addition of calcium chloride therefore concluded nanocochleates are more stable than the liposomes.

3.2.3 Differential scanning calorimetry analysis

As seen in Fig. 4(A), DSC thermogram of silymarin was seen at 148°C the temperature corresponding to the melting point of silymarin

which was found to be 146-150°C. Also, it confirmed physical compatibility of excipients and drug, as all the peaks corresponding to silymarin and excipients were observed. Whereas, DSC thermogram of final formulation SN5 revealed that silymarin was entrapped in the final formulation, as DSC thermogram of silymarin was not observed as seen in Fig. 4(B).

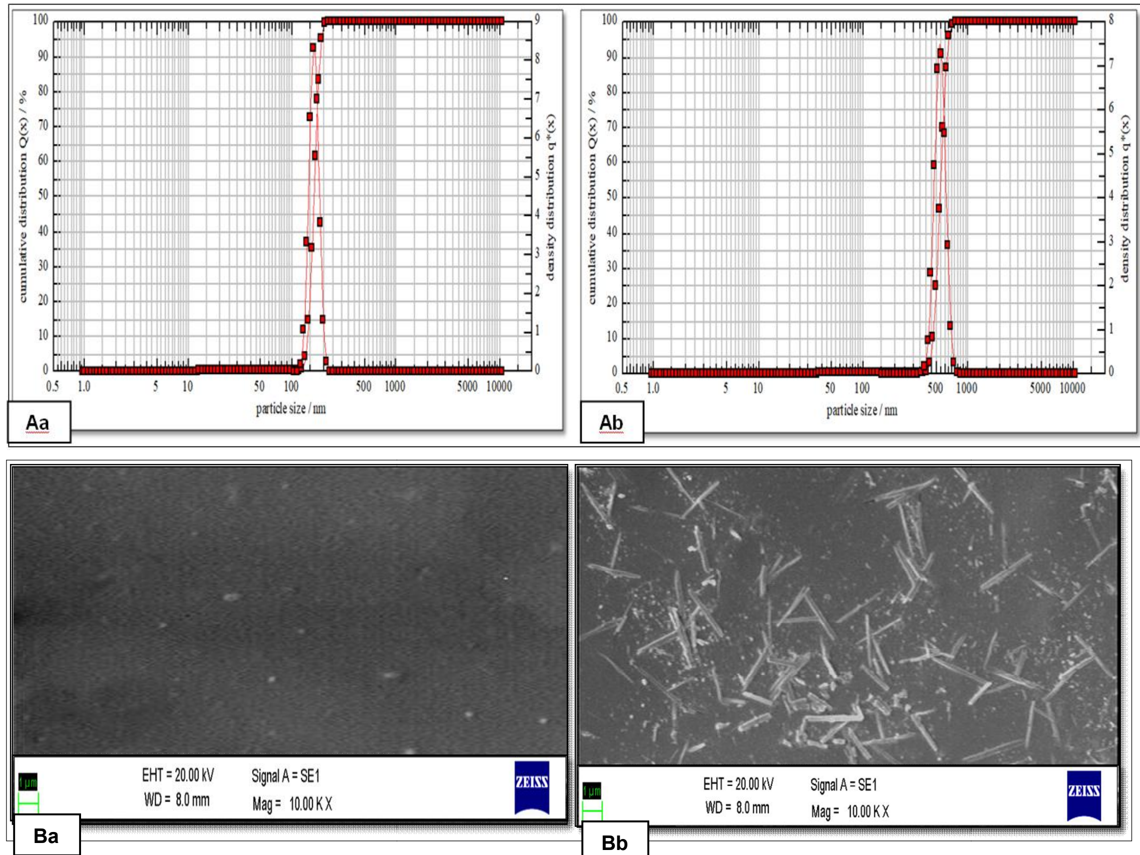

Fig. 2. Particle Size Analysis (PS) and Surface Morphology by SEM studies of Liposomes and Nanocochleates; (Aa) and (Ba) are PS and SEM images of Liposomes and (Ab) and (Bb) are PS and SEM images of Nanocochleates respectively

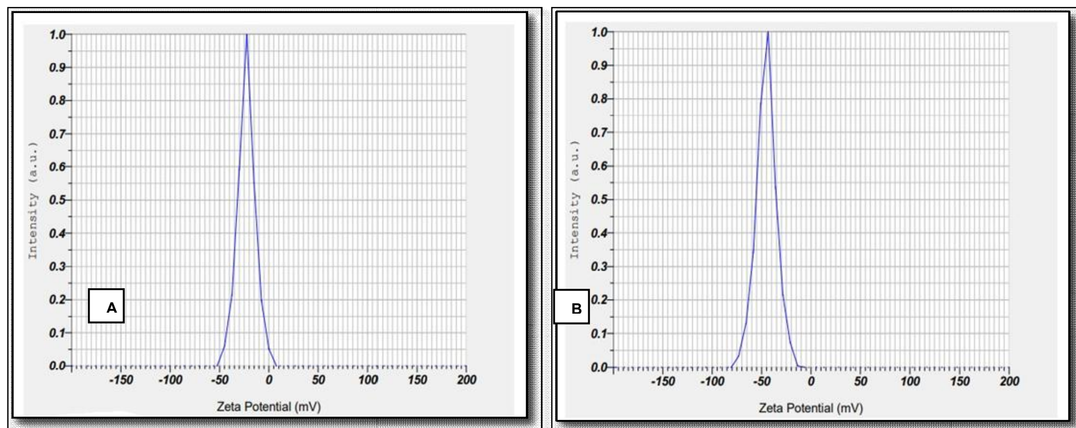

Fig. 3. Zeta potential measurements of Liposomes (A) and Nanocochleates (B)

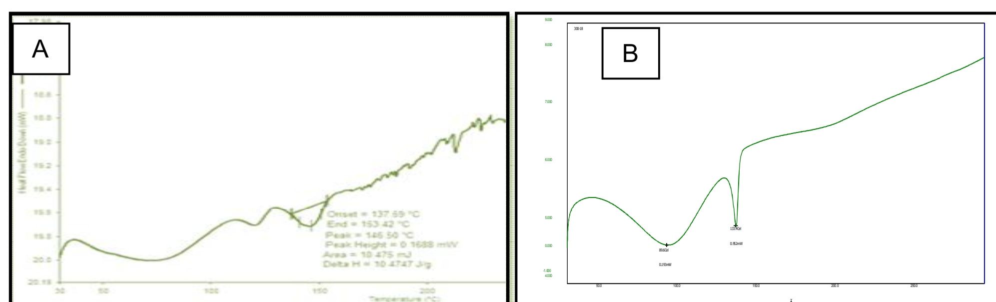

Fig. 4. DSC thermograms of Plain Silymarin (A) showing endothermic peak at 148°C and Nanocochleates (B) showing entrapment of silymarin in cochleates

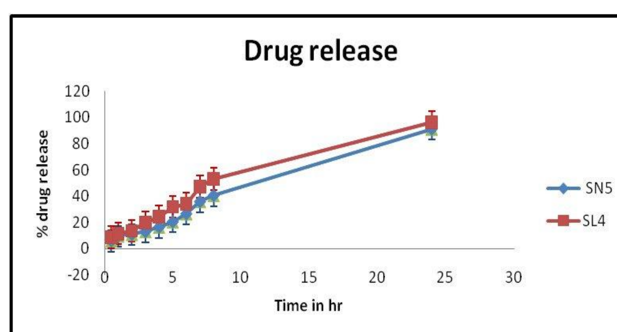

Fig. 5. Invitro drug release studies of Liposomes (SL4) and Nanocochleates (SN5)

3.2.4 In Vitro drug release from SL and SN

As seen in the Fig. 5, initially SL and SN showed burst release of silymarin within first hour which may be attributed to the enriched surface of SL and SN by the drug. Later, both the formulations showed controlled release of drug upto 24h as the drug had to diffuse out of the carriers. As compared to SL, the release of drug was more sustained from SN due to its coiled and compact structure.

3.2.5 Antimicrobial activity

Mechanism of killing microbes by silymarin is done by damaging the cell wall and causing leakage of cytoplasmic contents. Antimicrobial activity against the microbial strains, which are more prevalent in infectious lesions i.e *S.aureus and E.coli* was determined as if these remain untreated, may lead to delayed healing. The results for antimicrobial activity is shown in Table 6 As the drug was free in ethanol solution, it directly came in contact with microbial cell wall and showed activity whereas in other cases, the drug had to diffuse out of the liposomes or silymarin loaded nanocochleates (SN5). Silymarin loaded nanocochleates showed better activity as compared to SL4. This can be said due to presence of calcium ions in nanocochleates which help to have intimate contact with bacteria which are negatively charged.

3.3.6 Anti-proliferative activity on HaCaT cell lines using MTT assay

Present study deals with effect of silymarin loaded carriers for treatment of topical conditions. Silymarin, by virtue of its anti-inflammatory, antioxidant property can be utilized for the treatment of skin conditions like psoriasis, dermatitis etc. HaCaT cells are human spontaneous immortal keratinocyte cells and are often used as an effective model for in vivo skin irritation and psoriasis [28,29]. Hence, inhibition of hyperproliferation of these epidermal keratinocytes (which is one of the key mechanisms by which most of the antipsoriatic drugs act) was investigated using silymarin loaded liposomes and nanocochleates by the MTT assay.

Cytotoxicity study on HaCaT cell lines demonstrated the superiority of nanocochleates over liposomes as IC_{50} values for killing HaCaT cells were found to be 200 and 150 µg/mL for SL and SN respectively. Also, SN showed 80.24%

reduction in cell viability as compared to 69.99% showed by SL at the concentration of 250 µg/mL(Figure 6). This may be attributed to the interaction of calcium-rich nanocochleate membrane with cell membrane which might have caused perturbations of the membrane and increased the cytoplasm delivery of entrapped silymarin leading to cell death.

3.3.7 Stability studies

From the Table 7, it can be seen that there is significant decrease in %EE of SL with may be due to leakage of drug from the vesicles. Also, rapid release of silymarin within 12 h from liposomes was observed whereas nanocochleates could sustain the release of drug for 24 h even after 3 months. Increase in the particle size of liposomes after 3 months indicates fusion of vesicles upon storage. Liposomes and nanocochleates were lyophilized and it was seen that liposomal formulation was not stable, it formed agglomerates due to fusion of lipids while lyophilized nanocochleates were stable i.e, in the powder form. Thus it can be concluded that nanocochleates are more stable than liposomes.

Table 6. Antimicrobial Activity using Cup Plate Technique

Sr.No.	Antimicrobial activity	Zone of Inhibition(mm)	
		S.aureus	***E.coli***
1.	Ethanol Solution	06±0.4	05±0.3
2.	Ethanolic Silymarin Solution	11±0.3	08±0.5
3.	Silymarin liposomes (SL4)	18±0.4	13±0.5
4.	Silymarin Nanocochleates(SN5)	23±0.2	19±0.2

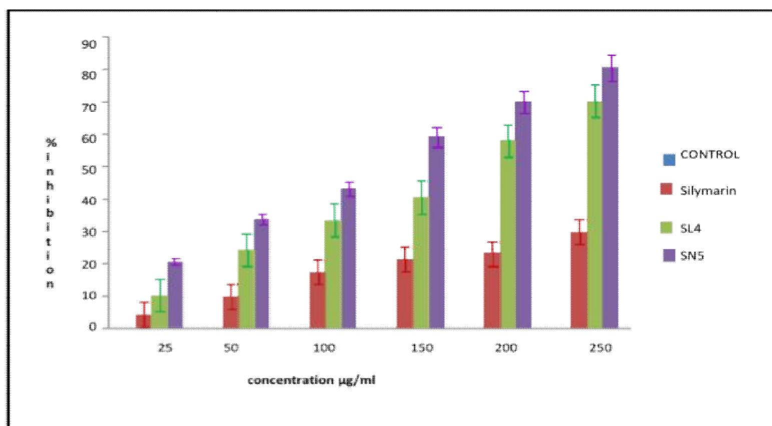

Fig. 6. *Anti-proliferative activity on HaCaT Cell Lines using MTT assay* of Control, Plain silymarin, Silymarin loaded Liposomes (SL4) and Nanocochleates (SN5)

Table 7. Stability Studies of Silymarin loaded Liposomes and Nanocochleates

Formulation	Days	Liposomes	Nanocochleates
EE (%)	**0**	73.30	87.41
	30	69.21	85.24
	60	65.44	82.74
	90	61.98	80.16
PS (nm)	**0**	161.16	536.01
	30	325.21	551.21
	60	459.01	560.84
	90	495.93	570.91
Appearance	**0**	Spherical	Rod shaped
	30	Spherical	Rod shaped
	60	Large aggregates	Rod shaped
	90	Large aggregates	Rod shaped

4. CONCLUSION

In this research work silymarin loaded nanocochleates composed of di myeristoyl phosphoryl glycerol (DMPG), cholesterol and calcium ions were prepared, optimized using factorial design approach and were compared with liposomes. Developed nanocochleates showed marked improvement in the stability and efficacy of delivering silymarin as compared to liposomes. The present study ascertained the role of nanocochleates in topical delivery of silymarin for the treatment of various skin ailments. This research also evokes the possibility of nanocochleates in the treatment of psoriasis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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