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RESEARCH ARTICLE

Implementing Central Composite Design for Developing Transdermal Diacerein-Loaded Niosomes: *Ex vivo* Permeation and *In vivo* Deposition

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Abstract: ***Background*:** Niosomes are surfactant-based vesicular nanosystems that proved their efficiency in transdermal delivery by overcoming skin inherent anatomic barrier; startum corneum. Central composite design is an efficient tool for developing and optimizing niosomal formulations using fewer experiments.

***Objective*:** The objective of this study was to prepare niosomes as a transdermal delivery system of diacerein using film hydration technique, employing central composite design, for avoiding its oral gastrointestinal problems.

***Methods*:** Three-level three-factor central composite design was employed for attaining optimal niosomes formulation with the desired characteristics. Three formulation variables were assessed: amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3). DCN-loaded niosomes were evaluated for entrapment efficiency percent (Y_1), particle size (Y_2), polydispersity index (Y_3) and zeta potential (Y_4). The suggested optimal niosomes were subjected to further characterization and utilized as a nucleus for developing elastic vesicles for comparative *ex vivo* and *in vivo* studies.

***Results*:** The values of the independent variables (X_1, X_2 and X_3) in the optimal niosomes formulation were 0 g, 150 mg and 5 parts, respectively. It showed entrapment efficiency percentage of 95.63%, particle size of 436.65 nm, polydispersity index of 0.47 and zeta potential of -38.80 mV. Results of *ex vivo* permeation and skin deposition studies showed enhanced skin permeation and retention capacity of the prepared vesicles than drug suspension.

***Conclusion*:** Results revealed that a transdermal niosomal system was successfully prepared and evaluated using central composite design which will result in delivering diacerein efficiently, avoiding its oral problems.

Keywords: Diacerein, central composite, niosomes, optimization, *ex vivo* permeation, skin deposition studies.

1. INTRODUCTION

Nanotechnology involves fabrication of nanoscale structures which are observed visibly under high resolution. These molecular assemblies are especially designed for attaining their target functions [1]. Based on their critical packing parameter and hydrophilic lipophilic balance (HLB), these molecules are self-assembled to various morphologies including micelles, sheets and vesicles (liposomes, exsosomes, niosomes, *etc*.) [2]. The concept of using liposomes as vesicular drug delivery systems had enriched various pharmaceutical fields including gene delivery and drug biomolecules. Delivering these agents using liposomes can alter their bioavailability, bio-distribution profiles and consequently enhances their therapeutic indices [3]. Exsosomes are nanoparticle extra-cellular vesicular carriers that are generated by different cells and can efficiently enter other cells. Unlike liposomes, they contain trans-membrane proteins that can guard against phagocytosis, enhance endocytosis and promote the delivery of their internal content [4]. Hence, exosomes, compared to liposomes, were found to be a promising approach for targeting cancer cells with high specificity and minimal circulatory clearance [4]. Furthermore, these vesicular formulations had been more exploited in the field of transdermal drug delivery [5]. They offer many advantages over conventional delivery systems like bio-compatibility, non-toxicity and ability to modify drugs' bioavailability [6]. In addition to the utilization of vesicular carriers for transdermal drug delivery, nanotransfection ap-

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proaches (TNT) have been recently introduced for topical and controllable delivery of reprogramming factors across the skin. These approaches allow delivery of controlling factors by applying intense and highly focused electric field using arrayed nano-channel. Hence, TNT can deliver the cargo to skin in rapid and non-invasive manner [7]. In this manuscript, the strategy of using niosomes as a vesicular nanocarrier has been selected and investigated for efficient transdermal delivering of drugs and bypassing their oral problems.

Niosomes are closed bilayer structures formed from self-assembly of non-ionic surfactants in aqueous medium. Hence, these systems can act as drug reservoirs which deliver the drug molecules across the skin at a predetermined rate over the period of treatment [8]. They are engineered to be site-specific and slowly degraded. As a result, drugs' encapsulation in these vesicular systems is predicted to prolong their existence in systemic circulation with resultant increase in their bioavailability [9]. Furthermore, non-ionic surfactants in niosomal constructs were shown to act as penetration enhancers which can loosen the most impervious skin anatomic barrier; stratum corneum (SC) [10]. Therefore, niosmes can be considered as a promising transdermal delivery system for enhancing the drug permeation and diffusion into the skin and avoiding the load placed on the liver and digestive tract as well as the gastrointestinal side effects caused by certain drugs after oral administration [11]. It had been demonstrated that the transdermal delivery of enoxicam [12], aceclofenac [13], minoxidil [14] and estradiol [15] could be enhanced *via* encapsulation in niosomes.

In developing any pharmaceutical formulation, designing a formulation with the optimal quality in a short time with the least number of experimental trials is the most important issue [16]. Response surface methodology (RSM) is an efficient technique for optimization of drug delivery systems by efficiently exploring the relationships between the investigated factors and measured responses using the minimum number of experimental trials [17]. Based on the experimental design, RSM encompasses the generation of polynominal mathematical equations and the mapping of measured responses over the experimental domain to select the optimal formulation. Central composite design (CCD) is one of the most applicable designs of RSM which consists of factorial and axial points as well as one point at least at the center of experimental region that provides properties like orthogonality and rotatability for fitting quadratic polynomials [18]. This diversity of points is useful for providing complete knowledge of responses using the least number of experiments [19].

Diacerein (DCN), is one of the recently introduced structural modifying osteoarthritis drugs (SMOADs) [20]. Because of its short half-life (4h), it is orally administrated frequently during the day [21]. Gastrointestinal side effects like diarrhea was observed in patients with the incidence of 20% to 40% within the first 2 weeks of oral DCN administration which would lead to treatment discontinuation and prohibition of its use in some countries [22]. Furthermore, DCN is a BCS class II drug with poor water solubility (3.197 mg/L) and low oral bioavailability (35%-56%) [23]. Hence, DCN is considered as a good candidate for transdermal niosomal formulation for maintaining continuous DCN delivery across the skin into systemic circulation, avoiding its oral problems and consequently improving the patient compliance.

Hence, our current study aimed at employing RSM to investigate the influence of different formulation variables on the feasibility of niosomes to be utilized as colloidal carriers for efficient transdermal DCN delivery. To achieve this goal, a rotatable three-level three-factor CCD was employed for preparing DCN-loaded niosomes to analyze the effect of the studied factors on the selected responses and to suggest the optimal production parameters for attaining a DCN niosomal formulation with the least particle size (PS) and polydispersity index (PDI) and the highest entrapment efficiency percent (EE%) and zeta potential (ZP) as absolute value. Furthermore, for the purpose of conferring more elastic properties to the suggested optimal niosomes, its composition was modulated using edge activators (EAs) for developing elastic vesicles with hypothesized ultra-deformability and consequently superior skin permeation potential and drug retention capacity [24]. To confirm this hypothesis, *ex vivo* permeation and *in vivo* skin deposition results of DCN from the suggested optimal niosomes were compared with the resultant elastic vesicles and drug suspension.

2. MATERIALS AND METHODS

2.1. Materials

Diacerein (DCN) was a kind gift from EVA Pharmaceutical Industries (Cairo, Egypt). Cholesterol (CH), Span 40 (sorbitan monopalmitate), Span 60 (sorbitan monostearate), Span 80 (sorbitan monooleate), Brij 58 (polyethylene glycol hexadecyl ether), Brij 72 (polyoxyethylene 2 stearyl ether), Brij S2 (polyoxyethylene (2) stearyl ether), Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbitan monooleate), orthophosphoric acid and acetonitrile (HPLC grade) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium taurocholate (STC) was acquired from BASF Co. (Florham Park, NJ, USA). Methanol, chloroform, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride were purchased from El-Nasr Pharmaceutical Chemicals Co. (Abu-Zaabal, Cairo, Egypt).

2.2. Preparation of DCN-Loaded Niosomes

DCN-loaded niosomes were prepared using film hydration technique [25] by varying the used hydration medium, amount of lipid used for bilayer formation (surfactant and CH) and number of surfactant parts (surfactant to CH ratio). In a 250 mL long-necked quick fit round-bottom flask, DCN (25 mg) and accurately weighted quantities of the surfactant (Span 40 or Span 60 or Span 80 or Tween 20 or Tween 80 or Brij 58 or Brij 72) and CH (150 or 200 or 250 mg) in varying ratios (2:1 or 4:1 or 6:1) were dissolved in 10 mL of chloroform-methanol mixture (7:3). The obtained clear organic solution was slowly evaporated at 60°C under reduced pressure using a rotary evaporator (Rotavapor, Heidolph VV 2000, Burladingen, Germany) for 30 min at 90 rpm. After solvent evaporation, the formed thin lipid film was then hydrated using 10 mL of ultra-pure distilled water containing different amount of salt (0 or 5 or 10 g sodium chloride) by rotating the flask in a water bath maintained at 60°C for 30 min at 150 rpm using the same apparatus under normal pressure to form milky dispersion of DCN-loaded niosomes. Glass beads were used during

the hydration step to increase the yield of the formed nanovesicles [26]. The niosomal dispersion was exposed to ultrasound bath sonication at 25°C (Model SH 150-41; USA) for different times (10 or 20 or 30 min) in order to reduce size [27]. Finally, the resultant fine-tuned dispersion was left to equilibrate overnight at 4°C for further investigation.

2.3. Experimental Design

A three-level three-factor rotatable CCD (3^3) was used for statistical optimization of DCN-loaded niosomes. Design-Expert® software (Version 7, Stat-Ease, Inc., Minneapolis, MN, USA) was employed for generation of mathematical relationship between input and output variables and for generation as well as evaluation of this experimental design. Different trial formulations of DCN-loaded niosomes were prepared based on the trial proposals of CCD according to the formula $N = 2^k + 2k + C_0$, where k is the number of variables and C_0 is the number of center points [18]. Hence, for 3 factors design, a total of 16 experiments were run; 6 axial points, 8 factorial points and 2 runs which were the replicates of the center point. In this design, amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) were selected as independent variables which were varied at three levels (low, medium, high) based on the preliminary experiments and feasibility of preparing DCN-loaded niosomes at these values. The EE% (Y_1), PS (Y_2), PDI (Y_3) and ZP (Y_4) were set as dependent variables. The independent and dependent variables are demonstrated in Table **1**. The design matrix including the composition of DCN niosomal formulae is shown in Table **2**.

Table 1. Three-level three-factor CCD used for optimization of DCN-loaded niosomes.

Factors (Independent Variables)	Levels		
	Low (-1)	Medium (0)	High (+1)
X_1: Amount of salt in hydration medium (g)	0	5	10
X_2: Lipid amount (mg)	150	200	250
X_3: Number of surfactant parts	2	4	6
Responses (dependent variables)	**Desirability Constraints**		
Y_1: EE%	Maximize		
Y_2: PS (nm)	Minimize		
Y_3: PDI	Minimize		
Y_4: ZP (mV)	Maximize (as absolute value)		

Abbreviations: EE%, entrapment efficiency percent; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

Table 2. Composition of the three-level three-factor CCD of DCN-loaded niosomes.

Run	Factors Levels in Actual Values		
	X_1: Amount of Salt in Hydration Medium (g)	X_2: Lipid Amount (mg)	X_3: Number of Surfactant Parts
Mid points			
N1	0	200	4
N2	10	200	4
N3	5	200	2
N4	5	200	6
N5	10	150	2
N6	5	250	4
N7	0	150	6
N8	0	250	6
N9	10	150	6
N10	10	250	2
N11	5	150	4
N12	0	250	2
N13	0	150	2
N14	10	250	6
Center points			
N15	5	200	4
N16	5	200	4

2.4. Characterization of DCN-Loaded Niosomes

2.4.1. Determination of DCN EE%

Centrifugation method was adopted for the determination of EE% of DCN. It was estimated indirectly by calculating the difference between the total amount of DCN added in the formulation and the free (unentrapped) DCN in aqueous medium [17]. The free DCN was separated from the prepared nanovesicles by centrifugation of 1 mL of the vesicular suspension at 25000 rpm for 1 h at 4°C using a cooling centrifuge (Sigma 3-30 KS, Sigma Laborzentrifugen GmbH, Germany). DCN content in the resultant supernatant was determined spectrophotometrically (Shimadzu, model UV-1601 PC, Kyoto, Japan) by measuring the ultraviolet (UV) absorbance at λ_{max} 258 nm. Each result was the mean of three determinations ± standard deviation (SD). Drug EE% was determined according to the following equation:

$$EE\% = \left[\frac{(\text{Total amount of DCN} - \text{Unentrapped DCN})}{\text{Total amount of DCN}}\right] \times 100 \quad (1)$$

2.4.2. Determination of PS, PDI and ZP

The average PS, PDI and ZP of the prepared DCN niosomes were determined by dynamic light scattering technique (photon correlation spectroscopy) using Zetasizer Nano ZS (Malvern Instrument Ltd., Worcestershire, UK). The procedure was carried out by employing a helium-neon laser with a wave length of 633 nm at 25°C in order to analyze the fluctuations in light scattering caused by the Brownian motion of particles in the dispersion. Before each measurement, the niosomal dispersions were properly diluted to ensure that the light scattering intensity is inside the instrument's sensitivity range. PDI is a dimensionless measure of the broadness of the PS distribution. ZP measurement was conducted by combination of Laser Doppler Velocimetry (LDV) and phase analysis light scattering using the same instrument to observe their electrophoretic mobility in the electric filed. The electrophoretic mobility was converted to ZP using the Smoluchowski equation [28, 29]. Three replicates were taken for each sample. The displayed results are the average value ± SD.

2.5. Optimization of DCN-Loaded Niosomes

Desirability values were calculated using Design-Expert® software for achieving optimal niosomes formulation by response surface analysis of the resultant data. Desirability function was applied to get the optimal niosomes formulation with the least PS and PDI and the highest EE% and ZP as absolute value. The calculated desirability values were considered differentiating parameters for comparing the prepared niosomal formulae [30]. To confirm the model efficacy, the suggested optimal niosomes were prepared, evaluated and compared with the predicted responses obtained by the software. Furthermore, for the purpose of comparison, elastic vesicles were formulated by the same method of preparation and using the same components of the optimal niosomes formulation in addition to different EAs (STC and Brij S2 in ratio 3:1).

2.6. Transmission Electron Microscopy (TEM)

For morphological examination, one drop of the optimal niosomes formulation was stratified on a carbon coated copper grid and allowed to dry at room temperature for 10 min. The air dried sample was then visualized using TEM (Joel JEM 1230, Tokyo, Japan) at different magnifications [31].

2.7. Differential Scanning Calorimetry (DSC)

Thermal analysis of pure DCN, surfactant, CH, physical mixture of DCN with niosomal components and the optimal niosomes formulation was performed using differential scanning calorimetry (DSC 60 Shimadzu, Japan) calibrated with purified indium (99.9). Accurately weighted samples (5 mg) were sealed in standard aluminum pans and heated from 10°C to 300°C at a scanning rate of 10°C /min under inert nitrogen flow (25 mL/min).

2.8. Animals Preparation for *Ex vivo* and *In vivo* Studies

The protocol of the studies (PI 1738) was assessed and approved by the Research Ethics Committee in the Faculty of Pharmacy, Cairo University, Egypt. The use and the handling of animals in all studies were in compliance with the EU Directive 2010/63/ EU for animal experiments.

2.9. *Ex vivo* Studies

2.9.1. Skin Preparation

Newly born rats weighing 70±20 g were sacrificed. The skin, free of bites or scratches, was carefully excised from animals after scarifying them. Subcutaneous tissues were removed by careful cleaning of the dermal surface with cotton swab in order not to damage the epidermal surface. The excised full thickness skin samples were equilibrated by soaking in phosphate buffered saline (PBS) solution pH 7.4 at 4-8°C about 1 h before beginning the experiment [32].

2.9.2. Ex vivo Permeation Study

The study was conducted using a vertical franz diffusion cell (1.76 cm^2) which was filled with 50 mL PBS pH 7.4 (receptor compartment). The skin samples were mounted on the glass joint of the diffusion cell with stratum corneum (SC) facing the ambient condition (donor compartment) and the dermal side fronting the receptor compartment. The temperature of receptor compartment was adjusted at 32°C with continuous stirring of the receiver medium (100 rpm) for preventing the boundary layer effects [33]. The donor compartment was charged with 1 mL of the investigated system (elastic vesicles or the optimal niosomes formulation or drug suspension) under non-occlusive condition. At predetermined time intervals, (0.5, 1, 2, 4, 6, 8 and 24 h), samples of 3 mL were withdrawn from the receptor fluid and the cell was refilled immediately by equal volume of freshly prepared receptor solution to maintain a constant volume and fulfill sink conditions. The experiment was conducted three times and the displayed results are average value ± SD. The withdrawn samples were analyzed using a validated HPLC method and then the concentration of DCN in each sample and the amount permeated across the skin was calculated. The cumulative amount of drug permeated through the skin per unit area (μg/cm^2) was plotted against time (h) [32]. The flux (J_{max}) at 24 h and the enhancement ratio (ER) were calculated according to the following equations [24]:

$$J_{max} = \frac{\text{Amount of drug permeated}}{\text{Time x Area of memebrane}} \quad (2)$$

$$ER = \frac{\text{Jmax of the nanovesicles}}{J_{max} \text{ of the drug suspension (control)}} \quad (3)$$

The flux values and the amount of DCN permeated from the prepared nanovesicles (elastic vesicles and optimal niosomes formulation) as well as drug suspension were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS software 17.0 (SPSS Inc., Chicago, USA). Post-hoc analysis was done using Tukey's HSD (honest significant difference) test. Difference at $P \leq 0.05$ was considered significant.

2.9.3. HPLC Determination

The permeated DCN was analyzed using an isocratic reported HPLC method with some modifications [34]. A Thermo Separation HPLC system (Fremont, California) equipped with a P4000 pump unit, an AS3000 autosampler including an injection valve with a sample loop of 50 µL volume and a UV2000 detector was used. A Zorbax Extend-C_{18} column (4.6 mm×250 mm) containing 3.5 µm size adsorbent as stationary phase (Agilent Technologies, Santa Clara, California) was utilized. The column was maintained at room temperature (25±2°C). The mobile phase consisted of a mixture of acetonitrile and phosphate buffer in the ratio of 65:35 and was delivered at a flow rate of 1 mL/min. The pH was adjusted to 4 by orthophosphoric acid. An amount of 20 µL of sample volume was injected into the column and detected at 254 nm. DCN was eluted at 12 min under these conditions. The assay procedures were validated in terms of linearity, precision and accuracy.

2.10. *In vivo* Skin Deposition Study

The experiment was conducted to investigate the potential of vesicular carriers to retain the entrapped drug within the skin compared to the drug suspension. A total of 72 male Wistar rats, weighing 150-200 g, were involved in the study. The animals were supplied with standard diet and tap water ad libitum and placed individually in cages with wide mesh wire bottoms to avoid coprophagy. On the experiment day, the rats were randomly separated into 4 groups with 18 animals in each group, where group I behaved as control while animals in groups II, III and IV received topical application of elastic vesicles, optimal niosomes formulation and drug suspension, respectively. Bottle caps that served as drug pools (4.15 cm^2) were stuck to rat dorsal skin which was shaved to remove hair with an electric clipper 24 h before application of the sample [35]. Half mL of each formulation was added non-occlusively into the drug pool. After different time intervals of application of treatments (1, 2, 4, 6, 8 and 10 h), 3 animals from each group were sacrificed by cervical dislocation method using an overdose of anesthetic ether and the dorsal rat skin that was in contact with the formulation was excised then immediately washed with 10 mL of normal saline in 2 divided portions. The excised skin sections were cut into pieces and sonicated in 5 mL dimethylsulfoxide (DMSO) for 30 min. The skin homogenate was then filtered through a 0.22 µm filter membrane and the concentration of DCN was determined using a validated HPLC method as previously discussed. The skin deposition of DCN was calculated from the obtained data. Statistical significance was analyzed by one-way ANOVA using SPSS software 17.0. Post-hoc analysis was done using Tukey's HSD test. Difference at $P \leq 0.05$ was considered significant. The destruction of animal carcasses was achieved by incineration at the end of experiment.

3. RESULTS AND DISCUSSION

3.1. Preparation of DCN-Loaded Niosomes

Based on preliminary studies, film hydration technique was the most appropriate method for the preparation of DCN-loaded niosomes. The solubility of DCN together with the lipid components (the surfactant and CH) was tested in different organic solvents. Only chloroform-methanol mixture in ratio (7:3) succeeded to obtain homogenous lipid solution using a total volume of 10 mL. The screening results demonstrated that this solvent mixture produced clear continuous film when evaporated for 30 min at 90 rpm.

Ten mL of the used hydration medium was the optimum volume for effective hydration of the formed film. In our study, the use of larger or smaller volume was shown to have negative effect on EE% (data not shown). This could be explained by the study conducted by Sankhyan *et al.* which concluded that using smaller volume would result in distorted bilayer formation due to less amble space available for vesicles' formation. On the other hand, increasing the volume of hydration medium would cause the materials to be dispersed in larger volume with resultant formation of smaller vesicles with low EE% [36]. During film hydration, the temperature was maintained at 60°C, above gel-liquid crystal transition temperature (Tc) of the used surfactants, to allow hydration of lipids in their fluid phase which has a significant impact on the shape and size of the vesicles and surfactants assembly into them [37]. Considering the sonication time, the resultant milky dispersions were sonicated at different times (10, 20, 30 min) and results showed that there was inverse relationship between the sonication time and PS of the prepared vesicles (data not shown). This might be due to cavitation (bubble formation) effect of the ultra sound on the lipid membrane of the prepared vesicles [38]. Preparing vesicles with smaller PS is advantageous for transdermal delivery as it strongly influences the penetration of the prepared vesicles through the skin [39]. Hence, sonication time was fixed at 30 min which resulted in the optimum PS without significantly affecting EE%.

Several non-ionic surfactants were used in the preliminary trials for preparation of DCN-loaded niosomes including Spans (Span 40, 60, 80), Tweens (Tween 20, 80), Brijs (Brij 58, 72). Span 80, Tweens and Brijs failed to form dry thin film at the flask round surface and therefore, they were not used for further study. Only Span 40 and Span 60 were able to give milky vesicular dispersion on hydration. This could be explained by their high Tc and their optimum HLB (4-8) which is suitable for preparation of vesicles [40]. In this study, Span 60 was chosen as it produced vesicles with relatively smaller PS (data not shown) due to the higher lipophicity of Span 60 (HLB 4.7) compared to Span 40 (HLB 6.7), which resulted in decreasing the mass transfer

within the droplets with consequent lower growth of nuclei and smaller PS [41].

3.2. Statistical Design Analysis

The choice of the suitable design is based on the experimental purpose. When the aim of the study is planning an optimum set of experimental conditions, factorial design would require large number of experimental trials which increase geometrically by increasing the number of tested factors [18]. Hence, one can resort to RSM combined with CCD as these statistically cognizant experimental designs involve combination of mathematical and statistical methods that can be employed for mapping the responses over the selected formulation variables to select the best experimental condition [19]. Therefore, a rotatable three-level three-factor CCD was employed in this study to analyze the effect of the studied factors on the selected responses using fewer experimental runs (16) compared to 27 runs in 3^3full factorial design [42]. Hence, CCD is considered as time-saving and cost-effective alternative to three-level full factorial design with a comparable performance. The measured responses of the prepared 16 niosomal formulae are shown in Table **3**.

Adequate precision measured the signal to noise ratio to make sure that the model can be used to navigate the design space [43]. A ratio greater than 4 is desirable which was observed in all responses except ZP which was a non-significant model term as shown in Table **4**. On the other hand, the predicted R-squared statistic was calculated to provide a good insight into how good the model can fit new data governed by the same relationship that had been modeled. The adjusted R-squared statistic is the modified version of R-squared which describes how good the model will fit the current observed data. Hence, the adjusted and predicted R^2 values are preferred to be close to each other in order to be in reasonable agreement [44]. If they are not, there could be a problem with either the data or the model [31]. It is worthy to mention that the predicted R^2 values were in a reasonable agreement with the adjusted R^2 in all responses except PDI (Table **4**). The negative predicted R^2 value of PDI implies that the overall mean is a better predictor of the response [45].

3.2.1. Effect of Formulation Variables on EE%

The ability of niosomes to entrap significant proportion of the drug is considered a critical parameter for optimum transdermal delivery. EE% of all DCN-loaded niosomes involved in the experimental design ranged from 21.6±0.9 to 95.8±0.2% (Table **3**). Fig. (**1a**) illustrates the response 3-D plots for the effect of amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) on EE% of DCN-loaded niosomes. ANOVA test for the ob-

Table 3. The measured responses of the prepared 16 DCN-loaded niosomes.

Run	Y_1:EE%[a]	Y_2:PS (nm)[a]	Y_3:PDI[a]	Y_4:ZP (mV)[a]
Mid points				
N1	94.0±0.9	786.8±177.0	0.4±0.3	-51.7±0.7
N2	31.1±1.5	1231.3±389.9	0.5±0.3	-63.2±1.8
N3	44.4±2.7	1262.0±304.1	0.7±0.0	-57.6±1.2
N4	22.5±3.5	336.4±95.6	0.4±0.0	-41.1±0.2
N5	21.6±0.9	1469.5±259.5	0.1±0.0	-62.1±0.9
N6	45.1±0.1	1136.0±451.2	0.5±0.3	-57.6±3.6
N7	90.5±0.4	448.5±55.9	0.5±0.0	-47.8±1.0
N8	95.5±0.1	899.5±123.2	0.5±0.2	-49.0±1.6
N9	24.5±2.1	290.8±3.8	0.4±0.1	-42.4±1.6
N10	44.1±2.7	1358.0±502.0	0.7±0.0	-72.3±1.9
N11	23.2±0.9	183.4±0.9	0.3±0.0	-44.3±2.0
N12	95.6±0.4	1189.2±528.6	0.4±0.3	-55.1±0.5
N13	95.8±0.2	1170.0±192.3	0.3±0.1	-47.4±1.3
N14	32.6±2.0	797.6±71.1	0.6±0.1	-56.3±1.0
Center points				
N15	41.5±3.5	814.0±66.5	0.3±0.3	-59.2±0.1
N16	35.5±4.9	619.0±9.7	0.6±0.1	-59.1±0.1

Abbreviations: EE%, entrapment efficiency percent; PS, particle size; PDI, polydispersity index; ZP, zeta potential.
[a] Data represented as mean ± SD (n = 3).

Table 4. Output data of the regression analysis of three-level three-factor CCD of DCN loaded niosomes.

Responses	Model	R^2	Adjusted R^2	Predicted R^2	Adequate Precision	P value	Significant Factors
Y_1: EE%	Quadratic	0.9758	0.9659	0.9505	26.731	<0.0001	X_1,X_2, X_3
Y_2: PS (nm)	Linear	0.5628	0.5158	0.4200	11.238	<0.0001	X_2, X_3
Y_3: PDI	2 FI	0.3893	0.2427	-0.0632	6.547	0.0393	X_2
Y_4: ZP (mV)	Linear	0.1034	0.074	0.0263	3.911	0.3750	-

Abbreviations: EE%, entrapment efficiency percent; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

served EE% demonstrated that the quadratic model was significant and fitting for the data. As shown in Table **4**, all of the three factors had significant effect on EE% of the prepared niosomal vesicles. The resulting equation in terms of coded factors was as follows:

$$EE\% = +35.90 - 31.73X_1 + 5.72X_2 - 3.60X_3 + 3.26X_1X_2 - 0.37X_1X_3 - 1.19X_2X_3 + 27.88X_1^2 - 0.47X_2^2 - 1.15X_3^2$$

The equation shows that increasing amount of salt in hydration medium (X_1) had significant negative effect on EE% (P<0.0001). By increasing amount of salt in aqueous medium, the added salts exerted stronger ionic effect which reinforced the vesicles to lose their water of hydration by means of ions competition with resultant quick release of their entrapped drug [46]. Furthermore, it was shown that by increasing the ionic strength of the hydration medium, vesicular erosion could occur and consequently more drug would be released in the dispersion medium [47]. The equation also reveals that that increasing lipid amount (X_2) had synergistic significant effect on EE% of DCN-loaded niosomes (P=0.0001). This might be attributed to that by increasing the amount of lipids available for hydration, more vesicles would be formed with increased lipophilic ambiance to accommodate high amount of the hydrophopic drug (DCN) with resultant increase in EE% [17, 48]. In other words, decreasing the total amount of lipids means insufficient bilayer materials for strong membrane formation and efficient drug encapsulation [36]. The negative coefficient of the term X_3, reveals that EE% decreased significantly by increasing number of surfactant parts (P=0.0077). At high surfactant level, the solubilization and diffusion of the drug into the aqueous media would increase during vesicular preparation with resultant decrease in EE% [27, 49]. Moreover, it was reported that increasing the surfactant amount beyond certain level would cause formation of leaky points within niosomal vesicles and consequently decrease DCN entrapment with consequent increase in its release [50].

3.2.2. Effect of Formulation Variables on PS of DCN-Loaded Niosomes

Preparing vesicular carriers with small PS was one of the major concerns in our study for enhancing the transdermal flux of DCN. PS of DCN-loaded niosomes ranged from 183.4±0.9 to 1469.5±259.5 nm (Table **3**). Fig. (**1b**) represents the response 3-D plots for the effect of amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) on PS of DCN-loaded niosomes. ANOVA test for the observed PS data showed that the linear model was significant and fitting for the data. The resulting equation in terms of coded factors was as follows:

$$PS = +877.42 + 65.34X_1 + 181.80X_2 - 367.59X_3$$

PS of DCN-loaded niosomes was not significantly affected by varying amount of salt in hydration medium (X_1) (P=0.3531). Oppositely, ANOVA results revealed that PS of DCN nanovesicles was significantly affected by the other two factors. The positive coefficient of the term, X_2, shows that increasing lipid amount caused significant increase in the vesicular PS (P=0.0138). The synergistic effect of lipid amount on PS of DCN-loaded niosomes could be related to the direct relationship between PS of the vesicles and the amount of drug entrapped within their vesicular bilayer. As previously mentioned, increasing lipid amount caused more drug to be incorporated in the vesicular hydrophobic region which consequently increased the distance between the lipid bilayers and led to larger PS [51, 52]. The equation also shows that number of surfactant parts (X_3) had significant negative effect on PS of DCN-loaded niosomes (P<0.0001). This might be due to formation of mixed micelles (instead of niosomal vesicles), at high surfactant level, which are smaller in PS [53, 54].

3.2.3. Effect of Formulation Variables on PDI of DCN-Loaded Niosomes

PDI of all DCN-loaded niosomes involved in the experimental design ranged from 0.1±0.0 to 0.7±0.0 (Table **3**). Fig. (**2a**) represents the response 3-D plots for the effect of amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) on PDI of DCN-loaded niosomes. ANOVA test for the observed PDI data indicated that two factor interaction (2 FI) model was significant and fitting for the data. The resulting equation in terms of coded factors was as follows:

$$PDI = +0.46 + 0.035X_1 + 0.11X_2 + 0.015X_3 + 0.082X_1X_2 - 6.375E\text{-}003X_1X_3 - 0.066X_2X_3.$$

ANOVA results revealed that only lipid amount (X_2) had significant impact on PDI (P=0.0065). The PDI increased with the increase in lipid amount. This could be attributed to the increased viscosity of the dispersion medium when the amount of lipid increased which would lead to formation of relatively larger particles with greater variation in size [28]. Furthermore, increasing the lipid amount resulted in increased amount of drug loaded inside the vesicles with resultant increase in the vesicles' heterogeneity [51, 55].

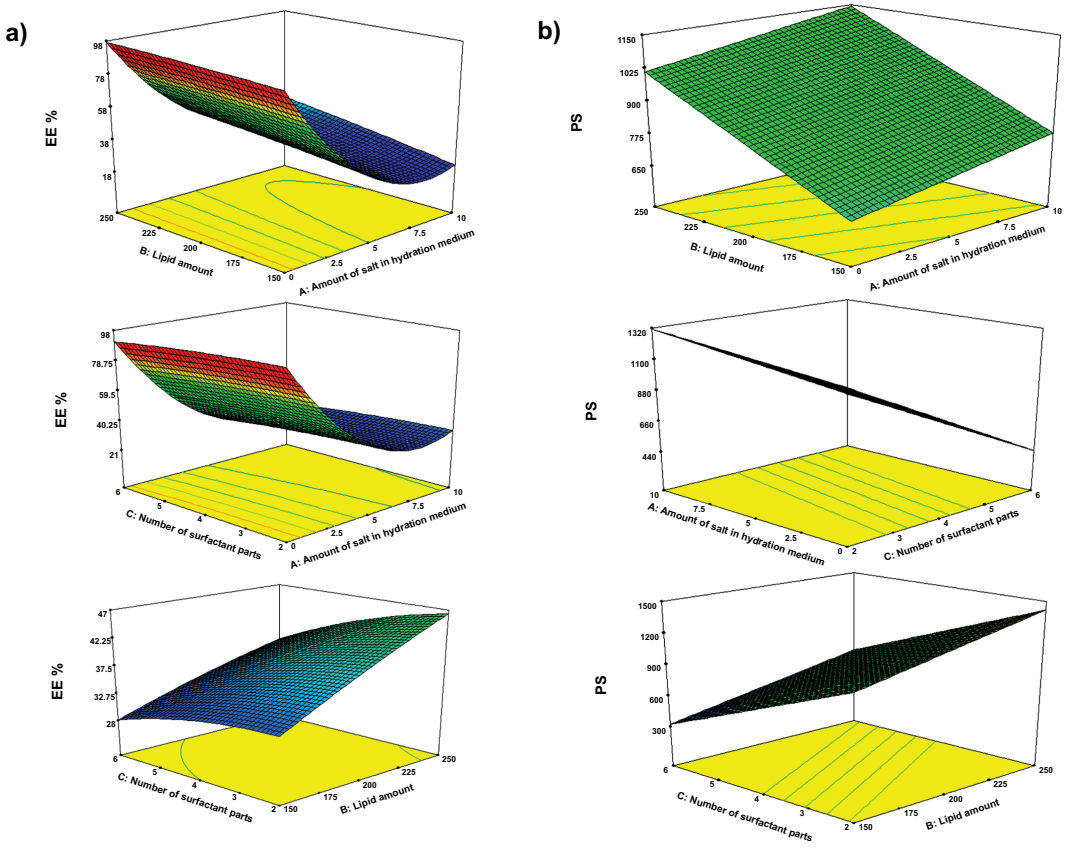

Fig. (1). Response 3-D plots for the effect of amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) on (**a**) entrapment efficiency percent (EE%) and (**b**) particle size (PS) of DCN-loaded niosomes.

3.2.4. Effect of Formulation Variables on ZP of DCN-Loaded Niosomes

ZP is the measure of the total charge acquired by the nanovesicles and considered as indirect measurement of the stability of niosomal dispersion [51]. The system with ZP value around ±30 mV is considered stable [56]. In this investigated design, it is worthy to note that all DCN-loaded niosomes obtained negative ZP values which ranged from -41.1±0.2 to -72.3±1.9 mV (Table **3**). Hence, DCN vesicles had sufficient charges to preserve them from aggregation and fusion and consequently maintain their stability and integrity [57]. Fig. (**2b**) represents the response 3-D plots for the effect of amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) on ZP of DCN-loaded niosomes. ANOVA results revealed that ZP of the prepared niosomal vesicles was not significantly affected by any of the studied factors. Linear model was selected and the resulting equation in terms of coded factors was as follows:

$$ZP = -50.41 - 4.50\ X_1 - 4.62\ X_2 + 5.79\ X_3$$

3.2.5. Optimization of CCD

The optimization of the pharmaceutical formulations is generally aimed at implementing systematic approaches for achieving the best possible combinations of the process characteristics from which a robust high quality product may be produced [27]. Attainment of this purpose calls for studying the effect of the selected variables on system responses by changing one variable at a time which had been shown to be costly, time-consuming and unfavorable for error fixation [16]. Furthermore, simultaneous attainment of all desired responses is almost impossible due to interference as the optimum condition reached in one response could have an opposite effect on other responses [58]. Hence, utilization of established statistical optimization tools was preferred for understanding the effect of different formulation variables on the quality of pharmaceutical formulations with subsequent attainment of the best performing one [16]. CCD is one of these talented tools that is widely used for optimization of drug delivery systems using the least number of experiments by quadratic response surface exploration and second order polynomial model construction [59].

After applying desirability constraints on EE%, PS, PDI and ZP (Table **1**), a numerical optimization analysis was then employed by Design-Expert® software using the desirability function to develop the optimal niosomes formulation with the desired responses [16]. The overall desirability of the optimal niosomes formulation was 0.751 which was suggested to be prepared using distilled water (0 g of salt in hydration medium) (X_1), lipid amount of 150 mg (X_2) and number of surfactant parts of 5 (X_3). Hence, it was prepared and evaluated. As shown in Table **5**, the residual between the expected and observed responses was small demonstrating the reasonableness of the optimization process. Thus, the optimal niosomes formulation was considered promising to be used in further investigation. In addition, the composition of optimal niosomes formulation was modulated using EAs

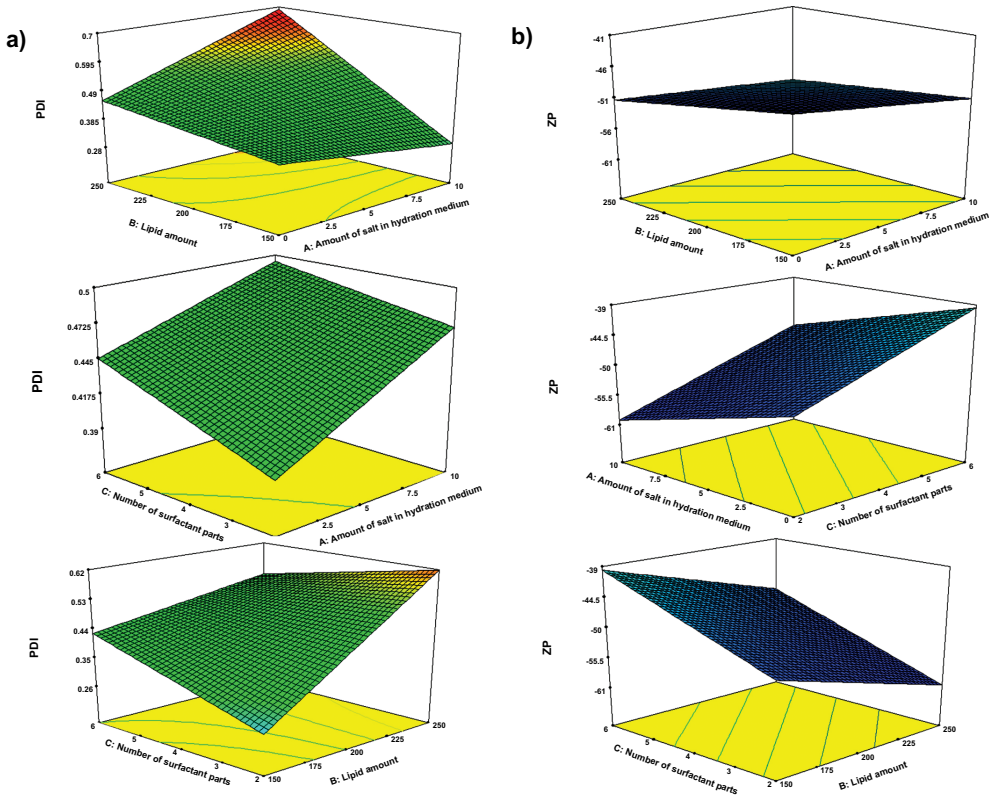

Fig. (2). Response 3-D plots for the effect of amount of salt in hydration medium (X_1), lipid amount (X_2) and number of surfactant parts (X_3) on (**a**) polydispersity index (PDI) and (**b**) zeta potential (ZP) of DCN-loaded niosomes.

Table 5. Expected and observed values for the optimal DCN niosomes.

Factor		Optimal Level	
X_1: Amount of salt in hydration medium (g)		0	
X_2: Lipid amount (mg)		150	
X_3: Number of surfactant parts		5	
Response	**Expected**	**Observed**	**Residual [a]**
Y_1: EE%	91.05	95.63	-4.58
Y_2: PS (nm)	426.39	436.65	-10.26
Y_3: PDI	0.44	0.47	-0.03
Y_4: ZP (mV)	-38.07	-38.80	0.73

[a] Residual = Expected - Observed.
Abbreviations: EE%, entrapment efficiency percent; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

as mentioned earlier to produce elastic vesicles for further comparative *ex vivo* permeation and *in vivo* skin deposition studies.

3.3. Transmission Electron Microscopy (TEM)

TEM analysis is used for determination of the shape of the vesicles and for confirming the results of photon correlation spectroscopy [60]. As illustrated in Fig. (**3**), the photomicrograph of the optimal niosomes formulation displayed non-aggregating, spherical vesicles with definite wall and smooth surface. Furthermore, the diameter of the vesicles observed by TEM micrographs was in a good agreement with the size obtained from the Zetasizer (Table **5**).

3.4. Differential Scanning Calorimetry (DSC)

Fig. (**4**) represents the thermograms of pure DCN, CH, Span 60, physical mixture of DCN with niosomal components and the optimal niosomes formulation. The DSC scan of pure DCN depicted a single endothermic peak at 256.19°C due to its crystalline nature [23]. The thermogram of CH showed sharp endothermic peak at 146.81°C because of degradation [61]. Span 60 showed endothermic transition

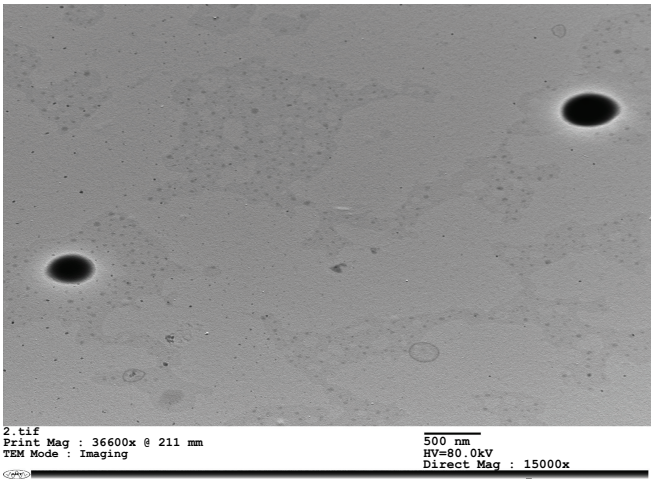

Fig. (3). Transmission electron micrograph of the optimal niosomes formulation.

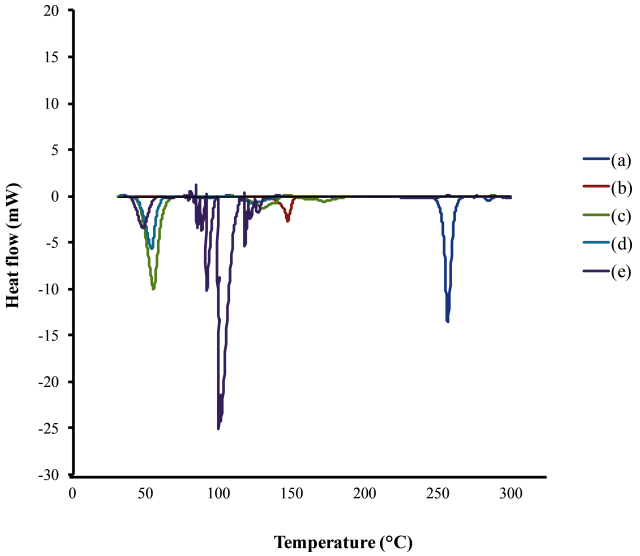

Fig. (4). DSC thermograms of (**a**) DCN, (**b**) CH, (**c**) Span 60, (**d**) physical mixture of DCN with niosomal components and (**e**) the optimal niosomes formulation.

at 55.01°C corresponding to its melting point [51]. With respect to the physical mixture of DCN with niosomal components, the endothermic transition of DCN was completely absent due to its dilution with the used excipients [23]. The complete absence of DCN endothermic transition was also observed in the thermogram of the optimal niosomes formulation confirming that DCN was entrapped in the niosomes with good interaction with the vesicular bilayer [51, 62]. Furthermore, the distinct endotherms of Span 60 and CH were shifted from 55.01°C and 146.81°C to 47.51°C and 99.62°C, respectively. The observed melting point depression might be attributed to that the components within the nanocarriers were arranged in less ordered lattice structure than the bulk excipients [53].

3.5. *Ex vivo* Permeation Study

To evaluate the potential of nanovesicles to aid in the permeation of DCN through the skin, comparative skin permeation study was conducted under non-occlusive condition using the excised rat's skin. The permeation profiles of DCN were studied from the prepared nanovesicles (elastic vesicles and optimal niosomes formulation) as well as drug suspension as shown in Fig. (**5**). Comparing the skin permeability parameters (Table **6**), it is worthy to note that the permeation of DCN was enhanced from the prepared nanovesicles compared to drug suspension. The ER was calculated to evaluate the extent of improvement in drug permeation through the vesicular formulae and it was more than 19 times for the elastic vesicles and more than 4 times for the optimal niosomes formulation compared to drug suspension. This might be attributed to the ability of the vesicular carriers to introduce DCN as fine dispersion, compared to the coarse PS of the drug suspension, which would result in increasing the surface area and consequently decreasing the diffusional path length of DCN through the skin [63]. Furthermore, as previously mentioned, the study was conducted under non-occlusive condition in order to create hydration gradient due to the difference in water content between the skin interior and its upper surface which enforced the vesicles to migrate deeply to water rich strata carrying drug molecules to secure adequate hydration condition [64]. Moreover, these vesicular systems can serve as penetration enhancers which could enter SC by modifying their constituting intercellular lipids and consequently raising their fluidity and weakness with resultant overcoming of the intrinsic skin barrier properties [24]. Furthermore, elastic vesicles showed significantly increased drug flux and resulted in higher amount permeated per unit area in 24 h than the optimal niosomes formulation ($P<0.05$) (Table **6**). The significant enhancement in DCN permeation through elastic vesicles was accredited to the presence of EAs in their constructs which imparts vesicular bilayer with high self-optimizing deformability that facilitates their penetration through skin pores that are much smaller than their size by fluidizing lipid domain of SC [24].

3.6. *In vivo* Skin Deposition Study

Skin deposition profiles of DCN from the elastic vesicles, optimal niosomes formulation and drug suspension are shown in Fig. (**6**). The extent of DCN deposited was expressed by $AUC_{0\rightarrow10}$ which was approximately 4 folds for elastic vesicles (65.8±17.6 µg h/cm^2) and 2 folds for the optimal niosomes formulation (32.7±8.1 µg h/cm^2) compared to drug suspension (16.7±5.6 µg h/cm^2). This could be attributed to that vesicular carriers, when applied on the skin under non-occlusive condition, could partition themselves through SC interstics by the influence of transcutaneous hydration gradient to form depot, from which drug could be released [65]. Furthermore, the presence of non-ionic surfactant in vesicular constructs could reduce the crystallinity of the skin intracellular lipid bilayer and consequently enhance the skin deposition [66]. Comparing the drug retention capacity of the prepared vesicular carriers, elastic vesicles showed significantly higher $AUC_{0\rightarrow10}$ than the optimal niosomes formulation ($P<0.05$). This might be related to the presence of bile salt (EA) in their composition which forms chemical compounds with keratocytes through interaction of the anionic head group with cationic sites of the skin proteins and consequently enhances the drug retention and deposition within the skin [67].

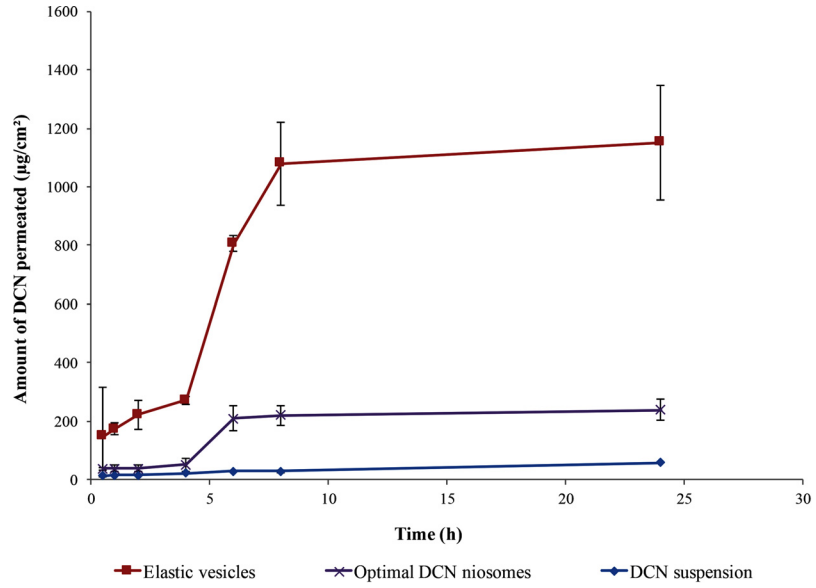

Fig. (5). Cumulative amount of DCN permeated per unit area across excised rat's skin *via* elastic vesicles and optimal niosomes formulation compared to drug suspension.

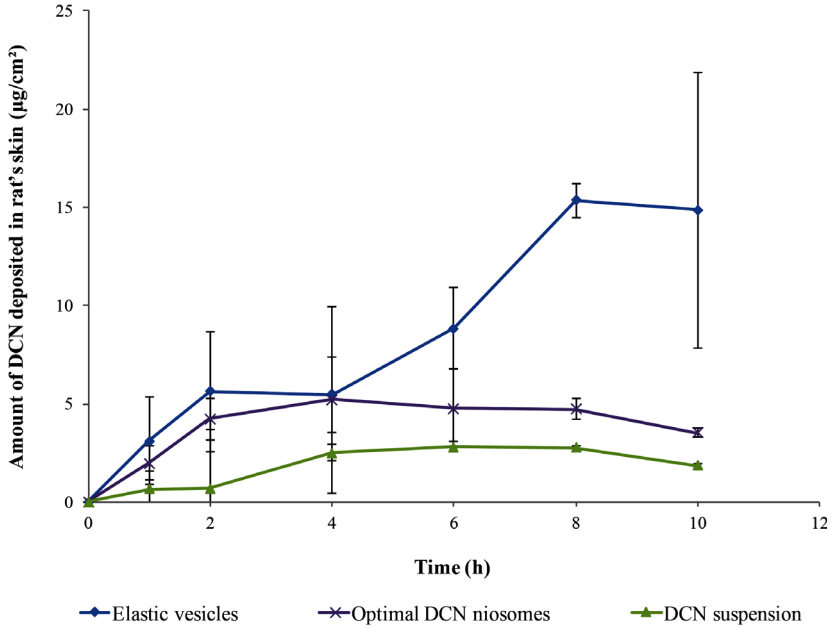

Fig. (6). *In vivo* skin deposition profiles of DCN versus times from elastic vesicles, optimal niosomes formulation and drug suspension after topical application.

Table 6. Skin permeability parameters of DCN after topical application of elastic vesicles, optimal DCN niosomes and drug suspension.

Skin Permeability Parameters	Elastic Vesicles	Optimal DCN Niosomes	Drug Suspension
Total amount of drug permeated per unit area in 24 h (µg/cm^2)[a]	1150.9±194.7	239.5±36.1	59.4±0.5
J_{max} (µg/cm^2/h)[a]	27.2±4.6	5.7±0.9	1.4±0.0
ER	19.4	4.1	1.0

Abbreviations: J_{max}, flux; ER, enhancement ratio.
[a] Data presented as mean ± SD (n = 3).

CONCLUSION

In this current work, niosomes were successfully prepared using film hydration technique for delivering DCN efficiently across the skin and avoiding its oral gastrointestinal problems. CCD was employed for optimization of the prepared niosomal formulae based on the desirability criterion. The suggested optimal niosomes were prepared and evaluated. It showed small PS, small PDI, high EE%, high ZP and spherical morphology under TEM. The internalization of DCN within the structure of the optimal niosomes formulation was confirmed by DSC studies. Furthermore, the composition of optimal niosomes formulation was modified to develop elastic vesicles for comparative evaluation. The results of *ex vivo* permeation and skin deposition studies ensured the superiority of vesicular carriers over drug suspension in enhancing DCN diffusion across the skin. Accordingly, the study supported that niosomes, optimized using CCD, could be a successful transdermal delivery system of DCN for avoiding its oral problems and consequently improving patient compliance.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol of the studies (PI 1738) was assessed and approved by the Research Ethics Committee in the Faculty of Pharmacy, Cairo University, Egypt.

HUMAN AND ANIMAL RIGHTS

No Human were used for studies that are base of this research. The use and the handling of animals were in compliance with the EU Directive 2010/63/ EU for animal experiments.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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