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PREPARATION, CHARACTERIZATION AND EVALUATION OF ANTIFUNGAL ACTIVITY OF CLOTRIMAZOLE NANOSTRUCTURED LIPID CARRIERS

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Keywords:

NLC, Clotrimazole, Compritol AT 888, labrasol, Kolliphor P188, Kolliphor EL, High shear homogenization

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ABSTRACT: Objectives: The aim of this work was the preparation of Clotrimazole (CM) loaded nanostructured lipid carriers (CM-NLC) and the examination of *in-vitro* antifungal activity of prepared formula. **Methods:** High shear hot homogenization method was used for CM-NLC preparation. Different ratios of Compritol AT 888 and Labrasol were used and combined with two types of surfactants: Kolliphor P188 or Kolliphor EL to stabilize NLC dispersion. The prepared formulae were tested for diameter, zeta potential, loading capacity, *in-vitro* release, differential scanning calorimetry (DSC), Transmission electron microscopy (TEM) and also for antifungal activity against strains of Candida albicans. **Results:** six formulae were successfully prepared with Z-average diameter ranges from 468.9 ± 3.7 down to 18.63 ± 0.964 nm, and high loading capacity of the drug (>50%) and Polydispersity index less than 1, indicating homogeneity of size. DSC images ensured drug solubility in the lipid matrix and dispersed in NLC in an amorphous state. While TEM images ensured that the CM-NLC formulation had a size below 30 nm and a spherical shape. *In-vitro* antifungal activity of formula NLC6 showed the largest inhibition zone, indicative of enhancement of drug activity. **Conclusion:** These results indicate that better homing of CM molecules and controlled drug release through NLC formulation may be a promising carrier for various anti-microbial and anti-fungal applications.

INTRODUCTION: Problems of solid lipid nanoparticles such as drug leakage during storage and insufficient total drug load can be overcome by nanostructured lipid carriers (NLC). It is formed of solid lipid at room temperature matrix with a variable content of liquid one [1]. This nano-drug delivery system has different advantages for the topical route application.

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The nature of being physiological and biodegradable lipids ensure low systemic toxicity and cytotoxicity [2]. Also, the used lipids are commercially approved as cosmetic excipients for topical pharmaceutical preparations. High drug penetration through stratum corneum is ensured due to nano range size of NLC.

Also, their solid lipid matrix help in maintaining the sustained release of entrapped drugs, which is of importance for prolonging the drug action and avoiding systemic absorption. Moreover, their ability in reducing the irritant effect of drugs and the formation of occlusive film [3-6]. CM is an imidazole anti-fungal agent, which is clinically administered both in orally and topical formulations. CM is used as a highly lipophilic model drug in the present study.

This research aimed to investigate the formulation and characterization of CM-NLC using different ratios of solid and liquid lipid ratios, with different surfactant types. Also, *in-vitro* antifungal activity of CM-NLC was investigated to elucidate the influence of nano-sizing of a particle on the activity of the drug.

MATERIALS: CM (gift from Pharco Pharmaceuticals, Alexandria, Egypt),Compritol® AT888 (Glyceryl behenate) (Gattefossé), Labrasol® (Caprylocaproyl polyoxyl-8 glycerides) (Gattefossé), Kolliphor® P188 (Sigma-Aldrich), Kolliphor® EL (Sigma- Aldrich), PHOSAL® 53 MCT (Lipoid- GmbH), all other chemicals and solvents are of HPLC grade.

METHODS:

Preparation of NLC with CM Model Drug: Different formulae were prepared according to Wissing *et al*., [7] with little modification. Briefly, 5 % w/w CM was dispersed in 10% w/w melted lipid mixture (different ratios of Compritol® AT888 and Labrasol® (60- 700).The dispersion medium (5 % w/w aqueous surfactant solution (either Kolliphor® P188 or Kolliphor® EL) [8] and 1% w/w Phosal® 53 MCT as co-surfactant) was heated to the same temperature and the hot lipid phase was emulsified in the dispersion medium by high-speed stirring, using an Ultra-Turrax homogenizer (Ultra- Turrax T–25, IKA, Germany) at 12, 000 rpm for 10 min, with 30 sec intervals every two minutes.

Detailed formulae codes and ratios are presented in **Table 1**. The dispersion thus obtained was allowed to cool to room temperature, forming lipid nanoparticles by recrystallization of the dispersed lipid.

TABLE 1: COMPOSITION OF NLC FORMULAE PREPARED WITH CLOTRIMAZOLE AS MODEL DRUG

Formula code	Lipid (Compritol /Labrasol)	Surfactant	PHOSAL® 53 MCT	Clotrimazole
NLC1	2:1	Kolliphor® P188	1 (% w/w)	5% w/w
NLC2	1:1			
NLC3	1:2			
NLC4	2:1	Kolliphor® EL		
NLC5	1:1			
NLC6	1:2			

Determination of Particle Size and Polydispersity Index (PDI): The particle size and polydispersity index of the formed NLC were determined by dynamic light scattering (DLS) using a photon correlation spectrometer (Zeta sizer, Malvern Instruments LTD, Malvern, UK) [9]. All measurements were done in triplicate, and the mean ± SD was calculated.

Zeta Potential Determination: The zeta potential of the NLC particles was measured using Zeta Sizer (Malvern Instruments LTD, Malvern, UK). Samples were placed in clear disposable cuvette, and results were recorded [10].

Drug Loading: The drug loading (D.L) refers to the percentage amount of drug entrapped in NLC according to the following equation:

DL = (Total amount of drug – amount of unbound drug) / (Nanoparticles weight) × 100

Samples were prepared in triplicate, and absorbances of CM were measured by spectrophotometrically at 243 nm using UV–Vis Spectrophotometer (Shimadzu double beam UV-visible spectrophotometer model UV- 1601PC connected to a Promax computer fitted with UPVC personal spectroscopy software version 3.7 (Shimadzu Corporation, Kyoto, Japan) [1].

***In-vitro* Drug Release Studies:** The release behavior of CM from prepared formulae was carried out using horizontal water bath shaker (Clifton water bath, USA) maintained at 60 cycles per min utilizing dialysis bag (Mw cut-off = 12 000 Da). The bags were soaked in distilled water for 12 h before use.

The release medium was 10 ml phosphate buffer (pH 5.5) [11]. The temperature was set at 32 ± 0.5 °C. A sample containing 5% drug was instilled in the bag held with two clamps at each end. At precise time intervals (0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h) the complete media were replaced by equal volumes of fresh buffer to maintain sink condition. The filtered samples were assayed for CM spectrophotometrically at 243 nm with Shimadzu double beam UV- visible spectrophotometer model UV- 1601PC connected to a Promax computer fitted with UPVC personal spectroscopy software version 3.7 (Shimadzu Corporation, Kyoto, Japan). The experiments were carried out as triplicate, and the mean values were calculated [12].

Statistical Analysis: Data were analyzed by using the program SPSS 17.0 (SPSS Inc., Chicago, IL, USA) one-way analysis of variance (ANOVA) test followed by post hoc multiple comparisons and(LSD) least significant difference formulae be significant at P<0.05.

Transmission Electron Microscopy (TEM): Formula showed the smallest particle size, and best release of CM was stained with phosphotungstic acid 2% w/v and placed on copper grids with Formvar films for viewing by a transmission electron microscope operated at 120 kV (JEOL-JEM-100CX EM) and operated using computer program named (AMT Image Capture Engine V601).

Differential Scanning Calorimetry (DSC): Samples (1-8) mg (CM, prepared formula, physical mixture, and Compritol AT888) were crimped in closed 40-µl aluminum pans. The heating rate of 10 °C /min under constant purging of nitrogen at 30 ml/ min and heated from 25.0 °C to 150.0 °C (except for samples of Compritol AT 888, it was heated to only 80 °C) using Shimadzu DSC-60, Kyoto, Japan, and Shimadzu DSC-60 data analysis. The references used for comparison were the same but empty aluminum pans.

Microbiological Assay of CM Nanostructured Lipid Carriers (NLC) (Disc Diffusion Method): Sterile discs were prepared for the CMNLC with the concentration 10 µg, 50 µg, and 100 µg/µl. In addition to, discs for the drug-free NLC and CM solution 5%. One drop from each concentration was placed in the pre-sterilized standard filter paper disc. The discs for each concentration were allowed to dry and kept in a glass Petri dish plates until use. Sabouraud Dextrose Agar (SDA) was weighted, dissolved, poured in tubes (10 ml) each and sterilized. SDA tubes were poured in plates and let harden. Fungal suspensions were prepared from fresh 24 h cultures. Five isolated colonies were suspended in sterile saline. After thorough mixing with a Vortex mixer, the turbidity of the suspension was adjusted to match that of a McFarland 0.5 turbidity standard at 530 nm [13, 14]. Inoculation of the plates was carried out by swabs impregnated in five *Candida albicans* (*C. albicans*) that isolated clinically(isolated and identified in the Department of Microbiology & Immunology, Faculty of Pharmacy, Suez Canal University), in addition to *C. albicans* ATCC 24433, in the surface of each plate. The drug discs were placed in the surface of the inoculated plates with the help of sterile forceps. SDA plates were incubated at 35 °C for 24 h and zones of inhibition were observed. Each isolate was tested in triplicates and the inhibition zone diameters were calculated by the average of the three readings for each concentration.

RESULTS:

Effect of Lipid Ratio on Physicochemical Properties of NLC: The change in lipid ratio (solid lipid to liquid lipid) affected Z-average diameter of formulated NLC as shown in **Fig. 1**.

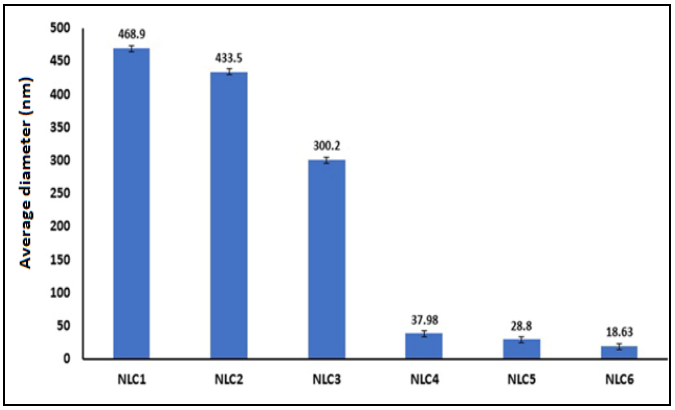

FIG. 1: Z-AVERAGE DIAMETER OF CM NLC

Increase amount of Labrasol (accompanied by meanwhile reduction of Compritol) or change ratio from 2:1 in NLC1 to 1:2 in NLC3 and also from 2:1 in NLC4 to 1:2 in NLC6, caused reduction in Z-average diameter from 468.9 ± 3.7 nm to 300.2 ± 2.97 nm for NLC prepared using Kolliphor® P188,

and from 37.98 ± 0.932 nm to 18.63 ± 0.964 nm for NLC prepared using Kolliphor® EL.

Meanwhile, as seen in **Table 2**, the polydispersity index (PDI) for all formulae showed values below 1, indicating homogeneity of formulae. Also, the table showed the values of zeta potential which indicated good stability and ranging from -13.8 ± 0.06 mV to -10.5 ± 0.5 mV for those prepared with Kolliphor® P188, while smaller values are noticed in case of those which prepared with Kolliphor® EL and ranging from 4.68 ± 0.03 to 6.05 ± 0.071 mV.

TABLE 2: P.D.I AND ZETA POTENTIAL VALUES OF CLOTRIMAZOLE NLC. * N=3

Formula code	Zeta potential (mV) means* ± S. D	Polydispersity index (P.D.I)
NLC1	13.8 ± 0.06	0.626
NLC2	12.1 ± 0.1	0.644
NLC3	10.5 ± 0.5	0.636
NLC4	4.68 ± 0.03	0.423
NLC5	6.89 ± 0.041	0.447
NLC6	6.05 ± 0.071	0.409

Loading capacity of CM into NLC was affected by lipid ratio as seen in **Fig. 2**, where it appears that percent of drug loading reduced from 78.3 ± 2.5 to 49.81 ± 1.46% for those which are prepared with Kolliphor® P188 and from 96.34 ± 4.2 to 57.3 ± 2.7% which prepared with Kolliphor® EL.

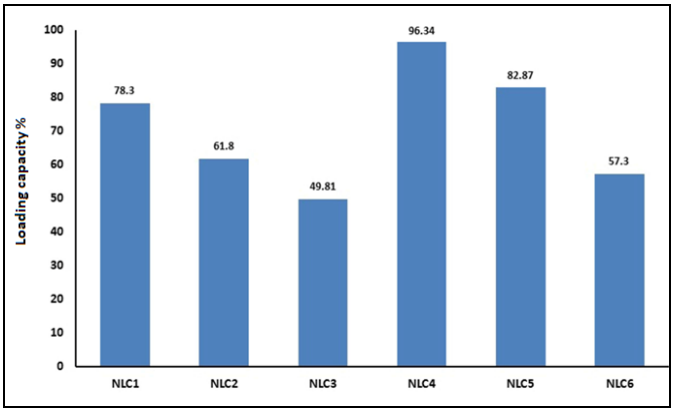

FIG. 2: LOADING CAPACITY OF CM INTO NLC

Effect of Surfactant Type on Physicochemical Properties of NLC: Two different surfactants were used in the formulation of CM NLC. Formulae (NLC1-NLC3) which are prepared with Kolliphor® P188 showed larger Z-average diameter more than (NLC4-NLC6) that prepared with Kolliphor® EL as regarded in **Fig. 1**, and so the values of zeta potential for (NLC1-NLC3) were higher than (NLC4-NLC6). On the other hand, CM was loaded in NLC prepared with Kolliphor® EL more than that prepared with Kolliphor® P188, as appeared in **Fig. 2**.

***In-vitro* Release Studies of CM:** It was notified from **Fig. 3** that release of CM from all prepared formulae was better than commercially available formulation as well as CM drug (negative standard). Formulae (NLC4-NLC6) showed better release efficiency that prepared using Kolliphor® EL, compared to formulae (NLC1-NLC3) that were prepared with Kolliphor® P188. Formula NLC6 showed complete *in-vitro* drug release.

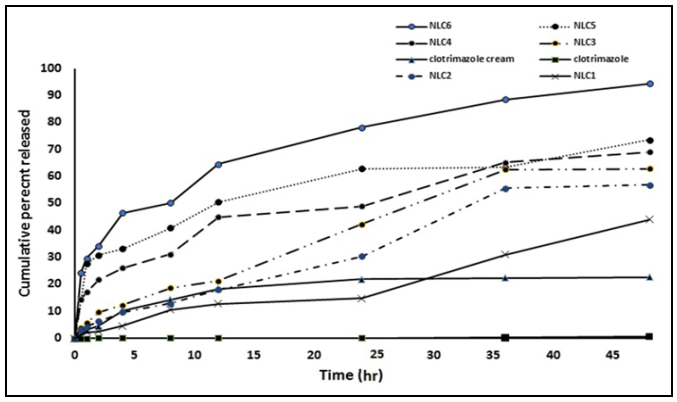

FIG. 3: EFFECT OF DIFFERENT FORMULATIVE FACTORS ON RELEASE OF CM

Differential Scanning Calorimetry of NLC (DSC): Formula NLC6, that showed the best release of CM, was further examined for its thermal stability. **Fig. 4A** showed DSC thermogram of Compritol AT888 (main constituent of NLC) with a sharp endothermic peak at 72.31 °C approximately, indicative of melting. **Fig. 4B** showed DSC thermogram of model drug CM with a sharp endothermic peak at 144 °C approximately. The physical mixture was prepared using model drug and Compritol with labrasol only with the help of hand mixing, tested for homogeneity of dispersion using drug-specific UV-spectroscopy. The DSC thermogram of the physical mixture in Figure 4C showed the characteristic peaks of both Compritol at 73°C with melting peaks of CM at 139 °C. **Fig. 4D** showed the DSC thermogram of NLC6prepared with 5% CM which was characterized by an initial endothermic peak at 73°C approximately which is characteristic for Compritol AT888 with an absence of characteristic endothermic peak of CM which may be due to absence drug in crystalline form and solubilization of it within lipid structure with enhanced stability.

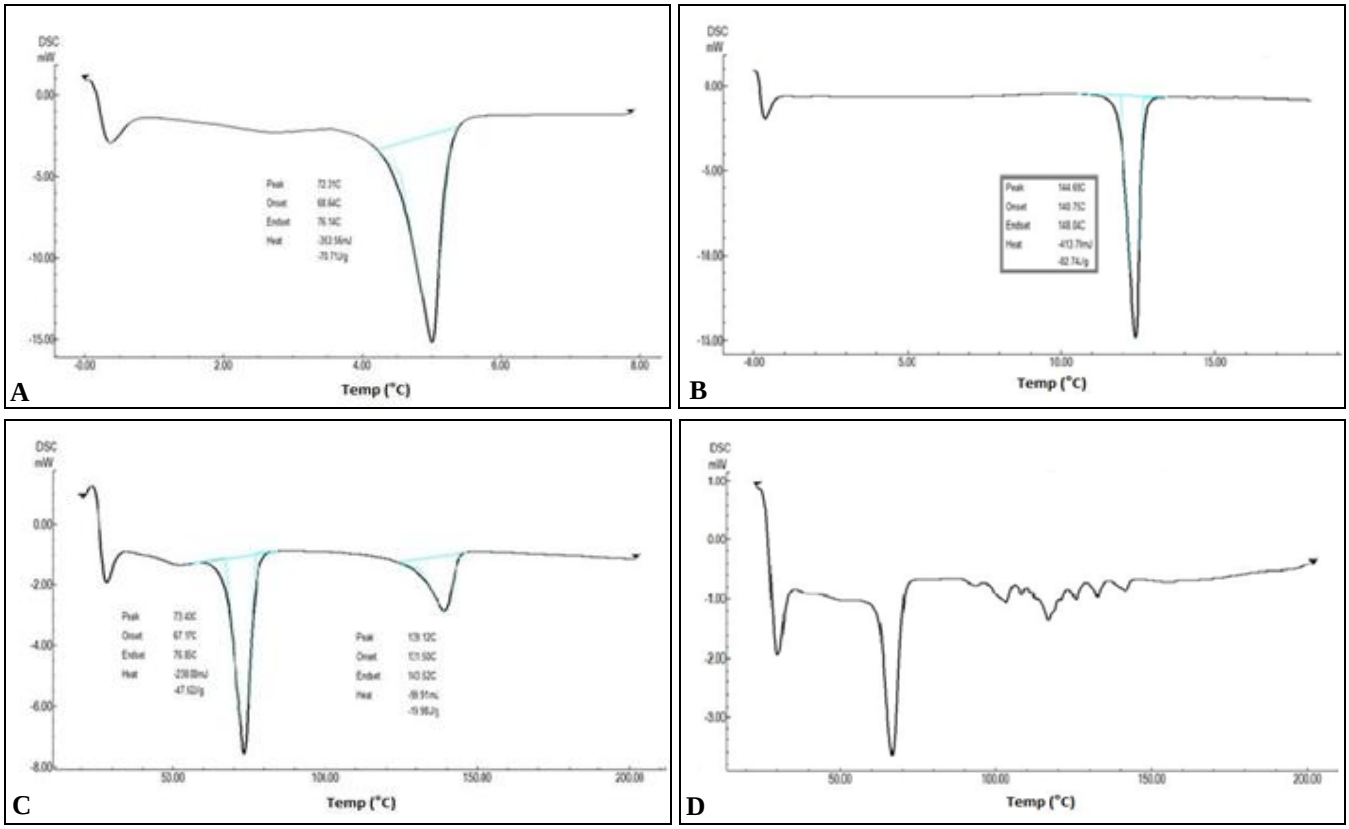

FIG. 4: DSC THERMOGRAMS OF (A) COMPRITOL AT888, (B) CM, (C) PHYSICAL MIXTURE OF NLC CONSTITUENTS AND DRUG, AND (D) NLC6 CONTAINING 5% CM

Transmission Electron Microscope imaging of NLC (TEM): Fig. 5 show the shape of the NLC6 entrapping CM. The prepared nanoparticles investigated reveal round and homogeneous shading; the micrographs also confirmed that the prepared NLC6 was less than 20 nm in size.

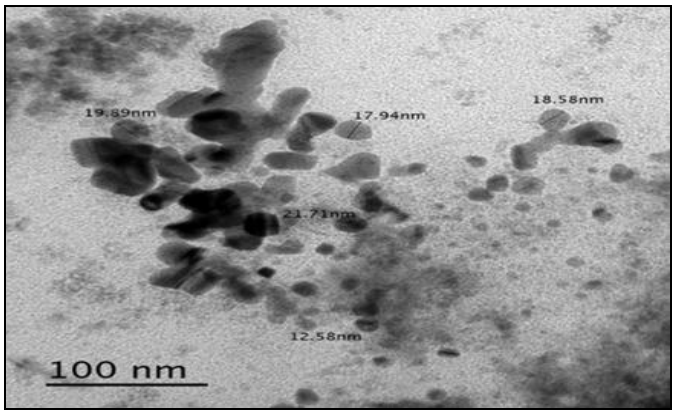

FIG. 5: TEM MICROGRAPH FOR NLC6 CONTAINING CM

Evaluation of *in-vitro* Antifungal Activity of CM NLC: Table 3 showed the results of *in-vitro* antifungal activity of NLC6 with 5% CM, NLC6 free from drug and also CM solution and diameters of inhibition zones for all the strains.

To compare the ability of both unprocessed CM and the nano-formulation of the drug (NLC6) to inhibit the growth of *C. albicans*, disk-diffusion assay was used. This method offers many advantages such as simplicity in procedures and results interpretation, low cost, and its ability to test several numbers of microorganisms and antimicrobial agents [14, 15]. CM in nano-formulation (NLC6) showed better inhibition of the tested organism with low dose compared to the unprocessed one. The drug used in this study was compared to the lowest concentration of the nano-formulation (as shown in **Table 3**).

TABLE 3: DIAMETER OF INHIBITION ZONES FOR ALL TESTED STRAINS UPON TREATMENT WITH DISCS IMPREGNATED IN FORMULA NLC6 (5% CLOTRIMAZOLE), NLC6 (DRUG-FREE) AND 5% CLOTRIMAZOLE SOLUTION

Tested organism	NLC6 with 5 % Clotrimazole			NLC6 (drug-free)	Clotrimazole 5% w/v
	10 µg	50 µg	100 µg		
ATCC strain	12 mm	13 mm	16 mm	None	12 mm
Clinical Isolate 1	12.5 mm	14 mm	17 mm	None	12.5 mm

Clinical Isolate 2	11.5 mm	13 mm	15.3 mm	None	11.7 mm
Clinical Isolate 3	12 mm	13.5 mm	15 mm	None	12 mm
Clinical Isolate 4	11.5 mm	12.7 mm	15.5 mm	None	11.3 mm
Clinical Isolate 5	12 mm	13 mm	16.5 mm	None	12.5 mm

DISCUSSION: Results showed the reduction of Z-average diameter with an increase of Labrasol amount and in the same time which prepared using Kolliphor EL, which may occur due to the higher solubility of CM as supported by Borhade *et al*., [16] who prepared CM nanoemulsion and studied different factors to enhance emulsification of lipids containing the drug. Also, our results were in full agreement with Gaba *et al*., results in [17] who prepared NLC of terbinafine HCl using labrasol and glyceryl monostearate by high-pressure homogenization and found that oil in NLC reduced particle size and PDI of prepared NLC, and also surfactant plays an important role in stabilization of formula. On the other hand, our results disagreed with Das *et al*., results in [18] who prepared NLC containing CM with Compritol AT888 and Labrafac using Cremophor EL as the surfactant, and found that increasing amount of solid lipid compared to liquid lipid, reduced Z-average diameter.

Loading capacity of CM into NLC increased inversely with particle size of formulae, whereas Z-average diameter reduced from NLC1 to NLC3, the drug loading increased in order of NLC1 > NLC2 > NLC3 and in the same manner NLC4 > NLC5 > NLC6 that may be due to that reduction in particle size, reduce spaces available for drug loading but in the same case, increasing the ratio of solid lipid content to liquid lipid, results in increasing drug loading due to prevention of drug escape. These explanations were fully augmented [19].

Moreover, we found that upon changing of surfactant from Kolliphor P188 to Kolliphor EL, Z-average diameter of NLC formulae reduced significantly with enhanced loading of CM, which agreed with Das *et al*., [18] who stated that non-ionic surfactant Cremophor EL with HLB value [12-14] is the optimum choice for production of stable nanoemulsion, while other polymer based surfactants like Pluronic F68 with high HLB (>24) which is structurally analog and of the same group of Kolliphor P188 (HLB =25-30), produced larger particle size. Also, drug loading was affected by using Kolliphor EL and 10% lipid, as this ensures high drug retention inside the lipid matrix by using a high concentration of surfactant (5%) [18]. Zeta potential values also were explained in favor of lipid concentration, type, and concentration of surfactant, and drug loading. Values of zeta potential showed values (< 20 mV) as seen in **Table 2**, that may be due to the using of non-ionic surfactants with concentration 5% and lipid concentration of 10%, with high drug loading [18]. Meanwhile zeta potential is not only the measure of nanoparticle stability as non-ionic surfactants can sterically stabilize the system by forming a coat around their surface although non-ionic surfactants do not contribute any charge to the particle; hence, but the charge also does not affect the stability of formed nanoemulsion [9, 19].

The results of the *in-vitro* release of CM from prepared formulae of NLC showed better release efficiency rather than the marketed formulation that had been tested against as well as unprocessed drug. Drug loading had an inverse effect on drug release, as NLC6 with the least loaded drug showed the best release among formulae prepared with Kolliphor EL, while NLC3 showed best release among formulae prepared with Kolliphor P188 [18-20]. The particle size of prepared formulae has major effect on release behavior of the drug, as formula NLC 6 has a smallest particle size, as mentioned earlier herein discussion, and showed the best release of CM due to increasing the surface area available for release of drug and smaller micelle size of Kolliphor EL [21-24]. From the above discussion, we considered NLC6 to be promising for further experiments. Thermal stability of NLC6 was confirmed through the loss of characteristic peak of CM, that ensures that drug lost its crystalline structure and completely dissolved in lipid matrix of NLC and converted to the amorphous state [18, 25]. Also, the peak heights and areas were further reduced, which may due to reduced crystallinity of the lipid matrix in NLCs, which should be due to the lipid mixture.

Images of transmission electron microscope showed that the size of particles of NLC6 lower than that obtained by laser diffraction technique;

this may be attributed to dehydration of NLC particles during preparation for TEM. The particle size analyzer measures the hydrodynamic radius of nanoparticles with hydrodynamic layers forming around them, leading to overestimation of the nanoparticles size [26-30].

The findings related to the *in-vitro* antifungal activity of NLC6 with CM 5% suggested the use of low dose CM NLC6 to treat susceptible *C. albicans* infections or to use a higher dose of it in treating the resistant *C. albicans* strains [24] with no dose increase compared to the CM itself or marketed formula. This may be especially useful in the case of the emergence of resistant *C. albicans* that causes mucosal candidiasis in HIV infected children, as the dose reduction mostly associated with the reduction of adverse drug reactions [31].

CONCLUSION: Nanostructured lipid carriers containing antifungal drug CM were successfully fabricated and showed spherical morphology with smaller particles size and no polymer-drug interaction. Lipid-based non-ionic surfactant Kolliphor EL produced smaller NLCs with better drug release rather than that prepared with the help of polymer-based Kolliphor P188. Drug loading of all prepared formulae was enhanced. Thermal stability of the prepared formula ensured complete solubility of the drug in lipid matrix with loss of crystallinity.

Transmission electron micrographs ensured the size of formula NLC6 less than 30 nm. All these physicochemical properties of the NLCs make them a suitable carrier system to deal with various microbial and fungal diseases. *In-vitro* antifungal activity of prepared formula showed better inhibition zone when tested versus unprocessed, that guarantee the activity of CM NLC with lower concentration.

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CONFLICTS OF INTEREST: There is no conflict of interest for all authors.

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