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Physalis peruviana oil based NLCs for dermal drug delivery

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ABSTRACT

Nanostructured lipid carriers (NLC) are a new progression in the area of lipid nanoparticles which are preferred for topical use. NLC possess reasonable solubility enhancement capacity of poorly soluble drugs, which create a high concentration gradient to aid the permeation of drug. The endeavor of this study was to develop and analyze *Physalis peruviana*oil based NLC. Theoilwas extracted by hot soxhlation using n-hexane as the solvent and the extracted oil wasutilized as an excipient (liquid lipid). Factorial design was adopted to optimize the amount of lipid blend (extracted oil and solid lipid), surfactant (Pluronic F-68) and sonication time to formulate NLC. The optimized formulations of fluconazole loaded NLC (PP-F-NLC) were evaluated by particle size, antifungal study, occlusion test, *in-vitro* skin permeation study and irritation test on animal skin. EE was found to be 99.96% and the optimized formulation showed 83% drug release in 24 hours. The formulated NLC showed better skin penetration, and potent antifungal activity against *Microsporum fulvum* as compared to marketed gel.It was concluded that the *Physalis peruviana* oil incorporated NLC is a promising formulation for fungal treatment and possesses high antioxidant potency.*Physalis peruviana* NLC can act as a potential carrier for anti-fungal/anti-inflammatory drugs to enhancethe purported pharmacological activity against various problems.

INTRODUCTION

Excipients are anintegral part of the drug delivery system. They play a major role in dosage forms such as, enhancing the dosage form volume, providing a pre-defined release (sustained/controlled/immediate), etc. A novel excipient is a substance which includes substances obtained through structural modification of an approved excipient(Swami, Gupta, Kymonil, & Saraf, 2010) and substance from a newer source, which has not been reported yet. Nanostructured lipid carriers (NLC) are colloidal lipoid systems that have been projected for numerous administration routes like oral, topical etc. NLC are produced by blending solid and liquid lipid,thus forming nanostructures with superior properties of drug loading, drug release profile and stability. Upon topical application, NLC show occlusive behaviour, adhesiveness and skin hydration effect (Desai, Patlolla, & Singh, 2010).

Physalis peruviana L. is a semi-shrub, herbaceous plant, the fruit of which is a juicy berry with an ovoid shape, protected by calyx and possesses many important medicinal properties. The major components of fruits are proteins, carbohydrates, lipids, vitamins, phytosterols, minerals, withanolides, physalins, etc (Puente, Pinto-Muñoz, Castro, & Cortés, 2011). *Physalis peruviana*oil can be extracted from whole berries, seed, pulp or peel of the fruit. Ramadan and Morsel (2003) reported the presence of 2% total lipid from whole berries, 1.8% from seed while 0.2% from pulp/peel. Most dominating lipid is linoleic acid. Oil also contains several other components such as β-carotene, vitamin K due to which it can be utilized for commercial purposes (M. F. Ramadan & Morsel, 2003). The current research envisages the utilization of *Physalis peruviana* oil as a novel excipient (liquid lipid). Fluconazole is taken as a model antifungal drug for evaluation parameters. The study mainly focuses on, whether the *Physalis peruviana* oil based nanostructured lipid carriers can be a good excipient-based approach for several biological problems.

MATERIALS AND METHOD

Physalis peruviana seeds and fluconazole were gifted from the Department of Horticulture, B.B.A. University,

Lucknow and Moraceae Pharmaceuticals respectively. Glycerol monostearate (GMS) was acquired from M/S Rolex Chemical Industries, Mumbai. Pluronic F-68 and polypropylene glycol were procured from M/S HiMedia labs pvt. Ltd, Dindori, Nashik. Chemicals utilized were of analytical grade. Fungus species *Microsporum fulvum* was purchased from IMTECH, Chandigarh.

Extraction of Physalis Peruviana Oil

Seeds of *Physalis peruviana* were dried and crushed to form powder. This powder was used for the extraction of oil by placing it inthe thimble and extraction was done by using n-hexane as a solvent in Soxhlet assembly. Solvent was then evaporated by stirring, pure oil obtained was stored in an air tight vial (Mohamed Fawzy Ramadan, Sitohy, & Moersel, 2008).

GC-MS of Physalis Peruviana Oil

0.1 gm of oil was dissolved in 2 ml n-hexane. 0.2ml of 2N methanolic potassium hydroxide was added and vortexed for 15min. The upper layer containing fatty acid methyl esters (FAME) was subjected to GC analysis. (Model: Perkin Elmer GC 480) 30m Elite-5 Columnusing Clarus flame ionization detector; oven temperature: 150℃ (1 min); 10psi nitrogen asCarrier gas with ramp 1: 5℃/ min to 230℃ for 5 min, Ramp 2: 150℃/min to 245℃ for 12 min; volume of injection: 1µl; internal standard: cetyl alcohol (Kaithwas, Mukerjee, Kumar, & Majumdar, 2011).

Antioxidant Property of Oil

The oil (1 ml) was taken and added in 1 ml of (2,2-diphenyl-1-picrylhydrazyl) DPPH (0.1 mM in ethanol) solution. This reaction mixture was incubated in dark environment for 20 minutes. It was then scanned through UV-visible spectrophotometer at 517 nm. DPPH solution was taken as a reference control. The free radical scavenging activity (in terms of percentage) was calculated by means of the formula given below (Gülçin, 2005; Neethu, Kumar, & Rao, 2015).

$$\%\text{DPPH scavenging effect} = 100 - \left[\frac{Absorbance_{Control} - Absorbance_{control}}{Absorbance_{Control}} \times 100\right]$$

Formulation of Physalis peruviana oil-fluconazole loaded NLC (PP-F-NLC)

Nine batches of NLC were designed (2^3 factorial design) using the software Design Expert 8.0.6.Glyceryl monostearate (solid lipid) and *Physalis peruviana*oil (liquid lipid) were heated at about 75°C to form a melt lipid blend. Fluconazole was added to the lipid blend and a clear melt blend (oil phase) was obtained. Pluronic F68 was dissolved in distilled water and heated to 75°C. Hot oil added to the aqueous phase with stirring (at 2000 rpm and temperature 75°C) for 1 hour by using mechanical stirrer (REMI 2MLH).Hot o/w emulsion formed was sonicated by Probe sonicator (Labsonic®M, 80amplitude, 9 cycles) for 30 to 60 minutes. PP-F-NLC were thus obtained by allowing it to cool at 4–8°C (Fang, Fang, Liu, & Su, 2008). PP-F-NLC1 to PP-F-NLC9 were made by varying the lipid (1-3%), surfactant (1-5%), sonication time (30-60 minutes) values at -1 and +1 levels respectively, keeping the dose of drug constant.

Characterization of PP-F-NLC

Particle size and Polydispersity Index (PDI)

PDI and sizeof PP-F-NLC was measured by Malvern Nanosizer ZS. PP-F-NLC samples were diluted 1/100 with double distilled water for an adequate light scattering intensity.

Entrapment efficiency (EE)

10 ml of mixture in the ratio of 1:1(7.4 pH phosphate buffer:ethanol) was prepared and to it, 1ml of dispersion of NLC formulation was dissolved.It was then allowed to stand for 15 minutes. Then the mixture was centrifuged (REMI CPR-24) at 15,000 rpm for 40 minutes at 25°C to separate the free drug.EE was measured by scanning supernatant in UV-visible spectrophotometer at 260nm and the drug entrapped in the NLC's was determined (Souto, Wissing, Barbosa, & Müller, 2004).

$$EE = \frac{\text{Initial amount of drug used} - \text{amount of free drug detected in the supernatant}}{\text{Initial amount of drug used}} \times 100$$

In-vitro Release Profile

In vitro drug release wascarried out as described by Jain *et al.*, 2008 with a little modification. The apparatus was washed and filled with pH 7.4 phosphate buffer at 37 ± 5℃. Formulation (1ml) was suspended in the sac made of cellophane membrane(12000-14000 Da MWCO) and tied to the paddle. At pre-determined time intervals, 5ml sample was taken and replaced with the fresh buffer. Aliquots were evaluated in UV-visible spectrophotometer at 260nm. The release studywas carried upto 24hours. The drug release data of the PP-F-NLC was fitted to various kinetic equations todeliberate the mechanism of drug release from NLC (Jain, Shah, Rajadhyaksha, Singh P. S, & Amin, 2008).

Electron Microscopy

The surface morphology was carried out using Transmission electron microscope (Model: JEM-2100), PP-F-NLC were diluted in Milli-Q water and sonicated for 2 minutes. It was then dropcasted on TEM grid, air dried and stored overnight. Sample was stained with uranyl acetate (1%w/v) and washed thrice with Milli-Q water. Sample was analyzed through the instrument at 7000 magnification and 200 kV accelerating voltage.

Occlusion Test

3 beakers (for PP-F-NLC, Zocon® and control) of 100 ml capacity with an internal diameter 3.9cm were selected. 50mL of water was added and sealed with Whatman filter paper. Undiluted aqueous PP-F-NLC dispersions and marketed preparation were uniformly spread over the filter paper, the beaker for control was left as such.

The samples were placed at 32 ± 1°C at 60 ± 5% RH for 48 hrs. Weights were noted after 6hrs, 24hrs, and 48 hrs. Beakers protected with filter paper lackingPP-F-NLC/ Zocon® served as reference. The experiment was carried out thrice.

$$\%F\ (occlusion\ factor) = \frac{A - B}{A} * 100$$

where A =water loss (reference), B = water loss (sample) (Okonogi & Riangjanapatee, 2015).

In vitro Antifungal study of PP-F-NLC

Antifungal activity of the formulation was evaluated by means of agar well diffusion method. Potato dextrose agar was poured into the sterile petri plates and was kept aside for few minutes within sterile condition till it solidified. Fungal culture was then uniformly spread above the mediawiththe help of sterile swabs. Wells (diameter = 6 mm) were made in the centerusing sterile cork-borer. Defined amount of PP-F-NLC lotion (being prepared in a cream base), saline (control) and marketed preparation were assigned separate wells. The petri plates were subsequently incubated at 27°C for 48 – 72 hrs. Presence of clear inhibition zones indicate antifungal efficacy. The cleared off zones were measured and recorded (Ambikapathy, Gomathi, & Panneerselvam, 2011).

Ex-vivo Studies

Institutional Animal Ethics Committee (Registration No. 876/AC/05/CPCSEA) approved protocol for the animal study was followed. Acclimatization of procured animals was carried out for at least seven days before experimentation. They were maintained on standard rat feed and water *ad libitum* (Abu-Elyazid, Kassem, Samy, & Gomaa, 2011).

Skin Irritation Study

The assessment for skin irritation was done on albino rats (weighing 150-200 g). The animals were kept randomly in three batches (standard, test and control), each batch containing six animals. One day before commencing the study, the dorsal hairs of rats were clipped off. Formulation was applied for 7 days continuously on the animals of batch used for test. Irritant used was 0.8% formalin. The animals were under observation for seven days for any signs of edema or erythema (Charde, Sharma, Choudhary, & Avari, 2014).

Permeation Studies

A patch of abdominal skin (2.5cm×2.5 cm) was excised and its hair, subcutaneous and connective tissue layers were detached. Diffusion cell with internal diffusion area of 1.54cm^2 was utilized to evaluate the rate and extent of skin permeation of drug from PP-F-NLC. Phosphate buffer; 25 ml; pH 7.4 was filled in receptor compartment. 5 mg drug equivalent sample was placedon the skin. Temperature was kept at 32 ± 0.5 ℃ and stirring condition was maintained at 200 rpm. 2ml samples were withdrawn from receptor zoneand replenished with buffer (Butani, Yewale, & Misra, 2014). Permeation flux was determined using the formula:

Permeation flux (J) = m/2;

where, m is the slope of curve

After 24 h, rat skin was taken off from the assembly, washed and made into small pieces. The acquired pieces were vortexed with methanol. They were soaked for 24hrs and the resulting mixture was then homogenized for 15 minutes and centrifuged for 10 minutes (at 10000 rpm; 25°C). Supernatant was collected and analyzed by UV spectrophotometer at 260 nm.

Evaluation for other applications of Physalis peruviana based NLC (PP-NLC)

Cytotoxicity Test (Against Human Skin Melanoma Cells)

Sulforhodamine B (SRB) assay on SK-MEL2 (a human melanoma cell line) was done. PP-NLC was diluted using Dulbecco's modified Eagle medium (DMEM), containing 2% inactivated FBS (fetal bovine serum), to prepare a stock of 5 mg/ml. 1ml diluted cell suspensionadded to each well in a 96-well plate. The supernatant was discarded after 24 h and defined amount of various concentrations of the test substance were added. 3-day-incubation was then done at 37°C. Trichloroacetic acid (TCA) at 4°C for 1hr was used in cell fixing and the excess was washed off. After 72 h, cells were washed and SRB solution was supplemented into the wells and was left to stain for 30 minutes and washed with acetic acid(1% v/v). 10mM tris buffer base (pH 10.5) was supplemented to each well. To extract the bound SRB gentle shaking of plates was done for 5 minutes. Triplicate readings were taken using a microplate reader. Growth inhibition of 50%, LC50 and total growth inhibition (TGI) values were determined (P. Singh *et al*., 2016).

RESULTS AND DISCUSSION

Physalis peruviana oil was successfully extracted using solvent extraction method. Extraction of oil using solvents had the advantage of greater oil recovery over aqueous extraction. In case of aqueous extracts, it is difficult to obtain good yield (Mohamed Fawzy Ramadan *et al*.,

2008). The GC graph of *Physalis peruviana* oil is depicted in Figure 1. The presence of saturated fatty acid, myristic acid, lignoceric acid, arachidonic acid and palmitic acid and unsaturated fatty acids such as, α-linolenic acid or esters (methyl lineolate, methyl tridecanoate) has been detected. These are the reported components of *Physalis peruviana* oil (M. F. Ramadan & Morsel, 2003).The free radical scavenging effect of the oil was found to be 55%. Based on this it can be said that oil acts as a free radical inhibitor (Gülçin, 2005).

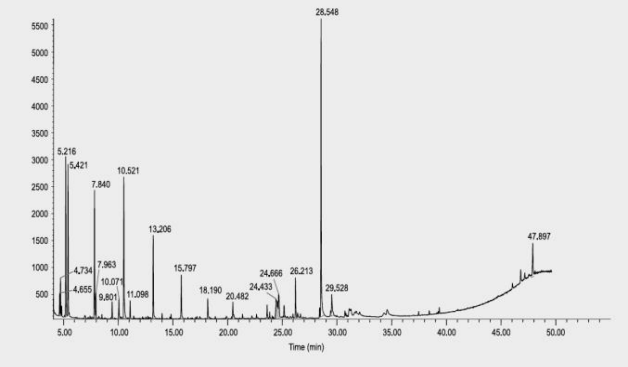

Figure 1: GC spectra of Physalis peruviana oil

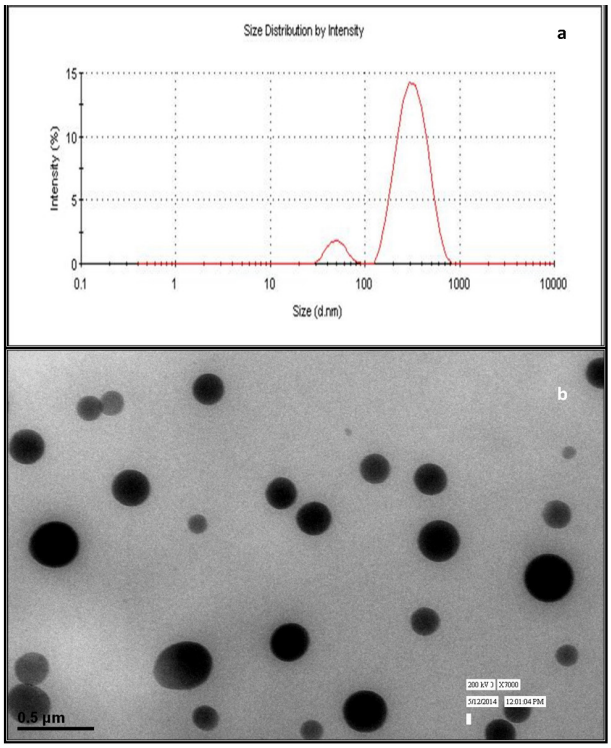

Figure 2: (a)Particle size distribution of optimized PP-F-NLC (b) TEM image of PP-F-NLC

The data for particle size, PDI and EE of all 9 batches is shown in (Table 1). Particle size criterion for selection was above 200 nm. The particle size and PDI of optimized batch was found to be 232.0 nm and 0.296 (Figure 2a). A PDI value below 0.5 was desired for homogeneity. EE of 99.96% was achieved in the optimized batch. The EE is mainly attributed to the affinity of the drug towards liquid and solid lipids. *Physalis* oil was found to have good miscibility with the lipids and surfactants used. The drug release profile of all the 9 batches is shown in Figure 3. The release of the drug depends on many factors, such as the nature of the excipient used, its concentration etc. The different batches of NLC showed drug release ranging from 64.96% to 93.75% over a span of 24 hours. Marketed preparation showed immediate release of more than 80% within 1.5 h, which was maximum remained constant till 24 h. PP-F-NLC showed sustained release (because of drug being entrapped within surfactant and lipid layer). Effect of each dependent parameter on independent parameters is shown by the help of contour plots (Figure 4) and overlay plots (Figure 5). A slight decrease in particle size was established with increase in lipid concentration and the lowest particle size was

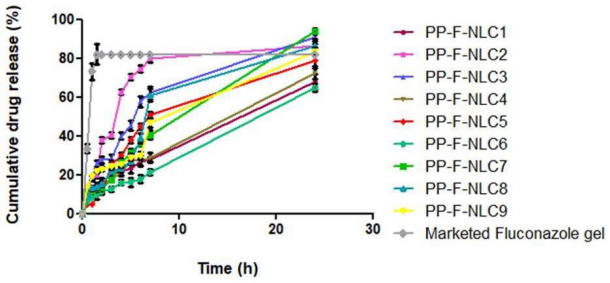

Figure 3: Release pattern of PP-F-NLC batches and Marketed gel

Table 1: Composition of *Physalis peruviana* oil and Fluconazole loaded NLC (PP-F-NLC)

Formulation	*Lipid (solid :liquid) 70:30(%w/v)*	*Surfactant (% w/v)*	*Sonication Time (min)*	*Particle size (nm)*	*Polydispersity Index*	*Entrapment Efficiency (%)*	*Cumulative drug release in 24 hrs (%)*
PP-F-NLC1	1	1	30	699.1 ± 50.4	0.064 ± 0.002	99.96 ± 0.02	67.98 ± 3.98
PP-F-NLC2	3	1	30	260.0 ± 18.9	0.329 ± 0.024	99.96 ± 0.01	86.3 ± 2.35
PP-F-NLC3	1	5	30	700.0 ±61.3	0.084 ± 0.006	99.96 ± 0.01	91.04 ± 3.63
PP-F-NLC4	3	5	30	238.3 ± 12.8	0.455 ± 0.042	99.95 ± 0.01	72.1 ± 2.64
PP-F-NLC5	1	1	60	172.8 ± 18.9	0.299 ± 0.016	99.96 ± 0.02	78.6 ± 2.97
PP-F-NLC6	3	1	60	183.4 ± 16.8	0.256 ± 0.062	99.96 ± 0.01	64.96 ± 2.1
PP-F-NLC7	1	5	60	273.3 ± 33.2	0.274 ± 0.073	99.95 ± 0.02	93.75 ± 1.43
PP-F-NLC8	3	5	60	234.7 ± 44.2	0.239 ± 0.033	99.96 ± 0.01	86.1 ± 3.52
PP-F-NLC9	2	3	45	232.0 ± 12.5	0.296 ± 0.024	99.96 ± 0.01	83.23 ± 1.93

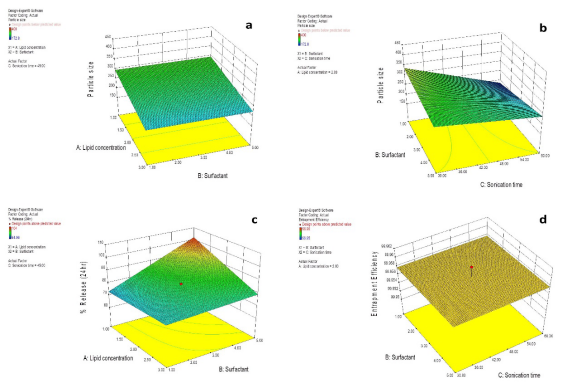

Figure 4: 3D plots showing the effects of various parameters on particle size, release and EE: (a)Effect of lipid concentration and sonication time on particle size; (b)Effect of surfactant concentration and sonication time on particle size; (c)Effect of lipid and surfactant concentration on EE; (d)Effect of lipid and surfactant concentration on drug release.

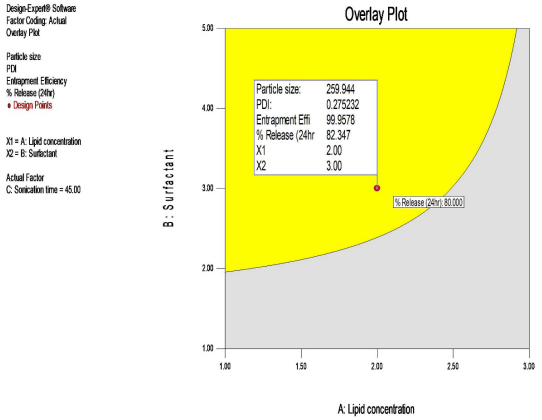

Figure 5: Overlay plot showing the optimized formulation

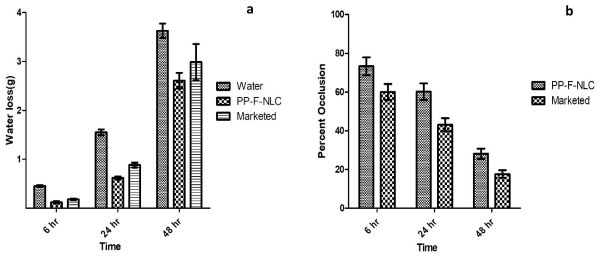

Figure 6: (a) Amount of water loss,(b) Percent occlusion of marketed formulation and PP-F-NLC

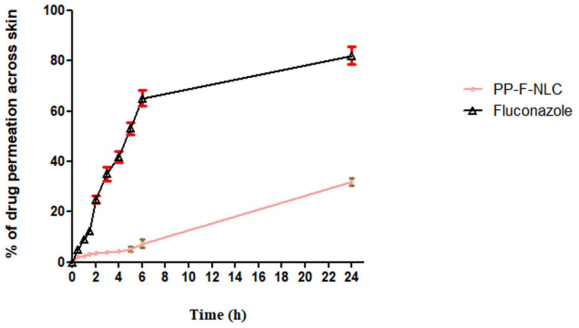

Figure 7: Permeation study of PP-F-NLC and Fluconazole

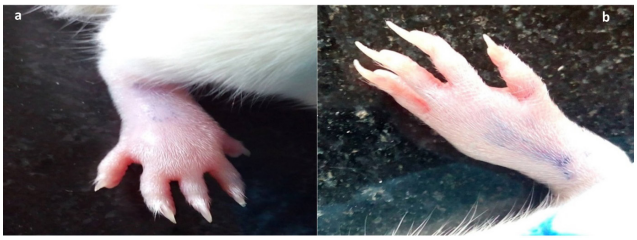

Figure 8: Improvement in the inflammatory reaction (a) before treatment, (b) PP-NLC treated

observed at higher level of sonication time.Sonication time increase leads to breakdown of coarser particles into smaller ones due to intense energy surge. The mean PDI values for the drug loaded NLC formulations were in the range of 0.064 to 0.455, hence all formulations expressed polydispersity. The EE remained constant at 99.96% to 99.98% for various factor level combinations. The drug release increased linearly at lower to middle level of lipid concentration on increasing the surfactant concentration. The surfactant concentration does not seem to have a much effect at a higher level of lipid concentration but as the lipid concentration was increased, drug release was found to decrease. The composition of optimized formulation was 2% lipid, 3% surfactant and 45 min sonication time. After analysis of each data, PP-F-NLC9 was selected as the optimized formulation for further studies. The optimized formulation PP-F-NLC9 followed higuchi model, which indicated that the release of drug from PP-F-NLC9 involved dissolution and diffusion both (Gouda, Baishya, & Qing, 2017). Transmission electron microscopy displayed smooth surface and spherical shape of PP-F-NLC and the particle diameters were between 200-250nm(Figure 2b).Occlusion property of lipidic nanoparticles increases the skin hydration. They possess good adhesiveness because of high van der Waal forces between the particles and contact surface. PP-F-NLC showed the high occlusion value during 24 h, and 48 h (Figure 6 a and b).PP-F-NLCshowed the highest occlusion factor (Hamishehkar *et al*., 2015). Permeation of drug fromPP-F-NLC through excised rat skin is given in Figure 7. It displays burst release of drug followed by a steady state. The permeation flux (J) was found to be 0.349 mg/cm^{2}h. The retention of drug on dermal layer of optimized PP-F-NLC was 47.03%, which gave an evidence of the good retention of drug in the skin. 31.94 % of the drug passed through the skin. The enhanced retention of fluconazole within skin layers in comparison to the receptor compartment is due to the sustained release properties of NLC, and increased contact with the corneocytes. The small size enables closer contact with superficial corneocyte junctions and channels between

the corneocytes and favors accumulation of drug for a longer duration (Rajinikanth & Chellian, 2016).

The anti-fungal efficacy of the developed PP-F-NLC lotion was significantly effective (Zone of inhibition = 2.733 ± 0.95 cm) in comparison to Zocon® (showed zone of inhibition = 1.86 ± 0.11 cm). In our knowledge, the efficacy of *P. peruviana* has been reported against several fungi but not against *Microsporum* sp (Singh, Singh, Tripathi, Arya, & Saraf, 2016). The results of skin irritation test revealed that, optimized PP-F-NLC and marketed gel safe for use, as there was no erythema (redness) or edema seen after application of PP-F-NLC lotion on the rat skin(Figure 8).Cytotoxicity i.e.,$LC_{50}$of *Physalis peruviana* NLC (without drug) was found to be 66.6 µg/ml. Total growth inhibition was observed at of 45.3 µg/ml and GI_{50} value was found to be 24 µg/ml.

CONCLUSION

Physalis peruviana based nanostructured lipid carriers were formulated (taking Pluronic F68 as a surfactant, GMS as solid lipid and model antifungal drug fluconazole for few studies). PP-F-NLC followed Higuchi kinetics and sustained the release of drug in concentration independent manner.The formulation not only proved to be effective against *Microsporum fulvum* but also gave desired anti-inflammatory activity and some cytotoxicity and growth inhibitory against human melanoma cancer cells. The GC results confirm the presence of saturated and unsaturated fatty acids in the extracted oil. The oil of *Physalis peruviana* seeds was utilized as liquid lipid during formulation of NLC in conjunction with GMS. During preliminary studies fluconazole was found soluble in the extracted oil. Solubility is an important criterion for selection of liquid lipid. The permeation study in comparison to pure drug also emphasizes the positive effects of fatty acids on skin permeation as the formulation possessed better permeation abilities than the fluconazole alone. It can be successfully concluded that *Physalis peruviana* oil based NLC can be a safe and suitable alternative for lipid-based formulations.

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