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Fabrication and functional attributes of nanotherapeutically engineered system for co-administration of adapalene and lycopene for enhanced acne management: an in vitro and in vivo investigation

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Abstract

Background Acne vulgaris is caused due to several different biological, environmental and specific pathological factors, resulting in secretion of unregulated amount of sebum by the sebaceous glands. Acne is thought to be a minor, self-restraint, puberty leading and hormone-induced cosmetic disorder. It significantly affects the quality of life leading to social disengagement, anxiety, depression, necessitating its therapeutic management in due course of time. Thus, despite not being fatal it has social and mental impact on individual's life. Pathogenesis of acne is complex and multifactorial involving diverse physiological, environmental factors and significant role of propionibacterium acne. Retinoids have been used as preferred first-line therapy for treatment of acne, but they have many limitations. Adapalene has found to be acting synergistically with other drugs like benzoyl peroxide, salicylic acid, clindamycin, etc., and various other topical antioxidants.

Results The study investigates the formulation and characterization of novel nanostructured lipid vesicles (NLVs) co-loaded with adapalene and lycopene, through topical gel. The high-pressure homogenization method was used to prepare nanostructured lipid vesicles followed by incorporation into hydrophilic gel vehicle. This novel system was scientifically tested for various evaluation parameters like size and vesicle size distribution, polydispersity index, drug entrapment efficiency and in vitro drug release characteristics, along with rheological behavior of formulation gel. Skin permeation and biodistribution characteristics through skin were also evaluated, and better localization of drug in dermis and reduced systemic penetration were the key observation in prepared formulation.

Conclusion The simultaneous co-administration of lycopene resulted in an additive effect in therapeutic management of acne. It can be predicted that this novel NLVs-based gel along with synergistic effect of lycopene will definitely result in better therapeutic efficacy and reduced systemic adverse effects of adapalene. This combination can be explored as future alternative for acne therapy.

Keywords Acne vulgaris, Adapalene, Lycopene, Rheology, Nanostructured lipid vesicles (NLVs), Topical delivery

1 Background

Acne vulgaris is a multifactorial disease caused due to over-production of sebum by sebaceous glands, hormonal change during adolescent growth, or by attack of bacteria *Propionibacterium acnes* [6]. Acne is thought to be

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common inconsequential, self-restrained minor cosmetic concern, which is mostly not very serious during its initial stage but on its progression results in reduced self-esteem, mental occupation for individual's appearance and many other emotional concerns. Although disease is not fatal, still it can lead to significant concerns, including despair, anxiety, social disengagement, etc., making its timely treatment important [7]. All trans retinoic acids (RA) have been effective in treating mild to moderate acne topically [22]. Natural or synthetic derivatives of vitamin A are known as retinoids and are still among the most effective comedolytic agents. Normalization of follicular keratinization and subsequent prevention of new microcomedos, comedos and inflammatory lesions are their principal mechanism of action [3]. Lycopene is useful as a complementary adjuvant assisting in synergistic effect of primary drug due to its anti-inflammatory as well as antioxidant activity along with it also neutralizes free radicals of skin, guards intracellular structures against oxidative stress and donates electrons in aqueous compartment of the cell [18].

Retinoids are natural or synthetic derivative of vitamin A. Being lipophilic, they easily penetrate epidermis and cause cellular differentiation and proliferation. They can also induce modulation of gene expression in diseased individual [31]. Although retinoids have been used historically but ever since their application, especially of first-generation retinoids, they have not been very safe and satisfactory due to burning or tingling sensation to skin. Their uses have also been limited due to their phototoxicity.

Adapalene (ADP) is a new third-generation polyaromatic synthetic naphthalene carboxylic acid derivative (having functional similarity with retinoid) approved in 1996 by USFDA for the treatment of acne. It selectively binds to retinoic acid receptors (RAR) β and RAR γ, reduces lipoxygenase activity and is capable of inducing changes in gene expression and mRNA synthesis. It has strong keratinocyte metabolism activity and is good modulator of keratinization of hair follicle and increased proliferation and hence preferred in acne therapeutics [33]. The selective binding to only nuclear retinoic acid proteins RAR β and Υ and not to cytosolic retinoic acid receptors results in greater and better tolerability of adapalene. Superior anti-inflammatory effect on comedos is due to inhibition of neutrophil chemotaxis and suppression of neutrophil lipoxygenase [2, 30]. Exact pathogenesis of acne is still under investigation. But abnormalities like acne involve presence of excessive free radical oxygen species, and various researches have justified the importance of antioxidant molecules in therapeutic management of acne and related disorders as second-line supportive treatment [21].

Lycopene is a red lipid-soluble unsaturated hydrocarbon. It is a tetraterpene non-pro vit A carotenoid. It is an excellent phytonutrient and found in abundance in tomatoes and tomato-based products [14]. It has been studied extensively for its strong antioxidant and antiaging effects that can alleviate damaging effects of oxidative stress. Lycopene has also been found effective in ameliorating tumor insurgencies, atherosclerosis and other cardiac complication, hepatic, neural and stress-mediated inflammatory disorders and various other dermatological disorders [10]. Lycopene is a dietary antioxidant which has limited natural bioavailability, and it has been observed that regular intake of lycopene in diseased skin conditions can improve morphological characteristics of skin [5].

Higher drug loading capacity, prolonged duration of action, better dermal localization as well as reduced systemic penetration in comparison with traditional cream/gel-based formulation are some key advantages which make these nanocarrier-based formulation as superior alternative delivery carriers for enhanced therapeutic management of acne [13, 17].

Nanostructured vesicles have been explored as most promising carriers comprising of non-toxic biodegradable lipids, having most desirable properties of formulation like flexibility of formulation, better localization to skin along with reduced toxicity and large-scale production capacity. Solid behavior is at both room and body temperatures [24, 26]. Better physical stability, possibility of modification of drug release and lesser sensitivity of encapsulated drug to chemical degradation are some important parameters that justify NLVs as a superior colloidal drug delivery carrier system compared to polymer-based micelles, liposomal vesicles, emulsified systems and polymeric nanoparticles [25]. The current study was done to explore possibility of newer system having encapsulated ADP and LYC inside nanocarriers which further incorporated into carbopol-based hydrogel to investigate simultaneous prolonged antiacne activity of ADP along with antioxidant benefits of lycopenes. Here, we have examined that this newer carrier of ADP and lycopene provided better drug protection of ADP inside carriers and enhanced pharmaceutical behavior and customized drug release of APIs in comparison with individual pharmaceutical moiety. Apart from these thorough rheological aspects of prepared hydrogel were also studied.

2 Methods

2.1 Materials

Drug adapalene was obtained from Glenmark Pharmaceuticals Ltd. (Nasik India). Phospholipid (100-S) was a kind gift from Lipoid, Ludwigshafen Germany. Lycopene, tristearin and triton X-100 were availed and purchased

from Sigma-Aldrich. Carbopol 940 was availed from CDH, New Delhi. Gattefosse, France, generously provided labrasol as sample. Dialysis bags of cellulose having molecular weight cutoff of 10 kDA bought from HiMedia (Mumbai, India). Certified nylon membrane filters (0.20 micron) were availed from Pall Gelman Sciences (USA). De-ionized and purified water was used throughout experimental protocol.

2.2 Preparation of ADP and LYC co-loaded nanostructured lipid carriers (NLVs-A-LYC)

Classic high-pressure homogenization method with reported protocol was used to prepare NLVs. Briefly to prepare 66.5gm of ADP and LYC co-loaded NLVs suspension (NLVs-A-LYC), 0.1 gm of ADP and 0.05gm LYC were carefully dissolved in perfectly molten lipid phase consisting of 1% w/v mixture of lipid tristearin and labrasol (4:1) with 0.3% w/v of phospholipid 100-S in 10 ml (1:1 v/v) acetone-ethyl alcohol mixture. 0.2% w/v of tween 80 dissolved in water to prepare aqueous phase. Now lipid phase and aqueous phase (50 ml previously prepared) heated individually at a temperature of 70 °C. After that, the lipid was slowly added into aqueous phase and homogenized (IKA) for approximately 30 min at 4000 rpm. Now weight of the mixture was adjusted with hot water up to 66.5 gm. After that above-prepared mixture was probe sonicated at 3 W for 2–4 min (Misonix-3000, QSonica, LLC, Newtown, CT). After completing homogenization, lipid mixture was further cooled to room temperature for rigidization of lipid droplets resulting in an aqueous NLVs dispersion of 0.15% of ADP. Only ADP-loaded NLVs (NLVs-A) were also prepared using the same method except addition of LYC which were further used to compare synergistic therapeutic effect of LYC.

2.3 Characterization of NLVs-A

2.3.1 Particle size and zeta potential of NLVs-A

Average particle size, polydispersity index (PDI) and zeta potential were analyzed by Zetasizer (PCS, Nano-ZS90, Malvern Instruments Corp, UK) at temperature 25 ± 0.1 °C keeping a fixed angle of 90° to incident beam 25 ± 0.1 °C. For this samples were appropriately dispersed and diluted with filtered demineralized water and observed for laser light diffraction and scattering pattern at fixed angle of 90°. Sample analysis was done in triplicate, and mean average values were reported.

2.3.2 Transmission electron microscopy (TEM)

Size and surface morphology of prepared system were analyzed by TEM using Philips CM 10 electron microscope at 200 kV voltage. Briefly, the sample drop was mounted on a carbon-coated copper grid to make a thin film on the grid. Before complete drying, this film was negatively stained with 1% phosphotungstic acid. At suitable magnification and voltage photomicrographs were taken.

2.3.3 Determination of entrapment efficiency of ADP and LYC

The above-prepared NLVs-A-LYC was placed inside dialysis bag (MWCO 10 kDa) and dialyzed extensively for 10 min against double-distilled water (DDW) using magnetic stirrer (50 rpm), and sink conditions were maintained during whole process to remove any traces of un-entrapped drugs from carrier vesicles. Samples were precisely collected in HPLC vials and diluted with solvent mixture (methanol and dimethylformamide). The drug ADP was analyzed by isocratic HPLC analysis using Merck RP-8 column (250 mm × 4.6 mm particle size 5 µm) as reported earlier [19] with slight modifications, column was previously degassed using ultrasonic bath, and acetonitrile–water (65:35 v/v; the pH adjusted to 2.5 with phosphoric acid) was used as mobile phase (flow rate, 1.3 ml/min). The volume of injectable was 20 µL for all solutions, and 321 nm was used as detection wavelength for adapalene [19]. The entrapment efficiency (EE) of adapalene was calculated using the equation given below:

$$\text{Entrapment Efficiency}(\%) = \frac{\text{Total amount of drug added} - \text{Amount of unentrapped drug in the collected sample}}{\text{Total amount of drug added}} \times 100$$

2.3.4 Determination of entrapment efficiency of lycopene

For determination of entrapment efficiency of LYC in NLVs-A-LYC, prepared NLVs-A-LYC formulation was placed inside dialysis bag (MWCO 10 kDa) and using a magnetic stirrer these dialysis bags containing NLVs-A-LYC were extensively dialyzed for 10 min against double-distilled water (DDW) and sink conditions were maintained during whole process to remove any traces of un-entrapped lycopene from carrier vesicles.

In brief, for chromatographic separation reversed phase C18 column (10 µm, 4.6 mm × 250 mm) was used. The mobile phase consisted of a 70:15:15, v/v/v blend of methanol: ethyl acetate: acetonitrile. The volume of injection was 5 µL, and the analysis was performed at 472 nm at 1.5 mL/min of flow rate [14]. The percent entrapment efficiency (PEE) was estimated by utilizing following formula

$$\text{PEE} = \frac{\text{Amount of LYC added in the formualtion} - \text{Amount of unentrapped LYC in collected sample}}{\text{Total amount of LYC added}} \times 100$$

2.3.5 In vitro drug release study

The concept of diffusion of drug through dialysis tube was used to estimate of in vitro release of entrapped drug from prepared formulation. This was accomplished by filling a dialysis membrane (MWCO 10–12 kDa) with 10 ml of NLVs-A-LYC dispersion free of any unentrapped medication. Both ends tied dialysis membrane was kept suspended in a separate beaker containing 20 ml 75% v/v Methanol: DMF (50:1) in PBS (pH 5.6) [12]. The beakers were continuously stirred at constant temperature (32 ± 1 °C) at 100 rpm in magnetic stirrer. One milliliter of the sample was drained out at specific time interval and was replaced with the same volume of fresh solvent in order to maintain sink conditions of system mixture in a receptor compartment [11, 34]. To quantify amount of ADP, samples were analyzed by earlier reported HPLC methods as described in Sect. 2.3.

In vitro drug release profile of Lyc was estimated in 0.1N HCl containing 1% tween-80 and phosphate buffer (10 mM; pH 5.6) containing little amount of DMSO (in 5: 1 ratio). In brief, NLVs-A-LYC (equivalent to 10 mg of LYC) was dispersed separately in 2 mL of release medium and located individually into dialysis bags (MWCO: 10 kDa). The bags were subsequently suspended into 100 mL of release medium keeping it continuously agitating. Aliquots of 500 µL were withdrawn intermittently for specified time interval. After each withdrawal, 500 µL of release medium replenished to recompense the volume of the receptor compartment. Amount of LYC released was measured by the validated HPLC technique, described previously. The experiment was performed in triplicate [14].

2.3.6 Estimation of viscoelastic behavior

Viscoelastic behavior of our final formulations NLVs-A gel was analyzed by amplitude sweep test method. Samples were subjected to increasing shear rate from 0.1 to 100 s^{-1}, and viscoelastic behavior of gel was noted. Kinetic parameters G′ (storage modulus), G″ (loss modulus) and shear stress (Ƭ) were measured by amplitude sweep test, and linear viscoelastic region (LVR) was analyzed. LVR tells about structural deformation of semisolid. The dynamic moduli (G′ and G″ in Pa) and shear stress were determined as function of shear strain ranging from 0.01 to 100 Pa and at a constant frequency of 1 Hz.

2.4 Preparation of adapalene-loaded NLVs gel (NLVs-A gel) and adapalene-loaded NLVs gel consisting lycopene (NLVs-A-Lyc gel)

Carbopol was selected as gel-forming material, and after preparing primary slurry of carbopol, NLVs dispersion was incorporated into it. In brief, concentrated Carbopol 940 gel base was prepared by dispersing Carbopol 940 in 25 g of water. For this purpose, 1 g carbopol 940 was dispersed slowly in doubled distilled water preheated at 40 °C under constant stirring (300 rpm). The mixture was allowed to stir for 30 min for complete hydration of Carbopol 940. Then, in this above-prepared carbopol gel slurry, prepared NLVs-A-LYC consisting 0.1 g of ADP and 0.05 g of lycopene was incorporated intimately and mixed for further 10 min. The pH of the mixture was adjusted by gradual addition of tri-ethanolamine under stirring (150 rpm). The pH of the gel was set to 6.2 ± 0.1 [29]. In a different vessel 0.1 g of butylated hydroxy toluene (BHT) was dissolved in 1 g of benzyl alcohol under stirring. The mixture of BHT and benzyl alcohol was added to the gel preparation and gel was mixed slowly for further 10 min.; then, weight of the preparation was made up to 100 g with double-distilled water, so the concentration of ADP and lycopene remained 0.1% w/w and 0.05%w/w, respectively. The same procedure was followed to prepare NLVs-A gel by using NLVs-A in gel base.

2.5 Apparent viscosity and rheological behavior of the gel formulations

At 25 °C, the apparent viscosity and rheological behavior of NLVs-A-LYC gel were assessed using a dynamic rheometer (Anton Paar, Germany) equipped with a cone and plate test geometry (cone diameter, 75 mm, cone angle 0.999°) and instrument software Rheoplus. Samples were mounted over the plate, and parameters were selected as per instruction of manufacturer. The apparent viscosity of the NLVs-A-LYC gel was then tested at a shear rate of 10/s. Flow curve test was used to determine flow behavior of the gel samples [4]. In brief the viscoelastic behavior of samples was measured as a function of shear rate (in s^{-1}, range 0.001 to 100 s^{-1}) by measuring viscosity (Pas) and shear stress (T).

In addition the overall performance of any semisolid formulation depends on rheological attributes of finished product, which in turn depend on internal microstructure as well as manufacturing conditions of product. Thus, to assure overall performance like spreadability

over skin and mucosal surface, a dynamic modular compact rheometer (Anton Paar, MCR-92) equipped with synchronous motor with a plate and plate test geometry (plate diameter, 50 mm) was used to analyze the rheological behavior of NLVs-A-LYC gel at 25 °C. Following equipment guidelines and SOPs samples were placed over plate. The apparent viscosity of the samples was recorded using instrument software Rheoplus, and flow behavior of sample was determined by flow curve test. The test was performed by measuring the viscosity (Pas) and shear stress (τ) as a function of shear rate (γ in s^{-1}, range from 0.1 to 100 s^{-1}). Our final formulation NLVs-A-LYC gel was subjected to different shear stress and shear rate conditions. The shear stress was increased linearly from 0.1 to 100 s^{-1}, and viscoelastic behavior of samples was recorded using amplitude sweep test. By measuring kinetic parameters G' (storage modulus), G'' (loss modulus) and shear stress (Ţ) through amplitude sweep test, the linear viscoelastic region (LVR) was analyzed. The dynamic moduli (G' and G'' in Pa) and shear stress were determined as function of shear strain ranging from 0.01 to 100 Pa and at a constant frequency of 1 Hz. The LVR provides critical information about viscoelastic flow behavior; it is important for determining the lowest strain of structural deformation of semisolid required to soften and to flow. Besides NLVs-A-LYC gel formulation, NLVs-A gel was also assessed for viscosity and rheology.

2.6 Skin permeation study

In vitro transdermal permeation potential of drugs was predicted by using relevant model of human skin and porcine ear skin [1, 9, 27]. Due to various concerns and compulsions both modal skin were available in limited amounts. Porcine ear skin was employed in the skin permeation studies in vitro due to its relatively higher permeation capacity and easier availability [8]. Thus, pig ears were procured from the local slaughter house, and all experimental protocols were approved by the Institutional Animals Ethical Committee, Bhagyoday Tirth Pharmacy College, Sagar, M.P., India (Ref. letter no.0604/BTPC/2021-22/12). CPCSEA guidelines were strictly followed during all the animal experimentation. From the selected excised thick pig ear skin adherent subcutaneous fat was removed carefully and then washed with saline [16]. Then in a Vertical Franz Diffusion (FD) cell this prepared skin was surmounted and 25 ml of solvent mixture having 75% (v/v) methanol and DMF (50:1) in PBS (pH 5.6) was filled in receptor compartment of this FD cell. The stratum corneum side of prepared skin was positioned toward donor (upward) compartment, and dermis part of skin was kept into the receptor (downward) compartment. The medium of receptor compartment was constantly stirred with magnetic stirrer and by using recirculating water bath. The whole assembly was maintained at 32 ± 0.5 °C [32]. The donor compartment facing stratum corneum side of excised skin was gently treated with marketed ADP gel formulation (ADPPEN gel, 0.1% w/w, INTAS), NLVs-A gel and NLVs-A-LYC gel (with an equivalent ADP). After treatment, intermittently 0.5 ml of the sample from the receiver compartment was collected and replaced with the same volume of PBS: methanol solution to maintain sink condition throughout the study [20]. These samples were then filtered through an aqueous 0.22-µm pore size cellulose membrane filter and quantitatively analyzed for the accumulated volumes of the ADP which permeated through the skins. The whole experiment was carried out for 24 h.

2.7 Skin distribution study: in vitro

The skin distribution study was carried out after completing of skin permeation study. Skin samples were carefully removed and scrapped to retrieve majority of adherent formulations. After recovery of formulation, this skin was then cleaned with lint-free cotton (previously soaked in de-ionized water) to remove any residual formulation and dried. Then with the help of tweezers epidermal and dermal skin layers were manually separated, and then they were chopped into pieces. After all this, these pieces were homogenized in 5 ml methanol: DMF (50:1) solvent mixture to extract out retained ADP [15]. Then these samples were filtered through a 0.22-µm membrane filters and analyzed by HPLC to examine permeated amounts of ADP.

2.8 Testosterone-induced acne in vivo model

To carry out further experiments, disease (acne) induction was done by creating hormonal change by the application of testosterone hormone in animals since due to sharp rise in testosterone level sebaceous glands produce excessive sebum which in turn induces acne, so testosterone was daily administered on the shaved dorsal side of animals [28]. Now five groups containing six male Wistar rats each were created. Sequence of treatment was designed as followed.

Group I treated with only testosterone, group II received testosterone and marketed adapalene gel, group III treated with testosterone and NLVs-A gel, group IV received testosterone and NLVs-A-LYC gel, and group V was selected as control to which no treatment was given. This line of treatment was given for 4 weeks daily for once-a-day application. Then any significant changes in skin or eruption of any type of acne were carefully observed to assure induction of acne

after testosterone treatment. After successful induction of acne, now diseased skin was precisely treated with prepared formulation. One group of treatment was not supplied with any formulation, and treatment efficiency of formulation was assured by comparing with this group of untreated animal. After 4th week of treatment shrinkage of papule density (4 cm^2 skin area) was visibly reported when compared with untreated group. After 4th week of treatment, the animals from all groups were euthanized and the treated skin samples were excised and microtomed. All the microtomed sections were further processed with paraffin embedding, and after H and E staining these sections were observed under microscope. The variation of size and number of sebaceous glands were observed, and the results were compared with untreated/control skin sections.

2.9 Data analysis

In vitro drug release and in vivo skin permeation as well as distribution of drug through formulation were statistically analyzed in triplicate, and observed analytical readings were expressed as mean ± SD. All the statistical analysis was completed by applying one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison post-tests using GraphPad InStat™ software (GraphPad Software Inc., San Diego, California).

3 Results

3.1 Formulation of ADP and LYC co-loaded nanostructured lipid vesicles (NLVs-A), NLVs-A gel, and NLVs-A-LYC gel

Classic high-pressure homogenization method with reported protocol was used to prepare NLVs. Gentle emulsification of lipid phase followed by vigorous diffusion of the lipid-solvent phase in an aqueous phase for specified time followed by evaporation of the solvent and subsequent cooling which results in increased rigidity of the lipid vesicles. Then these prepared NLVs were intimately dispersed into Carbopol® 940 gel vehicle to prepare 0.1% w/w gel strength. NLVs-A gel and NLVs-A-LYC gel were developed by incorporation of NLVs-A and NLVs-A and lycopene into a previously prepared concentrated Carbopol® 940 gel, respectively. Triethanolamine (TEA) was added to this acidic colloidal dispersion to neutralize it to produce a white, homogenous viscous gel. The prepared gel was grizzled, viscosity was adjusted, and acidic colloidal dispersion was finally neutralized with TEA.

3.2 Entrapment efficiency

NLVs-A-LYC showed a very promising entrapment efficiency (EE) of 81.31 ± 1.3% of ADP and 78.8 ± 1.4% of LYC. This may be due to lipophilic nature of drug and compatibility of ADP with lipid matrix of NLVs.

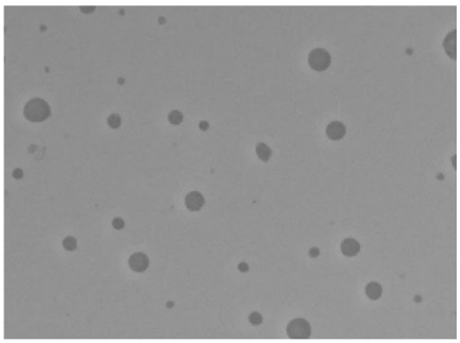

Fig. 1 TEM image of NLVs-A-LYC dispersion

3.3 Particle size, zeta potential and transmission electron microscopy

The particle/globule size of the any pharmaceutical formulation is one of the important parameters when it is intended for skin application. Close contact with the coenocytes of stratum corneum and penetration through intracellular route is favored by smaller size of particles/globules placing the amount of drug into viable skin [23]. The average size of ADP-loaded NLVs suspension was 208 ± 5.1 nm with a narrow size distribution (PDI-0.198). Spherical nature of globules was confirmed by TEM analysis (Fig. 1).

The long-term stability and cellular behavior for drug release were assessed by studying zeta potential as a function of surface charge of particles. The zeta potential of the NLVs-A dispersions was − 21.18 mv.

3.4 In vitro drug release study

We observed biphasic sustained release pattern of both ADP and LYC through NLVs-A-LYC after 48-h study. The initial burst release (40.19 ± 2.31% of ADP and 38.04% of LYC) from NLVs-A-LYC was obtained for 4 h followed by sustained release up to 48 h (Fig. 2). The release behavior of drug and lycopene is presented in Fig. 2.

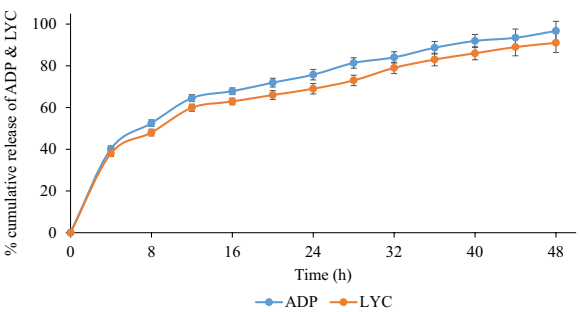

Fig. 2 Cumulative % ADP and LYC release from NLVs-A-LYC dispersion

The rapid release of any surface-adsorbed ADP and LYC or any drug right underneath the vesicle surface results in initial rapid release through NLVs-A-LYC, after that diffusion of ADP through lipid coat or degradation of lipid matrix in the vicinity of skin results in sustained release of ADP and LYC for next 48 h. For all the study period the amount of LYC in release media remained lower vis-a-vis ADP. This release behavior may be due to higher lipophilicity of lycopene in comparison with adapalene.

3.5 Analysis of rheological and textural profile of gels

After adjusting required phase and viscosity easy transportation and longer time storage of the gel formulation can be assured at given temperature range, if not adjusted with pharmaceutical excellence, any shear or stress can modify viscosity as well as structure and stability of prepared formulation gel. The apparent viscosity of NLVs-A gel and NLVs-A-LYC gel was reported as 12.01 ± 0.2 Pa and 12.04 ± 0.2 Pa, respectively, at 25 °C and 10 s^{-1} constant shear rate. Rheological flow behavior is presented in Fig. 3.

As the viscosity (Pas) is a function of shear rate (γ in s^{-1}, range from 0.1 to 100 s^{-1}), the viscoelastic behavior of the samples was analyzed using instrument software Rheoplus, and flow behavior of sample was determined by flow curve test. System is said to be shear thinning if the viscosity of the sample is decreased with increasing the shear rate.

The developed NLVs-A gel and NLVs-A-LYC gel were found to be easily spreadable. The present results suggest the correlation between reductions in viscosity with increasing shear rate. At 37 °C, the viscosity decreased up to 1.82 Pas from 14.10 Pas and to 1.90 Pas from 14.53 Pas with respect to shear rate from 10 to 100 s^{-1} for NLVsA gel and NLVs-A-LYC gel, respectively.

Amplitude sweep generally describes the deformation behavior of variety of system like food, medical, pharmaceutical as well as cosmetic substances in the non-destructive deformation range and at determining the upper limit of this range. Here statistical analysis is done by studying mathematical correlation between oscillatory frequency, angular strain and shear strain, etc. Thereafter flow point (where system start to flow), yield point (where deformation of internal structure initiates) and breakdown point of system (steep decrease in G' just after LVE) are analyzed. The linear viscoelastic region (LVE) signifies maximum amount of strain which can be applied on the sample without destroying the structure of the sample. In the LVE region if $G' > G''$, then sample shows a gel-like or solid structure and termed as viscoelastic solid material. However if $G'' > G'$, the sample displays fluid structure and can be termed as viscoelastic liquid. In LVE region, while $G' > G''$, higher the difference between G' and G'', higher will be the structural strength. For NLVs-A-LYC gel there was always $G' > G''$ signifying the gel samples are viscoelastic solid materials. The flow point of developed NLVs-A-LYC gel was found, which indicates that higher force will be required to change in the state of developed formulation (Fig. 4).

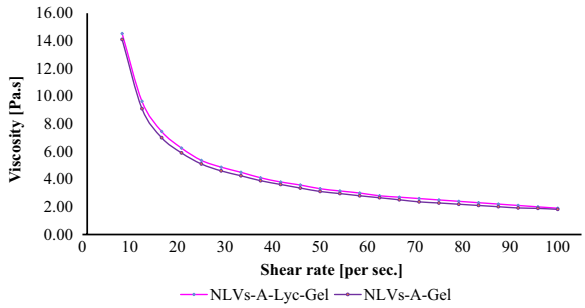

Fig. 3 Flow behavior of NLVs-A gel and NLVs-A-Lyc gel

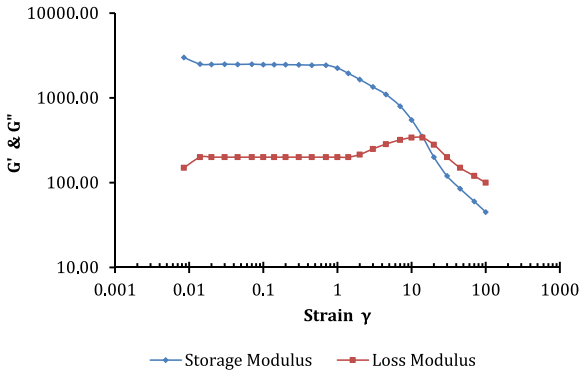

Fig. 4 Amplitude sweep and linear viscoelastic range of NLVs-A-LYC gel

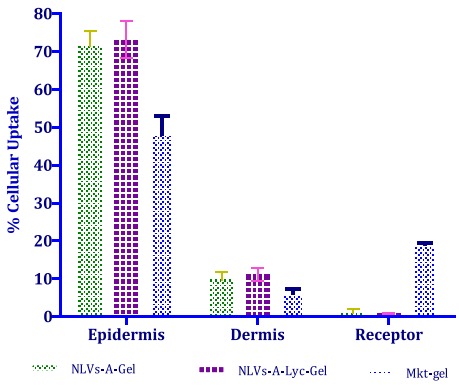

Fig. 5 Skin permeation and biodistribution of ADP indifferent skin layers and receptor compartment

3.6 Skin permeation studies

Skin uptake efficiency, targeting potential of NLVs along with efficiency of drug permeation across the skin from NLVs formulations into pig ear skin, was assessed by utilizing Franz Diffusion cells for 8-h study design. Marketed gel of ADP (0.1% w/w) was used as reference to evaluate the skin targeting potential of NLVs-A gel and NLVs-A-Lyc gel. In 8-h study there was no significant difference reported in amount of ADP in receptor chambers for NLVs-A gel and NLVs-A-LYC gel which is presented in Fig. 5. Interestingly from the marketed gel the amount of ADP in the receptor chamber showed a steady increase with time. Meanwhile, Fig. 5 indicates the inability of ADP from NLVs-based formulations to pass across the skin. Figure 5 also clearly elucidates differential penetration of drug from individual formulation in various skin compartments. Results suggest relatively higher drug penetration in receiver compartment from conventional gel having no carrier system.

3.6.1 Skin distribution study

The skin permeation and distribution of drug through skin were analyzed for quantification of drug across different formulations of NLVs-A gel, NLVs-A-LYC gel and marketed ADP product. After analysis of experimental results, it was observed that the NLVs-based formulation was able to deliver drug through epidermis, while a very insignificant concentration of drug was observed across dermis (Fig. 5). As compared to marketed gel ($p < 0.001$) substantial amount of ADP was retained across skin through NLVs-based gel. This was due to smaller size of NLVs which in turn might have improved penetration capacity of vesicles. Non-ionic surfactants were used in low concentration to increase penetration of drug through formulation.

3.7 Testosterone-induced acne model

Since overproduction of testosterone may lead to excessive sebum production, which in turn results in inflamed sebaceous glands and induce acne, this model was used to induce acne in test animals. For induction of acne, testosterone was topically applied daily on skin and carefully observed subsequently for development of inflamed sebaceous glands and any visible acne lesions. Effect of formulation on reduction of acne was observed by applying various formulation over testosterone-induced acne. After treatment for four week, there was noticeable lowering in the number of inflamed sebaceous glands in animals treated with NLVs-A gel as compared to marketed adapalene gel which clearly indicates superior efficiency nanogel formulation to reduce acne lesions (Fig. 6). During this study we also observed that formulation containing lycopene as adjuvant antioxidant has resulted in enhanced suppression of acne lesions which justifies the synergistic effect of lycopene with adapalene in treatment of acne (Fig. 6).

4 Discussion

The higher entrapment efficiency of drug has resulted due to lipophilic nature of drug, and it results in better protection of drug and improved localization of drug which in turn may reduce skin irritation.

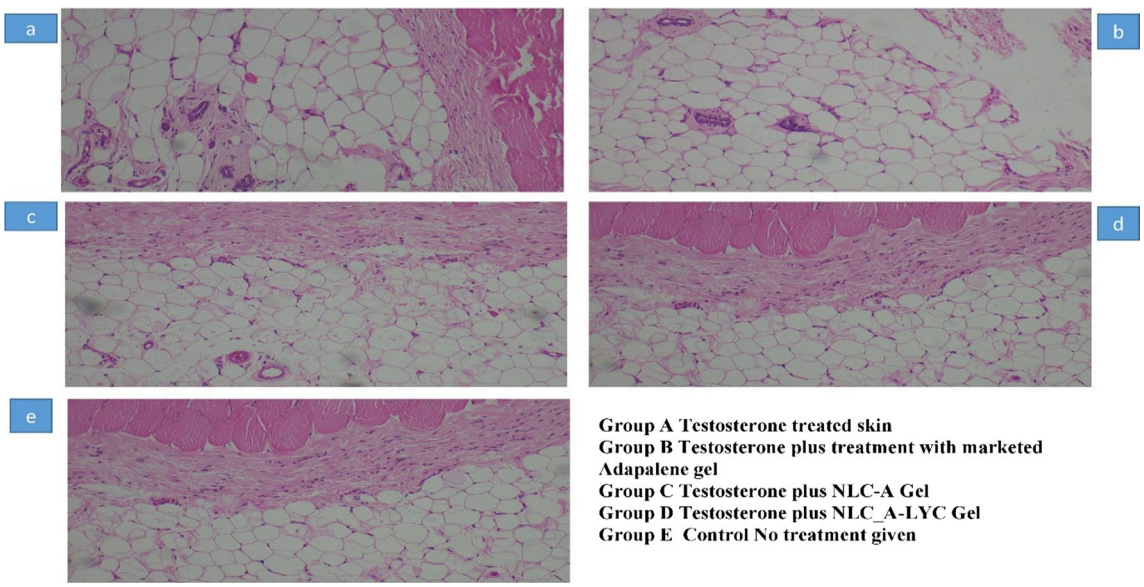

Fig. 6 Histopathological image of skin of animal treated with NLVs-A-Lyc gel

The stability of this dispersion is justified by negative value of zeta potential as well as low value of zeta potential approaching neutral also signifies about better permeability of NLVs across biological membrane.

TEM of formulation elucidates about spherical, uniform compact structure of vesicle without any visible drug crystal. The globule size obtained was 100–200 nm, showing the relevance of nanometric globules with regard to the topical application. Thus from size analysis and TEM study, it can be predicted that drug molecules can effectively access desired skin layers (stratum corneum, epidermis and dermis) retaining drug within the close vicinity of target site.

The rapid release of any surface-adsorbed ADP and LYC or any drug right underneath the vesicle surface results in initial rapid release through NLVs-A-LYC, after that diffusion of ADP through lipid coat or degradation of lipid matrix in the vicinity of skin results in sustained release of ADP and LYC for next 48 h.

For all the study period the amount of LYC in release media remained lower vis-a-vis ADP. This release behavior may be due to higher lipophilicity of lycopene in comparison with adapalene.

Analysis of rheological behavior and texture profile confirmed non-Newtonian flow behavior of both the gel formulation as depicted in Fig. 3. There was no difference in the viscosity in NLVs-A gel and of NLVs-A-LYC gel at equal shear rate, suggesting no contribution of Lyc to alter the viscosity of the vehicle.

Viscosity analysis of prepared formulation confirms the shear thinning behavior of system, and there was initial rise followed by decrease in viscosity upon increasing shear rate. The shear thinning behavior justifies the easy spreadability of formulation gel. In all the creams including marketed creams the flow behavior study confirmed structured liquids with pronounced non-Newtonian flow. Both the formulations showed the same flow behavior at similar shear ranges (Fig. 3). The only difference was slightly lower values of viscosity in NLVs-A gel vis-à-vis NLVs-A-LYC gel at the same shear rate. Since NLVs-A gel had slightly lower apparent viscosity compared to that seen with the NLVs-A-LYC gel, this behavior is in line with rheological behavior. Pseudoplastic flow was observed in both the formulation due to deformation of internal colloidal network microstructure of both the tested gel formulations. Besides pseudoplastic flow, thixotropic behavior was also observed in NLVs-A gel and NLVs-A-LYC gel which is a desirable flow characteristic of semisolid topical formulations.

Amplitude sweep and linear viscoelastic behavior of NLVs-A-LYC gel confirms the deformation behavior of formulation gel in non-destructive deformation range. For NLVs-A-LYC gel there was not any sharp decrease in viscosity beyond flow point, signifying that there was not any brittleness or cracking in all the tested samples after applying the shear.

Skin permeation and biodistribution study (Fig. 5) confirmed the higher concentration of ADP in the receptor compartment for samples treated with marketed gel was due to hydrophilic gel matrix and absence of any carrier system which can inhibit the penetration of drug across the skin by depositing it into skin layers. Therefore, it is assessed and can be predicted that NLVs-based formulation can minimize systemic uptake of drug, which is desired in such type of disease management where higher concentration of drug is needed in subepidermal region rather than in systemic circulation.

The observed distribution pattern of drug through marketed gel and NLVs-based gel suggests that there was relatively higher localization of drug in subepidermal region through NLVs-based gel due to sustained release behavior of drug through NLVs and higher skin contact time as drug was entrapped in lipid carrier vesicles and these carriers were incorporated in gel matrix so this array of arrangement has resulted in increased skin contact time for drug in subepidermal matrix, resulting in higher drug localization in this specific region. Across subepidermis the hydrophilic nature of dermis plays important role in restricting partitioning of hydrophobic drug in dermis region which is essential requirement to reduce systemic toxicity of this type disease management. Thus, occlusion of skin, enhanced retention of formulation drug and skin hydration were important factors for the enhanced penetration of drug into the skin. Therefore, on the basis of these favorable outcomes we can assertively predict NLVs to be a competent topical drug delivery carriers which can offer decent dermal localization and reduce systemic access of drug.

The study of testosterone-induced acne modal has clearly depicted formulation containing lycopene as adjuvant antioxidant has resulted in enhanced suppression of acne lesions which justifies the synergistic effect of lycopene with adapalene in treatment of acne.

5 Conclusions

The present study highlights importance of co-administration of a specific drug combination (ADP and Lycopene) of clinical relevance and compatibility into a single-dose regimen to reduce individual dose of drug and enhance its effectiveness by combination regimen. Various Efforts have been made to obtain greater amounts of ADP loaded into NLVs. In comparison with free drug, drug encapsulated in NLVs have shown better skin targeting potential and the adjuvant effect of antioxidant helped to magnify the therapeutic potential of ADP during the course of chronic therapy. The targeting potential of micellar

vesicles/nanoparticles/nanovesicles could be exploited further to improve the therapeutic efficiency of drug to treat acne. Testosterone-induced acne model studies were carried out to better understand the advantage of antioxidant over an individual drug. In a nut shell it can be expected that the developed formulation strategy presents great potential leading to therapeutic efficacy and safety profile of the combination regimen. We therefore believe that the NLVs-A-LYC gel as illustrated in the current investigation could prove to be comparatively advantageous over NLVs-A gel. The findings will put a future prospective into contributing to the broadening of future clinical possibilities and thereby provide a potential treatment strategy for skin disorders. The co-administration strategy may be an addition to acne therapeutic and interventional armory.

Abbreviations

ADP	Adapalene
API	Active pharmaceutical ingredients
EE	Entrapment efficiency
HPH	High-pressure homogenization
LYC	Lycopene
NLVs	Nanolipid vesicles
PDI	Polydispersity index
TEM	Transmission electron microscopy

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Author contributions
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Availability of data and materials
All the available data generated or analyzed in this work are presented here in this article.

Declarations

Ethics approval and consent to participate
The author asserts that all the procedures and studies contributing to this work were approved by the Institutional Animals Ethical Committee, Bhagyoday Tirth Pharmacy College, Sagar, M.P., India (Ref. letter no.0604/BTPC/2021-22/12). CPCSEA guidelines were strictly followed during all the animal experimentations.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interest of any type.

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