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ORIGINAL PAPER

Resveratrol-loaded nanoemulsion gel system to ameliorate UV-induced oxidative skin damage: from in vitro to in vivo investigation of antioxidant activity enhancement

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Abstract
In the present study resveratrol nanoemulsion gel was developed and optimized with the aim of enhancing the permeability and antioxidant activity against ultraviolet (UV)-induced oxidative skin damage. Droplet size, polydispersity index, drug permeation flux, permeability coefficient and drug deposition in skin of resveratrol-loaded nanoemulsion were found to be 65.00 ± 5.00 nm, 0.171 ± 0.082, 144.50 μg/cm^2/h, 2.90×10^{-2} cm/h and $45.65 \pm 4.76\%$, respectively, whereas drug permeation flux, permeability coefficient and drug deposition in skin from nanoemulsion gel were found to be 107.70 μg/cm^2/h, 2.06×10^{-2} cm/h and $62.65 \pm 4.98\%$, respectively. Confocal studies depicted deeper penetration of resveratrol from nanoemulsion gel. Differential scanning calorimetry and Fourier-transform infrared spectrophotometer studies confirmed that nanoemulsion gel enhanced fluidization of stratum corneum lipids and conformational disruption of lipid bilayer, thereby enhancing skin permeation of resveratrol. Histopathology study of skin revealed that resveratrol-loaded nanoemulsion gel inhibited UV-induced spongosis, edema and epidermal hyperplasia response. Levels of glutathione, superoxide dismutase, catalase and protein carbonyl in the skin of UV-irradiated rats were significantly ($p < 0.01$) improved in the skin of animals treated with nanoemulsion gel. Experimental results suggested that nanoemulsion gel could be explored as a promising carrier for topical delivery of resveratrol for prevention of UV-induced oxidative skin damage owing to its enhanced permeability and retention effect.

Keywords Nanoemulsion · Topical drug delivery · Nanoemulsion gel · Antioxidant · Skin permeation

Introduction

Long-term exposure of skin to UV radiation causes degenerative changes in cells. These destructive changes in the cells are induced by the generation of ROS [1, 2]. However, the skin possesses an endogenous antioxidant defense mechanism, but depletion in the level of components of endogenous antioxidant system due to excessive ROS leads to oxidative stress. Oxidative stress causes subsequent formation of products that can destructively react with cellular lipids, peptides, proteins and DNA [3]. Moreover, it may cause mutation in epidermal cells, upregulation of gene expression through the intracellular signal induction pathway, activation of transcription factor and nuclear factor-kB, and thus hyper cell proliferation [4–7].

Oxidative changes in the damaged skin can be managed by using antioxidants such as melatonin; vitamins A, C, D and E; oxytocin and polyphenolic phytoconstituents. Various herbal antioxidant drugs such as curcumin, quercetin and lycopene have been exploited to treat various skin diseases which are due to oxidative damage in the skin cells. This has gained a great attention of the health-care society, as these have multiple mechanisms of actions and are safe to use as compared to synthetic drugs [8, 9].

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Resveratrol is a herbal polyphonic acid and has a wide variety of pharmacological activity including anti-inflammatory, anticancer, antiviral, antiamyloid, antiarthritic and antioxidant properties [10, 11]. It can scavenge hydroxyl, lipid hydroperoxyl and superoxide anion. It can enhance antioxidant activity in skin cells by elevating the levels of CAT, SOD and other antioxidant enzymes. Resveratrol increases the levels of GSH, vitamin E, vitamin C and b-carotene, thereby playing a major role in preventing the oxidative stress that is known to contribute to skin carcinogenesis [12]. But its poor deposition in skin tissue after oral administration limits its clinical applications. Poor skin deposition of resveratrol after oral administration is due to low oral bioavailability. It is used as a topical antioxidant supplementation for the prevention and treatment of UV-induced oxidative skin damage. But resistance to permeation offered by the stratum corneum is a major obstacle in its topical delivery. However, several formulation strategies such as nanoparticles, liposomes, nanoemulsions and nanoconjugates have been investigated for enhancing the oral bioavailability of resveratrol, but very little attention has been given to the topical delivery of it using nanocarriers [13–18]. Teskac and co-workers developed solid lipid nanoparticles (SLN) for the topical delivery of resveratrol. SLN-encapsulated resveratrol exhibited higher permeability, good solubility, improved stability and anti-lipoperioxidative activity [19]. Pando and associates prepared resveratrol-loaded niosomes to increase the residence time of the drug in the uppermost layer of skin (stratum corneum) and viable epidermis [20]. Friedrich and co-workers developed resveratrol-loaded lipid-core nanocapsules to enhance the skin permeation of the drug [21]. Transferosomes which are a type of liposomes have also been developed for topical delivery of resveratrol [22]. But the infeasibility to scale up, high cost of production and comparatively less stability and more toxicity (skin irritation) of these nanocarriers are the major issues in their commercialization. Further, the problem of poor skin permeation and photodegradation (that reduces activity) of resveratrol could not be resolved effectively with conventional formulations [18].

Nanosized formulations are additionally advantageous in antioxidant therapeutics for the treatment of oxidative damaged skin. It is due to the enhanced permeability and skin retention [18]. Nanoemulsion has been reported as a novel carrier system to overcome most of the problems related to topical delivery of lipophilic molecules. Nanoemulsions use their ability to "hide" lipophilic molecules within the oil layer, which can then be used to transport lipophilic molecules through the stratum corneum into the deep layer of skin [23]. Nanoemulsions have the capability of excellent drug loading, exhibit excellent permeation through the biological membrane and cause negligible or low skin irritation [24]. Moreover, small droplets of nanoemulsions provide a large surface to volume ratio, which favors close contact with skin thereby providing more concentration gradient and improved drug permeation. Moreover', less surface tension ensures better adherence to the skin, and the disperse phase acts as a reservoir making it possible to transport drugs in a more controlled fashion [23]. They are commercially viable to formulate and are biocompatible [25, 26].

With this background, the present study was designed to formulate resveratrol-loaded nanoemulsion. Nanoemulsion of resveratrol was prepared using a high-pressure homogenization method and the formulation was optimized by employing a central composite rotable design (CCRD). For better topical applicability, optimized nanoemulsion was incorporated into a gel form. The suitability of resveratrol nanoemulsion gel for topical application was confirmed on Wistar rats. The depth of skin permeation of resveratrol was analyzed and the mechanism of skin permeation enhancement of the nanoemulsion gel was studied. Further, the efficacy of optimized resveratrol-loaded nanoemulsion gel for the prevention of UV-induced oxidative skin damage was checked on Wistar rats.

Materials and methods

Materials

Resveratrol was received as a gift sample from Lactonova, India. Sefsol 218® and Solutol HS 15 were received as gift samples from Nikko Chemicals Co. Ltd (Tokyo, Japan) and Signet Chemicals Corporation Pvt. Ltd, Mumbai, India, respectively. Almond oil and olive oil were purchased from Falcon, Bengaluru (India). Soybean oil was purchased from Sigma-Aldrich, Mumbai, India. Transcutol P and Labrasol were provided as gift samples from Gattefosse (Saint Priest, Cedex, France). Tween 20, Tween 80 and polyethylene glycol 400 (PEG 400) were purchased from S.D Fine Chemicals, New Delhi, India. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid and rhodamine-123 were purchased from Sigma-Aldrich Chemicals Pvt. Ltd, India. Propylene glycol was procured from Thomas Baker Chemicals, Mumbai, India. Thiobarbituric acid (TBA) and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Himedia, Mumbai (India) and Sisco Research Laboratories Pvt Ltd, Mumbai (India), respectively. Carbopol 940 was received as a gift sample from Unicure Pvt Ltd, India. All other chemicals and solvents employed during the studies were of analytical grade.

Formulation and optimization of resveratrol nanoemulsion formulation

Screening and selection of oil

Oils were screened on the basis of drug solubility [27]. The solubility of resveratrol was determined by adding an

excess amount of resveratrol in 2 ml of oils (Sefsol 218®, olive oil, almond oil and soybean oil) in 5 ml stopper vials and allowed to mix in a vortex mixer. After mixing, the samples were placed on a shaker for about 72 h to reach equilibrium. Finally, the samples were centrifuged for a time period of 15 min at 3000 rpm. The supernatant was taken and the concentration of the drug was determined by UV–visible double beam spectrophotometer (UV-1601, Shimadzu) at 306 nm in triplicate.

Selection of surfactant and co-surfactant

The criteria used for the selection of surfactant (Solutol HS, Labrasol, Tween 20 and Tween 80) were solubility and miscibility with the selected oil. On the other hand, the selection criteria used for selecting the co-surfactant was the miscibility with the selected oil and surfactant. Miscibility studies with oil or surfactant were carried out by mixing them in 1:1 ratio and vortexing for about 5 min, after which they were left to stand for 24 h at room temperature. Finally, the samples were evaluated for phase separation and color change. The mixtures exhibiting clarity were selected for preparation of nanoemulsion [3].

Pseudoternary phase diagram construction

Pseudoternary phase diagrams were constructed according to the procedure explained by Kumar and associates to identify the ratio of surfactant and co-surfactant required to formulate the nanoemulsion [25]. The selected surfactant and co-surfactant were mixed in different volume ratios (mixture was known as S_{mix}). S_{mix} ratios such as 1:0, 1:1, 1:2, 1:3, 2:1 and 3:1, 4:1 were prepared. The mixture of surfactant and co-surfactant is also known as S_{mix}. For the preparation of each phase diagram, oil and specific S_{mix} ratio was mixed in different volume ratios from 1:9 to 9:1 in glass vials making 16 different combinations (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1). Pseudoternary phase diagram was developed using the aqueous titration method as described by Kumar and associates [25]. Slow titration with water was performed for all ratios of S_{mix} and oil. After each 5% addition of water to the volume ratio, visible observation was made and recorded as follows: (a) if a transparent and easily flowable system was obtained, it were marked as nanoemulsion; (b) nanogel, if transparent gel was obtained; (c) if milky or cloudy appearance was obtained or phase separation was obtained, it was marked as emulsion, and (d) if milky gel was obtained, it was marked as emulgel. Nanoemulsion was marked on a phase diagram by using CHEMIX School software ver. 3.60 (USA).

Preparation and optimization of nanoemulsion by CCRD

The nanoemulsion was formulated using Sefsol 218® as oil, PEG 400 as co-surfactant, Tween 80 as surfactant and water as aqueous phase. Resveratrol was dissolved in the oil phase with continuous stirring at high speed using a magnetic stirrer at room temperature. Tween 80 and PEG 400 were added after the drug was completely solubilized in the oil. The preparation was continued by adding the required amount of water dropwise to the oily solution under continuous vortexing. The pre-homogenized emulsion produced was further subjected to homogenization using a high-pressure homogenizer (STANSTED® pressure Cell Homogeniser, Harlow, Essex CM19 5FN, UK) to produce a nanoemulsion.

CCRD was utilized to study the effect of composition variables including oil (2.5–4.5%) and S_{mix} (7.5–13.5%) and process variables including the number of homogenization cycles (3–11) and homogenization pressure (800–1600 bar) on the three response variables including droplet size (Y_1), transmittance (Y_2) and polydispersity index (Y_3). The range of composition variables was selected on the basis of phase diagrams showing more nanoemulsion area. On the basis of CCRD, a total of 29 experiments were run using Design Expert software (version 7.0, Stat ease Inc., Minneapolis, USA). Experimental data were analyzed by response surface regression procedure and the results were statistically analyzed by the corresponding analysis of variances (ANOVA). Equations for different variables were obtained by putting values of coefficients in the following equation:

$$\begin{aligned}\text{Response } (R) = {} & \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D \\ & + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 \\ & + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC \\ & + \beta_{24} BD + \beta_{34} CD,\end{aligned}$$

where A, B, C and D indicate oil, S_{mix}, number of homogenization cycles and homogenization pressure, respectively. β_0 represents intercept coefficient; β_1, β_2, β_3 and β_4 represent the linear coefficients. Quadratic coefficients are represented by β_{11}, β_{22}, β_{33} and β_{44}. Interaction coefficients are represented by β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34}.

On the basis of more desirability value, optimized nanoemulsion preparation was selected and post-optimization characterization was carried out for droplet size, PDI, refractive index, viscosity, pH, surface morphology and in vitro permeation studies.

Characterization and evaluation of nanoemulsion

Droplet size

Droplet size and size distribution of the selected nanoemulsion formulation were determined using Zetasizer (PCS, Zetasizer 1000 HAS, Malvern instruments, Worcestershire, UK). PDI, a measure of droplet size distribution in the sample, was measured in triplicate.

Surface morphology

The resveratrol nanoemulsion was studied for surface morphology with the help of transmission electron microscopy (TEM) operating at 200 kV (TECNAI G20, S-Twin, 200KV, HRTEM, FEI company Holland).

Percentage transmittance

The percentage transmittance of the formulation was evaluated in triplicate at 630 nm by employing UV–visible double beam spectrophotometer (UV-1601, Shimadzu) [25].

Viscosity

The viscosity of the nanoemulsion was measured using a Brookfield DV III ultra V6.0 RV cone and plate viscometer (Brookfield Engineering Lab, MA). All the measurements were carried out at a temperature of 25 ± 0.5 °C in triplicate using Rheocale V2.6 software.

Refractive index

The refractive index of the formulation was determined in triplicate using Abbe's type refractometer.

pH determination

Calibrated pH meter (Eutech Instruments, Singapore) was used to determine the pH of the nanoemulsion. pH determination was performed in triplicate.

Antioxidant activity

DPPH method

The DPPH method of evaluating antioxidant activity was based on the principle that DPPH accepts a hydrogen atom from the scavenger molecule, i.e., antioxidant, and leads to the reduction of the DPPH molecule to $DPPH_2$. This results in the change of the purple color to yellow with simultaneous decrease in absorbance at 515 nm with increase in concentration. The change in color was observed spectrophotometrically and used for the calculation of the parameters for antioxidant properties [3, 28]. Different concentrations (1, 2, 5, 10, 15, 20, 25 and 30 µg/ml) of resveratrol nanoemulsion, resveratrol solution and ascorbic acid (as standard antioxidant) in methanol were prepared. One milliliter of each diluted solution was added to 1 ml of 0.004% DPPH solution and kept at room temperature. The absorbance was observed at 515 taking methanol as a blank. The DPPH radical scavenging capacity was calculated using the following equation:

$$\%\text{Inhibition} = \frac{A_0 - A_1}{A_0} \times 100,$$

where A_1 and A_0 represent the absorbance of the resveratrol solution and blank, respectively.

Reducing power assay

Reducing power assay was performed to compare the antioxidant potential of resveratrol nanoemulsion formulation with drug resveratrol solution and ascorbic acid (as standard antioxidant). The reducing powers of all samples were determined as per previously established methods [28]. Different concentrations (5, 10, 15, 25, 35, 45, 55 and 65 µg/ml) of the sample solutions and ascorbic acid were prepared. One milliliter of each diluted solution was added to 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml of potassium ferricyanide. The resulting mixture was kept for 30 min in an oven at 50 ºC. About 2.5 ml of 10% trichloroacetic acid was added to the mixture. This was further subjected to centrifugation for 20 min at 2500 rpm. The supernatant solution (4 ml) was taken and mixed with 4 ml of distilled water. A further 0.8 ml of 0.1% ferric chloride solution was added and the absorbance was measured at 700 nm using phosphate buffer, pH 6.6, as a blank. The increase in the absorbance of the solution indicated more reducing power [3].

Preparation of resveratrol nanoemulsion gel

As nanoemulsions are easily washed by sweat, the nanoemulsion was transformed into gel for ease of application and to improve the topical contact time of drug with the affected area. Carbopol-940 (2 g) was dispersed in 98 ml of distilled water and allowed to stand in the dark for 24 h so that there was complete swelling of Carbopol-940. Then, resveratrol nanoemulsion was added slowly into the aqueous solution of Carbopol-940. Triethanolamine (0.05% v/w) was added to get a homogeneous dispersion of the gel and then the weight of the nanoemulsion gel was adjusted with placebo gel to get a gel of strength 1 mg resveratrol per gram gel.

Characterization of nanoemulsion gel

Viscosity

The viscosity of the nanoemulsion gel was measured without dilution using a Brookfield DV III ultra V6.0 RV cone and plate viscometer in triplicate.

pH

The pH of the optimized nanoemulsion gel was observed using a digital pH meter (pH Tutor Bench Meter, EUTECH Instruments, Singapore) in triplicate.

Homogeneity

The homogeneity of the prepared gel was determined by taking a small quantity of nanoemulsion gel and pressing between the thumb and index finger, whether homogenous or not and if any coarse particle appeared or detached on to the finger [29].

Spreadability

The spreadability of a sample of 1 g of nanoemulsion gel was determined by pressing it between two glass plates on which weights of 50, 100, 150 and 200 g were placed at intervals of 1 min. The diameters during each interval were taken as the area in cm^2. The variations of the area as a function of weight were then analyzed as response factor [30]. All the determinations were carried out in triplicate.

Extrudability

The extrudability test involves determining the amount of gel extruded from a collapsible tube on applying a constant weight. About 1 g of gel was filled in a 5 ml syringe and a constant weight was applied upon the syringe plunger, the amount of gel extruded was weighed and the extrudability was calculated in terms of gram per force applied (newton) in 1 min. The experiment was performed in triplicate.

Drug content

The drug content of the gel was determined by dissolving a specific quantity (100 mg) of gel in 100 ml of phosphate buffer (pH 6.8). This resulting gel solution was kept on a mechanical shaker for 2 h to solubilize the drug. This solution was filtered and the amount of drug was determined in triplicate using a UV spectrophotometer (UV-1601, Shimadzu) at 306 nm.

In vitro skin permeation study

In vitro permeation studies were performed to evaluate the permeation of resveratrol from nanoemulsion, aqueous dispersion, normal resveratrol gel and resveratrol nanoemulsion gel using the skin of male Wister rats and were compared to each other. The rats were obtained from the central animal facility at Jamia Hamdard, New Delhi (India). The skin was excised from the abdomen region and cleaned with normal saline using cotton buds to remove any adhering subcutaneous tissue and fat. The epidermal hair was removed using an electric razor. The skin was hydrated for 24 h in phosphate buffer (pH 6.8). The excised rat skin was cut into an appropriate size and mounted between two halves of Franz diffusion cell with the stratum corneum facing toward the donor compartment [31]. The exposed surface area of the release membrane was 0.64 cm^2. The receptor compartment was filled with 10 ml of phosphate buffer (pH 6.8) and maintained at 32 ± 1 °C. About 1.0 g of formulation was spread evenly in the donor compartment. 0.5 ml of diffusion media was withdrawn at regular intervals (0, 1, 2, 3, 4, 5, 6, 7 h) and replaced with the same volume of media. The amount of drug content was analyzed in triplicate using a UV spectrophotometer (UV-1601, Shimadzu) at 306 nm. The permeability coefficient (P_{app}) of resveratrol was calculated using the following equation [3]:

$$P_{app} = \left(\frac{dQ}{dt}\right)/SA_o,$$

where dQ/dt, S and A_o are the rate of resveratrol permeation, cross-sectional area of tissue and initial concentration of resveratrol in the donor compartment at $t = 0$, respectively.

Determination of drug deposition in skin

After completing the in vitro permeation experiment, the formulations remaining on skin were removed by using cotton swabs. The skin was then cut into small pieces. Methanol was added to the pieces and sonicated for 20 min at 25 ± 2 °C to extract resveratrol. Finally, the resulting mixture was centrifuged and the absorbance was determined in triplicate using a UV spectrophotometer (UV-1601, Shimadzu) at 306 nm. A blank of untreated skin was prepared using methanol in the same way of preparation of the sample of the treated skin. It was used to nullify the absorption due to lipid extraction from the skin tissue. The calibration curves of resveratrol (1–6 µg/ml) in filtered (filter of pore size of 0.45 µm) methanolic solution of epidermal tissue and dermal tissue were prepared separately. The methanolic solution of epidermal tissue and dermal tissue was taken as the blank.

Depth of permeation using confocal laser scanning microscopy (CLSM) study

The depth and visualization of skin permeation of resveratrol from nanoemulsion gel were evaluated using CLSM 410 invert-based CLSM system (Zeiss, Heidelberg, Germany). The formulation loaded with Rhodamine B dye (0.5 mg/g gel) having an excitation wavelength of 520 nm was applied on the skin mounted onto Franz diffusion cells (as per the procedure described in "In vitro skin permeation study"). After 7 h (duration of skin permeation study), the skin was removed and cleaned with ethanol to remove adhering residue of the formulation. Fixed slides of cleaned skin were prepared and observed under CLSM for the presence of fluorescence in the various layers of skin [32].

Determination of the mechanism of skin permeation

The mechanism of skin permeation enhancement was determined using DSC and FTIR studies of formulation-treated skin and untreated skin. The characteristic peaks corresponding to C–H stretching, C=O stretching and C=N stretching in the FTIR spectra and melting endothermic event of the DSC thermogram of treated and untreated skin can be used to predict the permeation dynamics.

DSC studies

Approximately, 5 mg of the treated and untreated skin was weighed separately and washed with phosphate buffer (pH 6.8). The washed skin was blotted over filter paper and hermetically sealed in aluminum pans. The DSC thermograms of the treated and untreated skin were recorded over the 30–300 °C range using DSC (PerkinElmer Inc., Waltham, MA, USA) at a scanning rate of 10 °C/min [32].

FTIR studies

After the skin permeation study, the skin disc of 0.64 cm^2 was removed from the Franz diffusion chamber and washed with phosphate buffer (pH 6.8). The disc was blotted over filter paper and FTIR spectra of skin was recorded in a range of 400–4000 cm^{-1} using FTIR (Perkin Elmer, Germany). The FTIR spectra of the hydrated untreated skin were also recorded and taken as control [32].

Skin irritation studies (Draize's test)

Skin irritation potential of resveratrol nanoemulsion gel was carried out in rats using the procedure explained previously [33, 34]. The experimental protocol was reviewed and approved by the Institutional Ethical Committee, Jamia Hamdard (India). Twelve rats of 200–300 g were taken and hair from the dorsal skin was removed. The optimized nanoemulsion gel was evenly applied once daily to a small area of skin (4 cm^2) and then the applied site was covered with bandage. After 24 h, the formulation was removed and the site was observed for any sign of skin irritation or reaction. The experiment was continued for 14 days. Scores such as 0, 0.1–0.4, 0.41–1.9, 2.0–4.9 and 5.0–8.0 were assigned to indicate non-irritation, negligible irritation, slight irritation, moderate irritation and severe irritation, respectively.

Induction of oxidative stress in animal and histopathology of skin section

Oxidative stress testing was performed to confirm the antioxidant potential of resveratrol in UV-induced oxidative skin damage. Oxidative stress was induced by UV radiation. The animals were divided into four groups (four rats in each group). The cages were marked and kept in a room maintained at a temperature of 22 ± 3 °C and relative humidity 30–70%. A prequalified UV radiation chamber was used for UV irradiation of rat. The UV source of irradiation consisted of a Philips TL40W/12 RS lamp emitting light spectrum between 270 and 400 nm and peak emission at 313 nm. It was mounted 10 cm above the rat-supporting apparatus. The irradiation rate was 0.27 mW/cm^2. The animals of group G-I were not exposed to UV radiation, whereas animals of the control group (G-II) were exposed to UV radiation for 6 h (total dose of 2.87 J/cm^2). Animals of group G-III and G-IV were treated with conventional gel and nanoemulsion gel of resveratrol, respectively, for 15 days, thereafter treated animals were also exposed to UV radiation for 6 h (total dose of 2.87 J/cm^2). The animals of group G-I, G-II, G-III and G-IV were killed and skin samples were stored with 10% formalin solution in phosphate buffer saline (pH 7.4). Skin sections were vertically cut down. Hematoxylin and eosin were employed for slide staining. The stained slides were observed under light microscope equipped with image processor (Motic, Nagoya, Japan) at 10X to determine any presence of damage to mucosa after application of the formulation.

Oxidative stress study: biochemical estimation

GSH

The GSH amount was calculated by the method explained by Pangeni and associates [15]. Briefly, 2 ml of skin homogenate was mixed with 2 ml of 0.02 M EDTA and 1.6 ml of cold distilled water. To this, 50% trichloroacetic acid (0.4 ml) was added and, after mixing, the mixture was centrifuged at 3500 rpm for 15 min. About 4 ml of 0.4 M Tris buffer (pH 8.9) was mixed with the supernatant (2 ml). Further 0.1 ml

of 0.1 M DTNB was added to it and the absorbance of the resultant solution measured at 412 nm.

Thiobarbituric acid-reactive substance (TBARS)

TBARS was used to estimate the rate of lipid peroxidation. Lipid peroxidation is a free radical-mediated reaction. The procedure described by Pandey and associates was used for the determination of TBARS content [15]. About 0.2 ml of the homogenate (0.2 ml) was mixed with 9 ml of potassium chloride (1.15%) and 0.2 ml of sodium dodecyl sulfate (8.1%). 1.5 ml of acetic acid (20%) was added to the mixture and the pH adjusted between 3 and 5 using NaOH solution. After adding 1.5 ml of 0.8% TBA, the solution was properly shaken and kept at 80˚C for 1 h. The mixture was cooled down for 10 min. Five milliliter of butanol and pyridine mixture in 30:2 ratios was added followed by addition of 1 ml of distilled water. The mixture was centrifuged and the absorbance of the supernatant was recorded at 532 nm. TBARS was calculated as nmol per mg of protein [35].

SOD

A known weight of skin (200 mg) was homogenized in 2 ml of potassium buffer, pH 6.8, and the homogenate was centrifuged at 1500 rpm for 10 min. About 100 µl of supernatant was added to Tris HCl buffer (pH 8.5). The final volume of 3 ml was made with Tris HCl buffer. About 25 µl pyrogallol was added to the mixture and the absorbance was measured at 420 nm.

Protein carbonyl estimation

The skin homogenate (0.5 ml) was mixed with 0.5 ml of 20% TCA which precipitated the protein. The solution was centrifuged at 4500 rpm for 5 min. The precipitate was extracted three times with 0.5 ml of 10% TCA which was dissolved in 2 ml of NaOH. The obtained solution was transferred to another tube and absorbance was taken at 360 nm against an appropriate blank.

Catalase

The skin homogenate (0.05 ml) was mixed with 2 ml of phosphate buffer, pH 7. Hydrogen peroxide (0.95 ml) was added to it and the absorbance was measured at 240 nm against the appropriate blank.

Results

Formulation development and optimization of the nanoemulsion

Selection of oils, surfactant and co-surfactant

Oil was selected on the basis of solubility of the required drug dose in the formulation. In Sefsol 218®, olive, almond and soybean oil resveratrol exhibited solubility of 165.45 ± 5.33, 24.26 ± 2.31, 14.69 ± 2.38 and 13.16 ± 3.29 mg/ml, respectively (Table 1). Surfactant selection was based on solubility and miscibility studies. The solubility of drug in different surfactants along with the miscibility with Sefsol 218® is listed in Table 1. From solubility studies data, it was observed that resveratrol exhibited maximum solubility in Tween 80 which was 46.27 ± 5.21 mg/ml and also had good miscibility with the selected oil, and therefore it was selected as the surfactant for the development of nanoemulsion. Co-surfactants play a very important role in reducing the interfacial tension as they fit themselves into the void space of the surfactant molecules. The selection of co-surfactant was done on the basis of miscibility studies with the selected oil and surfactant. Amongst the co-surfactants, polyethylene glycol 400 (PEG 400) exhibited miscibility with the oil as well as surfactant; hence, it was selected as the co-surfactant.

Construction of pseudoternary phase diagram

Phase diagram construction was carried out using oil (Sefsol 218®), co-surfactant (PEG 400), surfactant (Tween 80) and aqueous phase (water) (Fig. 1). Various S_{mix} ratios were tried

Table 1 Solubility of resveratrol in different excipients and miscibility of oil with surfactants

Solubility of resveratrol in oils		Solubility of resveratrol in surfactants		Miscibility of Sefsol 218® with surfactant
Oil	Solubility (mg/ml) ± SD ($n=3$)	Surfactant	Solubility (mg/ml) ± SD ($n=3$)	Observation
Sefsol 218®	165.45 ± 5.33	Solutol HS	33.82 ± 2.10	Clear
Olive oil	24.26 ± 2.31	Labrasol	2.82 ± 0.23	Turbid
Almond oil	14.69 ± 2.38	Tween 20	20.27 ± 2.67	Clear
Soybean oil	13.16 ± 3.29	Tween 80	46.27 ± 5.21	Clear

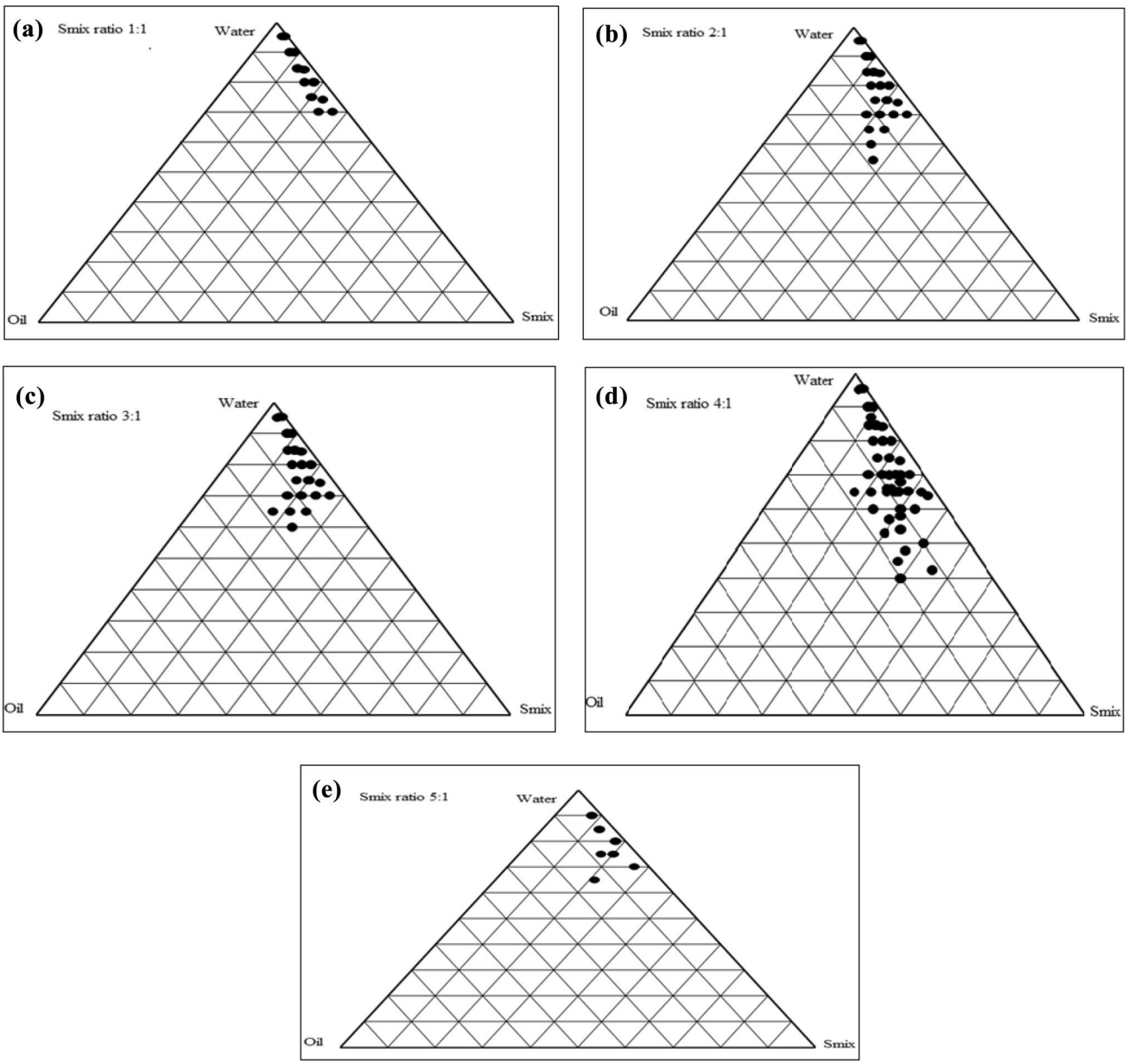

Fig. 1 Pseudo-ternary phase diagram showing o/w nanoemulsion region for S_{mix} ratio **a** 1:1, **b** 2:1, **c** 3:1, **d** 4:1 and **e** 5:1. The area of nanoemulsion region described by black dots indicates the emulsification potential of S_{mix}. Maximum emulsification was obtained with S_{mix} of 4:1 ratio

and it was found that the area of nanoemulsion isotropic region was different with each S_{mix}.

In S_{mix} ratio 1:1, the area was less in comparison to 2:1 ratio. It indicated that the oil phase solubilization by S_{mix} (1:1) was less. In S_{mix} ratio 4:1, the maximum area was found. The S_{mix} ratio 5:1 was also tried and it was found that on further addition of surfactant no more emulsification took place. S_{mix} ratio 2:1 was also tried and it gave an isotropic region less than the S_{mix} ratio of 1:1. Therefore, it was concluded that maximum nanoemulsion was achieved at 4:1 S_{mix} ratio. The ratio 4:1 ratio exhibited more nanoemulsion area because of its potential to solubilize the Sefsol 218®. Additionally, this ratio decreases the free energy of the system to very low level, which is a prerequisite for developing the nanoemulsion. Increased amount of Tween 80 resulted in decrease in interfacial tension as well as an increment in the oil–water interface fluidity, resulting in higher system entropy. Increasing the surfactant concentration relative to the co-surfactant enhanced water penetration into oil droplets, but further increase caused interfacial disruption and expulsion of oil droplets into the aqueous phase and formation of lesser micelles, resulting in decreasing the nanoemulsion area. Also, the high oil–surfactant interaction may lead

to increased adsorption of surfactants onto the oily droplets rejecting the water particles.

Screening and statistical analysis of variables

The oil and S_{mix} levels were determined from phase diagrams where there was more nanoemulsion area. Ranges of 2.5–4.5% and 7.5–13.5% were selected for oil and S_{mix}, respectively. The range for homogenization pressure and cycle was selected as 800–1600 bar and 3–11, respectively. The variables and their constraints in CCRD are listed in Table 2. Responses of the formulated batches are listed in Table 3.

Effect on droplet size (Y_1) It was observed that the droplet size of the prepared nanoemulsion batches was in the range of 24.03 ± 4.63 to 90.04 ± 2.94 nm. The effect of variables on droplet size can be predicted from the given quadratic polynomial equation suggested by CCRD. The linear term of S_{mix} had a significant effect on the droplet size of the nanoemulsion with $p < 0.0001$, followed by oil ($p < 0.0001$), homogenization pressure ($p < 0.05$) and number of homogenization cycles ($p < 0.05$). Oil concentration had a positive effect on the droplet size, whereas linear term of the surfactant, pressure and number of cycles had negative effect on it (Fig. 2).

$$\begin{aligned}\text{Droplet size } (Y_1) = {} & 55.73 + 9.44A - 9.47B - 1.19C \\ & - 4.51D - 1.10AB - 1.08AB - 1.16AD \\ & - 2.14BC + 1.17BD + 0.99CD + 2.24A^2 \\ & - 1.14B^2 - 2.93C^2 - 4.1D^2.\end{aligned}$$

Effect on transmittance (Y_2) Transmittance of the formulated nanoemulsion ranged from 84.00 ± 1.73 to $94.8 \pm 2.54\%$. The effect of variables on transmittance was best explained by the quadratic polynomial model as described in the given equation. The linear term of S_{mix} had a significant effect on transmittance of nanoemulsion with $p < 0.0001$, followed by oil ($p < 0.0001$), homogenization pressure ($p < 0.05$) and number of homogenization cycles ($p < 0.05$). The linear term of oil had a negative effect on transmittance, whereas the linear term of the surfactant, pressure and number of cycles had a positive effect on it (Fig. 2).

$$\begin{aligned}\text{Transmittance } (Y_2) = {} & 87.60 - 1.84A + 2.1B \\ & + 0.38C + 0.97D + 0.2AB \\ & + 0.80AC + 0.15AD + 0.95BC \\ & - 0.15BD - 0.16CD - 0.024A^2 \\ & + 0.68B^2 + 0.90C^2 + 1.03D^2.\end{aligned}$$

Effect on PDI (Y_3) Polydispersity basically is the ratio of standard deviation to the mean droplet size. The polydispersity values of the formulation ranged from 0.142 ± 0.021 to 0.420 ± 0.049, which indicated uniformity of droplet size within the formulation. A quadratic polynomial model for polydispersity size was suggested by CCRD. The linear term of S_{mix} had a significant effect on PDI of the nanoemulsion with $p < 0.0001$, followed by oil ($p < 0.0001$), homogenization pressure ($p < 0.05$) and number of homogenization cycles ($p < 0.05$). The linear term of oil had a positive effect on PDI, whereas the linear term of the surfactant, pressure and number of cycle had a negative effect on it (Fig. 2).

$$\begin{aligned}\text{PDI } (Y_3) = {} & 0.24 + 0.045A - 0.043B \\ & - 7.958C - 0.020D - 0.014AB \\ & - 0.016AC - 8.188AD - 0.015BC \\ & + 0.011BD + 3.312CD + 0.013A^2 \\ & + 1.802B^2 - 0.016C^2 - 0.013D^2.\end{aligned}$$

Characterization of optimized nanoemulsion

The optimized formulation had a droplet size 50.04 ± 3.24 with PDI 0.171 ± 0.082 (Fig. 3). The nanoemulsion was studied for the morphology of the droplet. TEM analysis of the nanoemulsion revealed spherical droplets with size

Table 2 Variables and their constraints in CCRD

Independent variable	Levels				
	− 2	− 1	0	1	2
A = oil (%)	2.5	3	3.5	4	4.5
$B = S_{mix}$ (%)	7.5	9	10.5	12	13.5
C = number of homogenization cycles	3	5	7	9	11
D = homogenization pressure (bar)	800	1000	1200	1400	1600
Dependent variable					Constraint
Y_1 = droplet size (nm)					Minimum
Y_2 = transmittance (%)					Maximum
Y_3 = PDI					Minimum

Table 3 Responses obtained by experimental runs generated by CCRD

Independent variables				Response		
A	*B*	*C*	*D*	Y_1	Y_2	Y_3
3	9.0	5	1400	43.20 ± 3.54	92.60 ± 2.54	0.172 ± 0.040
4	12	9	1400	42.00 ± 3.65	93.10 ± 3.50	0.153 ± 0.150
3	9.0	9	1400	48.00 ± 2.76	89.60 ± 1.76	0.210 ± 0.030
2.5	10.5	7	1200	46.17 ± 4.20	90.00 ± 2.18	0.206 ± 0.060
4	12.0	5	1400	49.60 ± 3.59	89.20 ± 1.92	0.260 ± 0.081
3.5	10.5	3	1200	50.65 ± 5.61	89.00 ± 2.05	0.218 ± 0.090
3	12.0	9	1000	34.80 ± 3.67	94.00 ± 1.17	0.160 ± 0.082
3	12.0	5	1000	36.34 ± 5.32	93.60 ± 1.56	0.161 ± 0.076
4	9.0	5	1000	81.03 ± 3.42	84.20 ± 2.67	0.390 ± 0.068
3.5	10.5	7	1600	50.04 ± 3.24	94.80 ± 2.54	0.171 ± 0.082
4	12.0	9	1000	44.51 ± 2.54	89.00 ± 2.10	0.182 ± 0.051
3.5	10.5	7	1200	55.73 ± 3.65	92.20 ± 2.10	0.242 ± 0.041
3.5	7.5	5	1000	77.06 ± 2.43	85.30 ± 2.31	0.380 ± 0.057
4	12.0	5	1400	52.00 ± 2.54	88.50 ± 2.12	0.236 ± 0.026
3	12.0	9	1000	32.30 ± 2.54	94.20 ± 1.61	0.152 ± 0.037
3	9.0	5	1400	51.00 ± 3.61	88.60 ± 2.02	0.228 ± 0.058
4	9.0	7	1200	52.45 ± 4.77	88.00 ± 2.18	0.238 ± 0.061
3.5	10.5	7	1200	50.73 ± 3.05	89.20 ± 1.10	0.272 ± 0.091
3.5	10.5	7	1200	51.07 ± 3.15	89.20 ± 2.08	0.292 ± 0.071
4.5	10.5	9	1000	90.04 ± 2.94	84.00 ± 1.73	0.420 ± 0.049
3.5	10.5	7	1200	49.03 ± 1.57	93.20 ± 2.08	0.190 ± 0.011
4	9.0	7	1200	68.49 ± 3.63	86.00 ± 2.10	0.320 ± 0.018
3.5	10.5	11	1200	44.19 ± 2.71	92.40 ± 1.68	0.176 ± 0.039
3.5	10.5	7	1200	48.03 ± 1.08	87.20 ± 1.58	0.290 ± 0.021
3	9.0	5	1000	45.12 ± 2.91	92.00 ± 1.59	0.183 ± 0.017
3.5	10.5	7	800	55.13 ± 3.26	87.60 ± 1.31	0.268 ± 0.061
4	9.0	9	1400	63.63 ± 2.59	86.20 ± 1.87	0.290 ± 0.030
3.5	13.5	7	1200	32.10 ± 4.39	94.36 ± 1.20	0.158 ± 0.029
3	12.0	9	1400	24.03 ± 4.63	91.00 ± 2.10	0.142 ± 0.021

A = oil (%); *B* = s_{mix} (%); *C* = number of homogenization cycles; *D* = homogenization pressure (bar); Y_1 = droplet size (nm); Y_2 = transmittance (%); and Y_3 = PDI

65.00 ± 5.00 as shown in Fig. 4. The refractive index of the optimized formulation was found to be 1.43 ± 0.02. The viscosity of the nanoemulsion was found to be 23.95 ± 2.19 mPas. The observed pH of the nanoemulsion was 6.82 ± 0.17.

Antioxidant studies

DPPH methods

The potency of resveratrol to reduce the DPPH radical was visually noticeable, as discoloration from purple to yellow took place and was also confirmed by observing the decrease in the absorbance at 515 nm. Figure 5 illustrates a significant reduction in DPPH radical due to the scavenging ability of resveratrol solution, nanoemulsion and the reference compound, ascorbic acid. The percentage inhibition obtained was maximum for ascorbic acid (97.25 ± 2.01%) compared to the nanoemulsion (83.93 ± 3.81%) and resveratrol solution (71.51 ± 3.82%).

Reduced power assay

The reducing power of the drug solution and resveratrol nanoemulsion was compared with that of ascorbic acid (standard). The reducing capacity of resveratrol was measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. The reducing capacity of a compound serves as a sign for potential antioxidant activity, thus resveratrol can be used as a potent antioxidant. It was observed that the reducing

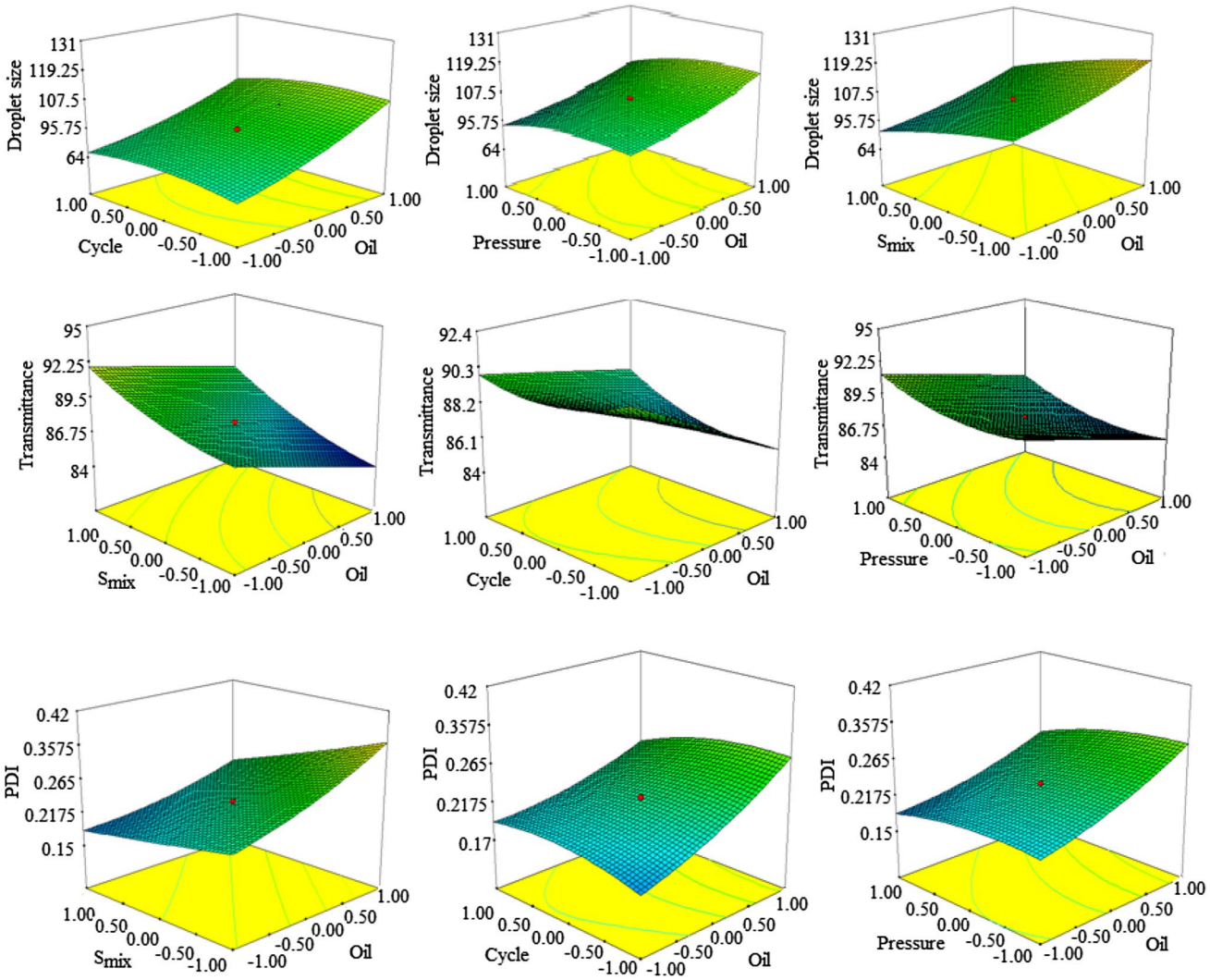

Fig. 2 Effect of oil, S_{mix}, homogenization cycle and pressure on the droplet size, transmittance and PDI of the formulation

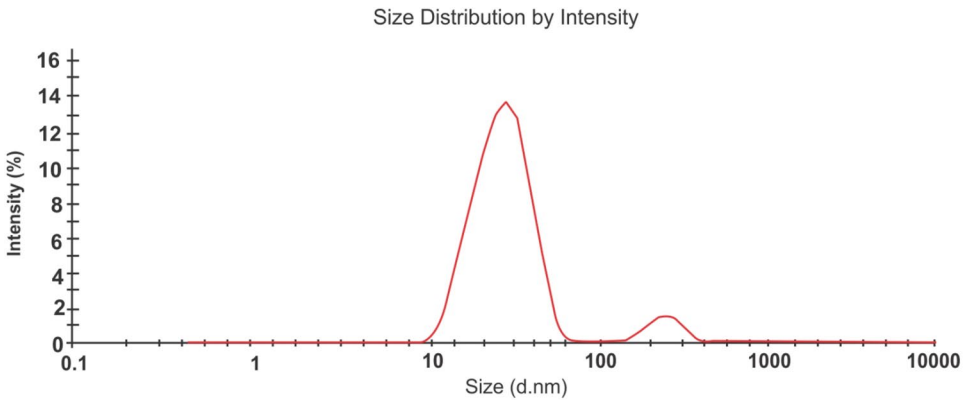

Fig. 3 Droplet size and PDI of optimized formulation. Plot indicates narrow monodispersed system with droplet size less than 100 nm

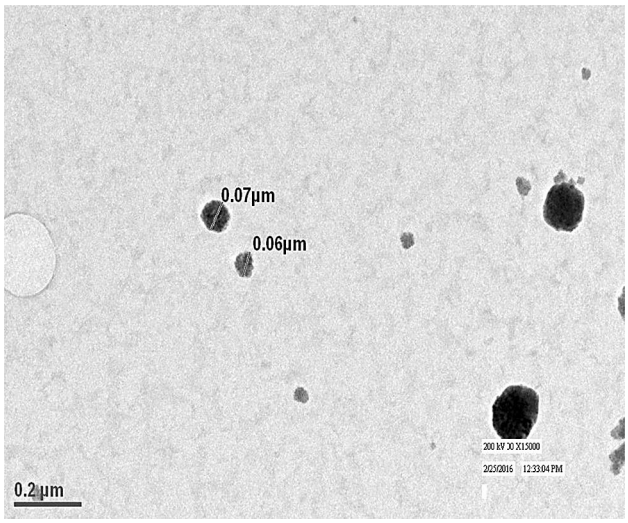

Fig. 4 TEM of nanoemulsion: almost spherical droplets with dark colour core

power of the nanoemulsion was higher than that of the drug solution as shown in Fig. 6.

Evaluation of nanoemulsion gel

The pH of the nanoemulsion-loaded gel was within the acceptable limit for topical products and compatible with the pH of skin. It was found to be 6.60 ± 0.04, which is well accepted to avoid skin irritation upon application to skin [36]. This was slightly less than that of the nanoemulsion due to addition of a drop of triethanolamine. The consistency and homogeneity of the gel were noticed by pressing a small amount of gel between the index and thumb finger. The gel did not contain any coarse particles or aggregates and was found to be homogenous and thus acceptable. Another important parameter is spreadability. The spreadability of the formulated resveratrol gels exhibited good spreading properties. Gel application on the affected skin would be more comfortable if it can be spread easily. The extrudability of the optimized nanoemulsion gel was found to be

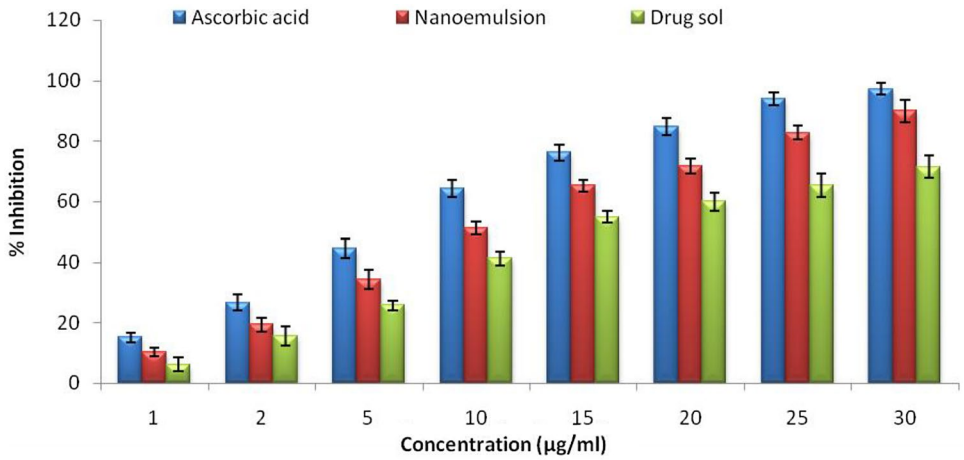

Fig. 5 Comparison of antioxidant activity of ascorbic acid, nanoemulsion and drug solution by DPPH methods

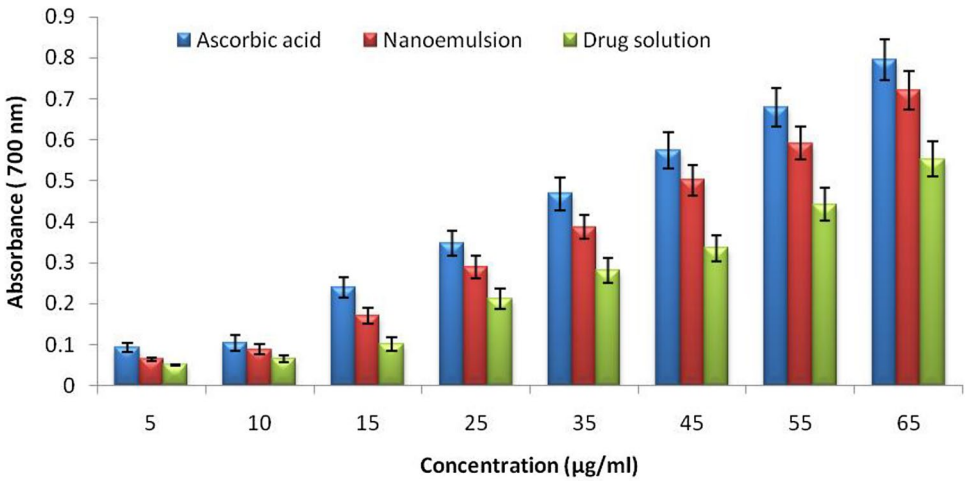

Fig. 6 Antioxidant activity of ascorbic acid, nanoemulsion and drug solution by reduced power assay

Table 4 In vitro skin permeation profile of resveratrol nanoemulsion, resveratrol aqueous dispersion, normal resveratrol gel and resveratrol-loaded nanoemulsion gel

Formulation	Drug permeated/area (μg/cm^2) ± SD time(h)							Flux (μg/cm^2/h)	P_{app} (cm/h)
	1	2	3	4	5	6	7		
Nanoemulsion	704.20 ± 3.60	806.40 ± 3.32	1061.20 ± 5.12	1226.40 ± 3.82	1421.00 ± 2.92	1645.70 ± 3.71	1890.00 ± 2.63	237.40	3.40×10^{-2}
Aqueous dispersion	144.52 ± 3.72	255.26 ± 5.62	358.49 ± 4.74	519.91 ± 3.74	700.09 ± 5.91	904.67 ± 4.54	978.34 ± 3.54	144.50	2.06×10^{-2}
Normal resveratrol gel	–	43.17 ± 3.18	128.00 ± 2.47	197.08 ± 3.19	238.37 ± 3.10	324.71 ± 2.91	384.77 ± 2.16	65.27	1.80×10^{-2}
Nanoemulsion gel	–	333.00 ± 3.03	414.40 ± 2.77	486.55 ± 2.84	540.20 ± 4.02	593.85 ± 3.08	666.74 ± 3.66	107.70	2.90×10^{-2}

5.03×10^{-4} g/N/min. This is a technique to evaluate the force needed to extrude a gel from the tube. The result revealed that minimum force was enough to extrude the gel out of the tube. The drug content test was carried out to evaluate the uniformity of content in each portion. The analysis was carried out in triplicate and the uniformity was evaluated in terms of percent drug content. There was no significant difference in three portions and the drug content of the optimized nanoemulsion gel formulation was found to be in the range of 98.66 ± 0.89–99.56 ± 0.29%.

In vitro skin permeation studies

The in vitro permeation profile of nanoemulsion showed that 1890 μg/cm^2 of resveratrol permeated through the rat skin in 7 h (Table 4). The permeation coefficient and flux were found to be 3.40×10^{-2} cm/h and 237.40 μg/cm^2/h, respectively. On the other hand, the in vitro permeation profile of aqueous dispersion of resveratrol showed that 978.34 ± 3.54 μg/cm^2 of resveratrol permeated through the rat skin in 7 h. The flux was found to be 144.50 μg/cm^2/h and the permeation coefficient was 2.06×10^{-2} cm/h. The permeation profile of nanoemulsion gel showed 666.74 μg/cm^2 amount of resveratrol permeation. Flux was found to be 107.70 μg/cm^2/h and the permeation coefficient was 2.9×10^{-2} cm/h. The in vitro permeation profile of normal gel showed that 384.77 ± 2.16 μg/cm^2 amount of resveratrol permeated through the skin. The flux was found to be 65.27 μg/cm^2/h and the permeation coefficient was 3.4×10^{-2} cm/h (Fig. 7).

Drug deposition in skin

The drug deposition in the rat skin treated with nanoemulsion, nanoemulsion gel, conventional gel and conventional dispersion of resveratrol was studied. Resveratrol deposition in rat skin from nanoemulsion, nanoemulsion gel, conventional gel and conventional dispersion was found to be 45.65 ± 4.76%, 62.65 ± 4.98%, 33.42 ± 2.14% and 22.42 ± 1.32%, respectively.

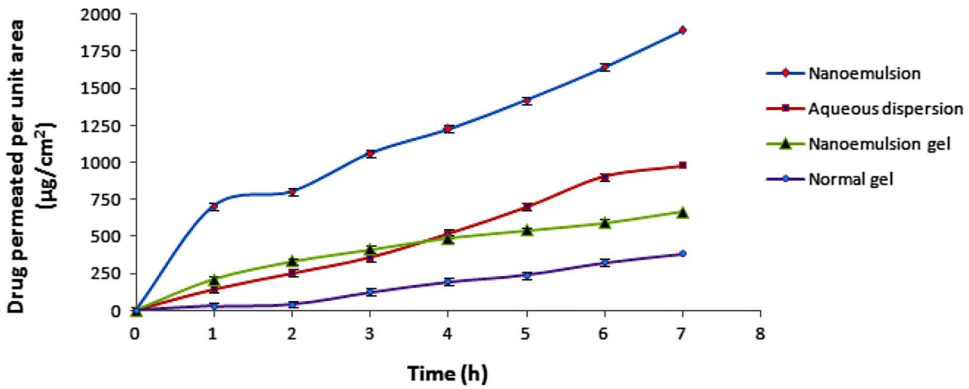

Fig. 7 Resveratrol permeated per unit time from nanoemulsion, aqueous dispersion, normal resveratrol gel and nanoemulsion gel (mean ± SD, $n = 3$). $p < 0.001$ highly significant, nanoemulsion vs. aqueous dispersion, nanoemulsion gel and normal gel. $p < 0.05$ significant, nanoemulsion gel vs. normal gel

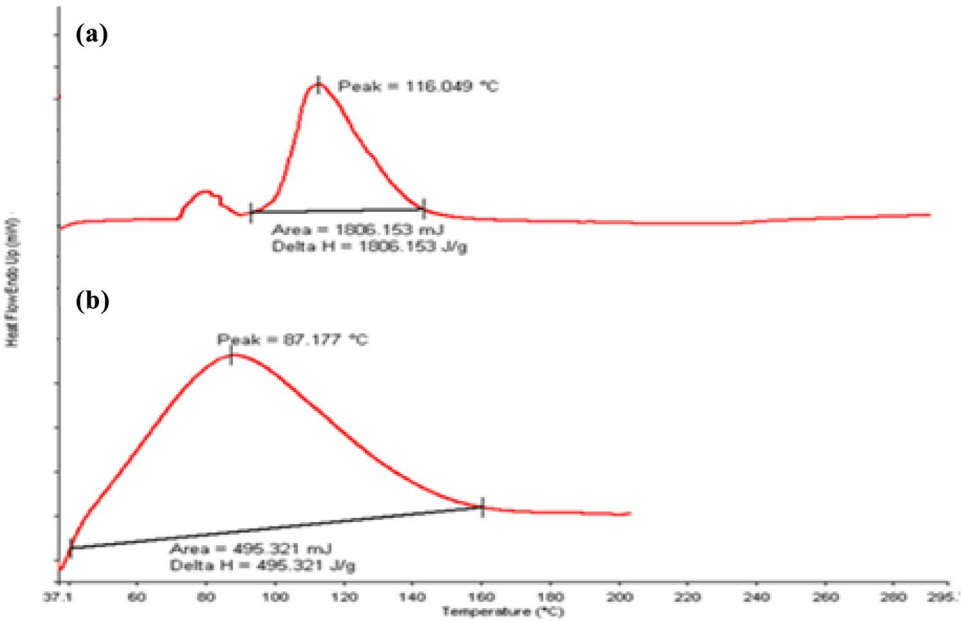

Fig. 8 DSC thermogram of **a** normal skin and **b** nanoemulsion gel treated skin. Peak-II (116.049 °C) shifted to lower temperature (87.177 °C) in skin treated with nanoemulsion gel that indicates denaturation of keratin protein

Mechanism of skin permeation

DSC

The mechanism of drug permeation was elucidated by measurement of the enthalpy changes in skin surface. The DSC thermogram of the nanoemulsion gel-treated skin was compared with the thermogram of normal rat skin. The DSC thermogram of normal skin showed two endotherm peaks at 85 °C (peak-I) and 116 °C (peak-II) (Fig. 8). Further, the DSC thermogram of the nanoemulsion gel-treated stratum corneum showed only a prominent sharp peak at 87.177 °C. It indicated that the treatment of skin with the nanoemulsion gel caused shifting of peak-II to 87.177 °C (lowered by 28.833 °C).

FTIR

The FTIR spectra of nanoemulsion gel-treated and -untreated skin were recorded and compared. The characteristic bands in the spectra of untreated skin appeared at 2923.14 and 2852.70 cm^{-1}. These peaks were due to asymmetric and symmetric –C–H stretching in lipids, respectively. Another band at 2958 cm^{-1} was due to asymmetric CH_3 vibrations and it related to the major stratum corneum lipid components (ceramides and cholesterol). Moreover, two strong bands at 1647 cm^{-1} and 1553 cm^{-1} occurred due to the amide I and amide II C=O stretching. The C=O stretching is related to protein conformation. In FTIR spectra of treated skin, split peak at 1651.14 cm^{-1} was observed. Moreover, the specific

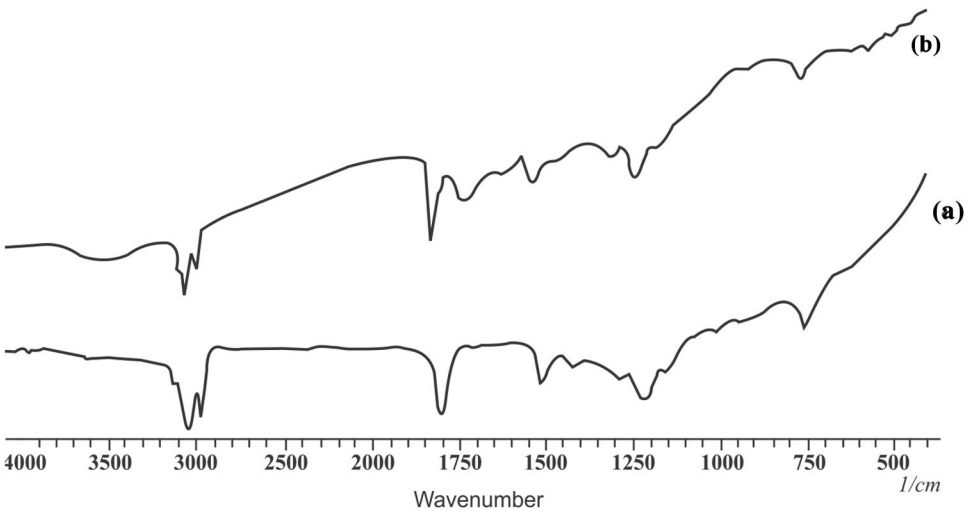

Fig. 9 FTIR of **a** normal skin and **b** resveratrol loaded nanoemulsion gel treated skin. A prominent decrease in peak area and height of characteristic asymmetric CH– stretching in the FTIR of formulation-treated skin indicates conformational disruption and fluidization of lipid bilayer of stratum corneum

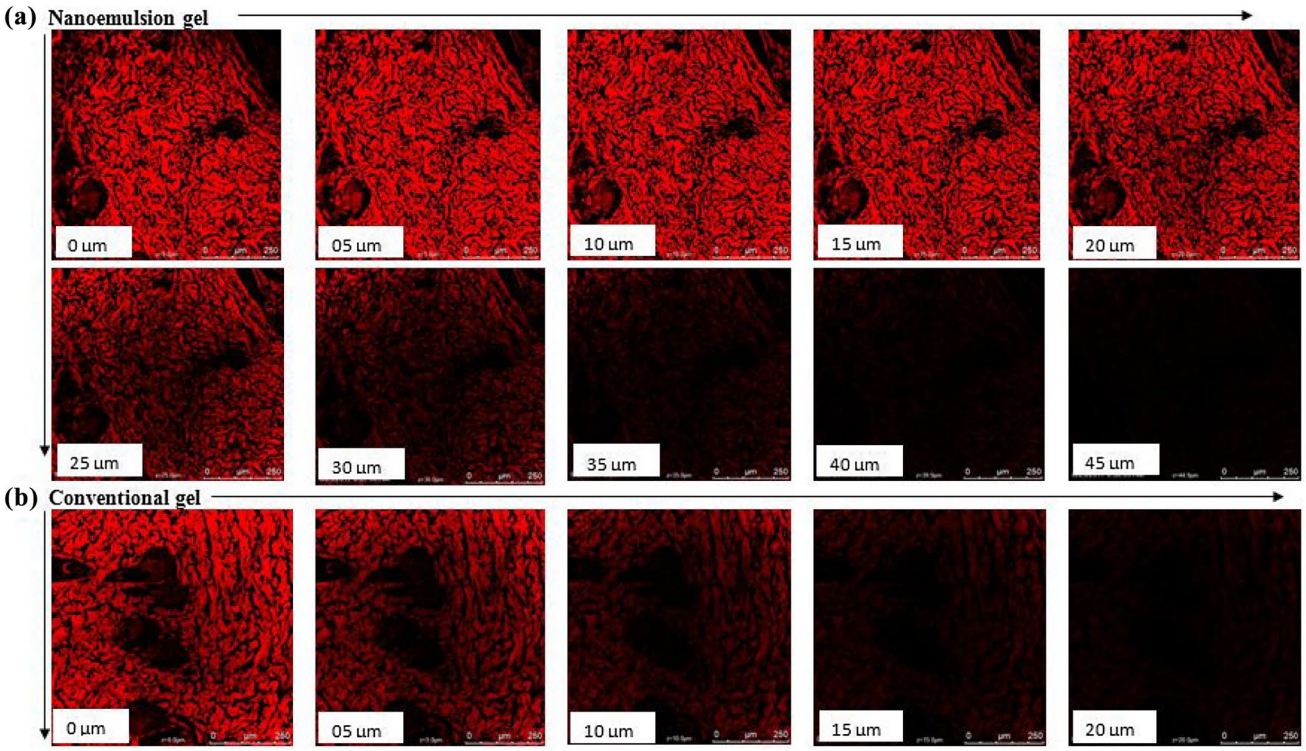

Fig. 10 Confocal laser microscopy images of rat skin treated with **a** nanoemulsion gel of resveratrol, **b** conventional gel of resveratrol. The intensity of red color is indicative of drug deposition in skin layers. Skin treated with normal gel showed maximum fluorescence intensity at the surface of the skin (0 µm), whereas skin treated with nanoemulsion showed drug deposition in viable epidermis (15–25 µm)

C–H stretching peaks at 2852.70 and 2923.14 cm^{-1} were of weaker intensity (Fig. 9).

Depth of permeation: CLSM study

The distribution of resveratrol in epidermal layers of rat skin was studied for nanoemulsion gel and plain gel (normal gel) using confocal microscope. The intensity of color produced by a fluorescence dye Rhodamine was measured in the epidermis and dermis layers of the skin. The confocal microscopic data revealed a significant difference in fluorescence intensity in nanoemulsion gel and normal gel-treated skin. The highest fluorescence intensity in skin treated with conventional gel of resveratrol was found to be at 0–5 µm. It means maximum drug was deposited at the surface of the skin and it was limited to the stratum corneum layer of skin (Fig. 10). Further, the fluorescence dye distribution in epidermis and dermis layer of skin from nanoemulsion gel was pronounced. The highest fluorescence intensity in skin treated with nanoemulsion gel of resveratrol was found to be at 15–20 µm. Furthermore, after 20 µm depth of skin, the fluorescence intensity decreased and became negligible after 40 µm.

Skin irritation study

The skin irritation test was performed to confirm the safety of the optimized nanoemulsion gel formulation of resveratrol. Optimized nanoemulsion gel was subjected to primary skin irritation test and the score obtained was zero, suggesting that the formulated nanoemulsion gel was nonirritant to skin.

Oxidative stress study: biochemical estimation

The skin-protective potential of conventional gel and nanoemulsion gel of resveratrol was evaluated on UV-induced oxidative damaged skin of rats. Oxidative damage was measured in terms of TBARS (MDA level), GSH, SOD, catalase and protein carbonyl. SOD, GSH, catalase MDA and protein carbonyl contents level in the normal group, control group, resveratrol gel-treated group and resveratrol nanoemulsion gel-treated group was 420.73 ± 31.82, nM/mg of protein, 102.83 ± 14.17 nM/mg of protein, 221.8 ± 17.02 nmol H_2O_2 consumed/min/mg protein, 654.29 ± 18.20 nM/mg of protein, 1.57 ± 0.5 nM/mg of protein, 220.25 ± 43.57 nM/mg of protein, 79.16 ± 9.91 nM/mg

Table 5 Effects of different formulation in GSH, TBARS, SOD, catalase and protein carbonyl

Group	GSH (nM min/mg of protein)	TBARS (nM/mg of protein)	SOD (μM/mg of protein)	Protein carbonyl (nmol carbonyl/mg protein)	Catalase (nmol of H_2O_2)
Normal	102.83 ± 14.17	654.29 ± 18.20	420.73 ± 31.82	1.57 ± 0.50	221.80 ± 17.02
Control	79.16 ± 9.91	1777.60 ± 61.59	220.25 ± 43.57	2.89 ± 0.59	132.38 ± 7.91
Normal gel treated	79.00 ± 10.30	1070.85 ± 42.41	314.62 ± 43.71	2.56 ± 0.12	155.81 ± 16.05
Nanoemulsion gel treated	90.00 ± 19.19	731.23 ± 21.59	405.45 ± 31.94	2.24 ± 0.24	202.13 ± 09.43

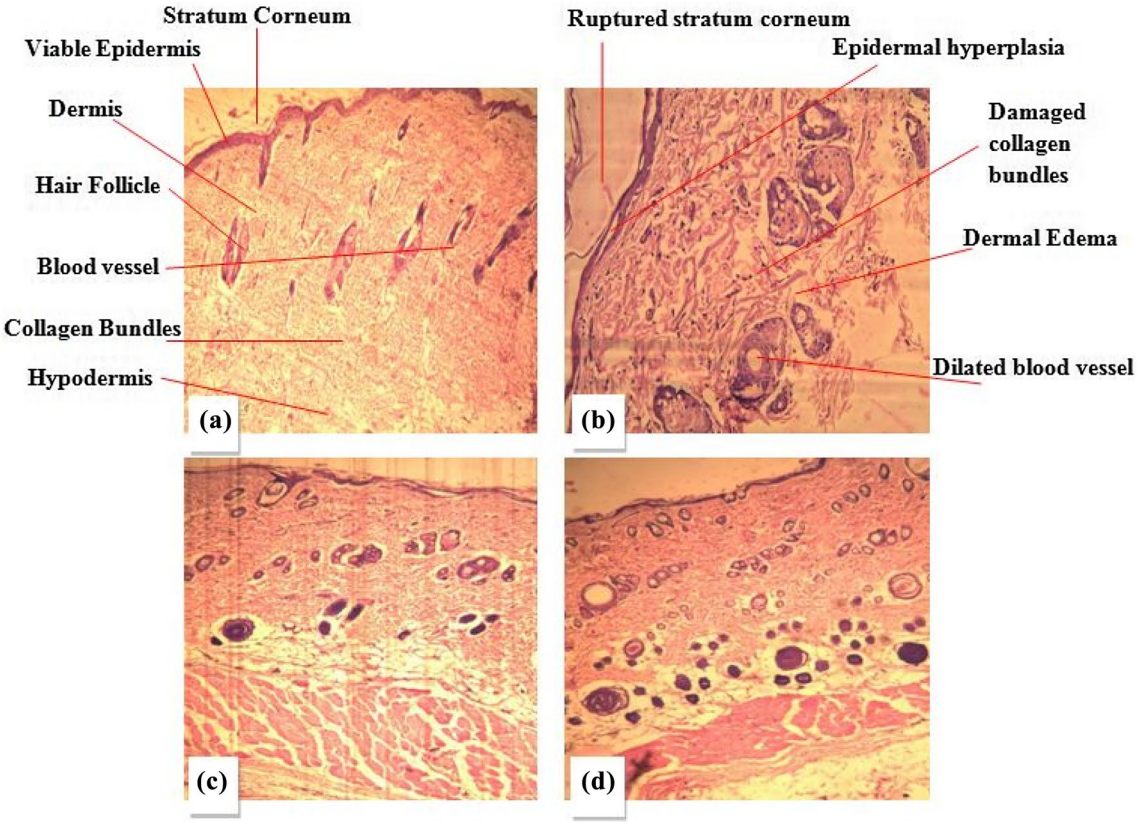

Fig. 11 Histopathology of rat skin **a** normal skin, **b** UV-irradiated skin, **c** skin treated with plain gel of resveratrol and **d** skin treated with nanoemulsion gel of resveratrol. Histopathology of the skin tissue of various groups indicates that resveratrol inhibits UV-induced spongosis, edema and epidermal hyperplasia response

of protein, 132.38 ± 7.91 nmol H_2O_2 consumed/min/mg protein, 1777.6 ± 61.59 nM/mg of protein, 2.89 ± 0.59 nM/mg of protein, 314.62 ± 43.71 nM/mg of protein, 79 ± 10.30 nM/mg of protein, 155.81 ± 16.05 nmol H_2O_2 consumed/min/mg protein, 1070.85 ± 42.41 nM/mg of protein, 2.56 ± 0.12 nM/mg of protein and 405.45 ± 31.94 nM/mg of protein, 90.00 ± 19.19 nM/mg of protein, 202.13 ± 9.43 nmol H_2O_2 consumed/min/mg protein, 731.23 ± 21.59 nM/mg of protein and 2.24 ± 0.24 nM/mg of protein respectively (Table 5).

Histopathological studies

Since the highest drug deposition was found with nanoemulsion gel, it was used for further investigation of histopathological changes in treated skin and findings were compared with the results of conventional gel of resveratrol. Histopathological analysis of the rat skin specimens was evaluated using light microscope (Motic Japan) at 10X. These studies were performed to determine the degenerative effect, erosion and other changes occurring in the epidermis and dermis of the skin after UV irradiation. The microscopic structure of normal skin, UV-irradiated skin and skin treated with

plain gel of resveratrol and nanoemulsion gel of resveratrol is shown in Fig. 11. Rat skin of the normal group (group 1) displayed a complete and clear structure with well-defined epidermal and dermal layers (Fig. 11a). The epidermis is composed of several layers of squamous cells and covered with a thin layer of stratum corneum on the top, whereas the dermis shows orderly arranged collagen bundles, fibroblasts and hair follicles. Altered histological characteristic features in UV-irradiated rat skin were detected. Figure 11b shows the histological appearance of UV-irradiated skin of rat in which UV irradiation caused transformation of the stratum corneum as well as changes in the cellular structure of the basal layer of epidermis. The dorsal skin of UV-irradiated rat showed moderate spongosis, intracellular edema and epidermal hyperplasia. Further, damaged collagen fibers, dense fibronoblast and collagen bundles were also observed in the papillary dermis of skin. However, the skin of rats treated with nanoemulsion gel of resveratrol showed well regular epidermis and dermis as compared to the skin of rats treated with normal gel. The epidermis consisted of multiple layers of squamous cells and was covered with stratum corneum. The dermal layer was occupied by sebaceous glands attached to hair follicles. In addition to it, an ordered arrangement of collagen bundles was also observed.

Discussion

The oil selected should be capable of dissolving the required quantity of the drug freely, as forced solubility with the incorporation of surfactants results in the precipitation of the drug over a period of time. The highest solubility in Sefsol 218® can be attributed to the non-polarity of resveratrol, which favors solubilization in median chain mono/di/triglycerides. Sefsol 218® has potential to solubilize the maximum amount of drug. Sefsol 218® consists of monoglyceride chains of propylene glycol and caprylic acid, which makes it an excellent solubilizer. It has the capability to penetrate the oil–water interface and enhance interaction of the surfactant at the interface to thus increase the emulsification potential of the surfactant [28]. Surfactants play an important role in the formation of stable nanoemulsion. Selection of surfactant is also critical, as the selected surfactant should not only lower the interfacial tension, but also be safe at the concentration used [26].Tween 80 is a polyoxyethylene derivative of sorbitan monooleate, and has good solvent capacity. It also facilitates oil emulsification at the oil–water interface to form a nanoemulsion and provide stability to the nanoemulsion droplets by incorporating the long chain fatty acids into the core of oil droplets. Tween 80 is a non-ionic surfactant with non-irritant characteristics on the skin. Moreover, a main thing regarding the surfactant to produce fine droplets is its molecular geometry. Molecular geometry is characterized by the packing parameter. Packaging parameter (P) is defined as the ratio of the tail group area to the head group area: $P = a_T/a_H$. Variation in the packing of surfactant at oil–water interface affects the mobility and surface tension, which play a key role in the spontaneous formation of oil droplets using low-energy emulsification techniques. Tween 80 exhibits a higher packing parameter (because of more a_T), thus it has optimum surfactant geometry required to promote the spontaneous formation of ultrafine dispersion [37]. From the pseudoternary phase diagram, it is clear that the region of nanoemulsion increases with increase in the concentration of Tween 80 in Smix. But there is no significant difference in the area of the nanoemulsion region of S_{mix} ratio 4:1 and S_{mix} ratio 5:1. As the maximum emulsification was obtained in S_{mix} ratio 4:1, this ratio was used for the preparation of nanoemulsion by high-pressure homogenization method.

The concentration of oil, S_{mix}, homogenization pressure and cycle were optimized by using response surface methodology. The droplet size increased with increase in oil composition, as on increasing the oil concentration, the globule disruption mechanism became more difficult as there was more flow resistance and thus the globule breakup rate became severely restricted. Secondly, part of the effect can be attributed to the increased rates of collision frequency, particularly at lower concentration, between the emulsion droplets followed by an ultimate increase of coalescence frequency, which subsequently led to a higher probability of coalescence of the droplets. On increasing the surfactant content, there was reduction in droplet size. This was because of the surfactant capability to decrease the interfacial tension, thereby decreasing the Laplace pressure and the stress required for droplet deformation [37]. Increment in pressure leads to decrease in the droplet size, because pressure significantly influences the properties of the formulation. Increase in homogenization cycle resulted in significant decrease in droplet size of the formulated nanoemulsion. Transmittance denotes the clarity of the formulation due to the smaller droplet size of the formulation which is required for the stability of formulation. Increase in concentration of oil significantly increased the droplet size of the formulation, which ultimately decreased the transmittance or vice versa. Increase in concentration of the surfactant reduces the droplet size of the formulation. This is due to the lowering of surface tension of the formulation due to small droplet size, resulting in more clarity (transmittance). It was also observed that transmittance increased with the increase in homogenization cycle and pressure. The formulation has droplet size in the nano range with low value of polydispersity. The higher value of PDI indicates low uniformity in the size of the formulation, whereas lesser value indicates good size distribution [24]. The PDI value ranges from 0.00 to 1.00 for monodispersed system to high dispersed system,

respectively. Smaller PDI values are indicative of the fact that the drug is uniformly dispersed in the oil phase and is not leaking into the aqueous phase of the system. The PDI value of the developed formulation indicates narrow size distribution and size uniformity of the optimized nanoemulsion formulation. It was observed that PDI increased with increase in oil concentration and decreased with increased S_{mix}. The nanoemulsion droplet appeared as a dark spot with bright surrounding. The droplet size obtained was compared with the droplet size obtained by using Malvern Zetasizer and was in the range with no aggregation. Refractive index is approximately equal to water, thus the obtained formulation was transparent in nature. Moreover, the refractive index of the nanoemulsion was not significantly changed in the optimized formulation and placebo formulation, suggesting that the prepared nanoemulsion was physically and chemically stable and remained isotropic in nature [25]. The pH of the gel required for the dermal delivery should be within the range of 6.8–7.4. The pH of obtained formulation signifies that the optimized nanoemulsion gel was well accepted to avoid skin irritation upon application to skin.

The obtained droplet size suggested that it can easily permeate through skin due to its smaller size [23]. The skin permeation data indicate that the drug rapidly permeated from nanoemulsion as compared to resveratrol dispersion. In topical delivery of drug, the permeation coefficient describes the ability of skin conductance to a drug from the vehicle into the stratum corneum and it depends on several factors such as solubilization of drug in vehicle and partitioning of drug from vehicle to stratum corneum. The increased permeation coefficient and flux of resveratrol in nanoemulsion indicated more solubilization and permeation of resveratrol in the lipid layer of skin. Further, improvement in permeation of resveratrol is due to hydrophilic and hydrophobic contents of nanoemulsion that altered the barrier structure. The lower permeation coefficient and flux of resveratrol in aqueous dispersion were due to poor solubilization and partition of the drug in the epidermal layer. Nanoemulsions are less viscous and have water-like consistency that limit its topical application. As they are removed from the applied area by sweat, the nanoemulsion was transformed to gel for ease of application and to increase the topical contact time of drug with the affected area. The lowered permeation of resveratrol from nanoemulsion gel as compared to nanoemulsion may be due to entrapment of resveratrol in the Carbopol matrix and hence deposition of resveratrol on the superficial covering of skin for a prolonged time. Furthermore, the low permeation of resveratrol from normal gel is due to poor solubilization of resveratrol in the hydrophilic matrix and low partitioning of it between the matrix and epidermal layer. The highest drug deposition was found in skin sample treated with nanoemulsion gel, followed by the skin sample treated with nanoemulsion. It may be due to enhanced solubilization of resveratrol in the hydrophilic matrix of Carbopol, improved partition coefficient as well as consistent release of resveratrol from the polymeric matrix. The dermal retention of resveratrol was due to greater contact with corneocytes, skin occlusion and sustained release owing to the characteristics of the nanoemulsion gel. Moreover, the relatively low drug deposition in nanoemulsion-treated skin is due to its low viscosity and hence low retention at the applied site. The result revealed that nanoemulsion-based gels are suitable for dermal use where local effect is required. The CLMS study indicated that the drug permeated between the epidermis and dermis and maximum drug was deposited in the viable epidermis. The enhanced penetration of drug to the epidermis and dermis layers of skin may be due to nanosized particles that enhanced solubilization of the drug. Moreover, it may also be due to the lipid component of the formulation vehicle that increases fluidization of the phospholipid bilayer of the stratum corneum [32].

Peak-I and peak-II are due to melting of stratum corneum lipids and denaturation of intracellular stratum corneum proteins (keratin), respectively. It was observed that peak-I completely disappeared in DSC thermogram of nanoemulsion gel-treated stratum corneum. Disappearance of peak-I was due to disruption of lipid bilayers of stratum corneum. This is because of fluidization of stratum corneum lipids by the interaction of Sefsol 218® (oil), Tween 80 (surfactant) and PEG 400 (co-surfactant) present in nanoemulsion. Shifting of peak-II suggests denaturation of stratum corneum protein and the possibility of intracellular permeation of resveratrol. All these findings indicated that the nanoemulsion enhanced fluidization of SC lipids, thereby enhancing the skin permeation of resveratrol [38]. There was clear difference in the FTIR spectra obtained with nanoemulsion gel-treated stratum corneum as compared to FTIR spectra of untreated skin. A prominent decrease in peak area and height of asymmetric CH– stretching was observed, due to the conformational disruption of the lipid bilayer and fluidization of stratum corneum lipids. In addition to this, the splitting of the peak at 1651.14 cm^{-1} was also observed which indicated breaking of hydrogen bonds. Treatment with nanoemulsion gel caused a change in the fluidity of stratum corneum lipids by disruption of the lipid bilayer and breaking of hydrogen bonds in ceramide [39].

In vitro antioxidant activity study indicates that the percentage inhibition of nanoemulsion was more than that of the drug solution, because the nanoemulsion resulted in greater solubilization of resveratrol, whereas in resveratrol dispersion the drug particle was not solubilized thus leading to limited inhibition [3]. Pangeni and associates have also reported that resveratrol exhibits high scavenging efficiency toward DPPH radicals [15]. Its phenolic hydroxyl groups have redox characteristics. Moreover, it has potential for

electron delocalization. Both these properties are responsible for its ROS scavenging activity.

SOD plays a defensive key role against oxidative damage. It catalyzes dismutation of free radicals such as O^-, OH^- and hydrogen peroxide. It has been reported earlier that SOD level in skin tissue is significantly decreased after exposure to UV radiation and hence the skin cells become more susceptible to oxidative damage. The result revealed that SOD level in the control group was lowered significantly ($p < 0.01$) when compared with normal. However, the SOD activity of normal gel and nanoemulsion gel-treated group was increased significantly ($p < 0.01$) as compared to the control group. Increased activity of SOD in animals treated topically with nanoemulsion gel of resveratrol indicates significant inhibition of UV-induced generation of ROS, demonstrating skin-protective potential of the developed formulation. GSH, which is an endogenous antioxidant, plays a defensive role against oxidative damage. Reduced glutathione is able to donate H^+ (a reducing equivalent) to reactive oxygen species (ROS) that is generated in oxidative damage and thereby neutralize them. Moreover, it also helps to maintain endogenous antioxidants (vitamin C and E) in active forms. A decreased glutathione concentration in skin cell indicates impaired defensive antioxidant enzyme mechanism that makes the skin sensitive to oxidative damage. The GSH concentration in skin tissue of UV-irradiated animals was decreased significantly ($p < 0.01$) as compared to normal. The GSH level increased significantly ($p < 0.01$) in skin tissue of animals treated with nanoemulsion gel of resveratrol. Further, no significant difference was observed in the level of GSH in the control group and normal gel group. Catalase is an important component of cellular antioxidant enzymes system that protects the epidermis from oxidative damage. It is responsible for the degradation of hydrogen peroxide generated in lipid peroxidation in oxidative stress. UV irradiation causes the formation of ROS and thereby generation of hydrogen peroxide. The generated hydrogen peroxide accelerates oxidation of cellular components in skin cells and also deactivates SOD. Catalase scavenge formed ROS mainly hydrogen peroxide. It protects SOD and hence protects skin cells from oxidative damage. Catalase activity in UV-irradiated rats was significantly ($p < 0.01$) lower as compared to the normal group. Further, catalase activity in animals treated with resveratrol formulation (plain gel and nanoemulsion gel) was significantly ($p < 0.01$) increased when compared to the UV-irradiated group. However, increased catalase activity was more prominent in animals treated with nanoemulsion gel of resveratrol. Nanoemulsion gel formulation of resveratrol increased the catalase activity by 52.68% as compared to the control group. This prominent effect of nanoemulsion gel of resveratrol is due to solubilization of resveratrol and enhanced permeability of resveratrol that increased its deposition in the epidermal layer. TBARS formation in UV-irradiated skin is a reliable indicator of free radical formation in the tissue and is used as an index of lipid peroxidation in biological systems [40]. It is expressed in terms of malondialdehyde (MDA). The increased level of MDA in skin tissue suggests increase in lipid peroxidation ultimately leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. However, topical pretreatment with nanoemulsion gel of resveratrol significantly reversed these changes and showed a significantly decreased MDA content after UV irradiation. This effect is due to antioxidant and skin-protective potential of resveratrol. Protein carbonyl content is greatly affected by UV irradiation and most commonly used marker of protein oxidation in oxidative damage. Carbonyl (CO) groups are produced on protein side chains when they are oxidized. Their derivatives can also be generated through oxidative cleavage of proteins or by secondary reaction of amino acid residues with aldehyde such as 4-hydroxy-2-nonenal, malondialdehyde and acrolein produced during lipid peroxidation. After the UV irradiation, the protein carbonyl content increased significantly in the control group ($p < 0.01$) as compared to normal, which shows that UV irradiation greatly induced the protein oxidation in the skin cells and caused cellular damage. Normal gel-treated and nanoemulsion gel-treated group decreased the formation of protein carbonyl content as compared to the control group. However, resveratrol nanoemulsion gel-treated animal showed significantly lowered content of protein carbonyl as compared to the control group. Further, treatment of animals with nanoemulsion gel of resveratrol before UV irradiation resulted in significant improvement in epidermal damage caused by oxidative stress in the skin cells. Nanoemulsion gel of resveratrol inhibits UV-induced spongosis, edema and epidermal hyperplasia response. This effect of resveratrol is due to antioxidant and skin-protective potential. The greater solubilization of resveratrol in nanoemulsion gel resulted in the epidermal deposition of resveratrol leading to significant improvement in photo damaged skin of rat. Slight improvement was noticed in animal treated with plain gel of resveratrol. The findings of this study indicates topical delivery of resveratrol via nanoemulsion gel may result in better treatment outcomes in diseases associated with oxidative stress in epidermal tissue.

Conclusion

Resveratrol nanoemulsion gel was developed, optimized and characterized for topical delivery in the management of UV-induced oxidative skin damage. In vitro skin permeation and skin deposition study indicated that nanoemulsion gel has potential to deliver drug to the upper layers of skin.

FTIR spectra and DSC thermograms of the nanoemulsion gel-treated skin provided an idea about the mechanism of the phenomenon of drug permeation through the stratum corneum. The result of histopathology studies have revealed the potential of nanoemulsion gel in the prevention of UV-induced oxidative skin damage, due to enhanced skin permeation and hence epidermal and dermal deposition of drug. Moreover, CLSM studies confirmed the deposition of resveratrol in the epidermis and dermis layers of skin. It can be concluded that topical delivery of resveratrol-loaded nanoemulsion gel is a better approach for the prevention of UV-induced oxidative skin damage.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

References

1. Schieber M, Chandel NS (2014) ROS function in redox signaling and oxidative stress. Curr Biol 24:R453–R462
2. Kryston TB, Georgiev AB, Pissis P, Georgakilas AG (2011) Role of oxidative stress and DNA damage in human carcinogenesis. Mutat Res 711:193–201
3. Sharma S, Narang JK, Ali J, Baboota S (2016) Synergistic antioxidant action of vitamin E and rutin SNEDDS in ameliorating oxidative stress in a Parkinson's disease model. Nanotechnology 27:375101
4. Choudhari SK, Chaudhary M, Gadbail AR, Sharma A, Tekade S (2014) Oxidative and antioxidative mechanisms in oral cancer and precancer: a review. Oral Oncol 50:10–18
5. Darr D, Fridovich I (1994) Free radicals in cutaneous biology. J Investig Dermatol 102:671–675
6. Kruk J, Duchnik E (2014) Oxidative stress and skin diseases: possible role of physical activity. Asian Pac J Cancer Prev 15:561–568
7. Wiseman H, Halliwell B (1996) Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 313:17–29
8. Addor FAS (2017) Antioxidants in dermatology. An Bras Dermatol 92:356–362
9. Ji H, Li XK (2016) Oxidative stress in atopic dermatitis. Oxidative Med Cell Longev 2016:1–8
10. Baur JA, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5:493–506
11. Singh G, Pai RS (2014) Recent advances of resveratrol in nanostructured based delivery systems and in the management of HIV/AIDS. J Control Release 194:178–188
12. Summerlin N, Soo E, Thakur S, Qu Z, Jambhrunkar S, Popat A (2015) Resveratrol nanoformulations: challenges and opportunities. Int J Pharm. https://doi.org/10.1016/j.ijpharm.2015.01.003
13. Joraholmen MW, Skalko-Basnet N, Acharya G, Basnet P (2015) Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections. Eur J Pharm Sci 79:112–121
14. Kim JH, Park EY, Ha HK, Jo CM, Lee WJ, Lee SS, Kim JW (2016) Resveratrol-loaded nanoparticles induce antioxidant activity against oxidative stress. Asian Australas J Anim Sci 29:288–298
15. Pangeni R, Sharma S, Mustafa G, Ali J, Baboota S (2014) Vitamin E loaded resveratrol nanoemulsion for brain targeting for the treatment of Parkinson's disease by reducing oxidative stress. Nanotechnology 25:485102
16. Park S, Cha SH, Cho I, Park S, Park Y, Cho S, Park Y (2016) Antibacterial nanocarriers of resveratrol with gold and silver nanoparticles. Mater Sci Eng C Mater Biol Appl 58:1160–1169
17. Sessa M, Balestrieri ML, Ferrari G, Servillo L, Castaldo D, D'Onofrio N, Donsì F, Tsao R (2014) Bioavailability of encapsulated resveratrol into nanoemulsion-based delivery systems. Food Chem 147:42–50
18. Vinardell MP, Mitjans M (2015) Nanocarriers for delivery of antioxidants on the skin. Cosmetics 2:342–354
19. Teskac K, Kristl J (2010) The evidence for solid lipid nanoparticles mediated cell uptake of resveratrol. Int J Pharm 390:61–69
20. Pando D, Caddeo C, Manconib M, Fadda AM, Pazos C (2013) Nanodesign of olein vesicles for the topical delivery of the antioxidant resveratrol. J Pharm Pharmacol 65:1158–1167
21. Friedrich RB, Kann B, Coradini K, Offerhaus HL, Beck RC, Windbergs M (2015) Skin penetration behavior of lipid-core nanocapsules for simultaneous delivery of resveratrol and curcumin. Eur J Pharm Sci 78:204–213
22. Immacolata S, DanielaDe S, Virginia C, Laura M, Rosa C, Gabriella F, Fabio A, Maria IR, GiuseppeDe R (2013) Nanocarriers for topical administration of resveratrol: a comparative study. Int J Pharm 440:179–187
23. Kumar D, Ali J, Baboota S (2016) Omega 3 fatty acid-enriched nanoemulsion of thiocolchicoside for transdermal delivery: formulation, characterization and absorption studies. Drug Deliv 23:591–600
24. Anton N, Vandamme TF (2011) Nano-emulsions and micro-emulsions: clarifications of the critical differences. Pharm Res 28:978–985
25. Kumar S, Ali J, Baboota S (2016) Design Expert® supported optimization and predictive analysis of selegiline nanoemulsion via the olfactory region with enhanced behavioural performance in Parkinson's disease. Nanotechnology 27:435101
26. Shakeel F, Baboota S, Ahuja A, Ali J, Aqil M, Shafiq S (2007) Nanoemulsions as vehicles for transdermal delivery of aceclofenac. AAPS Pharmscitech 8:191–199
27. Kumar A, Ahuja A, Ali J, Baboota S (2016) Curcumin-loaded lipid nanocarrier for improving bioavailability, stability and cytotoxicity against malignant glioma cells. Drug Deliv 23:214–229
28. Sharma S, Sahni JK, Ali J, Baboota S (2015) Effect of high-pressure homogenization on formulation of TPGS loaded nanoemulsion of rutin—pharmacodynamic and antioxidant studies. Drug Deliv 22:541–551
29. Shakeel F, Baboota S, Ahuja A, Ali J, Shafiq S (2008) Celecoxib nanoemulsion: skin permeation mechanism and bioavailability assessment. J Drug Target 16:733–740
30. Barakat NS (2010) Evaluation of glycofurol-based gel as a new vehicle for topical application of naproxen. AAPS Pharmscitech 11:1138–1146
31. Nguyen HX, Puri A, Banga AK (2017) Methods to simulate rubbing of topical formulation for in vitro skin permeation studies. Int J Pharm 519:22–33
32. Iqbal B, Ali J, Baboota S (2018) Silymarin loaded nanostructured lipid carrier: from design and dermatokinetic study to mechanistic analysis of epidermal drug deposition enhancement. J Mol Liq 255:513-529. https://doi.org/10.1016/j.molliq.2018.01.141
33. Ghate VM, Lewis SA, Prabhu P, Dubey A, Patel N (2016) Nanostructured lipid carriers for the topical delivery of tretinoin. Eur J Pharm Biopharm 108:253–261

34. Han F, Yin R, Che X, Yuan J, Cui Y, Yin H, Li S (2012) Nanostructured lipid carriers (NLC) based topical gel of flurbiprofen: design, characterization and in vivo evaluation. Int J Pharm 439:349–357
35. Pandey YR, Kumar S, Gupta BK, Ali J, Baboota S (2016) Intranasal delivery of paroxetine nanoemulsion via the olfactory region for the management of depression: formulation, behavioural and biochemical estimation. Nanotechnology 27:025102
36. Wan T, Xu T, Pan J, Qin M, Pan W, Zhang G, Wu Z, Wu C, Xu Y (2015) Microemulsion based gel for topical dermal delivery of pseudolaric acid B: in vitro and in vivo evaluation. Int J Pharm 493:111–120
37. Mayer S, Weiss J, McClements DJ (2013) Vitamin E-enriched nanoemulsions formed by emulsion phase inversion: factors influencing droplet size and stability. J Colloid Interface Sci 402:122–130
38. Naz Z, Ahmad FJ (2015) Curcumin-loaded colloidal carrier system: formulation optimization, mechanistic insight, ex vivo and in vivo evaluation. Int J Nanomed 10:4293–4307
39. Khurana S, Jain NK, Bedi PMS (2013) Nanoemulsion based gel for transdermal delivery of meloxicam: physico-chemical, mechanistic investigation. Life Sci 92:383–392
40. Harwansh RK, Mukherjee PK, Bahadur S, Biswas R (2015) Enhanced permeability of ferulic acid loaded nanoemulsion based gel through skin against UVA mediated oxidative stress. Life Sci 141:202–211