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Formulation, characterization and evaluation of an optimized microemulsion formulation of griseofulvin for topical application

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ABSTRACT

The main objective of the study was to develop a microemulsion (ME) formulation of griseofulvin for the treatment of dermatophytosis (Indian Patent Application 208/DEL/2009). The oil phase was selected on the basis of drug solubility whereas the surfactant and cosurfactant were screened on the basis of their oil solubilizing capacity as well as their efficiency to form ME from pseudo-ternary phase diagrams. The influence of surfactant and cosurfactant mass ratio (Smix) on the ME formation and its permeation through male Laca mice skin was studied. The optimized formulation (ME V) consisting of 0.2% (w/w) griseofulvin, 5% (w/w) oleic acid, 40% (w/w) Smix (1:1, Tween 80 and ethanol) possessed globule size of 12.21 nm, polydispersity index of 0.109 and zeta potential value of −0.139 mV. ME V exhibited 7, 5 and almost 3-fold higher drug permeation as compared to aqueous suspension, oily solution and conventional cream respectively. Besides this the formulation was also evaluated for drug content, pH, stability, dermatopharmacokinetics and antifungal activity against *Microsporum canis* using guinea pig model for dermatophytosis. Treatment of guinea pigs with ME V resulted in a complete clinical and mycological cure in 7 days. The formulation was observed to be non-sensitizing, histopathologically safe, and stable at 5 ± 3 °C, 25 ± 2 °C and 40 ± 2 °C for a period of six months.

1. Introduction

Griseofulvin is a heterocyclic benzofuran extracted from *Penicillium griseofulvum*. It is BCS class II drug having a log *P* value of 2.17 and is practically insoluble in water [1]. The conventional oral route of administration of griseofulvin is associated with issues of poor and highly variable bioavailability, numerous systemic side effects and long duration of treatment. The analysis of physicochemical characteristics reveals that the molecule possesses high melting point of 218–220 °C. This indicates that high energy is required to break the crystal lattice of the molecule in order to dissolve the drug. Literature reveals that despite possessing all the favorable molecular characteristics like the molecular weight (352.77 Da), lipophilicity (log *P* = 2.17), hydrogen bond donors (0) and acceptors (6), polar surface area (71.06 Å) and molar refractivity (87.85) [1,2]; the clinical performance of the drug is compromised just because of poor aqueous solubility. There are numerous reports pertaining to solubility and bioavailability enhancement of griseofulvin out of which micronized, ultramicronized and Gris-PEG™ (solid dispersion of griseofulvin and polyethylene glycol 8000) ultramicrosize tablets proved to be commercial success [3].

Literature also cites the usage of aprotic solvent(s), fugitive solvent(s) or their combination and some conventional formulations of griseofulvin for topical application [4]. In the case of topical drug delivery, the diffusion takes place mainly through the stratum corneum (lipoidal barrier). The drug follows different paths to permeate through the stratum corneum. Existing experimental models explain the existence of parallel path (lipid-only, aqueous-only) and series path (alternating lipid and aqueous). Owing to poor aqueous solubility griseofulvin cannot permeate through the skin due to its less solubility in water than required for crossing the skin barrier. Moreover the possibility of dependence of flux through parallel path exists only when log *P* < 0.8 [5]. Thus, for griseofulvin the optimum solubility in both aqueous and lipid phase is vital in order to maximize its flux through the series path.

Therefore, with an aim to enhance the solubility and eventually the dermal bioavailability of griseofulvin, microemulsion (ME) formulations were designed to increase the dermal penetration and permeation of the drug. Owing to the facile and low cost preparation ME system was opted over the other colloidal counterparts such as liposomes, niosomes, nanoparticles [6]. MEs are transparent, optically isotropic and thermodynamically stable liquid solutions; comprising of oil, water and amphiphile(s), in which either the oil globules are dispersed in water (o/w) or water globules are dispersed in oil (w/o). The globule size typically varies in the range of 10–100 nm [7]. Numerous investigations have revealed the pharmaceutical significance of MEs for dermal [8] as well as

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transdermal [9] administration of a wide variety of drug molecules. The components of microemulsion can interact with the lipid layers of stratum corneum and change its structural integrity leading to enhanced permeation of drug(s) without the need of any specific penetration enhancer(s) [10]. In view of all the above mentioned features of MEs, this system was explored for topical delivery of griseofulvin. Further, the optimized ME formulation was evaluated for *ex vivo* permeation, dermatopharmacokinetics and pharmacodynamic performance using *Microsporum canis* induced guinea pig model for dermatophytosis.

2. Materials and methods

2.1. Materials

Griseofulvin (Wallace Pharmaceuticals Ltd., Mumbai, India), Isopropyl palmitate, Eutanol GPH, Cetiol LC PH and Myritol 318 (Cognis GmbH, Düsseldorf, Germany), Captex 200, Captex 300, Captex 355 and Captex 1000 (Abitec, Janesville, WI, US), Labrafac CC and Labrafac Lipophile 1349 (Gattefossé, USA) and Carbopol® 980 NF (Lubrizol Advanced Materials India Pvt. Ltd., Mumbai, India) were received as gift samples. RPMI 1640 medium (Sigma–Aldrich Inc., MO, USA); HPLC-grade acetonitrile, acetic acid and methanol (Merck KGaA, Darmstadt, Germany) were also used in the study. Triple distilled water (TDW) was used throughout the study. All other chemicals and reagents were of analytical grade and were used without further purification.

2.2. Fungal strains

The standard strains of dermatophytes, *Microsporum gypseum* (MTCC no. 2830), *M. canis* (MTCC no. 2820), *Trichophyton mentagrophytes* (MTCC no. 7250) and *Trichophyton rubrum* (MTCC no. 296) were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.3. Animals

Male Laca mice 8–9 weeks old, weighing 30–35 g was obtained from Central Animal House, Panjab University, Chandigarh, India. These were housed in polypropylene cages and employed for performing *ex vivo* permeation, histopathology and dermatopharmacokinetic studies. Male albino guinea pigs (Duncan Hartley strain) 8–9 weeks old weighing between 350 and 400 g were obtained from disease free small animal house of College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India. Guinea pigs were housed in stainless steel metabolic cages and allowed to acclimatize for a minimum of 15 days before initiating the experiment. All the animals were kept at ambient temperature with a 12-h night/day cycle, and supplied with a standard pellet diet and water *ad libitum*. The protocols for animal use and care were approved by the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh, India (IAEC/97 dated 24.03.2011).

2.4. Screening of formulation ingredients

2.4.1. Screening of oils

The oil phase for developing MEs of griseofulvin was selected on the basis of solubility, surfactant efficiency; Smin [11] and water solubilization capacity; Wmax [12]. The solubility of griseofulvin in various oils (Table 1) was determined employing shake flask method [13] and drug content was analyzed using UV–visible spectrophotometer at 293 nm.

Smin % (w/w) was determined as the minimum amount of surfactant required for completely homogenizing equal masses of oil and TDW to form a single phase. Wmax % (w/w) was determined by titrating equal masses of oil and surfactant with TDW until the system became turbid.

2.4.2. Screening and selection of surfactants

Four different surfactants namely, Tween 20, Tween 40, Tween 60 and Tween 80 were screened. The solubilization capacity of surfactants for oleic acid was studied using 3 mL of 15% (w/v) aqueous solution of surfactants to which aliquots of 5 μL of oil was added with vigorous vortexing until the solution became cloudy [10]. Also, emulsification ability of above mentioned surfactants was screened. 500 mg of surfactant was added to 500 mg of oleic acid. The mixture was homogenized and then 100 mg of this isotropic mixture was accurately weighed and diluted with TDW (500 times) to yield fine emulsion. The emulsions were allowed to stand for 2 h and their transmittance was assessed at 650 nm by UV spectrophotometer using TDW as blank [14].

2.4.3. Screening and selection of cosurfactants

The selection of cosurfactants was done on the basis of ME region. Tween 80 was mixed with four types of cosurfactants, namely, ethanol, isopropyl alcohol, *n*-butanol and isobutyl alcohol. Smix ratio (1:1) was kept constant and pseudoternary phase diagrams were constructed. Twelve different combinations in different weight ratios of oil and Smix, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 1:0.7, 1:0.43 and 1:0.11 were taken so that maximum ratios were covered to explain the boundaries of phases formed in phase diagrams [15].

2.5. Influence of surfactant and cosurfactant mass ratio on ME formation

The selected surfactant and cosurfactant (Smix) were blended in the weight ratios of 3:1, 2:1, 1:0; 1:1, 1:2, and 1:3. Smix, ratios were chosen in decreasing concentration of surfactant with respect to cosurfactant and *vice versa* for a detailed insight into the phase diagrams. Different combinations in different weight ratios of oil and Smix, 1:9, 1:8, 1:7, 1:6, 1:5, 2:9, 1:4, 2:7, 1:3, 3:7, 1:2, 1:1, 1:0.7, 1:0.43, 1:0.25 and 1:0.11 were taken. Aqueous titration method was employed for the construction of the pseudoternary phase diagrams. Subsequently the mixtures were evaluated visually and ME phase was identified as the region in the phase diagram where clear, easily flowable, and transparent formulations were obtained.

2.6. Preparation and optimization of ME formulation

Griseofulvin loaded o/w ME was prepared by dissolving 0.2% (w/w) griseofulvin in 5% (w/w) oleic acid. Then required quantity of different Smix (Tween 80 and ethanol) ratios was added to oil phase and mixed with the aid of vortex mixer (Table 2). The mixture was made up to 100% (w/w) with slow addition of TDW with continuous stirring.

Microemulsion was optimized with respect to Smix ratios and effect of its concentration on *ex vivo* permeation characteristics. In order to alleviate the influence of composition of MEs and take into account the effect of Smix only, all other formulation and process variables were kept constant.

Optimized ME gel of griseofulvin was prepared using 0.2% (w/w) griseofulvin, 5% (w/w) oleic acid, 40% (w/w) mixture of Smix (1:1). This mixture was slowly added to 0.5% (w/w) Carbopol previously gelled in TDW and neutralized with triethanolamine and then TDW was added to make it 100% (w/w).

Also, griseofulvin (0.2% (w/w)) was incorporated in oleic acid to prepare oily solution, in aqueous dispersion (comprising of 0.5% (w/v) Carbopol in water) and o/w conventional cream (comprising of 6% sorbitan mono-oleate, 3% white bees wax, 36% white soft

Table 1
Screening of oils with respect to solubility, surfactant efficiency (Smin) and water solubilization capacity (Wmax).

Oil	Solubility (mg/mL)[a]	Smin (%, w/w)[a]	Wmax (%, w/w)[a]
Isopropyl myristate	0.299 ± 0.018	52.4	13.8
Oleic acid	2.245 ± 0.218	54.8	8.3
Isopropyl palmitate	0.370 ± 0.019	58.7	5.2
2-Octyl-1-dodecanol (Eutanol GPH)	0.729 ± 0.020	62.4	4.8
Ethyl oleate	0.149 ± 0.011	54.1	4.8
Cetiol LC PH (Cocoyl caprylocaprate)	0.241 ± 0.013	62.8	4.1
Captex 200	1.179 ± 0.109	62.5	5.1
Captex 300	1.958 ± 0.104	63.1	4.9
Captex 355	1.192 ± 0.245	64.2	5.0
Labrafac CC	1.105 ± 0.011	65.8	5.2
Labrafac Lipophile 1349	0.822 ± 0.018	65.3	4.9
Myritol 318	1.045 ± 0.025	63.0	5.1
Tricaprin (Captex 1000)	1.763 ± 0.145	69.1	3.8
Olive oil	1.436 ± 0.150	>90	<1
Sesame oil	0.653 ± 0.216	>90	<1
Castor oil	0.150 ± 0.015	>90	<1

[a] All the observations are an average of three experimental determinations.

paraffin, 15% liquid paraffin and 39.8% water) and evaluated for *ex vivo* permeation characteristics.

2.7. Ex vivo drug permeation and skin retention studies

The studies were performed using excised dorsal skin of Laca mice employing vertical Franz diffusion cell assembly (PermeGear, Inc., PA, USA) as described by Aggarwal et al. [16] with slight modifications. Phosphate buffer saline (PBS) pH 6.4 containing 2.0% (w/v) Tween 20 was used as receptor media and the cell contents were maintained at temperature of 32 ± 1 °C. 1 mL aliquot was periodically withdrawn at suitable time intervals from the sampling arm of receptor chamber and was replaced with fresh buffer. At the end of the permeation studies (24 h), the skin surface in the donor compartment was rinsed with ethanol to remove the excess drug. The receptor medium was then replaced with 50% (v/v) ethanol to extract the drug retained in the skin. Similar permeation and skin retention studies were performed using blank formulations (without drug) and the absorbance values were subtracted from test formulations to account for the effect of skin components as well as formulation excipients. The cumulative percent permeation, flux (Jss; μg/h/cm^2) and skin retention (μg/cm^2) were calculated.

The data of *ex vivo* permeation studies was statistically analyzed by one way analysis of variance (ANOVA) followed by Dunnett's method. Results were quoted as significant where p was <0.05.

2.8. Characterization and evaluation of optimized ME

The optimized ME was characterized for morphology (Hitachi H-7000 TEM), globule size, size distribution profile and zeta potential (Malvern Zetasizer, Malvern Instruments Ltd., Worcestershire, UK), thermodynamic stability against centrifugation at 3500 rpm for 30 min [17] and three freeze thaw cycles [18].

The optimized ME as well as its gel were characterized for total drug content (TDC), pH (Labindia Pico+, Mumbai, India).

The optimized ME gel was subjected to texture analysis for assessment of different rheological properties like work of shear, force of gel extrusion, stickiness and firmness [19].

The optimized ME gel was filled in lacquered aluminum collapsible tubes and stored at three different temperatures 5 ± 3 °C, 25 ± 2 °C and 40 ± 2 °C for a period of six months. Samples were withdrawn after specified intervals and evaluated for TDC, pH, transparency, clarity, non-grittiness and color change.

2.9. Skin sensitivity studies and histopathological examination

These studies were performed to evaluate any irritant potential of the developed formulation after topical application. The hair on the dorsal side (2 cm × 3 cm) of mice was removed with electric clipper in the direction of tail to head without damaging the skin. The control group was treated with normal saline and the optimized ME gel was applied to the treatment group three times a day for three days consecutively (n = 5). The animals were observed for any signs of itching or change in skin such as erythema, papule, flakiness and dryness. On the third day animals were sacrificed, the skin was incised and processed as reported by Azeem et al. [20].

2.10. Dermatopharmacokinetics

An area of 2 cm × 3 cm on the dorsal skin of mice was prepared as discussed under skin sensitivity studies. The animals were divided into six groups for sampling at different time points: 5 min, 15 min,

Table 2
Smix ratios of griseofulvin loaded MEs and comparison of permeation parameters of various formulations of griseofulvin (mean ± S.D.; n = 3).

Formulation code	Smix ratio	Mean percent drug permeation	Flux (μg/cm^2/h)	Skin retention (μg/cm^2)	Permeability coefficient (cm^2/h; ×10^{-2})	Enhancement ratio
Aqueous suspension (control)	–	9.69 ± 0.86	2.37 ± 0.11	0.74 ± 0.21	0.24	–
Oily solution	–	13.73 ± 0.78	3.34 ± 0.27	6.76 ± 1.26	0.33	1.41
Conventional cream	–	25.58 ± 0.61	4.35 ± 0.80	11.13 ± 0.42	0.44	1.84
ME I	1:0	40.82 ± 2.80	11.20 ± 0.44	13.66 ± 1.44	1.12	4.72
ME II	3:1	53.58 ± 1.69	13.82 ± 0.47	15.32 ± 1.42	1.38	5.83
ME III	2:1	65.34 ± 0.95	15.05 ± 0.60	23.29 ± 1.54	1.51	6.35
ME IV	1:1	67.48 ± 1.22	22.74 ± 0.65	29.03 ± 1.53	2.27	9.59
ME V[a]	1:1	70.96 ± 0.96	22.23 ± 0.66	35.28 ± 2.66	2.22	9.38
ME VI	1:2	61.73 ± 1.45	12.32 ± 0.46	18.61 ± 1.74	1.23	5.20
ME V Gel	1:1	64.73 ± 1.18	19.02 ± 1.12	36.06 ± 1.88	1.90	8.02

[a] ME V contains 40% Smix (1:1) rest other formulations contain 45% Smix.

30 min, 1 h, 2 h and 4 h ($n = 6$). An amount of 250 mg of optimized ME (equivalent to 500 μg of griseofulvin) was applied on the dorsal prepared region of animals. 500 μL of blood was collected from each animal at the specified time intervals and then they were sacrificed to collect the skin. The bio-samples were stored at −20 °C until analysis.

2.10.1. Reverse phase HPLC (RP-HPLC) conditions

HPLC method previously developed by Wei et al. (2008) was used with slight modifications [21]. Waters® 2695 Separation Module equipped with a 2996 Photodiode Array (PDA) detector and Waters Empower 2 software was employed for analysis. Chromatographic separation was performed using Hibar® 250 mm × 4.6 mm HPLC column (M/s Merck KGaA, Germany). The mobile phase consisted of a mixture of acetonitrile (ACN) and 0.1 M acetic acid (40:60%; (v/v)). The flow rate of mobile phase was kept at 1.5 mL/min. The temperature of the column was kept at 40 °C. A stock solution of griseofulvin (1 mg/mL) was prepared in ACN. The calibration curve standards were prepared by serial dilution of the stock solution in mobile phase in the concentration range between 0.5 and 20 μg/mL. All the samples were filtered through 0.22 μm nylon membrane filter before analysis. The injection volume employed for analysis was 20 μL and the wavelength of detection was 293 nm. The area under the peak was used to calculate the concentration of griseofulvin. Validation studies were executed according to the ICH and USFDA validation guidelines.

2.10.2. Preparation of skin homogenate and extraction of drug

Skin samples were treated with TDW at a temperature of 60 °C to make it free from subcutaneous fat [22]. Skin homogenates (10% (w/v)) were prepared in PBS pH 6.4 and methanol (1:1 (v/v)), using Teflon tissue homogenizer [23]. One part of skin homogenate was then treated with two parts of ACN (containing 0.5% (v/v); formic acid) and the contents were vortexed for 1 min followed by centrifugation for 10 min at 10,000 rpm at 4 °C. The supernatant was filtered through 0.22 μm nylon membrane filter and analyzed.

2.10.3. Processing of blood samples and extraction of drug

The blood samples were centrifuged for 10 min at 10,000 rpm to separate plasma. One part of plasma was extracted with two parts of ACN and analyzed as mentioned in Section 2.10.2.

2.11. Antifungal studies

The broth microdilution method was used to determine the minimal inhibitory concentration (m.i.c.) of griseofulvin against *M. gypseum, M. canis, T. mentagrophytes* and *T. rubrum*. The test were performed using RPMI 1640 medium supplemented with L-glutamine and without sodium bicarbonate buffered at pH 7.0 with MOPS [3-(*N*-morpholino) propanesulfonic acid] buffer. The agar plate diffusion method was performed to check the efficacy of optimized ME of griseofulvin against the above mentioned dermatophytes. The cultures were revived and inoculums were prepared as explained by Barros et al. [24].

2.11.1. Test procedure for broth microdilution method

The tests were performed in sterile, round-bottomed, 96-well microplates following Clinical and Laboratory Standards Institute (CLSI) M38-A protocol; using the drug concentrations between 0.039 and 16 μg/mL [16,24].

2.11.2. Antifungal assay using agar plate diffusion method

Standard plot of griseofulvin against all the dermatophyte strains were prepared in the concentration range 1–10 μg/100 μL using agar plate diffusion method [25]. 100 μL of the optimized ME equivalent to 10 μg of griseofulvin and its corresponding blank were placed in the agar plate wells and incubated for 48 h at 30 °C after pre-diffusion. After the incubation period, the zone of inhibition was measured and recorded.

2.12. Pharmacodynamic studies in guinea pigs

M. canis was selected as the infecting fungus because this zoophilic fungus can infect the skin, resulting in skin and hair root invasion.

2.12.1. Inoculum preparation

Stock inoculum suspensions of the fungi containing 1×10^7 fungal conidia of *M. canis* in 200 μL of sterile normal saline were prepared [24].

2.12.2. Animal inoculation and antifungal therapy

A total of 10 animals were taken and divided into control group treated with blank ME formulation and treatment group treated with optimized ME gel ($n = 5$). The inoculation of animals was done under general anesthesia. An area of 3 cm × 3 cm, on the guinea pigs back was made hair free, marked and abraded with sterile fine grit sandpaper. Then 200 μL of the prepared inoculum was applied to abraded skin [26]. The animals were observed on daily basis for signs of infection. The topical treatment was started after 7 days, after the appearance and confirmation of fungal hyphae on the skin of animals using potassium hydroxide microscopy [27]. ME gel was applied twice a day using a dose quantity of approximately 500 mg (containing 1 mg drug). The clinical parameters were evaluated every day and assessment was scored on a scale from 0 to 5 as follows: 0, no signs of infection; 1, few slightly erythematous places on skin; 2, well defined redness, swelling with few blistering hairs, bald patches with scaly areas; 3, large areas of marked redness, incrustation, bald patches and ulcerations; 4, partial damage to the integument, loss of hair and 5, excessive damage to the integument and complete loss of hair at the site of infection. The hair root invasion test was used to assess the mycological cure rate resulting from antifungal treatment [27]. Briefly, the area of infection was divided into four quadrants and 10 hairs per quadrant were uprooted and planted on the surface of PDA which was subsequently incubated at 30 °C for 48 h. After the incubation the number of hair exhibiting fungal filaments at the hair root was counted.

For histopathological examination, skin biopsy samples were obtained from one representative animal per group after completion of the treatment period. The tissue was fixed in 10% neutral buffered formalin, embedded in paraffin and processed for histopathological examination. The fungal elements were visualized using *Periodic acid-Schiff* (PAS) staining [28].

3. Results and discussion

3.1. Screening of formulation ingredients

3.1.1. Solubility in oils

The solubility of griseofulvin in different oils was determined and was found to be highest in oleic acid followed by Captex 300 (Table 1). The physicochemical properties of oils influence the area of existence of a ME; therefore, oils were also screened with respect to Smin and Wmax for developing the ME formulations (Table 1). It was observed that the vegetable oils (olive oil, sesame oil, castor oil) did not result in formation of ME even at extremely high surfactant concentration (Smin > 90%, (w/w)) which could be ascribed to their large molecular weight [29]. However, medium chain triglycerides were solubilized with lower surfactant concentration than vegetable oils. These results could be explained by the fact that in medium chain triglycerides about 95% of fatty acids are made of 8–10 carbon atoms and therefore their molecular weights are

less than vegetable oils [30]. Further low molecular weight fatty acid esters, isopropyl myristate, oleic acid and ethyl oleate, were solubilized completely with a lower concentration of Tween 80. In general it was concluded for both triglycerides and fatty acid esters that lower the molecular weight of oil, greater the surfactant efficiency.

Water solubilization capacity (Wmax) was highest for isopropyl myristate (13.8% (w/w)), followed by oleic acid (8.3% (w/w)). The results obtained indicated that Tween 80 showed a good efficiency and water solubilizing capacity in the presence of lower molecular weight fatty acid esters with a preferred chemical structure. Oleic acid was selected as oil phase because, it is reported to perturb the lipid barrier in the stratum corneum by forming separate domains which interfere with the continuity of the multilamellar stratum corneum and induce highly permeable pathways in the stratum corneum [31].

3.1.2. Screening of surfactants

Choice of the surfactant is critical for the formulation of MEs, as it helps in the reduction of the interfacial tension by forming a film at the oil–water interface resulting in the spontaneous formation of MEs [32]. There are literature reports regarding the selection of surfactant on the basis of drug solubility, however, the solubilization of oil with the surfactant is also an important factor. It is not necessary that the surfactant having good solubilizing property for drug would also have equally good affinity for the selected oil phase. [16]. Tween 80 solubilized maximum amount of oleic acid *i.e.* 1.67% (by weight), followed by Tween 20 (1.15%), Tween 40 (0.66%) and Tween 60 (0.50%). The emulsification ability of the surfactant in terms of percent transmittance was Tween 80 (94.2) > Tween 20 (91.6) > Tween 40 (90.1) > Tween 60 (89.1).

The differences between the Tween variants in terms of oil solubilization and emulsification capacity can be explained on the basis of structure of the alkyl chain group. Ideally the lipophilic chains of an amphiphile should be short or at least containing a fluidizing group such as double bonds in order to allow oil uptake [33]. The surfactants containing longer saturated alkyl chain (Tween 20, Tween 40 and Tween 60), may not exhibit the required fluidity for ME formation as compared to Tween 80 which contains a double bond in its lipophilic chain. Moreover, the tail group of Tween 80 comprising up of a long chain (C18) of unsaturated oleic acid is structurally similar to oleic acid. The structural similarity between the surfactant and the oil further supports the theory of "like dissolves like" and also explains the higher oil solubilization capacity of Tween 80 for oleic acid. Therefore, Tween 80 was selected as surfactant for formulating the MEs.

3.1.3. Screening of cosurfactants

The presence of cosurfactants decreases the bending stress of interface and allows the interfacial film sufficient flexibility to take up different curvatures required to form ME over a wide range of composition [34]. The emulsification capability of cosurfactants *i.e.* the ME region in the pseudo-ternary phase diagrams was used as the assessment criteria. These were compared at a fixed Smix (1:1), keeping the surfactant the same and replacing the cosurfactant (Fig. 1). It was established that, when the chain length was increased from ethanol (Fig. 1a) to isopropyl alcohol (Fig. 1b), there was very slight enhancement in the ME region. However, further increase in chain length to *n*-butanol (Fig. 1c) and isobutyl alcohol (Fig. 1d) led to decrement in the ME zone. The larger ME area in case of ethanol and isopropyl alcohol as compared to *n*-butanol and isobutyl alcohol may be attributed to the higher solubility of the former two in the aqueous phase.

Also, the dermal toxicity [LD_{50}] of ethanol and isopropyl alcohol in rabbits is reported to be 20,000 and 12,800 mg/kg respectively. Therefore, ethanol being 1.5 times safe as compared to isopropyl alcohol was selected as the cosurfactant for formulating MEs.

3.2. Effect of surfactant and cosurfactant mass ratio on ME formation

Pseudo-ternary phase diagrams were constructed using oleic acid as oil, Tween 80 as surfactant and ethanol as cosurfactant. The influence of surfactant and cosurfactant mass ratio on ME formation was assessed for further optimization of the system. It was observed that the surfactant alone was ineffective in reducing the o/w interfacial tension enough to provide a ME with desirable properties (Fig. 2a). A large ME gel area was obtained toward the surfactant rich apex and maximum concentration of oil that could be solubilized was 22% (w/w) at 56% (w/w) of Smix 1:0. Increasing the amount of co-surfactant with respect to surfactant *i.e.* Smix ratio 1:1, the maximum amount of oil that could be solubilized was 24% (w/w) with 45% (w/w) of Smix 1:1 at the maximum content of water (Fig. 2b). This might be due to the fact that the incorporation of cosurfactant could have enhanced the penetration of the oil phase in the hydrophobic zone of the surfactant monomers, which in turn reduced the interfacial tension and increased the flexibility and fluidity of the interface, ultimately leading to increased entropy of the system [35]. When cosurfactant concentration was doubled *i.e.* Smix ratio 1:2 (Fig. 2c) the maximum amount of oil that could be solubilized was 20% (w/w) with 48% (w/w) of Smix, whereas the total area of ME decreased as compared to 1:1. Further increment in cosurfactant concentration to 1:3 (Fig. 2d), led to a considerable decrease in ME area and only 11% (w/w) oil was solubilized with 55% (w/w) Smix. Higher concentration of cosurfactant appeared to have a destabilizing effect on the formation of ME resulting into substantial reduction of ME area.

In contrast, when surfactant concentration of Smix was increased from 1:1 to 2:1 (Fig. 2e) and 3:1 (Fig. 2f), depletion in ME region was observed. It might be due to insufficient cosurfactant concentration, required to reduce the interfacial tension and provide the flexibility of the interfaces and ME gel regions were also observed which may be due to increased concentration of surfactant. The literature also supports that the Smix 1:1 possesses the maximum ME area as compared to the other ratios indicating that surfactant and cosurfactant mass ratio (Smix) have pronounced effect on phase properties [36].

3.3. Ex vivo drug permeation studies

To study the influence of formulation ingredients on permeation of griseofulvin from aqueous dispersion, oily solution, conventional cream and MEs were investigated for a period of 24 h each and each sample was analyzed in triplicate (Table 2). The aqueous suspension of the drug exhibited only $9.69 \pm 0.86\%$, oily solution demonstrated $13.73 \pm 0.78\%$ and the cream base showed $25.58 \pm 0.61\%$ drug permeation in 24 h (Fig. 3). Comparison of cumulative permeation between MEs and the conventional formulation bases demonstrated that all the griseofulvin loaded MEs enhanced drug permeation significantly ($p < 0.05$); almost 2- to 7-fold when compared with conventional cream, oily solution and aqueous suspension (Fig. 3). Drug permeation from ME V ($70.96 \pm 0.96\%$) was significantly higher ($p < 0.001$) than the conventional cream (3 times), oily solution (5-fold) and aqueous suspension (7 times) of griseofulvin. Therefore ME V was selected for further studies. ME V gel depicted slightly lower drug permeation of $64.73 \pm 1.18\%$ respectively, compared to ME V which may be attributed to slow diffusion of drug through gel network. Besides providing the optimum structure and viscosity to microemulsion for topical application, Carbopol in ME gel offers an additional advantage of excellent adhering and constant releasing formulation

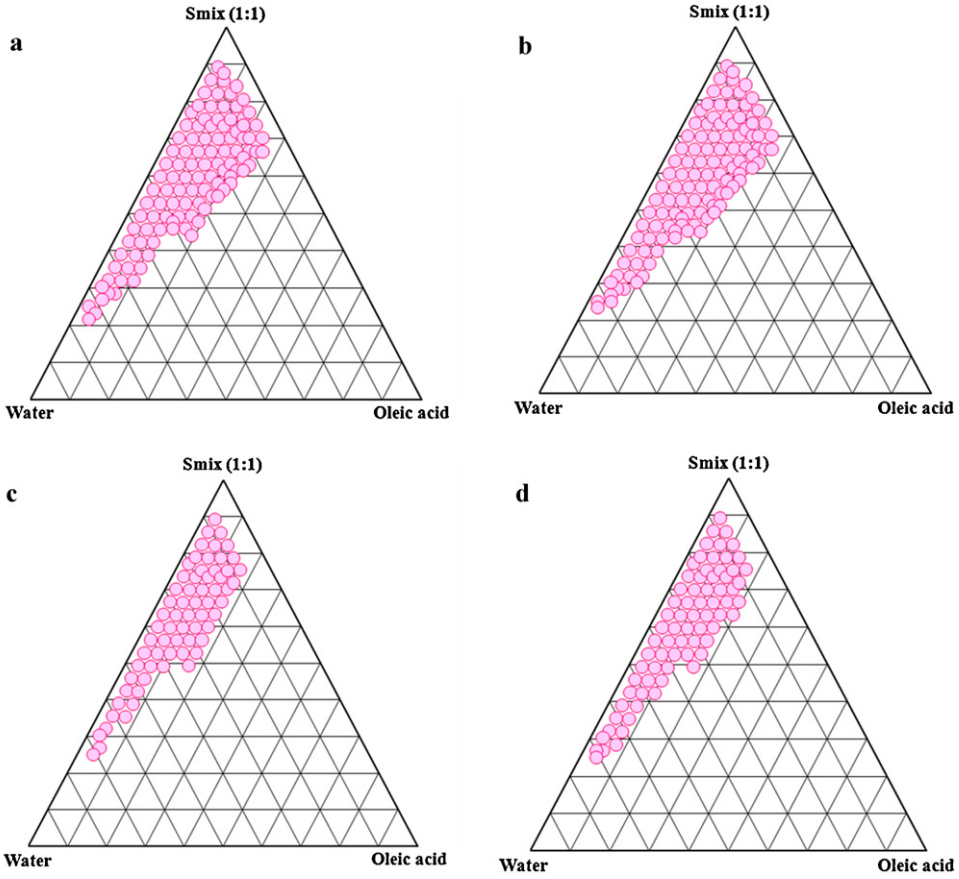

Fig. 1. Pseudoternary phase diagrams indicating o/w microemulsion region comprising of oleic acid, Tween 80 and different cosurfactants (a) ethanol, (b) isopropyl alcohol, (c) *n*-butanol and (d) isobutyl alcohol.

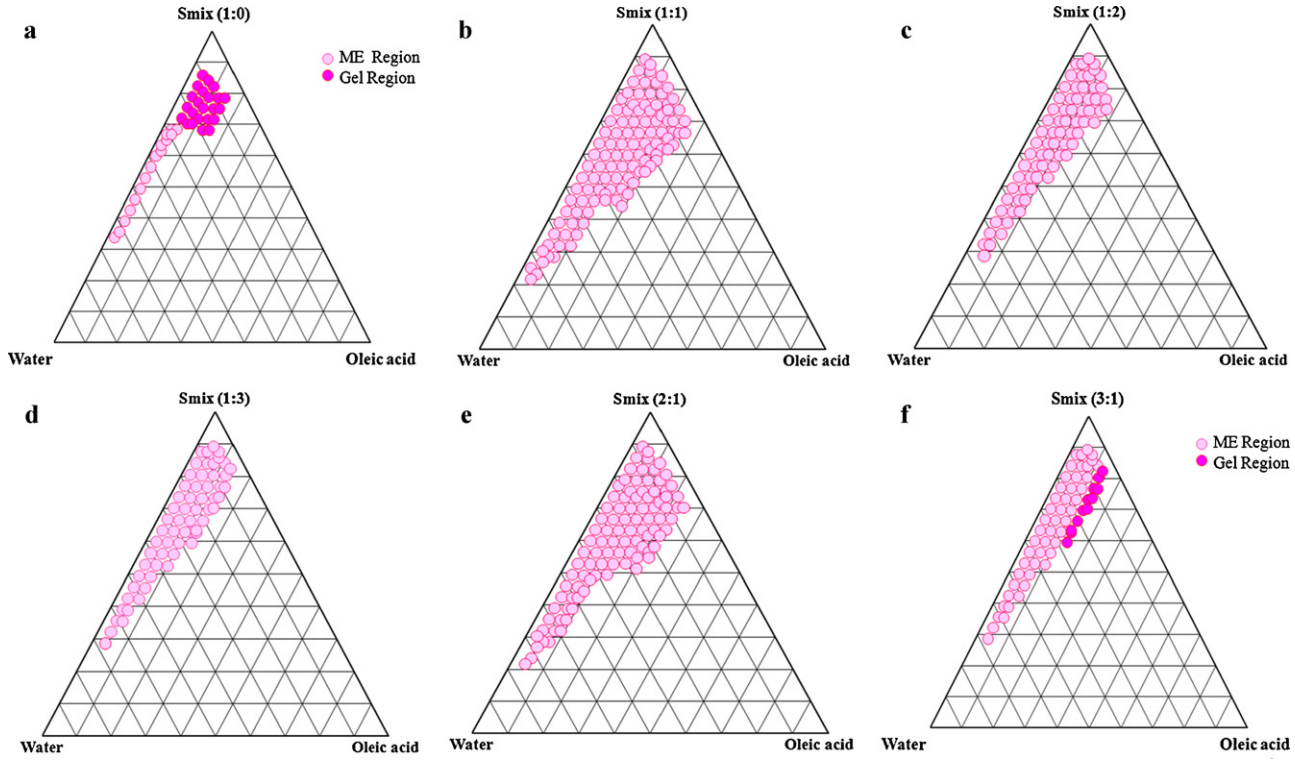

Fig. 2. Pseudoternary phase diagrams indicating o/w microemulsion region comprising of oleic acid, Tween 80 and ethanol at different S_{mix} ratios (a) 1:0, (b) 1:1, (c) 1:2, (d) 1:3, (e) 2:1 and (f) 3:1.

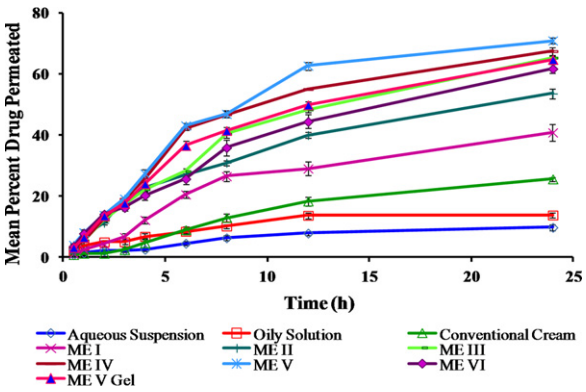

Fig. 3. Comparison of *ex vivo* permeation profiles of different formulations of griseofulvin through mice skin ($n = 3$).

[37]. The permeation kinetics revealed a non-Fickian drug permeation pattern for ME V and ME V gel, with a diffusional release exponent (n) value of 0.79 and 0.82 respectively [38].

The rate of permeation (flux) for all the MEs were found to range between 11.20 ± 0.44 and $22.74 \pm 0.6\ \mu g/cm^2/h$ (Table 2). These values were significantly higher ($p < 0.001$) than the oily solution ($3.34 \pm 0.27\ \mu g/cm^2/h$), conventional cream ($4.35 \pm 0.80\ \mu g/cm^2/h$) and aqueous suspension ($2.37 \pm 0.11\ \mu g/cm^2/h$) indicating that MEs resulted in considerable improvement in permeation of griseofulvin. Apart from the contribution of oleic acid in enhancing drug permeation in skin by disrupting the fluidity of the stratum corneum [31], the varying surfactant composition might be responsible for enhanced permeation from MEs. The non-ionic surfactants reportedly emulsify sebum, thereby enhancing the thermodynamic coefficient of the drug, allowing it to penetrate into the cells more effectively [39]. The present study highlights the multi-faceted role of ethanol as a cosurfactant as well as a permeation enhancer. The cosurfactants especially short chain alcohols are known to enhance the flux of ME formulations by altering the relative hydro/lipophilicity of the system [40]. The mechanism behind this phenomenon is the additional fluidity of the interfacial film due to penetration of surfactant monolayer and disruption of crystalline phases which are formed as a result of a rigid surfactant film. As a solubilizer and enhancer ethanol not only enhances drug solubility in vehicle but can also alter the structure of the bio-membrane by lipid extraction and increase the permeability of the drug. Another mechanism may be that ethanol is volatilized from the applied formulation and, consequently, increases the drug concentration to a supersaturated state with a greater driving force for permeation. Also, the water content of MEs may enhance the permeation because hydration of stratum corneum leads to the development and widening of channels in the keratin layer and distortion of lipid bilayer [10]. The studies revealed that ME V containing 40% surfactant (Smix ratio 1:1) exhibited maximum permeation, flux and drug retention in mice skin supporting the role of ethanol in permeation mechanics. The study illustrates and supports the importance of optimum levels of surfactant(s) as well as Smix ratio in a ME formulation.

The skin deposition of drug using aqueous suspension was only $0.74 \pm 0.21\ \mu g/cm^2$. This may be ascribed to the hydrophobic nature of the drug because the drug being insoluble did not penetrate the skin. However, cream based formulation of griseofulvin resulted in greater deposition ($11.13 \pm 0.42\ \mu g/cm^2$) of drug (Table 2). This may be credited to the presence of surfactants in the cream base that helped in the partial solubilization of the drug. ME V and its corresponding gel ME V Gel led to deposition of $35.28 \pm 2.66\ \mu g/cm^2$ and $36.06 \pm 1.88\ \mu g/cm^2$ drug respectively; which was nearly 48-fold, five times and 3-fold more than aqueous suspension, oily solution and conventional cream base respectively; significantly different at $p < 0.001$ (Table 2). Thus, it can be inferred that the optimized ME V could effectively make the drug molecules accessible within skin layers, retaining them within close vicinity of the target infection site and it was further subjected to dermatokinetic and pharmacodynamic evaluation.

3.4. Characterization studies

TEM micrograph depicted that the globules of optimized ME possessed spherical shape. ME V possessed mean globule size of 12.21 nm with a PDI of 0.109. The low value of PDI ratified the homogeneity and stability of the optimized microemulsion [41]. The zeta potential of the optimized microemulsion was found to be −0.139 mV that was near to neutral. It is reported that stability of ME and lipid emulsions containing non-ionic surfactants does not depend on zeta potential [42].

ME V exhibited no phase separation or breaking or drug precipitation indicating thermodynamic stability against centrifugation and freeze thaw cycles.

TDC for ME V was found to be 19.94 ± 0.05 mg ($99.72 \pm 0.28\%$) and for ME V gel it was found to be 19.87 ± 0.13 mg ($99.34 \pm 0.65\%$) illustrating uniform distribution and minimum drug loss during preparation of ME. The pH of ME V and ME gel was observed to be 6.41 and 6.56 respectively.

Texture analysis, revealed that the griseofulvin ME V gel possessed fairly good gel strength, ease of spreading and adequate cohesiveness; which are essential for application and retaining the formulation on the skin. Further, uniformity of texture curve, plotted employing Exponent 32® software, confirmed the smoothness of ME gel and absence of any grittiness or lumps.

ME V gel exhibited transparency, clarity and no drug precipitation or color change when it was subjected to stability study at $5 \pm 3\ ^\circ C$, $25 \pm 2\ ^\circ C$ and $40 \pm 2\ ^\circ C$ for 6 months. The organoleptic features like gel viscosity, gel firmness, gel strength, physical appearance were also observed and no significant change was found in these characters.

3.5. Skin sensitivity and histopathological studies

The mice skin treated with ME V gel on comparison with control established the safety of prepared formulation with no perceptible histopathological changes indicating the safety of the formulation for topical use. There was no apparent sign of edema, inflammatory cell infiltration, erythema, papule, flakiness and dryness on mice skin. Uniformly layered stratum corneum and loosely textured collagen in the dermis could be observed.

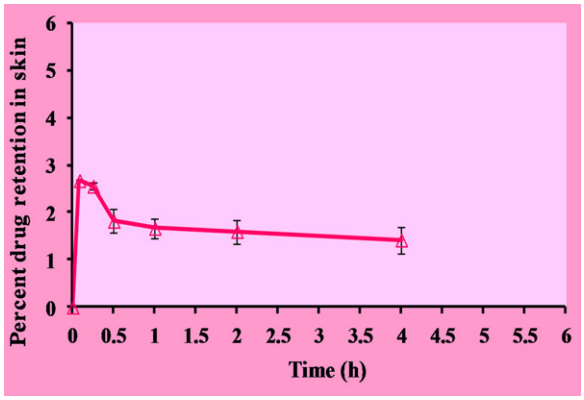

Fig. 4. Percent drug retention in mice skin at various time intervals after single topical application of ME V ($n = 6$).

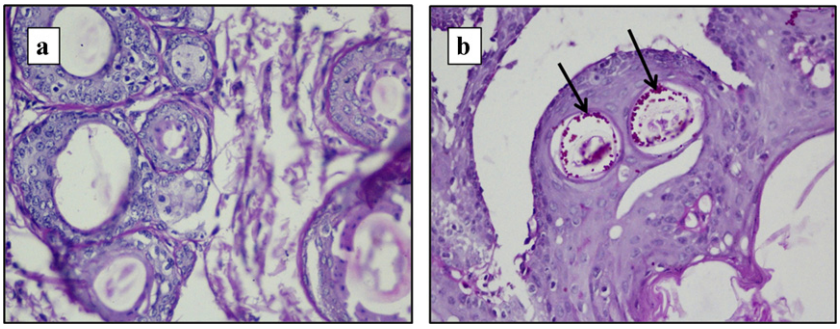

Fig. 5. Histopathology of skin of guinea pig infected with *M. canis* after treatment with (a) ME V showing complete absence of fungal elements (b) placebo, arrows show presence of spored hyphae in hair follicles ($n = 5$).

3.6. Dermatopharmacokinetics

Reverse phase and isocratic liquid chromatography method with PDA detector was developed for estimation of griseofulvin in the skin and plasma samples. Using ACN and 0.1 M acetic acid (40:60% (v/v)) as the mobile phase a well resolved and sharp peak of griseofulvin was obtained with a retention time of approximately 8–9 min and a total run time of 15 min with no interference with other components of the mobile phase, skin homogenate and plasma components. The method was linear for standard drug samples, skin homogenate and plasma samples, over the studied concentration range *i.e.* 0.5–20 μg/mL. Under these conditions, the limit of detection for griseofulvin was 0.05 μg/mL and the limit of quantification was 0.2 μg/mL in standard drug solution, skin homogenate as well as plasma samples. The recovery of drug from skin homogenates was found to be 98.31–103.49% showing good accuracy of the method. The recovery of drug from plasma samples varied between 98.57% and 102.13% again proving the accuracy of the method.

The kinetic studies revealed instantaneous penetration of the optimized ME V resulting in 2.67% (nearly 13 μg) drug retention in the skin in 5 min (Fig. 4). The initial concentration gradient would justify the huge drag force behind this response. The rapid penetration of griseofulvin may be credited to the direct partitioning of the drug from the continuous phase of ME into the skin [43]. This may be ascribed to the disruption of mortar–brick structure of stratum corneum by the surfactant(s) existing as free monomers in the formulation, facilitating in the instantaneous penetration of the drug. Another contributory factor could be the presence of drug in the solubilized form in the nano-sized ME micelles, resembling the 'pore' size of the stratum corneum allowing easy penetration of the drug in skin [7]. The presence of oleic acid in ME may also have contributed in the permeation process by fluidizing the intercellular domains of the stratum corneum, resulting in enhanced transport of encapsulated griseofulvin [31]. Thereafter, the drug penetration attained a plateau phase in 30 min with almost 8.16 μg drug retention until 4 h. The plasma samples indicated absence of drug signifying the restriction of drug to the dermal layers.

The study emphasizes the importance of topical delivery of griseofulvin, because after oral administration the drug is detectable at the base level of skin after 48–72 h. However, in the current investigation, a single topical application of ME showed an appreciable skin deposition of 13 μg of drug in 5 min.

3.7. Antifungal studies

The broth microdilution method revealed complete inhibition of *M. gypseum, M. canis, T. mentagrophytes* and *T. rubrum* at 0.5 μg/mL of the drug concentration. The blank ME did not exhibit any zone of inhibition indicating absence of any anti-fungal efficacy of the formulation excipients at the tested count of colony forming units (cfu/mL; Table 3). The agar plate diffusion protocol revealed the efficacy of griseofulvin ME against all the tested dermatophytes indicating the retention of antifungal efficacy of drug encapsulated in the ME (Table 3). The microbiological studies were observed to be synchronous with the *ex vivo* permeation studies supporting the steady diffusion of drug from the ME which would allow the drug to act on the fungus for a longer time period.

3.8. Pharmacodynamic studies

The infected guinea pigs were observed daily for the signs of infections. The first signs of infection were observed on the 3rd day after inoculation in all the animals manifested in the form of redness and scaling. These alterations became more evident around the 7th day with marked hair loss and brittle hair. The lesions progressively increased in diameter in the control group (treated with placebo) and were found to be covered with white-yellow crusts strongly adhered to the epidermis.

Redness and itching at the site of infection in the treatment groups was allayed in 2–3 days. It was also observed that there was shedding of the infected skin scales and appearance of healthy light pink colored skin 4–5 days after the initiation of treatment. It was followed by appearance of vellus (fine non-pigmented hair growth) and complete healing of the infected site in 7 days in the treatment group. Subsequently a fine uniform, smooth and healthy hair growth was observed at the site of infection. Skin biopsies were obtained from the test areas, skin sections were stained with PAS stain and histopathological examination of skin sections was performed to determine whether there was any skin tissue invasion by *M. canis*. The histopathogical results revealed complete absence of any fungal element in the skin biopsies of animals treated with ME V gel (Fig. 5a). However, in the animals treated with control,

Table 3
Antifungal efficacy of griseofulvin loaded optimized microemulsion (ME V) against different dermatophytes ($n = 4$).

Dermatophytes	Colony count (cfu/mL)	Zone of inhibition (cm)	Percent drug diffused
M. gypseum	1.0×10^6	2.75 ± 0.06	69.20 ± 1.87
M. canis	5.6×10^5	2.75 ± 0.13	68.32 ± 3.12
T. mentagrophytes	5.3×10^5	2.75 ± 0.13	69.17 ± 2.46
T. rubrum	5.4×10^5	2.73 ± 0.05	69.01 ± 2.03

fungal elements in the hair follicles were clearly visible (Fig. 5b). As discussed under the dermatopharmacokinetic studies the ME formulation might have led to efficient drug penetration in the upper layers of the stratum corneum where the drug must have bound efficiently to keratinocytes for its effects.

4. Conclusion

The results of the present investigations conclusively demonstrated the role of ME in effective dermal drug delivery of griseofulvin. The developed system may provide better remission from the disease due to localized delivery with minimal side effects. The future perspective includes elaborate stability and clinical studies for developing commercially viable topical ME formulation of griseofulvin.

Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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References

[1] A.I. Arida, M.M. Al-Tabakha, H.A.J. Hamoury, Chem. Pharm. Bull. 55 (2007) 1713.
[2] D.F. Veber, S.R. Johnson, H. Cheng, B.R. Smith, K.W. Ward, K.D. Kopple, J. Med. Chem. 45 (2002) 2615.
[3] B.L. Hainer, Am. Fam. Physician 67 (2003) 101.
[4] S. Goindi, N. Aggarwal, Int. J. Pharm. 326 (2006) 20.
[5] K. Yamaguchi, T. Mitsui, Y. Aso, K. Sugibayashi, J. Pharm. Sci. 97 (2008) 4391.
[6] F.T. Vicentini, M.M. Vaz, Y.M. Fonseca, M.V. Bentley, M.J. Fonseca, Drug Dev. Ind. Pharm. 37 (2011) 47.
[7] B. Kim, M. Won, K.M. Lee, C.S. Kim, Drug Deliv. 15 (2008) 465.
[8] M.H. Sakeena, F.A. Muthanna, Z.A. Ghassan, M.M. Kanakal, S.M. Elrashid, A.S. Munavvar, M.N. Azmin, J. Oleo Sci. 59 (2010) 223.
[9] Y. Zhang, J.G. Gao, H.T. Zheng, R. Zhang, Y.C. Han, Int. J. Nanomed. 6 (2011) 649.
[10] A. Azeem, Z.I. Khan, M. Aqil, F.J. Ahmad, R.K. Khar, S. Talegaonkar, Drug Dev. Ind. Pharm. 35 (2009) 525.
[11] V. Tchakalova, F. Testard, K. Wong, A. Parker, D. Benczedi, T. Zemb, Colloids Surf. A Physicochem. Eng. Asp. 331 (2008) 40.
[12] B.K. Paul, R.K. Mitra, J. Colloid. Interface Sci. 288 (2005) 261.
[13] A. Avdeef, Adv. Drug Deliv. Rev. 59 (2007) 568.
[14] A.A. Date, M.S. Nagarsenker, AAPS PharmSciTech 9 (2008) 138.
[15] A. Azeem, M. Rizwan, F.J. Ahmad, Z. Iqbal, R.K. Khar, M. Aqil, S. Talegaonkar, AAPS PharmSciTech 10 (2009) 69.
[16] N. Aggarwal, S. Goindi, S.D. Mehta, AAPS PharmSciTech 13 (2012) 67.
[17] A.S. Narang, D. Delmarre, D. Gao, Int. J. Pharm. 345 (2007) 9.
[18] J.B. Shukla, S.J. Patel, Int. J. Pharm. Pharm. Sci. 2 (2010) 143.
[19] A. Bhatia, K. Raza, B. Singh, O.P. Katare, J. Cosmet. Dermatol. 8 (2009) 282.
[20] A. Azeem, S. Talegaonkar, L.M. Negi, F.J. Ahmad, R.K. Khar, Z. Iqbal, Int. J. Pharm. 422 (2012) 436.
[21] B. Wei, D. Liang, R.B. Theodore, Anal. Chem. Insights 3 (2008) 103.
[22] M. Fujii, M. Bouno, S. Fujita, M. Yoshida, Y. Watanabe, M. Matsumoto, Biol. Pharm. Bull. 23 (2000) 1341.
[23] B. Kim, H. Doh, T.N. Le, W. Cho, C. Yong, H. Choi, J.S. Kim, C. Lee, D. Kim, Int. J. Pharm. 293 (2005) 193.
[24] M.E.S. Barros, D.A. Santos, J.S. Hamdan, J. Med. Microbiol. 56 (2007) 514.
[25] A. Ayanaba, S. Asanuma, D.N. Munns, Soil Sci. Soc. Am. J. 47 (1983) 256.
[26] C.R. Araujo, K.C. Miranda, O.F.L. Fernandes, A.J. Soares, M.R.R. Silva, Rev. Inst Med. Trop. Sau Paulo 51 (2009) 9.
[27] M.A. Ghannoum, M.A. Hossain, L. Long, S. Mohamed, G. Reyes, P.K. Mukherjee, J. Chemother. 16 (2004) 139.
[28] H. Alkhayat, N. Al-Sulaili, E. O'Brien, C. McCuaig, K. Watters, Bahrain Med. Bull. 31 (2009) 1.
[29] M. Bastić, L. Bastlć, J.J. Jovanović, G. Spiteller, J. Am. Oil. Chem. Soc. 55 (1978) 886.
[30] W. Warisnoicharoen, A.B. Lansley, M.J. Lawrence, Int. J. Pharm. 198 (2000) 7.
[31] W. Zhu, A. Yu, W. Wang, R. Dong, J. Wu, G. Zhai, Int. J. Pharm. 360 (2008) 184.
[32] V. Bali, M. Ali, J. Ali, Colloids Surf. B Biointerfaces 76 (2010) 410.
[33] J. Lawrence, Eur. J. Drug Metab. Pharmacokinet. 3 (1994) 257.
[34] A.K. Philip, K. Pathak, Drug Dev. Ind. Pharm. 34 (2008) 735.
[35] W. Warisnoicharoen, A.B. Lansley, M.J. Lawrence, AAPS PharmSci 2 (2000) 16.
[36] L. Hua, P. Weisan, L. Jiayu, Z. Ying, Drug Dev. Ind. Pharm. 30 (2004) 657.
[37] K. Bhaskar, M.C. Krishna, M. Lingam, M.S. Jagan, V. Venkateswarlu, R.Y. Madhusudan, K. Bhaskar, J. Anbu, V. Ravichandran, Drug Dev. Ind. Pharm. 35 (2008) 98.
[38] N.A. Peppas, J.J. Sahlin, Int. J. Pharm. 57 (1989) 169.
[39] I. Som, K. Bhatia, M. Yasir, J. Pharm. Bioall. Sci. 4 (2012) 2–9.
[40] M.R. Patel, R.B. Patel, J.R. Parikh, A.B. Solanki, B.G. Patel, AAPS PharmSciTech 10 (2009) 917.
[41] B. Biruss, R. Dietl, C. Valenta, Chem. Phys. Lipids 148 (2007) 84.
[42] T. Tadros, J. Cosmet. Sci. 57 (2006) 153.
[43] M. Grassi, N. Coceani, L. Magarotto, J. Colloid Interface Sci. 228 (2000) 141.