AMELIORATIVE EFFECT OF GRISEOFULVIN NANO EMULSION AS AN ANTIFUNGAL ACTIVITY

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Abstract:

Nanoemulsion-based drug delivery systems have emerged as a promising strategy for improving the therapeutic efficacy of various drugs. Nanoemulsions are colloidal dispersions composed of nanoscale droplets of oil or water stabilized by surfactants. Griseofulvin nanoemulsion comprising polymers like medium chain Tri-glycerides, Tween-80 and polyethylene glycol were tested against pathogenic Candida species to determine their antifungal activities. FT-IR, TEM were used to characterise Griseofulvin nanoemulsion, is in range of 10–23 nm. Generally, emulsions are water-in-oil or oil-in water type, but emulsions may contain polar liquid as one of the phase. Emulsions are useful in many situations where presence of water is not desirable, and formulation of active ingredients which undergo hydrolysis or oxidation in the presence of water. The study was to design a stable nanoemulsion (NE) using cosmetically approved ingredients as a vehicle for the water-sensitive active ingredients. An optimized NE was obtained through implementation of pseudo-ternary phase diagram. Pseudo-ternary phase diagram was constructed using surfactant and co-surfactant ratio (, 2:1, 3:1,) and nanoemulsion region was determined and further characterized for pH, rheology, globule size analysis, zeta potential and stability.

Keywords: Griseofulvin, Nanoemulsion, Medium Chain Triglycerides, Tween-80, Polyethylene glycol.

Introduction

Fungal infection mainly caused by microscopic organism readily available in the environment which can invade the epithelial tissue. Fungal infection commonly affects to the hair, nail and skin. In case of systemic infection fungal pneumonia can occur to individuals depending on the favorable climates for the proliferation of fungi. This fungal infection sometime leads to serious complication to the individuals and also life threatening

to the patient if care is not be taken immediately. The frequency and the change in growth of fungal infection have increased gradually over last few decades(1). Similarly, the treatment procedure and surgical care as well as new drug delivery system is more potent against the fungal growth. The fungal cell wall contains chitin and polysaccharides which make the outer cell very rigid and act as a barrier to prevent the invasion of the drug to the cell. Fungi cell contain ergosterol which control the efficacy of the drug and reduce its potency. Candidiasis is one of the threatening infection occur by candida fungi found worldwide showing high risk to the patient life. Candida first adheres to the host cell and releases some virulence factors which damage the host tissue. Similarly cryptococcal meningitis and invasive aspergillosis are also considered as one of the life threatening fungi infection. (2)Novel antifungal drugs are directly targeted sterol component of cell membrane of fungi either depleting the ergosterol or inhibiting the synthesis .Due to high antifungal resistance of the pathogens a research has been focused on the preparation of novel antifungal compounds. For the development of novel antifungal drug first we need to focus on the various important factors of infection and their basic mechanism could bea key factor to develop a new antifungal drug for targeted area.(3) Griseofulvin is a fungal secondary polyketide metabolite that is soluble in ethanol and methanol but has poor solubility in water. One notable feature of griseofulvin is its ability to tolerate heat stress and maintain its function at a high temperature of 121°C without losing functional properties. These properties are important to understand their medical applications because they affect absorption, transportation, excretion, and degradation. Medicine movement systems are methodologies which are used to ensure that drugs get into the body and accomplish the district where they are required. These systems must think about different prerequisites, stretching out from straightforwardness of movement to suitability of the drugs.(4) At the point when a medication is directed, the dose must be deliberately figured so the body can utilize the medication, which requires a drug delivery system framework which considers exact dosing. Drug delivery system frameworks additionally need to consider the manner by which a medication is utilized by the body. For instance, a few medications are wrecked in the intestinal tract, which implies that they can't be acquainted with the body thusly. Others might be unsafe in huge sums, which imply that a period discharge strategy ought to be utilized to convey the medication for quietsecurity. Topical drug delivery system frameworks include the acquaintance of a medication with the surface of the body, in a plan which can be ingested Solid lipid nanoparticles have the medication entrapped inside a strong lipid core of the matrix. Nanostructured lipid transporters have a

lattice made out of a blend of strong and fluid lipids. Solid lipid nanoparticles of clotrimazole and miconazole have demonstrated extendable and shows better retention properties over hydrogels Nano-emulsions are clear, steady, isotropic blends of oil, water, and surfactant, as often as possible in mix with a co-surfactant. Nanoemulsions have the benefits of improved tranquilize dissolvability, great thermodynamic solidness, and higher transdermal penetrability over customary plans such as oxiconazole, fluconazole and clotrimazole. Being an emerging transdermal delivery tool, nanoemulgel, has proved to show surprising upshots for the lipophilic drugs over other formulations. This lipophilic nature of majority of the newer drugs developed in this modern era resulting in poor oral bioavailability, erratic absorption and pharmacokinetic variations. Therefore, this novel transdermal delivery system has been proved to be advantageous over other oral and/or topical drug delivery to avoid such disturbances.(5)

2. Methods

Analytical grade materials were used for this study. Griseofulvin, Tween-80 (Loba Chemical, Mumbai, India), Medium Chain Triglycerides, and Polyethylene Glycerol monostearate (Research Lab Fine Chemical Industry, Mumbai, India) were purchased. Methanol, chloroform, distilled water, phosphate buffer pH (7.4) were also used throughout the study. All other chemicals and reagent were of analytical grade and were used without further purification.

2.1Construction of pseudo-ternary phase diagram

To investigate concentration range of components for the existing boundary of NE, pseudo-ternary phase diagram was constructed using the titration method.

2.2. Titration method

Pseudo ternary phase diagrams were constructed using aqueous titration method at ambient temperature comprising of surfactant, co-surfactant and oil. Each of them were plotted, representing an apex of the triangle. MCT as oil phase, TWEEN-80 as surfactant and Propylene glycol as co-surfactant were selected (based on solubility studies). The weight ratio of surfactant to co surfactant (Smix) was varied in the range, 2:1 and 3:1, for each pseudo- ternary phase diagram at a specific surfactant/co-surfactant weight ratio and this was mixed with oil at a various ratio. Water was added drop by drop to each oily surfactant mixture under magnetic stirrer at room temperature, after each addition the mixture was examined for the appearance.(6) The end point of the titration was the point where the solution becomes clear, cloudy or turbid. The quantity of the aqueous phase required to

make a mixture clear solution was noted. The concentrations of components were recorded in order to plot the pseudo-ternary phase diagrams using Tri-plot software Version 4.1.2. (Todd Thompson software). The total composition of the mixture or apex of the triangle represents 100% w/w. The resulting nanoemulsions were tightly sealed and stored at ambient temperature, and their physical stability was measured by observing periodically the occurrence of phase separation. The systems giving more area of nanoemulsion region from the four component systems were identified and selected for further studies.(7)\

2.3 Formulation of Nanoemulsion (NE)

Griseofulvin solubility was found maximum in MCT oil. Tween 80 and propylene glycol also showed high solubility of griseofulvin as compared to other surfactants and co-surfactants respectively. On the basis of this we have formulated following nanoemulsion systems. Formulation of nanoemulsion containing MCT as oil phase and tween 80 and propylene glycol as surfactant and co-surfactant respectively. Formulation and evaluation of nanoemulsion by phase inversion method(8)

Table1: Compositions of MCT - Tween-80 - Propylene glycol nanoemulsion.

S/Cos - (3:1)

Formulation Code	Oil (%w/w)	S/Cos mix (%w/w)	Water (%w/w)
NE1	3.37	30.40	66.26
NE2	14.28	57.14	28.57
NE3	21.27	53.19	25.53
NE4	31.25	46.87	21.87
NE5	41.66	41.66	16.66
NE6	53.57	35.71	10.71
NE7	64.81	27.77	7.40
NE8	74.07	18.51	7.40
NE9	86.53	9.61	3.84

Table: Indicates various formulation and their mixture ratio.

2.4 Characterization of Nanoemulsion

The droplet size of nanoemulsions is a critical parameter affecting their stability, drug loading

capacity, and biodistribution. Dynamic light scattering (DLS), laser diffraction, or microscopy-based techniques such as transmission electron microscopy (TEM) or atomic force microscopy (AFM) are commonly employed to measure the droplet size distribution and determine the mean droplet size of nanoemulsions. The zeta potential, measured using electrophoretic mobility techniques like electrophoretic light scattering (ELS), provides information about the surface charge and stability of nanoemulsion droplets.(9)

2.5 Physical Appearance of nanoemulsion

The physical appearance of a nanoemulsion can vary depending on its formulation and composition. Generally, nanoemulsions appear as translucent or transparent liquid systems. They are characterized by their ability to form stable and uniform colloidal dispersions of nanoscale droplets.The droplets in a nanoemulsion are typically in the range of 20 to 200 nanometers in diameter, which is significantly smaller than those found in traditional emulsions. Due to their small size, nanoemulsion droplets are often not visible to the naked eye, resulting in a clear or slightly hazy appearance.(10)

Table 2 Observation table for NE formulations using Oil and Smix

Oil phase and Smix (Tween 80 and Propylene glycol in 3:1)				
Formulation Code (Oil: Smix)	**Oil phase (ml)**	**Smix (ml)**	**Volume of distilled water at which system become translucent or turbid (ml)**	**After addition of distilled water up to 85% of total volume (total 8.5ml)**
NE1 (9:1)	1.35	0.15	0.2	Turbid system with phase separation within 1 hour
NE2 (8:2)	1.2	0.3	0.5	Turbid system with phase separation within 1 hour
NE3 (7:3)	1.05	0.45	0.85	Turbid system
NE4 (6:5)	0.9	0.6	1.25	Turbid system
NE5 (5:5)	0.75	0.75	1.7	Translucent system
NE6 (4:6)	0.6	0.9	2.4	Translucent system
NE7 (3.5:6.5)	0.53	0.97	3.2	Translucent system

NE8 (3:7)	0.45	1.05	Remains transparent up to 8.5 ml of water addition	Transparent system
NE9 (2.5:7.5)	0.38	1.12	Remains transparent up to 8.5 ml of water addition	Transparent system
NE10 (2:8)	0.3	1.2	Remains transparent up to 8.5 ml of water Addition	Transparent system
NE11 (1:9)	0.15	1.35	Remains transparent up to 8.5 ml of water Addition	Transparent system

Table showing various parameter and their inferences.

2.6 Average droplet size (ADS), polydispersity index (PDI) and zeta potential, determination

The average droplet size (ADS), polydispersity index (PDI), and zeta potential of a nanoemulsion can be determined using various characterization techniques.

2.7 Centrifugation test

Centrifugation is a laboratory technique used to separate components of a mixture based on their density using centrifugal force. It is widely employed in various scientific and clinical settings for various purposes, such as separating solids from liquids, isolating specific cell types, purifying biomolecules, and more. Accurately weighed 5 g of NANE was centrifuged (Remi Corp, Mumabai) at 3500 rpm for 30 min. After stipulated period, cream was observed for any signs of phase separation(11)

2.8 Average droplet size

The average droplet size in a nanoemulsion can vary depending on the specific formulation and processing methods used. However, nanoemulsions typically have droplet sizes in the range of 20 to 200 nanometers. This nanoscale range is a key characteristic of nanoemulsions and distinguishes them from conventional emulsions, which often have larger droplet sizes. The small droplet size in nanoemulsions offers several advantages. It's important to note that achieving and maintaining a small and uniform droplet size distribution is critical in nanoemulsion formulation. Various techniques, such as high-pressure homogenization, sonication, and microfluidization, are employed to produce nanoemulsions with the desired droplet size characteristics. Additionally, characterization techniques like dynamic light scattering (DLS), laser diffraction, or electron microscopy

can be utilized to measure and confirm the average droplet size in a nanoemulsion formulation.(12,13)

2.9 Poly Dispersity Index

Polydispersity index (PDI) is a parameter used to describe the distribution of particle sizes within a sample, including nanoemulsions. It provides an indication of the uniformity or heterogeneity of the droplet size distribution.

PDI is calculated based on the ratio of the standard deviation (σ) to the mean droplet size (μ) of the sample. Mathematically, it can be expressed as:$PDI = \sigma / \mu$

A PDI value of 0 indicates a monodisperse system where all particles have the same size, resulting in a very narrow size distribution. Conversely, a higher PDI value indicates a broader size distribution with a greater range of particle sizes.

In the context of nanoemulsions, a low PDI value is desirable as it suggests a more uniform and stable distribution of droplet sizes. A narrow size distribution can lead to enhanced physical stability, improved drug release kinetics, and better bioavailability.(14,15)

3. Zeta Potential

Zeta potential is an important parameter used to characterize the surface charge of colloidal particles, including the droplets in a nanoemulsion. It provides information about the electrostatic stability and potential for aggregation or dispersion of the particles.Zeta potential is measured by applying an electric field to the dispersion and observing the movement of charged particles. The electric field causes the particles to migrate towards the oppositely charged electrode, and the velocity of this movement is measured. This velocity is then used to calculate the zeta potential using electrophoretic mobility equations, such as the Smoluchowski equation or the Henry equation.The zeta potential is typically reported in units of millivolts (mV) and represents the electric potential at the slipping plane between the particle surface and the surrounding medium. A higher magnitude of zeta potential indicates a higher surface charge, which leads to greater electrostatic repulsion between particles, resulting in improved dispersion and stability.

Table 3: Evaluation of drug-loaded Griseofulvin NE formulations

Sr. no.	Formulation code no.	ADS (nm)	PDI	ZP	RI	PT (%)	Centrifugation test	H/C cycle test

1	F8	39.53	0.534	-5.78	1.345	92.83	No phase separation or creaming	Stable
2	F9	29.23	0.745	-5.73	1.345	98.26	No phase separation or creaming	Stable
3	F10	23.09	0.293	-6.56	1.345	98.75	No phase separation or creaming	Stable

Table showing characteristics features of drug loaded Griseofulvin nanoemulsion formulation

3.1 Drug content

One mL of nanoemulsion was pipetted from the nanoemulsion and was lysed with methanol. It was further diluted with 7.4 pH phosphate buffer and the samples were analyzed spectrophotometrically at 272 nm.

3.2 *IN VITRO* DRUG RELEASE STUDY

In- vitro diffusion study was carried out by using a dialysis technique. Dialysis membrane (Dialysis membrane-150; LA401-5MT; average diameter 25.4 mm and average flat width 42.44 mm purchased from HiMedia Laboratories, Mumbai, India) was first washed with running water for 2 hrs. then immersed in releasing medium (0.1N HCl) for 24 hrs. The activated dialysis membrane was tied with thread at one end. 1 ml of SNEDDS preconcentrate was filled in dialysis membrane through another end and diluted up to 15 ml with 0.1N HCl. Then other end was also tide similarly and checked to ensure no leakage. Then this sealed dialysis bag was submerged in 250 ml of 0.1N HCl at 200 rpm speed and at temperature of 370 ± 0.5 °C. 5 ml samples were withdrawn at specified time interval (15 min, 30 min,1 hr., 2 hrs., 3 hrs. and so on).(16)

Table 4: *In-vitro* drug diffusion study from NE formulations in 0.1N HCl

	Cumulative percentage drug release (%) in 0.1N HCl

Time (Hours)	**Formulation F8** (Mean±SD), n=3	**Formulation F9** (Mean±SD), n=3	**Formulation F10** (Mean±SD), n=3
0.25	8.65±0.58	10.29±0.71	10.91±0.66
0.5	14.97±0.26	15.61±0.87	19.76±1.01
1	30.86±0.49	32.71±1.21	35.98±0.95
2	47.32±0.85	51.11±0.78	50.09±0.75
3	59.24±0.73	62.20±1.41	64.69±1.16
4	69.25±0.98	73.41±1.39	76.34±1.02
5	78.15±1.06	82.06±0.95	82.88±0.86
6	83.19±0.92	87.35±1.13	87.97±0.92
7	87.78±1.43	88.46±1.55	90.76±1.28
8	88.06±1.12	90.10±1.23	91.49±1.43
9	88.42±1.16	90.66±1.34	91.73±1.49

3.3 In vitro Anti-Fungal Activity

In-Vitro antifungal activity was performed against Candida albicans NCIM 3471, with the objective to determine the effect of process parameter on the strength (potency) of griseofulvin and to study the antifungal activity of griseofulvin in terms of zone of inhibition. From the present investigation the test sample with the strength equivalent to 500 µg/ml showed the greater zone of inhibition (32 mm) when compared with the standard 500 µg/ml ketoconazole solution (30 mm) as shown in table. Figure shows antifungal activity study showing Petri plates. The study was carried out in triplicate and the same results were obtained for the zone of inhibition(17)

Table 5: Concentrations of nanoemulsion of griseofulvin and relative zone of inhibition.

Concentration (µg/ml)	Zone of inhibition (mm)
0.1	**0**
1	**0**
3	**0**
50	**21**
100	**25**
250	**28**

500	**30**
1000	**32**
Nanoemulsion 8	**29.5**

Table showing various concentration of nanoemulsion of griseofulvin and relative zone of inhibition.

4.Results and discussion

4.1Screening of surfactant

Conventional emulsion shows better stability when having the optimum HLB with hydrophilic and lipophilic groups. Initially, a combination of surfactants was screened using hydrophilic and hydrophobic surfactants in 1:1 ratio. But it was observed that single surfactant shows more stability in formulation of NE i.e.,2:1 and 3:1 are more preferred. All surfactants produce stable nanoemulsion, viz. Tween 20, 40, 60 and 80 and able to formulate a NE having milky appearance at 10 % surfactant concentration but phase separation occurred within a week. Hydrophobic surfactants were found to be more efficient; Span 20 and 40 produced emulsions which were stable up to 3 weeks. Span 80 and 85 produced NE at even lower surfactant concentrations which were stable for a period more than 4 weeks. So these formulations were considered as stable for formulating NE of griseofulvin.

4.2. Pseudo-ternary phase diagram

The chemix school (3) pseudo-ternary phase diagram was used for the selection of optimized batch on basis of emulsification region. The phases were identified by visual inspection. A nanoemulsion is optically clear and transparent and the samples with transparent appearance were separated for further investigations. Pseudo-ternary phase diagrams were constructed using olive oil, GMS (surfactant) and ethanol (co-surfactant) mass ratio (Figs. 1, 2, 3, 4). It was observed that the surfactant alone was ineffective in reducing the g/o interfacial tension enough to provide a NE with desirable properties.

4.3. Effect of surfactant and co-surfactant ratio on NE

A large NE was obtained through the surfactant-rich apex, maximum concentration of oil that could be solubilized and increased amount of co-surfactant with respect to surfactant. In Smix 3:1 ratio maximum amount of oil that could be solubilized was 15 % (w/w). This might be due to the incorporation of co-surfactant and resembles for enhanced penetration of the oil phase in the hydrophobic zone of the surfactant monomer, which in turn reduced the interfacial tension and increased the flexibility and fluidity of the interface, ultimately leading to increased entropy of the system. When co-surfactant ratio reaches up to 2:1, the

total area of NE decreased. Therefore, as the surfactant ratio increased, and nanoemulsion area decreased. In contrast when surfactant concentration of Smix was increased from 1:1, 2:1, 3:1, 4:1, depletion in nanoemulsion region was observed. It might be because of insufficient co-surfactant concentration, resembling for reduction in an interfacial tension and provides the flexibility to the interface and nanoemulsion region. The literature [5] also supports that the Smix 1:1 possesses the maximum NANE area as compared to the other ratio indicating that surfactant and co-surfactant mass ratio has effect on phase ratio

4.4. Globule size analysis

A key distinctive property of nanoemulsion is its nanoscale particle size. The size distribution analysis of selected NE was performed using Malvern nanosizer. shows a broader globule size distribution and globule size intensity ranges from 5.59 to 33.63 nm. The droplet size resembles for rate and extent of drug release, absorption and stability of NE. As the distance from the surface of nanoemulsion increases, the potential gradually decreases. The zeta potential can be related to the stability of colloidal dispersions for molecules and particles that are small enough; a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds the repulsion and dispersion will break out to flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate. So, the nanoemulsion shows the zeta potential -0.787 having low potential but having good stability.

4.5. Stability studies

Nanoemulsion droplets exhibit Brownian movement and no coalescence of droplets takes place unless droplets impinge upon each other owing to their Brownian movement. Agitation can contribute to the energy with which two droplets impinge upon each other. After agitation on a reciprocating shaker for 24 h, there was no phase separation in NE indicating that it has good stability and can withstand the mechanical forces during the transportation and handling. Centrifugation was carried out to examine the effect of gravity on the NE. NE showed no phase separation after centrifugation for 30 min at 3500 rpm indicating that cream has a good stability over the gravitational forces Freeze–thaw cycle is a stability test in which emulsions are subjected to two extreme temperature conditions. For the freeze–thaw cycling, samples were placed alternately at -10 and 25 C for 48 h at each temperature. There was no phase separation after three freeze–thaw cycles indicating a good thermal stability of the formulation. Also, there were no significant changes in viscosity measured before and after performing the test. Non-aqueous nanoemulsion was

monitored for changes in color, viscosity and drug content for the period of 3 months. During studies, formulation was kept at low temperature (5 C), moderate-temperature (25 C) and high-temperature (40 C) conditions. It was observed that formulation was not sensitive to the low temperature. Drug content of the NE was found to be decreased from 98.13 ± 0.56 to 97.50 ± 0.35 % within 3 months at 5 C with no significant change in the chemical composition of the formulation. But there were some extents of changes in the viscosity of the formulation. Initial viscosity of the formulation was found to be 11043cPs up to 1 month; thereafter decrease in the viscosity was 10351 cPs. Initial increase in viscosity might be due to the gelation, because GMS at higher concentration causes the gelation. Color of the formulation did not change at the low temperature. So, it was observed that NE was stable at low temperature. Stability study at 25 C observed for color, drug content and viscosity. There was decrease in the drug content at 25 C from 98.75 ± 0.13 to 97.50 ± 0.13 % within 3 months. As seen in the case of low temperature, there was an initial increase in viscosity of 11043 cPs followed by a decrease up to 9396 cPs. Greater decrease in the viscosity was observed due to increase in the temperature. Also, there was change in the color of the formulation; color was changed from fresh white to dull white. At 40 C, a distinct phase separation occurs within 48 h because of rise in temperature resembling for decrease in viscosity of formulation, leads to formation of larger globule sizes; therefore, stability study at higher temperature was terminated. From the stability studies, it was evident that NE is stable at moderate and low temperatures. The globule size of NE and aqueous formulation was found to be significant throughout the stability study.

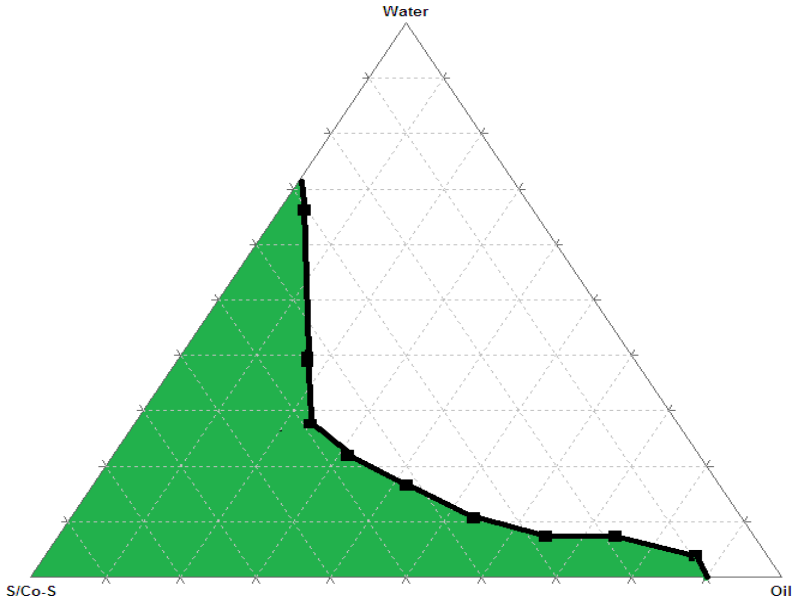

Figure : Pseudo-ternary phase diagrams of nanoemulsions composed of oil (MCT), Smix (surfactant: Tween-80, co-solvent: Propylene glycol) and water atoil/Smixratios, 3:1

Table 6: Compositions of MCT - Tween-80 – Propylene glycol nanoemulsion.
S/Cos - (2:1)

Formulation Code	Oil (%w/w)	S/Cos mix (%w/w)	Water (%w/w)
ME1	3.4	30.82	65.75
ME2	10.25	40.51	49.23
ME3	21.53	49.74	28.71
ME4	35.71	53.57	10.71
ME5	44.80	44.44	10.75
ME6	56.07	36.44	7.47
ME7	67.30	28.84	3.84
ME8	78.92	19.05	1.97
ME9	88.32	9.715	1.96

5. Conclusion

During formulation, it was found that emulsification was achieved when single surfactant was used, rather than surfactant combination, and hydrophobic surfactants was found to be more efficient than hydrophilic surfactants. Stable NE can be obtained using MCT as dispersed phase, polyethylene glycol as continuous phase and Tween 80 as surfactant. This emulsion has improved the stability of griseofulvin.

6. Conflict of interest

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

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