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Screening of nanoemulgels for physicochemical stability and antifungal efficacy

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Abstract. The nanoemulgel was prepared to induce a synergistic effect along with higher efficacy. Nine sets of macroemulsion were made in which liquid paraffin was stabilized by the two non-ionic surfactants, Tween® 80 and Span® 80. Comparative stability analysis of the macroemulsions was used to determine the effective surfactant concentrations that gave the most stable systems (NE 2, NE3, NE4, NE5). High-speed homogenization was then applied. The final formulation was evaluated for globule size and polydispersablity index, physical properties (color, homogeneity, consistency, syneresis), pH, viscosity, spreadability with 200 g and 500 g weight, conductivity, drug content, stability, skin irritation, antifungal efficacy. Zeta size analysis confirmed the nanosize of the droplets in NE2 (284.8 nm), NE3 (79.89 nm), NE4 (194 nm) but not NE5 (632.8 nm), which was outside the nanoemulsion range. The antifungal assay exhibited zone of inhibition for NE3 (43±1.0 mm) and NE4 (42±1.7 mm), a marketed cream (33±1 mm), fluconazole alone (35±1 mm) and terbinafine alone (35.0±1.7 mm). The zone of inhibition of nanoemulgels increased compared with the drugs when used individually and when compared a placebo.
Keywords: antifungal assay; effective surfactant blend concentration; nanoemulgel high-speed homogenization

1. Introduction

The antifungal therapies currently available on the market have become less effective and fungal infections are widely spreading due to increased multiple resistant pathogens, toxicities and drug-drug interaction. Therefore, the demand for alternative therapeutic agents and effective health care plans has risen significantly (Cuenca-Estrella 2004). The combination of two or more antifungal agents can achieve synergistic or true additive effects (Fishman 2002). Hence monotherapy may be substituted by combination therapy to treat invasive mycosis, speed up recovery, better clinical outcomes, reduce toxicity as well as provide true additive, synergistic effects and improve the pharmacokinetics of the active ingredient to target same pathogen at multiple sites of the body concurrently via different mechanisms (Fishman 2002).

In conventional topical delivery, the poor permeability of drugs like terbinafine hydrochloride and fluconazole leads to prolonged therapy, thereby contributing to the increased cost of therapy and decreased patient compliance and increased risks of relapse. Nanosystems can augment the skin diffusion of such molecules (Nastiti *et al*. 2017).

Nanoemulsions (NE) are transparent or milky, monophasic, optically isotropic and kinetically stable colloidal dispersions composed of oil, water, a surfactant and co-surfactant, with a droplet size of 50-500 nm (Jasmina *et al*. 2017, McClements 2012, Jafari and Bhandari 2006). Nanoemulsion-based gels offer a dual control release benefit. Gel-based topical dosage forms increase the contact time and mean residence time. Hydrophilic as well as hydrophobic active pharmaceutical agents can be incorporated in gels (Kumar 2013).

The objectives of this study were to: (1) prepare the nanoemulgel using a high speed-homogenizer, (2) determine the physicochemical attributes of the nano-emulgel, (3) evaluate the synergistic interactions between fluconazole and terbinafine hydrochloride in the nanoemulgel.

2. Methods and materials

2.1 Equipment and materials

UV visible spectrophotometer (CECIL CE7400S), Homogenizer (Heidolph Silent Crusher M), pH digital meter (HI 2210 HANNA instruments), Hot plate magnetic stirrer (AM 4 multiple heating magnetic stirrer VELP Scientifica), Conductivity meter (Ecoscan con5 eutech

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Table 1 Composition of combination of fluconazole and terbinafine hydrochloride loaded nanoemulgels

Ingredients (W/W% in grams)	NE 1	NE 2	NE 3	NE4	NE5	NE6	NE7	NE8	NE9
Terbinafine hydrochloride	1	1	1	1	1	1	1	1	1
Fluconazole	1	1	1	1	1	1	1	1	1
Carbopol 934	1	1	1	1	1	1	1	1	1
Triethanolamine	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Propylene glycol	5	5	5	5	5	5	5	5	5
Liquid paraffin	6	6	6	6	6	6	6	6	6
Span 80	0.421	0.6315	0.842	1.05	1.263	1.48	1.684	1.89	2.11
Tween 80	0.579	0.8689	1.158	1.447	1.737	2.02	2.316	2.605	2.89
Methyl paraben	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Propyl paraben	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Ethanol 94 %	10	10	10	10	10	10	10	10	10
Purified Water	74.0	73.5	73.0	72.6	72.1	71.6	71.1	70.6	70.1

instruments), Dynamic light scattering technique (HSA 3000 Malvern Instruments Ltd., UK), Viscometer (Brookfield DV –II+ Pro viscometer Ltd). Terbinafine hydrochloride (Saffron Pharmaceuticals, Pakistan), Fluconazole (Medicina Pharma Lahore, Pakistan), carbopol 934 and propylene glycol (Saffron Pharmaceutical Pvt Ltd), methyl paraben, propyle paraben, ethanol, triethanolamine, liquid paraffin (Sigma Aldrich, USA), span 80 (Daejung chemicals, Korea), tween 80 (Daejung chemicals, Korea).

2.2 HLB matching

Nine macroemulsions were made in which liquid paraffin was the hydrophobic phase with a required HLB of 10.0. An oil-in-water colloidal system, the macroemulsions were stabilized by two non-ionic surfactants, Tween® 80 (polyoxyethylene 20 sorbitan monooleate /polysorbate 80; HLB = 15.0) and Span® 80 (sorbitan monooleate; HLB = 4.3). The effective surfactant blend (ESB) was determined by calculating the proportion of each surfactant required to produce a surfactant mixture with a HLB value matching the HLB requirement of liquid paraffin, according to the following formula:

$$\text{HLB of mix} = \frac{(\text{proportion of tween} \times \text{HLB tween}) + (\text{proportion of span} \times \text{HLB Span})}{(\text{proportion of tween} + \text{proportion of span})} \quad (1)$$

2.3 Method of preparation

The proportions Tween® 80 and Span® 80 were 57.9% and 42.1%, respectively. For nanoemulsion from 1% up to 5%, an increment of 0.5 of blend was used and nine formulations (NE1, NE2, NE3, NE4, NE5, NE6, NE7, NE8, NE9) were developed.

2.3.1 Preparation of macroemulsion

1. To prepare the oil phase of NE1 blank, 0.421g Span® 80 was weighed in a 100 ml beaker. Liquid paraffin (6g) was weighed in another beaker and poured into the beaker containing the Span® 80, while the latter was being stirred at 250-280 rpm and 70-80℃ on a hot plate magnetic stirrer.

2. To formulate the aqueous phase of NE1, 37.9 g of water was weighed in a in a 250 ml beaker and added to 0.579 g of Tween® 80, which had been weighed separately. The mixture was stirred with a 30 mm magnetic stirrer bar at 250–300 rpm and 70–80°C. An amount of water sufficient for 100% w/w formulation ranging from 76 to 78 g w/w was divided into two halves – one half was used to make the gel phase, the other was used to make the aqueous phase.

3. On a glass watch plate, 0.03 g methylparaben and 0.01 g propylparaben were measured and added to a beaker containing 5 g of propylene glycol.

4. In a measuring cylinder, absolute ethanol and was diluted with distilled water to obtain the desired volume of 94% of ethanol. From this, 10 g of 94% ethanol was transferred to a beaker and covered with cling film.

5. Propylene glycol containing the preservatives (methylparaben and propylparaben) was added gradually to ethanol (94% v/v) to avoid clouding. The oil phase containing Span® 80 was added dropwise to the aqueous phase with stirring at 350–450 rpm but without heating, over 15 minutes (D. L. Kumar, 2013).

6. Fluconazole (1 g) was added to the mixture containing propylene glycol, methylparaben and propylparaben, with stirring until a homogenous mixture was achieved. Terbinafine hydrochloride (1 g) was added to 94% v/v ethanol as it was freely soluble in it.

7. The mixture was placed in a 100 ml beaker and the homogenizer horn inserted to an appropriate depth, such that air entrapment and splashing would be avoided. The mixture was homogenized at 10,000 rpm over 2 hours to produce the macroemulsion (Karri *et al*. 2015; Gupta *et al*. 2016).

2.3.2 Preparation of microgel

Water (37.0 g) was added to Carbopol® 934 (1 g) in a 250 ml beaker, with stirring at 400–500 rpm and the heating turned off. As the viscosity of the mixture increased, the stirring speed was reduced to facilitate stirring. The process continued until a homogenous, lump-free viscous material

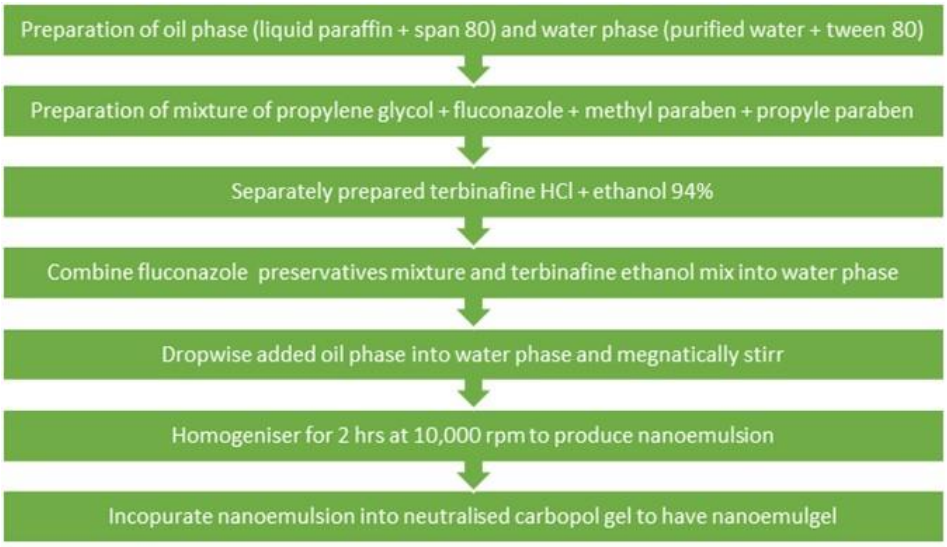

Fig. 1 Flowchart of the fabrication method

was obtained. To induce gelling, 0.9 g triethanolamine (water-soluble organic amine) was added to the mixture using a syringe to produce a highly cross-linked microgel (Ubaid *et al*. 2016).

2.3.3 Preparation of nanoemulgel

The homogenized nanoemulsion was added gradually into the microgel using a glass stirrer. Each portion was added only after the previous portion had completely infiltrated the gel. The process continued till the nanoemulsion had been fully incorporated into the microgel, and the product was smooth in appearance. Gelation was initiated immediately before the nanoemulsion was ready to be incorporated, since prolonged shearing of carbopol would alter its viscosity (Lubrizol 2011, Matsaridou *et al*. 2012).

2.4 Characterization studies

2.4.1 Optimization of blank macroemulsions on the bases of phase behavior and morphology

The phase behaviors and the time at which instability started to appear was noted. This was used as the primary parameter to identify appropriate formulations to take forward. The other morphological parameters were compared using light microscopy and the bottle test (Mahreen *et al*. 2023).

2.4.2 Physical examination of nanogel

A visual test of prepared nanoemulsion loaded gels of terbinafine hydrochloride and fluconazole was done, to ensure any separation of phases, or syneresis (extrusion of water from a gel), consistency (i.e., thickness, firmness, elasticity, plasticity, and tackiness), homogeneity, color, and foreign matter if present. The visual test was done with naked eye.

2.4.3 Globule size

The polydispersity index (PDI) was used to assess the homogeneity of the nanoemulgel formulations (Nastiti *et al*. 2017). The PDI was calculated from the droplets size distribution as measured using dynamic light scattering (Malvern zetasizer Nano Series Zen 3600, Malvern UK) by utilizing a green laser and a scattering angle of 173°.

2.4.4 Measurement of pH

The pH of the nanoemulgel formulations was determined using a digital pH meter. 1 g of the nanoemulgel was dissolved in 100 ml of distilled water and stirred on a magnetic stirrer until completely dissolved. The resultant solution was covered with aluminum foil and set aside for 2 hours until use. The pH probe was then inserted into the beaker. The experiment was performed in triplicate (Helal *et al*. 2012).

2.4.5 Viscosity estimation

The viscosity of true nanoemulgel was determined, without any dilution, using a viscometer. The nanoemulgel (100 ml) was placed in a 100 ml beaker. Spindle 6 was introduced perpendicularly into the center of the beaker filled with the formulation without touching the bottom. The speed was set at 6 rpm and the setup was allowed to equilibrate. Once the reading had stabilised, the reading on the dial was recorded (Dadkari *et al*. 2019).

2.4.6 Electrical conductivity

The electrical conductivity of the nanoemulgels was used to determine the emulsion type (Nastiti *et al*. 2017). Electrical conductivity was measured using a conductivity meter (Ecoscan Con 5, Eutech Instruments) at 25°C. The nanoemulgel formulation was placed in a 100 ml beaker and the electrode immersed in the product. The setup was allowed to equilibrate until the reading stabilised. The stabilized reading was recorded as the electrical conductivity of the nanoemulgel. The experiment was conducted in triplicates.

2.4.7 Spreadability

0.2 gram of each formulation was placed into a circle (diameter: 1 cm, measured with a ruler) in the middle of a

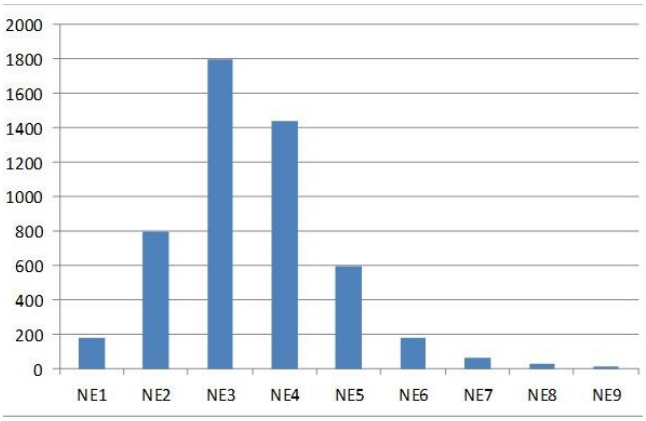

Fig. 2 Comparative stability analysis of macroemulsion

glass plate. Another glass plate was placed over the formulation in the glass plate. The positions of the glass plates relative to each other were maintained across all experiments to minimize inter-sample variability. After 60 seconds, the spread of the formulation was noted from 3 different points and the average was calculated as the initial diameter (D1).

A weight of 200 g was placed on top of the glass plates. The diameter of the formulation was taken from 3 different points and the average was calculated and then divided by the initial diameter (D1) to attain spreadability. Next, a weight of 500 g was placed on top of the plates the and diameter of the formulation was recorded from 3 different points after 5 minutes (Zheng *et al*. 2016).

The method above was applied to all 3 nanoemulgels, NE2, NE3, NE4 and spreadability was calculated according to the equation: (Kenechukwu *et al*. 2018).

$$spreadability = final\ diameter(D2) \div initial\ diameter(D1) \quad (2)$$

$$\%\ spreadability = (increase\ in\ diameter \div initial\ diameter)100 \quad (3)$$

2.4.8 Drug content

The drug content of the formulations was determined by UV/Vis spectroscopy (Chaudhary *et al.*, 2011). To construct the standard curve, standard solutions for fluconazole and terbinafine were prepared in methanol. For the drug assays, 1 g of the formulation (NE2, NE3 and NE4) was weighed into a 100 ml beaker. Methanol (50 ml) was added and the beaker was covered with aluminum foil. The formulation was mixed with methanol on a magnetic stirrer for at least 15 minutes, until the formulation had completely dissolved. The dispersion was then filtered through a 0.45µm syringe filter to obtain a clear filtrate. This filtrate (5 ml) was transferred into a 25 ml volumetric flask, made up to 25 ml and mixed by repeatedly inverting the stoppered flask. The absorbance of the standard solutions and the samples was measured in a UV/Vis spectrophotometer at 261 nm (fluconazole) and 283 nm (terbinafine). The drug concentration (x), and thus the drug content, was determined from the linear calibration plot according to the following equation:

$$Y = mx + c \quad (4)$$

Y = absorbance,
m = slope of curve,
c = Y − intercept

2.4.9 Skin irritation studies

This study was carried out on 40 healthy volunteers in a single-blinded study. 16 individuals received NE3, 16 received NE4, and 4 received the placebo formulation (nanoemulgel without terbinafine HCl and fluconazole). The formulation was applied on hair-free skin of the arm for 24 hours (Ubaid *et al*. 2016). Skin irritation score board was used to assess skin irritation (Kore *et al*. 2011).

2.4.10 Stability testing

Stability studies of the optimized formulations, NE3 and NE4 were carried out according to the guidelines of the International Conference on Harmonization (ICH). The nanoemulgel formulations were filled into well-closed aluminum tubes and stored at 40±2°C and 75% relative humidity for 90 days. The nanoemulgel formulations were then evaluated for their physicochemical properties (including appearance, color, consistency, any phase separation, pH and drug content) in a hot air oven after 2 months (Ubaid *et al*. 2016).

2.4.11 Antifungal studies

The antifungal assay was done using the agar well method. Sabouraud dextrose agar (SDA, 16.25 g) was weighed into a 500 ml conical flask. Purified water (250 ml) was added with some heating to dissolve it completely and allowed to solidify upon cooling. The inoculum was introduced via the streaking method to the solidified SDA at room temperature. Wells were created in a petri dish with the help of a sterile steel borer (internal diameter: 6 mm). The solvent was introduced and measured quantity of standard drug was poured in the well another technique which was used in this practice to make the solution of standard drug in solvent then introduced by the micropipette. The comparative analysis was made among each standard drug (fluconazole and terbinafine hydrochloride, optimized nanoemulgels (NE3 and NE 4), marketed gel (Terbisil by Saffron Pharma) and placebo. The petri dishes were incubated for 48 hours at 37°C. The antifungal activities of the formulations were quantified in terms of the radius of the zone of inhibition.

3. Results and discussion

3.1 Phase behavior

The initial instability (Fig. 2) indicates that the surfactant coverage of the oil globule surfaces was incomplete, due to the concentration being below the critical micelle concentration (CMC) and the relatively high interfacial surface tension. As the surfactant concentration increased towards the CMC, the stability of the formulations improved. Upon further increases of the surfactant

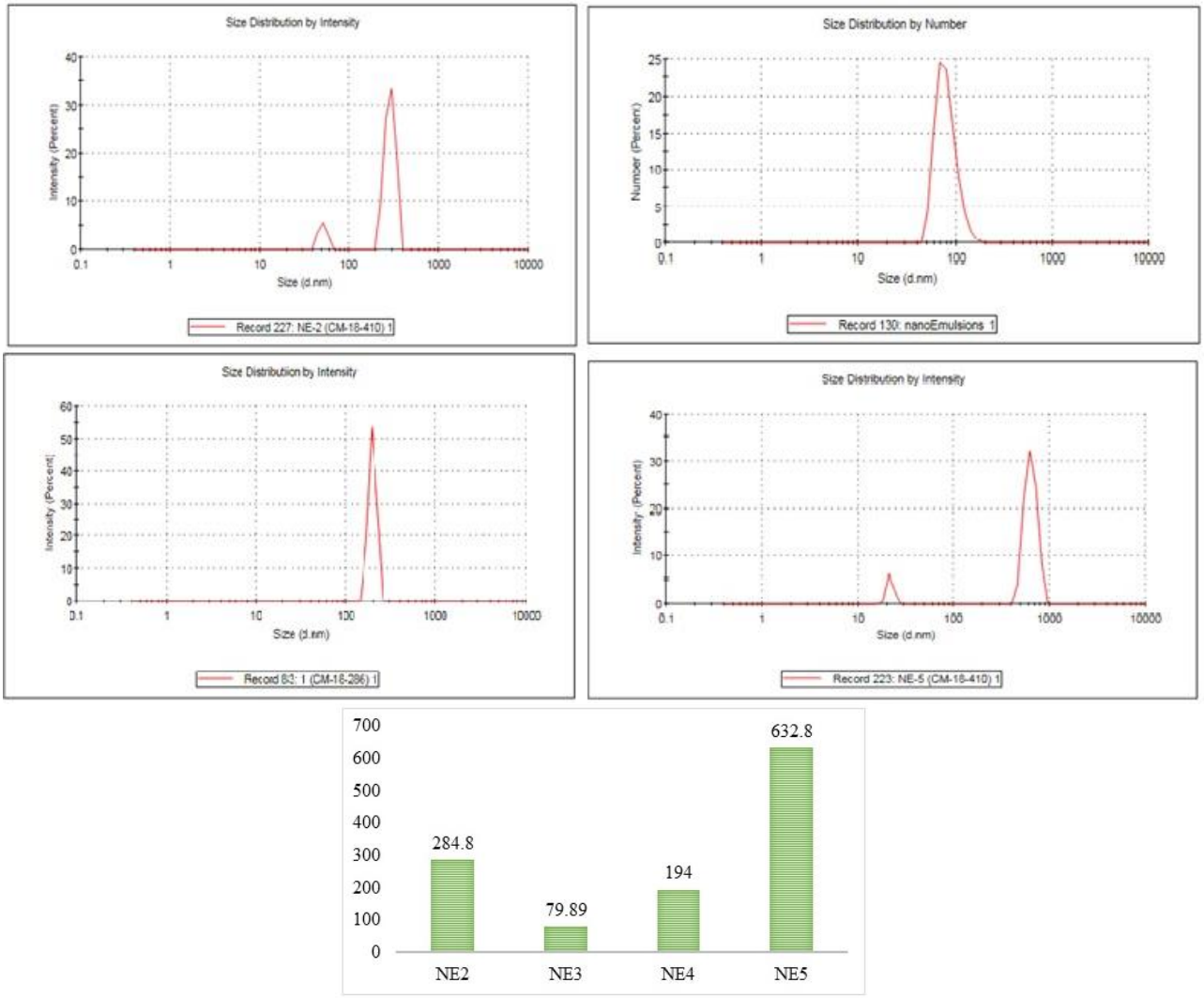

Fig. 3 Zeta size analysis of nanoemulgel

Table 2 The physical characteristics and stability of the optimized nanoemulgels, NE3 and NE

Nanoemulgel	Parameters	0 days	90 days
NE 3	Homogeneity	+++	+++
	Colour	Milky white	No change
	Appearance	No lumps or clogs, phase separation,weeping or syneresis.	No change
	pH	6.65	6.45
	Drug content	TBH 97.35% FLU 95.29%	No change
NE 4	Homogeneity	+++	+++
	pH	6.43	5.90
	Appearance	No lumps, phase separation OR syneresis	No alternation
	Drug content	TBH 90.36% FLU 92.4%	No appreciable change

concentration above the CMC, the surfactant molecules tended to knock out each other due to competitive desorption, as the surfactant molecules favoured the oil-water interface (Sonia *et al*. 2018), hence instability was observed in NE6–NE9.

3.2 Physical examination of nanogels

All nanogels showed excellent textural characteristics, i.e., excellent homogeneity and no syneresis or weeping (Table 2).

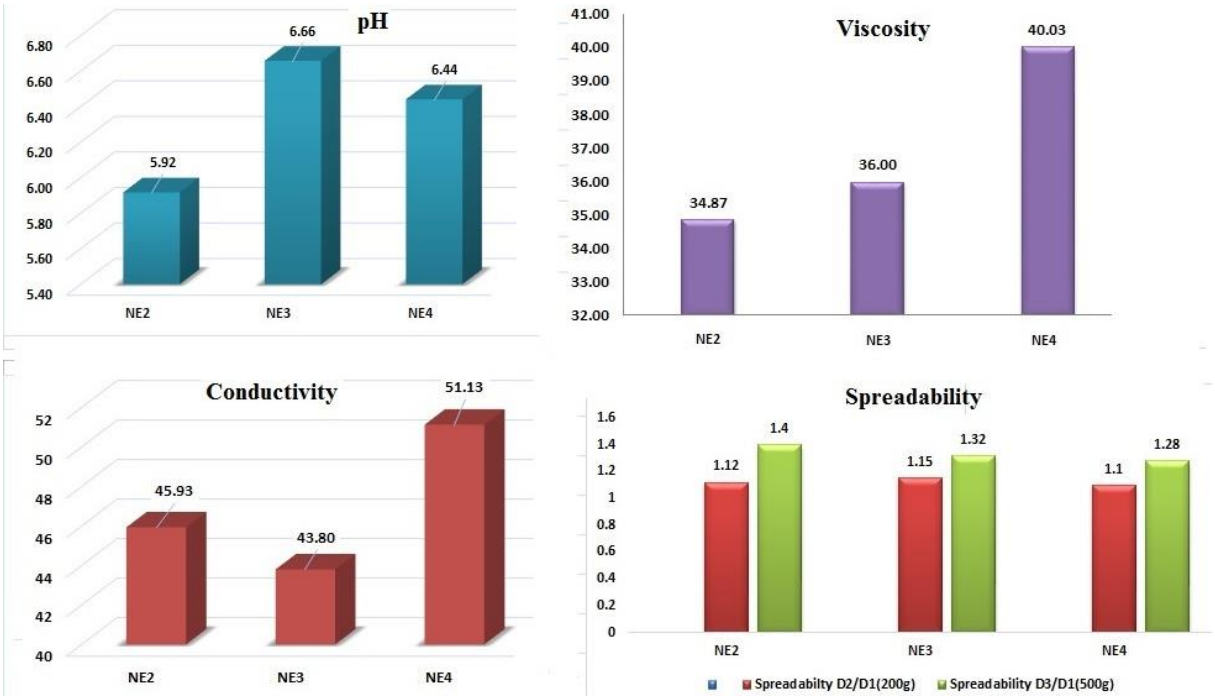

Fig. 4 Viscosity, pH, conductivity and spreadability trend of nanoemulgels

3.3 Globule size and polydispersity index (PDI) determination

The mean globule size of the macroemulsions was 284.8 nm (NE2), 79.89 nm (NE3) and 194.0 nm (NE4). The macroemulsions were formed by homogenization at high shear. However, the particle size of NE5 fell outside the particle size range of macroemulsions, even though it was subjected to the same shear intensity and duration as the other formulations.

3.4 pH

The pH of NE2, NE3 and NE4 was close to the pH of human skin. Therefore, they are likely to be suitable for application to the skin without severe irritation (see Section 2.4.9). There was little variation in pH between the formulations, probably due to their similar chemical compositions. The stable pH may also be important for the overall physicochemical stability of the formulations, e.g. emulsion stability, rheological behavior and effectiveness of the preservatives.

3.5 Viscosity

The viscosity of the nanoemulgels increased from NE2 to NE4 (Fig. 3) as the concentration of the surfactant increased and the water content decreased proportionately. The droplet size of NE3 was smaller than that of NE2, giving a smaller effective free volume, as more of the continuous phase was confined to the interfacial layers Thus, the viscosity of NE3 and NE4 was higher than that of NE2. The decreasing interfacial tension from NE2 to NE4 could have also produced greater frictional forces, thus enhancing the viscosity (Nastiti *et al*. 2017).

The viscosity of the nanoemulgel is important because it determines the retention time of the formulation at the site of administration. A greater viscosity can prolong the contact time of the drug with the target tissue (Kumar and Verma 2010). It also influences the stability and the drug release rate of the nanoemulgel.

These observations confirm the textural and mechanical properties of the nanoemulgels, support their spreadability and ease of application, and promise good esthetic qualities and user satisfaction. The viscosity of NE2, NE3, NE4 indicates that these formulations will be easy to apply on both hairy and non-hairy tissue surfaces, as well as being easy to remove from the container. These qualities help assure the clinical effectiveness and consumer acceptability of these nanoemulgels (Demartine and Cussler 1975).

3.6 Electrical conductivity

All nanoemulgels showed similar electrical conductivity (Fig. 3), since their chemical compositions were very similar. The high values of conductivity of NE2, NE3 and NE4 are characteristic of oil-in-water emulsions, thus suggesting that water was the external phase in these formulations.

3.7 Spreadability

The nanoemulgels exhibited similar spreadability (Fig. 3). The spreadability is inversely related to the concentration of the gelling agent. Since all the nano-emulgels tested contained the same concentration of gelling agent (1% w/w Carbopol® 934), their spreadability was expected to be similar. The nanoemulgels maintained their structural integrity under shear forces of 200 g and 500 g, as no phase separation, syneresis or weeping was observed. Consistent

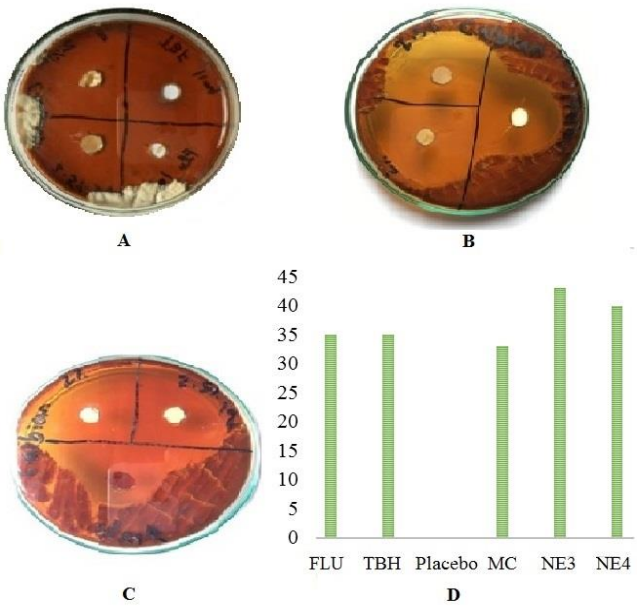

Fig. 5 Viscosity, pH, conductivity and spreadability trend of nanoemulgels

with previous reports (Kenechukwu, Franklin C., 2018), the spreadability of the nanoemulgels increased when the shear force increased from 200 g to 500 g. These results confirmed that the nanoemulgels could be spread with little application of shear.

The efficacy of topical therapy relies on the ability of the patient to spread the formulation in an even layer to deliver a standard dose. The optimum consistency and homogeneous, lump-free nature of the nanoemulgels are therefore important to ensure that a suitable dose can be delivered to the target site. Low spreadability can result in uneven application of the formulation, while excessive spreadability could lead to an insufficient dose being delivered despite an even application being achieved. Both of these situations could lead to suboptiomal therapy and side effects.

Viscosity and spreadability are inversely related (Kumar and Verma 2010). If the cohesiveness is high in the formulation, the flowability of the formulation will be low. Thus, increasing viscosity could also reduce spreadability.

3.8 Drug assay

The terbinafine content in NE2, NE3 and NE4 was 87.3%, 97.35% and 90.36%, respectively. The fluconazole content was 86.0%, 95.29% and 92.4% in NE2, NE3 and NE4, respectively. Both drugs were compatible and stable in the nanoemulgel formulations.

3.9 Skin irritation

NE3 and NE4 showed no skin irritation (defined as a skin irritation score of 2 or below). 40 participants showed no irritation (skin irritation score = 0), while 2 showed faint, barely visible erythema (skin irritation score = 0.5). All 8 participants in the placebo group also showed no signs of skin irritation (skin irritation score = 0).

3.10 Stability study

The optimized nanoemulgels (E3 and NE4) showed no significant change in physicochemical characteristics after 90 days in storage (Table 2). Microscopic examination revealed no crystal or lump formation in the nanoemulgels, indicating that all ingredients had remained fully dispersed and solubilized in the semisolid formulation. Taken together with the other physicochemical characteristics, including the unchanged pH, appearance (no clog or lumps present), homogeneity and drug content, the results confirm that the optimized nanoemulgel formulations exhibited good overall stability (Ubaid *et al*. 2016).

3.11 Antifungal assay

The antifungal assay (Fig. 4) confirmed a synergistic effect in the nanoemulgels containing both APIs compared with the effects of the individual APIs when introduced separately. The optimized nanoemulgels containing both APIs showed a greater zone of inhibition than that observed with the marketed cream. Both nanoemulgels also showed superior antifungal activity, assessed from the size of the zone of inhibition, compared to the placebo. The placebo (nanoemulgel without any API) showed no antifungal activity. Carbopol is known to be inert (Kore *et al*. 2011).

4. Conclusions

In this study we concluded that:

• A nanoemulgel with a globule size below 100 nm was successfully produced with a lab-scale mixer.

• The nanoemulgel was an oil-in-water nanosystem rationally resigned to improve antifungal activity.

• The nanoemulgel formulation combining two established antifungals, terbinafine HCl and fluconazole, exhibited a synergistic antifungal effect, which could broaden their respective antifungal activity to target both superficial and invasive mycosis.

• The optimized nanoemulgel formulations were stable and did not cause skin irritation. Their physicochemical properties also promise effective topical delivery.

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