Self-nanoemulsifying drug delivery system (SNEDDS) with enhanced solubilization of nystatin for treatment of oral candidiasis: Design, optimization, in vitro and in vivo evaluation

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A B S T R A C T

The aim of the present study is to develop and optimize self-nanoemulsifying drug delivery systems (SNEDDSs) to improve the per-oral bioavailability of poorly soluble polyene antifungal drug, nystatin (NYS), and to evaluate its in vitro and in vivo performance. Solubility of NYS was estimated in various vehicles to select proper components combinations. Oleic acid (oil), Tween® 20 (Tw20) and Tween® 40 (Tw40) (surfactants) as well as dimethyl sulfoxide (DMSO) and propylene glycol (PG) (co-surfactants) were employed to construct pseudo-ternary phase diagrams. Thermodynamic stability, dispersibility and robustness to dilution tests were performed to optimize formulations from phase diagram. Five optimized formulations composed of oleic acid, Tw20 and DMSO or PG at Smix ratios (1:1, 2:1 or 3:1) were selected. They were spherical in shape of mean droplet size (∼100 nm with negatively charged zeta potential < −15 mV. The in vitro release profile of NYS-SNEDDSs was found significant in comparison to the plain NYS suspension. In vitro and in vivo evaluations against Candida albicans depicted promoted antifungal efficacy of selected NYS-SNEDDS formulations compared to marketed and plain NYS suspensions. The results indicate that NYS loaded SNEDDSs, with enhanced solubilization and nanosizing, has potential to improve the absorption of drug and increase its oral antifungal efficacy.

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1. Introduction

Gastrointestinal candidiasis (GIC) is one of the most common mucosal infections which may become pathogenic in patients with human immunodeficiency virus (HIV) or acquired immune deficiency syndrome (AIDS) [1,2]. Oropharyngeal candidiasis (OPC) is a common human fungal infection characterized by an overgrowth of Candida species in the superficial epithelium of the oral mucosa [3,4]. The vast majority of these infections are caused by Candida albicans [5]. Immunocompromised patients, including those with HIV infection or cancer, are at an enhanced risk of OPC [4,6]. In addition, OPC can be triggered in healthy patients by transient risk factors such as antibiotic, corticosteroid treatment or dental prosthesis [7,8]. The infection can be complicated by esophageal candidiasis and, in the worst cases, fungal septicemia [3]. OPC is not a lethal disease but must be treated to avoid chronicity, other tissue invasions or systemic infection [9]. Although it is infrequent, disseminated candidiasis has a mortality rate of 47% [6,10].

Nystatin (NYS) and fluconazole are the most widely employed antifungal agents in the treatment of OPC [5]. NYS is a polyene antifungal antibiotic, one of the oldest antifungal drugs, produced by Streptomyces noursei strains [8,11], commonly used for the prophylaxis and treatment of candidiasis. It acts by interfering with the fungal cell membrane of the antibiotic-sensitive organism by binding to sterols, chiefly ergosterol, and the formation of barrel-like membrane spanning channels [12]. NYS possesses a broad antifungal spectrum; it has been reported to be effective against azole-resistant strains of Candida and, in some cases, amphotericin B-resistant strains of C. albicans [13]. NYS is a yellow or slightly brownish hygroscopic substance [1,4], practically insoluble in water and alcohol, slightly soluble in methanol, and freely soluble in dimethyl formamide and dimethyl sulfoxide [14]. NYS is poorly absorbed from the gastrointestinal tract, and detectable blood concentrations are not obtained after usual doses [15]. Following oral administration, NYS is excreted almost entirely in feces as an unchanged drug [15]. It is not absorbed through the skin or mucous membranes when applied topically [15,16]. The chemical structure of NYS reveals formulation challenges, because it is characterized by the presence of a large lactone ring containing several double bonds conferring an amphiphilic and amphoteric nature [17,18], which in turn contributes to its low solubility in aqueous media and poor bioavailability. Special care needs to be taken for the delivery of this drug as it cannot be simply introduced into an aqueous solution because it forms aggregates that lead to formulation challenges [19]. NYS aggregates formed in aqueous media are non-selective and able to disrupt the integrity of both fungal and mammalian cell membranes, inducing toxicity and host cell death.
Therefore, it is proposed that NYS delivery in an un-aggregated form may improve its therapeutic index [19]. For this reason, the controlled-release delivery of this drug is a complex task. To overcome this problem, several approaches have been reported [11]. NYS has been formulated in micellar gels [18], mucoadhesive devices for topical use [21,22], liposomes [23], niosomes [24], nanoemulsions [19,25], lipid intravenous emulsions [11], intralipids [26], nanosuspensions [5], microparticles [27], pellets [1], solid lipid nanoparticles [28] and nano-structured lipid carriers [29].

The use of formulation such as conventional microemulsion and self micro/nanoemulsifying drug delivery systems (SMEDDS/SNEDDS) have generated much academic and industrial interest as potential formulations for improving the oral bioavailability of drugs [30] and representing a unique solution to delivery of poorly soluble compounds [31]. The advantages of SNEDDS over the conventional emulsions or other lipid carriers are the significantly reduced energy required for their preparation, their physical stability upon storage [32] and easier to manufacture in a large scale [33]. The SNEDDS can reduce the limitation of slow and incomplete dissolution of poorly water soluble drugs and facilitate the formation of solubilized phases from which absorption might take place [34]. Hydrophobic drugs can be dissolved in these systems, enabling them to be administered as a unit dosage form for per-oral administration. This leads to in situ solubilization of drug that can subsequently be absorbed by lymphatic pathways, by passing the hepatic first-pass effect [35]. The rationale to use SNEDDS for the delivery of poorly soluble drugs is that, they are presented in the form of pre-concentrated solution. Hence, the dissolution step required for solid crystalline compounds shall be avoided [33,36]. In addition, the formation of a variety of colloidal species on dispersion, and subsequent digestion of SNEDDS facilitates drug absorption [37]. SNEDDS have been described in the literatures as homogenous (transparent) complex systems consisting of oils, surfactants, co-solvents and water, which are thermodynamically stable [34]. SNEDDS provide ultra low interfacial tensions and large o/w interfacial areas. Therefore, SNEDDS have the advantages in possessing higher solubilization capacity than simple micellar solutions, leading to the incorporation of poor water-soluble pharmaceutical inside the oil phase [38]. They rapidly form oil in water (o/w) nanoemulsion when exposed to aqueous media upon gentle agitation or digestive motility of GI tract [35,39]. When a SNEDDS is introduced into the body, it is rapidly dispersed to form droplets of approximately nano-size range (<200 nm) [40]. Furthermore, these particulate delivery systems show a prolonged residence time on mucosal membranes [41] and they could reach greater mucosal surface areas, resulting in a comparatively higher drug uptake [40,42]. Because of the small droplet size, nanoemulsions keep the transparency after aqueous dilution [34].

On the basis of these considerations, the major aim of the present study is the development, optimization, characterization and evaluation of NYS-SNEDDS, with objectives of enhanced solubilization of the poorly soluble drug, NYS, for the treatment of oral fungal infections.

2. Materials and methods

2.1. Materials

NYS was kindly donated by GlaxoSmithKline Co., Cairo, Egypt. Oleic acid (extra-pure, 99%) was purchased from Alpha Chemika, India. Tween® 20 (Tw20) (extra-pure, 99%) was obtained from VWR International, France. Tween® 40 (Tw40) (extra-pure, 99%) and Tween® 60 (Tw60) (extra-pure, 99%) were bought from Sisco Research Laboratories (SRL) Pvt. Ltd., India. Tween® 80 (Tw80) (extra-pure, 99%) was procured from Riedel-de Haën, Italy. Dimethyl sulfoxide (DMSO) (HPLC grade, 99.5%) was purchased from Tedia, USA. Dimethyl formamide (DMF) (99.5%) was obtained from S.D. Fine-Chem Ltd., India. Propylene glycol (PG) (99.5%) was bought from BDH Laboratory, UK. Methanol (HPLC grade, 99.7%) was procured from Fisher Scientific, UK. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Solubility study

The solubility of NYS in various oils (Isopropyl myristate, Isopropyl palmitate, Oleic acid, Methyl laurate and Miglyol), surfactants (Tw20, Tw40, Tw60, Diacetin, Captex 200 and Span® 80) and cosurfactants (PG, DMSO, Polyethylene glycol (PEG) 400 and Ethanol) was determined. An excess amount of NYS (≈50 mg) was added to 2 g of each component in screw-capped glass vials. The obtained mixtures were mixed continuously for 2 min using vortex mixer (Julabo, Paramix II, Germany) to facilitate proper mixing of NYS with the vehicles. The mixtures were shaken (100 rpm) for 72 h at 25 ± 0.5 °C in a thermostatically controlled shaking water bath (Memmert, SV 1422, Germany) followed by equilibrium for 24 h [31,43]. The equilibrated samples were removed and centrifuged at 5000 rpm for 30 min. The supernatant solution was then filtered through a Millipore membrane filter (0.45 µm) and then suitably diluted with methanol. The concentration of NYS was determined spectrophotometrically using UV–Visible recording spectrophotometer (Shimadzu, UV-2401 PC, Japan) at 304 nm using methanol as a blank [14,24]. The experiment was repeated in triplicates.

2.2.2. Preliminary screening of surfactants for their emulsification ability

Different surfactants for the per-oral use were screened for emulsification ability according to the method described by Date and Nagarsenker [44]. Briefly, 300 mg of each selected surfactant (Tw20, Tw40, Tw60 or Tw80) was added to 300 mg of the chosen oily phase (oleic acid). The mixtures were gently heated at 50 °C for homogenization of the components. Fifty mg of each mixture was then diluted with double distilled water to 50 ml in a stoppered volumetric flask to yield fine emulsion. Ease of emulsification was judged by the number of flask inversions required to yield homogenous emulsion. The resulting emulsions were allowed to stand for 2 h and their % transmittance was determined. An excess amount of NYS (~50 mg) was added to 2 g of each component in screw-capped glass vials. The obtained mixtures were mixed continuously for 2 min using vortex mixer (Julabo, Paramix II, Germany) to facilitate proper mixing of NYS with the vehicles. The mixtures were shaken (100 rpm) for 72 h at 25 ± 0.5 °C in a thermostatically controlled shaking water bath (Memmert, SV 1422, Germany) followed by equilibrium for 24 h [31,43]. The equilibrated samples were removed and centrifuged at 5000 rpm for 30 min. The supernatant solution was then filtered through a Millipore membrane filter (0.45 µm) and then suitably diluted with methanol. The concentration of NYS was determined spectrophotometrically using UV–Visible recording spectrophotometer (Shimadzu, UV-2401 PC, Japan) at 304 nm using methanol as a blank [14,24]. The experiment was repeated in triplicates.

2.2.3. Construction of pseudo-ternary phase diagrams

Self-nanoemulsifying systems form fine o/w emulsions when introduced into aqueous media with gentle agitation. Surfactant and cosurfactant get preferentially adsorbed at the interface, reduce the tension, and provide mechanical barrier to prevent the globules from coalescence. The decrease in free energy required for emulsion formation consequently improves the thermodynamic stability [47]. On the basis of the solubility studies (Section 2.2.1) and preliminary screening of surfactants (Section 2.2.2), oleic acid was selected as the oil phase, Tw20 and Tw40 as the surfactants and DMSO and PG as the cosurfactants. Double distilled water was used as the aqueous phase for construction of phase diagrams. Pseudo-ternary phase diagrams of mixed surfactant and co-surfactant (Smix), oil, and water but without drug incorporation were plotted, and each of them represents a side of the triangle. For any mixture, the total of surfactant, co-surfactant and oil concentrations always added to 100% [46]. Ternary mixtures with varying compositions of surfactant, co-surfactant, and oil were prepared resulting in a total amount of 1 g. Surfactant and cosurfactant were mixed in three ratios, namely: 1:1, 2:1 and 3:1 (Smix, w/w). For each phase diagram, oil and specific Smix ratio was mixed thoroughly in nine different weight ratios from 1:9 to 9:1 in different glass vials. The nine different combinations of oil and Smix; 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1, were made so that maximum ratios were covered for the study to delineate the boundaries of phase precisely formed in the phase diagrams. Pseudo-ternary phase diagrams were developed using the aqueous titration method. Slow titration with...
aqueous phase was done to each weight ratio of oil and Smix. The total water consumed was noted in terms of w/w during titration of oil—Smix ratio and observations were made for phase clarity [47]. The formation of the nanoemulsion was visually observed as clear/transparent and easily flowable/dispersible with low viscosity o/w nanoemulsion and marked on the pseudo-ternary phase diagram [38,48]. The point at which the mixture appeared turbid, viscous, or separated in phases was considered as the end point of titration. The amount of each component (oil, Smix and water) at this point was recorded and presented in a pseudo-ternary phase diagram [34]. The phase diagram was plotted using ternary plot software (SigmaPlot for windows, version 11.0, Systat Software, Inc., Ca, USA). All the studies were repeated thrice, with similar observations being made between repeats.

2.2.4. Preparation of NYS-SNEDDS

Once the self-emulsifying region was identified, the desired component ratios of SNEDDS were selected (Table 1) for drug incorporation and further optimization. Ten mg of NYS was dissolved in the Smix, at their determined ratios, and mixed continuously for 2 min using the vortex mixer. The oil phase was then finally incorporated into the mixture containing drug and mixed using the vortex mixer to obtain a homogenous mixture. The prepared NYS-SNEDDSs were kept in a tightly closed glass bottles at 25 °C and from these, the stable formulations were subjected to further studies.

2.2.5. Optimization of formulations

2.2.5.1. Thermodynamic stability studies. The objective of these tests was to evaluate the stability and phase integrity of NYS-SNEDDS under different conditions of temperature variation and centrifugal force [34]. Three different tests were carried out in this study [49]:

2.2.5.1.1. Centrifugation study. Formulations were centrifuged at 5000 rpm for 30 min and were then checked visually for instability such as phase separation, creaming, cracking or drug precipitation. The formulations that did not show any signs of instability were chosen for heating—cooling cycle.

2.2.5.1.2. Heating and cooling cycle. Heating cooling cycle so performed involved three cycles between 4 °C and 45 °C with storage at each temperature for not less than 48 h. The formulations that passed at these temperatures, without undergoing any creaming, cracking, coalescence, phase separation or phase inversion, were chosen for freeze thaw stress test.

2.2.5.1.3. Freeze thaw cycle (accelerated aging). Freeze thaw cycle involved three freeze thaw cycles at temperatures between −20 °C and +25 °C with storage at each temperature for not less than 48 h. The formulations were then visually observed for phase separation. Only formulations that were stable to phase separation were selected for dispersibility study.

2.2.5.2. Dispersibility study. The dispersibility studies were carried out to observe the self-emulsification efficiency and self-emulsification time [47]. Self emulsification time is the time required by the pre-concentrate to form a homogeneous mixture upon dilution, when disappearance of SNEDDS is observed visually [33]. Briefly, 0.5 ml of each formulation was added drop wise to 250 ml of double distilled water in a glass beaker with gentle agitation by placing it on a hotplate magnetic stirrer at 100 rpm with temperature adjusted to 37 °C ± 0.5 °C [47]. The time required for the disappearance of the SNEDDS was recorded [50]. The efficiency of self-emulsification of SNEDDS was visually assessed using the five grading system [30,51].

<table>
<thead>
<tr>
<th>Grade Inference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Passed</td>
</tr>
<tr>
<td>B</td>
<td>Failed</td>
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<tr>
<td>C</td>
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</tr>
<tr>
<td>D</td>
<td>Failed</td>
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<tr>
<td>E</td>
<td>Failed</td>
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</table>

Formulations those passed this test in grade A or B were selected for further optimization.

2.2.5.3. Robustness to dilution. Robustness of selected NYS-SNEDDS formulations to dilution was studied by diluting it 50, 100 and 1000 times with various diluents i.e. double distilled water, 0.1 N HCl and phosphate buffer (pH 6.8). The diluted samples were stored for 24 h and observed for any signs of physical changes i.e. phase separation or drug precipitation [33,45].

2.2.6. Characterization of formulations

Based on optimization results, five NYS-SNEDDS formulations were selected to perform characterization and further investigations. NYS-SNEDDS formulations were characterized for their cloud point, electrical conductivity, optical clarity, droplet size, polydispersity index, zeta potential and morphology.

2.2.6.1. Cloud point (Tcloud) determination. The effect of temperature on the phase behavior of NYS-SNEDDS was evaluated through measurement of the cloud point value (Tcloud) [45,49]. Each optimized

Table 1 Composition, thermodynamic stability and dispersibility studies of prepared NYS-SNEDDS formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Oil (%)</th>
<th>Surfactant (%)</th>
<th>Co-surfactant (%)</th>
<th>Smix (%)</th>
<th>Water (%)</th>
<th>Oil/Smix</th>
<th>Centrifugation study</th>
<th>Heating and cooling cycle</th>
<th>Freeze–thaw cycle</th>
<th>Dispersibility study</th>
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<td>F1</td>
<td>9</td>
<td>54</td>
<td>–</td>
<td>–</td>
<td>27</td>
<td>2:1</td>
<td>81</td>
<td>10</td>
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<td>Passed</td>
<td>A</td>
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</tr>
<tr>
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<td>–</td>
<td>–</td>
<td>20.25</td>
<td>3:1</td>
<td>81</td>
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<td>Passed</td>
<td>Passed</td>
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<td>Passed</td>
</tr>
<tr>
<td>F3</td>
<td>9</td>
<td>40.5</td>
<td>–</td>
<td>–</td>
<td>40.5</td>
<td>1:1</td>
<td>81</td>
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<td>Passed</td>
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<tr>
<td>F4</td>
<td>9</td>
<td>54</td>
<td>–</td>
<td>–</td>
<td>27</td>
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<td>10</td>
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<td>Passed</td>
<td>A</td>
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<tr>
<td>F5</td>
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<td>–</td>
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<td>–</td>
<td>24</td>
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<td>–</td>
<td>40.5</td>
<td>1:1</td>
<td>81</td>
<td>10</td>
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<td>Passed</td>
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<td>54</td>
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<td>27</td>
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<td>10</td>
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<tr>
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<td>F13</td>
<td>18</td>
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<td>36</td>
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<td>72</td>
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<td>2:8</td>
<td>Passed</td>
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</table>
formulation was diluted with double distilled water in the ratio of 1:100 and placed in a water bath with gradual increase in temperature (5 °C increments). Each sample was equilibrated at the specified temperature for at least 2 min. At the end of each measurement the samples were visually assessed for optical transparency, signs of phase separation or drug precipitation [52].

2.2.6.2. Electrical conductivity measurement. The electrical conductivity of selected NYS-SNEDDS, before and after 50-fold dilution with double distilled water, was determined at 25 °C using advanced electrochemistry multi-parameter meter (Thermo Fisher Scientific Inc., Orion VSTAR 92, USA).

2.2.6.3. Spectroscopic characterization of optical clarity. Nanoemulsion is defined as “a system of water, oil, and surfactant (or amphiphile) which is a single optically isotropic and thermodynamically stable solution” [31]. Therefore, to meet the isotropic parameter, optical clarity investigation is important. The optical clarity of the aqueous dispersions of the selected NYS-SNEDDS formulations was measured spectrophotometrically upon dilution [43,53]. Briefly, 1 ml of each SNEDDS was diluted with 50 ml double distilled water. The absorbance of each solution was measured at 638 nm using double distilled water as a blank.

2.2.6.4. Droplet size analysis. The droplet size of the selected formulations was determined by dynamic light scattering (DLS) using Zeta-Sizer (Malvern, Nano Series ZS90, Malvern Instruments, Ltd., UK). A He–Ne laser beam at 632 nm wavelength was used and light scattering was monitored at 25 °C at a fixed angle of 90°. The analytical sensitivity range of the analyzer is 1 nm to 6 μm. The selected formulations were diluted at 1:50 (v/v) employing double distilled water and mixed for 1 min using a magnetic stirrer to assure the homogeneity. After complete dispersion, aliquots were transferred to a cuvette and loaded into the apparatus for measurement. The polydispersity index (PDI) reflects the uniformity of particle diameter and it can be used to depict the size distribution of nanoemulsion. All measurements were done in triplicate from three independent samples.

2.2.6.5. Zeta potential measurement. The nanoemulsion stability is directly related to the magnitude of the surface charge [33,54]. The zeta potential of the selected formulations was determined by laser diffraction analysis using Zeta-Sizer (Malvern, Nano Series ZS90, Malvern Instruments, Ltd., UK). The samples were diluted at a ratio of 1:50 (v/v) with double distilled water and mixed for 1 min using a magnetic stirrer to guarantee the homogeneity. After complete dispersion, aliquots were transferred to a folded capillary cell and loaded into the apparatus for investigation. All studies were repeated in triplicate at 25 °C.

2.2.6.6. Transmission electron microscopy (TEM). The morphology of the selected NYS-SNEDDS was observed by TEM (JEOL Co., JEM-2100, Tokyo, Japan), under a high tension electricity of 160 kV. After sample dilution with double distilled water (1:50), a sample drop was placed on a carbon coated copper grid and air-dried for 10 min at room temperature. The excess was instantly drawn off with a filter paper. Samples were subsequently negatively stained with 1% (w/v) phosphotungstic solution for 60 s and excess of solution was also removed, before loading in the microscope. Subsequently, the shape and surface characteristics were evaluated at appropriate magnifications.

2.2.7. In vitro drug release study

The release profile study of selected NYS-SNEDDS formulations was performed using the dialysis bag method [45,55] with some modifications. In all release experiments the dialysis bag (Dialysis tubing cellulose membrane, Sigma Co., USA; Molecular weight cutoff 12,000–14,000) was used. One g of NYS-SNEDDS containing 2 mg NYS were instilled into the dialysis bag, firmly sealed with dialysis clamp and placed in a 100 ml screw-capped glass container filled with methanol/DMF/water (55:15:30, v/v/v) [8,11,19,25,27] as the release medium. DMF had been utilized by others authors in dialysis membranes [56]. This release medium allows maintaining sink conditions in the whole experiment. In this way, the released NYS would be soluble in the release medium in contrast to other previous investigations [57,58], in which water was utilized as release medium, wherein NYS is practically insoluble. The experiment was conducted in a thermo-stated shaking water bath (Memmert, SV 1422, Germany) equipped at 37 °C ± 0.5 °C and 100 rpm. Samples were drawn at predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h) and replenished with the same volume of fresh release medium. The samples were filtered through 0.45 μm pore-sized nylon syringe filter and NYS concentrations were determined spectrophotometrically at 304 nm using the regression equation of a standard curve developed in the same medium. The release profile of NYS from SNEDDS formulations was compared to plain NYS suspended in phosphate buffer, containing the same quantity of drug. The cumulative release percentages were calculated as the ratio of the amount of drug released to the initial amount of drug in the dialysis bag. All measurements were performed in triplicate from three independent samples.

2.2.8. Stability study

Long-term stability was assessed by keeping the optimized NYS-SNEDDS formulations into sealed amber glass vials at ambient room temperature (20–25 °C) and refrigeration temperature (4–8 °C) for 6 months. The physical stability of the optimized formulations was evaluated by monitoring the time-dependent change in the physical characteristics (e.g., drug precipitation, phase separation and clarity) in addition to the drug content [31,38]. Samples were withdrawn at specified time intervals of 15, 30, 60, 90, 120 and 180 days and the drug content analysis was carried out spectrophotometrically at 304 nm after appropriate dilutions using methanol. All measurements were repeated for three independent samples.

2.2.9. In vitro antifungal activity

2.2.9.1. Organism. C. albicans ATCC 60193 was used for the induction of oral candidiasis in mice. C. albicans was cultured, from a stored stock at −80 °C, on Sabouraud dextrose (SD) agar plate and incubated at 37 °C for 24 h. Then, yeast cells were harvested into 6 ml SD broth and left overnight in a shaker incubator at 37 °C. The overnight broth was centrifuged and the pellet was diluted using sterile saline to optical density (OD) = 1.6 which corresponds to a cell count of 3 × 10⁸ colony forming units (CFU)/ml. The cell count was verified by viable count of serial 10-folds dilutions spotted on SD agar plate incubated at 37 °C for 48 h.

2.2.9.2. Determination of minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism [19,25]. The MICs of the 5 optimized formulations (F1–F5) compared to marketed NYS suspension, containing the same quantity of drug, as well as plain NYS suspended in phosphate buffer, were assessed against C. albicans using 2-fold serial dilution method [59]. A disk impregnated with 10 μl of each dilution was applied on the surface of SD agar media inoculated by an overnight culture of C. albicans. The plates were incubated at 25 °C for 24 h. The lowest concentration that gave considerable inhibitory zone was recorded.

2.2.9.3. Time-kill test. The time needed for NYS-SNEDDS formulations, to achieve their lethal effect against C. albicans, compared to marketed NYS suspension, containing the same quantity of drug, as well as plain NYS suspended in phosphate buffer, was calculated according to Clancy et al. [60] with some modifications. Concentrations of 236 and 512 μg/ml of each NYS-SNEDDS, suspensions of marketed NYS and plain NYS were inoculated by 5% v/v of an overnight culture of C. albicans adjusted to an OD of 1.6 and incubated at room temperature in glass test tubes. A
volume of 100 μl of each test tube was collected after contact time of 0, 5, 10, 15 and 20 min. The antifungal effect of the formulations and NYS suspensions was neutralized by dilution into Dey–Engley neutralizing media. A volume of 10 μl of the previous mixture was spotted on SD agar plates and incubated at upright position at 25 °C for 24 h. The least contact time that showed a total absence of any sign of fungal growth for each formula was recorded.

2.2.10. In vivo antifungal activity

2.2.10.1. Animals. All the experiments involving animals were conducted in accordance with the institutional ethical and regulatory guidelines and were approved prior by the Institutional Animal Ethics Committee (Medical Research Ethics Committee (MREC) of the National Research Centre, Cairo, Egypt). Fifty male albino Swiss mice weighing 20–25 g were employed in the present study. The mice were kept in polyethylene cages, fed with pelleted food and tap water ad libitum, and exposed to alternating 12 h of light and darkness. The care of the rats was in accordance with the institutional guidelines.

2.2.10.2. Experimental oral candidiasis in mice. Based on the in vitro release study, long-term stability study and in vitro antifungal activity experiments, two NYS-SNEDDS formulations (F1 and F2) were chosen to investigate their in vivo antifungal activity. The study of experimental oral candidiasis in mice was performed according to the method described in previous reports [61,62] with some modifications. All mice were immunosuppressed with three subcutaneous injections of prednisolone (Depo-Medrol®, EIPICO Pharmaceutical Co., Egypt, under license of Pfizer Co., USA) at a dose of 100 mg/kg of body weight at days −1, +1 and +3 of C. albicans infection. Tetracycline hydrochloride (0.1%) (Tetracid®, CID Pharmaceutical Co., Egypt) was given in the drinking water beginning 1 day before the infection and maintained for the viable count of the yeast cells. The end of the cotton swab was using a cotton swab at days 1, 3, 5 and 7 post infection, and analyzed by inoculating the whole oral cavity including the buccal mucosa, rinsed gently with normal saline and processed separately for microscopic examination. Briefly, the tongues were fixed in 20% formalin solution followed by embedding in paraffin. Six-μm-thick sections were taken from paraffin blocks and stained with hematoxylin and eosin (HE) for histopathological examinations.

2.2.11. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using (SPSS® statistics for windows, version 17.0). The significance of differences between the mean values of the in vivo evaluation was determined by one-way analysis of variance (ANOVA) followed by Fisher LSD’s post-hoc test, and a P-value <0.05 was considered statistically significant.

3. Results and discussion

Poor water solubility and consequent restricted absorption is a major limitation with many drugs despite their good efficacy. SNEDDS provides an opportunity for the improvement of the in vitro and in vivo performance of poorly water soluble drugs and thus serves as an ideal carrier for the delivery of drugs belonging to Biopharmaceutics Classification System (BCS) classes II and IV [54]. The current study was performed to define the role of self-nanoemulsifying formulations to enhance the solubility and bioavailability of the antifungal drug, nystatin. SNEDDS represents a possible alternative to the more traditional oral suspensions for lipophilic compounds.

3.1. Solubility study

Solubility of drug in excipients plays an important role in determining stability of formulation, as many formulations undergo precipitation before undergoing in situ solubilization [31]. In addition, for a successful formulation of NYS loaded SNEDDS, the entire dose of NYS should be soluble in SNEDDS ingredients. The formulations of self-emulsifying property should be carried out following combination of oils, surfactants, and co-surfactants. This mixture should be clear, isotropic, monophasic liquid at room temperature [63] and should have good solubilizing capacity to incorporate dose of drug in minimum volume of the mixture [64]. The solubility of NYS in various oils, surfactants and co-surfactants is presented in (Table 2). Among various vehicles screened, oleic acid was selected as the oil phase showing the highest solubilization capacity (2.111 ± 0.042 mg/g). Tw40 (1.792 ± 0.053 mg/g), Tw40 (1.987 ± 0.039 mg/g), Tw60 (1.425 ± 0.028 mg/g) and Tw80 (1.369 ± 0.019 mg/g) were used as surfactants possessing the presence of C. albicans. Oral infection was performed by means of a cotton swab rolled twice over all parts of the mouth. The animals were then after randomly divided into five groups (n = 10 per group): Group I was treated with marketed NYS suspension, group II with plain NYS suspended in phosphate buffer, group III with F1, group IV with F2 and group V with phosphate buffer, as a control. All treatment groups were employed in the present study. The mice were kept in polyethylene cages, fed with pelleted food and tap water ad libitum, and exposed to alternating 12 h of light and darkness. The care of the rats was in accordance with the institutional guidelines.

Microbiological evaluation of progression of the infection was carried out as follows: the whole oral cavity including the buccal mucosa, tongue, soft palate and other oral mucosal surfaces was swabbed using a cotton swab at days 1, 3, 5 and 7 post infection, and analyzed for the viable count of the yeast cells. The end of the cotton swab was then cut off and placed in a tube containing 1 ml sterile saline. After mixing on a vortex mixer, to release Candida cells from the swab into the saline, serial 10-fold dilutions of the cell suspension were spotted on SD agar plates and incubated at 37 °C for 48 h. The CFU of Candida colonies were counted. All results were expressed as log10 CFU/ml. Mice were monitored once daily to evaluate their survival during the 7 days treatment period.

2.2.10.3. Histopathological findings. At the end of the experiment (24 h after the last administration of treatments), 3 mice from each group were sacrificed by cervical dislocation. The tongues were removed by incision at the base, rinsed gently with normal saline and processed separately for microscopic examination. Briefly, the tongues were fixed in 20% formalin solution followed by embedding in paraffin. Six-μm-thick sections were taken from paraffin blocks and stained with hematoxylin and eosin (HE) for histopathological examinations.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Solubility of NYS (mg/g) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oils</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>2.111 ± 0.042</td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>0.063 ± 0.003</td>
</tr>
<tr>
<td>Isopropyl palmitate</td>
<td>0.053 ± 0.002</td>
</tr>
<tr>
<td>Methyl laurate</td>
<td>0.075 ± 0.005</td>
</tr>
<tr>
<td>Miglyol</td>
<td>0.194 ± 0.009</td>
</tr>
<tr>
<td>Surfactants</td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td>1.792 ± 0.053</td>
</tr>
<tr>
<td>Tween 40</td>
<td>1.987 ± 0.039</td>
</tr>
<tr>
<td>Tween 60</td>
<td>1.425 ± 0.028</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1.369 ± 0.019</td>
</tr>
<tr>
<td>Dicetin</td>
<td>0.797 ± 0.014</td>
</tr>
<tr>
<td>Captex 200</td>
<td>0.135 ± 0.008</td>
</tr>
<tr>
<td>Span 80</td>
<td>1.227 ± 0.022</td>
</tr>
<tr>
<td>Co-surfactants</td>
<td></td>
</tr>
<tr>
<td>Polyethylene glycol 400</td>
<td>3.625 ± 0.067</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>4.097 ± 0.096</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.956 ± 0.025</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>&gt;50 mg</td>
</tr>
</tbody>
</table>
the highest solubilities. Additionally, DMSO (≥ 50 mg/g) and PG (4.69 ± mg/g) were chosen as the co-surfactants due to their efficient and moderate solubilizing effect, respectively. Oleic acid was reported for its enhanced intestinal absorption of drugs [64,65]. Tweens are non-ionic surfactants providing formulation benefits in a number of pharmaceutical applications having long standing food and pharmacopeia approval [66]. DMSO and PG are safe co-surfactants previously reported as SNEDDS excipients for oral administration [30].

3.2. Preliminary screening of surfactants for their emulsification ability

Emulsification process and efficiency are controlled by multiple variables including lipid–surfactant affinity, hydrophilic–lipophilic balance (HLB) value of surfactant and viscoelasticity of the emulsion base [67]. HLB value of surfactant affects the spontaneity of emulsification and emulsion droplet size. Surfactants with HLB > 10 are suitable for formation of o/w nanoemulsions [68] and hence all screened surfactants have HLB > 10 (Table 3). Non-ionic surfactants are often considered for pharmaceutical applications and nanoemulsion formulations since these are typically have lower critical micelle concentration compared to their ionic counterparts [67,69]. They are usually accepted for oral ingestion [70]. The selected surfactants were compared for their emulsification efficiencies using oleic acid as the oily phases. It has been reported that well formulated SNEDDS is dispersed within seconds under gentle stirring conditions [70]. Transmittance values as well as number of flask inversions of different mixtures are demonstrated in (Table 3). The results inferred that highest % transmittance, i.e., highest emulsification efficiency, is acquired by Tw20 (HLB 16.7) followed by Tw40 (HLB 15.6) then Tw80 (HLB 15.0) and Tw60 (HLB 14.9) in the same order. The observed difference in their emulsifying ability could be attributed to the HLB values of the examined surfactants, where Tw20 possessed the highest value and Tw60 the lowest, in addition to the difference in their structure and chain length [44,46]. Higher HLB surfactants lead to the formation of more stable nanoemulsion upon exposure to water [68]. Tw20 and TW40 showed highest solubility as well as emulsification efficiency (yielded clear nanoemulsions requiring shorter time for nanoemulsification). Thus, both were selected for further investigations.

3.3. Construction of pseudo-ternary phase diagrams

Ternary phase diagrams were constructed in the absence of NYS to identify the self-nanoemulsifying region and to select a suitable concentration of oil, surfactant and co-surfactant for the development of liquid SNEDDS formulations. These phase diagram plays important role in studying phase behavior of the formed nanoemulsions [31,33]. A simple ternary phase diagram comprises oil, water, and Smix, where each corner in the phase diagram represents 100% of that particular component. Based on the data obtained from the solubility study and preliminary screening of surfactants, oleic acid was used as the oil phase, Tw20 and Tw40 as surfactants and PG and DMSO as co-surfactants for constructing different phase diagrams. Fig. 1 shows the constructed phase diagrams, where the translucent and low viscosity nanoemulsion area is presented as shaded area in the phase diagrams. It was clearly shown that the use of DMSO as co-surfactant at Smix ratio (1:1) with Tw20 could not form clear isotropic nanoemulsion region while at Smix ratio (2:1) and (3:1) could form nanoemulsion but with not more than 20% oil. Employing Tw40 could not form any nanoemulsion region with DMSO as co-surfactant. The use of PG as co-surfactant gave clear isotropic regions employing not more than 30% of oil for all constructed phase diagrams except for Tw20/PG (3:1) system which could emulsify up to 40% of oil. Tw20 showed larger self-emulsification region compared to Tw40, due its higher HLB and hence more hydrophilic nature. Higher HLB value is required for forming a good o/w emulsion [68,71] as higher hydrophilicity property favors faster emulsification of the oil–surfactant mixture in contact with water [47,72]. Self-nanoemulsification is spontaneous and the resulting dispersion is thermodynamically stable [38,73]. Free energy of nanoemulsion formation depends on the extent to which the surfactant lowers the surface tension of the oil–water interface and the change in dispersion entropy [69], and the present results demonstrated that increasing surfactant proportion (Smix 1:1, 2:1 and 3:1) led to a more favorable formation of nanoemulsion. The surfactant forms a layer around oil globule in such a way that polar head lies toward aqueous and non-polar tail pull out oil and thereby reduces surface tension between oil phase and aqueous phase [73,74]. Further, increasing the concentration of surfactant increased the spontaneity of the self-emulsification process [47]. The increase in co-surfactant decreases the region of emulsion formation, as co-surfactants have very little effect on reducing the interfacial tension directly rather they help the surfactants to reduce the interfacial tension [68]. Results obtained indicated that apart from HLB value and type of surfactant, other factors such as structure and relative length of hydrophobic chains of co-surfactant had influence on nano-emulsification and therefore nanoemulsion area [31]. Moreover, since the drug is co-surfactant more soluble (especially DMSO), co-surfactant concentration is an important factor to solubilize required amount of drug dose.

3.4. Optimization of formulations

3.4.1. Thermodynamic stability studies

The main difference between emulsions and nanoemulsions is kinetic stability, reflecting the thermodynamic stability of the two systems. SNEDDS undergoes in situ solubilization to form nanoemulsion system, and it should have stability such that it does not undergo precipitation, creaming or cracking. However, in many cases, prolonged storage might cause the drug to precipitate from the nanoemulsion; seed crystals start to appear and might grow to large crystalline materials that will precipitate out at the bottom of the vessel [31]. Therefore to check the stability, formulation was exposed to centrifugation study, heating and cooling cycle and freeze thawing cycle in order to eliminate the metastable ones. Fourteen formulations passed the test (Table 1) i.e. there was no sign of phase separation, turbidity or drug precipitation observed. One formulation (F9), containing oleic acid in a concentration more than 25%, were unstable and showed phase separation as well as turbidity, which may be due to the coagulation of the internal phase which led to phase separation [43,53]. The stable formulations were further tested for dispersibility.

3.4.2. Dispersibility study

As SNEDDSs are released in the lumen of the gastrointestinal tract, it disperses to form a fine nanoemulsion with the aid of GI fluid. Thus, it is important that formed nanoemulsion does not undergo precipitation following phase separation with infinite dilution in the GI fluids. It is

Table 3

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Chemical name</th>
<th>HLB value</th>
<th>No. of inversions</th>
<th>% transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween® 20</td>
<td>Polyoxyethylene sorbitan monolaurate</td>
<td>16.7</td>
<td>3</td>
<td>58.479</td>
</tr>
<tr>
<td>Tween® 40</td>
<td>Polyoxyethylene sorbitan monopalmitate</td>
<td>15.6</td>
<td>4</td>
<td>42.853</td>
</tr>
<tr>
<td>Tween® 60</td>
<td>Polyoxyethylene sorbitan monostearate</td>
<td>14.9</td>
<td>4</td>
<td>37.670</td>
</tr>
<tr>
<td>Tween® 80</td>
<td>Polyoxyethylene sorbitan monoooleate</td>
<td>15.0</td>
<td>5</td>
<td>41.115</td>
</tr>
</tbody>
</table>
observed more prominently with drugs having poor aqueous solubility or nanoemulsion which undergoes phase transition [31]. The SNEDDS should disperse completely and quickly when subjected to dilution under mild agitation of GIT due to peristaltic activity [31,33]. It has been reported that self-emulsification mechanism involves the erosion of a fine cloud of small droplets from the monolayer around emulsion droplets, rather than progressive reduction in droplet size [31,75]. The ease of emulsification was suggested to be related to the ease of water penetration into the colloidal or gel phases formed on the surface of the droplet [76].

Accordingly, dispersibility study in double distilled water was conducted. In the present study, double distilled water was used as a diluent for self-nanoemulsification test because it is well known that there is no significant difference in the SNEDDS prepared using pharmaceutically acceptable surfactants, dispersed in either water or GI fluids [77,78]. All SNEDDSs that passed this test either in grade A or B were selected for further investigation, because grade A or B formulations will remain as SNEDDS when dispersed in GI fluids [48] (Table 1). It was clearly revealed that only SNEDDSs prepared using Tw20 as the surfactant at ratio oil/Smix (9:1) i.e. F1–F5, could pass the test. This might be explained by the higher HLB value (16.7) possessed by Tw20 and consequently higher hydrophilicity compared to Tw40 (15.6). Higher HLB value is required for forming a good o/w emulsion [68,71] since higher hydrophilicity property favors faster emulsification of the oil–surfactant mixture in contact with water [47,72], in addition to reduced free energy of the system [79]. It was also observed that the increase in oil concentration (F6–F8 and F13–15), required more time for emulsification. The reason that can be put forward is the increase in interfacial tension between larger volume of oil and aqueous phase with a net decrease in surfactant system, which makes the emulsification time longer [80]. Pouton found that the formulations containing more hydrophilic components was superior in self-nanoemulsifying ability and provided smaller droplets than those containing more lipophilic components [81]. Eventually, five formulations (F1–F5), which passed dispersibility study, were selected for further optimizations as these formulations were certain to form nanoemulsion upon dilution in the aqueous environment.

3.4.3. Robustness to dilution

Uniform emulsion formation from SNEDDS is very important at different dilutions because drugs may precipitate at higher dilution in vivo which affects the drug absorption significantly [32,33]. Optimized SNEDDS formulations were exposed to different folds of dilution in different media to mimic the in vivo conditions where the formulation would encounter gradual dilution. Hence, each formulation was subjected to 50, 100 and 1000 times dilution in double distilled water, 0.1 N HCl, and phosphate buffer (pH 6.8). Even after 24 h, all investigated formulations showed no signs of precipitation, cloudiness or separation which ensured the stability of the reconstituted emulsion. This reveals that all media were robust to dilution. These findings will ensure the prospect of uniform drug release profile in vivo.
3.5. Characterization of formulations

3.5.1. Cloud point (T_{cloud}) determination

Temperature dependent phase behavior is one of the major problems associated with nanoemulsions, especially when dealing with non-ionic surfactants [49,52]. An ideal formulation should remain as a one-phase transparent system at its storage temperature and at the temperature of its intended use. The cloud point (T_{cloud}) is the temperature above which the formulation clarity turns into cloudiness [45]. At higher temperatures, phase separation can occur due to the decrease in the solubility of the surfactant in water, the dehydration of the head group and phase separation [52]. Since both drug solubilization and the solubility of the surfactant in water, the dehydration of the head groups and phase separation [52]. Since both drug solubilization and

3.5.2. Electrical conductivity measurement

Table 4 shows the electrical conductivity results of the selected formulations, where the values ranged between (0.54–2.25 μS/cm) and (20.17–36.02 μS/cm) before and after 50-fold dilution using double distilled water, respectively. It was clearly depicted that before and after dilution, the higher the co-surfactant concentration the higher the conductivity value (Smix 3:1, 2:1 and 1:1). Such increase in the electrical conductivity may be attributed to greater interaction of water molecules with the polar co-solvents (DMSO and PG) that can highly incorporate into water [53]. In addition, increasing the amount of water upon dilution led to noticeable increase in the conductivity values. This finding agrees with a previous report [83]. Furthermore, conductivity values −1 μS/cm have been reported to be indicative of bicontinuous or solution type systems which possess ultra low interfacial tension [83]. These dynamic structures include water and oil pseudo-domains which rapidly exchange [84]. In the presence of these structures, the conductivity is comparable to that of electrolyte solutions and decreases with decreasing water content [83].

3.5.3. Spectroscopic characterization of optical clarity

Lower spectroscopic absorbance should be obtained with optically clear solutions because cloudier solutions will scatter more of the incident radiation, resulting in higher absorbance [79]. To assess the optical clarity quantitatively, UV–Visible spectrophotometer was used to measure the amount of light of a given wavelength (638 nm) transmitted by the solution. Absorbance values of optimized formulations F1–F5, upon dilution with double distilled water (1 × 50) varied between 0.034–0.189 (Table 4). As expected, the compositions with the lower absorbance values showed the smallest droplet size values (Table 4) because aqueous dispersions with small absorbance values are optically clear, and oil droplets are thought to be in a state of finer dispersion [46,53]. It was observed that the spectroscopic clarity (i.e. droplet size) was inversely proportional to the concentration of surfactant in the Smix (1:1, 2:1 and 3:1). Such a decrease in droplet size may be the result of more surfactants being available to stabilize the oil–water interface [79]. Furthermore, the decrease in the droplet size behavior reflects the formation of a better closed packed film of the surfactant at the oil–water interface, thereby stabilizing the oil droplets [68].

3.5.4. Droplet size analysis and polydispersity index

Droplet size is one of the most important characteristics of nanoemulsion for stability evaluation and a critical step in the pathway of enhancing drug bioavailability [33,38]. Smaller droplet size leads to larger interfacial surface area for drug absorption and hence may lead to more rapid absorption and improved bioavailability [33]. Hence, the droplet size of the nanoemulsion may govern the effective drug release [31,63,79]. The droplet sizes of optimized formulations are given in Table 4. The average droplet size of the five investigated formulations at 50 times dilution ranged from 26.45–85.94 nm, which indicated that emulsion droplets are in nanometric range (∼100 nm). The droplet size decreased as the concentration of surfactant in the Smix increased i.e. F2 < F1 and F5 < F4 < F3. This could be attributed to an increased surfactant proportion relative to co-surfactant which is probably explained by stabilization of the oil droplets as a result of the localization of the surfactant molecules at the oil–water interface [31,74]. The surfactant may cause the interfacial film to condense and stabilize, resulting in smaller droplet diameters, whereas the addition of the co-surfactant may cause the film to expand [43,53]; thus, the relative proportion of surfactant to co-surfactant has varied effects on the droplet size [85]. These observations are in consistent with earlier author reports [43, 49,53]. Polydispersity index (PDI) of all formulations was less than 0.5, indicating uniform globule size distribution [33,48].

3.5.5. Zeta potential measurement

Emulsion droplet polarity is also a very important factor in characterizing emulsification efficiency [31,86]. Zeta potential is the potential difference between the surface of tightly bound layer (shear plane and electroneutral region of the solution). The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability i.e. the solution or dispersion will resist aggregation [31,33]. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized. In the present study, the zeta potential of the nanoemulsion was determined after 50-fold dilution of the selected NYS-SNEDDS with double distilled water (Table 4). The results show that the optimized formulations were negatively charged, with values ranging from −15.3 to −23.9 mV, indicating stable system and well separated emulsion globules [49]. The electrostatic repulsion forces between the negatively charged droplets get an avoidance of the coalescence of the nanoemulsion [63]. Non-ionic surfactants produce a negatively charged interface at neutral pH because of differential adsorption of the hydroxyl ion (OH\(^{-}\)) and hydrated oxonium ion (H\(_{3}\)O\(^{+}\)) [87]. This might explain the negative zeta-potential values of all the SNNEDS formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cloud point (°C)</th>
<th>Conductivity (μS/cm)</th>
<th>Spectroscopic absorbance</th>
<th>Droplet size (nm) ± S.D.</th>
<th>PDI</th>
<th>Zeta potential (mV) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>90</td>
<td>2.25</td>
<td>26.45 ± 0.25</td>
<td>0.008</td>
<td>19.5 ± 4.20</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>90</td>
<td>1.2</td>
<td>31.59 ± 0.91</td>
<td>0.854</td>
<td>19.8 ± 4.08</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>90</td>
<td>0.91</td>
<td>31.59 ± 0.91</td>
<td>0.854</td>
<td>19.8 ± 4.08</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>90</td>
<td>0.73</td>
<td>28.68 ± 0.87</td>
<td>0.154</td>
<td>19.8 ± 4.08</td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>90</td>
<td>0.54</td>
<td>20.17 ± 0.79</td>
<td>0.079</td>
<td>19.8 ± 4.08</td>
<td></td>
</tr>
</tbody>
</table>
3.5.6. Transmission electron microscopy (TEM)

TEM was used to explore the structure and morphology of formed nanoemulsions of two selected NYS-SNEDDS formulations (F1 and F2), after dilution using double distilled water. The TEM images are depicted in Fig. 2a and b. The images clearly indicate the spherical nature of nanoemulsions, with no signs of coalescence. The nanoemulsion droplets emerged as dark spheres, with smooth surface, and the surroundings were found to be bright. Furthermore, the droplets appeared distinct and non-aggregated with no signs of drug precipitation inferring the physical stability of formed nanoemulsions [31,63,79].

3.6. In vitro drug release study

When SNEDDS encounter aqueous media, the drug consequently exists in the system in different forms including a free molecular form, or mixed in micelles or entrapped in the nanoemulsion droplets. Under these circumstances, it is necessary to separate free drug molecules from those entrapped in the nanoemulsion droplets or micelles to assess the real release pattern [38,45,50]. Thereby, conventional release testing is not adequate for this system. Recently, dialysis bag method was recently adopted for SNEDDS in vitro release study [69,88]. In the designing of colloidal drug-carrier formulations, the study of the rate at which the drug is released from the vehicle is particularly important, because it can be used as quality control data to predict in vivo behavior, or to study the structure and release mechanism of the system [11]. Previous studies [8,11,19,25,27] demonstrated the ability of the mixture methanol/DMF/water (55:15:30, v/v/v) to maintain sink conditions in NYS release experiments and excellent compatibility with cellulose membranes. Fig. 3 shows NYS release profiles from their respective SNEDDS formulations at 37 °C for 48 h. The results show that NYS-SNEDDS prepared using DMSO as co-solvent (F1–F2) possessed higher release compared to those prepared employing PG as the co-surfactant (F3–F5). In addition, considering F1 and F2, it was observed that the higher the co-surfactant (DMSO) concentration in the formulation (Smix 2:1 and 1:1), the higher the release rate. This might be explained by the higher solubilization of NYS in DMSO compared to PG, which contributes in higher availability of NYS in the release media. On the contrary, in case of F3–F5, it was found that the higher the co-surfactant (PG) concentration in the formulation (Smix 3:1, 2:1 and 1:1) the lower the release rate. This could be clarified by the moderate solubilization efficiency of PG compared to DMSO, where another factor may governs the drug release; the droplet size [63]. As the droplets size of the nanoemulsion decreases, a larger interfacial surface area is provided [34]. Therefore, the drug release from small droplets will be faster and more frequent than from the large oil droplets [89]. Fig. 3 also demonstrates comparison of drug release from SNEDDS formulations to plain NYS suspended in phosphate buffer, containing the same quantity of drug. Release patterns reveal that NYS release was significantly higher from SNEDDS formulations compared to plain drug suspension. Drug release from suspension did not exceed 47% after 48 h, whereas, the drug was 98% released from F1 after 48 h. These results contend the role of SNEDDS formulation to improve NYS solubilization and in vitro release. The faster drug release from SNEDDS is attributed to the spontaneous formation of nanoemulsion due to low surface free energy at oil–water interface, which causes immediate solubilization of drug in the release medium [47]. During emulsification with water, the nanoemulsion components (oil, surfactant and co-surfactant) effectively swell and decreases the globule size, which leads to increase in surface area and decrease of surface free energy, thus eventually
increases the drug release rate [47]. Thus, it can be concluded that the formulated SNEDDS improved the solubilization and release rate of NYS.

### 3.7. Stability study

All investigated NYS-SNEDDSs were physically stable with no obvious change in visual appearance i.e. no precipitation, phase separation or color change when being stored at ambient room temperature (20–25 °C) and refrigeration temperature (4–8 °C) for 6 months. The initial concentrations (time 0) of NYS were referenced as 100% and all subsequent concentrations were expressed as percentages of the initial concentration. The stability of NYS was assessed by evaluating the percent change in NYS concentrations from time 0. A decrease of >10% of the initial concentration was considered to represent a significant loss of drug [90,91]. At ambient room temperature (with light protection) the concentrations of NYS decreased below 90% of the initial value after 15 days for all investigated formulations. On the other hand, at refrigeration temperature, the concentrations of NYS decreased below 70% of the initial value after 15 days for all investigated formulations. On the other hand, at refrigeration temperature, the concentrations of NYS decreased below 90% of the initial value after 15 days (Table 5). These findings are in accordance with previous reports [92–94], where NYS was found to be more stable at refrigeration temperature (up to 15 days) in contrast to ambient room temperature (up to 4 days). It was clearly shown, that the formulations prepared using DMSO (F1 and F2) as the co-surfactant, revealed better stability profiles compared to their counterparts (F3–F5) prepared employing PG as the co-surfactant. This might be explained by the higher solubilization power of DMSO toward NYS in contrast to PG. Also, it was obviously depicted that increasing amounts of Tw20 in the Smix ratio (1:1, 2:1 and 3:1) has led to better NYS stability. As reported in the droplet size analysis (Section 3.5.4), the nanomulsion droplet size was influenced by surfactant concentration [67]. Increase in surfactant concentration produced smaller emulsion droplet size. Increased availability of the surfactant to adsorb around oil–water interface may decrease emulsion droplet size owing to decreased interfacial tension of the system [39]. Moreover, decrease in droplet size indicates close packed arrangement of surfactant at the oil–water interface and improved stabilizing effect on oil droplets by forming a strong mechanical barrier to coalescence, which consequently led to higher entrapped NYS stability [68].

NYS is practically insoluble in water and is easily degraded by light, heat, oxygen, moisture, humidity and extreme pH values [93,94]. NYS should be stored in airtight containers at a temperature of between 2 and 8 °C and protected from light [92], and at 25 °C maximum after opening for 7 days [16]. Some ready-to-use commercial NYS suspensions (100,000 IU/ml) containing preservatives have expiration dates of many weeks [15,94]. On the contrary, extemporaneously prepared oral suspensions of NYS powder should be used immediately after mixing and should not be stored when this product contains no preservatives [15,94].

The explanation of the observed rate of degradation would require detailed kinetic studies, which were beyond the scope of this investigation. The reaction mechanisms involved may be very complicated, as there are six double bonds in the NYS molecule [93]. In conclusion, the previous findings postulate the necessity for a detailed stability study, to obtain the maximum benefit of drug delivery. The addition of preservatives and stabilizing agents to NYS-SNEDDSs is noticeably needed, in light of the obtained data.

### 3.8. In vitro antifungal efficacy

#### 3.8.1. Determination of minimum inhibitory concentration (MIC)

The 2-fold serial dilution method was used to assess the in vitro antifungal activity of NYS-SNEDDSs and NYS suspensions against C. albicans. SNEDDS excipients showed no inhibitory effect on the yeast grown, proving that the excipients did not interfere the test. By assessing the MIC of the 5 selected NYS-SNEDDSs, only 2 formulations, namely F1 and F2, showed a comparable MIC (256 μg/ml) to that of the marketed NYS suspension. All other formulations (F3–F5), in addition to plain NYS suspension, showed higher MIC values. F1 and F2 contain DMSO as co-surfactant in contrast to F3–F5 containing PG as the co-surfactant.

#### Fig. 4. Microbiological study of therapeutic efficacy of selected NYS-SNEDDS formulations and NYS suspensions against oral candidiasis in mice. Data presented as mean ± S.D. (n = 10). Same letters in the same time interval are statistically non-significant different and different letters in the same time interval are statistically significant different at P < 0.05.
3.8.2. Time-kill test

Marketed NYS suspension showed the shortest time-kill values (less than 10 min at 256 μg/μl and less than 5 min at 512 μg/μl) followed by F1 and F2 (less than 20 min at 256 μg/μl and less than 15 min at 512 μg/μl). All other investigated formulations as well as plain NYS suspensions revealed more than 20 min time-kill values. Marketed NYS suspension exhibited a much quicker effect, probably due to the direct contact with the yeast on the culture medium [8].

3.9. In vivo antifungal efficacy

The present study assesses the antifungal activity of selected NYS-SNEDDS formulations administered per-oraly against oral candidiasis with an immunosuppressed-mouse model, compared to marketed NYS suspension, containing the same quantity of drug, as well as plain NYS suspended in phosphate buffer. In this model, the severity of Candida infection was estimated both by measuring the number of viable Candida cells in the oral cavity and by manifestation of histopathological findings on the tongue.

3.9.1. Microbiological evaluation of the fungal burden

At day 1 after oral inoculation of C. albicans (just before beginning of treatment) sample swabs were taken from mice mouths and cultured to confirm the presence of C. albicans and to quantify the number of CFU in the oral cavity. The results demonstrated in Fig. 4 confirmed the yeast presence with a non-significant difference (P > 0.05) between all treatment groups. At day 3; F1, F2 and marketed NYS suspension

![Microscopic observations of the tongues of mice with oral candidiasis on (day 8) after infection stained with HE with scale bar 20 μm. (A) Section of control mice tongue showing degeneration of the filiform papillae and the keratinized spine (asterisk); hemorrhagic area (arrow) and inflammatory infiltration (arrowhead) in lamina propria. (B) Section of control mice tongue showing large hemorrhagic areas (arrows) in the lamina propria. (C) Section of mice tongue treated with plain NYS suspension showing dislocation of the filiform papillae (arrow). (D) Section of mice tongue treated with marketed NYS suspension showing the filiform papillae (arrow); lamina propria (arrowhead); and transverse muscular fibers (asterisk). (E) Section of mice tongue treated with F1 showing slight disturbance of the filiform papillae and overlapping of the keratinized spine (arrow). (F) Section of mice tongue treated with F2 showing the filiform papillae (arrow); lamina propria (arrowhead) and transverse muscular fibers (asterisk).](image-url)
significantly (P < 0.05) reduced the oral burden of C. albicans compared to the plain NYS suspension as well as the control group (Fig. 4). Indeed, F1 and F2 showed a significant reduction (P < 0.05) in oral C. albicans burden, in contrast to marketed NYS suspension, at day 5 of treatment. Afterwards, both treatments (F1 and F2) were statistically superior to all other treatment groups. Of particular interest, the NYS-SNEDDS formulations were significantly more efficacious than the marketed NYS suspension in reducing the oral burden of C. albicans on treatment days, 5 and 7 (Fig. 4). Mice were assessed for survival during the treatment period, where no mortality was recorded.

Both F1 and F2 showed the highest release rate, long-term stability and in vitro antifungal efficacy compared to F3–F5. As discussed previously, the presence of DMSO as co-surfactant in F1 and F2, led to higher solubilization and hence better physico-chemical properties was reported. In addition, formed nanoemulsions possessed small droplet size (~100 nm), which is one of the most important characteristics of emulsion for stability evaluation and a critical step in the pathway of enhancing drug bioavailability [33,38]. Smaller droplet size leads to larger interfacial area for drug absorption and hence may lead to more rapid absorption and improve bioavailability. Furthermore, these particulate delivery systems are reported to prolong residence time on mucosal membranes [41] and they could reach greater mucosal surface areas, resulting in a comparatively higher drug uptake [40,42].

3.9.2. Histopathological findings

Untreated (infected) control mice as well as mice treated with plain NYS suspension consistently showed significant histopathologic changes of the stratified squamous epithelium of the entire dorsal area of the tongue such as abundant inflammatory cell infiltrates, hemorrhagic areas, degeneration of the filiform papillae, intercellular edema, and detachment of the cell layers, leading to disorganization of tissue structure (Fig. 5a–c). These histopathologic changes may be directly related to the lesions induced by Candida colonization in the dorsal epithelium [95]. In contrast, in mice treated with either marketed NYS suspension or the two selected NYS-SNEDDS formulations, the histopathologic changes in the epithelium were sharply reduced, and, in some areas, changes are entirely absent (Fig. 5d, e). It was not possible to distinguish marketed NYS suspension from the NYS-SNEDDS formulations due to the qualitative nature of histopathology investigation.

4. Conclusions

In the present study, self-nanoemulsiﬁying drug delivery systems of nystatin were prepared, and in vitro and in vivo evaluated. Following optimization, ﬁve nystatin SNEDDS formulations, composed of oleic acid as the oily phase, Tween 20 as surfactant, dimethyl sulfoxide or propylene glycol as co-surfactants, were selected. The formulations were robust to dilution in different media using different dilution folds, exhibiting no signs of precipitation or separation. The prepared SNEDDS showed cloud point of 90 °C and their electrical conductivity increased as the water content increased. The globule size was found to be in the nanometric range (~100 nm) and the zeta potential ranged from −15.3 to −23.9 mV indicating good stability. The morphology investigation revealed spherical shaped globules using transmission electron microscopy. In vitro release proﬁles in methanol/DMF/water (55:15:30, v/v/v) depicted a gradual release pattern with 100% release after 48 h. Nystatin loaded SNEDDS formulations showed a signiﬁcant increase in release rate compared to the plain drug suspension under the same conditions. Long-term stability revealed that the formulations were physically stable with no obvious change in visual appearance but a remarkable decay of nystatin after 6 months storage at ambient room and refrigeration temperatures was reported. In vitro antifungal efﬁcacy depicted the superiority of F1 and F2 NYS-SNEDDSs containing oleic acid, Tw20/DMSO (2:1) and (3:1), respectively. In vivo antifungal efﬁcacy in immunosuppressed mice with oral candidiasis showed supremacy of F1 and F2 compared to marketed NYS suspension after a 7-day treatment period as well as improved histopathological ﬁndings. In conclusion, solubilization and nano-scaling by means of SNEDDS provided a promising approach for increasing the efﬁcacy of topically administered NYS in the treatment of OPC. This novel application of nano-emulsiﬁcation could lead to the development of improved formulations of other antimicrobial agents.

Declaration of interest

The authors report no declarations of interest.

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