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Development of synergistic antifungal in situ gel of miconazole nitrate loaded microemulsion as a novel approach to treat vaginal candidiasis

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Limited solubility is the main cause of the low local availability of anti-candidiasis drug, miconazole nitrate (MN). The study's objective was to develop and characterize microemulsion (ME) based temperature-triggered in situ gel of MN for intravaginal administration to enhance local availability and antifungal activity. The solubility of MN was initially studied in different oils, surfactants, and co-surfactants. Then, pseudo-ternary phase diagrams were constructed to select the best ratio of various components. The ME formulations were characterized by thermodynamic study, droplet size, polydispersity index (PDI), viscosity, and in-vitro antifungal mean inhibition zone (MIZ). Selected MEs were incorporated into different in situ gel bases using a combination of two thermosensitive polymers (poloxamer (PLX) 407 and 188), with 0.6% of hydroxypropyl methylcellulose (HPMC K4M) and gellan gum (GG) as mucoadhesive polymer. ME-based gels (MG) were investigated for gelation temperature, gelation time, viscosity, spreadability, mucoadhesive strength, in vitro release profile, and MIZ test. Furthermore, the optimum MG was assessed for in vivo animal irritation test and FESEM investigation. Tea tree oil, lavender oil, tween 80, and propylene glycol (PG) were chosen for ME preparation for the optimal formulation; formulation ME7 and ME10 were chosen. After incorporation of the selected formulation into a mixture of P407 and P188 (18:2% w/w) with 0.6% mucoadhesive polymer, the resultant MG formulation (MG1) revealed optimum gelation temperature (33 ± 0.01℃) and appropriate viscosity with enhanced sustained release (98%) and retention through sheep vaginal mucosa, MG1 exhibited a better MIZ compared to the 2% MN gel formulation and the marketed MN product, and no rabbit vagina irritation. In conclusion, the miconazole nitrate-loaded MG-based formula sustained the duration of action and better antifungal activity than the marketed miconazole nitrate formulation.

Keywords Miconazole nitrate, Microemulsion, Essential oil, Antifungal action, Solubility enhancement, In situ gel

Vaginal candidiasis (VVC) is considered one of the most troublesome genital tract infections caused by *Candida albicans* where up to 75% of women could be infected no less than once in their lifetime[1]. *Candida albicans* is normally found in the vagina as a part of normal flora and could turn pathogenic if any change in the vaginal condition occurs[2].

Miconazole nitrate (MN) is an azole derivative used to treat VVC. Its antifungal activity is related to the permeability alteration of the membrane of fungal cells[3,4]. MN is categorized as class II in the biopharmaceutical classification system (BCS) with low aqueous solubility and high permeability. This hydrophobic nature of MN lowers its therapeutic efficiency due to lower local availability at the application site[5]. Accordingly, it is necessary to enhance MN solubility to develop a drug delivery system with compulsory local availability and improved therapeutic efficiency[6].

Various methods were employed to enhance the solubility of lipophilic active ingredients; for instance, particle size reduction, development of salt form, co-crystallization, nano-crystallization, solid dispersions, complexation, amorphous dispersions, and microemulsion[7–9]. Microemulsions are homogenous transparent

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thermodynamically stable liquid dispersions of aqueous and oil phases that are stabilized by the addition of comparatively large amounts of a surfactant as well as a co-surfactant. The dispersed droplet diameter varies from 1 to 100 nm[10]. The development of this micro-size droplet enhances lipophilic drug water solubility by dissolving the drug into an oil phase[11,12].

Plant-origin essential oils (EO) are generally complex mixes of both polar and non-polar natural compounds that are recognized for their antiseptic and therapeutic properties for instance antifungal activity. Tee tree oil (TTO), lavender oil (LO), and eucalyptus oil (ECO) are examples of such EO which are known for their well-recognized broad-spectrum antimicrobial effect and have been specified for fungal and bacterial vaginal infections[13,14]. Consequently, a novel formulation comprising the two antimicrobials (MN, EO) is expected to work synergistically with an exceptionally broad spectrum of antimicrobial action[15].

Commercially, numerous conventional vaginal antifungal formulation are employed for local therapy for example solutions, gels, creams, capsules, and vaginal ovules. These dosage forms are of limited use vaginally due to moderate leakage, issues of drug stability, poor residence time, deprived drug release as well as distribution via the vaginal cavity[16,17]. Furthermore, repeated application is required due to the self-washing manner and the availability of vaginal fluids which enhances the elimination of the dosage form[16,17].

Thermo-responsive in situ gels are liquid formulations that form a solid-like gel after application in response to physiological temperature, allowing prolonged system permanence and drug distribution into vaginal mucosa[18]. Poloxamer (PLX), is a synthetic triblock copolymer with thermoresponsive properties. They have beneficial features of sustained release of the active ingredient and excellent compatibility nonetheless low mucoadhesive properties. Consequently, mucoadhesive polymers should be implemented to enhance mucoadhesion and extend the residence time of in situ gel formulations[19].

The objective of this study was to enhance the solubility of MN by developing stable ME taking advantage of synergistic antifungal effect by utilizing EOs comprising antifungal activity and incorporating the optimum ME to in situ mucoadhesive gel as a novel approach to enhance the residence time and reducing the limitation of ordinary vaginal dosage form.

Materials and methods

Materials

Safa Pharmaceutical Industries, Iraq, gifted Miconazole Nitrate while all other components were purchased. Tween 60, Tween 80 (Merck Schuchardt, Germany), Span 20 (Energy Chemicals, China), Propylene glycol (AXO Industry, Belgium), Eucalyptus oil, Oleic acid, Lavender oil, Tee tree oil (Caeser and Loretz, Germany), Poloxamer 407, Poloxamer 188, hydroxyl propyl methyl cellulose, gellan gum (Sigma Aldrich, USA), methanol (analytical grade), acetic acid, Sodium acetate, and all other chemicals were of analytical grade. The current study defines room temperature as a temperature range of 25 ± 2 ℃.

Solubility studies

Choosing a proper oil phase, surfactant, and co-surfactant blend with a good capacity to solubilize MN to formulate an ME formula is crucial. Screening of numerous oils (LO, TTO, ECO, and oleic acid (OAO)), surfactants (tween 60 and tween 80), and co-surfactants (propylene glycol (PG), span 20) was employed for MN solubility. An excess amount of MN was positioned in 10 mL of each vehicle. The resultant mixtures were then vortexed for 5 min and continuously stirred at 100 rpm for two consecutive days at 37 ± 1 °C using a magnetic stirrer (APOPS, MS300HS, China) to assist the solubilization. The collected 10 mL sample was centrifuged at 6000 rpm for 5 min. The supernatant was then filtered using a syringe filter (0.45 µm, micropore) to eliminate undissolved MN particles. The solubility of MN was studied spectrophotometrically (UV-3000 spectrophotometer, Germany) at 230 nm[20].

Construction of pseudo-ternary phase diagram

The aqueous titration technique was used to construct pseudo-ternary phase diagrams to find the ingredients and their concentration ranges that can consequences in the wide existence zone of ME. By combining various ratios of tween 80: PG (Smix) (1:1 and 1:2) with OAO, LO, and TTO, except ECO (1:1) Smix ratio was employed.

The mixtures with ratio of oil: Smix; 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 were placed in glass vials. Water was added dropwise to each mixture under vigorous stirring at ambient temperature (25 ± 2℃) until turbidity was observed. A ternary diagram generator (chemix school 10.0 application) was utilized to create the pseudo-ternary phase diagram based on the percentage of various components in each vial[5].

Preparation of MN-loaded ME

After recognizing the ME existence zone in the phase diagram, the ME formulation were designated at the required constituent ratios. MN-loaded ME formulation were prepared by primarily dissolving a pre-established amount of MN in a surfactant and co-surfactant mixture (at a ratio of 1:1–1:2). The oil phase was then added to the drug mixture and followed by the addition of water gradually in a dropwise way with continual stirring. Finally, the mixture was vortexed for 5 min and preserved till a clear homogenous solution was obtained[21].

According to the pseudo-ternary phase diagram, area size, and ME formulation stability of each oil, 12 ME formulation were conducted, as shown in Table 1.

Characterization of ME formulation

Assessment of macroscopic appearance

Visual inspection at room temperature was performed to assess the prepared MEs' color, consistency, and homogeneity[22].

Formula	Percent of each ingredient							Smix ratio
	MN w/w	OAO w/w	ECO w/w	TTO w/w	LO w/w	Smix w/w	Water w/w	
ME1	4	10	–	–	–	75	15	1:1
ME2	4	10	–	–	–	65	25	1:1
ME3	4	15	–	–	–	70	15	1:1
ME4	4	–	8	–	–	52	40	1:1
ME5	4	–	10	–	–	50	40	1:1
ME6	4	–	12	–	–	48	40	1:1
ME7	4	–	–	5	–	46.05	48.95	1:2
ME8	4	–	–	5	–	45	50	1:2
ME9	4	–	–	5	–	43	52	1:2
ME10	4	–	–	–	7	45	48	1:2
ME11	4	–	–	–	7	42	51	1:2
ME12	4	–	–	–	7	40	53	1:2

Table 1. Composition of different MN ME.

Thermodynamic stability study
Thermodynamic experiments, such as centrifugation and heat-cold cycles, have examined the formula's stability.

Centrifugation Centrifugation was employed under stressed conditions to assess the thermodynamic stability of ME formulation. The formula was centrifuged at 3500 rpm for 15 min at room temperature, and cloudiness signs, sedimentation, and phase separation were checked[23].

Heating-cooling test The heating-cooling test assesses cracking, precipitation, and phase separation effects on stability. All 12 formulations were heated at 45℃, followed by cooling at 4℃ for 24 h at each temperature for two cycles[24]. Successful formulation were further analyzed as follow:

Assessment of droplet size and polydispersity index (PDI)
ME formulation' average droplet size and PDI were assessed utilizing a Malvern zeta-sizer (Malvern instruments, Nano ZS 632.8, USA). ME formulation were diluted properly to ten times by distilled water before the measurement[25,26].

Assessment of pH
The pH of MN-loaded ME formulation was measured using a pH meter (HANNA® Instruments, HI 2210 and EC214, USA) by immersing the pH meter electrode directly in 100 mL of prepared formulation[27].

Assessment of drug content The ME formulation containing 400 mg of MN were diluted with absolute alcohol (methanol) and spectrophotometrically examined for MN content at 230 nm compared to absolute methanol as a blank and employing a constructed calibration curve. Drug content was then calculated using Eq. 1 to determine drug homogeneity[28].

$$\text{Drug-content}\,(\%) = \frac{\text{Analyzed drug content}}{\text{Theoretical drug content}} \times 100 \quad (1)$$

Assessment of viscosity
The viscosity MN-loaded ME formulation were estimated at room temperature utilizing a Brookfield viscometer (DV-II Pro, USA) with spindle 63 at pre-defined speeds 60[29].

Assessment of in vitro antifungal activity
The selected ME formulation were evaluated for their antifungal activity against *candida albicans* by agar well diffusion technique. Two mL of blank ME (without drug) formula were diluted with distilled water to demonstrate the oils' antifungal activity. Each solution was then inoculated to agar plates (Sabouraud-dextrose), previously seeded with 100 µL of clinically isolated *candida albicans*, then incubated at 37℃ for 24 h. After the incubation, the mean diameters of inhibition zones around each will be were measured[30].

Selection of ME optimum formula
The ME optimum formula was chosen based on the outcomes of the thermodynamic stability study, droplet size, PDI, PH, drug content, viscosity, and in vitro antifungal activity. The chosen optimum formula will then be incorporated to develop a temperature trigger in situ gel.

Thermosensitive in situ gel formulation

Preliminary studies for preparation of MN-based in situ gels

The cold method was employed to achieve an optimal gel formula that has a relevant gelation temperature (Tgel) range between 31 and 36℃ for vaginal delivery. We conducted a pilot study to obtain the best in situ gel formula components; the best formulation contain poloxamer (PLX) PLX-407, PLX-188, hydroxypropyl methylcellulose K4M (HPMCK4M), and gellan gum (GG) as mucoadhesive polymers. In detail, initially, we examined the best concentration of PLX-407 and PLX-188 in terms of their Tgel (Table S1) and Formula FP10, which contain 18% (w/v) PLX-407 and 2% (w/v) PLX-188.

MN, as well as bioadhesive polymers (HPMC-K4M, HPMC-K15M, and GG), were dispersed in 30% of the total volume of deionized water (DW) with continuous stir by magnetic stirrer at 60℃ and then cooled down to 25℃. PLX-407 and PLX-188 were concurrently mixed at 100 rpm with the remaining 60% of the total volume of DW at 4℃ till completely dissolved. Both solutions were mixed, and the volume was measured and adjusted using cold DW. Finally, the resultant formulation were kept in the refrigerator overnight at 4℃ to confirm thorough solubilization until a transparent clear solution was acquired[31]; two formulation, IG2 and IG8, were selected based on their Tgel and drug release (Table S2). These formulation contain 18% PLX-407, 2% PLX-188, and 0.2% (w/v) MN, in addition to either 0.6% (w/v) HPMC-K4M (Formula IG2) or 0.6% (w/v) GG (Formula IG8) to formulate the blank (pilot) components of the in-situ gel base formula, which will be incorporated with the microemulsion formulation of the miconazole nitrate.

Preparation of MN-loaded ME-based gels

MN in situ gel (IG) was prepared by directly adding a predetermined MN weight to the polymer solution with continuous stirring. Microemulsion gel (MG) was prepared by adding the chosen MN-loaded ME formula dropwise at the specified ratio with mild stirring by a magnetic stirrer. Optimum MG formulations with acceptable Tgel were selected for further investigations[32]. Table 2 demonstrates the composition of ME-loaded gel and in situ gel.

Assessment of ME-loaded thermosensitive vaginal in situ gelling formulation

Assessment of gelation temperature

The tube inversion technique with a magnetic bar was employed to detect the Tgel of the prepared formulation.

Each formula was accurately weighed to get 5 g, placed into a transparent vial (20 mL), and then submerged in a thermos-balanced water bath at room temperature. The temperature was elevated gradually with a rise of 1℃ from 25 to 40℃ until the gelation occurred. The temperature remained constant for 5 min at each point, and the flow of gels and stopping of the magnetic bar were observed after the immediate inversion of the test tubes, at time point the Tgel was recorded. Formulations that passed the Tgel tests were selected for the following studies[33].

Formula	ME formula	Mucoadhesive in situ gel formula	Dilution ratio (ME to in situ gel)
MG1	ME10	IS-I	1:1
MG2	ME10	IS-I	1:2
MG3	ME10	IS-I	1:3
MG4	ME10	IS-I	1:4
MG5	ME10	IS-I	1:5
MG6	ME7	IS-I	1:1
MG7	ME7	IS-I	1:2
MG8	ME7	IS-I	1:3
MG9	ME7	IS-I	1:4
MG10	ME7	IS-I	1:5
MG11	ME10	IS-II	1:1
MG12	ME10	IS-II	1:2
MG13	ME10	IS-II	1:3
MG14	ME10	IS-II	1:4
MG15	ME10	IS-II	1:5
MG16	ME7	IS-II	1:1
MG17	ME7	IS-II	1:2
MG18	ME7	IS-II	1:3
MG19	ME7	IS-II	1:4
MG20	ME7	IS-II	1:5

Table 2. Composition of MN- ME in situ gel formulation. IS-I: Mucoadhesive in situ gel (18% PLX 407, 2% PLX 188 and 0.6% HPMC K4M). IS-II: Mucoadhesive in situ gel (18% PLX 407, 2% PLX 188 and 0.6%GG).

Assessment gelation time (gt)
To assess the gelation time (Gt), 5 mL of each formula was sited in a see-through vial (20 mL) with a magnetic bar inside. In a water bath, the vial was submerged at 36℃, and the formula underwent continuous stirring at a rate of 100 rpm. The Gt of the gel formulations was recorded as the magnetic bar completely stopped[34].

Visual assessment for clarity
The prepared formulation were checked for color, clarity, and grittiness in contrast to a white background. The presence of any precipitate, grittiness, or aggregates was also detected by spreading smears of prepared formulation on glass slides[35].

Assessment of pH, drug content, and viscosity
The chosen prepared formulation pH values and drug content were assessed using the same procedure implemented for ME formulae. The viscosity was also assessed at room temperature (liquid form) as well as at body temperature (gel form) by the same method previously used for ME with different rotation speed[34].

Assessment of gel spreadability
To determine the spreadability of prepared in situ gel formulation, one gram of the formula was positioned at the center of the glass plate with dimensions of (10 × 23 cm). Subsequently, another glass plate was placed over the former one, and 1 kg weight was sited attentively on the top of the upper plate (without sliding) for 30 min till gel spreading was completed. The increase in the circle diameter of the gels was documented as the spreadability value[36].

Assessment of mucoadhesive force
The sheep were acquired from the Mustansiriyah Research Centre, Baghdad, Iraq, and accommodated before the study was established. Following the AVMA 2020 guideline for animal care[37]; the euthanasia procedure involved the intravenous administration of pentobarbital sodium (100 mg/kg) to healthy female sheep at the age of 6 months (as an anesthetic agent and primary method for euthanasia). Following total anesthesia by pentobarbital sodium, secondary means of ensuring death were utilized pneumatic Captive Bolt Guns. Subsequently, the sheep's vaginal mucosal tissue was harvested.

A modified balance technique was employed to assess the mucoadhesive strength of the formulation by quantifying the force necessary to separate the formulation from a sheep's vaginal mucosal tissue. Ten grams of in situ gel were secured to a platform provided by a small glass container set at the inferior surface of the right-side pan. A section of vaginal tissue was fixed using adhesive to a portable wooden platform at the balance right arm, as shown in Fig. S1. Before mucoadhesion assessment, the exposed gel sample was immersed in 1 mL of acetate buffer (pH 4.1) for half a minute to provide initial hydration. Consequently, the platform was relocated upwards to bring the hydrated gel in contact with the tissue surface. A preload of 20 g was located on the right pan for exactly 3 min to deliver the initial pressure. The preload was removed from the right pan, and a steady weight (using water) was added to the left pan until the gel was completely separated from the tissue surface. The total weight needed for the thorough detachment of the gel was documented, and mucoadhesion was found to be measured in grams[38].

Assessment of in-vitro drug release
An in-vitro drug release study was achieved using a dialysis bag in modified USP dissolution apparatus II to mimic in vivo drug release from the prepared MG formulation. An accurately measured gel sample equivalent to a single dose of MN (200 mg) was placed inside the semipermeable membrane tube with molecular weight 8000–14,000 D (HiMedia Laboratories LLC, USA), formerly immersed in a dissolution medium overnight.

Both sides of the dialysis tube were then secured utilizing rubber bands to prevent leakage and then held to the paddle rod of the dissolution apparatus. The tube was dipped in a previously filled dissolution jar with 100 mL of acetate buffer (pH 4.1) maintained at 36 ± 0.5℃ with a stirring speed of 50 rpm. At a predetermined time interval (30, 60, 120, 180, 240, 300, 360, 420, 480, 540, and 600), a 5 mL sample of dissolution medium was obtained to analyze the MN release spectrophotometrically at 230 nm after being filtered using a 0.45 µm Millipore filter syringe; each time the dissolution media was taken, it was substituted by an equal volume of dissolution medium to preserve the sink condition. The amount of MN released was estimated using a constructed calibration curve[39].

The same procedure was implemented for MN-loaded IG formulation, in which 200 mg of MN was incorporated as a pure powder into the selected gel formula (considered a control formula in addition to the marketed product). In vitro drug release of MN from the marketed suppository was accomplished using the USP paddle method in the same conditions as the non-membrane technique[40].

In vitro antifungal activity
Antifungal activity of MN-loaded MG formula, suppository marketed product dissolved in 0.5% dimethyl sulfoxide (DMSO), and MN-loaded IG formula was evaluated against *Candida albicans* by using agar-well diffusion method by the same technique used for antifungal activity assessment for ME. The mean zone of inhibition was recorded for all the test samples[41,42].

Selection of MG selected formula
The Tgel, Gt, pH, viscosity, mucoadhesive force, gel spreadability, in vitro drug release study, and in vitro anti-fungal activity provided the choice of MN-loaded MG selected formula. The selected formula was then subjected to further evaluation tests.

Primary vaginal irritation studies

The possibility of MN-loaded MG and MN-loaded IG formulation to produce vaginal irritation was assessed in rabbits employing a method reported previously[43].

Animal handling and care during the experimental technique were following the "Experimentation on animals in the course of medical research and education" (CPCSEA) guidelines and AVMA 2020 guidelines[37]. The Animal Ethical Committee of Mustansiriyah University, College of Pharmacy, permitted the experimental rules.

White albino female rabbits weighing 2.5–3 kg was acquired from Mustansiriyah Research Centre, Baghdad, Iraq, and accommodated before the study was established. The animals were categorized into three groups ($n=3$): the control group (without treatment), the pure drug in situ gel group (optimal MN-loaded in situ gel (IG-2 formula)), and the ME group (MN-loaded ME in situ gel (MG-1 formula).

All formulation were administered via a 5 mL plastic syringe, as shown in Fig. 1. After a single vaginal application, the vaginal cavity was perceived for 48 h for any indication of potential irritation (i.e., erythema and edema) of the vaginal mucosa.

The mean erythemal outcomes were documented (from 0 to 4) reliant on the degree of erythema as follows: no erythema = 0, slight erythema (barely perceptible-light pink) = 1, moderate erythema (dark pink) = 2, moderate to severe erythema (light red) = 3, and severe erythema (extreme redness) = 4 [44].

Determination of MN-loaded ME and MN-loaded MG formulation morphology by FESEM

Field emission scanning electron microscopy (FESEM) using an inspect f 50, Fei company, Netherlands; FESEM was made at an accelerating voltage of 30 kV[45].

Statistical analysis

Statistical analysis for all experimental data was achieved using IBM SPSS statistic software. Data were stated as mean values with their standard deviation (SD). ANOVA test (one-way analysis of the variance) and *post hoc* Tukey test for pair-wise comparison were applied for statistical analysis. Significant statistical differences were considered when ($p \leq 0.05$)[47].

Fig. 1. Insertion of in situ gel formula into rabbit vagina.

Results and discussion

Assessment of MN-loaded ME formulations

Solubility of drug

It is crucial to select an ME component with the highest solubility capacity for the drug mainly to ensure that no precipitation of drugs will occur and to maintain them in solubilized form within formulation[48]. The drug's solubility was determined in each component of ME (oils, surfactants, and co-surfactants). The results, as demonstrated in Fig. 2A, declared that the drug solubility was in the following order: ECO > OAO > LO > TTO with a significantly ($P \leq 0.05$) high solubility in ECO as compared to other oils. However, all oils undergo a pseudo-ternary phase diagram study since all have a satisfactory solubilizing effect for the drug and to explore the one that provides a potentiated antifungal activity ME[49].

Concerning surfactants, which are used to reduce the surface tension between two immiscible phases and provide a stable ME, Tween 80 showed a significant effect on MN solubility in comparison to tween 60 (Fig. 2B). This result could be attributed to the chemical structure of Tween 80, which has a larger number of hydrophobic and polar groups (OH– and –OCH2CH2–) than Tween 60. Additionally, Tween 80 was considered nontoxic and had an optimum HLB value of 15. Consequently, it was selected as an optimum surfactant[50].

Regarding co-surfactant, which is mainly employed in ME formulation as a stabilizing agent and depending on the obtained solubility results, PG was selected. Significantly higher ($P \leq 0.05$) MN solubility in PG was observed compared to span 20 (Fig. 2C). This observation may be attributed to the higher amphiphilic nature and higher (–OH) groups in the PG structure compared to the span. This nature may increase the possibility of interaction of MN functional groups with the hydroxyl group in PG, thereby increasing its solubility[51].

Construction of the pseudo-ternary phase diagram

Generating ternary phase diagrams for various oils to map the best drug-loaded formulation. From the obtained diagrams and as illustrated in Fig. 3, a higher concentration of Tween 80 and HLB value was implemented when the Smix ratio was 1:1 compared to when the Smix ratio was 1:2 and consequently a higher ME area (colored area). These results match the fact that the ME region tends to narrow down with increasing the ratio of surfactant to co-surfactant (1:1–1:2) and higher HLB value of the system[52].

As a result, a Smix ratio of 1:1 was better used to prepare MN-loaded ME when OAO and ECO were used. In contrast, there are no significant differences between the pseudo-ternary region areas for TTO and LO. As a result, a 1:2 Smix ratio pseudo-ternary plot was chosen; this could be attributed to the higher co-surfactant concentration that decreases the interfacial tension to the limit that enables the formation of smaller and stabilized drug-oil droplets[53].

Macroscopic appearance

Visual transparency was determined to ensure the absence of turbidity, a single-phase ME system, and complete drug dissolution without any precipitation[54]. All formulation (ME1-ME12) were translucent and light yellowish in color, and no turbidity was observed when examined. Table 3 shows that clear formulations are labeled as passed, and turbid formulations are labeled as failed.

Thermodynamic stability

The thermodynamic stress test reflects the dispersibility of the ME components and, hence, its stability. The formulations that passed the centrifugation and heating-cooling cycle proved their thermodynamic stability. All formulation except ME3, ME6, ME9, and ME11, as shown in Table 3, showed no flocculation, aggregation, phase separation, creaming, or coalescence when the surfactant, co-surfactant, oil, and water were mixed in the specific quantities for ME formulation[55].

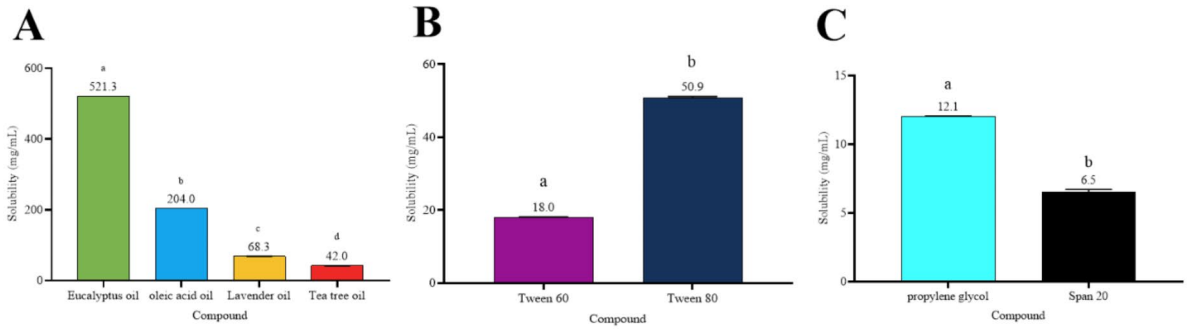


Fig. 2. MN solubility in (**A**) different oils, (**B**) surfactants, and (**C**) co-surfactants. Data presented as mean ± SD ($n = 3$). Columns with similar letters indicate no significant difference (a vs. b, c, d indicates p-value ≤ 0.05, b vs. c, d indicates p-value ≤ 0.05, and c vs. d indicates p-value ≤ 0.05).

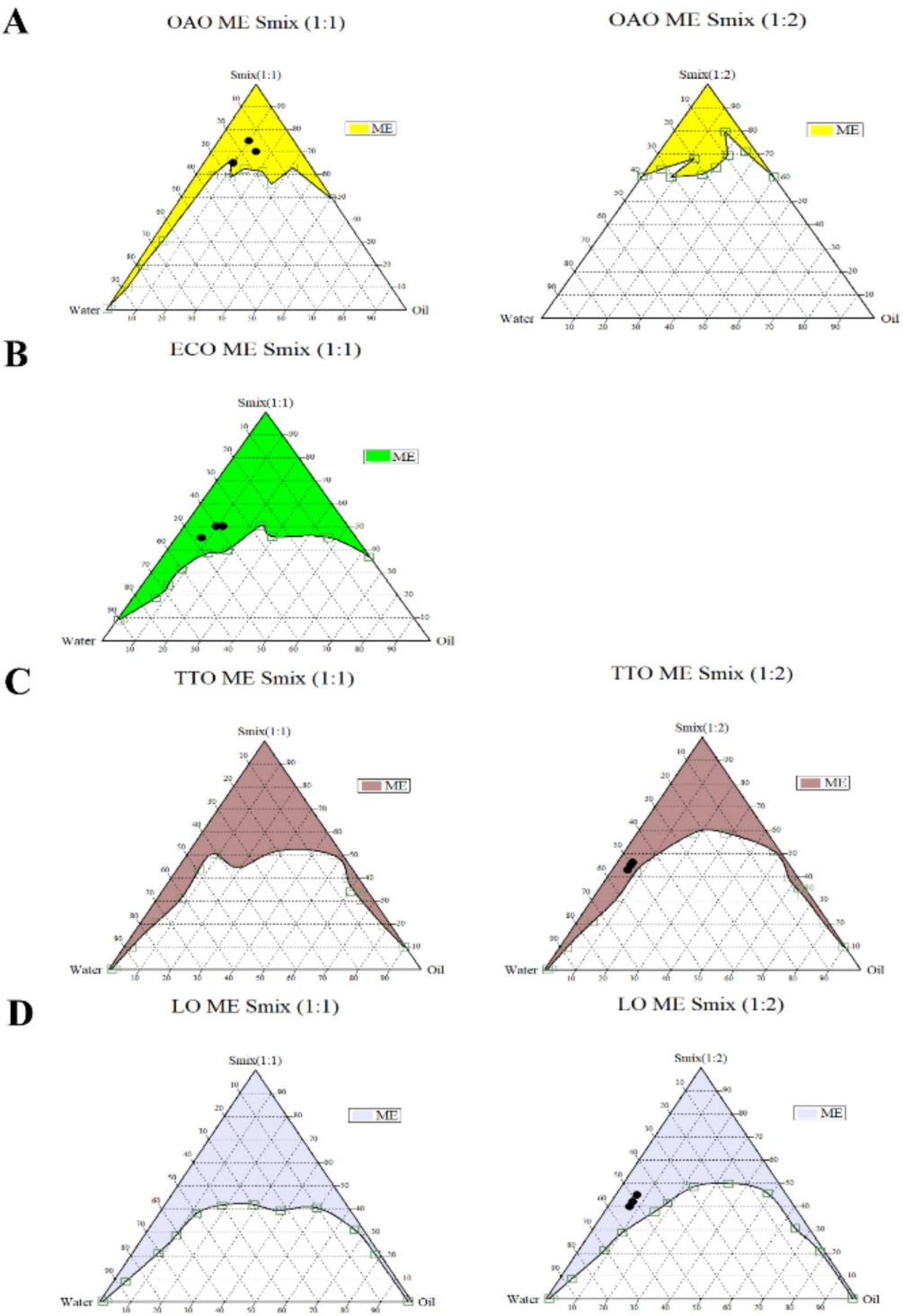


Fig. 3. Pseudo-ternary phase plots of different oils. (**A**) OAO with Smix (1:1) and (1:2), (**B**) ECO with Smix (1:1), (**C**) TTO with Smix (1:1) and (1:2), and (**D**) LO with Smix (1:1) and (1:2). Smix represents by Tween 80 as surfactant and Propylene glycol as co-surfactant. ECO with a Smix ratio of 1:2 failed to construct a pseudo-ternary diagram.

Droplet size and polydispersity index (PDI)

The size of microparticles is crucial for enhancing the dissolution rate of active ingredient[56]. Figure 4A and G show that the average globule size of the prepared formulation ranged from 16.00 to 95.31 nm, indicating that all globules were micro-sized and occurred within the ME region range (10–100 nm). The obtained results

Formula code	Visual appearance	Centrifugation test	Heating–cooling cycles
ME1	Passed	Passed	Passed
ME2	Passed	Passed	Passed
ME3	Passed	Passed	Failed
ME4	Passed	Passed	Passed
ME5	Passed	Passed	Passed
ME6	Passed	Failed	–
ME7	Passed	Passed	Passed
ME8	Passed	Passed	Passed
ME9	Passed	Passed	Failed
ME10	Passed	Passed	Passed
ME11	Passed	Passed	Failed
ME12	Passed	Failed	–

Table 3. Visual and thermodynamic stability of MN-loaded ME-prepared formulation.

demonstrated that as the oil content increased with a fixed Smix ratio, the globule size increased significantly, where the size was 27.5 nm and 35.11 nm for ME4 and ME5, respectively, as the oil content increased from 8 to 10% (w/w). These outcomes could be justified by the decrease in the Smix concentration in the formulation[57].

The results also demonstrated that the particle size significantly increased as the concentration of Smix decreased with a fixed amount of oil content (ME1, ME2, ME7, and ME8). This could be due to the accumulation of surfactant molecules at the interface of two immiscible phases, providing better stabilization against droplet accumulation and lowering the flocculation rate. Additionally, a greater penetration of the oil phase in the hydrophobic region of the surfactant leads to a reduction in the droplet size[58].

Furthermore, the obtained results showed low values of PDI (< 0.7), indicating a uniform distribution, stability, and homogeneity of micro-sized droplets within the preparation (Fig. 4B)[59].

Determination of pH

Any deviation from the normal pH value of the vagina may cause discomfort and patient noncompliance upon administration. All prepared MEs showed pH values within the range of (5.1–5.7) as shown in Fig. 4C, and lie within the normal pH values, indicating no possibility for irritation and good gel formulation compatibility[60].

Drug content results

The obtained results, as shown in Fig. 4D, signified that MN was loaded successfully in the oil phase of all the prepared MEs formulation and met the British Pharmacopeia range requirement of 95–110%[61].

Viscosity measurement

Viscosity is a crucial ME characteristic for signifying the stability and efficient drug release of ME[62]. The results obtained, as shown in Fig. 4E, demonstrated that as the concentration of the Smix decreased in ME1 and ME2 as well as ME7 and ME8, the viscosity of ME decreased significantly. This could be due to the water molecules' entrapment in cross-linking surfactant chains and the more rigid dispersion medium resulting from the highest surfactant concentration[63].

In vitro antifungal activity

The anti-fungal activity of selected ME formulation was examined to determine the most potent formula against candida fungus, which was consequently incorporated into selected in situ gel formulation.

Figure 4F demonstrated that ME10 has the most potent anti-fungal activity supported by a 12 mm inhibition zone diameter, as pointed out in Fig. 4H. This could be justified by the antifungal action of LO, which is rich in different components, mainly 1,8-cineole, linalool, and linalyl acetate. All these groups act through different mechanisms of action against candida albicans[14,64]. In addition to the smaller oil droplet size, it permeates easily through the candida membrane and potentiates the anti-fungal activity of the LO active group. The results also showed no significant differences in antifungal activity between ME7 and ME10 since the highly anti-fungal active groups terpinene-4-ol and 1–8 cineole are also the major constituents of TTO[65].

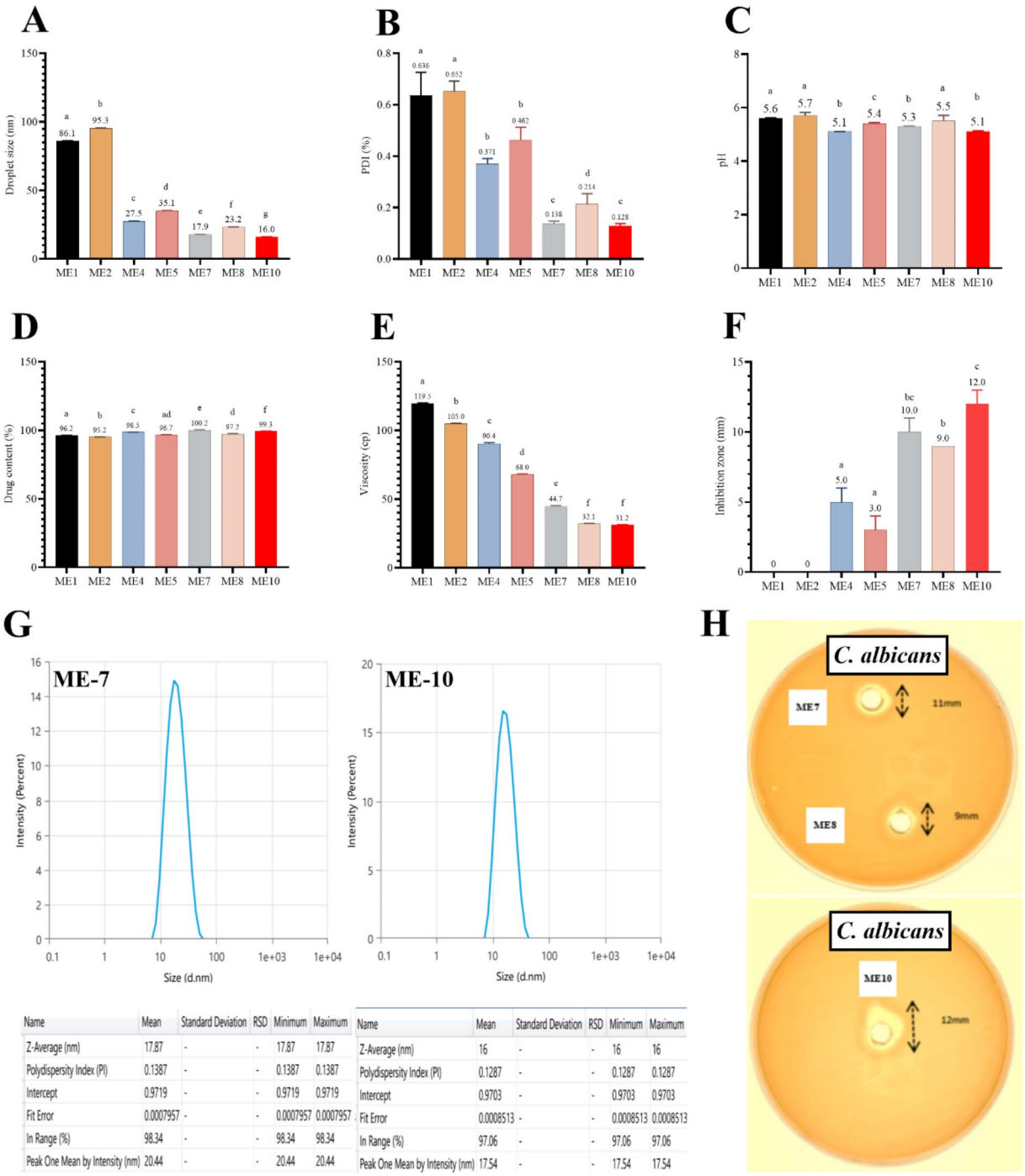
ME evaluation results show that ME7 and ME10 were chosen for further incorporation study due to their extremely high anti-fungal activity with appropriate ME characteristic parameters.

Assessment of ME-loaded thermosensitive vaginal in situ gelling formulation

Gelation temperature

Tgel is the temperature at which the liquid phase transforms to gel. An ideal in situ gel should neither have a Tgel lower than 25℃ nor higher than 37℃ to maintain its solution form at room temperature and to assure gel formation upon application, resulting in the avoidance of the drainage of the formula from the vagina at an early stage. Consequently, an optimum Tgel for in situ vaginal formulation should be in the range of 31–36℃[66].

The results shown in Fig. 5A showed a significant reduction in Tgel after incorporating the selected ME formula. The results also demonstrated that as the incorporation ratio of ME: gel was increased (from 1:1 to 1:5), the Tgel deviated from the required Tgel range opposing to gels prepared using 1:1 incorporation ratio, which



Name	Mean	Standard Deviation	RSD	Minimum	Maximum
Z-Average (nm)	17.87	-	-	17.87	17.87
Polydispersity Index (PI)	0.1387	-	-	0.1387	0.1387
Intercept	0.9719	-	-	0.9719	0.9719
Fit Error	0.0007957	-	-	0.0007957	0.0007957
In Range (%)	98.34	-	-	98.34	98.34
Peak One Mean by Intensity (nm)	20.44	-	-	20.44	20.44

Name	Mean	Standard Deviation	RSD	Minimum	Maximum
Z-Average (nm)	16	-	-	16	16
Polydispersity Index (PI)	0.1287	-	-	0.1287	0.1287
Intercept	0.9703	-	-	0.9703	0.9703
Fit Error	0.0008513	-	-	0.0008513	0.0008513
In Range (%)	97.06	-	-	97.06	97.06
Peak One Mean by Intensity (nm)	17.54	-	-	17.54	17.54

Fig. 4. Characterization of ME; (**A**) ME droplet size (nm), (**B**) PDI (%), (**C**) pH, (**D**) drug content (%), (**E**) viscosity (cp.), (**F**) anti-fungal activity for blank ME, (**G**) Droplet size and PDI for ME7 and ME10, and (**H**) anti-fungal activity for *candida albicans* inhibition zone for blank ME of ME7, ME8 and ME10. Data presented as mean ± SD. Columns with similar letters indicate no significant difference (a vs. b, c, d, e, f indicates p-value ≤ 0.05, b vs. c, d, e, f indicates p-value ≤ 0.05, and c vs. d, e, f indicates p-value ≤ 0.05; d vs. e, f indicates p-value ≤ 0.05; e vs. f indicates p-value ≤ 0.05).

provides the required Tgel as in MG1, MG6, MG11 and MG16, which showed Tgel values 33 ± 0.01, 33 ± 0.015, 31 ± 0.021 and 31 ± 0.05 ℃ respectively. This outcome is in agreement with what was reported previously[67,68].

Further assessment tests were employed for MG1, MG6, MG11, and MG16 since they achieved the required Tgel (31–36℃) to form a gel upon administration intravaginally. At the same time, the rest of the MN-loaded

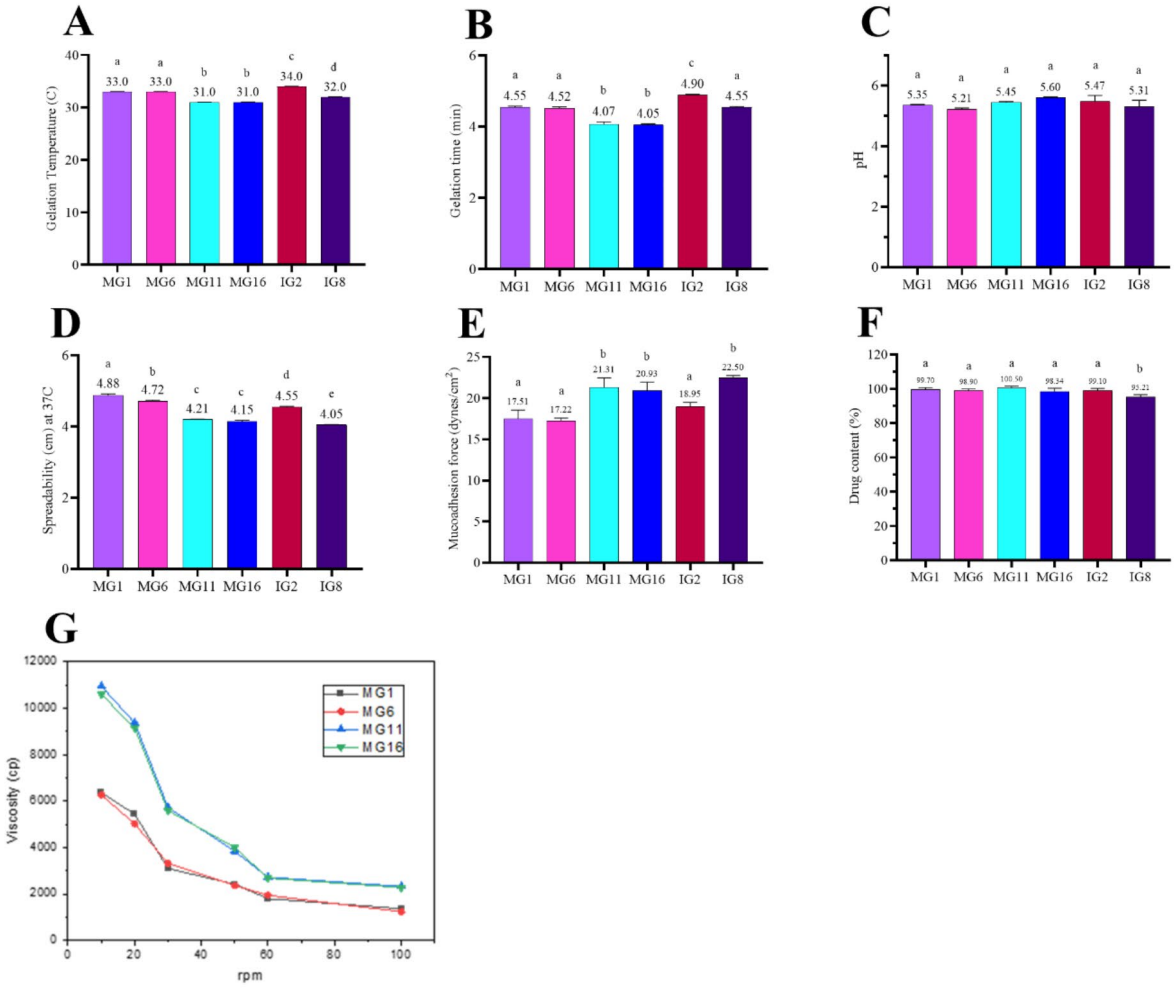


Fig. 5. Assessment of ME-loaded thermosensitive vaginal in situ gelling formulation; (**A**) Tgel, (**B**) Gt, (**C**) pH, (**D**) spreadability, (**E**) Mucoadhesion force, (**F**) drug content of prepared gel formulation, and (**G**) Viscosity study with different share rates at 37 °C. Data presented as mean ± SD. Columns with similar letters indicate no significant difference (a vs. b, c, d indicates p-value ≤ 0.05, b vs. c, d indicates p-value ≤ 0.05, and c vs. d indicates p-value ≤ 0.05).

MG formula failed to achieve the required Tgel (i.e., other formulation showed Tgel of less than 30℃). As such, no further assessment was carried out.

Gelation time

The Gt is another crucial factor since it assures drainage prevention from the site of application leading to extended retention of the active substance on the mucosal vaginal tissue. The result, as shown in Fig. 5B, showed that the Gt of the prepared MN-loaded ME in situ gel formulations was found to be around 5 min, which is consistent with results obtained by previously published literature[69].

Visual assessment for clarity

The clarity, homogeneity, and grittiness of prepared formulation should be examined to confirm the desired requirements for intra-vaginal formulation of being clear, homogenous, and free from any undissolved particulates[70]. All selected formulation, MG1, MG6, MG11, and MG16, showed a clear and homogenous gelling system that could be applicable for vaginal administration.

Assessment of pH

The pH of formulation is one of the important parameters for vaginal compatibility, formulation stability, and patient acceptance. Normal physiological vaginal pH is around 3.5–4.5 [71]; the pH of the formulation was in the range of 5.21–5.60, as demonstrated in Fig. 5C, in which all formula's pH is close to the normal pH range and hence acceptable for vaginal application.

Gel spreadability

Good spreadability is an essential criterion for MG formulations since it assures ease of application, delivery of the correct dose of the loaded drug, and excellent formula distribution in the vaginal cavity[72]. As shown in Fig. 5D, the spreadability increased significantly when GG was replaced by HPMC-K4M (MG1 and MG 11), which showed spreadability values of 4.88 ± 0.04 and 4.21 ± 0.001 cm, respectively. This result could be due to the higher viscosity produced by GG in comparison to HPMC-K4M as a result of the higher cross-linking density of GG[73].

Mucoadhesive force

The mucoadhesive force is a vital characteristic for in situ forming vaginal gels since it assures the extension of its retention time in the vagina without drainage[74]. The obtained outcomes showed a significantly higher mucoadhesive force when GG was employed as a mucoadhesive polymer than HPMC-K4M in the same concentration (MG1 and MG11), as seen in Fig. 5E. This could be related to the chemical structure of GG, which could provide more adhesive sites and polymer chains for entanglement with mucin, resulting consequently in increasing mucoadhesive strength[75].

Drug content results

The obtained results for drug content were around (98.9 ± 0.026–100.5 ± 0.021%) as shown in Fig. 5F. These values are within the standard range according to USP[76]. Non-significant variances were observed among the samples' upper, middle, and lower points. This finding specifies that the formulation process implemented was able to produce gels with minimum variability and even drug distribution[77].

Viscosity measurement

The viscosity of in situ gel formulations is crucial since it controls their flowability, spreadability, release of drug, and residence time in the vaginal mucos[78].

The viscosity results for the selected formulation (MG1, MG6, MG11, and MG16) were 1124 ± 4.21, 1209 ± 6.3, 1389 ± 1.66, and 1431 ± 2.81 cp.: respectively. At room temperature, all formulation are presented in a solution form. A significant rise in viscosity was perceived by increasing the temperature to physiological temperature. This could be justified by the existence of gel-forming thermosensitive polymers that develop gel and raise viscosity once the temperature is elevated. In which, at a specific concentration of the PLXs and temperature, polymer molecules in an aqueous solution will form spherical micelles by self-assembling with a dehydrated poly propylene oxide core closed by hydrated swollen poly ethylene oxide chains, entanglements, and packing of micelles with an increase of temperature and as a results gel formed[79].

Additionally, it was observed that increasing the spindle speed or shear rate, as seen in Fig. 5G, reveals the pseudoplastic flow behavior of the prepared MG formulation (shear thinning liquids) due to viscosity reduction[80].

In vitro drug release

Figure 6 illustrates the in vitro release profiles of MN from MG1, MG6, MG11, and MG16, as well as IG2, IG8, and the marketed product. The results demonstrated that about 98% and 93% of the drug was released from MG1 and MG11 (MN-loaded ME in liquid suppository formula utilizing the same concentration of HPMC K4M and GG), respectively, within 12 h. This non-significant reduction in drug release could be attributed to the effect of the mucoadhesive polymer employed. The higher the cross-linking polymer (GG), the higher the viscosity of the gel network and, consequently, the slower drug release[81].

The results also demonstrated that a significant enhancement in drug release was observed when the drug was incorporated as ME rather than incorporated as received; 98% and 79% of the drug were released for MG1 and IG2, respectively, and 83% and 15% for MG11 and IG8, respectively, within 12 h. This outcome could be explained by the higher aqueous solubility of MN when prepared as ME compared to plain MN due to the tendency of drug release is increased as the particle size of the nanoparticle decreases and, subsequently, higher dissolution[82]. The results also showed that a complete drug release was obtained in 30 min for the marketed product, which provides faster release compared to MG1 and IG2 which showed 98% and 79%, respectively, within 12 h. This could be justified by the effect of both thermosensitive and mucoadhesive polymers employed in the formation of a gel with high viscosity that retard MN release from in situ gel formulation when compared to ordinary suppository bases[40].

In vitro antifungal activity

Candidiasis albicans was employed to assess the in vitro antifungal activity of MN-loaded MG-prepared formulation. The plates' mean inhibition zone (MIZ) was calculated by measuring the mean diameter of MIZ after applying MN-loaded MG (2% w/w) in the wells.

Higher antifungal activity was observed by MN-loaded MG1, which showed an inhibition zone of 31 mm, compared to MN-loaded IG2, which showed 26 mm, and the marketed product showed 12 mm MIZ (Fig. 7A). This could be due to the synergistic effect of both MN, which creates cellular membrane abnormalities by inhibiting cytochrome P450 (inhibiting ergosterol synthesis) and EO used, causing alteration in the cellular membrane permeability of candida[83]. Additionally, the micronizing of MN facilitated its contact with *candida albicans* and enhanced its penetration to fungi membranes efficiently[84].

The obtained results for the in vitro release profile, anti-fungal activity, and other evaluation tests for the selected MG formulation showed that MG1 was the optimum formula due to its superior sustained release (98% within 12 h) and anti-fungal activity (inhibition zone of 31 mm). Consequently, it underwent further analysis, including irritation tests and FESEM imaging.

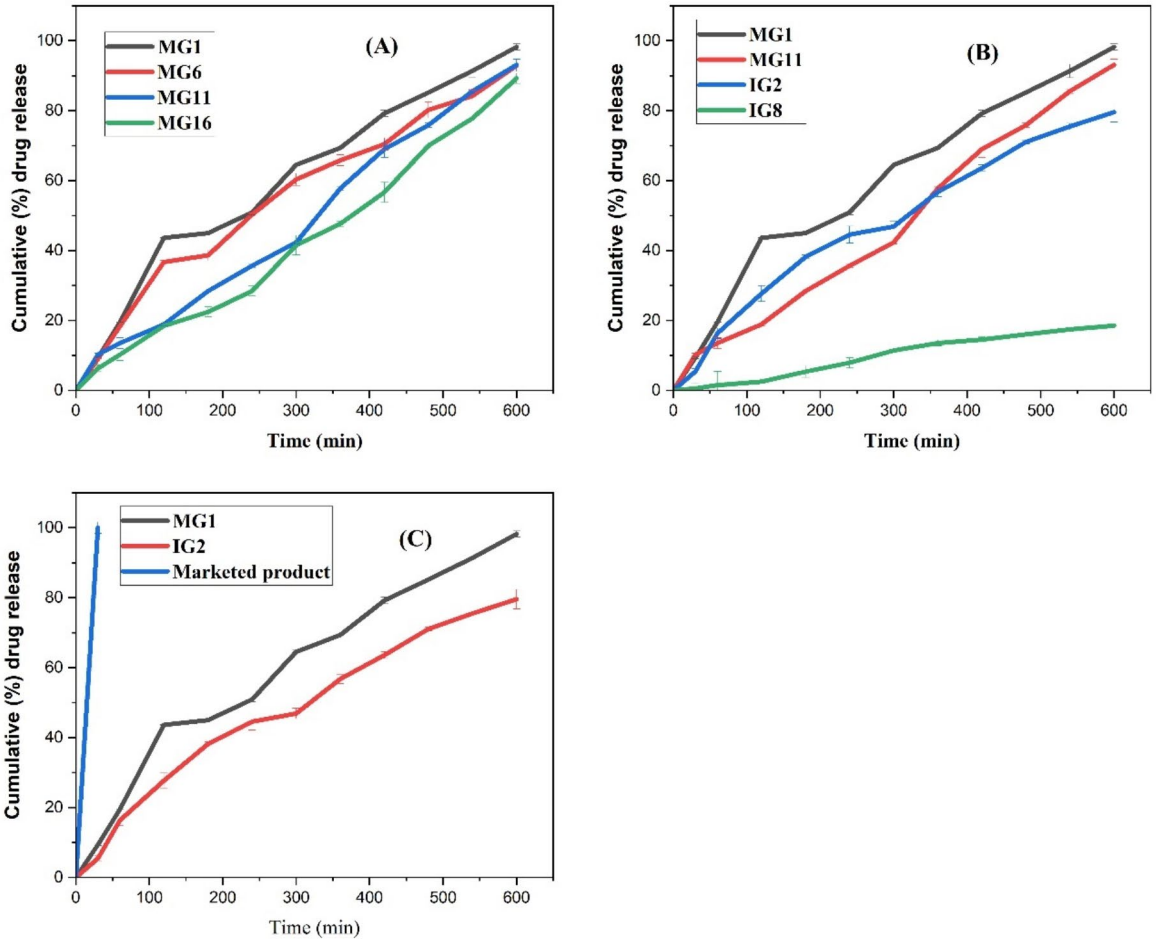


Fig. 6. MN formulations release profile in acetate buffer (pH 4.10). (**A**) Release of MG1, MG6, MG11 and MG16; (**B**) release of MG1, MG11, IG2 and IG8; and (**C**) release of MG1, IG2, and marketed product. Data presented as mean ± SD.

Primary vaginal irritation studies

The vaginal irritation study was carried out to evaluate the tolerability of the MN-loaded MG1 afterward application. The results show that the rabbits well tolerated MG1, and no signs of erythema and/or edema were seen even after 2 days. Studies indicated that IG2 was also well tolerated by the rabbits and showed no irritation, as shown in Fig. 7B.

FESEM size and morphology rationalization

The size and morphology of the MN-loaded ME (ME10) and MN-loaded MG (MG1) were confirmed by employing imaging with the high accuracy of the FESEM[85]. The findings approved that the ME globules are spherical and fall within the micro range, as pointed out in Fig. 7C.

Conclusion

This work demonstrated the feasibility of developing intra-vaginal formulations using microemulsions of essential oil. Seven microemulsions of oleic acid and essential oil containing tea tree oil, lavender oil or eucalyptus oil, surfactant Tween 80, and co-surfactant propylene glycol have been successfully developed and selected via pseudo-ternary phase diagram, droplet size, PDI, and anti-fungal activity. The essential oils used to formulate microemulsions were multifunctional ingredients that served as the oil phase of microemulsion and exhibited antimicrobial properties. All seven active-free microemulsions tested for anti-fungal activity showed the inhibition level of ME-7 and ME-10 as high as 11mm and 12mm inhibition zone against *Candida albicans*. These findings suggest the potential of microemulsions of essential oil as a safer alternative to synthetic antifungals; Fig. 8 summarizes the study workflow.

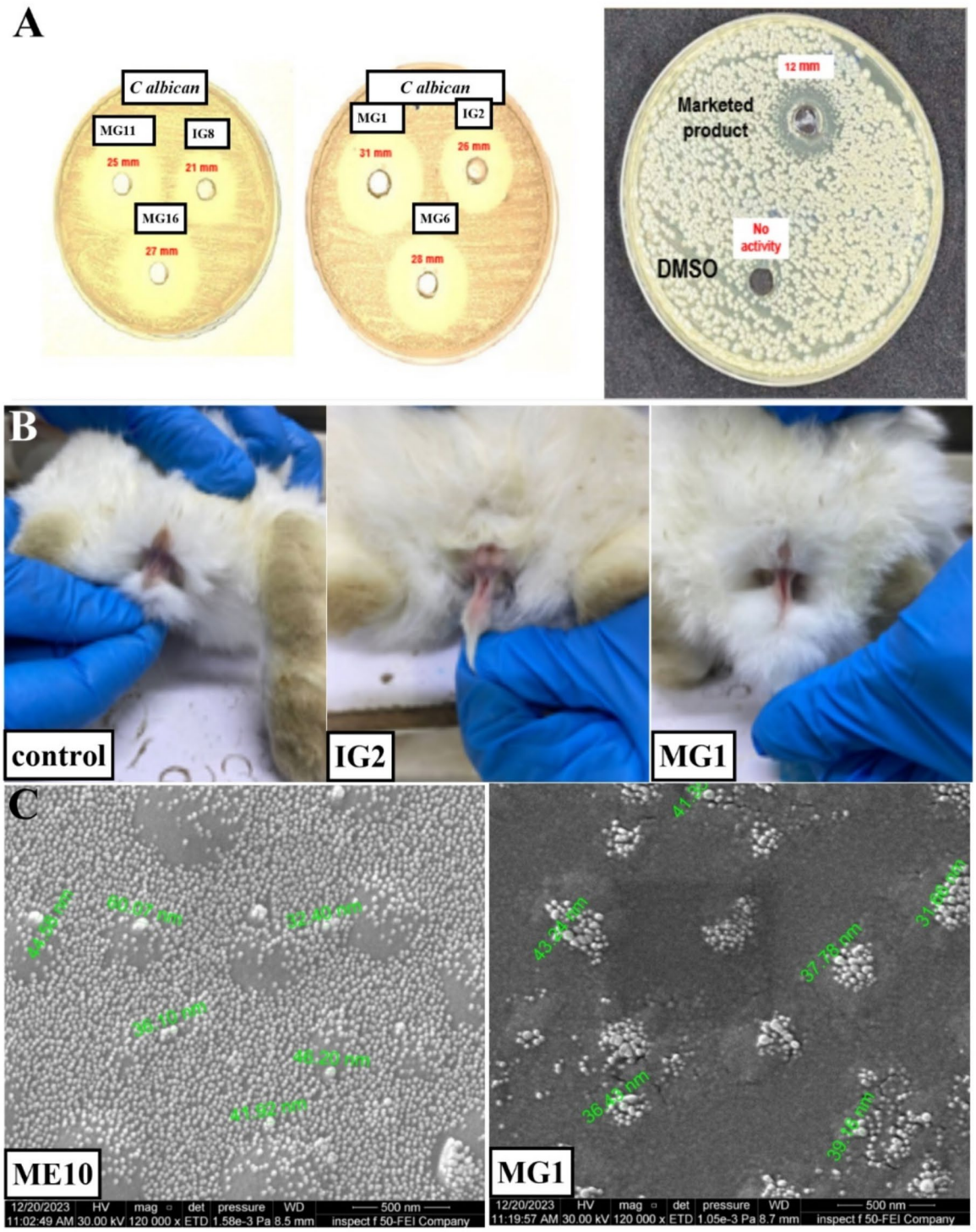


Fig. 7. Selected MN-loaded formula. (**A**) Antifungal activity of selected MN-loaded MG formulations, ordinary gel (IG formula), marketed product, and negative control (0.5% DMSO). (**B**) Results of primary vaginal irritation studies after 2 days, and (**C**) FESEM images.

When loaded with 4% (w/w) of poorly water-soluble MN and formulated into thermosensitive in situ gel form, these MN-loaded microemulsion-gel formulations (MG1, MG6, MG11, and MG16) demonstrated a significantly appropriate gelation temperature that close to vagina ambient temperature at which solution converted to gel take place and higher release of miconazole nitrate and better anti-fungal activity as compared with MN plain in situ gel and marketed product. Therefore, MG1 was the best formula that achieved superior

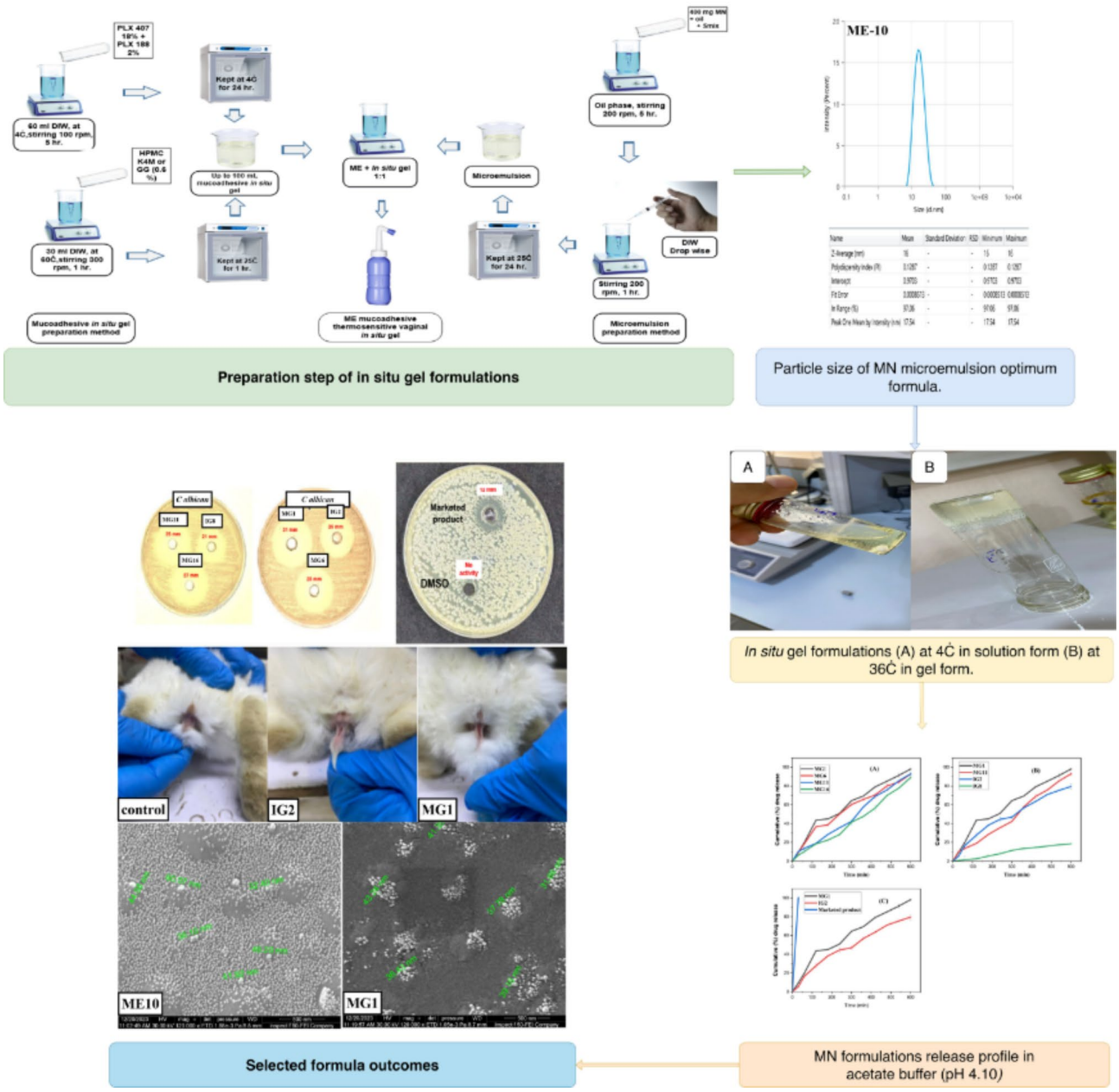


Fig. 8. Schematic presentation of the study work.

sustained drug release (98% with 12 h), extreme inhibition zone 31 mm, no in vitro rabbit vagina irritation, and good FESEM pictures. The studies indicated that MN with lavender oil microemulsion-based vaginal gel could be a viable alternative to the current topical formulations available for the treatment of vaginal candidiasis. In vivo, human clinical study should be carried out in the future to improve formula safety during fungal vaginitis management.

Data availability

Data are available upon required from the corresponding author.

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

All experimental protocols were approved by the Research Ethics Committee of the College of Pharmacy, Mustansiriyah University (approval number: 20, Reference number: 98, date of approval: 31st December 2023). All methods were carried out following the "Experimentation on animals in the course of medical research and education" (CPCSEA) guidelines and AVMA 2020 guidelines for animal care[37]. The authors complied with the ARRIVE 2.0 guidelines[46].

Additional information

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