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Research Article

Bioadhesive-Thermosensitive *In Situ* Vaginal Gel of the Gel Flake-Solid Dispersion of Itraconazole for Enhanced Antifungal Activity in the Treatment of Vaginal Candidiasis

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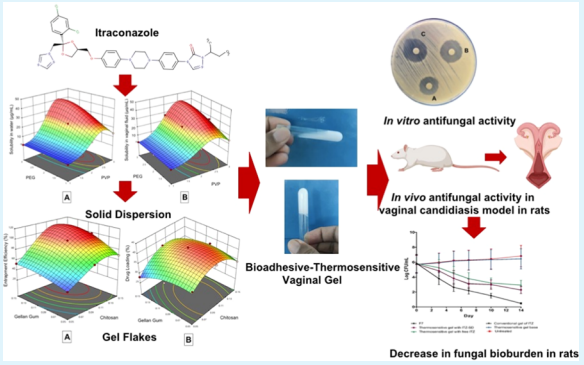
ABSTRACT: The poor solubility of itraconazole (ITZ) has limited its efficacy in the treatment of vaginal candidiasis. Accordingly, the improvement of ITZ solubility using a solid dispersion technique was important to enhance its antifungal activity. Besides, as the purpose of this research was to develop local-targeting formulations, bioadhesive-thermosensitive *in situ* vaginal gel combined with the gel-flake system was found to be the most suitable choice. To obtain optimum solubility, entrapment efficiency, and drug-loading capacity, optimization of solid dispersion (SD) and gel-flake formulations of ITZ was performed using a composite central design. The results showed that the optimized formulation of SD-ITZ was able to significantly enhance its solubility in both water and simulated vaginal fluid to reach the values of 4.211 ± 0.23 and 4.291 ± 0.21 mg/mL, respectively. Additionally, the optimized formulation of SD-ITZ gel flakes possessed desirable entrapment efficiency and drug-loading capacity. The *in situ* vaginal gel containing SD-ITZ gel flakes was prepared using PF-127 and PF-68, as the gelling agents, with the addition of hydroxypropyl methylcellulose (HPMC) as the mucoadhesive polymer. It was found that the obtained *in situ* vaginal gel provided desirable physicochemical properties and was able to retain an amount of more than 4 mg of ITZ in the vaginal tissue after 8 h. Importantly, according to the *in vivo* antifungal activity using infection animal models, the incorporation of the solid dispersion technique and gel-flake system in the formulation of the bioadhesive-thermosensitive *in situ* vaginal gel led to the most significant decrease of the growth of *Candida albicans* reaching <1 log colony-forming units (CFU)/mL or equivalent to <10% of the total colony after 14 days, indicating the improvement of ITZ antifungal activity compared to other treated groups. Therefore, these studies confirmed a great potential to enhance the efficacy of ITZ in treating vaginal candidiasis. Following these findings, several further experiments need to be performed to ensure acceptability and usability before the research reaches the clinical stage.



KEYWORDS: *vaginal candidiasis, itraconazole, solid dispersion, bioadhesive, thermosensitive in situ vaginal gel*

1. INTRODUCTION

Candida albicans (CA) is one of the fungi found as normal human microbiota in several parts of the body such as the oral cavity, reproductive, and gastrointestinal tract.[1] However, since CA is known as an opportunistic pathogen, its colonization in human tissues and organs can cause serious and invasive infection, especially in the event of immune compromising conditions.[2] It has been reported that CA was one of the major causes of hospital-acquired systemic infections in the United States, responsible for up to 50% of the mortality rate. Essentially, there are two types of infections caused by CA, namely, superficial infections (oral or vaginal candidiasis) and life-threatening systemic infections.[1] As a type of CA infection, vaginal candidiasis is one of the highest prevalence of candidiasis suffered by around 75% of women over the world.[3] In normal conditions, the vagina has its defense mechanism against CA through the presence of lactobacilli and the specific immune responses. This immune system is able to tolerate and maintain the presence of the commensal Y-cells of CA in a low number. This further prevents the change of the Y-type of CA to the pathogen H-type of CA, which causes vaginal candidiasis.[4] However, the failure of this tolerance

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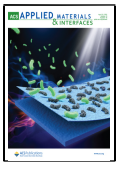

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mechanism leads to the overgrowth of this pathogen, resulting in inflammation in the vaginal epithelium.[5]

Currently, antifungals are used as the main therapy for vaginal candidiasis treatment, especially azole derivative groups.[6] Itraconazole (ITZ), one of the third generation of azoles, is mostly preferred to be applied for vaginal candidiasis compared to other types of azole antifungals. For this role, ITZ has several advantages compared to other azole antifungals, for instance, a better pharmacokinetic profile, lower nephrotoxic effects, and the ability to treat resistant types of fungal infection.[6,7] ITZ, which selectively inhibits the synthesis of the fungal cell membrane, is recently available in oral dosage form with a dose of 200 mg and the administration is twice daily for vaginal candidiasis treatment.[8,9]

The currently available treatment for vaginal candidiasis is intravaginal and oral dosage forms. Intravaginal administration includes the application of cream and vaginal pessaries.[10,11] Despite the fact that cream is one of the most favorable topical formulations due to its good spreadability and ease of use, the cream is not appropriate for vaginal use due to its greasiness, resulting in discomfort for the patients.[12] Additionally, the use of vaginal pessaries leads to several complications, such as inconvenience and allergic issues in patients.[13] In the case of oral administration, this system provides several drawbacks such as systemic adverse effects, namely, cholestatic and hepatocellular damage.[9] Accordingly, to enhance the antifungal effect for treating the vaginal candidiasis and to avoid the side effects, topical dosage forms could be an option as it provides direct and local effects on the target site. Previously, several studies have been conducted to develop local targeting of ITZ, including microarray patches for cutaneous candidiasis,[7] cream formulations for cutaneous mycoses,[14] and *in situ* gel for vaginal candidiasis.[15] However, the development of ITZ for vaginal infection was not widely explored in the previous studies. In addition, the lack of specific evaluations regarding the drug delivery to the target site was missing, which was considered as an essential matter. As an alternative for topical administration, the gel-based formulation is preferred compared to cream as it provides nonsticky properties and has a simple manufacturing process.[12] Unfortunately, for vaginal application, the water-based topical formulation can be easily rinsed by the vaginal physiological fluid. To overcome this problem, *in situ* gel is developed using thermogelling components, which provide the transformation of a free-flow solution into a solid gel at body temperature. Therefore, *in situ* vaginal gel is proposed in this study, as expected, to provide longer contact with the vaginal tissue, which finally increases the absorption of the drug and efficacy of the treatment.[16]

The physicochemical properties of ITZ, including high lipophilicity and low solubility in water, were found to be a challenge in the development of prolonged release topical preparation. Accordingly, in the present work, several novel formulation strategies were applied to develop gel-based formulations that can generate the sustained release profile of ITZ. First, due to the low aqueous solubility of ITZ, solid dispersion was developed to improve the aqueous solubility of ITZ using water-soluble polymers.[17] Second, the development of ITZ containing solid dispersion powder into the gel-flake system. Gel flakes have the shape of thread-like forms with polygonal structures, which offer a good advantage for ITZ to be entrapped within the highly folded epithelial surfaces of the vagina, leading to extended contact with vaginal mucus and controllable drug release.[5] Moreover, the gel system in this study was formulated using the thermosensitive polymer to prepare a liquid formulation that can transform into gel due to the higher temperature in the vagina. Hence, in this paper, for the first time, we proposed the combination of solid dispersion, gel flakes, and *in situ* gel formulation technologies to generate local targeting of ITZ for the vaginal candidiasis treatment. In addition, several comprehensive evaluations were performed following the gel formulations, namely, physicochemical characterization, *in vitro* permeation and retention test using vaginal tissue, and *in vivo* studies using suitable animal models.

2. EXPERIMENTAL SECTION

2.1. Materials. Itraconazole (purity, ≥98%) of analytical grade was purchased from Tokyo Chemical Industry (Tokyo, Japan). Gellan gum, chitosan, Pluronic F127 (PF-127), poly(vinylpyrrolidone) (PVP) K-30, and poly(ethylene glycol) (PEG) 6000 were purchased from Sigma-Aldrich Pte Ltd. (Singapore, Singapore). Pluronic F68 (PF-68) was kindly gifted by BASF SE (Jakarta, Indonesia). All other chemicals were of pharmaceutical grade.

2.2. Preparation of Solid Dispersion of ITZ (SD-ITZ). Certain amounts of ITZ, PEG 6000, and PVP K-30 were dissolved in 25 mL of chloroform. The solvent was subsequently evaporated at 45 °C using a water bath. The mixture was then stored in a vacuum oven at room temperature for 48 h to remove the solvent residue. Afterward, the dry solid dispersion powder was milled and sieved to obtain a fine powder of SD-ITZ. The powder was stored in desiccators prior to characterization. The amount of all materials was determined based on the optimized formulation. A formulation that resulted in the highest solubility in both solvents was determined as the best formulation.

2.3. Optimization of SD-ITZ Formulations. The SD-ITZ formulations were optimized using a composite central design (CCD) with Design-Expert Software version 11 (State-Ease, Minneapolis, MN). Two main factors affecting the SD-ITZ formulations were selected as the variables in the optimization process, namely, PVP and PEG 6000 concentrations. Both factors were analyzed at three different levels. The level of PVP and PEG 6000 concentrations were 1% w/v, 2% w/v, and 3% w/v for low, medium, and high, respectively. Additionally, the solubility of the formulations in water and simulated vaginal fluid was recorded as the responses. The amount of ITZ, 1 g, was kept constant in all formulations.

2.4. Characterization of SD-ITZ Formulations. The solubility tests were performed in water and simulated vaginal fluid. Excess amounts of the optimized SD-ITZ formulations and free ITZ were dissolved in both solvents under stirring at 200 rpm at 37 °C. After 24 h, the mixture was filtered using a 0.2 μm syringe filter to obtain a clear solution. The filtrate was subsequently analyzed using a validated high-performance liquid chromatography (HPLC) method.

Any chemical interaction between the drug and the excipients was observed using Fourier transform infrared (FT-IR) spectroscopy (Accutrac FT/IR-4100 Series, Perkin Elmer). The spectra of free ITZ, physical mixture of ITZ and the excipients (PM), and the optimized SD-ITZ formulation were recorded between 400 and 4000 cm^{-1} using 32 scans with a resolution of 4.0 cm^{-1} at room temperature.

The crystallinity of free ITZ, PM, and the optimized SD-ITZ formulation was observed using an X-ray diffractometer (Rigaku Corporation, Kent, England).

The thermal properties of free ITZ, PM, and the optimized SD-ITZ formulation were evaluated using a DSC Q100 (DSC 2920, TA Instruments, Surrey, U.K.). A specific amount of sample was heated in a sealed aluminum pan from 0 to 300 °C with a heating rate of 10 °C/min.

The morphologies of free ITZ and SD-ITZ powder were investigated using a scanning electron microscope (SEM) (JEM-1400Plus; JEOL, Tokyo, Japan).

2.5. *In Vitro* Release Studies of SD-ITZ. *2.5.1. Preparation of Simulated Vaginal Fluid.* The simulated vaginal fluid was used as the

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release medium to mimic the vaginal environment. The simulated vaginal fluid was prepared according to Owen and Katz.[18,19] The compositions of the stimulated vaginal fluid were sodium chloride (3.51% w/v), potassium hydroxide (1.4% w/v), calcium hydroxide (0.22% w/v), bovine serum albumin (0.018% w/v), lactic acid (2% w/v), acetic acid (1% w/v), glycerol (0.16% w/v), urea (0.4% w/v), and glucose (5% w/v). The pH of the final solution was 4.6.

2.5.2. In Vitro Drug Release Evaluation. This evaluation aimed to compare the release profiles of free ITZ and SD-ITZ formulations. The release studies were performed using a dialysis method with the simulated vaginal fluid with the addition of 1% Tween 80 as the release medium. The addition of Tween 80 aimed to ensure the sink conditions during the experiment. The free ITZ and SD-ITZ powder, equivalent to 50 mg of ITZ, was each dispersed into the Spectra-Por, 12 000−14 000 MWCO dialysis membrane (Spectrum Medical Industries, Los Angeles, CA). The bags were subsequently placed in 100 mL of the release media at 37 °C in an orbital shaker at 100 rpm. Aliquots of 1 mL of sample were taken at the predetermined time and replaced by 1 mL of the fresh release medium to maintain the sink conditions. To calculate the amount of drug released, the samples were analyzed using the validated HPLC method. Moreover, the obtained drug release profiles were further fitted to different mathematic kinetic models, namely, zero-order, first-order, Higuchi, Korsmeyer−Peppas, and Hixson−Crowell.[20] The model parameters were calculated using the DDSolver.

2.6. Fabrication of SD-ITZ Gel Flakes. The gel flakes containing solid dispersion of ITZ were prepared using a drop−stir technique. Initially, a certain amount of gellan gum was stirred continuously in 50 mL of water for 15−20 min at a boiling temperature. The obtained mixture was subsequently placed at room temperature allowing the mixture to cool down. SD-ITZ with a concentration of 1% w/v was added into the gellan gum solution. Afterward, the obtained solution was dropped into 50 mL of chitosan solution in 1% acetic acid solution. This dropping process was performed under continuous vigorous stirring. The composition of the gel-flake formulation was determined by the optimization process.[5]

2.7. Optimization of SD-ITZ Gel-Flake Formulations. The optimization of SD-ITZ gel-flake formulations was carried out using a charge-coupled device (CCD) with Design-Expert Software version 11. There were two main factors used in this process, namely, chitosan and gellan gum concentrations. Both of these variables were expected to affect the SD-ITZ gel-flake formulations in terms of entrapment efficiency and drug loading, which were recorded as responses. All factors were analyzed at three different levels. The level of chitosan and gellan gum concentrations were 0.05% w/v, 0.1% w/v, and 0.15% w/v for low, medium, and high, respectively. The amount of SD-ITZ, 1 g, was maintained in all formulations. A formulation, which resulted in the highest entrapment efficiency and drug loading, was determined as the best formulation.

2.8. Characterization of SD-ITZ Gel Flakes. To determine encapsulation efficiency (EE), each formulation was first filtered using a 0.2 μm syringe filter. Subsequently, the filtrate was measured using the validated analytical HPLC method. The percentage of EE was calculated using eq 1[5]

$$\%\mathrm{EE} = \frac{W_0 - W_f}{W_0} \times 100 \quad (1)$$

where W_0 is the amount of drug added in the formulation and W_f is the amount of drug measured in the filtrate.

The drug-loading (DL) capacity of ITZ in the gel-flake formulation was calculated by initially dissolving 10 mg of gel flakes in 10 mL of water. This process was followed by sonicating the suspension using a bath sonicator for 1 h. Subsequently, the suspension was centrifuged at 14 000 rpm for 15 min. The supernatant was taken out and quantified using HPLC. The percentage of drug loading was calculated using eq 2[21]

$$\%\mathrm{DL} = \frac{\text{amount of entrapped ITZ}}{\text{total weight}} \times 100 \quad (2)$$

Any chemical interaction between the drug and the excipients was observed using FT-IR spectroscopy. The spectra were recorded between 400 and 4000 cm^{-1} using 32 scans with a resolution of 4.0 cm^{-1} at room temperature.

Differential scanning calorimetry (DSC) was used to evaluate the thermal properties of each formulation. A given amount of formulation, sealed in an aluminum pan, was heated from 0 to 300 °C with a heating rate of 10 °C/min.

The morphology of the obtained gel-flake formulation was observed using a SEM.

2.9. *In Vitro* Antimicrobial Activity Test of SD-ITZ Gel Flakes. The antimicrobial activity test of free ITZ, SD-ITZ, and SD-ITZ gel flakes was conducted using a diffusion agar method. Initially, the growth medium of Sabouraud agar containing 50 mg/mL chloramphenicol was prepared in a Petri dish. The inoculum of *C. albicans* was subsequently spread to cover all of the surfaces of the agar. An amount that was equal to 2.5 μg/mL of free ITZ, SD-ITZ, and SD-ITZ gel-flake solutions was dropped onto the paper discs, which were set up on the agar surface. The Petri dish was subsequently incubated at 32 °C for 48 h. The diameter of the inhibitory zone of each sample tested was measured using digital vernier calipers.

2.10. Preparation of Itraconazole Flake-Loaded Thermosensitive *In Situ* Gel. The thermosensitive gel loaded with SD-ITZ flake formulations was prepared using the cold method.[22] An amount of PF-127, as the gelling agent, was dispersed in cold water (5 °C) at room temperature (20 °C). Separately, benzalkonium chloride (BKC), hydroxypropyl methylcellulose (HPMC), and hydroxyethyl cellulose (HEC) were each dissolved in water and then added into the PF-127 solution. The mixture was then gently mixed and placed in a refrigerator at 10 °C for 24 h. The SD-ITZ flakes were then added into the PF-127 solution while stirring using a magnetic stirrer at a speed of 200 rpm for 10 min. The thermosensitive composition of the gel containing the SD-ITZ gel flakes is shown in Table 1.

Table 1. Composition of Gel Flakes Loaded with SD-ITZ Formulations (% w/v)

formula	SD-ITZ gel flakes (equal to pure ITZ)	PF-127	PF-68	BKC	HPMC	HEC
F1	1	20.00		0.01		
F2	1	17.50	2.50	0.01		
F3	1	15.00	5.00	0.01		
F4	1	12.50	7.50	0.01		
F5	1	10.00	10.00	0.01		
F6	1	15.00	5.00	0.01	0.20	
F7	1	15.00	5.00	0.01	0.40	
F8	1	15.00	5.00	0.01	0.60	
F9	1	15.00	5.00	0.01		0.20
F10	1	15.00	5.00	0.01		0.40
F11	1	15.00	5.00	0.01		0.60

2.11. Characterization of SD-ITZ Flakes *In Situ* in Vaginal Gel Formulations. *2.11.1. Gelation Temperature ($T_{sol-gel}$) Measurement.* The determination of the gelation temperature was carried out using a test tube inverting method.[23] This evaluation was subjected to two different conditions, such as with and without dilution. A volume of 2 mL of each gel formulation at 4 °C was taken and then placed in a closed test tube. After that, the test tube was immersed in water at 20 °C and then the temperature was gradually increased by 1 °C each time to reach 65 °C. Each formulation was visually observed by turning the tube over to 90 °C for each temperature. The gelation temperature was recorded as the lowest temperature at which the solution turned into a gel and could not flow over 30 s when inverted. The same technique was applied to the dilution gel with the addition of 0.25 mL of vaginal fluid prior to evaluation.

2.11.2. Mucoadhesion Strength. The measurement of mucoadhesion strength was performed using a modified physical balance

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according to Morsi et al. with a slight modification.[24] A freshly excised cow vaginal tissue was fixed onto two glass vials with the mucosa faced outside. One vial was connected to the balance in the inverted position, while the second vial was placed on a height-adjustable pan. The gel was placed onto the mucosa of the first vial. Subsequently, the second vial was adjusted until both of the mucosa contacted tightly and this condition was kept for 2 min. Weights were added onto the pan until both vials got detached. The minimum weight used to detach both vials was recorded, and the mucoadhesive strength was calculated using eq 3

$$\text{mucoadhesive strength (dyne cm}^2) = \frac{m.\ g}{A} \quad (3)$$

where m is the weight used for detachment (g), A is the area of mucosa exposed (cm^2), and g is the acceleration due to gravity (980 cm/s^2).

2.11.3. Mucoadhesion Time. Mucoadhesion time of the *in situ* thermovaginal gel was evaluated using USP dissolution test apparatus 2, also known as paddle apparatus.[25] Initially, the freshly excised cow vaginal mucosa was flattened and clamped on the paddle surface. A certain amount of gel was placed onto the mucosa prior to immersion in the vessel containing simulated vaginal fluid. The experiment was performed at 37 °C, and the rotation of the paddles was set at 100 rpm. The mucoadhesion time was recorded as the residence time of gel before being completely dissolved in the medium.

2.11.4. pH Measurement. The pH of the formulations was measured using a pH meter (Horiba Scientific, Kyoto, Japan) in triplicate at 25 °C.

2.11.5. Viscosity and Rheological Study. The rheology of the *in situ* thermosensitive SD-ITZ gel flakes was measured using a DV-III viscometer (RV model, Brookfield). The viscosity of the gel was measured at different temperature conditions: storage temperature (4 °C), test temperature (25 °C), and vaginal physiological temperature (37 °C). The viscometer is adjusted to a spindle speed of 50 rpm using spindle 07.

2.11.6. Drug Content Analysis. Fifty milligrams of the thermosensitive gel loaded with SD-ITZ gel flakes was dissolved in methanol up to 100 mL in a volumetric flask. This solution was subsequently sonicated for 4 h. Afterward, the solution was centrifuged at 14 000 rpm for 15 min. The supernatant was analyzed by HPLC.[21]

2.12. *Ex Vivo* Permeation Studies. The *ex vivo* permeation studies of the optimized thermosensitive vaginal gel loaded with SD-ITZ flakes were performed using the vertical Franz cell with a diffusion area of 4.9 cm^2. Cow vaginal mucosa was used in this work to facilitate the drug permeation from the donor to the receptor compartments. The receptor compartment was filled with 24 mL of simulated vaginal fluid and stirred at 100 rpm with the temperature maintained at 37 ± 1 °C. A total of 1 mL of each formulation was placed on the donor compartment. An aliquot of 1.5 mL was taken from the receptor compartment at the predetermined time points until 8 h and then replaced with the same volume of release medium to maintain sink conditions. All samples were further analyzed using HPLC.[25]

2.13. *Ex Vivo* Recovery and Extraction Assays. After completing the permeation studies, the vaginal mucosa was taken from the cells and soaked for cleaning in 0.05% solution of sodium dodecyl sulfate. The tissue was finally rinsed using distilled water. The extraction of ITZ from the mucosa was performed using a mixture of methanol−water (70:30 v/v) and sonicated for 20 min. Subsequently, the solution was centrifuged at 5000 rpm for 30 min. The supernatant obtained was measured using HPLC.[25]

2.14. *In Vivo* Antifungal Activity in the Model of Infection on Rats. *2.14.1. Preparation of the Candidiasis Model on Rats.* Prior to the inoculation of *Candida albicans* (CA), eighteen female Wistar rats were placed in an individual cage and fed with enough food and water for at least 7 days. Afterward, all of the rats were treated with estradiol benzoate with a dose of 25 mg/kg subcutaneously every 2 days during the experiment. It was aimed to keep all of the animals in pseudoestrus conditions. For the infection technique, CA was first inoculated in sterile saline to obtain a concentration of 10^8 colony-forming units (CFU)/mL. Subsequently, intravaginal inoculation of CA was applied to the animals.[26]

2.14.2. In Vivo Antifungal Activity. The *in vivo* antifungal activity of the optimized formulation was tested on 18 infected models of female Wistar rats. The animals were divided into six groups of different treatments, namely, an optimized thermosensitive gel containing SD-ITZ flakes, thermosensitive gel containing SD-ITZ, thermosensitive gel containing pure ITZ, conventional gel containing ITZ, thermosensitive gel without the drug, and negative control without any treatment. One gram of each gel was applied to the vagina of the animals. At the specified time points, 1 μL of the vaginal fluid was taken and diluted further to be inoculated in Sabouraud agar containing 50 mg/mL chloramphenicol. The inoculum was incubated at 32 °C for 48 h before CFU counting.[27] This experiment was performed over 14 days and based on ethical approval from the Ethical Committee from the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, with a protocol number UH20070336.

2.15. Instrumentation and Chromatographic Conditions for the ITZ Analytical Method. The analysis of ITZ in all of the samples was performed using HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan) using a Phenomenex Luna C_{18} (ODS1) column (150 mm × 4.6 mm id with 5 μm particle size). The mobile phase consisted of 25 mM ammonium acetate buffer, pH 5, as the aqueous phase, and acetonitrile, as the organic phase with a ratio of 65:35 v/v. The flow rate was set at 1 mL/min, and the drug was detected at 270 nm using a UV detector. The injection volume was 50 μL, and the analysis was carried out at ambient temperature. This method was validated according to the International Committee on Harmonization (ICH) guidelines 2005.[7] The analytical method validation was performed in the concentration range of 0.25−50 μg/mL. The limit of detection and limit of quantification values were found to be 0.27 and 0.56 μg/mL, respectively.

2.16. Statistical Analysis. All of the work was done in triplicates and presented as mean ± SD. Statistical difference was tested using analysis of variance (ANOVA; GraphPad Prism 6.0, GraphPad, San Diego, CA). Student's *t*-test was used to compare the mean values between groups. The obtained $p < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Preparation of SD-ITZ. Solid dispersion is a technique to enhance the solubility of poorly water-soluble drugs using the water-soluble polymer. In the present work, the solvent evaporation method was used to fabricate the SD-ITZ powder due to avoidance of heat, which might cause drug degradation. Additionally, a more homogeneous mixture was obtained using this method.[28] PVP and PEG were found to be the most commonly used solubility enhancer due to their high water solubility and low toxicity.[29] Therefore, the combination of PVP and PEG 6000 in different ratios was chosen in the preparation of SD-ITZ. To determine the exact ratio of both materials and to obtain the maximum improvement in the water solubility of ITZ, optimization was performed software-aided CCD. As shown in Table S1, there were nine formulations suggested for the optimization with three different concentrations of PVP and PEG. The results showed that the ITZ solubility in water and simulated vaginal fluid were in the range of 0.04−2.64 and 0.07−2.71 mg/mL, respectively. Based on the fit statistical analysis results, the difference between the predicted R^2 value and adjusted R^2 value was less than 0.2, which met the requirement to be optimized. Additionally, the values of adequate precision were 84.783 and 42.989 for ITZ solubility in water and simulated vaginal fluid, respectively. These values were found to be desirable as the recommended value was higher than 4. The

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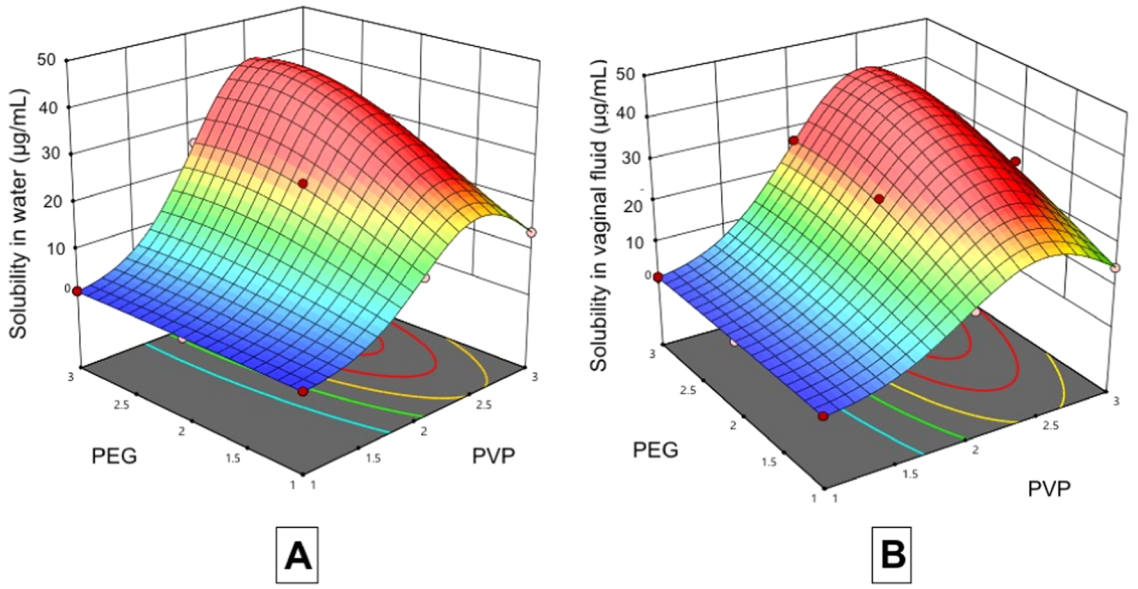

Figure 1. Representative response surface plots showing the effect of the selected factors on the solubility of ITZ in (A) water and (B) simulated vaginal fluid.

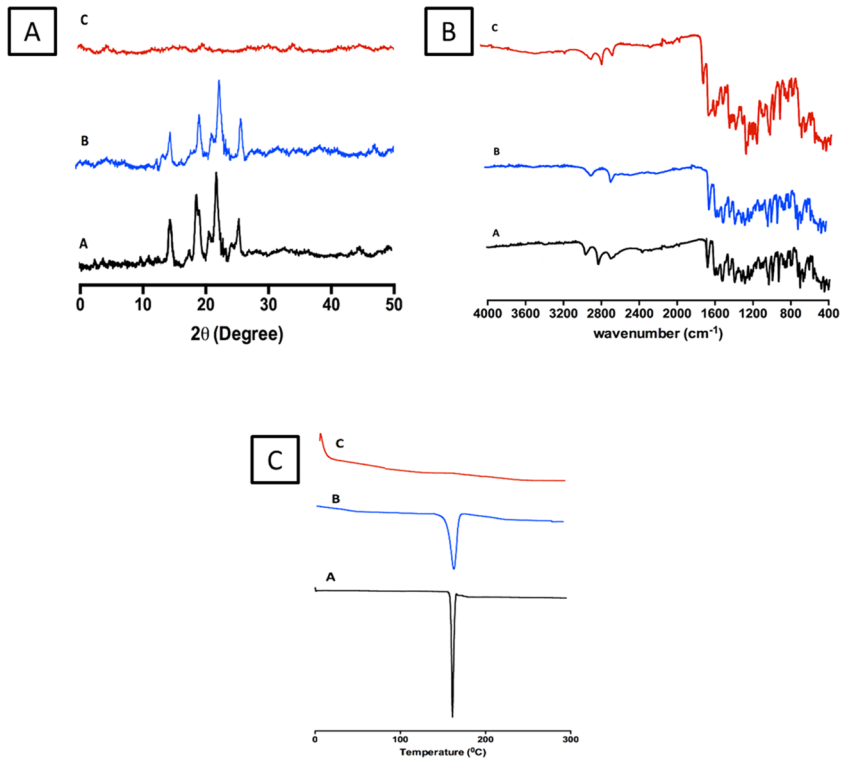

Figure 2. Results of (A) X-ray diffraction (XRD), (B) FT-IR, and (C) DSC analysis of free ITZ (A, black line), physical mixture of ITZ and other excipients (B, blue line), and SD-ITZ (C, red line).

results of these parameters showed that this model was acceptable to design the optimum formulation of SD-ITZ. The results of the effect of the concentrations of PVP and PEG on the solubility of ITZ are shown in Table S1.

3.2. Optimization of SD-ITZ Formulations. The solubility of ITZ in water and simulated vaginal fluid was the parameter observed in this optimization process. The results showed that ITZ solubility in both solvents fitted to the quadratic model. It was found that the *F*-values of the analysis were 1418.91 and 360.61 for water solubility and simulated vaginal fluid solubility, respectively. Additionally, both of the *p*-values were <0.001, indicating that the optimization in this process had a significant role in ITZ solubility. The representative three-dimensional (3D) graph illustrating the effect of selected factors on the solubility of ITZ in water and simulated vaginal fluid is shown in Figure 1. Specifically, the *p*-values analyzed from the effect of PVP and PEG on ITZ solubility in water and simulated vaginal fluid were found to be <0.001 and >0.05, respectively. Therefore, despite the increase in the solubility of ITZ with PEG, the effect of PEG was found

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to be nonsignificant. Moreover, the *p*-value analyzed from the combination of PVP and PEG was <0.001. From these models, it can be clearly seen that the solubility of ITZ was directly proportional to the combination of PVP and PEG. The increase of the amount of PVP and PEG in the formulation was able to generate higher solubility of ITZ. In the 3D graph presented in Figure 1, it can be seen that although the solubility of ITZ increased with the increase of the PVP concentration, the use of PEG was able to produce higher solubility of ITZ in both fluids when combined with PVP. This might be caused by the properties of both substances as polymeric carriers, possessing greater dispersibility and ability to inhibit the recrystallization phenomenon of the drug.[30] Finally, based on the software analysis, the concentrations of 2.487 and 2.294% of PVP and PEG, respectively, were recommended for SD-ITZ formulation to obtain the optimum solubility of ITZ.

3.3. Characterization of SD-ITZ Formulations. According to the optimization process, several solutions were recommended by the software and ranked based on the desirability factor. The most recommended formulation was prepared in triplicates. Subsequently, the formulation was evaluated to determine ITZ solubility in water and simulated vaginal fluid. The solubility of ITZ in water and simulated vaginal fluid was 4.211 ± 0.23 and 4.291 ± 0.21%, respectively. The comparison between the actual and predicted values is presented in Table S2. It was found that the difference between the obtained and predicted solubility in both solvents was less than 15%, indicating that the computationally designed optimization processes had been successful. Since ITZ is practically insoluble in water (1 ng/mL),[9] the results obtained revealed that the solid dispersion technology was able to significantly enhance the ITZ solubility. Basically, there were several mechanisms of solid dispersion to enhance the solubility, including a reduction in the drug particle size, formation of an amorphous state, increasing the porosity, and improving wettability.[31] The use of hydrophilic polymers with a strong ability to prevent the aggregation of drug crystals further increased the wettability of ITZ and transformed the ITZ into an amorphous state.[17,30]

Several peaks were observed, which were attributed to the specific chemical groups of ITZ. A major peak was found at 1689 cm^{-1}, corresponding to the imine group C═N stretch (1690−1650 cm^{-1}) and N−C═O stretch (1680−1630 cm^{-1}). Another peak at 1511 cm^{-1} was attributed to C═C, characteristic of the aromatic ring of ITZ (1600−1475 cm^{-1}). Additionally, a peak, observed at 1221 cm^{-1}, corresponded to C−N stretching (1350−1000 cm^{-1}). Other signals found at 1192 and 823 cm^{-1} can be attributed to the C−O functional group (1300−1000 cm^{-1}) and the para-substituted aromatic ring (870−800 cm^{-1}), respectively. Finally, a peak at 685 cm^{-1} was assigned to the C−Cl group present in ortho- and para-substituted aromatic ring (785−540 cm^{-1}).[7] From Figure 2, it can be seen that all of the characteristic peaks of ITZ were also present in the spectra of PM and SD-ITZ formulations. These results confirmed that there was no interaction between ITZ and other excipients used in the SD-ITZ preparation.

XRD analysis was performed to observe the crystalline properties of ITZ before and after SD preparation. Figure 2A,B presents the spectra of pure ITZ, PM, and SD-ITZ. The results showed sharp peaks observed at 2θ values of 14.87, 17.95, 20.35, and 23.67 in ITZ and PM, respectively.[7] However, these peaks were not found in SD-ITZ spectra. This might be explained as follows: in the solid dispersion system, the drug was molecularly dispersed in the polymer matrix, creating an amorphous polymer matrix.[32] Regarding the solubility, the absence of crystalline lattice in the amorphous state of the drug results in lower energy required in the solubilization process.[33] This means that the preparation of SD-ITZ was able to change the form of ITZ from a highly crystalline to an amorphous state, which had a significant role in enhancing the water solubility of ITZ.

The thermal properties of pure ITZ, PM, and SD-ITZ were evaluated using DSC, as shown in Figure 2C. A sharp endothermic peak at 165 °C, representing the melting point of ITZ, was observed in the spectra of pure ITZ and PM. The same endothermic peak was not found in the SD-ITZ thermogram. This was in good agreement with the obtained XRD results related to the crystallinity of ITZ after SD preparation. As the form of ITZ changed to an amorphous form due to the SD preparation process, the endothermic peak was no longer detected.

To identify the effect of the solid dispersion system on the morphology of ITZ, the obtained SD-ITZ powder was observed using a SEM. From Figure 3A, it can be clearly seen that the pure ITZ was in the crystalline form. In contrast, the SD-ITZ powder was seen in an irregular form, indicating the amorphous state of ITZ. This agreed with the results of the previous evaluation, confirming that the solid dispersion technique was able to change the form of ITZ, which subsequently affect its solubility.

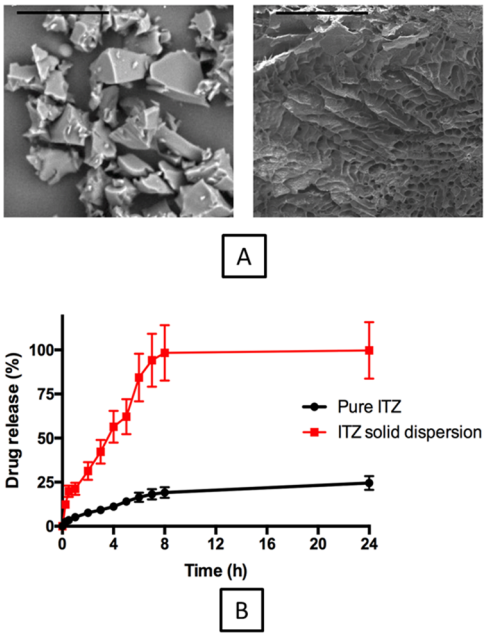

Figure 3. (A) Morphologies of free ITZ (left) and SD-ITZ (right) observed under SEM with the scale representing 50 μm. (B) Drug release profiles of pure ITZ (black line) and SD-ITZ (red line) (mean ± SD, n = 3).

3.4. *In Vitro* Release Studies of SD-ITZ. The *in vitro* release profiles of ITZ compared to SD-ITZ formulation are shown in Figure 3. Based on the results obtained, it was found that the SD formulation was able to significantly increase the release behavior of ITZ. After 24 h, the amount of ITZ released from SD-ITZ formulation was 99.76 ± 15.97%.

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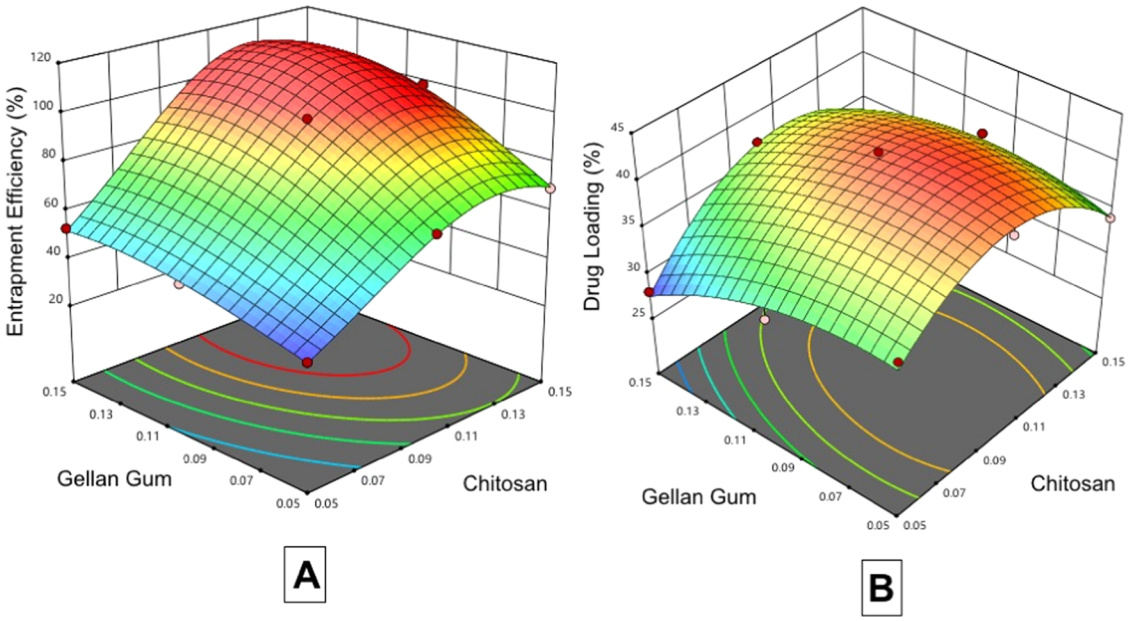

Figure 4. Representative response surface plots showing the effect of the selected factors on (A) EE and (B) DL of the optimized formulations.

Contrastingly, the free ITZ only reached 23.76 ± 3.92%, which was around four times lower than the release behavior of SD-ITZ formulation. These values were statistically different, which clearly confirmed that the increase in ITZ solubility had a significant effect on the release profiles ($p < 0.005$). It was as expected that the reduction of particle size, the transformation to the amorphous state, and the increase of wettability and dispersibility were able to decrease the interfacial tension between the drug and the dissolution medium, resulting in a better release behavior.[31,34]

Several models of mathematical kinetics were then applied to the release profiles to investigate the release mechanism of ITZ from SD formulation. The results showed that the correlation coefficients for zero-order, first-order, Higuchi, Korsmeyer−Peppas, and Hixson−Crowell were found to be 0.53, 0.96, 0.71, 0.83, and 0.61, respectively. As the release mechanism model was selected based on the correlation coefficient value, the first-order kinetic model was considered as the appropriate model. Accordingly, it can be concluded that the concentration of ITZ in the SD affected the release of ITZ.[20]

3.5. Fabrication of SD-ITZ Gel Flakes. The drop−stir technique was utilized to prepare SD-ITZ gel flakes as it provides a rapid and easy way without many external interventions such as high temperature or organic solvents.[5] Gellan gum is one of the anionic heteropolysaccharide derivatives containing gluconic acid. Chitosan is a cationic polysaccharide produced by the deacetylation of chitin. Both of these substances were extensively used in pharmaceutical research due to their biodegradability and biocompatibility.[35] In this part of work, gellan gum and chitosan were the most important factors in the preparation of gel flakes. Hence, these two parameters were selected to be optimized using CCD with Design-Expert software. As a result, the responses expected by varying these factors were entrapment efficiency and drug loading. Nine formulations were provided to be optimized with three different concentrations of gellan gum and chitosan, as shown in Table S3. The results exhibited that the entrapment efficiency and drug loading of ITZ in the gel flakes were in the range of 37.65−98.91 and 28.05−42.11%, respectively. In terms of statistical analysis, it was found that the difference between predicted R^2 and adjusted R^2 of both responses was less than 0.2, which means that the models designed were acceptable to be optimized. Furthermore, the adequate precision values, which measure the ratio of signal-to-noise in the analysis, were 51.220 and 14.148 for entrapment efficiency and drug loading, respectively. As the requirement of these values should be greater than 4, it means that the values obtained were desirable for the optimization process. The results of the effect of the concentrations of chitosan and gellan on EE and DL of ITZ are shown in Table S2.

3.6. Optimization of SD-ITZ Gel-Flake Formulations. The results showed that the EE and DL of all optimization processes followed the quadratic model. The *F*-values obtained were 362.23 and 19.30 for the responses of EE and DL, respectively. Additionally, $p < 0.001$ was found in all models, indicating that the concentration of gellan gum and chitosan, as the variables, had a significant role in affecting the amount of drug entrapped in the gel flakes, in terms of EE and DL. The relationship between the factors selected and the expected responses is shown in Figure 4. From this graph, it can be seen that the increase of gellan gum and chitosan concentration was followed by the improvement in EE and DL. The higher concentration of gellan gum and chitosan would allow more intense cross-linking, resulting in a higher amount of drug entrapped.[36] Finally, the composition of 0.094% of gellan gum and 0.092% of chitosan was the suggested formulation to obtain the maximum EE and DL percentage.

3.7. Characterization of SD-ITZ Gel-Flake Formulations. In the gel-flake system, ITZ was entrapped in a gel layer caused by the gelation of gellan gum in a cationic environment (chitosan solution). This could occur due to ionic gelation between carboxylate ions of gellan gum and cationic moieties of the amino groups of chitosan.[5] The percentage of EE represented the measured amount of drug entrapped in the gel-flake system in comparison to the theoretical amount. The optimum formulation, recommended by the software, was prepared and used for further investigation. The predicted

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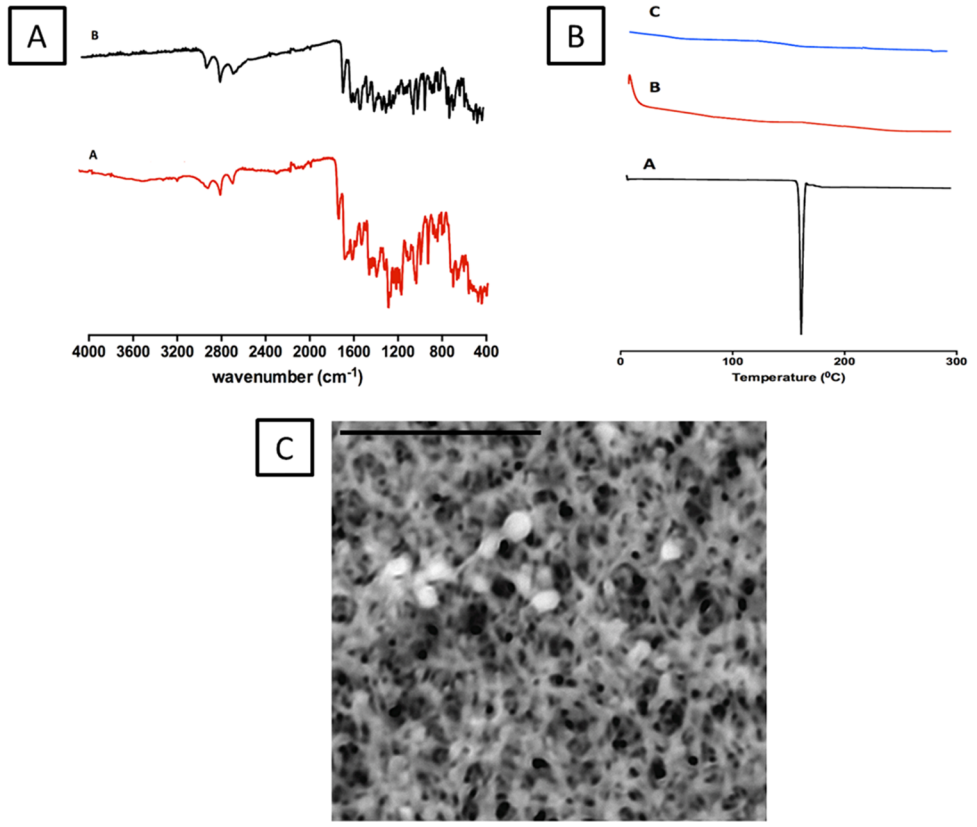

Figure 5. (A) FT-IR results of free ITZ (A, red line) and SD-ITZ gel flakes (B, black line). (B) DSC results of free ITZ (A, black line), SD-ITZ (B, red line), and SD-ITZ gel flakes (C, blue line). (C) Morphology of SD-ITZ gel flakes observed under SEM with the scale representing 50 μm.

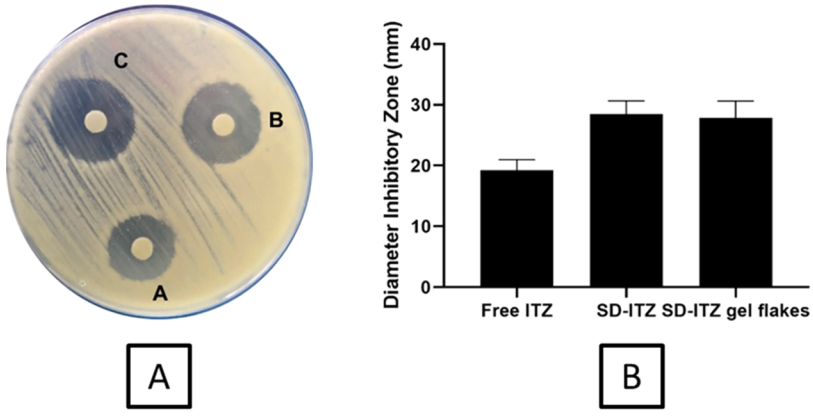

Figure 6. (A) Image of the inhibitory zone of free ITZ (A), SD-ITZ (B), and SD-ITZ gel flakes. (B) Results of antimicrobial activity of free ITZ, SD-ITZ, and SD-ITZ gel flakes in the bar graph (mean + SD, n = 3).

value of EE was 98.978%. Indeed, the actual value obtained was 98.87 ± 7.42% (Table S4). The bias between the predicted and actual values was less than 15%, indicating that the software was successful to suggest the optimum formulation.

The DL measurement aimed to determine the amount of drug contained in the gel-flake system. The actual value of drug loading obtained was 42.98 ± 1.98%. This value had less than 15% bias from the predicted value (Table S4). These results suggested that the software utilized in the optimization processes was reliable to provide the formulation to obtain the optimum value of DL.

FT-IR spectroscopy was utilized to assess the chemical groups of ITZ in the gel flakes compared to the pure ITZ. The results, as shown in Figure 5A, revealed that the characteristic peaks of ITZ were present in the spectra of SD-ITZ gel flakes. A major peak, which was found at 1689 cm^{-1}, corresponded to the imine group C═N stretch (1690−1650 cm^{-1}) and N−C═O stretch (1680−1630 cm^{-1}). Another peak that appeared at 1511 cm^{-1} was attributed to the double bond group, C═C, characteristic of the aromatic ring of ITZ (1600−1475 cm^{-1}). Furthermore, a peak was observed at 1221 cm^{-1}, corresponding to C−N stretching (1350−1000 cm^{-1}). Other signals found at 1192 and 823 cm^{-1} were attributed to the C−O functional group (1300−1000 cm^{-1}) and para-substituted aromatic ring (870−800 cm^{-1}), respectively. Finally, a signal at 685 cm^{-1} was assigned to the C−Cl group presented in the ortho- and para-substituted aromatic ring (785−540 cm^{-1}).[7]

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Therefore, the results indicated that the preparation of gel flakes had no effect on the change of the ITZ chemical structure.

The DSC analysis results of free ITZ, SD-ITZ, and SD-ITZ gel flakes are shown in Figure 5B. The results showed that the melting point of ITZ, marked by a sharp endothermic peak, was 165 °C. This peak appeared only in the thermogram of pure ITZ, which was caused by the high crystalline part contained in the ITZ. However, there was no peak detected in SD-ITZ and SD-ITZ gel flakes due to the amorphous form of ITZ contained in both preparations. There was no difference found in the thermogram of SD-ITZ and SD-ITZ gel flakes, meaning that the preparation of gel flakes loaded with SD-ITZ did not result in any changes in terms of the degree of crystallinity.

The gel flakes were prepared using the drop−stir technique. It was reported that the vigorous stirring involved in the process played an important role to form the flakes. The absence of the stirring process would result in the form of gel spheres. Due to the morphology of the gel flakes, which provided a higher surface area, the application of gel flakes for the intravaginal purpose was preferred over the spheres.[5] The morphology of the prepared gel flakes loaded with SD-ITZ is shown in Figure 5C. It was observed that the fabricated flakes were small with polygonal structures. This form was found to be suitable to interpenetrate and spread into the highly folded vaginal surfaces.

3.8. *In Vitro* Antimicrobial Activity Test of SD-ITZ Gel Flakes. The *in vitro* antimicrobial activity test was carried out to investigate the ability of ITZ to inhibit fungal growth in different forms. The results are presented in Figure 6, showing the inhibitory zone formed as a result of antimicrobial activity of free ITZ, SD-ITZ, and SD-ITZ gel flakes. The diameter of the inhibitory zone of free ITZ was 19.23 ± 1.76 mm. Interestingly, the diameter of the inhibitory zone of 28.49 ± 2.17 mm was obtained by increasing the solubility of ITZ using the solid dispersion technique. SD-ITZ, which possessed higher solubility, would allow the drug to diffuse to the growth medium and was able to provide better permeability to the microbial cells, resulting in better antimicrobial activity.[37] Additionally, the SD-ITZ gel flakes also possessed a similar antifungal activity, showing a diameter of 27.89 ± 2.72 mm, which was not significantly different compared to SD-ITZ ($p < 0.005$). It was clear to describe that the preparation of SD-ITZ was able to enhance the antimicrobial activity of ITZ and the incorporation of SD-ITZ into the gel-flake system had no significant influence on ITZ antimicrobial activity.

3.9. Preparation of SD-ITZ Gel-Flake-Loaded Thermosensitive *In Situ* Gel. The preparation of *in situ* gel containing SD-ITZ gel flakes was performed to deliver ITZ to the target site, in the vaginal mucosa, as the treatment of vaginal candidiasis. As the presence of vaginal fluid in the vaginal physiology system is able to rinse the topical preparation applied to the vagina, the addition of the mucoadhesive polymer was important to increase the residence time. Pluronics, also known as poloxamers, are synthetic triblock copolymers containing poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO−PPO−PEO). In the present work, PF-127 and PF-68 were used as thermosensitive polymers due to their ability to undergo sol-to-gel transition at a certain temperature.[38] HPMC and HEC were natural cellulose derivative polymers used as the mucoadhesive polymers. Additionally, BKC was used as the preservative.[39] Prior to the addition of the mucoadhesive polymer, five formulations, F1−F5, were prepared and evaluated to obtain the optimum formulation, which possessed the most acceptable thermosensitive gel properties.

3.10. Characterization of SD-ITZ Gel Flakes in *In Situ* Vaginal Gel Formulations. *3.10.1. Gelation Temperature ($T_{sol-gel}$) Measurement.* Gelation temperature was determined using the test tube inverting method. This research aimed to prepare a solution transforming into gel at body temperature. Figure S1 shows the representative images of *in situ* gel formulation at room temperature (solution) and at gelation temperature (gel). It was important to note that the presence of vaginal fluid during the application of the gel might be able to dilute the gel, resulting in poor gelation properties. Additionally, the dilution with simulated vaginal fluid was able to mimic the actual conditions where the gel would be applied. Therefore, this evaluation was performed in two conditions, namely, with and without dilution with the simulated vaginal fluid. The results of this study are shown in Table 2. The results showed that F3 containing 15% PF-127 and 5% PF-68 transformed into a gel at body temperature, 37 °C, both before and after dilution, which was desirable ($p > 0.005$). It can also be observed that the higher concentration of PF-127 results in the lower $T_{sol-gel}$ temperature, which might be caused by the longer triblock chain of PF-127. It can also be seen that the $T_{sol-gel}$ of F6 and F7, with the addition of 0.2 and 0.4% of HPMC, respectively, was not significantly different both with and without dilution, which was at 37 °C ($p > 0.005$). Similar results were obtained by F9 and F10, containing 0.2% and 0.4% of HEC, respectively ($p > 0.005$). Interestingly, the increase in the concentration of the mucoadhesive polymers, HPMC and HEC, resulted in the decrease of gelation temperature. Both F8 and F11, containing 0.6% of HPMC and HEC, respectively, possessed significantly lower $T_{sol-gel}$ temperatures compared to other formulations containing mucoadhesive polymers ($p < 0.005$). It was confirmed that the concentration of mucoadhesive polymers was important to be considered in the thermosensitive *in situ* gel preparation.

Table 2. Gelation Temperature of Thermosensitive *In Situ* Gel Containing SD-ITZ Gel Flakes (Mean ± SD, $n = 3$)

	$T_{sol-gel}$ (without dilution)	$T_{sol-gel}$ (with dilution)
F1	23.12 ± 2.59	25.21 ± 2.82
F2	29.31 ± 3.28	31.52 ± 3.53
F3	37.05 ± 4.26	37.21 ± 4.28
F4	39.11 ± 4.42	41.21 ± 4.66
F5	49.52 ± 5.45	51.78 ± 5.70
F6	37.01 ± 4.44	37.21 ± 4.47
F7	36.94 ± 4.40	37.19 ± 4.43
F8	32.18 ± 3.80	31.92 ± 3.77
F9	36.91 ± 4.80	37.17 ± 4.83
F10	36.83 ± 4.46	37.09 ± 4.49
F11	30.12 ± 3.37	29.52 ± 3.31

3.10.2. Mucoadhesion Strength and Mucoadhesion Time. The mucoadhesion strength determined the ability of the gel to attach to the mucosa. Basically, the formulations without the addition of mucoadhesive polymers exhibited mucoadhesive properties. Table 3 shows the mucoadhesion strength and time of different formulations of *in situ* vaginal gels. It can be seen that PF-127 showed stronger mucoadhesive ability compared

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Table 3. Obtained Values of Mucoadhesion Strength and Time of *In Situ* Vaginal Gel (Mean ± SD, n = 3)

	mucoadhesion strength (dyne cm^2)	mucoadhesion time (h)
F1	21.12 ± 4.01	4.32 ± 0.82
F2	18.23 ± 3.04	3.14 ± 0.52
F3	16.98 ± 2.65	2.98 ± 0.46
F4	13.98 ± 2.77	2.16 ± 0.43
F5	11.76 ± 1.78	1.98 ± 0.30
F6	35.12 ± 5.79	5.98 ± 0.99
F7	43.12 ± 7.72	8.54 ± 1.53
F8	44.87 ± 7.76	8.74 ± 1.51
F9	29.32 ± 5.54	5.31 ± 1.00
F10	37.41 ± 7.00	6.17 ± 1.34
F11	39.31 ± 7.47	6.31 ± 1.41

to PF-68. Despite these results, to obtain better mucoadhesive properties, HPMC and HEC were added into the formulations. The mechanism of adhesion was due to the formation of the H bond between the carboxylic acid group of cellulose polymers and the glycoprotein of mucin.[40] The results showed that HPMC has stronger adhesion properties than HEC. The results obtained showed that F7 possessed higher mucoadhesive strength compared to F10, however, based on the statistical analysis, both of these values were not significantly different (p > 0.005). It was also found that the formulations containing 0.4 and 0.6% of both HPMC (F7 and F8) and HEC (F10 and F11), respectively, generated similar mucoadhesion strength (p > 0.005). Besides possessing the same strength, the increase of the HPMC and HEC concentration to 0.6% provided lower gelation temperatures, as mentioned previously. Therefore, the concentration of 0.4% of both HPMC and HEC resulted in the most acceptable mucoadhesive and gelation properties. Furthermore, mucoadhesion time was also taken into consideration as the parameter of the residence time. The results showed that the adhesion strength was directly proportional to the adhesion time. The formulation that possessed the highest mucoadhesion strength was able to adhere to the mucin for the longest duration. In this case, it was found that the mucoadhesive time of F7 was significantly higher than F10 (p < 0.005), despite the similar mucoadhesive strength provided by both formulations. Therefore, it can be confirmed that F7 containing 0.4% of HPMC generated the most appropriate mucoadhesive properties.

3.10.3. pH Measurement. The results showed that all of the formulations had acidic pH in the range of 4.81−5.82 (Table 4). The pH of the normal vagina is in the range of 4.5−5.5.

Table 4. Results of the Measured pH and Drug Content of *In Situ* Vaginal Gel (Mean ± SD, n = 3)

	pH	drug content
F1	5.81 ± 0.65	98.21 ± 11.00
F2	5.79 ± 0.65	99.11 ± 11.10
F3	5.82 ± 0.67	100.02 ± 11.50
F4	5.78 ± 0.65	98.43 ± 11.12
F5	5.80 ± 0.64	99.29 ± 10.92
F6	5.12 ± 0.61	99.11 ± 11.89
F7	5.02 ± 0.60	99.72 ± 11.87
F8	4.92 ± 0.58	98.98 ± 11.68
F9	5.09 ± 0.66	98.19 ± 12.76
F10	5.01 ± 0.61	98.29 ± 11.89
F11	4.81 ± 0.54	99.09 ± 11.10

This acidic condition is maintained by the presence of lactobacilli, which converts glycogen into lactic acid.[41] These results were appropriate to the normal vaginal environment, which confirmed that the formulations would not change the natural environment of the vagina nor caused any irritation in the vagina.

3.10.4. Viscosity and Rheological Study. The viscosity of each formulation was measured under three different conditions, including cold temperature (4 °C), room temperature (25 °C), and body temperature (37 °C). The desirable formulation was the one forming free-flow liquid at cold and room temperature to facilitate the application while transforming into a gel at body temperature to increase the residence time. In terms of the flow properties, the gel system exhibited pseudoplastic behavior; therefore, the *in situ* gelling system should generate shear-thinning conditions in both liquid and gel forms. Shear-thinning behavior is defined as the decrease of viscosity by increasing the shear rate. The application of a high rate of shear (high speed of the spindle) is able to break the three-dimensional structures, resulting in lower viscosity.[42] Figure 7 depicts the rheology of the prepared

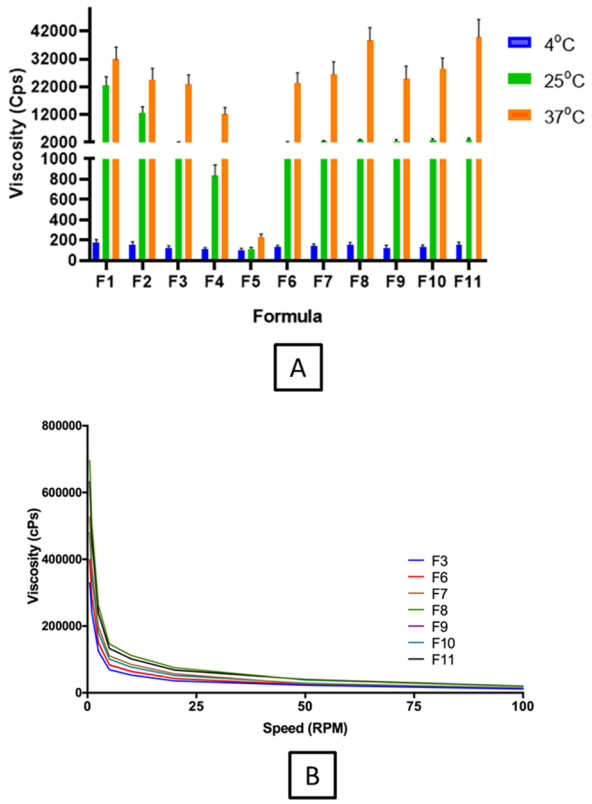

Figure 7. Results of (A) measured viscosity at 4, 25, and 37 °C (mean ± SD, n = 3) and (B) the rheology behavior of bioadhesive-thermosensitive *in situ* vaginal gel.

formulations. The results showed that all of the formulations indicated this expected behavior. Additionally, it was clear that the transition from solution to gel resulted in an increase in the viscosity of the formulations due to the micelle formation of PF-127 and PF-68, following the increase in the temperature. It was found that the ratio of the gelling agents, PF-127 and PF-68, affecting the viscosity of the formulation was obtained. PF-127 possesses a higher number of triblock chains, followed by a higher micelle size and number compared to PF-68. This

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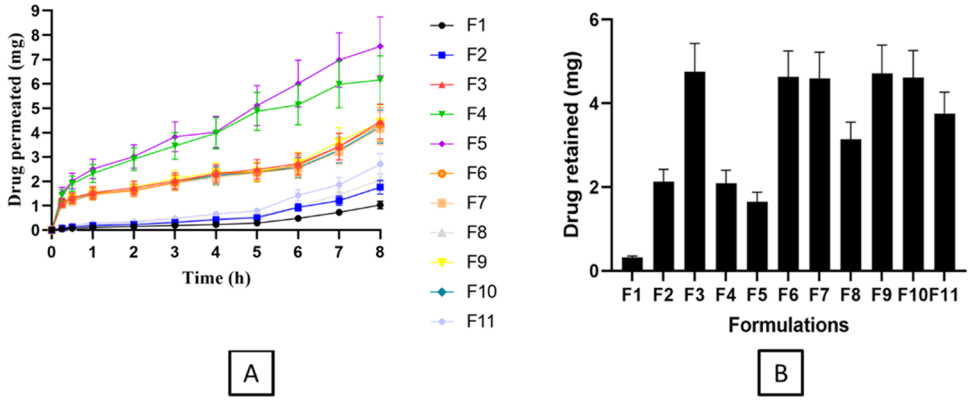

Figure 8. (A) Release profiles of ITZ permeation studies using cow vaginal mucosa (mean ± SD, $n = 3$) and (B) the amount of ITZ retained in the vaginal mucosa (mean + SD, $n = 3$).

resulted in an increased interaction of micelles causing the formation of more viscous gels.[43] Consequently, the formulations containing a higher concentration of PF-127 exhibited a more viscous solution. Based on the results obtained, F1 and F2 were found in the gel state at room temperature. Besides, F5 was not able to fully transform into the gel state at body temperature remarked by the low viscosity. Both of these conditions did not meet the requirement of thermosensitive gel. Therefore, F3 was found as the most desirable formulation performing as a free-flow liquid at room temperature and transforming into a gel at body temperature. Furthermore, the concentration of mucoadhesive polymers affected the viscosity of the formulations. It can be seen that F8 and F11, containing 0.6% of HPMC and HEC, respectively, had a significantly higher viscosity than other mucoadhesive formulations at 37 °C. Nevertheless, the addition of 0.2 and 0.4% of mucoadhesive polymers had no significant effect on the viscosity. This confirmed that 0.4% of mucoadhesive polymers was found to be the optimum concentration to obtain the desirable physicochemical properties of the thermosensitive *in situ* gel.

3.10.5. Drug Content Analysis. The analysis of the drug content was performed to calculate the amount of drug contained in the *in situ* gel formulation. The results exhibited that the percentage of drug content was in the range of 98.19−100.02% (Table 4), indicating that the method used was able to provide a homogeneous mixture. Additionally, the process involved in the preparation of thermosensitive *in situ* gel did not affect the amount of ITZ entrapped in the gel-flake system. The obtained values of the drug content percentage complied within the range of the requirements recommended by ICH related to recovery percentage, which was 95−105%.[44]

3.11. *Ex Vivo* Permeation Studies. The release behavior of thermosensitive *in situ* gel loaded with SD-ITZ gel flakes was carried out using cow vaginal mucosa over 8 h. Figure 8 depicts the results of this evaluation. It can be seen that F4 and F5 released the highest amount of ITZ, reaching 6.16 ± 0.99 and 7.54 ± 1.21 mg, respectively, after 8 h. This was caused by the high concentration of PF-68, resulting in lower viscosity. Lower release rate profiles were exhibited by F3, F6, F7, F8, and F9 with a total of ∼4 mg ITZ at the end of the experiment. As this evaluation involved no external intervention, such as vaginal physiology, meaning that the gel was maintained on the mucosa in the donor compartment, the amount of ITZ released by F3, containing no mucoadhesive polymers, was not significantly different from the mucoadhesive formulations. Additionally, these results also showed that 0.2 and 0.4% of the mucoadhesive polymers added into the formulations generated a nonsignificant difference in the drug release. However, the increase of the mucoadhesive concentration into 0.6% resulted in an almost twice lower amount of drug released after 8 h, indicating that the gel viscosity affected the release behavior. The other formulations, F1 and F2, which possessed the highest viscosity amongst all formulations, exhibited the lowest release rate. The high viscosity of the gel caused the drug retention in the gel system, resulting in less amount of drug delivered to the receptor compartment.

3.12. *Ex Vivo* Recovery and Extraction Assays. The purpose of this research was to prepare thermosensitive *in situ* gel for local targeting of CA vaginal candidiasis. In this case, the drug was intended to retain in the vaginal mucosa rather than to permeate to the acceptor compartment expressing systemic circulation. It was reported that CA was able to penetrate into the vaginal tissue in the severe conditions of vaginal candidiasis.[4] Hence, it was important to evaluate the amount of drug retained in the vaginal mucosa to investigate the ability of ITZ to permeate the tissue, which further affects the treatment efficacy. The results showed that F1 showed the lowest amount of ITZ by retaining less than 1 mg of ITZ in the vaginal mucosa. This indicated that the drug was entrapped in the gel system and due to the high viscosity of F1, ITZ was not able to permeate to both vaginal tissue and the acceptor compartment in the release studies, as shown by the low amount of drug detected. F2, F4, and F5 possessed a higher amount of drug, which was ∼2 mg, maintained in the vaginal mucosa. In contrast, F3, F6, F7, F9, and F10 provided a significantly higher amount of drug in the vaginal tissue, reaching 4.76 ± 0.67, 4.63 ± 0.62, 4.59 ± 0.63, 4.72 ± 0.67, and 4.61 ± 0.65 mg, respectively, which were contradictory with the release behavior. This indicated the ability of the formulation to release the drug from the gel system and retain more drug locally in the vaginal tissue. Other formulations containing 0.6% of mucoadhesive polymers, F8 and F10, exhibited a lower amount of drug retained, showing 3.14 ± 0.41 and 3.76 ± 0.51 mg, respectively. Therefore, considering the mucoadhesive strength and time, as well as other characterization methods, F7 was selected as the best formulation for further antifungal activity tests.

3.13. *In Vivo* Antifungal Activity in the Model of Infection on Rat. The antifungal activity of ITZ in the thermosensitive *in situ* gel was evaluated by inoculating the vaginal fluid of the infection animal models in suitable media.

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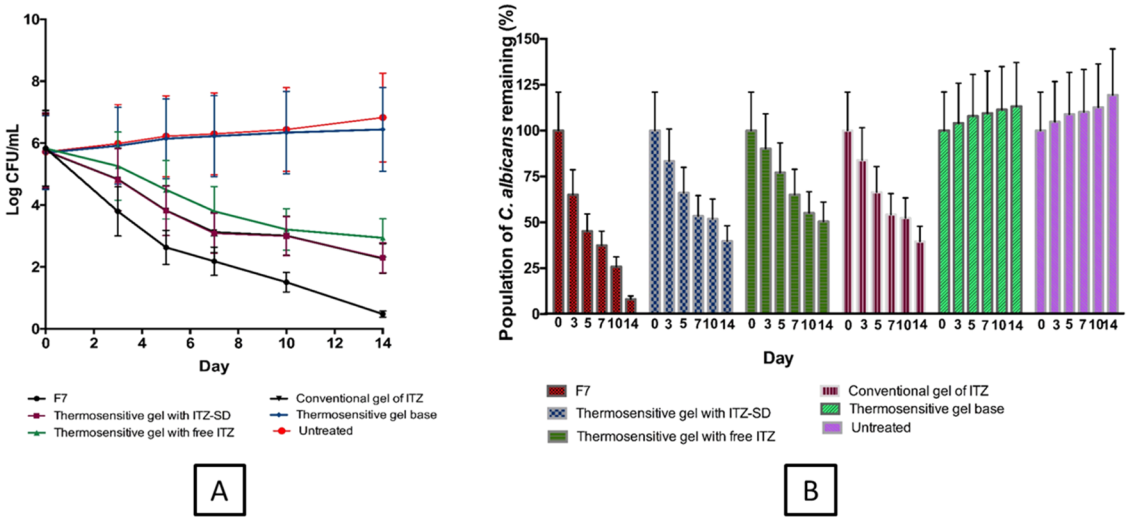

Figure 9. Results of *in vivo* antifungal activity studies expressed in (A) log CFU/mL and (mean ± SD, n = 3) (B) percentage of *C. albicans* population remaining (mean + SD, n = 3).

As shown in Figure 9, there were six groups with different treatments, namely, the optimum formulation F7, thermosensitive gel loaded with ITZ-SD, thermosensitive gel loaded with free ITZ, conventional gel of ITZ, thermosensitive gel base, and untreated control group. F7 exhibited the highest reduction of the CA colony, reaching less than 1 log CFU/mL or equivalent to less than 10% of the total colony after 14 days. In contrast, the untreated group and the group treated with thermosensitive gel base possessed a similar trend with the increase of 25% of the colony after 14 days, reaching almost 7 log CFU/mL, which indicated that the excipients used in the formulations had no antifungal activity. It is also important to note that the growth of CA in the untreated group confirmed that the infection model of the Wistar rats was successfully developed. Despite the group of thermosensitive gel with ITZ-SD also possessed antifungal activity with the decrease of 60% of the total colony (2.29 ± 0.48 log CFU/mL), this result was significantly higher than the value provided by F7. It was clearly explained that the incorporation of the mucoadhesive polymer in the thermosensitive gel formulation was able to enhance the antifungal activity of ITZ. On the other hand, the other groups, namely, thermosensitive gel with free ITZ and conventional gel of ITZ, also exhibited antifungal activity, proved by the decrease of more than half of the initial fungal colony, which were 2.94 ± 0.61 and 2.27 ± 0.47 CFU/mL, respectively. However, the obtained values were significantly higher compared to the mucoadhesive formulation, F7, which contained SD-ITZ gel flakes. As the limitation of ITZ was the poor solubility in an aqueous medium, the attempt to increase its solubility using the solid dispersion technique was found to be a successful approach to enhance the antifungal activity of ITZ by increasing the drug diffusion rate.[45] Furthermore, the application of the gel-flake system was able to improve the spreadability of gel at the target site, resulting in more effective antifungal activity. Therefore, the combination of the solid dispersion technique, gel-flake system, and thermosensitive *in situ* gel was able to significantly improve the antifungal activity of ITZ.

According to the findings presented here, it is clear that the development of bioadhesive-thermosensitive *in situ* vaginal gel containing SD-ITZ gel flakes was able to provide great potential for the improvement of local-targeting vaginal candidiasis treatment. The major advantage provided by the delivery approach presented here compared to the conventional ITZ vaginal gel was the ability to retain the drug at the target site due to the mucoadhesive properties, resulting in improved antifungal activity. Besides, the increase of ITZ solubility and good spreadability properties were other key points supporting these promising results. Moving forward, further studies, such as toxicity and biocompatibility tests, are now required to be performed to ensure acceptability and usability before proceeding to the clinical stage.

4. CONCLUSIONS

In the present work, bioadhesive-thermosensitive *in situ* vaginal gel for local targeting of vaginal candidiasis treatment was successfully developed. Initially, the application of the solid dispersion technique was able to increase ITZ solubility up to ~4 mg/mL, leading to an improved release behavior and antifungal activity compared to free ITZ. Subsequently, to obtain high spreadability on the highly folded vaginal surfaces, the gel-flake system containing SD-ITZ was prepared. Gellan gum and chitosan solution were found to be able to entrap the drug in the gel-flake system, resulting in the desirable entrapment efficiency and drug-loading capacity. Finally, the formulation of *in situ* vaginal gel using a combination of PF-127 and PF-68 with HPMC exhibited higher residence time, providing a higher amount of drug retained in the vaginal tissue. This was proven to affect the ITZ antifungal activity as shown by the most significant decrease of the growth of CA among all of the treated groups of infected animal models. This could be a great promise for the improvement of vaginal candidiasis treatment, which could be applied clinically in the future. However, prior to the clinical studies, several further experimental studies regarding the safety and efficacy must now be performed to maximize the potential impact of this research.

■ ASSOCIATED CONTENT

Supporting Information

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Details on the solubility of ITZ in water and stimulated vaginal fluid following the optimization process using CCD; predicted and actual values of ITZ solubility in water and simulated vaginal fluid following the optimization process using CCD; the entrapment efficiency and drug loading of ITZ in gel-flake formulations following the optimization process using CCD and PDI; predicted and actual values of entrapment efficiency and drug loading of ITZ in gel flakes following the optimization process using CCD; and representative images of *in situ* gel formulation at room temperature (solution) and at gelation temperature (gel) (PDF)

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Notes

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REFERENCES

(1) Mayer, F. L.; Wilson, D.; Hube, B. *Candida albicans* Pathogenicity. *Virulence* **2013**, *4*, 119−128.

(2) Hernday, A. D.; Noble, S. M.; Mitrovich, Q. M.; Johnson, A. D. *Genetics and Molecular Biology in Candida Albicans*, 2nd ed.; Elsevier Inc., 2010; Vol. *470*.

(3) Gonçalves, B.; Ferreira, C.; Alves, C. T.; Henriques, M.; Azeredo, J.; Silva, S. Vulvovaginal Candidiasis: Epidemiology, Microbiology and Risk Factors. *Crit. Rev. Microbiol.* **2016**, *42*, 905−927.

(4) Cassone, A. Vulvovaginal *Candida albicans* Infections: Pathogenesis, Immunity and Vaccine Prospects. *BJOG: Int. J. Obstet. Gynaecol.* **2015**, *122*, 785−794.

(5) Abd Ellah, N. H.; Abdel-Aleem, J. A.; Abdo, M. N.; Abou-Ghadir, O. F.; Zahran, K. M.; Hetta, H. F. Efficacy of Ketoconazole Gel-Flakes in Treatment of Vaginal Candidiasis: Formulation, in Vitro and Clinical Evaluation. *Int. J. Pharm.* **2019**, *567*, No. 118472.

(6) Shafiei, M.; Peyton, L.; Hashemzadeh, M.; Foroumadi, A. History of the Development of Antifungal Azoles: A Review on Structures, SAR, and Mechanism of Action. *Bioorg. Chem.* **2020**, *104*, No. 104240.

(7) Permana, A. D.; Paredes, A. J.; Volpe-Zanutto, F.; Anjani, Q. K.; Utomo, E.; Donnelly, R. F. Dissolving Microneedle-Mediated Dermal Delivery of Itraconazole Nanocrystals for Improved Treatment of Cutaneous Candidiasis. *Eur. J. Pharm. Biopharm.* **2020**, *154*, 50−61.

(8) Zhang, A. Y.; Camp, W. L.; Elewski, B. E. Advances in Topical and Systemic Antifungals. *Dermatol. Clin.* **2007**, *25*, 165−183.

(9) *Martindale: The Complete Drug Reference*, 36th ed.; Sweetman, S. C., Ed.; Pharmaceutical Press: London, 2009.

(10) Sobel, J. D. Factors Involved in Patient Choice of Oral or Vaginal Treatment for Vulvovaginal Candidiasis. *Patient Prefer. Adherence* **2013**, *8*, 31−34.

(11) Committee, J. F. British National Formulary. http://www.medicinescomplete.com (accessed Feb 15, 2021).

(12) Patil, P. B.; Datir, S. K.; Saudagar, R. B. A Review on Topical Gels as Drug Delivery System. *J. Drug Delivery Ther.* **2019**, *9*, 989−994.

(13) Jones, K. A.; Harmanli, O. Pessary Use in Pelvic Organ Prolapse and Urinary Incontinence. *Rev. Obstet. Gynecol.* **2010**, *3*, 3−9.

(14) Kim, H.; Jung, S.; Yeo, S.; Kim, D.; Na, Y. C.; Yun, G.; Lee, J. Characteristics of Skin Deposition of Itraconazole Solubilized in Cream Formulation. *Pharmaceutics* **2019**, *11*, No. 195.

(15) Karavana, S. Y.; Rençbe, S.; Şenyiğit, Z. A.; Baloğlu, E. A New In-Situ Gel Formulation of Itraconazole for Vaginal Administration. *Pharmacol. Pharm.* **2012**, *03*, 417−426.

(16) Patel, P.; Patel, P. Formulation and Evaluation of Clindamycin HCL in Situ Gel for Vaginal Application. *Int. J. Pharm. Invest.* **2015**, *5*, 50.

(17) Vasconcelos, T.; Sarmento, B.; Costa, P. Solid Dispersions as Strategy to Improve Oral Bioavailability of Poor Water Soluble Drugs. *Drug Discovery Today* **2007**, *12*, 1068−1075.

(18) Owen, D. H.; Katz, D. F. A Vaginal Fluid Simulant. *Contraception* **1999**, *59*, 91−95.

(19) Falavigna, M.; Pattacini, M.; Wibel, R.; Sonvico, F.; Škalko-Basnet, N.; Flaten, G. E. The Vaginal-PVPA: A Vaginal Mucosa-Mimicking in Vitro Permeation Tool for Evaluation of Mucoadhesive Formulations. *Pharmaceutics* **2020**, *12*, 1−15.

(20) Permana, A. D.; McCrudden, M. T. C.; Donnelly, R. F. Enhanced Intradermal Delivery of Nanosuspensions of Antifilariasis Drugs Using Dissolving Microneedles: A Proof of Concept Study. *Pharmaceutics* **2019**, *11*, 1−22.

(21) Permana, A. D.; Mir, M.; Utomo, E.; Donnelly, R. F. Bacterially Sensitive Nanoparticle-Based Dissolving Microneedles of Doxycycline

for Enhanced Treatment of Bacterial Biofilm Skin Infection: A Proof of Concept Study. *Int. J. Pharm. X* **2020**, *2*, No. 100047.
(22) Deshkar, S. S.; Palve, V. K. Formulation and Development of Thermosensitive Cyclodextrin-Based in Situ Gel of Voriconazole for Vaginal Delivery. *J. Drug Delivery Sci. Technol.* **2019**, *49*, 277−285.
(23) Mo, F.; Ma, J.; Yang, X.; Zhang, P.; Li, Q.; Zhang, J. In Vitro and in Vivo Effects of the Combination of Myricetin and Miconazole Nitrate Incorporated to Thermosensitive Hydrogels, on C. albicans Biofilms. *Phytomedicine* **2020**, *71*, No. 153223.
(24) Morsi, N.; Ghorab, D.; Refai, H.; Teba, H. Ketoroloac Tromethamine Loaded Nanodispersion Incorporated into Thermosensitive in Situ Gel for Prolonged Ocular Delivery. *Int. J. Pharm.* **2016**, *506*, 57−67.
(25) Sanz, R.; Clares, B.; Mallandrich, M.; Suñer-Carbó, J.; Montes, M. J.; Calpena, A. C. Development of a Mucoadhesive Delivery System for Control Release of Doxepin with Application in Vaginal Pain Relief Associated with Gynecological Surgery. *Int. J. Pharm.* **2018**, *535*, 393−401.
(26) Yun Chang, J.; Oh, Y. K.; Soo Kong, H.; Jung Kim, E.; Deuk Jang, D.; Taek Nam, K.; Kim, C. K. Prolonged Antifungal Effects of Clotrimazole-Containing Mucoadhesive Thermosensitive Gels on Vaginitis. *J. Controlled Release* **2002**, *82*, 39−50.
(27) Mirza, M. A.; Ahmad, S.; Mallick, M. N.; Manzoor, N.; Talegaonkar, S.; Iqbal, Z. Development of a Novel Synergistic Thermosensitive Gel for Vaginal Candidiasis: An in Vitro, in Vivo Evaluation. *Colloids Surf., B* **2013**, *103*, 275−282.
(28) Tran, P.; Pyo, Y. C.; Kim, D. H.; Lee, S. E.; Kim, J. K.; Park, J. S. Overview of the Manufacturing Methods of Solid Dispersion Technology for Improving the Solubility of Poorly Water-Soluble Drugs and Application to Anticancer Drugs. *Pharmaceutics* **2019**, *11*, No. 132.
(29) Patel, R. P.; Patel, D. J.; Bhimani, D. B.; Patel, J. K. Physicochemical Characterization and Dissolution Study of Solid Dispersions of Furosemide with Polyethylene Glycol 6000 and Polyvinylpyrrolidone K30. *Dissolution Technol.* **2008**, *15*, 17−25.
(30) Zhang, X.; Xing, H.; Zhao, Y.; Ma, Z. Pharmaceutical Dispersion Techniques for Dissolution and Bioavailability Enhancement of Poorly Water-Soluble Drugs. *Pharmaceutics* **2018**, *10*, No. 74.
(31) Sareen, S.; Joseph, L.; Mathew, G. Improvement in Solubility of Poor Water-Soluble Drugs by Solid Dispersion. *Int. J. Pharm. Invest.* **2012**, *2*, 12.
(32) Huang, Y.; Dai, W.-G. Fundamental Aspects of Solid Dispersion Technology for Poorly Soluble Drugs. *Acta Pharm. Sin. B* **2014**, *4*, 18−25.
(33) Pandi, P.; Bulusu, R.; Kommineni, N.; Khan, W.; Singh, M. Amorphous Solid Dispersions: An Update for Preparation, Characterization, Mechanism on Bioavailability, Stability, Regulatory Considerations and Marketed Products. *Int. J. Pharm.* **2020**, *586*, No. 119560.
(34) Guleria, R.; Sharma, V.; Kapoor, A.; Kaith, N. S.; Singh, R. Polyethylene Glycol Enhances Solubility of Domperidone through Solid Dispersion. *Am. J. PharmTech Res.* **2012**, *2*, 629.
(35) Picone, C. S. F.; Cunha, R. L. Chitosan-Gellan Electrostatic Complexes: Influence of Preparation Conditions and Surfactant Presence. *Carbohydr. Polym.* **2013**, *94*, 695−703.
(36) Sabadini, R. C.; Martins, V. C. A.; Pawlicka, A. Synthesis and Characterization of Gellan Gum: Chitosan Biohydrogels for Soil Humidity Control and Fertilizer Release. *Cellulose* **2015**, *22*, 2045−2054.
(37) Mesallati, H.; Umerska, A.; Paluch, K. J.; Tajber, L. Amorphous Polymeric Drug Salts as Ionic Solid Dispersion Forms of Ciprofloxacin. *Mol. Pharm.* **2017**, *14*, 2209−2223.
(38) Russo, E.; Villa, C. Poloxamer Hydrogels for Biomedical Applications. *Pharmaceutics* **2019**, *11*, No. 671.
(39) *Handbook of Pharmaceutical Excipients*, 6th ed.; Rowe, R. C.; Sheskey, P. J.; Quinn, M. E., Eds.; Pharmaceutical Press: London, 2009.
(40) Chatterjee, B.; Amalina, N.; Sengupta, P.; Mandal, U. K. Mucoadhesive Polymers and Their Mode of Action: A Recent Update. *J. Appl. Pharm. Sci.* **2017**, *7*, 195−203.
(41) Mirza, M. A.; Panda, A. K.; Asif, S.; Verma, D.; Talegaonkar, S.; Manzoor, N.; Khan, A.; Ahmed, F. J.; Dudeja, M.; Iqbal, Z. A Vaginal Drug Delivery Model. *Drug Delivery* **2016**, *23*, 3123−3134.
(42) Deshkar, S. S.; Patil, A. T.; Poddar, S. S. Development of Thermosensitive Gel of Fluconazole for Vaginal Candidiasis. *Int. J. Pharm. Pharm. Sci.* **2016**, *8*, 391−398.
(43) Shaarani, S.; Hamid, S. S.; Kaus, N. H. M. The Influence of Pluronic F68 and F127 Nanocarrier on Physicochemical Properties, in Vitro Release, and Antiproliferative Activity of Thymoquinone Drug. *Pharmacogn. Res.* **2017**, *9*, 12−20.
(44) Walfish, S. Analytical Methods: A Statistical Perspective on the ICH Q2A and Q2B Guidelines for Validation of Analytical Methods. *BioPharm Int.* **2006**, 1−6.
(45) Kumar, M.; Shanthi, N.; Mahato, A. K.; Soni, S.; Rajnikanth, P. S. Preparation of Luliconazole Nanocrystals Loaded Hydrogel for Improvement of Dissolution and Antifungal Activity. *Heliyon* **2019**, *5*, No. e01688.

https://doi.org/10.1021/acsami.1c03422
ACS Appl. Mater. Interfaces 2021, 13, 18128−18141