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30-Mar-2021

Journal: ACS Applied Materials & Interfaces

Manuscript ID: am-2021-03422u.R2

Title: "Bioadhesive-Thermosensitive In Situ Vaginal Gel of Gel Flakes-Solid Dispersion of Itraconazole for Enhanced Antifungal Activity in The Treatment of Vaginal Candidiasis"

Authors: Permana, Andi Dian; Utomo, Emilia; Pratama, Muhammad Rezky; Amir, Muhammad Nur; Anjani, Qonita Kurnia; Mardikasari, Sandra Aulia; Sumarheni, Sumarheni; Himawan, Achmad; Arjuna, Andi; Usmanengsi, Usmanengsi; Donnelly, Ryan F.

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16-Mar-2021

Journal: ACS Applied Materials & Interfaces

Manuscript ID: am-2021-03422u

Title: "Bioadhesive-thermosensitive in situ vaginal gel of gel flakes-solid dispersion of itraconazole for enhanced antifungal activity in the treatment of vaginal candidiasis"

Author(s): Permana, Andi Dian; Utomo, Emilia; Pratama, Muhammad Rezky; Amir, Muhammad Nur; Anjani, Qonita Kurnia; Mardikasari, Sandra Aulia; Sumarheni, Sumarheni; Himawan, Achmad; Arjuna, Andi; Usmanengsi, Usmanengsi; Donnelly, Ryan F.

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Reviewer(s)' Comments to Author:

Reviewer: 1

Recommendation: Major revisions needed as noted.

Comments:

Minor comments:

1. Kindly make the use of complete word/abbreviation similar throughout the text.
2. A few grammar errors need revisiting

Major comments:

1. In a statement "Contrastingly, in a certain abnormal condition, the change in vaginal physiology leads to the overgrowth of this fungus, resulting in inflammation in the vaginal epithelium". Kindly provide a brief account of abnormal conditions.
2. In a statement "From these models, it can be clearly seen that the solubility of ITZ was directly proportional to the concentration of PVP and PEG. By increasing the amount of PVP and PEG in the formulation was able to generate higher solubility of ITZ". However, the graph figures do not comply with the above statement, the effect of PEG is not clear. Kindly check and provide a suitable response.
3. In a statement "This means that the preparation of SD-ITZ was able to change the form of ITZ from high crystalline to amorphous state, which had a significant role to enhance the water solubility of ITZ". Kindly explain in detail how and also add a suitable reference.
4. In a statement "It was clear to describe that the preparation of SD-ITZ was able to enhance the antimicrobial activity of ITZ". Kindly explain in brief with suitable reference.
5. Kindly provide few more points of discussion in the result section or make a separate section of the discussion. (refer to journal guidelines for the same).
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Are the literature references appropriate and up to date?: No

Provides significant insight into or the development of an important application: Fair

Work is original and significant: Fair

Conclusions adequately supported by data: Fair

Clarity of presentation: Fair

Potential for impact in materials science and engineering: Fair

Reviewer: 2

Recommendation: Publish after minor revisions noted.

Comments:

Comments to the Author

A very nice and well-designed study about the bioadhesive-thermosensitive in situ vaginal gel of gel flakes-solid dispersion of itraconazole. Authors have fully characterized the prepared formulations while their conclusions are supported by their results. However, the some comments must be taken into consideration for the improvement of the study. I'm sending my comments and recommendations.

1. Please carefully check the text for minor grammar and syntax errors.
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Materials Science & Engineering C

Bioadhesive-thermosensitive in situ vaginal gel of gel flakes-solid dispersion of itraconazole for enhanced antifungal activity in the treatment of vaginal candidiasis --Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	vaginal candidiasis, itraconazole, solid dispersion, bioadhesive, thermosensitive in situ vaginal gel
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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 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 gel flakes system was found to be the most suitable choice. In order to obtain the optimum solubility, entrapment efficiency and drug loading capacity, optimization of solid dispersion and gel flakes formulations of ITZ were performed using a composite central design. The results show that the optimized formulation of SD-ITZ was able to significantly enhance its solubility in both water and simulated vaginal fluid to reach the value 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 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 hours. Importantly, according to the in vivo antifungal activity using infection animal models, the incorporation of solid dispersion technique and gel flakes system in the formulation of bioadhesive-thermosensitive in situ vaginal gel was able to generate the most significant decrease of the growth of <i>C. albicans</i> reaching $< 1 \log$ CFU/mL or equivalent to less than 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 treating vaginal candidiasis. Following these findings, several further experiments need to be performed to ensure acceptability and usability before reaching the research in the clinical stage.
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The Editor

Materials Science & Engineering C

16th February 2021

Dear Sir/Madam,

I wish you to consider our manuscript entitled: “**Bioadhesive-thermosensitive *in situ* vaginal gel of gel flakes-solid dispersion of itraconazole for enhanced antifungal activity in the treatment of vaginal candidiasis**” for publication in Materials Science & Engineering C.

Candida albicans (CA) is one of the fungus found as normal human microbiota in several part of the body such as oral cavity, reproductive and gastrointestinal tract. As a part of CA infection, vaginal candidiasis is one of the highest prevalent of candidiasis suffered by around 75% of women over the world. Itraconazole (ITZ), one of the third generation of azoles, is mostly preferred to be applied for vaginal candidiasis compared to other type of azole antifungals. However, the physicochemical properties of ITZ including high lipophilicity and low solubility in water was found to be a challenge in the development of prolonged release topical preparation. Accordingly, *in situ* vaginal gel containing solid dispersion of ITZ entrapped in gel flakes system is proposed in this study, as expected, to increase the drug solubility and provide longer contact to the vaginal tissue which finally increase the absorption of drug and efficacy of treatment.

In the present work, we developed the formulations of ITZ solid dispersion (SD-ITZ) and gel-flakes using a composite central design to obtain the optimum solubility, entrapment efficiency and drug loading capacity. The results show that the optimized formulation of SD-ITZ was able to significantly enhance its solubility in both water and simulated vaginal fluid. Additionally, the optimized formulation of SD-ITZ gel flakes possessed desirable entrapment efficiency and drug loading capacity. Subsequently, the *in situ* vaginal gel containing SD-ITZ gel flakes prepared using PF-127 and PF-68, as the gelling agents, with the addition of HPMC and HEC, as the mucoadhesive polymers provided desirable physicochemical properties and was able to retain the drug in the vaginal tissue after 8 hours. Importantly, according to the *in vivo* antifungal activity using infection animal models, the incorporation of solid dispersion technique and gel flakes system in the formulation of bioadhesive-thermosensitive *in situ* vaginal gel was able to generate the most significant decrease of the growth of *C. albicans* reaching < 1 log CFU/mL or equivalent to less than 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 treating vaginal candidiasis.

We believe that this work will be of great interest to the readers of Materials Science & Engineering C, particularly those working on applications of polymer, *in situ* gel formulations,



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drug delivery for topical administration especially for local-targeting, antimicrobial activity testing using infected animal models.

The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

I hope that you consider this manuscript worthy of publication in Materials Science & Engineering C and look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

Department of Pharmaceutics, Faculty of Pharmacy, Hasanuddin University, Makassar,
Indonesia

Email: andi.dian.permana@unhas.ac.id

Bioadhesive-thermosensitive *in situ* vaginal gel of gel flakes-solid dispersion of itraconazole for enhanced antifungal activity in the treatment of vaginal candidiasis

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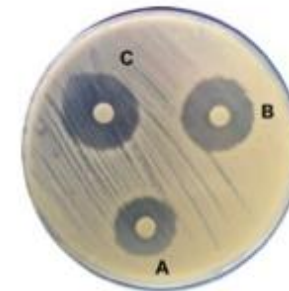
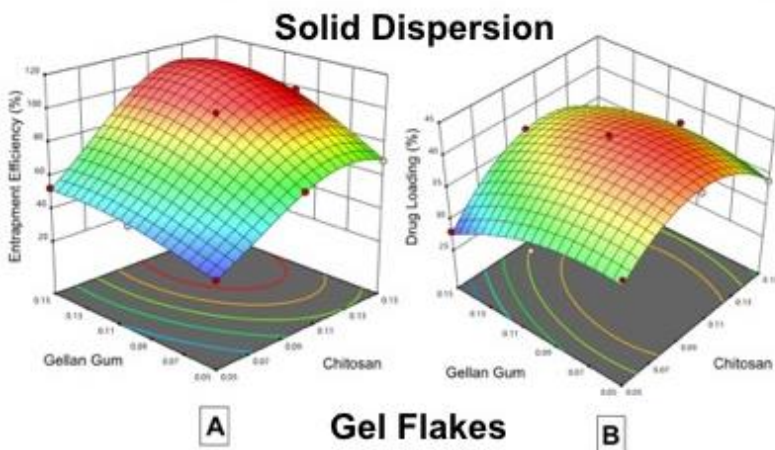
Department of Pharmaceutics, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia

Abstract

The poor solubility of itraconazole (ITZ) has limited its efficacy in the treatment of vaginal candidiasis. Accordingly, the improvement of ITZ solubility using 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 gel flakes system was found to be the most suitable choice. In order to obtain the optimum solubility, entrapment efficiency and drug loading capacity, optimization of solid dispersion and gel flakes formulations of ITZ were performed using a composite central design. The results show that the optimized formulation of SD-ITZ was able to significantly enhance its solubility in both water and simulated vaginal fluid to reach the value 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 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 hours. Importantly, according to the *in vivo* antifungal activity using infection animal models, the incorporation of solid dispersion technique and gel flakes system in the formulation of bioadhesive-thermosensitive *in situ* vaginal gel was able to generate the most significant decrease of the growth of *C. albicans* reaching < 1 log CFU/mL or equivalent to less than 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 treating vaginal candidiasis. Following these findings, several further experiments need to be performed to ensure acceptability and usability before reaching the research in the clinical stage.

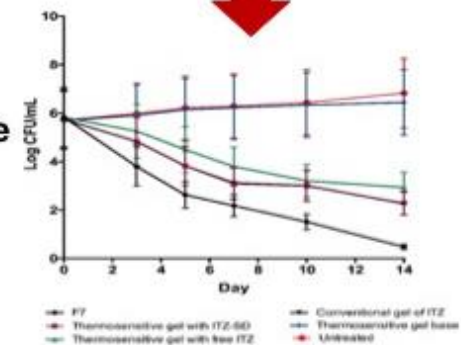
Keywords: vaginal candidiasis, itraconazole, solid dispersion, bioadhesive, thermosensitive *in situ* vaginal gel

Itraconazole

CC(C)C1=CN(C(=O)N1c2ccc(cc2)N3CCN(CC3)c4ccc(cc4)CO[C@@H]5O[C@H](C[C@H]6C=CC=C6)[C@@H](C[C@@H]7C=CC(=C7)Cl)O5)

In vitro antifungal activity

***In vivo* antifungal activity in vaginal candidiasis model in rats**



Decrease in fungal bioburden in rats

1. Introduction

Candida albicans (CA) is one of the fungus found as normal human microbiota in several parts of the body such as oral cavity, reproductive and gastrointestinal tract [1]. However, since CA is known as an opportunistic pathogen, its colonization in human tissue and organ can cause serious and invasive infection, especially in the event of immune compromising [2]. It has been reported that CA was one of the major causes of hospital-acquired systemic infections in the United States, providing 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 infection [1]. As a part of CA infection, vaginal candidiasis is one of the highest prevalence of candidiasis suffered by around 75% of women over the world [3]. In the normal condition, the vagina has its defence mechanism against CA through the presence of *lactobacilli* and the specific immune responses. Therefore, this can control the growth of CA and prevent vaginal candidiasis. Contrastingly, in a certain abnormal condition, the change in vaginal physiology leads to the overgrowth of this fungus, resulting in inflammation in the vaginal epithelium [4].

Currently, antifungals are used as the main therapy for vaginal candidiasis treatment, especially azole derivative groups [5]. 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, has a better pharmacokinetic profile, lower nephrotoxic effects, and owing to the ability to kill resistant types of fungal infection [5,6]. ITZ, which selectively inhibits the synthesis of the fungal cell membrane, is recently available in oral dosage form with the dose of 200 mg and the administration is twice daily for vaginal candidiasis treatment [7,8].

The currently available treatment for vaginal candidiasis is intravaginal and oral dosage forms. Intravaginal administration includes the application of cream and vaginal pessaries [9,10]. 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 [11]. Additionally, the use of vaginal pessaries leads to several complications, such as patient inconvenient and allergic issues [12]. In the case of oral administration, this system provides several drawbacks such as systemic adverse effect, namely cholestatic and hepatocellular damage [8]. 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 effect in the target site. Previously, several studies have been conducted to develop local targeting of ITZ including microarray patches for cutaneous candidiasis [6], cream formulations for cutaneous mycoses [13] and *in situ* gel for vaginal candidiasis [14]. However, the development of ITZ for vaginal infection was not widely explored in the previous studies. Not only that, the lack of specific evaluations regarding the drug delivery to the target site was found as the gap, which was considered as essential matters. As an alternative for topical administration, the gel-based formulation is preferred compared to cream as it provides non-sticky properties and has simple manufacturing process [11]. Unfortunately, for vaginal application, the water-based topical formulation can be easily rinsed by 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 increase the absorption of drug and efficacy of treatment [15].

The physicochemical properties of ITZ including high lipophilicity and low solubility in water was 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. Firstly, due to the low aqueous solubility of ITZ, solid dispersion was developed in order to improve the aqueous solubility of ITZ by using water-soluble polymers [16]. Secondly, the development of ITZ containing solid dispersion powder into the gel flakes system. Gel flakes has the shape of thread-like forms with polygonal structures, which offer a good opportunity for ITZ to be entrapped within the highly folded epithelial surfaces of the vagina, leading to extended contact to vaginal mucus and the drug release is controllable [4]. Moreover, the gel system in this study was formulated using thermosensitive polymer in order to prepare a liquid formulation which can transform to 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 vaginal candidiasis treatment. In addition to that, 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. Materials and methods

2.1. Materials

Itraconazole (purity, $\geq 98\%$) of analytical grade was purchased from Tokyo Chemical Industry (Tokyo, Japan). Gellan gum, chitosan, Pluronic® F127 (PF127), and PVP K-30, PEG 6000 were purchased from Sigma–Aldrich Pte Ltd, (Singapore, Singapore). Pluronic® F68 (PF68) 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)

A certain amount 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 hours to remove the solvent residue. Afterwards, 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 characterizations. The amount of all materials was determined based on the optimized formulation. A formulation which 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, USA). 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 (low, medium and high). 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.

Table 1. The experimental design of SD-ITZ formulations

Factors	Level		
	low	medium	high
PVP concentration (% w/v)	1	2	3
PEG 6000 concentration (%w/v)	1	2	3

2.4. Characterization of SD-ITZ Formulations

The solubility tests were performed in water and simulated vaginal fluid. An excess amount 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 HPLC method.

Any chemical interaction between the drug and the excipients was observed using FT-IR spectroscopy (Accutrac FT/IR-4100™ Series, Perkin Elmer, USA). The spectra of free ITZ, physical mixture of ITZ and the excipients (PM), and the optimized SD-ITZ formulation were recorded between 400 – 4000 cm⁻¹ using 32 scans with the 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 was evaluated using DSC Q100 (DSC 2920, TA Instruments, Surrey, UK) and. An amount of sample was heated in a sealed aluminium pan from 0°C to 300°C with the heating rate of 10°C/min.

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

2.5. *In vitro* release studies of SD-ITZ

2.5.1. *The preparation of simulated vaginal fluid*

The simulated vaginal fluid was used as the release medium in order to mimic the vaginal environment. The composition of the simulated vaginal fluid is presented in Table 2, according to Owen and Katz (1999) [17,18]. The pH of the final solution was 4.6.

Table 2. The compositions of simulated vaginal fluid

Name	Concentrations (%w/v)
Sodium chloride	3.510
Potassium hydroxide	1.400
Calcium hydroxide	0.222
Bovine serum albumin	0.018
Lactic acid	2.000
Acetic acid	1.000
Glycerol	0.160
Urea	0.400
Glucose	5.000

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 dialysis method with simulated vaginal fluid with the addition of 1% Tween 80 as the release medium. The addition of Tween 80 aimed to ensure the sink condition 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, USA). 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 fresh release medium in order to maintain the sink condition. 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 [19].

$$\text{Zero-order : } Q_t = Q_0 + K_0 t$$

$$\text{First-order : } \ln Q_t = \ln Q_0 + K_1 t$$

$$\text{Higuchi : } Q_t = K_H \sqrt{t}$$

$$\text{Korsmeyer-Peppas : } Q_t = K_P n$$

$$\text{Hixson-Crowell : } Q_0^{1/3} - Q_t^{1/3} = K_S t$$

Where Q_t is the percentage of drug released at the time t , Q_0 is the initial amount of drug, t is the time, n is the diffusion release exponent, K_0 , K_1 , K_H , K_P , and K_S are the release constants of the relevant kinetic models. The model parameters were calculated using DDSolver.

2.6. Fabrication of SD-ITZ Gel flakes

The gel flakes containing solid dispersion of ITZ was prepared using a drop-stir technique. Initially, a certain amount of gellan gum was stirred continuously in 50 mL water for 15-20 min at 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. Afterwards, the obtained solution was dropped into 50 mL chitosan solution in 1% acetic acid solution. This dropping process was performed under continuous vigorous stirring. The composition of the gel flakes formulation was determined by optimization process [4].

2.7. Optimization of SD-ITZ Gel flakes Formulations

The optimization of SD-ITZ gel flakes formulations was carried out using a CCD with Design Expert Software version 11 (State-Ease, Minneapolis, MN, USA). 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 flakes formulations in terms of entrapment efficiency and drug loading which were recorded as the responses. All factors were analyzed at three different levels (low, medium, and high). 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.

Table 3. The experimental design of SD-ITZ gel flakes formulations

Factors	Level		
	low	medium	high
Chitosan concentration (% w/v)	0.05	0.10	0.15
Gellan gum concentration (%w/v)	0.05	0.10	0.15

2.8. Characterization of SD-ITZ Gel flakes

In order 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 Equation (1) [4]

$$\%EE = \frac{W_o - W_f}{W_o} \times 100 \quad \text{Equation (1)}$$

Where W_o is the amount of drug added in the formulation and W_f is the amount of drug measured in the filtrate.

Drug loading (DL) capacity of ITZ in the gel flakes formulation was calculated by initially dissolving 10 mg of the 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 and quantified using HPLC. The percentage of drug loading was calculated using Equation (2) [20]

$$\%DL = \frac{\text{Amount of entrapped ITZ}}{\text{Total weight}} \times 100 \quad \text{Equation (2)}$$

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

DSC was used to evaluate the thermal properties of each formulation. An amount of formulation, sealed in an aluminium pan, was heated from 0°C to 300°C with the heating rate of 10°C/min.

The morphology of the obtained gel flakes 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 diffusion agar method. Initially, the growth medium of Saboraud agar containing 50 mg/mL of chloramphenicol was prepared in a petri dish. The inoculum of *C. albicans* was subsequently spread to cover all the surface of the agar. An amount which was equal to 2.5 µg/mL of free ITZ, SD-ITZ, and SD-ITZ gel flakes 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 flakes-loaded thermosensitive *in situ* gel

The thermosensitive gel loaded with SD-ITZ flakes formulations were prepared using the cold method [21]. An amount of PF-127, as the gelling agent, was dispersed in cold water (5°C) at room temperature (20°C). Separately, BKC, HPMC and HEC was 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 minutes. The thermosensitive composition of the gel containing the SD-ITZ gel flakes is shown in Table 4.

Table 4. The 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 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 [22]. 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 placed into immersion water at 20°C and then the temperature was gradually increased per 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 gel and could not flow over 30 s when was inverted. The same technique was applied to the dilution gel with the addition of 0.25 mL of vaginal fluid prior to the evaluation.

2.11.2. Mucoadhesion strength

The measurement of mucoadhesion strength was performed using a modified physical balance according to Morsi, *et al.* (2016) with slight modification [23]. A fresh excised cow vaginal tissue was fixed onto two glass vials with the mucosal faced outside. One vial was connected to the balanced in inverted position while the second vial was placed on a height adjustable pan. The gel was put onto the mucosal of the first vial. Subsequently, the second vial was adjusted until both of the mucosal contacted tightly and this condition was kept for 2 min. Weights were added onto the pan until both of vials got detached. The minimum weight used to detach both vials was recorded and the mucoadhesive strength was calculated using the Equation (3):

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

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 [24]. Initially, fresh 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 completely dissolved in the medium.

2.11.4. pH measurement

The pH of the formulations was measured by a pH meter (Mettler Toledo MP 220, Greifensee, Switzerland) in triplicate at 25°C .

2.11.5. Viscosity and Rheological study

The rheology of the *in situ* thermosensitive gel flakes SD-ITZ was measured using a DV-III Ultra viscometer (RV model, Brookfield, USA). 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 (Abd Ellah et al., 2019).

2.11.6. Drug content analysis

An amount of 50 mg 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. Afterwards, the solution was centrifuged at 14,000 rpm for 15 min. The supernatant was analyzed by HPLC [20].

2.12. Ex vivo permeation studies

The *ex vivo* permeation studies of the optimized thermosensitive vaginal gel loaded with SD-ITZ flakes was performed using vertical Franz cell with the 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 $37 \pm 1^\circ\text{C}$. 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 pre-determined time points until 8 h and then replaced with the same volume of release medium to maintain sink condition. All samples were further analyzed using HPLC [24].

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 conducted 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 [24].

2.14. *In vivo* antifungal activity in model of infection on rat

2.14.1. *Preparation of candidiasis model on rat*

Prior to the inoculation of *C. albicans*, eighteen female Wistar rats were placed in an individual cage and fed with enough food and water for at least 7 days. Afterwards, all 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 the animals in pseudoestrus condition. For the infection technique, *C. albicans* was first inoculated in sterile saline to obtain the concentration of 10^8 CFU/mL. Subsequently, intravaginal inoculation of *C. albicans* was applied to the animals [25].

2.14.2. *In vivo* antifungal activity

The *in vivo* antifungal activity of the optimized formulation was tested on eighteen infected models of female Wistar rats. The animals were divided into six groups of different treatments, namely optimized thermosensitive gel containing SD-ITZ flakes, thermosensitive gel containing SD-ITZ, thermosensitive gel containing pure ITZ, conventional gel containing ITZ, thermosensitive gel without drug, and negative control without any treatment. An amount of 1 g of each gel was applied in the vaginal of the animals. At the specified time points, 1 μ L of the vaginal fluid was taken and diluted for further to be inoculated in Saboraud agar containing 50 mg/mL of chloramphenicol. The inoculum was incubated at 32°C for 48 h before CFU counting [26]. This experiment was performed over 14 days and based on the ethical approval from the Ethical Committee from the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

2.15. Instrumentation and chromatographic condition for ITZ analytical method

The analysis of ITZ in all the samples was performed using HPLC (Shimadzu Prominence, Shimadzu, Kyoto, Japan) using a Phenomenex[®] Luna C₁₈ (ODS1) column (150 mm x 4.6 mm i.d. with 5 μ m 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 the 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 μ L and the analysis was carried out at ambient temperature. This method was validated according to the International Committee on Harmonization (ICH) guidelines 2005 [6].

2.16. Statistical analysis

For *in vitro* studies, all the work was done in triplicates and presented as mean $\pm$ SD. Statistical difference was tested using ANOVA followed by Tukey's test (GraphPad Prism 6.0, GraphPad, San Diego, CA, USA). The clinical data were analyzed using SPSS version 21 (Chicago, IL, SA). Student's *t*-test was used to compare the mean values between groups. The obtained *p*-value < 0.05 was considered 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 water-soluble polymer. In the present work, solvent evaporation method was used to fabricate SD-ITZ powder due to avoidance of heat which might cause drug degradation. Additionally, a more homogeneous mixture was able to be obtained using this method [27]. PVP and PEG were found to be the most commonly used solubility enhancer due to their high water solubility and low toxicity [28]. Therefore, the combination of PVP and PEG 6000 in different ratios was chosen in the preparation of SD-ITZ. In order to determine the exact ratio of both materials to obtain the maximum improvement in the water solubility of ITZ, optimization was performed with software-aided using CCD. There were 9 formulations suggested for the optimization with three different concentrations of PVP and PEG. The composition of each formulation and the responses of the variables were presented in Table 5. The results showed that the ITZ solubility in water and simulated vaginal fluid were in the range of 0.04-2.64 mg/mL and 0.07-2.71 mg/mL, respectively. Based on the fit statistical analysis results, the difference between predicted R^2 value and adjusted R^2 value was less than 0.2, which met the requirement to be

optimized. Additionally, the value of adeq precision was 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 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 5.

Table 5. Central composite experimental design and the response values for the SD-ITZ formulations

Run	Factors		Responses	
	PVP Concentration (%w/v)	PEG Concentration (%w/v)	Solubility in water (mg/mL)	Solubility in vaginal fluid (mg/mL)
1	3	1	1.37	1.43
2	2	1	1.32	1.39
3	3	2	2.51	2.63
4	2	2	2.43	2.57
5	1	3	0.07	0.11
6	2	3	2.55	2.69
7	3	3	2.64	2.71
8	1	1	0.04	0.07
9	1	2	0.06	0.09

3.2. Optimization of SD-ITZ formulations

The solubility of ITZ in water and simulated vaginal fluid were the parameters 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-value of the analysis was 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 factors selected in this process had a significant role in the ITZ solubility. The representative 3D graph illustrating the effect of selected factors on the solubility of ITZ in water and simulated vaginal fluid are shown in Figure 1. From these models, it can be clearly seen that the solubility of ITZ was directly proportional to the concentration of PVP and PEG. By increasing the amount of PVP and PEG in the formulation was able to generate higher solubility of ITZ. This might be caused by the properties of both substances as polymeric carriers, possessing greater dispersibility and able to inhibit the recrystallization phenomenon of the drug [29]. Finally, based on the software analysis, the concentration of 2.487% and 2.294% of PVP and PEG, respectively, were recommended for SD-ITZ formulation to obtain the optimum solubility of ITZ.

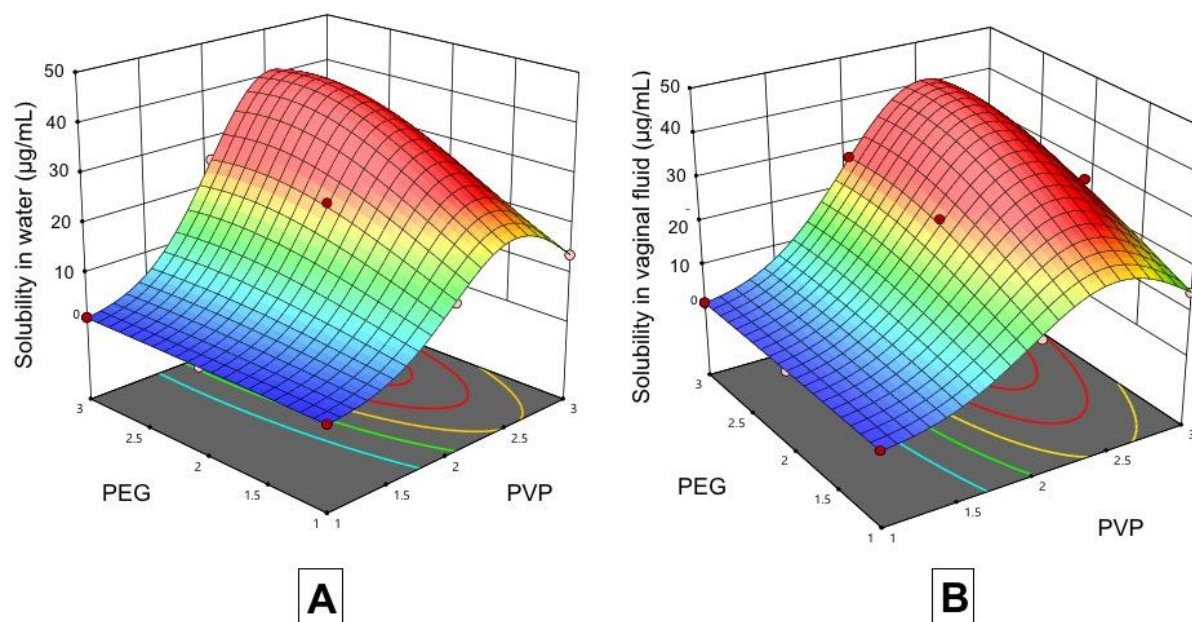


Figure 1. Representative response surface plots showing the effect of the selected factors on the solubility of ITZ in (A) water and (B) simulated vaginal fluid.

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 were $4.211 \pm 0.23\%$ and $4.291 \pm 0.21\%$. The comparison between the actual and predicted values is presented in Table 6. 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) [8], 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 reduction in the drug particle size, formation of amorphous state, increasing the porosity, and improving wettability [30]. The use of hydrophilic polymers with a strong ability to prevent the aggregation of drug crystals, which further increased the wettability of ITZ and transformed the ITZ into the amorphous state [16,29].

Table 6. The predicted and actual values of ITZ solubility in water and simulated vaginal fluid (mean $\pm$ S.D., $n=3$)

	Predicted values (mg/mL)	Actual values (mg/mL)	Bias (%)
Solubility in water	4.182	4.21 $\pm$ 0.23	-0.693
Solubility in simulated vaginal fluid	4.342	4.29 $\pm$ 0.21	1.174

Several peaks were observed which attributed to specific chemical groups of ITZ. A major peak was found at 1689 cm^{-1} , corresponding to 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 from 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 cm^{-1} and 823 cm^{-1} can be attributed to C–O functional group (1300–1000 cm^{-1}) and *para* aromatic ring-substituted (870–800 cm^{-1}), respectively. Finally, a peak at 685 cm^{-1} referred to C–Cl group presented in *ortho* and *para*-substituted in the aromatic ring (785–540 cm^{-1}) [6]. From Figure 2, it can be seen that all the characteristic peaks of ITZ were also presented 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 crystallinity properties of ITZ before and after SD preparation. Figure 2(A) and (B) present 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 [6]. However, these peaks were not found in SD-ITZ spectra. This means that the preparation of SD-ITZ was able to change the form of ITZ from high crystalline to amorphous state, which had a significant role to enhance the water solubility of ITZ.

Thermal properties of pure ITZ, PM and SD-ITZ was evaluated using DSC, as shown in Figure 2(C). 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 a good agreement with the obtained XRD results related to the crystallinity of ITZ after SD preparation. As the form of ITZ changed to amorphous form due to the SD preparation process, the endothermic peak was no longer able to be detected.

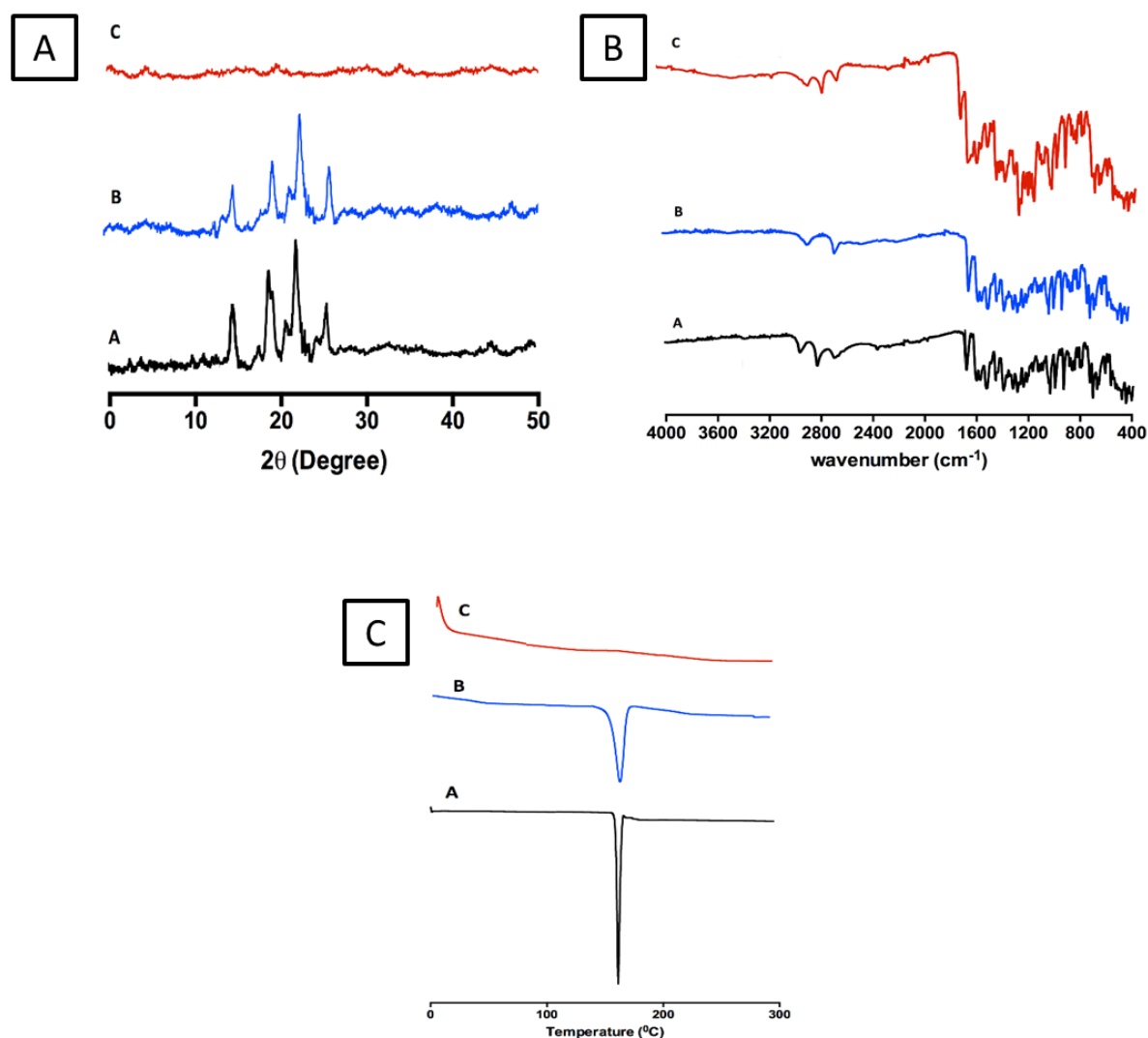


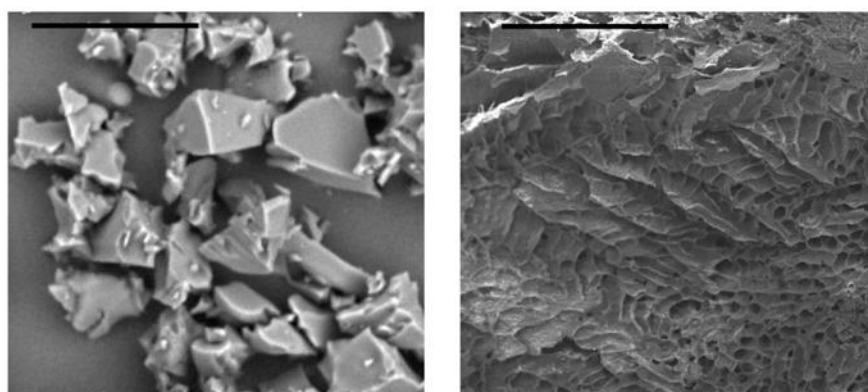
Figure 2. The results of (A) XRD, (B) FT-IR, and (C) DSC analysis of free ITZ (A, black line), physical mixture of ITZ and other excipients (B, blue line), and SD-ITZ (C, red line)

In order to identify the effect of solid dispersion system against the morphology of ITZ, the obtained SD-ITZ powder was observed using SEM. From Figure 3(A), it can be clearly seen that the pure ITZ was in 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 previous evaluation, confirming that the solid dispersion technique was able to change the form of ITZ which subsequently affect its solubility.

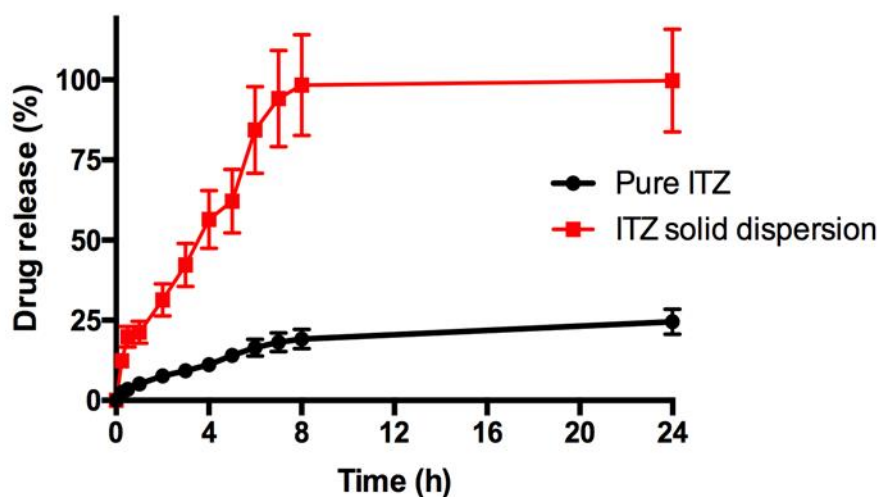
3.4. *In vitro* release studies of SD-ITZ

The *in vitro* release profiles of ITZ compared to SD-ITZ formulation is shown in Figure 3. Based on the results obtained, it was found the SD formulation was able to significantly increased the release behaviour of ITZ. After 24 h, the amount of ITZ released from SD-ITZ

formulation was $99.76 \pm 15.97\%$. Contrastingly, the free ITZ only reached $23.76 \pm 3.92\%$ which was around four times lower than the release behaviour 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 ($p\text{-value} < 0.005$). It was as expected that the reduction of particle size, the transformation to the amorphous state, the increase of wettability and dispersibility was able to decrease the interfacial tension between the drug and the dissolution medium resulting in better release behaviour [30,31].



A



B

Figure 3. (A) The morphologies of free ITZ (left) and SD-ITZ (right) observed under SEM with the scale representing 50 μm . (B) The drug release profiles of pure ITZ (black line) and SD-ITZ (red line) (mean $\pm$ S.D., $n=3$)

Several models of mathematic kinetic were then applied to the release profiles to investigate the release mechanism of ITZ from SD formulation. The results showed that the correlation

coefficient 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, 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 [19].

3.5. Fabrication of SD-ITZ gel flakes

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 [4]. Gellan gum is one of the anionic heteropolysaccharide derivatives containing gluconic acid. Chitosan is a cationic polysaccharide, generated by deacetylation of chitin. Both of these substances were extensively used in pharmaceutical research due to its biodegradability and biocompatibility [32]. 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 utilizing Design Expert software. As a result, the responses expected by varying these factors were the 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 7. 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 term of statistical analysis, it was found that the difference between predicted R^2 and adjusted R^2 of both responses were less than 0.2, which means that the models designed were acceptable to be optimized. Furthermore, adeq precision value, which measures the ratio of signal to noise in the analysis, was 51.220 and 14.148 for entrapment efficiency and drug loading, respectively. As the requirement of this value 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 7.

Table 7. Central composite experimental design and the response values for the gel flakes formulations

Run	Factors		Responses	
	Chitosan concentration (%w/v)	Gellan gum concentration (%w/v)	Entrapment efficiency (%)	Drug loading (%)
1	0.05	0.15	52.75	28.05
2	0.05	0.10	48.14	32.43
3	0.10	0.15	98.14	37.03
4	0.10	0.05	67.85	40.12
5	0.15	0.15	98.91	33.11
6	0.15	0.10	98.42	37.03
7	0.05	0.05	37.65	35.69
8	0.10	0.10	97.76	42.11
9	0.15	0.05	69.43	34.11

3.6. Optimization of SD-ITZ gel flakes formulations

The results showed that the EE and DL of all optimization processes followed the quadratic model. The F-value obtained was 362.23 and 19.30 for the responses of entrapment efficiency and drug loading, respectively. Additionally, the p -value of <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 entrapment efficiency and drug loading. The relation between the factors selected and the expected responses was 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 entrapment efficiency and drug loading. The higher concentration of gellan gum and chitosan would allow more intense cross-linking resulting in a higher amount of drug entrapped [33]. Finally, the composition of 0.094% of gellan gum and 0.092% of chitosan was the suggested formulation to obtain the maximum entrapment efficiency and drug loading percentage.

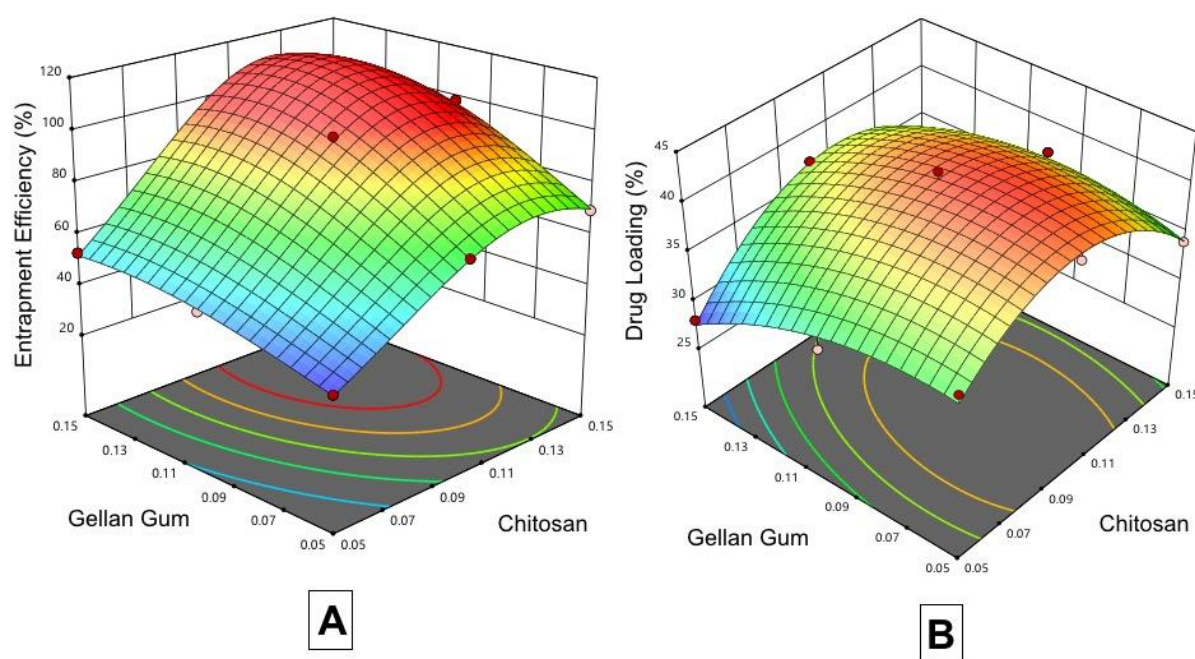


Figure 4. Representative response surface plots showing the effect of the selected factors on (A) EE and (B) DL of the optimized formulations

3.7. Characterization of SD-ITZ gel flakes formulations

In the gel flakes system, ITZ was entrapped in a gel layer caused by the gelation of gellan gum in a cationic environment (chitosan solution). This could happen due to ionic gelation between carboxylate ions of gellan gum and cationic moieties of the amino groups of chitosan [4]. The

percentage of entrapment efficiency represented the measured amount of drug entrapped in the gel flakes system in comparison to the theoretical amount. The optimum formulation, recommended by the software, was prepared and used for further investigation. The predicted value of entrapment efficiency was 98.978%. Indeed, the actual value obtained was $98.87 \pm 7.42\%$. As 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 flakes system. The actual value of drug loading obtained was $42.98 \pm 1.98\%$. This value had a less than 15% bias from the predicted value. These results suggested that the software utilized in the optimization processes was reliable to provide the formulation to obtain the optimum value of drug loading.

Table 8. The predicted and actual values of entrapment efficiency and drug loading of SD-ITZ gel flakes (mean $\pm$ S.D., $n=3$)

	Predicted values (% w/w)	Actual values (% w/w)	Bias (%)
Entrapment efficiency	98.978	98.87 ± 7.42	-0.109
Drug loading	43.631	42.98 ± 1.98	-1.492

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 5(A), 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 imine group C=N stretch ($1690\text{--}1650\text{ cm}^{-1}$) and N-C=O stretch ($1680\text{--}1630\text{ cm}^{-1}$). Another peak appeared at 1511 cm^{-1} was attributed to the double bond group, C=C, characteristic from the aromatic ring of ITZ ($1600\text{--}1475\text{ cm}^{-1}$). Furthermore, a peak was observed at 1221 cm^{-1} , corresponding to C-N stretching ($1350\text{--}1000\text{ cm}^{-1}$). Other signals found at 1192 cm^{-1} and 823 cm^{-1} were attributed to C-O functional group ($1300\text{--}1000\text{ cm}^{-1}$) and *para* aromatic ring-substituted ($870\text{--}800\text{ cm}^{-1}$), respectively. The last, a signal at 685 cm^{-1} referred to C-Cl group presented in *ortho* and *para*-substituted in the aromatic ring ($785\text{--}540\text{ cm}^{-1}$) [6]. 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 were shown in Figure 5(B). The results showed that the melting point of ITZ, marked by a sharp endothermic peak, was at 165°C . This peak only appeared in the thermogram of pure ITZ, which 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. As 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 *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 morphology of gel flakes which provided higher surface area, the application of gel flakes for the intravaginal purpose was preferred over the spheres [4]. The morphology of the prepared gel flakes loaded with SD-ITZ is shown in Figure 5(C). It was observed that the fabricated flakes were small and in polygonal structures. This form was found to be suitable to interpenetrate and spread into the highly folded vaginal surfaces.

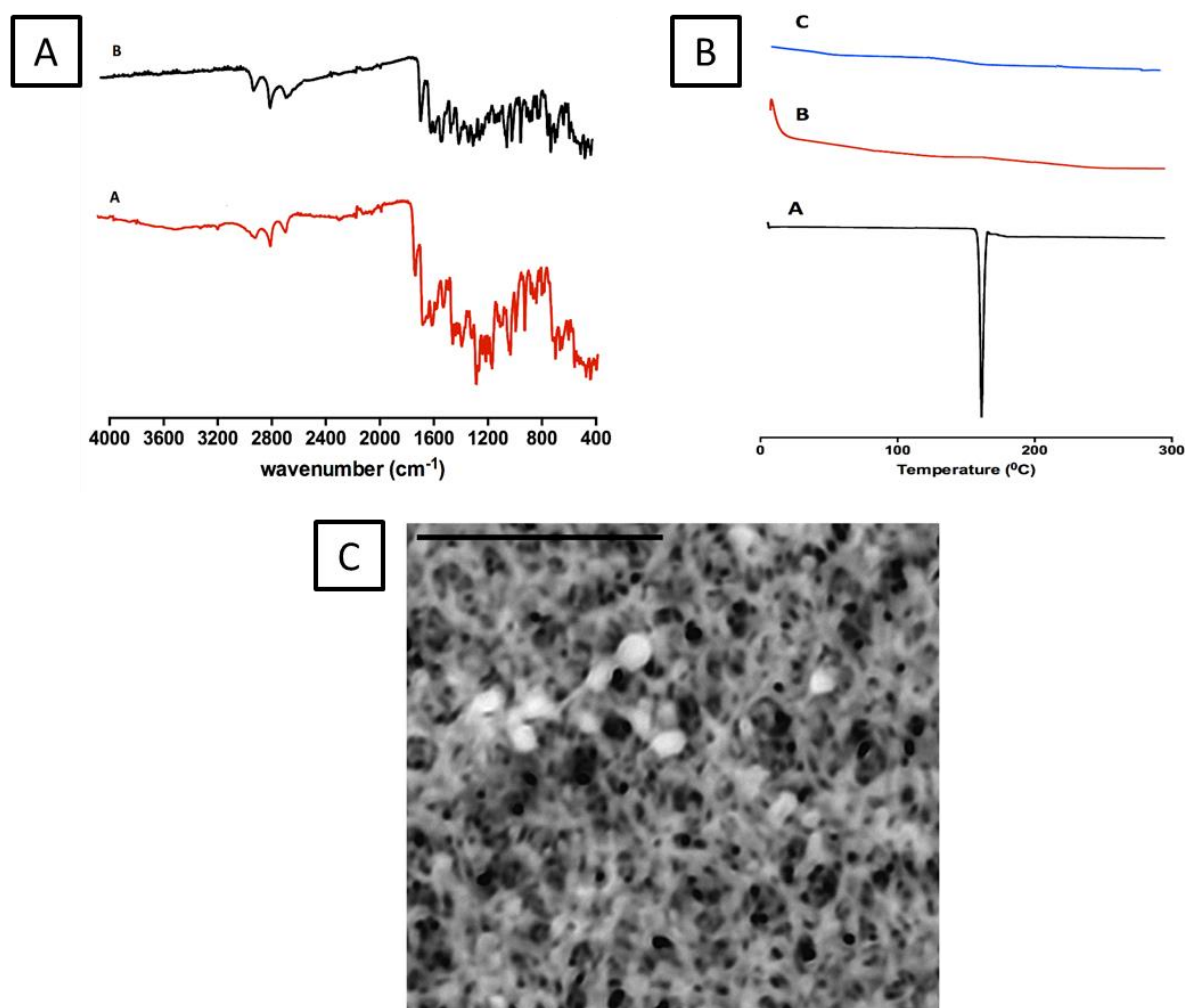


Figure 5. (A) FT-IR results of free ITZ (A, red line) and SD-ITZ gel flakes (B, black line). (B) DSC results of free ITZ (A, black line), SD-ITZ (B, red line), and SD-ITZ gel flakes (C, blue line). (C) The morphology of SD-ITZ gel flakes observed under SEM with the scale representing 50 μm .

3.8. *In vitro* antimicrobial activity test of SD-ITZ gel flakes

The *in vitro* antimicrobial activity test was carried out in order to investigate the ability of ITZ to inhibit the fungal growth in different forms. The results were 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, by increasing the solubility of ITZ using solid dispersion technique was able to provide the diameter of the inhibitory zone of 28.49 ± 2.17 mm. Higher solubility would allow the drug to diffuse to the growth medium resulting in better antimicrobial activity. Additionally, the SD-ITZ gel flakes also possessed a similar antifungal activity, showing the diameter of 27.89 ± 2.72 mm which was not significantly different compared to SD-ITZ (p -value <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 flakes system had no significant influence on ITZ antimicrobial activity.

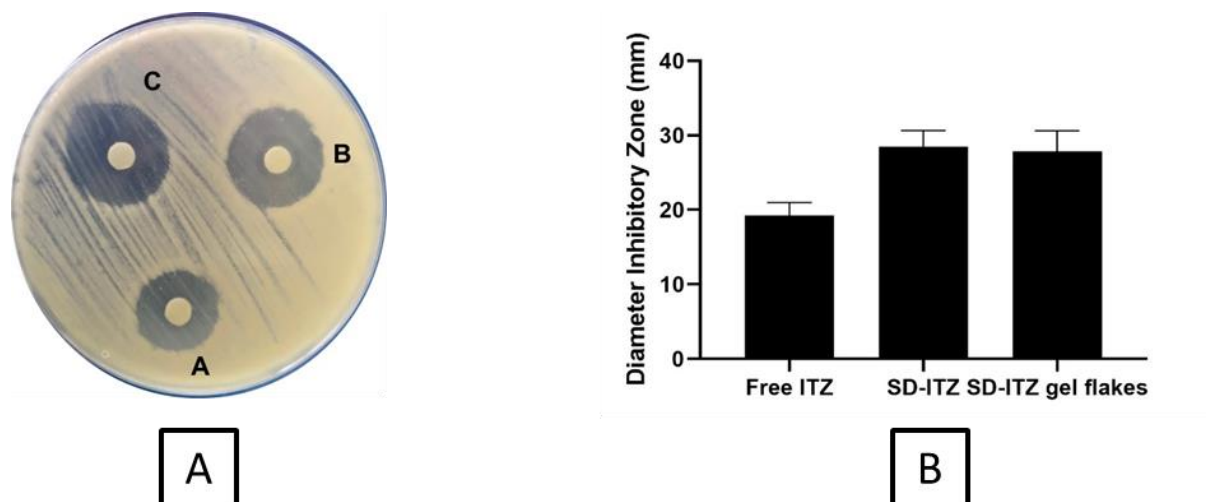


Figure 6. (A) The image of inhibitory zone of free ITZ (A), SD-ITZ (B), and SD-ITZ gel flakes. (B) The results of antimicrobial activity of free ITZ, SD-ITZ, and SD-ITZ gel flakes in bar graph (mean + S.D., $n=3$).

3.9. Preparation of SD-ITZ gel flakes-loaded thermosensitive *in situ* gel

The preparation of *in situ* gel containing SD-ITZ gel flakes was for the purpose 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 in the vaginal, the addition of mucoadhesive polymer was important in order 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 the thermosensitive polymers due to its ability to undergo sol-to-gel transition at a certain temperature [34]. HPMC and HEC were natural cellulose derivatives polymers used as the mucoadhesive polymers. Additionally, BKC was used as the preservative [35]. Prior to the addition of 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 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 solution transforming to gel at body temperature. Figure 7 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 vaginal simulated fluid was able to mimic the actual condition where the gel would be applied. Therefore, this evaluation was performed in two conditions, namely with and without dilution with simulated vaginal fluid. The results showed that F3 containing 15% PF-127 and 5% PF-68 transformed into gel at body temperature, 37°C both before and after dilution which were desirable (p -value>0.005). It also can be observed that higher concentration of PF-127 resulting in the lower $T_{\text{sol-gel}}$ temperature, which might be caused by the longer triblock chain of PF-127. It also can be seen that the $T_{\text{sol-gel}}$ of F6 and F7, with the addition of 0.2% and 0.4% of HPMC, respectively were not significantly different both with and without dilution, which were at 37°C (p -value>0.005). The similar results were obtained by F9 and F10, containing 0.2% and 0.4% of HEC, respectively (p -value>0.005). Interestingly, by increasing the concentration of the mucoadhesive polymers, HPMC and HEC, resulted in the decrease of gelation temperature. Both F8 and F11, contained 0.6% of HPMC and HEC, respectively, possessed significantly lower $T_{\text{sol-gel}}$ temperature compared to other formulations containing mucoadhesive polymers (p -value<0.005). This was confirmed that the concentration of mucoadhesive polymers was important to be considered in the thermosensitive *in situ* gel preparation.

Table 9. The gelation temperature of thermosensitive *in situ* gel containing SD-ITZ gel flakes (mean $\pm$ S.D., $n=3$)

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

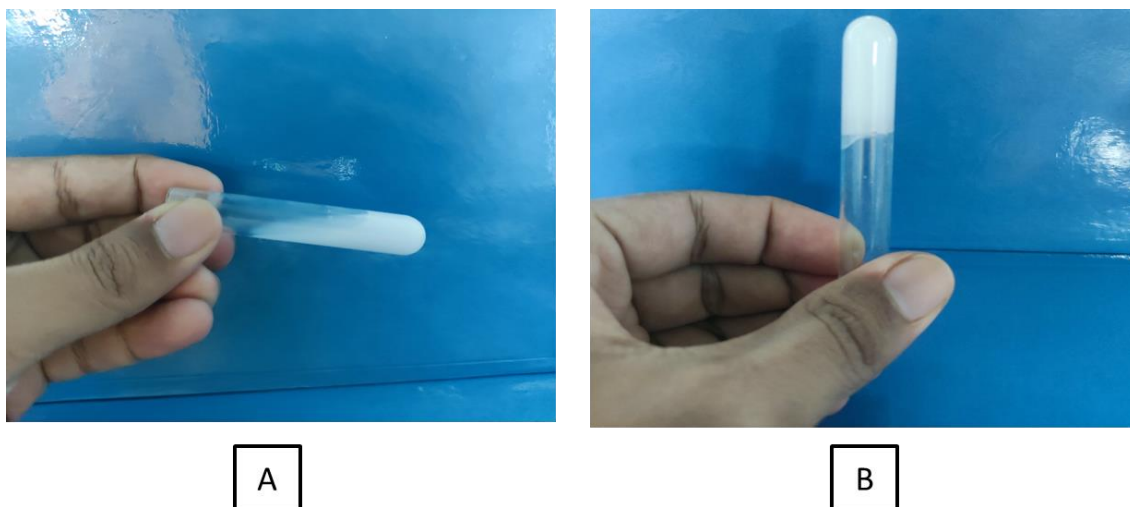


Figure 7. The images of *in situ* gel formulation (A) at room temperature (solution) and (B) at gelation temperature (gel)

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. It can be seen that PF-127 showed stronger mucoadhesive ability compared to PF-68. Despite these results, in order 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 glycoprotein of mucin [36]. 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 -value >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 -value >0.005). Besides possessing the same strength, the increasing of HPMC and HEC concentration to 0.6% provided lower gelation temperature 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 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 -value <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.

Table 10. The obtained values of mucoadhesion strength and time of *in situ* vaginal gel (mean $\pm$ S.D., $n=3$)

	Mucoadhesion strength (dyne.cm²)	Mucoadhesion time (h)
F1	21.12 $\pm$ 4.01	4.32 $\pm$ 0.82
F2	18.23 $\pm$ 3.04	3.14 $\pm$ 0.52
F3	16.98 $\pm$ 2.65	2.98 $\pm$ 0.46
F4	13.98 $\pm$ 2.77	2.16 $\pm$ 0.43
F5	11.76 $\pm$ 1.78	1.98 $\pm$ 0.30
F6	35.12 $\pm$ 5.79	5.98 $\pm$ 0.99
F7	43.12 $\pm$ 7.72	8.54 $\pm$ 1.53
F8	44.87 $\pm$ 7.76	8.74 $\pm$ 1.51
F9	29.32 $\pm$ 5.54	5.31 $\pm$ 1.00
F10	37.41 $\pm$ 7.00	6.17 $\pm$ 1.34
F11	39.31 $\pm$ 7.47	6.31 $\pm$ 1.41

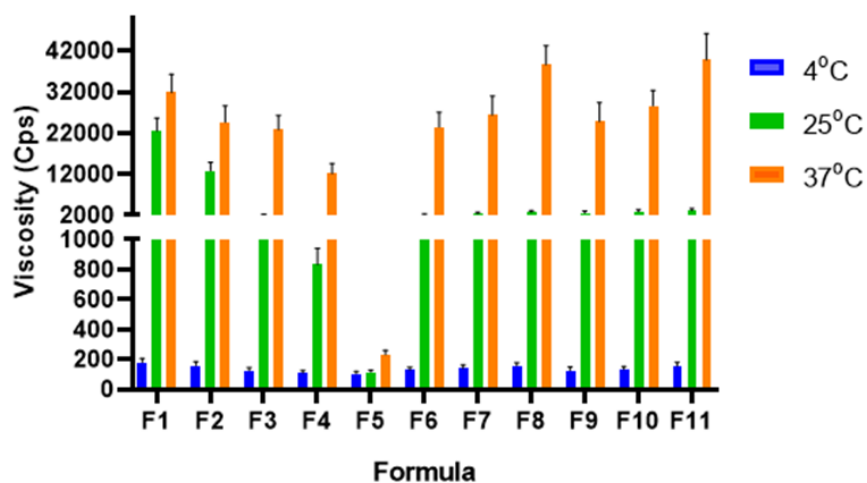
3.10.3. pH measurement

The results showed that all the formulations had acidic pH in the range of 4.81 – 5.82. These results corresponded to the acidic pH of the vaginal environment which confirmed that the formulations would not change the natural environment of the vagina nor caused any irritation in the vagina.

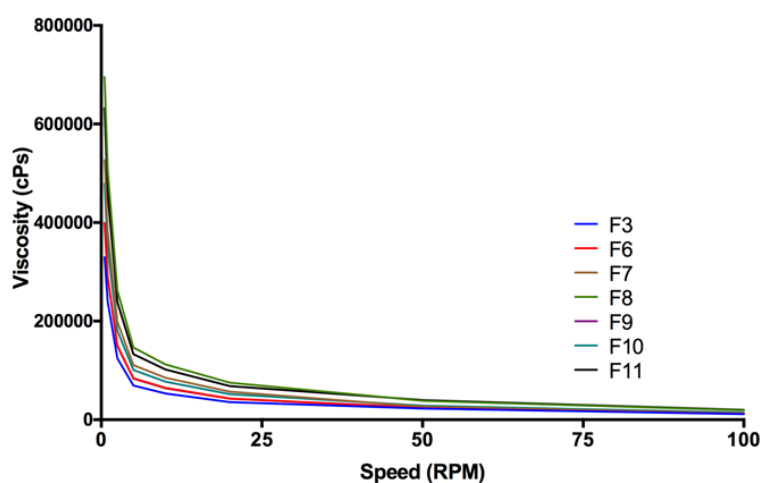
3.10.4. Viscosity and rheological study

The viscosity of each formulation was measured under 3 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 in order to facilitate the application while transforming into gel at body temperature to increase the residence time. In terms of the flow properties, as the gel system exhibited pseudoplastic behavior, therefore, the *in situ* gelling system should generate shear thinning condition 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 the lower viscosity [37]. Figure 8 depicts the rheology of the prepared formulations. The results showed that all the formulations indicated this expected behavior. Additionally, it was clear that the transition from solution to gel resulted in the increase in 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 obtained. PF-127 possesses a higher number of triblock chains followed by higher micelle size and number

compared to PF-68. This resulted in increased interaction of micelles causing the formation of more viscous gels [38]. 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 the room temperature. Besides, F5 was not able to fully transform to 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 free-flow liquid at room temperature and transforming into 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 0.2% and 0.4% of mucoadhesive polymers had no significant effect in 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.



A



B

Figure 8. The results of (A) measured viscosity at 4°C, 25°C, and 37°C (mean $\pm$ S.D., $n=3$) and (B) the rheology behavior of bioadhesive-thermosensitive *in situ* vaginal gel

3.10.5. Drug content analysis

The drug content analysis 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%, indicating that the process involved in the preparation of thermosensitive *in situ* gel did not affect the amount of ITZ entrapped in the gel flakes system.

Table 11. The results of measured pH and drug content of *in situ* vaginal gel (mean $\pm$ S.D., $n=3$)

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

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 hours. Figure 9 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 mg and 7.54 ± 1.21 mg, respectively, after 8 hours. 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 upon the mucosa in the donor compartment, the amount of ITZ released by F3, containing no mucoadhesive polymers, was not significantly different to the mucoadhesive formulations. Additionally, these results also showed that 0.2% and 0.4% of the mucoadhesive polymers added into the formulations generated non-significant 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 hours, indicating that the gel viscosity affected the release behaviour. The other formulations, F1 and F2, which possessed the highest viscosity amongst all formulation, 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 *C. albicans* vaginal candidiasis. In this case, the drug was intended to retain in the vaginal mucosal rather than to permeate to the acceptor compartment expressing systemic circulation. It was

reported that *C. albicans* were able to penetrate into the vaginal tissue in the severe condition of vaginal candidiasis [39]. Hence, it was important to evaluate the amount of drug retained in the vaginal mucosa to investigate the ability of ITZ permeating the tissue which further affects in the treatment efficacy. The results showed that F1 performed 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 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 were ~ 2 mg, maintained in the vaginal mucosa. Contrastingly, F3, F6, F7, F9, and F10 provided a significantly higher amount of drug in the vaginal tissue, reaching 4.76 ± 0.67 mg, 4.63 ± 0.62 mg, 4.59 ± 0.63 mg, 4.72 ± 0.67 mg, 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 mg and 3.76 ± 0.51 mg, respectively. Therefore, considering the mucoadhesive strength and time as well as other characterizations, F7 was selected as the best formulation for further antifungal activity test.

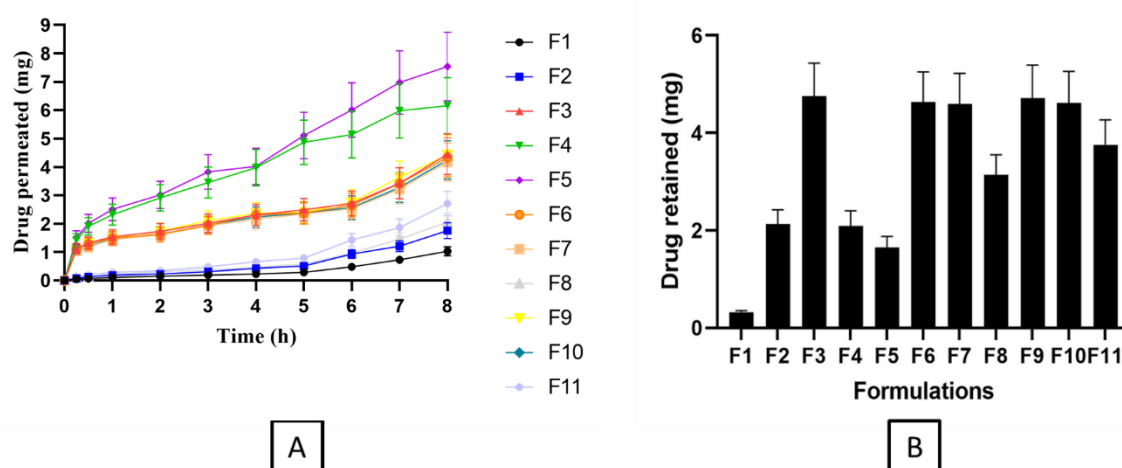


Figure 9. (A) The release profiles of ITZ permeation studies using cow vaginal mucosa (mean $\pm$ S.D., $n=3$) and (B) the amount of ITZ retained in the vaginal mucosa (mean $\pm$ S.D., $n=3$)

3.13. *In vivo* antifungal activity in 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 the suitable media. As shown in Figure 10, there were 6 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 *C. albicans* 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 the 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 was also important to note that the growth of *C. albicans* 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 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 CFU/mL 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 solid dispersion technique was found to be a successful approach to enhance the antifungal activity of ITZ by increasing the drug diffusion rate [40]. Furthermore, the application of gel flakes system was able to improve the spreadability of gel at the target site resulting in more effective antifungal activity. Therefore, the combination of solid dispersion technique, gel flakes system, and thermosensitive *in situ* gel was able to significantly improve the antifungal activity of ITZ.

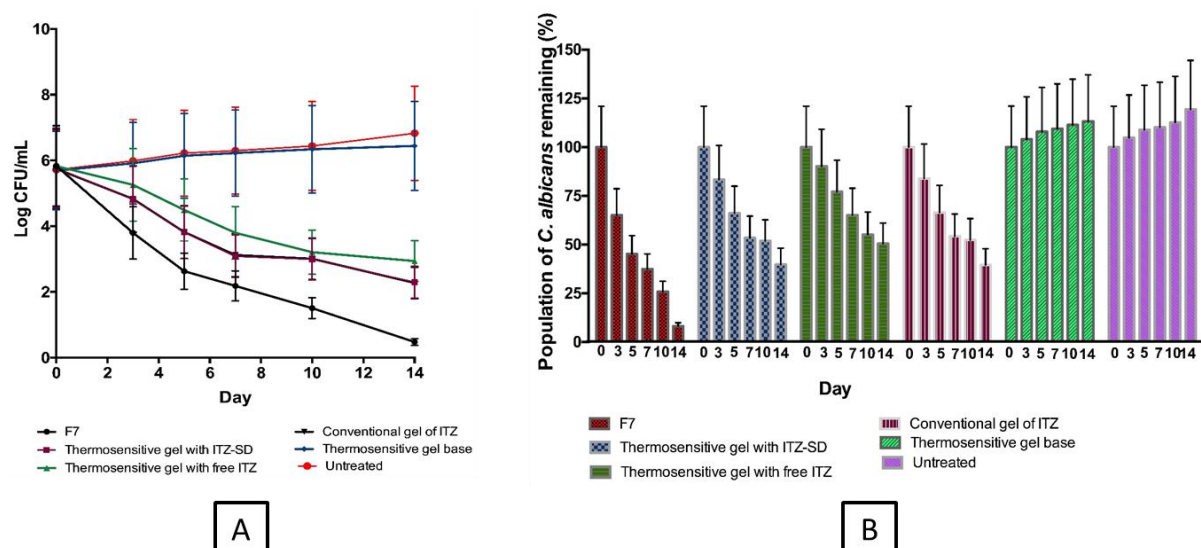


Figure 10. The results of *in vivo* antifungal activity studies expressed in (A) log CFU/mL and (mean $\pm$ S.D., $n=3$) (B) percentage of *C. albicans* population remaining (mean + S.D., $n=3$).

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 a 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 on 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 forwards, further studies, such as toxicity and biocompatibility tests, are now required to be performed to ensure the acceptability and usability before proceeding to the clinical stage.

4. Conclusion

In the present work, bioadhesive-thermosensitive *in situ* vaginal gel for local-targeting of vaginal candidiasis treatment was successfully developed. Initially, the application of solid dispersion technique was able to increase ITZ solubility up to ~ 4 mg/mL leading to improved release behavior and antifungal activity compared to free ITZ. Subsequently, in order to obtain high spreadability on the highly-folded vaginal surfaces, the gel flakes system containing SD-ITZ was prepared. Gellan gum and chitosan solution were found to be able to entrap the drug in the gel flakes system resulting in the desirable entrapment efficiency and drug loading capacity. Finally, the formulation of *in situ* vaginal gel using the 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 *C. albicans* 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 works regarding the safety and efficacy must now be performed to maximize the potential impact of this research.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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30-Mar-2021

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Title: "Bioadhesive-Thermosensitive In Situ Vaginal Gel of Gel Flakes-Solid Dispersion of Itraconazole for Enhanced Antifungal Activity in The Treatment of Vaginal Candidiasis"

Authors: Permana, Andi Dian; Utomo, Emilia; Pratama, Muhammad Rezky; Amir, Muhammad Nur; Anjani, Qonita Kurnia; Mardikasari, Sandra Aulia; Sumarheni, Sumarheni; Himawan, Achmad; Arjuna, Andi; Usmanengsi, Usmanengsi; Donnelly, Ryan F.

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Wed, Mar 31, 2021 at 3:30 AM

Dear Ryan,

Thank you very much for all your help and suggestions with this manuscript. It has been accepted for publication in ACS Applied Materials & Interfaces. 😊

Best wishes,
Dian

[Quoted text hidden]

Ryan Donnelly <R.Donnelly@qub.ac.uk>
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Wed, Mar 31, 2021 at 3:41 AM

Well done Dian,

Another for your ever-growing collection 😊

Congratulations!

Ryan

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Thank you very much Ryan.

I hope we can keep continuing working together 😊

Best wishes,
Dian
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Wed, Mar 31, 2021 at 4:06 AM

Of course Dian,

You can also come back here any time 🤔

Ryan

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Sat, Apr 3, 2021 at 9:30 AM

Thank you very much Ryan 😊😊

Dian
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