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DESIGNING, DEVELOPMENT AND IN-VITRO FUNCTIONALITY ASSESSMENT OF ANTICANADIAL MUCOADHESIVE VAGINAL GEL: A NOVEL THERAPEUTIC APPROACH

RAVAL HG[1,2*], SHAH UA[3] AND THAKKAR VT[4]

1: Research Scholar, Sankalchand Patel University, S.K. Campus, Kamana Cross Road, Visnagar – 384315, Gujarat, India

2: Department of Pharmaceutical Quality Assurance, Anand Pharmacy College, Anand – 388001, Gujarat, India

3: Department of Quality Assurance and Pharmaceutical Chemistry, Nootan Pharmacy College, Sankalchand Patel University, S.K. Campus, Kamana Cross Road, Visnagar – 384315, Gujarat, India

4: Department of Pharmaceutics, Anand Pharmacy College, Anand – 388001, Gujarat, India

***Corresponding Author: Mr. Hitesh G. Raval: E Mail: hitesh2530@rediffmail.com**

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ABSTRACT

Background: *Candida albicans* is the most commonly detected yeast in patient samples. Vaginal thrush, or vulvovaginal candidiasis (VVC), results from the excessive growth of *C. albicans* in the vagina. The formation of *C. albicans* biofilms presents a significant challenge in treating candidiasis due to their resistance to conventional antifungal drugs. Research indicates that Fluconazole (FCZ) resistant *C. albicans* requires novel treatments, either as standalone therapies or in combination with FCZ. FCZ and Quercetin (QCT) may work synergistically to effectively treat resistant *C. albicans* infections. This study addressed the issue by developing mucoadhesive in-situ vaginal gel formulations containing both fluconazole (FCZ) and quercetin (QCT), aimed at the treatment of resistant strains of *Candida albicans*. The mucoadhesive vaginal gel formulation was developed and optimized using 3^2 Full Factorial Design (FFD) with reference to optimization of mucoadhesive and thermosensitive polymers. The optimized batch consists of a thermosensitive polymer composed of PLX188 (10%w/w) and PLX407 (25.51%w/w), as well as mucoadhesive polymers, namely HPMCK4M (0.82%w/w). The formulation has an appropriate viscosity and gel strength, making it ideal for the desired phase transition

temperature. The optimized formulation exhibited a gelation temperature of 32.83 ºC, a rapid gelation time of 24 seconds, and a bioadhesive strength of 84.85 Kg.m/S^2. The results also showed that the FCZ had a medication release rate of 98.61% within 60 minutes, whereas the QCT had a drug release rate of 92.03% within 180 minutes in the optimized batch. The drug release kinetics results indicated that FCZ followed zero-order kinetics, whereas QCT exhibited first-order kinetic release from the optimized mucoadhesive vaginal gel formulation. The developed mucoadhesive vaginal formulation provide synergistic effect against Itraconazole resistant *Candida albicans*. The mucoadhesive in-situ vaginal gel outperforms oral dosage forms with lower dosage requirements, enhanced bioavailability of quercetin, and targeted effectiveness against drug-resistant *Candida albicans*. It also improves patient adherence and remains effective when fluconazole alone is ineffective.

Keywords: Fluconazole (FCZ), Quercetin (QCT), Mucoadhesive vaginal gel, 3^2 Full Factorial Design (FFD), Antifungal activity

INTRODUCTION

Despite medical advancements, fungal diseases remain a serious threat to human health, with actual mortality rates estimated to be five times higher than reported **[1, 2]**. Over 90% of fungal infections are caused by Candida strains, predominantly *Candida albicans*, which affects skin and mucous membranes **[3-7]**.

Vulvovaginal Candidiasis (VVC), commonly referred to as vaginal thrush, is a prevalent gynaecological condition resulting from the overgrowth of yeast in the vagina, primarily caused by *Candida albicans* **[8, 9]**. Vaginal thrush, or vulvovaginal candidiasis (VVC), caused by *C. albicans*, affects 75% of women, with over 85% of VVC cases attributed to this pathogen **[8-10]**. Extensive studies show free-floating Candida cells and biofilm formation contribute to antifungal resistance, complicating treatment **[11-17].**

Fluconazole (FCZ) is the first-line drug frequently employed in the clinical prophylaxis and treatment of both mucosal and invasive Candida infections, including VVC. The widespread use of Fluconazole is attributed to its favourable bioavailability and lower toxicity **[18-20]**. Research indicates that the resistance rate of *C. albicans* to FCZ among patients with Vulvovaginal Candidiasis ranges from 10-20%. The excessive and indiscriminate clinical application of FCZ has led to the emergence of multiple-drug-resistant (MDR) strains of *C. albicans* **[21-23]**. Consequently, there is a pressing need to explore novel therapies that can be used either alone or in conjunction with FCZ for treating FCZ-resistant *C. albicans* isolated from VVC.

Quercetin (QCT), a dietary flavonoid, has shown weak antifungal activity that can help manage clinical *Candida albicans* biofilms and enhance the susceptibility of FCZ-

resistant *Candida albicans* isolates to FCZ **[24, 25]**.

Furthermore, the study indicates that following treatment with QCT and FCZ, fungal load was reduced, hyphal forms disappeared, and the inflammation of mucosal epithelial cells was significantly alleviated, suggesting a potential synergistic effect between FCZ and QCT in treating resistant *Candida albicans*-associated infections **[26]**.

Conventional vaginal delivery methods work pretty well, but they have some problems that need to be fixed before they can be used to give effective antifungal medicine: The disadvantages connected with creams, suppositories, simple tablets and gels include discomfort, leakage, and messiness. The medications are not retained well in the vaginal epithelium, and frequent administration of the pharmaceuticals leads to poor patient compliance. Additionally, the drugs have low oral bioavailability because of first-pass metabolism, and their release pattern is not optimal, which affects the effectiveness of the treatment. Patients may also experience alienation, discomfort, and reluctance, which may result in a reduced desire to take the medication **[27-30]**.

A mucoadhesive thermosensitive in situ vaginal gel is an advanced drug delivery system designed for vaginal administration of therapeutics. Its primary features include Mucoadhesiveness, which ensures adhesion to the vaginal mucosa for prolonged retention and targeted drug release, and thermosensitivity, allowing the gel to remain in a liquid state at lower temperatures for ease of application. Upon exposure to body temperature, the gel transitions to a solid state, ensuring localized delivery of the medication at the intended site.

There is no any reported data about the formulation for the combined dose form of FCZ and QCT, according to the literature study.

The present investigation more emphasizes for development mucoadhesive In-situ vaginal gel using FCZ and QCT to address the current issue. The employment of mucoadhesive vaginal gel formulation also full fill the limitation of the conventional dosage form and enhance the performance characteristic upon binding with the mucus membrane of the vaginal cavity **[31-32]**.

In current investigation, the mucoadhesive vaginal gel formulation was developed and optimized using 3^2 Full Factorial Design with reference to optimization of mucoadhesive and thermosensitive polymers. The formulated gel underwent a comprehensive evaluation, focusing on parameters such as gelation temperature, gelation time, gelation strength, pH, viscosity, and texture analysis. Additionally, in vitro assessments included drug diffusion

studies, kinetic analyses, assay of the formulated product, and evaluation of antifungal activity against resistant strains of *Candida albicans*.

MATEIRALS AND METHODS

Materials

The Fluconazole (FCZ) standard was given by Rutu chemicals, Panoli, Gujarat. Quercetin, featuring 98.0% purity (HPLC-grade, molecular weight 338.27, CAS No. 6151-25-3), was obtained from Sigma-Aldrich, Mumbai. Poloxamer (PLX) 188 (Kolliphor® 188) and Poloxamer (PLX) 407 (Kolliphor® 407) were gifted by BASF Corporation, New jersey, USA. HPMCK4M, HPMCK100M, HPMCK15M, polycarbophil, propylene glycol and benzalkonium chloride was procured from S.D. Fine chemicals, Vadodara, Gujarat, India and distilled water was prepared inhouse at the college.

Instrumentation

FTIR Spectrophotometer, FTIR6100 – JASCO, USA; Differential Scanning Calorimetry DSC7020, Hitachi, Japan; QTS Texture Analyzer – QTS 25, Brookfield Engineering Laboratories, India; Brookfield Viscometer, LVDV-2+ Pro, Germany ; pH meter, Weltronix PM100, EIE instrument Pvt. Ltd., India; UV-1800, UV Probe 2.35, Shimadzu, Japan; HPLC, Shimadzu LC-2010C HT, Japan; HPTLC, CAMAG, Switzerland; Ultrasonic Cleaner (USC 300), Model No. – USC 300, Capacity - 5.5 Liter, MultiTech Enviro Analytical Pvt. Ltd, India; Digital Magnetic Stirrer, MS-500, Remi Equipment, India; Steam bath, Elie Instrument Ltd. India, Water Distillation Unit, Riviera glass Pvt. Ltd., India (Capacity: 2 L/h) were used in formulation development and testing of mucoadhesive vaginal gel. AUW 220D Electronic balance (Shimadzu Corp., Japan) weighed all substances.

Formulation and Development of Mucoadhesive In-Situ Vaginal Gel

Modifications were made to the cold procedure to develop the mucoadhesive vaginal gel **[33-35]**. The quantity of PLX 188 and PLX 407 was transferred into a 100 mL beaker. A consistent stirring motion was maintained while introducing the required volume of cold deionized water. The gels underwent a 24-hour incubation period at 4 ℃ to produced solution that exhibited transparency. The requisite quantity of HPMCK4M was subsequently added during agitation, and the solution was allowed to stand undisturbed overnight at a temperature of 4 ℃, leading to the formation of a liquid polymeric solution with a translucent appearance. Fluconazole, Quercetin, and Benzalkonium chloride were dissolved using a desired quantity of propylene glycol. The liquid obtained was further agitated for 5 minutes until a transparent solution was attained. The polymer solution was gradually added to a mixture consisting of

Propylene Glycol, Fluconazole, Quercetin, and Benzalkonium chloride, with continuous stirring. The vaginal gel, which exhibited transparency and pale yellow colour, was subjected to a storage temperature of 20 °C. The optimized formulation comprises the active components enumerated in the **Table 1**.

Table 1: Composition of Optimized Mucoadhesive In-Situ Vaginal Gel

Drugs and Excipients	Amount (%w/w)	Role of Drugs and Excipients in formulation
Fluconazole	0.5	Antifungal agent
Quercetin	0.125	Antifungal and biofilm inhibitors
Poloxamer 188 (PLX 188)	10.63	Thermosensitive gelling agents and surfactants
Poloxamer 407 (PLX 407)	25.51	Thermosensitive gelling agent
HPMCK4M	0.82	Mucoadhesive polymers
Propylene Glycol	3	Solvents and permeation enhancer
Benzalkonium chloride	0.01	Preservative
Distilled water	59.4	Solvent for polymers

Optimization of Mucoadhesive In-situ Gel Formulation Using 3^2 Full Factorial Design (FFD)

As part of quality by design (QbD), a design of experiments (DoE) approach was employed to systematically and scientifically determine the empirical relationship between critical material attributes (CMAs) and critical quality attributes (CQAs). A full factorial design involving two variables at three levels (3^2) was developed to explore all possible combinations of factors at every level. The experimental design consisted of nine experiments, utilizing Design Expert 11.0.4.0 software (Stat-Ease, Inc., Minneapolis).

In this study, a 3^2 full factorial design was applied for optimization of polymers composition in vaginal gel. The independent variables included the concentration of Thermosensitive Polymers (PLX 188 and PLX 407: X1=25-45%w/w) and the concentration of the Mucoadhesive Polymer (HPMCK4M: X2=1-3%w/w). During the investigation, all other material attributes and process parameters were kept constant. The response factors examined were gelation temperature (Y1=33-37ºC), mucoadhesive strength (Y2=10-30 sec), and gelation time (Y3=25-80 sec) **[36]**.

Evaluation Parameters of Mucoadhesive In-Situ Vaginal Gel

Gelation Temperature (Tsol−gel)

The gelation temperature was determined using the test tube inversion method. Two millilitres of gel formulation were placed in a closed test tube at 4 °C. The tube was then immersed in water bath at 20°C, with the temperature gradually increased. At each temperature, the tube was rotated 90 degrees to visually inspect the formulation. The gelation temperature was recorded as the lowest temperature at which the solution solidified into a gel and stopped flowing

when inverted for over 30 seconds. This test was performed three times.

Gelation Time

Gelation time was measured using the test tube inversion method. After adding 2 mL of the formulation and 0.25 mL of freshly prepared SVF (pH 7.0) to the test tube, it was placed in a water bath at the specified gelation temperature. The tube was then inverted to check the sample's flowability. Gelation time was determined by observing if the gel flowed or remained in place when inverted **[37]**. The test was repeated three times.

Gelation Strength

Gelation strength is measured by the time it takes for a 1 g weight to penetrate 5 centimetres into a 50 g gel. This time, recorded in seconds, indicates the gel's strength. The experiment was carried out using laboratory-modified 'Gel strength equipment'. A 50 g mucoadhesive gel was placed into a 100 mL measuring cylinder, and the process of turning into a gel was started. Afterward, the piston, weighing 1 gram, was placed upon the gel. The gel strength was evaluated by recording the time (in seconds) needed for the piston to descend 5 cm into the gel **[38-40]**. This procedure was repeated three times.

pH

It was measured three times using a calibrated pH meter (Weltronix PM100, EIE instrument Pvt. Ltd., India) at 25°C. For optimal compatibility, it is desirable for the pH of the formulation to closely match the pH of the vaginal environment, which should ideally fall within the range of 3.5 to 4.5.

Clarity

Clarity is an essential aspect in gel formation, and it is necessary that the solution be devoid of turbidity or particulate debris. The in-situ gel formulation was tested on both a white and black backdrop, and no presence of particle matter was observed in the formulation.

Viscosity

The viscosity of the gel was determined using the Brookfield Viscometer DV-I-Prime, manufactured by AMETEK Brookfield. A 100 mL sample was placed in a beaker and the viscosity was measured using spindle number 62. The dial reading was recorded for each speed. The viscosity of the gel was measured three times at a speed of 50 revolutions per minute.

Texture Analysis

The gel texture profile was analysed by QTS-25 Texture Analyzer (Brookfield Engineering Labs., Mumbai, India) to measure mechanical characteristics like adhesiveness, hardness, force, and bioadhesive strength. Formulations were placed in an 80 mL cavity at 37±1°C. A 1.2 cm diameter probe was inserted twice into each sample to a depth of 20mm at 30mm/min, with a 15-second recovery time.

A trigger force of 5 grams was used. Each sample underwent at least six duplicate analyses at 37±1°C. Data were processed using Texture Pro software, version 2.1 (Biozon Food Innovations GmbH).

In-Vitro Drug Diffusion Study

The in vitro drug release was examined using a Franz diffusion cell. The experiment included applying a 2 mL mucoadhesive vaginal gel formulation to the donor compartment, followed by introducing 75 mL of SVF (pH 7.0) to the acceptor compartment. The cellophane membrane was positioned between the ends of both compartments on the Franz diffusion cell. The rotational velocity was set at 50 revolutions per minute. The temperature of the SVF was maintained at a precise range of 37±0.5 °C using a magnetic stirrer **[41-44]**.

The drug penetration study included by collecting 1 mL of the sample at certain time intervals, namely at 5, 10, 20, 30, 45, 60, 90, 120, 150, and 180 minutes. The samples were collected for each sampling event. After removing a specific volume of the sample, it was adjusted with an equal amount of SVF at pH 7.0 **[41, 44, 45]**. The analysis of each sample was conducted utilizing developed first-order UV-Spectrophotometric method **[46]**. A blank solution containing SVF at a pH of 7.0 was used as a reference solution. Three repetitions of the drug permeation study were performed, and the mean of all data was used to estimate the drug release profile using the regression equation derived from the first order derivative spectrophotometric method **[46]**.

Determination of In Vitro Kinetic Release

In order to determine the process by which drugs are released from sol-gel, it is essential to gather in vitro data on drug release. An investigation was carried out to analysed the release kinetics of a produced mucoadhesive in situ gel utilizing several models, such as the Zero order model, First order model, Higuchi model, and Korsmeyer Peppas model **[47, 48]**.

Drug Content Study

The mucoadhesive gel was tested for drug content using an in-house developed and validated first-order UV spectrophotometry technique published in our previous studies **[46]**.

Drug Polymer Compatibility Study

Compatibility studies aim to identify and predict drug-excipient interactions and assess their effects on the final product's manufacturability, quality, and performance. With reference to same, Fourier Transform Infrared (FTIR) Spectroscopy Study and Differential Scanning Calorimeter (DSC) Study was performed for developed mucoadhesive vaginal gel.

Fourier Transform Infrared (FTIR) Spectroscopy Study

The Fourier Transform Infrared Spectrophotometer (FTIR) (Jasco FTIR 6100 Type-A, Japan) was used to obtain infrared spectra of individual drugs and drug-polymer physical mixtures to detect drug-polymer interactions. Samples were dispersed in KBr and gently mixed. The spectrum was recorded at a resolution of 16 cm^{-1} using a Triglycine Sulfate (TGS) detector, with a scanning speed of 2 mm/sec, covering a frequency range of 4003.5 to 397.264 cm^{-1}. The KBr background spectrum was used as a blank.

Differential Scanning Calorimeter (DSC) Study

The thermal behaviour of the samples was examined using a Differential Scanning Calorimeter. The studies were conducted under a desiccated nitrogen environment. The specimens underwent heating at a rate of 10°C per minute from the surrounding temperature until reaching the melting point. A vacant aluminium pan served as a point of reference. Both individual drugs and drug-polymer physical mixtures were subjected to DSC spectroscopy study.

Antifungal Studies of Mucoadhesive Vaginal Gel Formulation

The broth microdilution technique assessed the minimum inhibitory concentrations (MICs) of FCZ alone and combined with QCT against Itraconazole-resistant *C. albicans* isolates using 96-well microtiter plates. The fungal suspension was diluted to 10^3 CFU/ml in RPMI 1640 medium, with FCZ and QCT at a 4:1 ratio in developed formulation. RPMI 1640 was added to each well to a final volume of 200 µL. A drug-free well served as the control, and wells with only RPMI 1640 were negative controls. Plates were incubated at 35°C for 24 hours. Growth inhibition was assessed visually and quantified by measuring optical density at 492 nm. The MIC80 is the minimum concentration inhibiting 80% of yeast growth relative to the control.

RESULTS AND DISCUSSIONS

For 3^2 factorial designs, a total of nine experiments were performed for two factors at three levels each. All the nine formulations were evaluated for gelation temperature, gelation time (sec) and gelation strength (sec).

The relationship between the two independent variables (X1 and X2) and three dependent variables (Y1, Y2, and Y3) in the factorial design was determined using Design-Expert® 11.0.4.0 through multiple regression analysis (MLR) and ANOVA. The optimal mathematical model was selected based on correlation coefficients (R^2), coefficient values, and Fisher's ratio with P values, with statistical significance determined at a 5% level ($p < 0.05$).

The nine experimental runs conducted are outlined in **Table 2**, which also includes the design matrix presented in actual values.

The data of all the three responses were fitted to different statistic models. Responses Y2 and Y3 confirmed highest adjusted and predicted R^2 values in linear model, whereas response Y1 confirmed highest values in quadratic model to study the interaction effect between selected variable and response. Adequate precision was > 4 in all the three responses. P value was < 0.05. A higher Model F-value indicates the significance of the model. The Correlation coefficient (R^2) value close to 1 indicates a good fit. Therefore, reliability of model was confirmed. Regression statistical analysis of the three responses is represented in **Table 3**.

2D and 3D plots were drawn as shown in **Figure 1** to estimate the effect of independent variable on each response. The data represented that Factor X1 (concentration of thermosensitive polymers PLX188 and PLX 405) and Factor X2 (concentration of mucoadhesive polymer HPMCK4M) have a significant impact on the variables Y1 (Gelling Temperature), Y2 (Gelling time), and Y3 (Gelling Strength). Factor X1 had a greater impact on the response variable of the formulation compared to Factor X2.

Validation of design model

The overlay plot, created by superimposing contour plots, highlights the common area for generating standard checkpoint batches **(Figure 2)**. The yellow coloured region was identified in the overlay plot and considered optimal. Three checkpoint sets were selected, and the mathematical models for three batches were validated for selected CQAs related to anticipated values, and the % prediction error was calculated. The % prediction error for all responses was less than 10% **(Table 4)**, which ascertains the high predictive ability of the developed model **[49, 50]**.

The checkpoint analysis **(Table 4)** showed a negligible percentage error, below 5%, indicating a close match between observed and predicted values. The selected batch was optimized based on key formulation criteria: Gelation Temperature, Gelation Time, and Gelation Strength. The ideal Gelation Temperature should between 33°C to 37°C, aligning with body temperature. To prevent leakage from the vaginal canal post-application, reducing Gelation Time is crucial. Increasing Gelation Strength is crucial to ensure the gel remains intact on the vaginal mucosal barrier for an extended period.

Evaluation Parameters of Mucoadhesive Vaginal Gel (Check Point Batch)

The optimal temperature range for the effectiveness of vaginal gel formulation is between 25°C to 37°C **[51]**. Below 25°C, the gel may solidify at room temperature, complicating manufacture and administration. If the gelation temperature

exceeds 37°C, the gel remains liquid at body temperature, leading to inadequate drug release and shorter retention. The checkpoint batch gelation temperature ranged from 32.83 to 33°C, suitable for vaginal usage **(Table 4)**. Gelation time, crucial for creating a thermosensitive hydrogel, ranged from 24.49 to 25.70 seconds, aligning with the literature **[51]**. Gelation strength, essential for firm barrier formation and prolonged drug retention, ranged from 26.80 to 27.62 seconds. The checkpoint data of the Critical Quality Attributes (CQA) demonstrated that all data aligned with the constraints specified in the design. The combination of polymers in the gel formulation achieved ideal viscosity for easy administration, enhanced adherence, and somewhat longer drug release. The validation checkpoint batches confirmed that increased polymer concentrations raised viscosity, and the formulation's pH (5.67 to 5.73) was close to the normal vaginal pH range (3.8 to 4.5) **(Table 5)**.

To optimize vaginal gel formulations, they should possess strong adhesion, easy application, low hardness, and excellent retention at the site **[52]**. Texture Profile Analysis (TPA) was used to assess the mechanical properties of the validated batches, as shown in **Table 5**. The hardness of the batches was ranked as F2 > F3 > F1, indicating that Batches F1 and F3 are ideal due to their low hardness and compressibility, which facilitate easy removal from the container and even application. Mucoadhesive strength, essential for effective drug delivery and prolonged therapeutic effects, was ranked as F3 > F2 > F1. These findings suggest that Batch F3 has strong mucoadhesive properties and should be considered ideal. Therefore, the F3 batch is considered optimal due to its moderate hardness and strong mucoadhesive properties.

In-Vitro Drug Diffusion Study

The drug permeation evaluation results for FCZ and QCT from the optimized mucoadhesive vaginal gel formulation (Batch F3) graphically represented in **Figure 3** (FCZ) and **Figure 4** (QCT). The findings demonstrated that the FCZ exhibited a drug release rate of 98.61% during 60 minutes, whereas the QCT exhibited a drug release rate of 92.03% for 180 minutes.

Four models were used to evaluate the kinetics of FCZ and QCT in an optimized batch (F3 batch). A zero-order kinetic model, A first-order kinetic model, The Higuchi kinetic model, and The Korsmeyer-Peppas kinetic model. The data analysis showed that FCZ followed Zero-order kinetics (R^2 = 0.9990) **(Figure 5a)**, whereas QCT displayed First-order kinetic release (R^2 = 0.9926) **(Figure 5b)**.

Kinetic Models of Fluconazole (FCZ) (F3 batch)

Drug Content Study

The developed and validated first-order UV spectrophotometry method yielded an assay range for FCZ of 97.97% to 100.26% w/w, while for QCT, it was found to be 98.66% to 100.79% w/w in the developed mucoadhesive vaginal gel **[46]**.

FTIR Study

The FTIR data of the mucoadhesive vaginal gel formulation is shown in **Figure 6**. The FTIR data for QCT showed the disappearance of peaks at wavenumber 3409.53 cm^{-1} (C-OH group stretching, phenol), 1662.2 cm^{-1} (C=O stretching, strong, conjugated ketone, cyclohexanone), and 1365.35 cm^{-1} (C-OH bending, phenol) in the sample formulation. Similarly, the FTIR data for FCZ showed the disappearance of the peak at wavenumber 1137.8 cm^{-1} (C-F stretching, strong) and a reduction in peak intensity at wavenumber 1276.65 cm^{-1} (C-N stretching, strong) in the sample. These results indicate a significant interaction between QCT and FCZ with the polymers and excipients, as evidenced by the absence of several functional group peaks of both compounds in the sample.

DSC Study

The DSC spectrum of the mucoadhesive vaginal gel formulation **(Figure 7)** revealed significant interactions between QCT (melting point 323°C) and FCZ (melting point 139°C) with the polymers and excipients, as indicated by the absence of their endothermic peaks. A peak corresponding to PLX188 and PLX407, with a melting point around 52°C, was observed. Additionally, a distinct signal for HPMCK4M, with a melting point of 230°C, was detected. The DSC thermogram of pure QCT showed two endothermic peaks: a glass transition (Tg) at 119°C, indicating dehydration, and a second transition at 323°C, reflecting the rapid liquefaction of quercetin and confirming its crystalline structure.

Antifungal Activity:

The MIC80 is the minimum concentration inhibiting 80% of yeast growth relative to the control. The MIC for Itraconazole-resistant *Candida albicans* was 1000 µg/mL for both FCZ and QCT standards. The mucoadhesive vaginal gel formulations exhibited an MIC of 500 µg/mL against this strain. The MIC value for developed mucoadhesive vaginal gel formulation was half that of the individual drugs, demonstrating a synergistic effect.

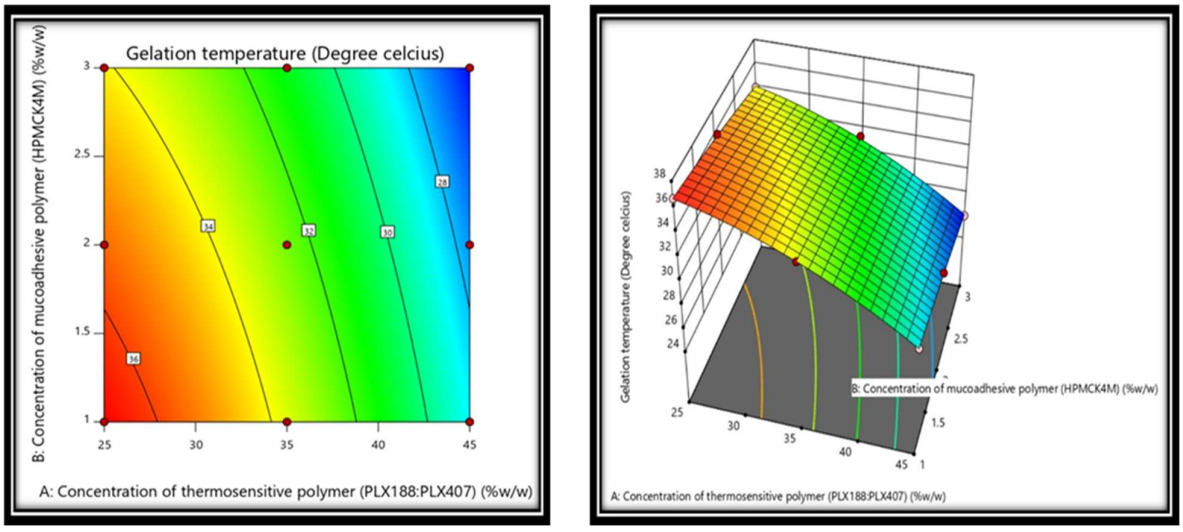
Table 2: 3^2 Full Factorial Design Batches

Batch No.	Coded Value		Non coded Value		Avg. Gelation Temp. (^OC)* ± SD (Y1)	Avg. Gelation Time (Sec)* ± SD (Y2)	Avg. Gelation Strength (Sec)* ± SD (Y3)
	Factor 1	Factor 2	Factor 1	Factor 2			
	Conc. of thermos sensitive polymer (PLX188:PLX407)	Conc. of mucoadhesive polymer (HPMCK4M)	Conc. of thermos sensitive polymer (PLX188:PLX407) (%w/w) (X1)	Conc. of mucoadhesive polymer (HPMCK4M) (%w/w) (X2)			
B1	-1	-1	25*	1	36.67 ± 0.577	36.33 ± 0.577	8 ± 1.155
B2	-1	0	25*	2	35.67 ± 0.577	32.67 ± 0.577	13 ± 1.000
B3	-1	1	25*	3	34.00 ± 1.000	31.33 ± 1.528	19 ± 1.000
B4	0	-1	35#	1	33.67 ± 0.577	26.67 ± 0.577	24 ± 1.000
B5	0	0	35#	2	32.33 ± 0.577	21.00 ± 2.000	27 ± 0.577
B6	0	1	35#	3	31.33 ± 0.577	18.00 ± 1.000	31 ± 1.528
B7	1	-1	45$	1	28.67 ± 0.577	15.00 ± 1.000	47 ± 1.528
B8	1	0	45$	2	27.67 ± 0.577	12.00 ± 1.000	64 ± 1.528
B9	1	1	45$	3	26.00 ± 1.000	10.00 ± 1.000	79 ± 1.528

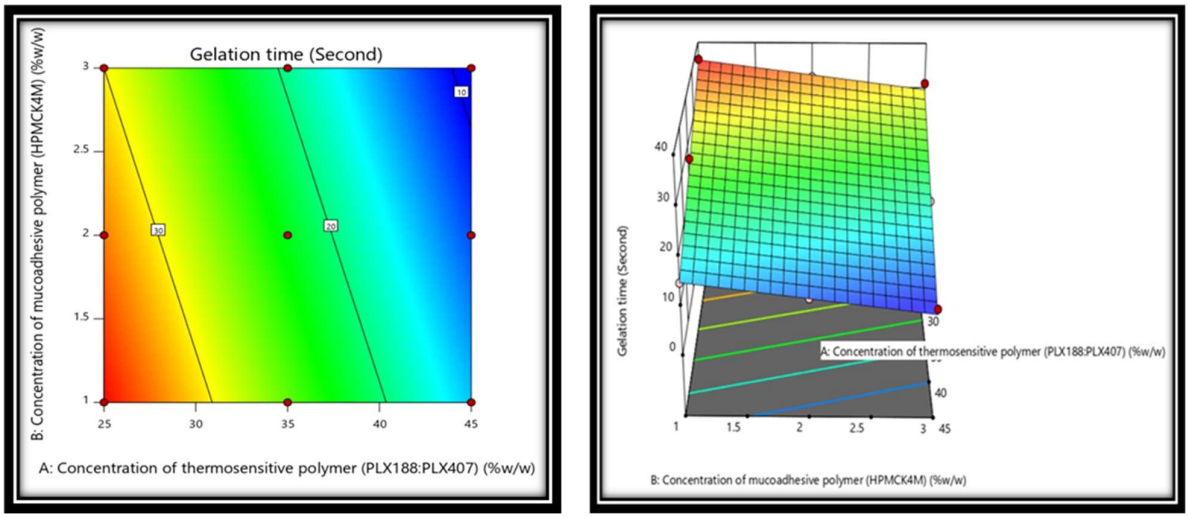
*PLX188(7.35%w/w): PLX407(17.65%w/w); #PLX188 (10.29%w/w): PLX407(24.71%w/w); $PLX188(13.23%w/w): PLX407(31.77%w/w) -the composition is pre-optimized on the basis of preliminary investigation in the lab.

Table 3: Regression Statistical Analysis for the Observed Responses of 3^2 Full Factorial Design (FFD)

Statistical Parameters	Response Y1	Response Y2	Response Y3
Suggested Model	Quadratic	Linear	Linear
R^2	0.9986	0.9881	0.9078
Adjusted R^2	0.9963	0.9842	0.8770
Predicted R^2	0.9881	0.9739	0.7781
Adequate Precision	57.908	39.237	13.747
PRESS Value	1.29	19.18	1018.32
F-Value	432.52	249.53	29.53
P-Value	0.0002	0.0001	0.0008
Polynomial Equation	$Y= 32.55-4A-1.28B-0.9967A^2-0.1667B^2$	Y= 22.56-10.56A-3.11B	Y= 34.67+25A+8.33B



(a)



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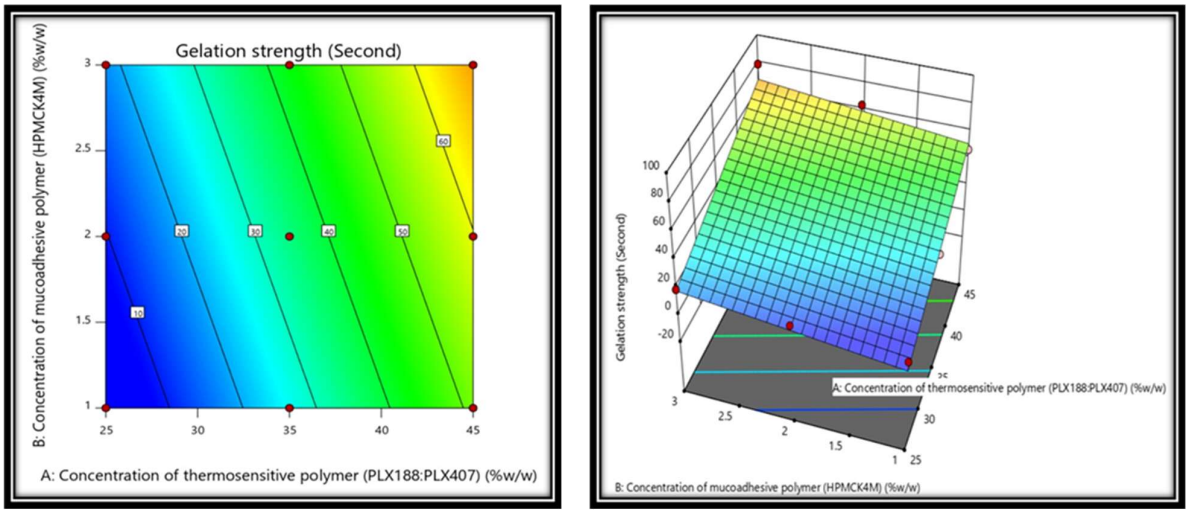


(c)

Figure 1: 2D and 3D contour plot for: a) Y1response, b) Y2 response, c) Y3 response

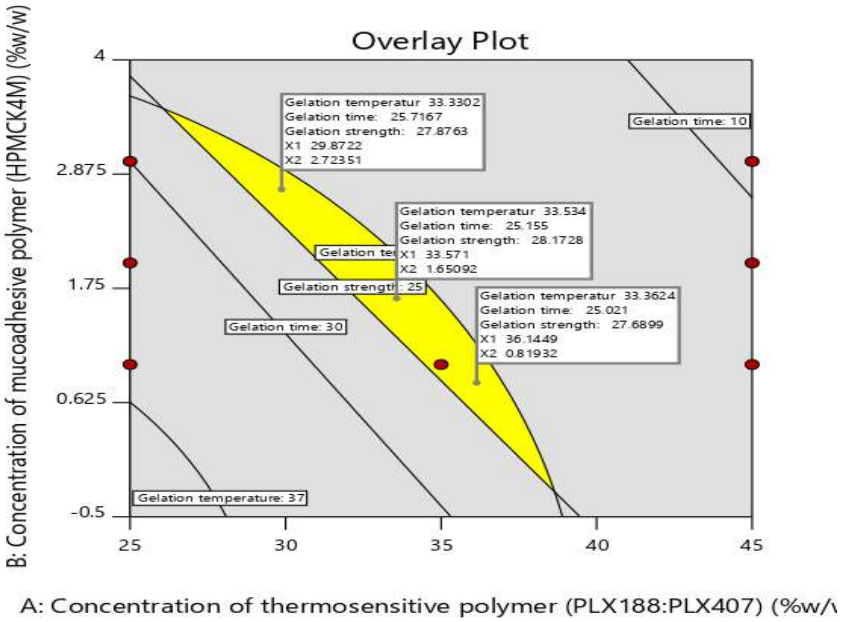


Figure 2: Overlay Plot Created by Design Expert Software

Table 4: Validation Data of Check Point Batches Generated from Design Space

Check point batches	Gelation Temperature (°C)			Gelation Time (sec)			Gelation Strength (sec)		
	Predicted Value	Experimental value* ±SD	% Error	Predicted Value	Experimental value* ± SD	% Error	Predicted Value	Experimental value* ± SD	% Error
F1	33.33	32.83 ± 0.289	1.50	25.71	25.70 ± 0.479	0.04	27.87	26.83 ± 0.086	3.73
F2	33.53	33.00 ± 0.500	1.58	25.155	24.61 ± 0.340	2.17	28.17	27.62 ± 0.176	1.95
F3	33.36	32.83 ± 0.764	1.59	25.02	24.49 ± 0.339	2.12	27.68	26.80 ± 0.087	3.18

Table 5: Evaluation Study of Mucoadhesive Vaginal Gel (Check Point Batch)

Check point batches	Average Gelation Temperature (°C) * ±SD	Average Gelation Time (sec)*±SD	Average Gelation Strength (sec)* ±SD	Average Viscosity (cP)* ±SD	Average pH*±SD	Texture Analysis Parameters			
						Hardness (g)	Adhesiveness (g*s)	Adhesion Force (g)	Bioadhesive Strength ($Kg.m/S^2$)
F1	32.83 ± 0.289	25.70 ± 0.479	26.83± 0.086	393.4± 0.265	5.73 ± 0.058	1001	-434.7	-761	74.65
F2	33.00 ± 0.500	24.61 ± 0.340	27.62± 0.176	412.7± 0.153	5.70 ± 0.000	4457	-469.24	-820	80.44
F3	32.83 ± 0.764	24.49 ± 0.339	26.80± 0.087	428.4± 0.289	5.67 ± 0.058	1708	-454.16	-865	84.85

*n=3

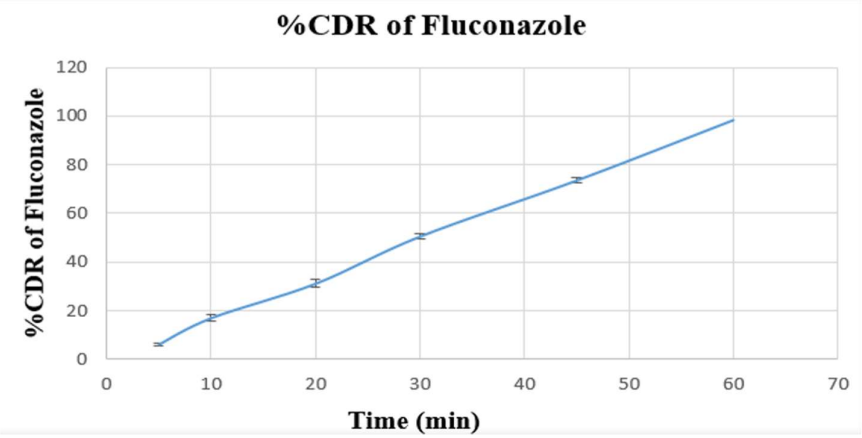


Figure 3: % Cumulative Drug Released (CDR) Profile of Fluconazole (FCZ) (F3 batch)

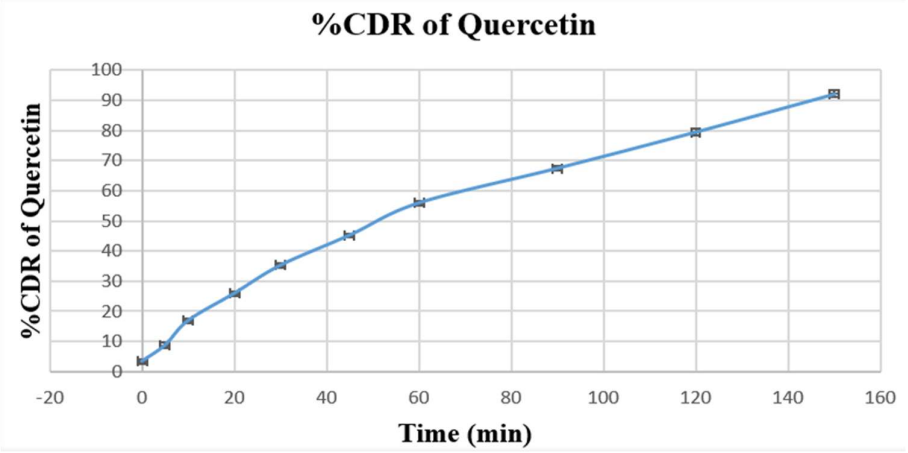
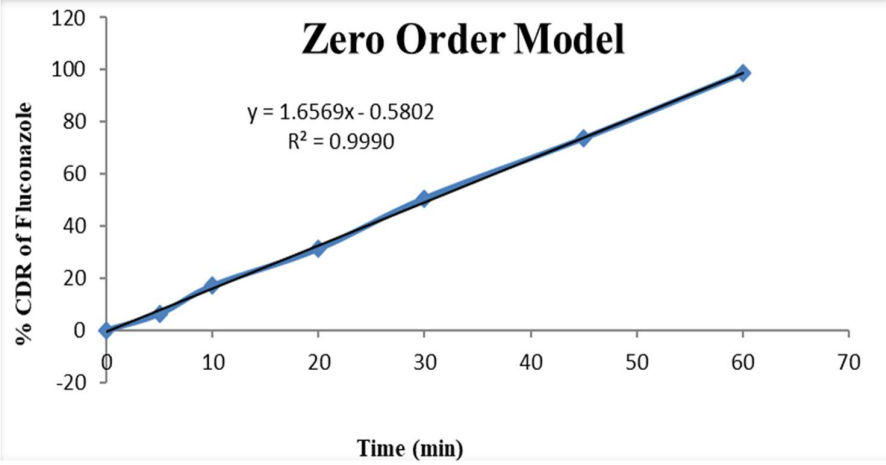
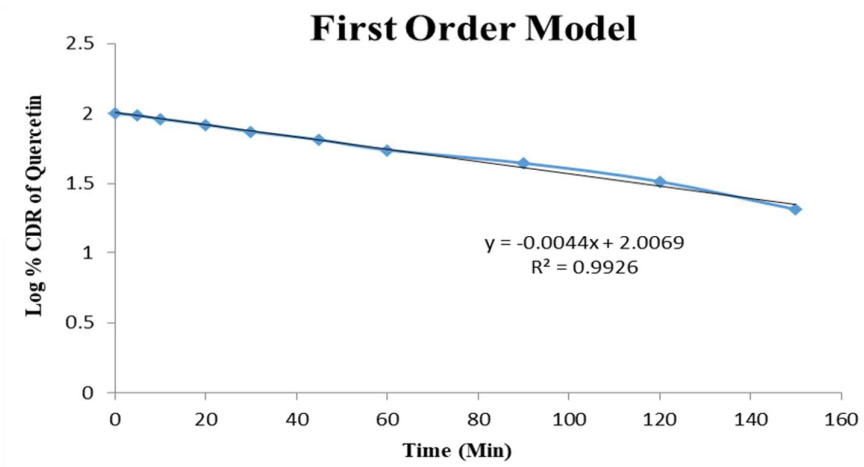


Figure 4: % Cumulative Drug Released (CDR) Profile of Quercetin (QCT) (F3 batch)

Determination of In-Vitro Kinetic Release



a) Zero order kinetic model for FCZ



b) First Order Kinetic Model for QCT

Figure 5: Kinetic Models for Batch 3 Formulation: a) Zero Order Kinetic Model for FCZ b) First Order Kinetic Models for QCT

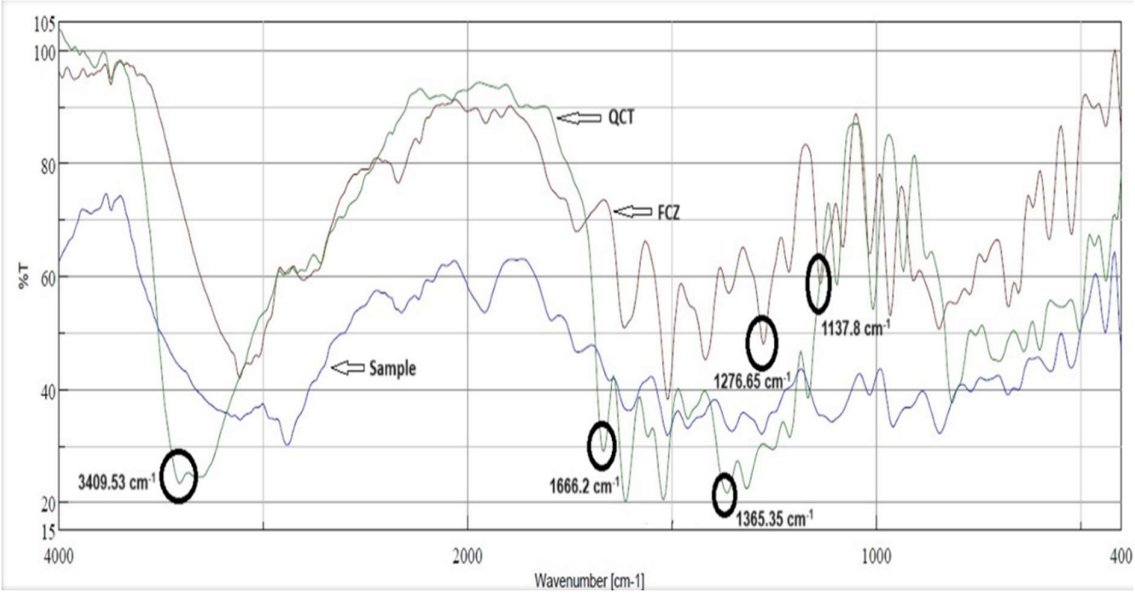


Figure 6: FTIR Spectrum of FCZ, QCT and Sample

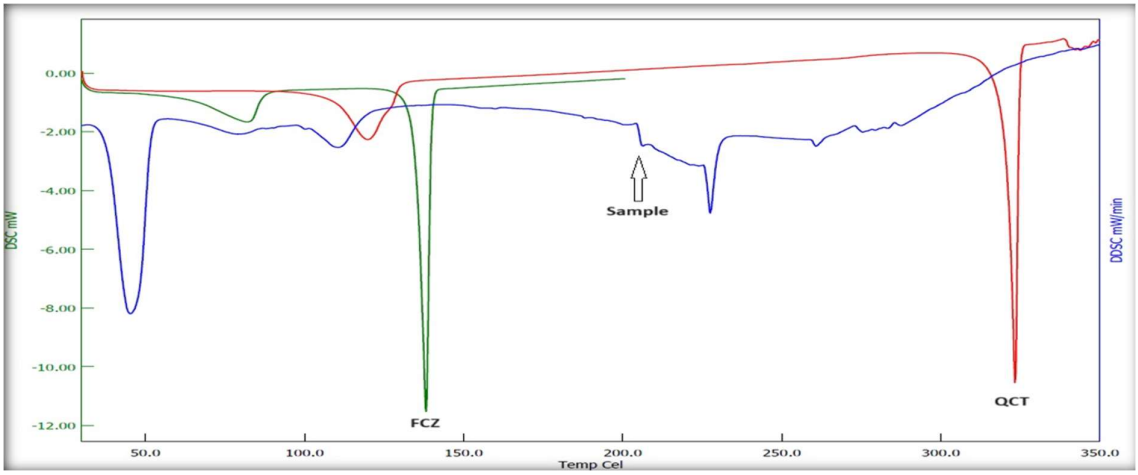


Figure 7: DSC Spectrum of FCZ, QCT and Mucoadhesive vaginal gel sample

CONCLUSION

Candida albicans forms a well-defined biofilm that resists antifungal treatment. FCZ is effective in 71% of VVC patients, with a success rate of up to 90.6% for FCZ-sensitive *C. albicans*. However, the failure rate can reach 100% in FCZ-resistant strains **[53]**. Therefore, exploring new pharmaceuticals, either alone or with FCZ, is crucial for managing FCZ-resistant *C. albicans* from VVC **[54-55]**. Both in vitro and in vivo studies suggest that QCT is a promising antifungal agent that can enhance FCZ's effectiveness against *C. albicans* by inhibiting biofilm production **[56, 57]**. Combining FCZ and QCT could significantly advance anticandidal therapies. The MIC value for developed formulation was half that of the individual drugs, indicating a synergistic effect. QCT shows synergy with FCZ against both free-floating and biofilm forms of *C. albicans*, including Itraconazole-resistant strains, reducing fungal burden. The proposed mechanism studies reveal that QCT induces cell death, disrupts cell structure, breaks down the cell membrane, increases reactive oxygen species, impairs mitochondrial function, and damages DNA when used with FCZ **[58]**. This study suggests a novel approach to overcoming resistant fungal infections by combining traditional antifungal therapy with biofilm inhibitors.

The development of a thermosensitive vaginal gel combining FCZ and QCT was shown to be suitable for vaginal administration to obtain rapid onset of action and somewhat longer the drug's residence time in the vaginal cavity. The gel's thermo-reversible qualities made it easier to handle and administer. The formulation has a suitable viscosity and gel strength, making it suitable for the required phase transition temperature range of 33 to 35 ^{o}C, which closely resembles the human body temperature. The mucoadhesive In-situ vaginal gel surpasses oral dosage forms in certain aspects when it comes to focused delivery of medicine. The benefits of this formulation involve: targeted drug delivery, decreased dosage administration, reduced drug metabolism, cosmetic acceptability, improved patient compliance, scalability, and specific activity against resistant strains of *Candida albicans.* Therefore, this formulation can also be used in cases where fluconazole alone is ineffective. The optimized formulation comprises the active components enumerated in the **Table 1**.

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