ORIGINAL RESEARCH

Enhancing Anti-fungal Activity and Bioavailability of Optimized Clotrimazole Transfersomal Gel for the Treatment of Transdermal Fungal Infection

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ABSTRACT

Objective: Clotrimazole (CTM) is an anti-fungal drug which is used in the treatment of candidiasis. The CTM's poor skin permeability makes it a good candidate to be formulated as Transfersomes (TF) to increase skin permeability of CTM and enhance its anti-fungal activity.

Methods: The CTM-TF was formulated by using the lipid film hydration method. Different CTM-TF formulations have been characterized for the polydispersity index (PDI), vesicle size, zeta potential, entrapment efficiency (EE %), elasticity and quantity of CTM permeated through the skin to determine the optimized formula. The optimized formula was incorporated into the Hydroxypropyl methylcellulose (HPMC E15) gel base and compared to the reference product Canesten® cream 1% for spreadability, pH, viscosity, drug content, in-vitro and in-vivo anti-fungal activity, and bioavailability.

Results: Different CTM-TF formulations have shown a small vesicle size varied from (64.52 ± 0.24 nm) to (84.42 ± 0.78 nm) with a high EE % ranged from (68.55 ± 0.45 %) to (90.56 ± 0.62%). The in-vitro release of the different formulae was inversely proportional to EE %. The kinetic analysis of the release data was best fitted to Higuchi's diffusion model. CTM-TF gel showed higher anti-fungal activity compared to Canesten® cream 1%. The in-vivo pharmacokinetic study has proved that, CTM-TF gel had improved its bioavailability compared to Canesten® cream 1% through the skin.

Conclusion: CTM-TF gel can penetrate a stratum corneum barrier with improved anti-fungal activity and bioavailability compared to market product.

Keywords: Clotrimazole, Transfersomes, Anti-fungal activity, HPMC E15, Bioavailability.

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INTRODUCTION

Fungal infections are one of most widespread infections that can affect nails, mucous membrane and skin. Candidiasis is a common type of skin infection and might penetrate into deeper tissue in case of immune system disease. It always affects warm, sweaty and furrowed areas like stratum corneum and subcutaneous areas.[1]

The topical treatment of candidiasis is typically most popular than the systemic anti-fungal therapy, because the drug is delivered on to affect area with minimal adverse effects and satisfaction of the patient.[2] Stratum corneum, i.e. the outer skin layer, is considered the barrier for drug penetration. Therefore, it is important to develop a new technique for the treatment of fungal infection that has the possibility to pass the stratum corneum.[2,3] The CTM is an imidazole anti-fungal drug that can be used in the treatment of candidiasis. Poor aqueous solubility and hepatic first-pass metabolism leading to reduced systemic efficacy of CTM.[4] The mode of action of CTM is depending on the inhibition of ergosterol biosynthesis leading to the lyse of the fungal cell membrane and inhibition of peroxidase, resulting in the aggregation of peroxide in the cell and leading to the death of the cell.[5]

The CTM has several problems in the treatment of skin infections due to poor penetration of the skin. Topical formulae are administered in large doses to resolving these problems and give a therapeutic effect.[6] Recently, lipid vesicles are used as carriers for topical drugs that have the strategy to overcome skin barrier properties.[7]

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The TF ia s liposomal structurs formed of bilayer structure elastic vesicles; each vesicle is composed of phospholipid and surfactancalleded edge activator (EA). The elasticity of the vesicle is due to the presence of EA, which can be weakening the structure of the stratum corneum and squeezing through intracellular lipid of the skin.[8] The important benefits of vesicle structure are increasing the bioavailability of the drug, prolonging the duration of action, reducing its toxicity, and guarding the drug against metabolic degradation.[9]

The current study's objective was to design and evaluate CTM-TF gel to increase skin permeability and enhance anti-fungal activity of CTM -TF gel compared to marketed products (Canesten® cream 1%).

MATERIALS AND METHODS

Materials

CTM was bought from Pharco Company (Alexandria, Egypt). Canesten® cream 1% was purchased from Alexandria/Bayer company (Alexandria, Egypt). Phospholipon 90 G (soybean L-α-phosphatidylcholine (94% purity)) was purchased from Avanti Polar Lipids (Birmingham, England). Span 80 and Tween 80 were bought from Merck Specialties Pvt. Ltd. (Mumbai, India). Hydroxypropyl methylcellulose (HPMC E15) (viscosity 2% in water 12-18 cP) was purchased from JRS pharma (Rosenberg, Germany). Methanol, formic acid 85% (HPLC grades), and dimethyl sulphoxide (DMSO) were bought from Merck (Merck, Germany). Methyl tert-butyl ether, sodium hydroxide, dichloromethane and methanol (analytical grade) were bought from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Methods

Preparation of CTM-Loaded TF

The CTM-TF different formulae were formulated by lipid film hydration method with a slight modification.[3,10] Depending on the composition of different formulae shown in Table 1, CTM (10 mg), lipid, and EA were dissolved in methanol (10 mL), the lipid ratios: EA were 90:10 and 80:20, respectively. These mixtures were then sonicated by using a water bath sonicator (LUC power sonicator, Lab tech, Korea) for 10 minutes to ensure dissolving all components. The organic solvent has been removed by rotary evaporator (Rocket Synergy, Thermo, China) under vacuum at 45°C.

A thin film of lipids was developed on an internal surface of the flask during rotation. This film of lipid was maintained under vacuum overnight in vacuum oven (Thermo, China) for the removal of the organic solvent completely. The dried film has been hydrated in rotary evaporator water bath with phosphate buffer saline (PBS) (pH 7.4, 10 mL) at a temperature of (2-20°C), and ice has been added to the water bath to ensure complete cooling of suspension. The obtained CTM-TF suspension was then sonicated by the aid of water bath sonicator for 30 minutes.

Characterization of the Prepared CTM-TF Formulations

The CTM-TF different formulae have been evaluated for vesicle size, PDI, zeta potential, EE %, elasticity measurement, and *in-vitro* permeation of CTM from different formulations. Each trial was done in triplicate with a calculation of mean and standard deviation.

Determination of Vesicle Size, PDI and Zeta Potential

Vesicle size, PDI, and zeta potential were determined for all formulated CTM-TF formulations. Briefly, one milliliter of each formula has been suspended in distilled water and measured by using the dynamic light scattering technique (Zetasizer, Malvern Instruments Ltd., Malvern, UK).[11]

Determination of EE %

One milliliter from different formulae suspensions has been centrifuged using a centrifuge (Sigma Laboratory centrifuge, Germany) at 14,000 rpm for 1-hour at 25°C. The supernatant has been removed, and sediment has been dissolved in methanol and determined using UV spectrophotometer (CF20R, Thermo, China) at wavelength 260 nm. The EE % of CTM in CTM-TF different formulations were determined using the following equation:[12]

$$EE\ (\%) = (C_{total} - C_{free\ drug}/C_{total}) \times 100$$

Where C_{total} is the total amount of the drug-loaded and C free drug is the amount of free drug in the supernatant.

Table 1: Composition of the prepared CTM-TF formulations (F1-F8)*

Formula No.	*CTM (mg)*	*Lipid concentration (mg)*	*Edge activators (EA) (mg)*		*Lipid: EA ratio*
		*PL $_{90G}$ **	*Span 80*	*Tween 80*	
F1	10.00	600.00	66.67	-	90:10
F2	10.00	600.00	150.00	-	80:20
F3	10.00	300.00	33.33	-	90:10
F4	10.00	300.00	75.00	-	80:20
F5	10.00	600.00	-	66.67	90:10
F6	10.00	600.00	-	150.00	80:20
F7	10.00	300.00	-	33.33	90:10
F8	10.00	300.00	-	75.00	80:20

* PL_{90G} means phospholipon 90 G.

Elasticity Measurement

The test was done by using the extrusion method through a 0.2 µm polycarbonate syringe filter (Sigma chemicals, UK) at 7.5 psi pressure. One milliliter of each formula has been diluted to 20 mL with PBS (pH 7.4) and pumped through a polycarbonate syringe filter for 10 minutes. The following equation:13 has determined the elasticity of each formula

$$E = P_{flux} (S_y/S_p)^2$$

Where E is the elasticity value of TF (mg/s.cm^2), P_{flux} is the penetration rate through the permeable membrane (mg/s.cm^2), s_v is the vesicle size after extrusion and s_p is the pore size of the filter membrane.

***In-vitro* Permeation of CTM from Formulated TF**

The release of CTM from TF different formulae have been determined by Franz's diffusion cell apparatus (Copley scientific, UK) (area=1.7 cm^2) with a slight modification. A cellophane membrane of a molecular weight of 12,000 Dalton has been fixed between the donor and receptor compartments of Franz's cell. The receiver phase was composed of 20 mL methanol: PBS (pH 7.4) (1:1 v/v).

The temperature has been adjusted at 37 ± 0.5 °C, and the media has been stirred at 100 rpm for 24 h CTM-TF formula, equivalent to 2000 µg of CTM, has been placed in the donor compartment. Dissolution media (1mL) have been collected at predetermined intervals of time (0, 1, 4, 7, 10, 12, 15, 18, 21, and 24 hours) and have been substituted by an equal quantity of fresh dissolution media to maintain trial condition. Each trial has been done in triplicate with the calculation of mean and standard deviation. The released quantity of CTM was measured spectrophotometrically at a wavelength of 260 nm against methanol: PBS (pH 7.4) (1:1 v/v) as a blank.[14]

Kinetic Analysis of the Released Data

In-vitro release data of CTM-TF different formulae have been kinetically analyzed for determining the suitable order of drug release.[15]

Morphological Study of Optimized CTM-TF Formula

The morphology of CTM-TF optimized formula has been studied by a transmission electron microscope (TEM) (Model HT7700, Hitachi, Japan). One drop of CTM-TF optimized formula has been applied to a collodion-coated copper grid and allows standing for 2 min for complete drying. The sample was stained with uranyl acetate solution and examined after 5 min by TEM.[16]

Fourier Transforms Infrared Spectroscopy (FTIR)

FTIR of CTM, a physical mixture of CTM-phospholipid, and a physical mixture of CTM-EA of optimized formula were determined and compared with FTIR of CTM-TF optimized formula to ensure the compatibility between TF optimized formula components by using FTIR spectrometer (FTIR-435U-04, Shimadzu, Japan). Each physical mixture has been mixed with potassium bromide, pressed into a tablet, and scanned at range from 4000 cm^{-1} to 400 cm^{-1}.[17]

Differential Scanning Calorimetry (DSC)

Thermal analysis of CTM and excipients of optimized formula have been performed using DSC (TA-50 ESI, Shimadzu, Japan). DSC have been applied to CTM, a physical mixture of CTM-phospholipid, and a physical mixture of CTM-EA of optimized formula and compared with DSC of CTM-TF optimized formula. The DSC study was performed at temperature varied from zero to 250°C at a scanning rate of 10°C/min.[6]

Preparation of CTM-TF Gel

CTM-TF gel, 1%, was formulated by using Hydroxypropyl methylcellulose (HPMC E15) 2% as a gelling agent. Accurately 2g of HPMC E15 was dispersed into a glass beaker containing 60 mL hot distilled water and stirred at 50 rpm for 2 hours using magnetic stirrer (IKA, Germany). Gel base was cooled to room temperature.

A CTM-TF suspension optimized formula, equivalent to 1% CTM, was added to gel base with stirring at 50 rpm for 10 minutes till a homogenous gel was obtained.

Benzyl alcohol was added to the gel formula as preservative with stirring for 10 minutes. The weight of the formula has been completed to 100 g by distilled water and stirred at 50 rpm for 10 minutes The formula has been placed for the whole night in a glass beaker to allow removal of air bubbles.[18]

Evaluation of Optimized CTM-TF Gel Formula

The CTM-TF gel optimized formula has been evaluated and compared to Canesten® cream 1% for homogeneity, spreadability, pH, viscosity, drug content, and in-vitro permeation.[16] Each trial was done in triplicate with the calculation of mean and standard deviation.

Spreadability

Spreadability of both optimized formula and marketed product have been determined by pressing one gram of each formula between two colorless circular glass plates. The spreading value was the diameter of the circle formed between plates after leaving them for 5 minutes.[1]

Homogeneity

This was done by pressing one gram of each formula between index and thumb fingers. The consistency of each formula was considered as an indicator for homogeneity.[19]

pH Measurement

One gram of each formula was placed in 25 mL distilled water and the pH value has been determined by a pH meter (Jenway, UK).[14]

Viscosity Measurement

The viscosity of each formula has been measured by Brookfield viscometer (Brookfield Inc., USA). One gram of each sample was placed in the cup of the viscometer. The apparatus was rotated at a speed of 5 rpm for 10 sec by using spindle CC 14.[1]

Drug content

One gram of each sample has been dissolved in 100 mL methanol: PBS (pH 7.4) (1:1 v/v) and the sample has been

filtered through 0.20 μm syringe filter. The drug content has been measured spectrophotometrically at a wavelength of 260 nm.[6]

In-vitro Permeation of CTM-TF Gel

In-vitro permeation of both the CTM-TF optimized formula and the marketed product gels have been determined by Franz's diffusion cell as mentioned before for In-vitro permeation of CTM-TF suspension. The values of permeation have been kinetically analyzed to identify the suitable order of drug permeation.[20] Permeability coefficient (KP) of CTM through the cellophane membrane has been calculated by the following equation.

$$KP = J_{SS}/C_0$$

Where J_{SS} is the slope of the curve between the quantity of CTM released per unit area (μg/cm^2) versus time and C_0 is the initial concentration of CTM.[3]

In-vitro Anti-fungal Activity

In-vitro anti-fungal activity of optimized CTM-TF (test) was evaluated against Canesten® cream 1% (standard) after authorization of the Ethics Committee for Faculty of Pharmacy-Mansoura University (authorization number 115-11-06) under the supervision of the Microbiology Department of the college.

The anti-fungal activity of optimized CTM-TF (test) was detected against Canesten® cream 1% (standard) by the cup plate method.[21] Standard and test formulations have been dissolved in dimethyl sulphoxide (DMSO) and evaluated at a concentration of 100 μg/mL against *Candida Albicans*. Sterile media of sabouraud's dextrose agar plate have been used and 0.1 mL from *Candida Albicans* was spread uniformly. Both standard and test formulations were taken into cups of 10 mm diameter and bored into the previously prepared *Candida Albicans* agar media. The media plate has been maintained at 4°C for 1-hour for diffusion of both formulations, and then incubated at 28°C for 48 hours for the growth of 10 generation. The zone of inhibition of microbial growth for both formulations have been measured in mm.[19,21]

In-vivo Anti-fungal Activity

The In-vivo anti-fungal activity of the optimized CTM-TF (test) has been evaluated against Canesten® cream 1% (standard) after authorization of the Ethics Committee for Faculty of Pharmacy-Mansoura University (authorization number 115-12-06) under the supervision of the Pharmacology Department of the college.

Design of Experiment

Male Sprague Dawley rats ranged from 200–250 g have been obtained from the animal house at the Faculty of Pharmacy-Mansoura University. All animals have been kept for one week under animal house standard conditions before starting the experiment protocol. The rats have been equally divided into four groups; each contains six rats. Group I, represented the negative control (normal rats); Group II, represented the positive control (infected without treatment); Group III, received topical Canesten® cream 1%; and Group IV received topical treatment of optimized CTM-TF gel. Animals of groups II, III and IV were immunosuppressed by intravenous injection of dexamethasone sodium phosphate at a dose of (5 mg/kg)[21] for 3 days before induction of candidiasis. An area of about 4 cm^2 was shaved in the back of each rat 24 h before the induction of infection.[22]

Preparation of Candida Albicans

Candida Albicans has been allowed to grow on YPD broth (Yeast extract 10 g/L, peptone 20 g/L, and dextrose 20 g/L) containing 10% fetal bovine serum (FBS) for a period ranged from 14–18 hours at a temperature of 30°C. The cells have been collected, washed, and dispersed in sterile normal saline solution at a concentration of 10^7 CFU/mL.[23]

Induction of Candida Albicans Infection

Each rat of groups II, III and IV was injected intradermal with *Candida Albicans* suspension 100 μL at a concentration of 10^7 CFU/mL in the midpoint of the bleached area. The area of injection has been rubbed to prevent edema. *Candida* infection has been determined in the bleached area after 72 hours.[22]

Treatment of Candida Albicans Infection

Rats of groups III and IV have been topically treated through the application of Canesten® cream 1% and CTM-TF gel optimized formula, respectively for a period of 10 days. During the period of treatment, medical examinations have been applied.

Rats of groups III and IV were examined visually daily at the infected site for the presence of nodules, erythema, ulceration, and crusting. Rats were considered to be cured when features of infection disappeared.[23]

Pharmacokinetic Study

Subjects

The study was applied by parallel experimental design to twelve non-smokers healthy male volunteers with body mass index (BMI) ranged between 18–30, and age ranged between 20–35 years old. The volunteers had stopped any therapeutic medication for one week before having been submitted to the study. All of them agreed to sign a written authorization before starting the study. Besides, the study has been approved by the Ethics Committee, Faculty of Pharmacy-Mansoura University (authorization number 115-13-06) under the supervision of the Clinical Pharmacy Department of the college with respect to ethical principles and regulations for clinical research related to human volunteers approved by the World Health Organization (Helsinki, 1964).

Study design

The volunteers have been equally divided into two groups; each contains six volunteers. Group I received CTM-TF gel optimized formula (equivalent to 10 mg of CTM) while group II received reference marketed product (Canesten® cream 1%, equivalent to 10 mg of CTM).[24] The dose was applied over 25 cm^2 of abdomen skin by the aid of a plastic stick. The skin was shaved and cleaned by alcoholic swap 24 hours before starting the study.

Samples Collection and Handling

Blood samples were collected from intravenous route via a cannula placed in the forearm in the following intervals: 0 (pre-dos) 1, 4, 7, 10, 12, 15, 18, 21, and 24 hours after the dose application. Blood samples were collected in heparinized Eppendorf tubes then centrifuged at 1,000 rpm for 15 min (Heraeus Biofuge Primo, Thermo Scientific, USA) and then stored at – 20 °C until analysis.[24]

Samples Preparation

Sodium hydroxide solution (1 mol/L, 100 µL) and a solvent mixture of methyl tert-butyl ether and dichloromethane (4:1 v/v, 4 mL) were added to 500 µL plasma sample and then vortex mixed for 3 minutes The sample was centrifuged at 2500 rpm for 3 min, and 3 mL from each organic phase were transferred to a glass tube and evaporated under a stream of nitrogen at 40 °C till it dried.

The residue was dissolved in a aqueous methanol solution (50 ng/mL, 200 µL) and the sample was detected for CTM by a validated method of HPLC.[25]

Chromatography

Plasma concentration of CTM was analyzed by HPLC (Thermo 1100, China) using C_{18} column (250 x 4.6 mm, Phenomenex, USA) and a mobile phase composed of methanol-formic acid 85% (85:15 v/v) was pumped at a rate of flow (1 mL/min). The analysis has been carried out using UV detector at 260 nm and the retention time was 6.5 minutes.[25]

Statistical Analysis

Data have been calculated as mean ± SD (n = 6) and statistically treated by the unpaired student 't' test and the one-way analysis of variance (ANOVA). Significance has been applied at p-value < 0.05. Pharmacokinetic data have been calculated by the standard non-compartmental program (WinNonlinR®, Pharsight Corporation, USA) to determine the pharmacokinetic parameters.

RESULTS AND DISCUSSION

Preparation of CTM-loaded TF

CTM-TF different formulae have been prepared by using the lipid film hydration method. The lipid concentration, type of EA and the lipid: EA ratios were used as variables to obtain eight different formulae, as shown in Table 1. Formulations prepared with Tween 80 were off-white to yellow while formulations prepared with Span 80 were yellow to brown in color.

The alteration in color was due to variation in optical density of each formula, as explained by *McClements*.[26] Vesicle size, PDI, zeta potential , elasticity and in-vitro permeation have been measured for each formula.[27]

Characterization of the prepared CTM-TF formulations

Vesicle size, PDI and Zeta Potential of the Formulated CTM-TF Formulations

The different CTM-TF formulae have been tested for vesicle size, PDI and zeta potential, as shown in Table 2. The vesicle size varied from (64.52 ± 0.24) for F1 to (85.42 ± 0.78) for F8. From the results of vesicle size, all formulations have vesicle sizes lesser than 100 nm, which are efficient for transdermal use.[28] The results have shown that the vesicle size of F1, F2, F3, and F4 formulated with Span 80 have smaller vesicle sizes than F5, F6, F7, and F8 formulated with Tween 80. These results can be explained according to the rule: as the hydrophilic-lipophilic balance (HLB) of surfactant decreased, the vesicle size also decreased.[29] CTM-TF formulated with Span 80 (lipophilic surfactant, HLB = 4.3) had smaller particle sizes than CTM-TF formulated with Tween 80 (hydrophilic surfactant, HLB = 15). The relationship between the HLB value of surfactant and vesicle size can be illustrated by the increase in surface free energy obtained from the high lipophilicity. The same reason can also be applied to the reduction in the vesicle size as the quantity of lipid increased from 300–600 mg.

The values of vesicle size were increased when the phospholipid-surfactant ratio decreased from 90:10–80:20. This is may be due to the reduction in the amount of surfactant resulting in incomplete formation of the vesicle to produce small vesicle size.[2]

Zeta potential is a parameter representing the stability of CTM-TF dispersion, as it represents the degree of electrostatic repulsion between particles that prevent particles' aggregation during storage. Zeta potential more than +20 mV or less than -20 mV are enough for the stability of CTM-TF dispersion.[30] Surfactants as Span 80 and Tween 80 increase the stability of CTM-TF dispersion due to their stearic hindrance characters.[31]

Table 2: Characterization of the prepared CTM-TF formulations (F1-F8)*

Formula No.	*Vesicle size (nm)*	*Zeta potential (mV)*	*PDI*	*EE (%)*	*Elasticity (mg/s.cm²)*	*Flux (µg/cm²/ h)*
F1	64.52 ± 0.24	-16.24 ± 1.36	0.365 ± 0.73	90.56 ± 0.62	12.57 ± 1.23	92.53 ± 1.23
F2	69.43 ± 0.37	-34.87 ± 0.65	0.433 ± 0.54	70.42 ± 1.25	14.67 ± 1.42	94.17 ± 0.64
F3	71.66 ± 0.61	-20.43 ± 0.55	0.423 ± 0.65	83.66 ± 0.75	17.61 ± 0.73	96.55 ± 0.55
F4	73.76 ± 0.43	-24.82 ± 0.27	0.387 ± 0.44	87.53 ± 0.88	21.62 ± 1.33	98.87 ± 0.87
F5	72.87 ± 0.25	-17.66 ± 1.23	0.426 ± 1.23	85.62 ± 0.37	31.57 ± 0.54	99.78 ± 1.27
F6	74.68 ± 0.64	-22.15 ± 0.78	0.394 ± 0.53	68.55 ± 0.45	36.41 ± 0.64	101.64 ± 0.54
F7	83.64 ± 1.25	-27.43 ± 0.77	0.317± 0.95	79.29 ± 0.36	41.57 ± 1.22	103.22 ± 0.43
F8	84.42 ± 0.78	-31.89 ± 0.87	0.469 ± 0.65	77.68 ± 0.73	44.72 ± 0.48	104.76 ± 0.45

* Results are represented as mean ± SD, n = 3

As shown in Table 2, CTM-TF formulations have negative zeta potential values ranging from (-16.24 ± 1.36) for F1 to (-34.87 ± 0.65) for F2, which increase the stability of the developed formulations during storage.

PDI measures the homogeneity of the formula and ranges from 0.00 to 1.00. Formulae with PDI values closer to zero are more homogenous than formulae with values closer to 1.00. As represented in Table 2, all formulations with PDI values of lesser than 0.50 indicate homogenous and high stable dispersions.[32]

Entrapment Efficiency of CTM in the Formulated TF

As represented in Table 2, CTM-TF different formulations have shown a high EE %, ranged from (68.55 ± 0.45) for F6 to (90.56 ± 0.62) for F1. TF prepared with lipophilic surfactant (Span 80) has had a high EE % than those formulated with hydrophilic surfactant (Tween 80(which could be due to the HLB values of the used surfactants. The HLB value of Span 80 was 4.3 while HLB value of Tween 80 was 15; thus, the affinity of phospholipid (PL_{90G}) was higher to lipophilic surfactant (Span 80) compared to hydrophilic one (Tween 80).[2,20]

Formulations F1 and F5 had a high EE % (90.56 ± 0.62) and (85.62 ± 0.37) respectively, which may be due to higher lipid content (600 mg) and higher lipid: EA ratio (90:10) compared to the other formulations, which resulted in hardness of lipid bilayer and decrease in the ratio of the aqueous volume in comparison to lipid volume of the formed vesicle.[16] These interpretation has been explained by *Abdallah et al.* who formulated nystatin TF and noticed that EE % was higher in TF formulated with Span 80 compared to TF prepared with Tween 80.[20]

Elasticity of CTM-TF Vesicles

As shown in Table 2, CTM-TF prepared with Tween 80 had high elasticity values compared to those prepared with Span 80; this may be due to the higher viscosity value of Span 80 (1000-2000 mPa.s) compared to Tween 80 (375-480 mPa.s).[33] Formula F8 had a higher elasticity value (104.72 ± 0.48) compared to formula F7 (103.57 ± 1.22) while formula F6 had a higher elasticity value (101.41 ± 0.64) compared to formula F5 (99.57 ± 0.54), which could be illustrated by the decrease in the ratio of the lipid – surfactant (80:20) in the formed vesicle for formulations F8 and F6 compared to formulations F7 and F5, respectively, which had lipid-surfactant ratio (90:10).[16] These interpretations make the formulations prepared with Tween 80 and had a lipid-surfactant ratio of (80:20) more elastic and alter their shape under stress while TF prepared with Span 80 were viscous and their flexibility was significantly ($p < 0.05$) lower than TF prepared with Tween 80.

In-vitro Permeation of CTM from Formulated TF

Figure 1 represented in-vitro release of CTM from TF different formulae. It was shown that the release of CTM was achieved in two stages. The initial stage showed a fast drug release which continued for about 10 hours because of the desorption of CTM from surface of TF, while the remaining amount of CTM released slowly for a period of about 14 hours as a result of the release of CTM from lipid bilayer of TF.

In-vitro permeation of CTM from different TF formulations was applied to estimate the transdermal flux, which ranged from (78.53 ± 1.23μg/cm^2/hr) for F1 to (104.76 ± 0.45μg/cm^2/hr) for F8, as shown in Table 1. The permeation of CTM was higher with Tween 80 than with Span 80; this could be due to the hydrophilicity of Tween 80, which acts as a solubilizer for CTM and enhances its release.[20] The release rate increased as the total lipid decreased, which could be attributed to the decreased bilayer hydrophobicity; hence, the release increased. When the lipid-surfactant ratio decreased from 90:10 to 80:20, the rate of release increased, which may be due to the decrease in the amount of lipid causing a decrease in the ratio and an increase in the diffusion of bilayer structure.[3]

According to the results of EE% and in-vitro release, it has been shown that there is an inverse relation between them: the lower the EE% of CTM in the formulated TF, the higher the in-vitro release.

As represented in Table 3, the higher values of Higuchi's diffusion pattern's correlation coefficient showed that the permeation of CTM from different TF formulations follows Higuchi's diffusion model.

Formula F8 has been chosen as the optimized formula for applying further studies and in-vivo studies as it exhibited 100% CTM permeation, which increases CTM level stability in-vivo.

Optimized formula F8 was composed of CTM, Phospholipon 90 G, and Tween 80 by quantities 10, 300, and 75.00 mg, respectively, while the lipid: EA ratio was 80:20.

Morphology of Optimized CTM-TF Formula

Figure 2 showed TEM photograph of optimized formula F8. TF were spherical and unilamellar nanovesicles. Figure 3 showed the vesicle size and PDI of the optimized formula.

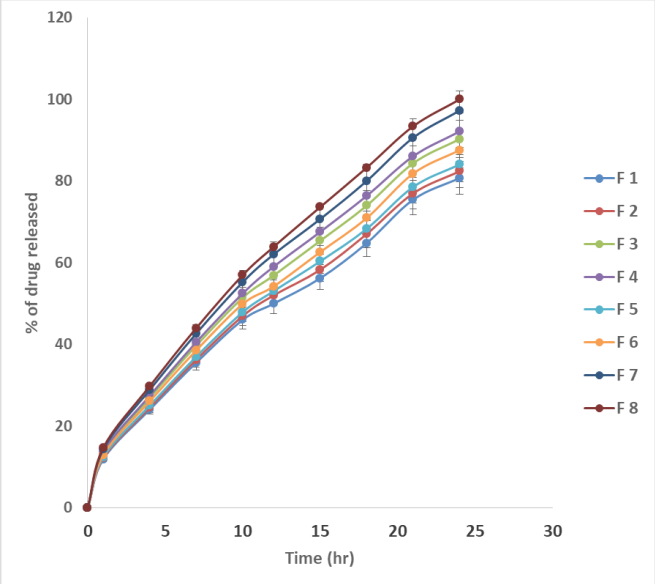

Figure 1: In-vitro permeation of CTM from formulated TF (F1–F8), (results are illustrated as mean ± SD, n = 3)

Fourier Transforms Infrared Spectroscopy (FTIR)

FTIR spectroscopy has been used to detect drug excipients interaction. Figure 4 showed the IR spectrum of CTM, the physical mixture of CTM-phospholipid, the physical mixture of CTM-Tween 80, and the physical mixture of optimized formula F8. The FTIR of CTM was distinguished by bands at 3342 cm^{-1} (imidazole C-N), 3106 cm^{-1} (aromatic C-H), 1502 cm^{-1} (aromatic -C-C-), 1123 cm^{-1} (C-N stretch) and 632 cm^{-1} (C-Cl).[17]

All the peaks shown in IR spectrum of CTM were similar to those obtained from IR spectra of the physical mixture of CTM-phospholipid, CTM-Tween 80, and optimized formula F8, which ensure there was no interaction between CTM and used excipients.

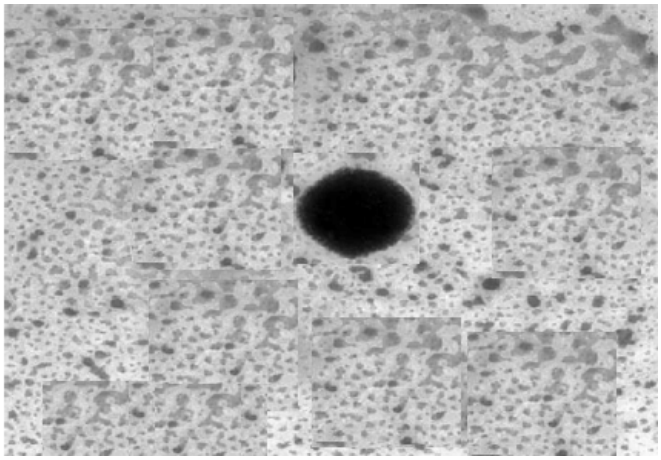

Figure 2: TEM photograph of optimized CTM-TF formula (F8)

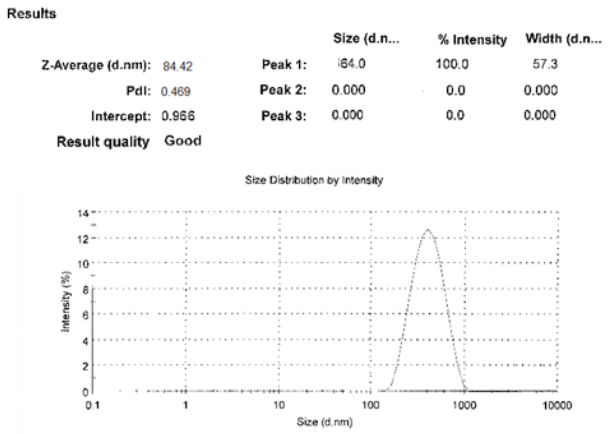

Figure 3: Mean vesicle size and PDI of optimized CTM-TF formula (F8)

Differential Scanning Calorimetry (DSC)

DSC is a calorimetric technique used to identify the physical state and solubility of drug in phospholipid vesicle. Figure 5 showed DSC the thermograms of CTM, the physical mixture of CTM-phospholipon 90 G, the physical mixture of CTM-Tween 80, and the physical mixture of optimized formula F8. The DSC thermogram of CTM showed an endothermic peak at 148.6 °C, which represents the melting point of CTM.[6] This endothermic peak vanished in thermograms of the drug with phospholipid, Tween 80, and optimized formula. The vanishing of the endothermic peak of CTM indicated CTM's presence in a more amorphous and soluble state.[33] The variation in the melting manner of CTM could be the result of depression of its crystalline form and the increase of its solubility in TF. Thus, CTM used in the formulation of TF was in an amorphous state. The transformation of CTM to an amorphous state led to an augmenting solubility.[6]

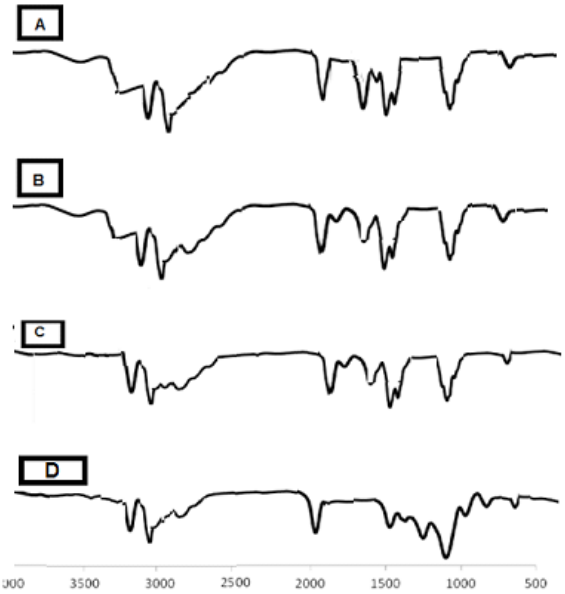

Figure 4: IR spectra of (A) CTM, (B) physical mixture of CTM-phospholipid, (C) physical mixture of CTM-Tween 80 and (D) physical mixture of optimized formula (F8)

Table 3: The correlation coefficient (r) values for the in-vitro permeation of CTM from different TF formulations using different kinetic orders

Correlation Coefficient (r)						
Formula No.	*Zero*	*First*	*Second*	*Diffusion*	*Hixon-Crowell*	*Baker-Lonsdale*
F1	0.9855	-0.9715	0.9083	0.9882	0.9838	0.9455
F2	0.9863	-0.9691	0.8997	0.9897	0.9828	0.9438
F3	0.9825	-0.9506	0.8392	0.9884	0.9751	0.9330
F4	0.9860	-0.9433	0.8166	0.9876	0.9722	0.9294
F5	0.9864	-0.9662	0.8899	0.9879	0.9816	0.9419
F6	0.9814	-0.9586	0.8649	0.9877	0.9785	0.9373
F7	0.9853	-0.9030	0.7021	0.9899	0.9563	0.9135
F8	0.9822	-0.7366	0.5589	0.9866	0.9088	0.8899

Preparation of CTM-TF Gel

CTM-TF gel was prepared by using HPMC E15 2% as gelling agent. The quantity of CTM in the formulated gel was 1% w/w as in Canesten® cream 1%.

Evaluation of Optimized CTM-TF Gel and Canesten® Cream 1%

Both CTM-TF gel and Canesten® cream 1% were homogenous and smooth. The spreadability values of both CTM-TF gel and Canesten® cream 1% were 11.2 ± 0.24 and 7.8 ± 0.18 cm, respectively, which referring to CTM-TF gel can be spread readily on the skin. The pH values were 5.48 ± 0.76 and 5.89 ± 0.54 for both CTM-TF gel and Canesten® cream 1%, respectively, which were considered to be within a usual range for topical formulations. Both CTM-TF gel and Canesten® cream 1%' viscosity were 3.68 ± 0.34 and 6.58 ± 0.75 Pa/s, respectively. The drug content was 99.42 ± 0.64 % for CTM-TF gel, while drug content for Canesten® cream 1 % was 99.37 ± 0.75 %, which showed a good content uniformity (Table 4).

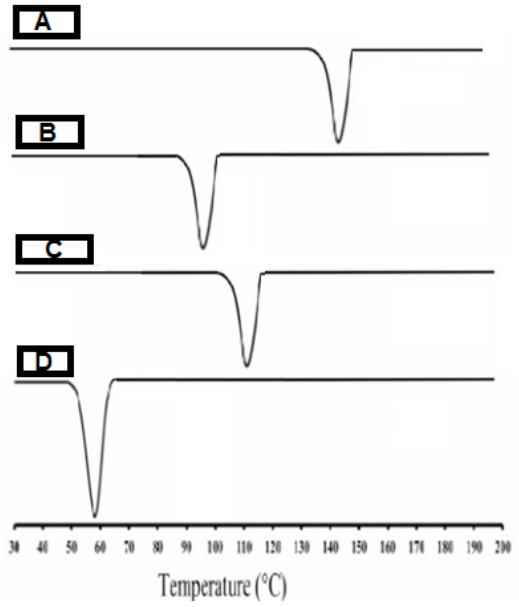

Figure 5: DSC thermograms of (A) CTM, (B) physical mixture of CTM-phospholipid, (C) physical mixture of CTM-Tween 80 and (D) physical mixture of optimized formula (F8)

Table 4: Evaluation of CTM-TF gel in comparison to Canesten® cream 1 % *

Formulation	*CTM-TF gel*	*Canesten® cream 1 %*
Homogeneity	Good	Good
Spreadability (cm)	11.2 ± 0.24	7.8 ± 0.18
pH	5.48 ± 0.76	5.89 ± 0.54
Viscosity (Pa/s)	3.68 ± 0.34	6.58 ± 0.75
Drug content (%)	99.42 ± 0.64	99.37 ± 0.75 %

* Results are represented as mean ± SD, n = 3

In-vitro permeation of CTM-TF gel and Canesten® Cream 1%

The release of CTM from both TF gel and Canesten® cream 1 % has been shown in Figure 6. It has been shown that the amount of CTM released from CTM-TF gel was 2250.24 µg/cm^{2,} which was significantly greater than the quantity delivered from Canesten® cream 1 %, which was 1775.25 µg/cm^2 ($p < 0.05$). The higher release of CTM from the formulated gel could be due to the flexibility of TF vesicles; thus, they can overcome the barrier function of the skin by squeezing through lipid in the stratum corneum.[34] However, after the topical application of TF on the skin, they penetrate a dry stratum corneum to a hydrated layer by the aid of the osmotic gradient and the use of EA in the structure of TF, which aids to dissolve the lipid in stratum corneum leading to high permeability of TF.[7]

As represented in Table 5, the correlation coefficient values were high with Higuchi diffusion pattern; thus, the permeation of CTM-TF gel and Canesten® cream 1% were best fitted to Higuchi diffusion pattern.[1]

Permeation data analysis

As represented in Table 6, the steady-state flux was greater for CTM-TF gel in comparison to Canesten® cream 1%. The steady-state flux for CTM-TF gel and Canesten® cream 1% after 24 h were 88.942 and 73.234 µg/cm^2.h, respectively. As shown in Table 6, there was a direct relationship between permeability coefficient and steady-state flux.

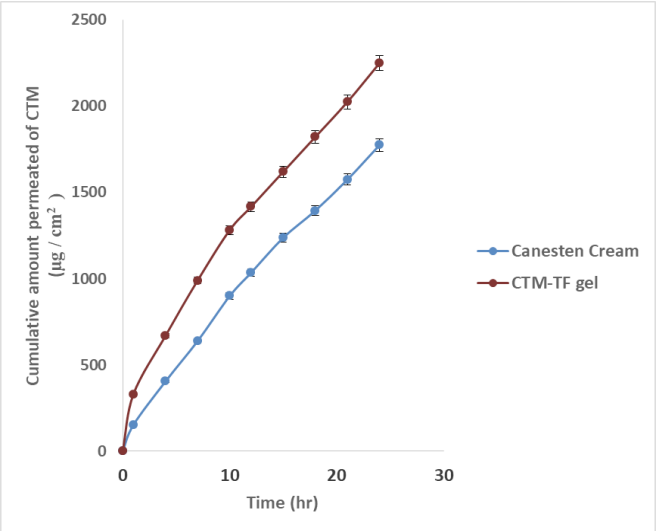

Figure 6: The cumulative amount of permeated CTM using CTM-TF gel in comparison with Canesten® cream 1 % (results are expressed as mean ± SD, n = 3)

Table 5: The correlation coefficient (r) values for the in-vitro permeation of CTM from TF gel and Canesten® cream 1% using different kinetic orders

Correlation Coefficient (r)						
Formula No.	*Zero*	*First*	*Second*	*Diffusion*	*Hixon-Crowell*	*Baker-Lonsdale*
CTM-TF gel	0.9823	-0.9717	0.9115	0.9943	0.9752	0.9302
Canesten® cream 1 %	0.9856	-0.7752	0.5463	0.9976	0.9076	0.8507

The permeability coefficient of CTM-TF gel was higher than Canesten® cream 1%, which may be due to TF's flexibility; thus, it can overcome skin barrier.[20]

In-vitro Anti-fungal Activity

The cup plate technique was used to detect anti-fungal activity of CTM-TF gel (test) against *Candida Albicans* in comparison to Canesten® cream 1% (standard).[1] The zone of inhibition was taken as a response to the comparison between standard and test preparations.[35] Figure 7 represented the results of zone of inhibition; it was shown that the zone of inhibition of CTM-TF gel (20 mm) was higher than that of Canesten® cream 1% (11 mm), which could be due to the flexibility of TF, enabling its permeation through fungal cell wall and resulting in ergosterol biosynthesis inhibition that is leading to the lysis of cell membrane and cell death.[20]

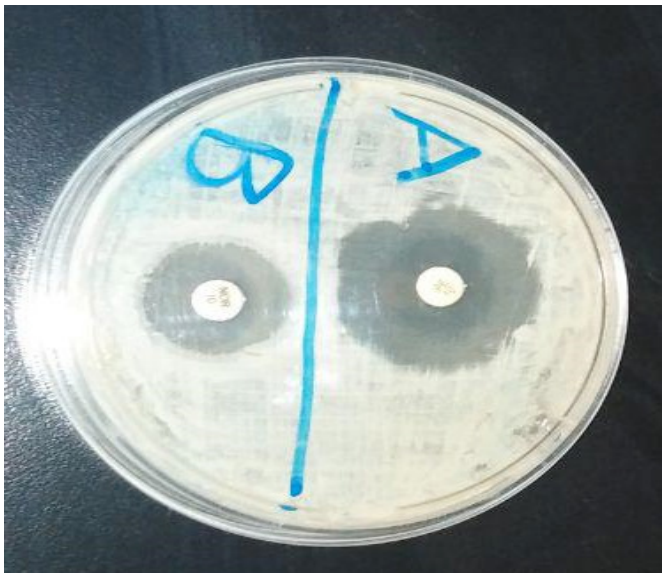

Figure 7: Anti-fungal activity test representing inhibition zone of CTM-TF gel (A) and Canesten® cream 1% (B)

In-vivo Anti-fungal activity of CTM-TF Gel

The anti-fungal activity may be evaluated by using *Candida Albicans*. Before the cutaneous fungal infection has been induced, all rats' skin structure should be usual without any signs of fungal infection, like color change, edema, inflammation, and cracking, as represented in Figure 8. After the fungal infection has been induced, the rats showed purple or brownish batches, edema, inflammation and skin cracking. After the topical application of Canesten® cream 1%, the edema and inflammation vanished, but few wounds were still present. The rats treated with CTM-TF gel were cured with little inflammation,[36] as showed in Figure 8.

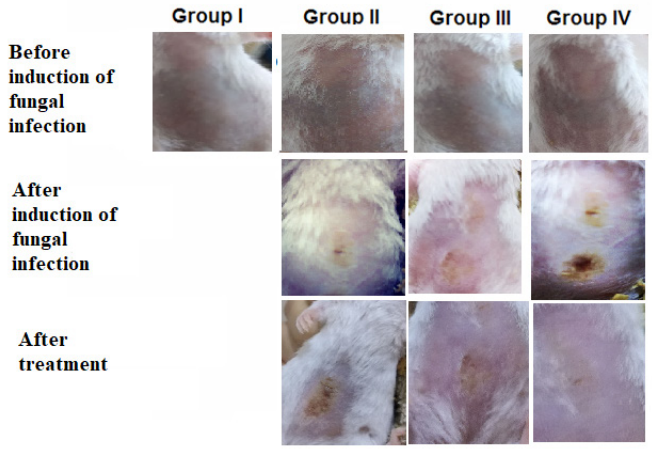

Figure 8: In-vivo anti-fungal activity of Canesten® cream 1% (Group III) and CTM-TF gel (Group IV)

In-vivo Pharmacokinetic Study

All volunteers had been done with the experiment without any side effects. The results of plasma concentration after the application of CTM-TF gel and Canesten® cream 1% were shown in Figure 9. By comparing peak plasma concentration, C_{max} of CTM after the application of CTM-TF gel and Canesten® cream 1%, which were 297 ± 10.5 and 188 ± 8.7 ng/mL respectively, the statistical difference between them has been shown in Table 7 to be ($p < 0.050$). The peak plasma concentration of CTM-TF gel increased slowly to reach its maximum after 10 ± 0.76 h then the permeation of CTM sustained for a duration of 14 h in all volunteers while the release of CTM from Canesten® cream 1% reach the maximum concentration after 6.5 ± 0.56 h then declined to release all of CTM after about 20 hours as shown in Table 7. The time to reach a maximum concentration t_{max} was slower with CTM-TF gel than that with Canesten® cream 1% and, hence, was taken to be a reasonable parameter to compare the absorption rate

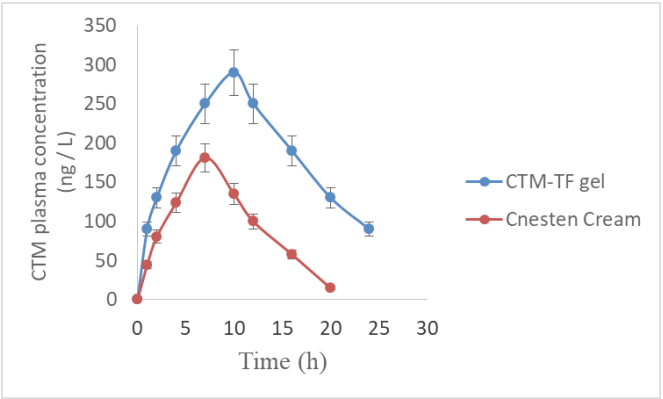

Figure 9: Plasma concentration time profile for CTM in CTM-TF gel and Canesten® cream 1% (results are represented as mean ± SD, n = 6)

Table 6: Permeation parameters of CTM-TF gel compared to Canesten® cream 1%*

Permeation Parameters	*CTM-TF gel*	*Canesten® cream 1%*
Steady state flux (J_{SS}, μg/cm^2.h)	88.942 ± 0.45	73.234 ± 0.68
permeability coefficient (*KP*, cm/h)	0.0245 ± 0.23	0.0186 ± 0.36

* Results are represented as mean ± SD, n = 3

Table 7: Pharmacokinetic parameters of CTM-TF gel and Canesten® cream 1%*

Parameter	*CTM-TF gel*	*Canesten® cream 1%*
AUC (ng.h/mL)	2314 ± 107	1348 ± 97
C_{max} (ng/mL)	297 ± 10.5	188 ± 8.7
t_{max} (h)	10 ± 0.76	6.5 ± 0.56
K_{el} (h^{-1})	0.024 ± 0.06	0.087 ± 0.03
$t_{½}$ (h)	± 0.15	3.4± 0.17

* Results are represented as mean ± SD, n = 6

of the drug from both formulations. It accordingly indicates the sustained release of CTM from TF than Canesten® cream 1%. In addition, the plasma level of CTM from CTM-TF gel remained detectable for 24 hours; thus, proved the sustained release action of CTM-TF gel.

The data of the in-vivo study were compatible with that of the in-vitro release for both formulations. The area under the curve (AUC) is a measure for the quantity of the drug absorbed into circulation, the AUC for CTM-TF gel and Canesten® cream 1% were 2314 ± 107 and 1348 ± 97 ng.h/mL, respectively, as represented in Table 7. This is maybe due to the flexibility of TF vesicle, which can penetrate the skin's stratum corneum barrier. Moreover, the presence of EA in TF structure dissolved lipids in stratum corneum layer leading to a high penetration of CTM [7,34]. The CTM-TF gel release exhibited a flip-flop phenomenon associated with a sustained release formulation and characterized by a decrease in the elimination rate constant and an increase in elimination half-life.[37] The elimination rates constant K_{el} for CTM-TF gel and Canesten® cream 1% were 0.024 ± 0.06 and 0.087 ± 0.03 h^{-1}, respectively, while the elimination half-life was 5.2 ± 0.15 h for CTM-TF gel and 3.4 ± 0.17 hours for Canesten® cream 1%, see Table 7. These results are a good indicator for the sustained release action of CTM-TF gel.

CONCLUSION

CTM was formulated as TF to enhance skin permeability and increase its anti-fungal activity. The developed TF had small vesicles size, small PDI, high zeta potential, high EE %, high elasticity and high transdermal flux. The optimized formula has been formulated as a TF gel, which had the ability to increase the anti-fungal activity of CTM compared to Canesten® cream 1%. Besides, the pharmacokinetic study of CTM-TF gel in healthy volunteers has shown an improvement in the CTM-TF gel's bioavailability and sustained the release of CTM compared to Canesten® cream 1%.

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AUTHORS CONTRIBUTIONS

The design of experiments has been applied by the contribution of all authors. The formulation, characterization and analysis of the data of CTM-TF different formulae have been achieved by Jamal Alyossef Alkkrad and Rashad M. Kaoud. The HPLC analysis has been performed by Eman J. Heikal. The In-vitro permeation, in-vitro anti-fungal activity and the in-vivo pharmacokinetic study of optimized formula have been achieved by the contribution of all authors. The manuscript has been written and submitted to the journal by Rashad M. Kaoud and Jamal Alyossef Alkkrad.

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