

Synergistic antifungal potential of fluconazole-based cardamom oil oral microemulsion formulation against *C. albicans*

Nisha TIWARI ^{1*} , Amravati SIVAKUMAR ¹ 

¹ School for Advanced Sciences, Vellore Institute of Technology, Vellore-14. Tamil Nadu. India

* Corresponding Author. E-mail: svkmr7627@gmail.com (A.S.); Tel. +91-7598218192.

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ABSTRACT: Microemulsion has grabbed a lot of attention in the last few decades as a unique candidate for drug delivery owing to its low viscosity, limpidness, ease of preparation, and good thermodynamic stability. The potential to incorporate drugs from unlike lipophilicity and its spontaneous formation makes it a suitable candidate for pharmaceutical application. The objective of the current study is to enhance the solubility of Fluconazole, an antifungal drug by formulating a microemulsion made up of cardamom oil, tween 20, and water (5:15:80). The formulations were checked for their physiochemical properties and thermodynamic stability. An antimicrobial activity like zone of inhibition, minimum inhibitory concentration and kill kinetics assay for optimized formulations were checked against *C. albicans*. The droplet size of the system and FLZ loaded microemulsion was found to be 7.8 and 8.6nm respectively. *In vitro* drug release from optimized microemulsion formulation showed efficient release (78.45%) compared to the bulk API (7.98%) in simulated intestinal fluid. The cytoplasmic release for CDMM FLZ ME at 0.5X, 1X and 5X was 43.24%, 82.11%, 91.73% respectively. *In vitro* toxicity demonstrated a lower toxicity rate with lower surfactant concentration against human lymphocytes and its safer usage. SEM analysis depicts the morphological distortion in *C. albicans* that occurred due to the interaction with microemulsion. The results indicate cardamom oil-based microemulsion is an efficient drug delivery vehicle intended for oral usage.

KEYWORDS: Antifungal; Minimum Inhibitory concentration; Oral drug delivery; Microemulsion; Thermodynamic stability.

1. INTRODUCTION

Fluconazole (FLZ), a synthetic fluorinated bis-triazole derivative, is associated with the subclass of triazole antifungal agents [1]. It is a broad-spectrum antimycotic agent with a bioavailability of nearly 90%, the plasma protein binding is found to be low (11-12%), which results in renal excretion of 80% of the drug in the unchanged form [2]. The drug is found to be fungistatic rather than fungicidal, hence resulting in resistance against certain fungi [3]. The mode of action of the drug on fungal cytochrome P450 is by inhibiting the formation of 14 α -demethylase and interferes with the biosynthesis of vital sterol present in the fungal membrane [4]. FLZ is a widely used drug against fungal species like *Cryptococcus*, *Histoplasma*, *Trichophyton*, *Blastomyces*, *Candida*, and *Microsporum* [5]. Fluconazole is a hydrophobic drug and possesses dissolution-related issues [6]. FLZ is used to treat meningitis and helps in the treatment of yeast infection in patients infected post-chemotherapy and radiation therapy [7]. Adverse effects include liver damage, hormonal imbalance, long QT intervals, and skin allergies [8]. The route of elimination is through urine hence the patients with renal failure possess an uncertainty of drug overdose [9]. The drug is vulnerable to the chemicals present in the body and possesses tremendous chances of drug interaction leading to unwanted by-

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products [10]. Thus, it's a necessity to formulate a suitable drug delivery vehicle to reduce the unwanted drug interaction, enhance the solubility of hydrophobic moiety and enhance the efficacy of the drug.

In recent years, microemulsion (ME) has gathered a lot of importance as an efficient drug delivery vehicle. The existence of compartmentalized hydrophobic and lipophilic domains aids in the incorporation of polar and non-polar components [11]. MEs are optically transparent, thermodynamically stable, and have low viscous colloidal dispersion of oil and aqueous phase stabilized by a surfactant, sometimes in combination with cosurfactant [12]. The ease of preparation, low interfacial tension, enhanced solubilization, and high surface area make them a prospective drug delivery system [13]. The fine droplets of oil due to reduced interfacial tension encourage the extensive distribution of drugs through the intestine and hence help reduce the irritation encountered with the prolonged contact of drugs and the gut wall [14]. The unwanted drug interaction is reduced and hence the potential for drug delivery is enhanced which was earlier exposed to enzymatic degradation in the gastrointestinal tract (GIT) [15].

Essential oils (EO) are a complex mixture of varying bioactive compounds with different chemical and biological natures [16]. Since the ancient era, they are known for their physiological and therapeutic activity, which may work either alone or in synergy with other components [17]. Essential oil-based microemulsion formulation appeared suitable to enhance the biopharmaceutical properties of these bioactive compounds and proved to have immense potential to treat numerous health diseases [18]. The major components of cardamom essential oils (CDMM oil) are alpha-terpinyl acetate, linalool, and 1,8-cineole [19]. Cardamom extracts have exhibited antifungal activity against oral pathogens like *C. albicans* [20]. This study aims at formulating an oral microemulsion to enhance the solubility and drug activity of FLZ compared to conventional drugs. The inherent activity of essential oil-based microemulsion should work synergistically with the bulk drug and help reduce the dose-dependent side effects for oral drug delivery. A lot of studies on transdermal studies have been performed, but the ones limiting oral delivery are still very much restricted.

2. MATERIALS AND METHODS

2.1 Materials

Fluconazole (FLZ) was procured from Microlabs, India. The EO of Cardamom was purchased from falcon, India. Surfactants like polysorbate 20 and polysorbate 80 were acquired from Sigma- Aldrich, Bangalore, India. Sorbitan monolaurate, Sorbitan monooleate, RPMI-1640, sabouraud dextrose agar (SDA), and Mueller Hinton agar (MHA) were acquired from HiMedia Laboratories Ltd, India. Throughout the experimentations, ultrapure water (Cascada™ Biowater System, Pall Corporation, USA) and other chemicals were used without any further purification. *C. albicans* (ATCC 90029) was obtained from ATCC for performing the anti-fungal testing.

2.2 Methods

2.2.1 Solubility Studies

The solubility testing was done for FLZ in different oil systems by dissolving an extra quantity of FLZ in 1mL of each selected essential oil in a 5 mL stoppered vial. The drug and oil mixture were vortexed and allowed for shaking (Orbitek, Scigenics Biotech, India) at $37 \pm 1^\circ\text{C}$ for a time interval of 72 h to reach equilibration. The equilibrated mixtures were subjected to centrifugation at 3000 rpm for 15 min and the supernatant was carefully collected using a 0.45 μm membrane filter. The unknown dissolved amount of FLZ was determined in the supernatant by using a UV double beam spectrophotometer (UV-Vis Systronics 2201, Ahmedabad, India) at 260 nm [19].

2.2.2 Surfactant Selection

The selection of surfactant for formulating the FLZ loaded O/W microemulsion was done by testing the emulsification efficiency of the surfactant against various oil phases. Non-ionic surfactant was preferred over ionic surfactant due to relatively lesser toxicity and typically higher CMCs. Hydrophilic lipophilic balance (HLB) suitable for the formation of O/W ME was selected resulting in clear and transparent emulsions. Ease of emulsification was determined using % transparency for the selected oil phase (based on solubility) for various surfactants at 600nm. An equal volume (500 μ L) of surfactant was mixed with the (500 μ L) of an oil phase and was homogenized by manual agitation followed by gentle heating (45-50°C). Eventually, the mixture was diluted to a 1:100 ratio in a 50 mL falcon tube and checked for the numbers of inversions required to get a homogeneous emulsion. The lesser the number of inversions denoted the ease of emulsification of surfactant against a particular oil phase [21].

2.2.3 Pseudo Ternary Phase Diagram for Optimizing the Components

Based on the solubility (oil phase) and ease of emulsification (surfactant), the optimum concentration range of the clear microemulsion region was determined by plotting the phase diagram. The various ratio of oil: surfactant ranging 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 was mixed in a beaker under magnetic stirring (5ML DX Remi, India) and titrated by conventional water titration method to allow equilibration under gentle stirring. Milli Q water was added dropwise to the oil: surfactant mixture and visually observed to see phase behavior such as the formation of the microemulsion, gels, and coarse emulsions. The endpoint of composition was determined by visual inspection of the sample turning from turbid to transparent. Spontaneous formation of clear and less viscous samples was considered a monophasic system indicating the clear microemulsion region, any turbid mixture resulting in phase separation was designated as a biphasic region [22]. The ratios at which clear and transparent microemulsions were formed were noted and plotted in the ternary graph. To ensure reproducibility, the experiments were performed in triplicates.

2.2.4 Microemulsion Preparation

Microemulsions were prepared using cardamom essential oil (CDMM oil) as the dispersed phase, tween 20 as the surface-active agent, and water as the continuous phase. A determined quantity of Fluconazole (1% w/v) was dissolved into the oil phase and was used to prepare the FLZ-loaded microemulsion. A set of formulations were prepared with ratios ranging from 1:1 to 1:9 (oil: surfactant) and titrated with water. The oil and surfactant ratio were allowed to mix in a beaker using a magnetic stirrer (5ML DX Remi, India) for 10 min. Further water was added to the mixture to obtain the set ratios and was allowed to equilibrate overnight at room temperature. Drug-loaded (CDMM FLZ ME) and drug-free microemulsions (CDMM ME system) were formulated and inspected for their optical observation.

2.2.5 Thermodynamic Stability Study

The impact of temperature, sunlight exposure, and humidity may result in the reduced shelf life of microemulsions hence to avoid issues related to metastable formulations the thermodynamic stability tests are performed. Formulation sets of unloaded cardamom system (CDMM system) and FLZ loaded cardamom microemulsion (CDMM FLZ ME) were subjected to thermal and kinetic stress settings. The samples were centrifuged at 3500 rpm for 30 mins and checked for any phase separation. The clear samples were then imposed to six cycles of heating (45°C) and cooling (4°C) for 48 h each. Further, the samples were exposed to three alterations of freeze (-21°C) and thaw (+25°C) for 48 h each [23]. The sample with the minimum concentration of surfactant which remained clear with no phase separation, creaming, or cracking was chosen as optimized ME and was further taken for other experiments.

2.2.6 Physiochemical Characterization

2.2.6.1 pH

The pH value for both the sets of CDMM system and CDMM-FLZ ME were measured at $25 \pm 1^\circ\text{C}$ by immersing the pre-calibrated pH meter (Systronics, India) in the undiluted samples. The pH readings were measured in triplicates.

2.2.6.2 Conductivity

The measurement of electrical conductivity (σ) is a key method to differentiate between oil continuous (w/o) or aqueous continuous (o/w) microemulsion system. Moreover, it is considered a reliable means to investigate phenomena like percolation or phase inversion [24]. The measurements were carried out in triplicates at $25 \pm 1^\circ\text{C}$ for the CDMM system and CDMM-FLZ ME with a conductivity meter (CM180, Elico, India).

2.2.6.3 Hydrodynamic Size Analysis

The droplet hydrodynamic size and distribution of the optimized ME were investigated using the dynamic light scattering (DLS) method by a nanoparticle analyzer (SZ-100, Horiba Scientific, Japan). The optimized ratio of formulations was diluted (1:3) for both the CDMM system and CDMM FLZ ME with ultrapure water and examined for the variability in light scattering as a function of time for the droplets at a scattering angle of 90° at $25 \pm 1^\circ\text{C}$ [25]. The software with the system performed the data analysis and the measurements were conducted in triplicates to ensure reproducibility.

2.2.7 FTIR Drug Excipient Compatibility

FTIR spectra of CDMM oil, Tween 20, CDMM system, CDMM FLZ, and FLZ were recorded using an FTIR spectrophotometer (IR Affinity-1, Shimadzu). The optimized formulations and bulk API were prepared for analysis by the KBr disk method and the measurement was performed from the range $600\text{--}4000\text{cm}^{-1}$. The major peak for functional groups was shown in the spectra and was compared to check the congruity between the drug and the excipient.

2.2.8 In Vitro Dissolution Study

The drug release of FLZ from optimized CDMM FLZ ME formulation was carried out *in vitro* using Franz diffusion apparatus (Tritech instruments private limited, Mumbai, India). Bulk FLZ (API) suspension in ultrapure water and newly formulated FLZ loaded microemulsion of predetermined concentration (2mg/mL) were placed into the donor compartment. The dialysis membrane (110 Avg. flat width- 31.33 mm , Avg. diameter- 21.5 mm , capacity approximately 3.63 mL/cm , HiMedia, India) was carefully positioned between the donor and the receptor compartment to avoid any air bubbles. Simulated gastric fluids (SGF) and simulated intestinal fluid (SIF) with a pH adjusted to 1.2 and 6.4 respectively were placed in the receptor compartment (5mL) at $37 \pm 1^\circ\text{C}$ and 50 rpm. Aliquots ($200\text{ }\mu\text{L}$) were drawn out of the receptor compartment at predetermined intervals and were quantified for the concentration of FLZ by quantifying the absorbance (260 nm) using simulated fluid as blank by UV-Visible spectrophotometer. The volume of the recipient compartment was maintained by refilling an equal amount of fresh simulated fluid to the same compartment [26].

2.2.9 Drug Release Kinetics

The *in vitro* release kinetics of the drug from optimized ME formulation were checked using various mathematical models such as Korsemeyer-Peppas, zero order, first order, and Higuchi's diffusion model. The most satisfactory model was selected according to the goodness-of-fit test.

2.2.10 Antifungal activity

2.2.10.1 Inoculum Preparation

The fungal strain of *C. albicans* ATCC 10231 (American Type Culture Collection, Rockville, MD) was maintained at -80°C and was revived by subculturing it aseptically at Sabouraud's Dextrose Agar (SDA) media plates followed by incubation for 48 h at 37°C . The isolated *C. albicans* colonies were picked up and resuspended into 0.85% normal saline, to obtain a definite concentration of 1×10^8 CFU mL^{-1} for further investigation.

2.2.10.2 Antifungal Susceptibility Testing (AST)

Antifungal susceptibility testing (AST) of the optimized microemulsion and its components was carried out *in vitro* against *C. albicans* by agar well diffusion technique. This protocol was followed according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). Mueller Hinton Agar (MHA) was additionally enriched with 2% (w/w) glucose and methylene blue (0.5 mg/l), adjusted to pH 7.2 was autoclaved at 121°C for 15 min, and poured aseptically onto sterile Petri plates. The fungal culture (24 h old) was adjusted to 1×10^5 CFU/mL with normal saline and swabbed for a uniform lawn of growth onto the MHA plates. The wells were punched using a sterile borer and 100 μL of cardamom oil, tween 20, CDMM system, CDMM FLZ ME, and FLZ were added to the wells [27]. The results for the zone of inhibition were measured after incubation of 48 h at 37°C and specified in $\text{mm} \pm \text{SD}$.

2.2.10.3 Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentration of test samples (CDMM oil, tween 20, CDMM system, CDMM FLZ ME, FLZ) was tested against *C. albicans* using a modified broth microdilution technique following the guidelines provided by CLSI (M27-A3) for yeast. The fungal inoculum was adjusted to 10^6 CFU/mL with RPMI 1640. The plates were incubated for 48 h at $37 \pm 1^{\circ}\text{C}$. The absorbance values were determined with the PowerWave XS2 microplate reader (BioTek Corp. USA) at 600 nm. Inhibition in 80% of the fungal growth compared to the growth control was considered to be the MIC at that sample concentration [28]. The experiment was recorded in triplicates.

2.2.10.4 Live/Dead Cell Viability Analysis

The degree of fungal membrane impairment caused due to interaction of *C. albicans* with ME was detected using Fluorescence microscopy. Fresh *C. albicans* culture was picked up from an SDA plate, to obtain a fungal inoculum of cell density 10^6 cell /ml. This inoculum was allowed to interact with 1 X MIC of CDMM FLZ ME for 1 h. The sample was centrifuged at 3000 rpm for 15 min and washed with PBS thrice. The fungal pellet was interacted using 4 μL each of acridine orange (AO) and ethidium bromide (EtBr) with a concentration of 15 $\mu\text{g}/\text{mL}$ and 50 $\mu\text{g}/\text{mL}$ respectively for 10 min. The samples were centrifuged to remove an excess unbound dye and were resuspended in PBS. The optical representation was captured using the Leica DFC-295 camera (Leica- application suite 3.8 processor).

2.2.10.5 Kill Kinetics Assay

The time-kill kinetics assay helps in determining the time and concentration-dependent impact of the antimicrobial agents on the test samples. Three different concentrations of MIC (0.5X, 1X, and 5X) of FLZ loaded optimized CDMM ME and bulk FLZ (API) was allowed to interact against *C. albicans* according to the CLSI guidelines (M27-A3, Broth microdilution method). The samples and growth control were prepared using RPMI-1640 for all time points in a 96-well microtiter plate and incubated at $37 \pm 1^{\circ}\text{C}$. The three known concentrations of MIC were prepared by using RPMI-1640 in microtiter wells. A growth control was included for drug-loaded and bulk FLZ (API) to monitor its activity and was incubated at $37 \pm 1^{\circ}\text{C}$. A ten-fold serial dilution of test samples at respective concentrations was used to estimate the Colony Forming Unit (CFU) by plating it onto a BHIA plate at different time points [29]. The Petri plates were analyzed after the incubation of 48h at $37 \pm 1^{\circ}\text{C}$ for viable colony count (CFU/mL) and were performed in duplicates.

2.2.10.6 Membrane Integrity Assay

Fungal culture of 24-48 h incubated at 37°C was cultured on an SDA plate and re-suspended using phosphate-buffered saline (PBS) to adjust for a final cell density of 10^8 CFU/ml. The fungal culture of *C. albicans* was allowed to interact with the CDMM ME system, CDMM FLZ ME, and bulk FLZ (API) at three various concentrations of MIC (0.5x, 1x, and 5x). Media control was 9.5 mL of PBS and 0.5 mL of fungal suspension and growth control was 9.5 mL of Triton X 100 with 0.5 mL of fungal suspension. The samples for membrane integrity analysis were allowed to homogenize and the discharge of cytoplasmic content was analyzed at a predetermined time interval by centrifuging for 15 min at 6000 rpm. The absorbance was recorded at 260 nm using a UV- Vis spectrophotometer by using negative control as blank [30]. The percentage release of cytoplasmic content was determined employing the absorbance value in the test/ standard X100 formula. The results were recorded in triplicates to ensure reproducibility.

2.2.11 In vitro Toxicity

Isolation of human lymphocytes from healthy volunteers' blood was done using lymphocyte segregation fluid HiSep™ LSM 1077 (HiMedia, India) using the ficol density gradient technique. The buffy coat layer was relocated into a sterile falcon and was rinsed using PBS twice. The extracted lymphocytes were resuspended in RPMI 1640 media supplemented with 10% FBS and was incubated at 37° C with 5% CO₂. The cells were enumerated for viability using trypan blue and hemocytometer. The test samples (CDMM oil, tween 20, CDMM system, CDMM FLZ ME, FLZ) at different concentrations of 12.5, 25, and 50% were treated with human lymphocytes (1×10^4 cells) in a 96 microwell plate, followed by overnight incubation at 37° C with 5% CO₂. The samples interacted with 20 µL/well of MTT (5mg/mL) at 37° C for 4h later 50 µL/well of DMSO was added and allowed to mix gently [31]. The samples were done in triplicates to ensure reproducibility. The optical density was recorded at 600nm using microplate reader PowerWave X2 (BioTek Corp., USA).

2.2.12 SEM

The morphological changes or the cellular damage that occurred to *C. albicans* post-treatment with 1X MIC CDMM-FLZ ME for 24 h was observed [32]. The negative control was a *C. albicans* culture suspended in PBS. The treated and untreated cell suspension of 0.5 McFarland was centrifuged for 10 mins at 3500 rpm, the pellet was collected and smeared onto a coverslip. The smear was maintained to get dried up under ambient conditions and was immobilized using glutaraldehyde (2.5%) for 30min. The samples were later dehydrated with a gradient wash of 50%, 70%, and 100% ethanol for 10 min each. The smear slides were layered with an ultra-thin coating of Au/Pd sputtering and examined using a high-resolution SEM (EV018 Research, Zeiss, Germany).

3. Results and Discussion

3.1 Solubility Studies

Hydrophobic drug molecules when incorporated into oil in water microemulsion system, help in enhancing the solubility, stability, and better *in vitro* efficacy [33]. The oil phase helps in solubilizing the drug. The solubility of FLZ in various oils is represented in **Table 1**. The solubility of FLZ was found to be maximum in cardamom oil (268mg/mL). The components of microemulsion are preferred from the generally regarded as safe (GRAS) class, certain specific pharmaceutical excipients blends result in efficient formulations.

Table 1 Solubility of FLZ in various oils

Name of the oil	Solubility of FLZ (mg/mL)
Rose Oil	134
Olive Oil	8
Cardamom Oil	268
Black Pepper Oil	18
Rosemary Oil	82

Oregano Oil	197
Lemongrass Oil	28
Turmeric Oil	24

3.2 Surfactant Selection

The selection of surfactant for cardamom oil was done based on its emulsification efficiency performance. The higher emulsification efficiency was denoted by the maximum transparency with the least number of flask inversions. Sorbitan esters (Span 20 and span 80), polysorbates (Tween 20 and Tween 80), Brij 93, and Cremophor EL were screened for higher diffusion of a hydrophobic particle in the aqueous phase (**Table 2**). HLB values from 12-16 help in the formation of O/W microemulsion [34]. Among the various surfactants tested, tween 20 exhibited maximum ease of emulsification with cardamom oil. The literature favors the use of nonionic surfactants in drug delivery systems because of their biocompatibility, lesser toxicity, and low hemolytic damage and does not affect the physiological pH of the system [35].

Table 2 Emulsification efficiency of various surfactants and cosurfactants for Cardamom oil

S.No	Surfactant/ Co-surfactant	CDMM Oil	
		No. of Inversions	Transparency (%)
1	Tween 20	2	99.90%
2	Tween 80	6	99.90%
3	Span 20	20	99.92%
4	Span 80	20	99.88%
5	Cremophor EL	10	99.95%
6	Brij 93	18	99.94%
7	Triton X 100	5	99.66%
8	PEG-400	3	99.92%
9	Propylene glycol	9	99.98%

3.3 Pseudo Ternary Phase Diagram for Optimizing the Components

Microemulsion systems are formed spontaneously, where the surfactants help reduce the interfacial surface tension [36]. The microemulsion region in the pseudo ternary phase diagram signifies the clear and transparent mixtures with a maximum proportion of oil phase stabilized by the lowest concentration of the surfactant to give a homogeneous ME system. Tween 20 with cardamom oil was able to give the maximum ME region based on its visual lucidity, and transparency and was plotted on the trilateral coordinate (**Figure 1**).

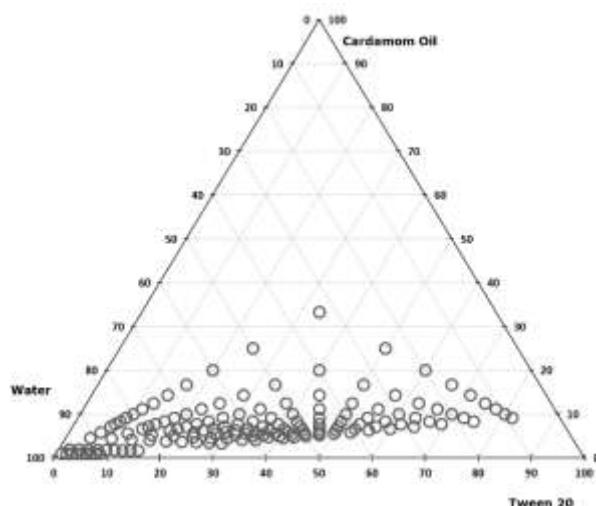


Figure 1. Pseudo ternary phase diagram demonstrating cardamom oil, tween 20, and water

3.4 Preparation of Microemulsion

FLZ drug (1% w/v) was allowed to dissolve in CDMM oil. A distinct set of FLZ CDMM ME and CDMM ME systems were formulated using tween 20 and water. The optical observation of two sets of the drug-loaded and unloaded system of the varied composition of CDMM oil: Tween 20: water (**Figure S1**). It is important to determine that the least surfactant concentration can stabilize the microemulsion. Beyond its critical micellar concentration, the higher surfactant concentration leads to increased viscosity and toxicity serving as an obstacle to various routes to delivery [37]. The set was clear and transparent from the ratio 1:2:17 to 1:9:10 (oil: surfactant: water). To find the optimized ratios, the formulations should be checked for phase separation via thermodynamic stability testing and other characterizations.

3.5 Thermodynamic Stability Studies

The absence of creaming, cracking, or phase separation at a given fraction of oil, surfactant, and water signifies the formation of a thermodynamically stable system. The optical observation of formulations was inspected after subjecting it to centrifugation, heating cooling, and freeze-thaw cycle (**Table 3**). Samples from 1:3:16 to 1:9:10 were found to be stable monophasic ME and were taken for further characterization. Similar results were obtained for thermodynamic studies performed with FLZ-loaded microemulsions.

Table 3 Optical observation and thermodynamic testing of Cardamom oil-based formulation from 1:1 to 1:9

Tube name	Dilution (CDMM:Tween20)	O:S:W (%)	Optical observation	Centrifugation	H/C	F/T
C1	1:1	5:5:90	Transparent	Phase separation	-	-
C2	1:2	5:10:85	Transparent	Phase separation	-	-
C3	1:3	5:15:80	Transparent	Clear	Clear	Clear
C4	1:4	5:20:75	Transparent	Clear	Clear	Clear
C5	1:5	5:25:70	Transparent	Clear	Clear	Clear
C6	1:6	5:30:65	Transparent	Clear	Clear	Clear
C7	1:7	5:35:60	Transparent	Clear	Clear	Clear
C8	1:8	5:40:55	Transparent	Clear	Clear	Clear
C9	1:9	5:45:50	Transparent	Clear	Clear	Clear

(O- Cardamom oil; S- Tween 20; W- water; H/C- heating-cooling cycle; F/T- Freeze-thaw cycle)

3.6 Physiochemical Characterization

3.6.1 pH

The pH value of CDMM FLZ ME and CDMM ME system were analyzed by immersing the pH probe directly into the absolute samples by a pre-calibrated pH meter at $25 \pm 1^\circ\text{C}$ (**Figure S2**). All the measurement was recorded in triplicates to ensure reproducibility. The non-ionic surfactants ensure that the pH of the system is having insignificant variation upon long-term storage [38]. The pH of the optimized formulation i.e. 1:3 of the CDMM system was 6.34 ± 0.02 and for CDMM FLZ was 6.29 ± 0.04 .

3.6.2 Conductivity

The non-ionic surfactants used for the development of microemulsion formulation results in lower electrical conductivity due to the absence of charged species. But the o/w microemulsion has higher electrical conductivity in comparison with the w/o microemulsion because of the presence of the outer aqueous pseudo phase. The conductivity value for optimized formulation (1:3) of the CDMM system was $0.201 \pm 0.001 \mu\text{S}/\text{cm}$ and for CDMM -FLZ ME was $0.199 \pm 0.001 \mu\text{S}/\text{cm}$ (**Figure S3**). The conductivity of the formulation is not affected by the presence of a free basic drug, but if they dissociate from their salt forms may lead to a surge in the conductivity of the formulation.

3.6.3 Hydrodynamic Size Analysis

The concept of DLS analysis is a correlation between the time measurements as a function of the scattering intensity. The hydrodynamic radius is calculated using the diffusion coefficient due to Brownian motion for colloidal dispersions [39]. The mean droplet size of optimized CDMM- FLZ was 8.6 nm. The smaller droplet size of microemulsion serves a vital role in the drug delivery system, as it determines the degree of drug release and a higher rate of drug absorption [40]. The FLZ-loaded and unloaded CDMM systems have a similar hydrodynamic radius and both possess low PDI, which clearly explains the homogeneity and stability of formulation (**Figure S4, Table 4**).

Table 4 Droplet size analysis of the optimized ratio of CDMM ME and CDMM FLZ ME

Sample	Z-Avg.	PI	Peak size
CDMM ME System (1:3)	7.8	0.449	10.2
CDMM FLZ ME (1:3)	8.6	0.204	11.1

3.7 FTIR Drug Excipient Compatibility

The FTIR spectra of CDMM oil, Tween 20, CDMM system, CDMM FLZ ME, and FLZ was recorded (**Figure 2**). The major structural peak of API (FLZ) depicted =C-H stretching from $3010\text{--}3100 \text{ cm}^{-1}$, C-H (alkane) stretching from $2850\text{--}3000 \text{ cm}^{-1}$, C=C stretching from $1620\text{--}1680 \text{ cm}^{-1}$, C=C (aromatic) stretching from $1400\text{--}1600 \text{ cm}^{-1}$, C-N stretching from $1080\text{--}1360 \text{ cm}^{-1}$, C-F stretching from $1000\text{--}1400 \text{ cm}^{-1}$. The structural bands present in the drug-loaded microemulsion demonstrated the presence of O-H stretching from $3200\text{--}3600 \text{ cm}^{-1}$, and other peaks of the drugs were not affected by the components of ME; which signifies no structural modification.

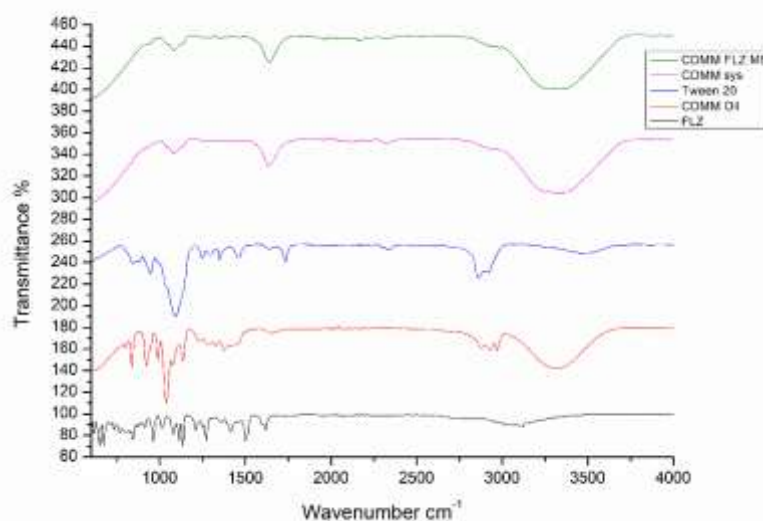


Figure 2. FTIR spectra of cardamom oil, tween 20, Unloaded and FLZ loaded cardamom ME, FLZ

3.8 *In vitro* Dissolution Study

FLZ-loaded CDMM ME and bulk FLZ API in aqueous suspension was examined for its cumulative release in simulated fluids using Franz apparatus for 8 h at fixed time points (**Figure 3**). With reference to simulated intestinal fluid 78.45% and 7.98% of the drug were released from CDMM FLZ ME and FLZ API aqueous suspension respectively. Whereas in simulated gastric fluid the release was found to be slower with 34.08% and 12.76% of drug release from the CDMM FLZ ME and FLZ aqueous suspension. A similar trend from a lemongrass-based formulation showed up to 80% of drug (FLZ) in 6 h and its applicability for topical administration was studied [41]. Another study, of FLZ-loaded nanoemulsion lipid carriers (NLCs), demonstrated a drug release of 37.34% for 72h [42]. The drug release is found maximum in the intestinal fluid which helps with the drug absorption in the lymphatic system and reduces the dosage of the drug [43].

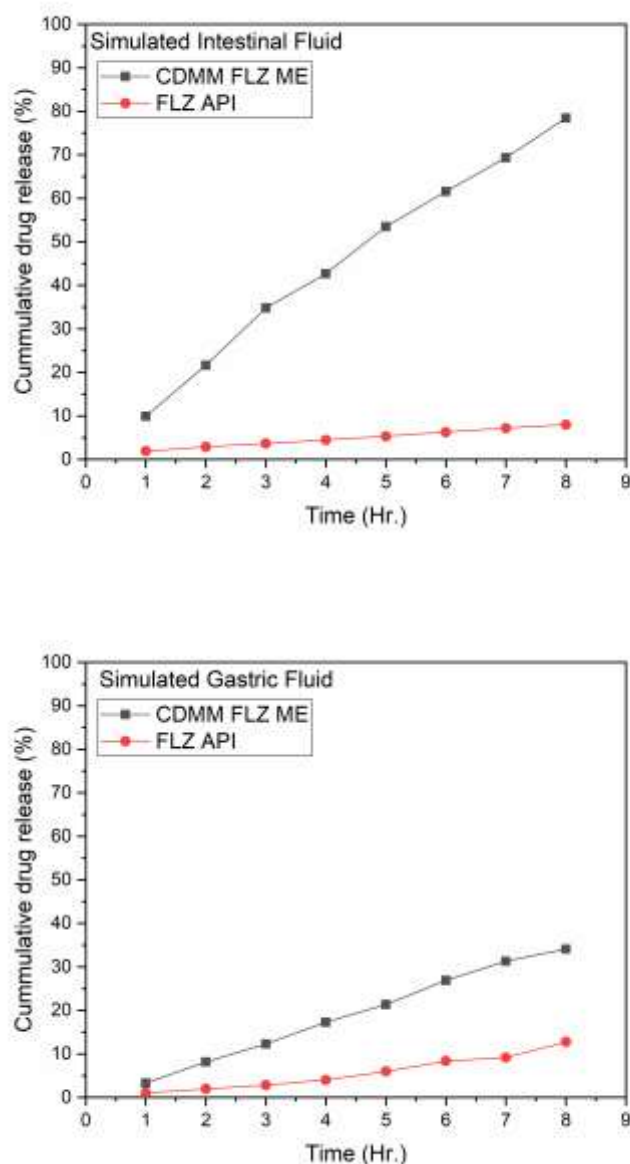


Figure 3. *In vitro* drug release of FLZ from CDMM FLZ ME and FLZ API in an aqueous form in simulated fluids.

3.9 Release Kinetics Study

In order to understand the optimal kinetic model which best suits the obtained diffusion data, some of the kinetic models like Korsemeyer- Peppas, zero-order, first-order, and Higuchi diffusion models were used (Table 5). From the results, it was evident that the *in vitro* drug release followed the zero-order kinetic model for the FLZ-loaded cardamom oil ME formulation.

Table 5 Release kinetics profile for FLZ loaded CDMM oil-based ME formulation

Simulated Gastric Fluid	Simulated Intestinal Fluid
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Release kinetics model	CDMM FLZ ME	FLZ	CDMM FLZ ME	FLZ
Korsemeyer-Peppas	0.9522	0.8582	0.9711	0.9217
Zero-order	0.9972	0.9564	0.9967	0.9786
First order	0.9916	0.9512	0.9912	0.9756
Higuchi	0.8851	0.7996	0.9244	0.9759

3.10 Antifungal Activity

3.10.1 Antifungal Susceptibility testing (AST): Antifungal susceptibility testing was performed for the CDMM system, CDMM FLZ ME, FLZ, and the components of ME up against the fungal culture of *C. albicans* by agar well diffusion technique (Table 6). The zone of inhibition explains the efficient antifungal activity of ME formulation with a zone similar to the bulk API of FLZ. The system has only 5% of oil in the formulation and surfactant (tween 20) displays negligible activity against *C. albicans*. Hence the oil possesses inherent antifungal activity, which comes into synergistic action when incorporated with the drug [44]. A similar study for FLZ-loaded lemongrass-based emulsion was studied against candida to evaluate the antifungal potential for topical administration and demonstrated a relatively smaller inhibition zone [45].

Table 6 Zone of Inhibition (mm) of various components of ME and bulk drug

Test Sample	Clear zone (in mm)
CDMM Oil	20
Surfactant (Tween 20)	08
CDMM ME System	20
CDMM FLZ ME	24
API (FLZ)	28
Ethanol	06
FLZ Control	28

(CDMM oil: cardamom oil; CDMM ME system: unloaded cardamom microemulsion; CDMM FLZ ME: fluconazole loaded cardamom microemulsion, FLZ: fluconazole bulk form)

3.10.2 Minimum Inhibitory Concentration (MIC)

The MIC is the least concentration of a chemical entity (drug) that prevents the evident growth of microbes. The MIC values for the FLZ, CDMM system, CDMM FLZ ME and each component of ME were tested against *C. albicans*. The test range of FLZ was 16 to 0.0078 µg/ml. The MIC value attained for FLZ was 1 µg/mL, whereas for Cardamom oil was found to be 1.5% of oil concentrate. There is a one-fold decrease for the CDMM system and a two-fold decrease in CDMM FLZ ME activity against *C. albicans* compared to the FLZ (bulk drug). Surfactant (Tween 20) didn't demonstrate antifungal properties against *C. albicans*. The growth control and media control exhibited satisfactory results. The results can be correlated with the fact that the nanosize of the droplets helps enhance the penetration and consequently delivers an effective growth inhibition for the fungal culture [46]. Treatment of vulvovaginal candidiasis due to *C. glabrata* and susceptible *C. albicans* strains were tested against azole antifungals and demonstrated the MIC range for fluconazole to lie between 32 to 0.5 µg/mL [47].

3.10.3 Live/ Dead Cell Viability Analysis

The fungal culture of *C. albicans* interacted with 1 X MIC of CDMM FLZ ME and was analyzed for cell viability (**Figure 4**). Staining dyes like acridine orange (AO) and ethidium bromide (EtBr) are used distinctively for determining the viability of cells and any morphological changes in the cell membrane of the microbial strains using fluorescence microscopy. AO is a basic dye taken up by both live and dead cells and appears to be fluorescent green. EtBr appears to be red and is taken up by the dead cells [43]. The microemulsion droplets fuse with the lipid bilayer and impair the membrane integrity resulting in the lysis and death of the treated cells. The treated cells appeared to be red upon interaction because of cellular disruption and release of the intercellular component. A similar study with AO/EB concluded the structural alterations and cellular release from fluconazole-resistant *C. albicans* using Ascorbic acid for the fluorescence pattern of cells [48].

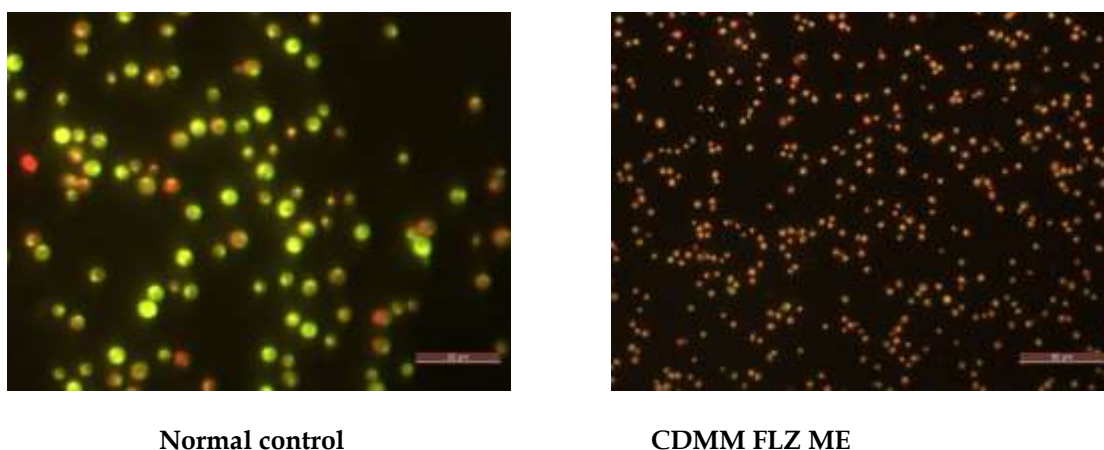


Figure 4. Fluorescence images of *C. albicans* a) untreated; b) interacted with 1 X CDMM FLZ ME

3.10.4 Kill Kinetics Assay

The optimized CDMM FLZ ME and FLZ were tested at three distinct concentrations of its MIC (0.5X, 1X, 5X) concentration and reported the time-kill kinetics at different time points (**Figure 5**). In the beginning, the viable count of fungal culture was found to be 10^6 CFU/ml. The test samples were diluted to twice the predetermined MIC concentrations against *C. albicans* using RPMI 1640. The 5X MIC concentration of CDMM FLZ ME interacted with *C. albicans* at 8th h showing a 4-log reduction in the viability. Whereas a 2-log reduction is found for the 5X FLZ treated culture. This elucidates that the exposed fungal strains were more susceptible to the optimized CDMM FLZ ME at 1X and 5X concentrations compared to FLZ (bulk drug) at a similar MIC. The reason may be attributed to nano-sized droplets and better penetration and interaction with fungal membrane compared to bulk drug. The dynamics of killing are fungistatic rather than biocidal for the bulk drug but microemulsion treatment at 5X was found to be fungicidal. A CFU reduction of < 3-logs was said to be fungistatic compared to the initial inoculum, and ≥ 3 -logs was considered fungicidal [49]. A similar study with *A. indica* leaf and *C. roseus* flower extract in synergy with fluconazole demonstrated a fungistatic effect against *C. albicans* at 8X concentration of its MIC [50]. Our FLZ-loaded CDMM demonstrated a better activity at a relatively lower concentration of MIC.

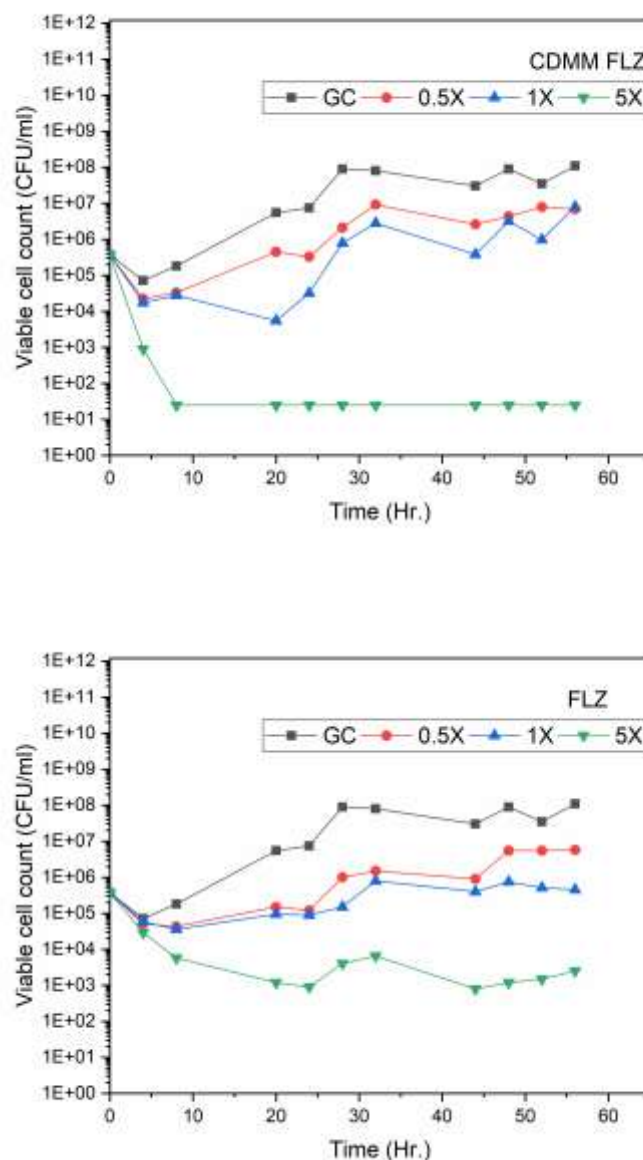


Figure 5. Kill kinetics of *C. albicans* treated with CDMM FLZ ME and FLZ

3.10.5 Membrane Integrity Assay

Cytoplasmic release was determined after interacting *C. albicans* culture with the CDMM ME system, CDMM FLZ ME, and FLZ bulk API with three distinct concentrations of minimum inhibitory concentration (0.5X, 1X, and 5X) at 260nm. The samples were allowed to interact for 1 h. The cytoplasmic release for CDMM FLZ ME at 0.5X, 1X and 5X was 43.24%, 82.11%, and 91.73% respectively much higher compared to bulk FLZ, which was 4.57%, 9.84%, 13.25% at a given concentration (**Figure S5**). The microemulsions depicted a dose-dependent impact on the fungal cell membrane. At all the tested concentrations, significant damage to the fungal cell wall was observed in the form of intracellular material (DNA/RNA) to extracellular media within 1 hr of treatment. The cytoplasmic release was found to be more efficient in comparison with the bulk API drug (FLZ) the reason might be attributed to the nano-scale size and larger surface area, which may facilitate the enhanced penetration hence rupturing the cell membrane leading to cytoplasmic release [51].

3.11 In Vitro Toxicity

The defense mechanism of the body is majorly played by human lymphocytes; hence the cultured lymphocytes were treated with formulated microemulsion and its components. The percentage of viable cells post-interaction gives an account of the cytotoxicity of ME [52]. The lymphocytes were checked for dose-dependent cytotoxicity by interacting with 50%, 25%, and 12.5% of test samples (**Figure 6**). The cytotoxicity of ME was shown by a decline in cellular viability in a dose-dependent manner. A similar study carried out by Sundaramoorthy et.al, demonstrated the impact of oil in water microemulsion loaded with permethrin has a dose-dependent impact on the cell viability of the human lymphocytes [53]. The interaction of human lymphocytes with ME even at the highest concentration shows more than 50% of viable cells. Individual components of the ME were not found to be toxic at the lower concentrations. The results demonstrate LD₅₀ of FLZ-loaded ME formulation has lesser toxicity even at its highest concentration, which features its safer oral dosage form.

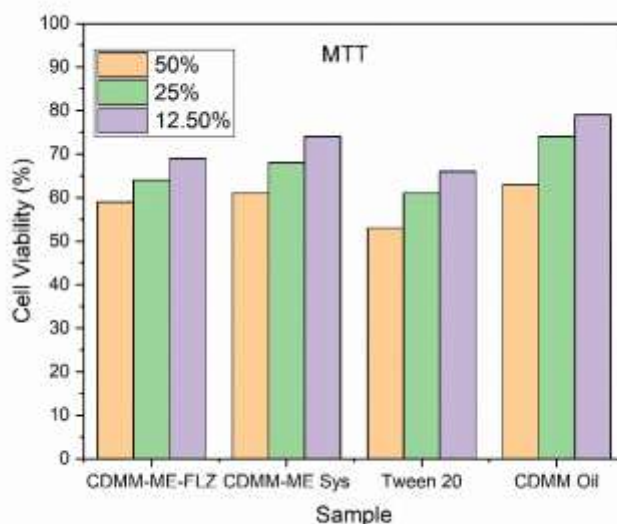


Figure 6. MTT assay on human lymphocytes by CDMM ME and its components

3.12 SEM

Fungal culture of *C. albicans* interacted with 1X MIC of CDMM-FLZ ME demonstrates morphological changes in the structure in comparison with the untreated *C. albicans* strain in the SEM micrographs (**Figure 7**). Visual observation of cell structure damage to *C. albicans* depicts the evidence of the antifungal action of the formulated FLZ CDMM ME against the fungal culture [45]. The morphology of untreated control cells shows the intact cell membrane structure with a well-defined membrane structure and few budding yeasts. Whereas, the ME-treated cells showed irregularity in shape and structure, which clearly explains the leakage of the cytoplasmic material of the fungal culture and inhibition of cell division. Similar results were demonstrated by Ebenazer et.al, where itraconazole loaded thyme oil-based microemulsion shows structural deformities post-treatment for the fungal strain of *C. albicans* [32].

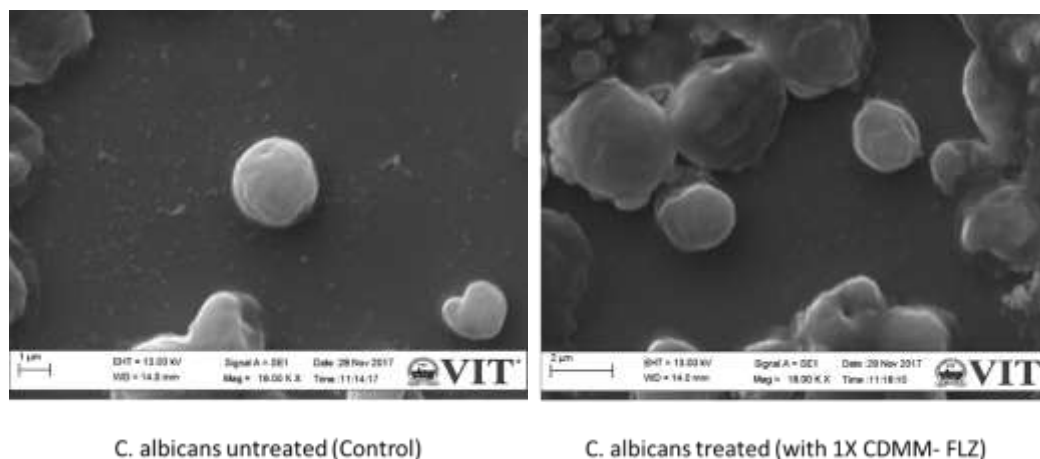


Figure 7. SEM micrographs of *C. albicans* a) untreated; b) treated with 1 X CDMM FLZ ME

4. CONCLUSION

The present study describes fluconazole-loaded cardamom oil-based microemulsion formulation, formed spontaneously without any external energy requirements to attain nano-scaled droplets of homogeneous size, low concentration of the surface-active agent, and enhanced antifungal activity for therapeutic pertinence as a drug delivery tool to cure fungal infections orally caused by *C. albicans*. The nanosized drug formulation provides enormous surface area and boosted intestinal absorption. The cumulative drug release in simulated intestinal fluid demonstrated a release of 78.45% from FLZ loaded cardamom microemulsion compared to 7.98% of bulk drug release over 8 hrs. The optimized formulation demonstrated a fungistatic effect against *Candida albicans* at a 5x concentration. The better antifungal activity of FLZ-loaded microemulsion demonstrates the synergistic behavior achieved using cardamom oil. The toxicity studies for the optimized ME confirms the oral intake to be safer and thus helps in enhancing the efficacy of hydrophobic drug through oral delivery routes. Novel drug delivery systems proved to be a better approach to enhance the effectiveness of the antifungals and enhance patient compliance by reducing the adverse effect.

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