Turkish Online Journal of Qualitative Inquiry (TOJQI)
Volume 12, Issue 7, July 2021: 3538-3558

Research Article

FORMULATION OF FLUCONAZOLE TOPICAL GEL FOR IMPROVED ANTIFUNGAL EFFECT

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Introduction

Healing with medicinal plants is as old as mankind. Ever since ancient times, in search for rescue of diseases, the people looked for drugs in nature. Medicinal plants have always had an important place in the therapeutic armory of mankind. According to WHO, in Africa, Asia and Latin America up to 80% of population relies on medicinal plants in order to meet their primary health care needs (Burton, Smith, & Falkenberg, 2015). Medicinal plants as life – saving pharmaceutical agents are becoming essential component in the search for new medicines to combat various diseases. Out of the 350,000 plant species known so far, about 35,000 (some estimate up to 70,000) are used worldwide for medicinal purposes and less than about 0.5% of these have been investigated for their phytochemical and pharmacological potential, yet many issues are to be addressed (Shasany, Shukla, & Khanuja, 2007).

Plants are considered as the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediated and chemical entities for synthetic drugs(Burton et al., 2015). Over 50% of all allopathic drugs are made up of natural products and thus, natural product play an important role in drug development programs of the pharmaceutical industry (Patwardhan, Vaidya, & Chorghade, 2004). In this regard, present study involves a medicinal plant named *Mallotusphillipinensis*in combination of antifungal agent i.e. fluconazole (FLZ) for the development of topical gel formulation. Two approaches had been considered; surfactant i.e. sodium dodecyl sulfate (SDS)-aided and ethosomal based topical gel formulations for the improved residence time, stability and antifungal activity ofFLZ.

Topicalgels

The field of pharmaceutical science has been developing steadily over the years, and become invaluable to keep us healthy and prevent disease. An avenue of research that has progressed a great deal in the past few decades is the treatment of diseases via biomolecules such as drugs, proteins etc. Initially these could only be administered in limited manner, due to limitations of drug delivery through harmful environments in the body. Thus limited mobility reduced the effectiveness of administered drug(Peppas, Bures, Leobandung, & Ichikawa, 2000).

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The nature of these carriers progressed over the years from ceramics to natural and then synthetic materials (Russell, Axel, Shields, & Pishko, 2001). Factors such as integrity, biocompatibility and flexibility were considered and helps the use of hydrophilic three dimensional matrices as carrier materials which are known as *gels*.

There have been concerns related to the conventional topical dosage forms such as lotions, creams, ointment and powder in terms of drug diffusion or release from the vehicle and delivery through the skin. Creams and lotions often provide poor bioavailability of the drug because they are rapidly cleared from the skin and poorly release the drug from the base. Non-hydrophilic ointments are oleaginous, greasy and are not convenient to patients, and also medicated powders for topical application have short residence time on the skin(Quiñones & Ghaly, 2008).

Topical gels are soft, solid-like, or semisolid in nature. These consist of at least two components, one is liquid that is present in a substantial quantity and another is macro/nano molecule uniformly dispersed with no apparent boundary between the dispersed macro/nano molecule and liquid. Although the liquid content is high, on a time scale of seconds, a gel should not flow under the influence of its own weight. Gels combine the cohesive properties of solids and the diffusive transport characteristics of liquids (Escobar-Chávez et al., 2006). Among the drug delivery systems and pharmaceutical dosage forms, topical gels are most preferred choice for both systemic and local administration of drugs. Topical gels have several characteristics such as non-flowing material, excellent unprimed adhesion, low viscosity for easy and rapid room temperature dispensing, no heat generated (no exotherm) during cure and low toxicity (Bonacucina, Cespi, Mencarelli, Giorgioni, & Palmieri, 2011).

Medicinal plant for fungal infection

Infectious diseases, particularly skin and mucosal infections, are common in most of the tribal inhabitants due to lack of sanitation, potable water and awareness of hygienic food habits (Hassawi & Kharma, 2006). It has been estimated that skin diseases account for 34% of all occupational diseases. As the primary interface between the body and external environment, the skin provides the first line of defense against broad injury by microbial and chemical agents. And many more factors other than trauma and primary skin disease have been identified as contributory to skin infections and these include immune deficiency diseases, diabetes mellitus and systemic or topical use of steroids. The most damaging consequence of disruption to the skin is invasion by pathogenic microorganisms(Chanda & Baravalia, 2010).

Skin diseases can be caused by a variety of the microbes and the skin is a haven for many microbes. In skin and soft tissue infections, the commonest bacterial agents are *Staphylococcus aureus*, *Streptococcus pyogenes*(Group A haemolytic streptococcus), *Clostridium perfringes*and the bacteriodes group. Others are *Mycobacterium tuberculosis, Mycobacterium leprae, Neisseria gonorrhea, Pasturellatulurensis, Bacillus antracis and Pseudomonas aeruginosa(Stevens et al., 2005).* The common fungi which cause skin infections are *Candida albicans, Candida neoformans, Epidermophytonflocossum, Trychophytontonsurans, Melassezia furfur,* etc. The yeast *Candida albicans*and *Candida krusei*may occur in low frequency on skin and mucous membranes without causing symptoms. As opportunistic pathogens they may overgrow the normal flora and cause skin diseases like impetigo and candidiasis in diabetics, adipose and immunodeficient subjects(Perfect et al., 2003).

Mallotusphilippenesis

*Mallotusphilippenesis*belongs to the euphorbiaceae family. It is an important medicinal plant with various curative properties attributed to almost every part of plant. It is a woody tree species consisting of herbs and shrubs (Fig. 1.1) widely distributed throughout tropical India along with the beat of Himalaya from Kashmir eastwards up to 5000 ft all over the Punjab, Uttar Pradesh, Bengal, Assam, Burma, Singapore and from Sind, southwards to Bombay and Caylon. It it is also reported as growing in China, the Malaya Islands, Australia, Pakistan and Andaman Ialands. The

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Bengal, Assam, Burma, Singapore and from Sind, southwards to Bombay and Caylon. It it is also reported as growing in China, the Malaya Islands, Australia, Pakistan and Andaman Ialands. The plant is known by various names like kamala, kampallaka, Raktanga, and Spoon Wood (Jaya, Ranjana, & Alka, 2012). It is also known Red Kamala, due to the fruit covering, which produces a
red dye (Siva, 2007).

Fig. 1.1 Images of Mallotusphilippenesis

Fungal infections include common skin diseases. The most frequently observed species of fungal infection in clinical practice are *candida (Pappas et al., 2009). Candida albicans*are mostly responsive for *candida* infections. It is a regular commensal in the mouth, gastrointestinal tract and vagina in healthly individuals but prevalence of carriage is higher in hospitalized patients. *Candida* skin infection is frequently restricted to body folds including the inter-digital spaces of the hands or feet. Soreness and white patches on erythematous background (plaque type) are typical symptoms and signs of mouth *candida* and vaginal *candida (Naglik et al., 2003).*

Drugs are rarely administered in their pure forms. So they have to be necessarily admixed with various kinds of adjucts resulting in their transformation into so called ‘dosage form’ or ‘drug delivery sysytems’ (Pouton, 2006). Although the physical forms of medication have not changed dramatically, the attitude of the scientist has been changed in developing the drug into

a formulation. In this regard fluconazole (FLZ) (Fig. 1) had been chosen for the present study. FLZ (2, 4-difluoro-1', 1'-bis (1H-1, 2, 4-triazol- 1-ylmethyl) benzyl alcohol -$C_{13}H_{12}F_2N_6O$ (Fig. 1.2) is a trizolefungistatic agent used for dermal infection caused by various species of *candida* such as *C. albican*and *C. glabratra(Kathiravan et al., 2012)*.

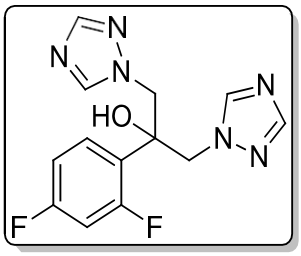


Fig. 1.2 Structure of Fluconazole

FLZ can treat both fungal skin infection as well as serious systemic fungal infections. FLZ antifungal class has been the most successful in terms of numbers of different agents that have entered clinical use. This triazole, FLZ inhibit CYP P450 14 α- demethylase in fungi. This enzyme is involved in the conversion of lanosterol to ergosterol (Fig. 1.3). Other P450s in sterol biosynthesis may be affected. The basic nitrogen of the azole ring forms a tight bond with the heme iron of the fungal P450 preventing substrate and oxygen binding. Inhibition of the C14α-demethylase results in accumulation of sterols still bearing a C14 methyl group changing the exact shape and physical properties of the membrane causing permeability changes and malfunction of membrane imbedded proteins. They have a lower affinity for mammalian P450's. The effect is fungistatic, but may be fungicidal at higher concentrations (Ong, Coulter, Birkett, Bhasker, &Miners, 2000).

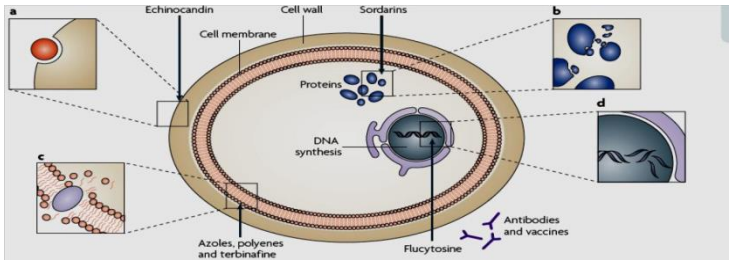


Fig. 1.3 Mechanism of action of azoles derivatives and other antifungal agents

FLZ is commercially available as tablets and injections despite its well- know adverse effects including GIT and taste disturbances. Other adverse effects include headache, dizziness, leucopenia, thrombocytopenia,hyperlipidermias, hepatotoxicity and raised liver enzyme values (Zhang, Camp, & Elewski, 2007). This embraces FLZ to be formulated only for topical use in order to avoid adverse effects or bioavailability problems that limit its potential as systemic agent. Although, topical FLZ formulation avoid the adverse effect associated with commercially available tablets and injections yet it also associated with insufficient residence time to provide adequate therapeutic effects. Hence, the development of an effective drug delivery system with prolonged contact time with skin surface and provide sustained drug release would be beneficial. Therefore, in this present thesis we tried to develop two topical gel formulations of

FLZ using two approaches; surfactant-aided and ethosomal drug delivery. In order to develop best formulations utilizing above two cited approaches we must go through the detailed investigation of surfactants and ethosomes.

Chemicals and Reagents

Water: Water being one of the major solvent in the study which is also employed in calibration of instruments or apparatus was obtained by double distillation process. By volume, 1000 ml of pure water was collected from the double distillation unit (Harco& Co.) which was further subjected to distillation on acidified $KMnO_4$ over a 750 mm long fractionating column. Different fractions of distilled water were collected having specific conductivity and pH, □ (S cm^{-1}), ~ 1-2 $\times 10^{-7}$ S cm^{-1} and 6.75–6.95respectively.

Solvents: Absolute alcohols i.e. methanol and ethanol was obtained from Merck Chemicals with purity ≥ 99.9 %. Other solvents in experimental and lab processes such as acetone, sulfuric acid and hydrochloric acid etc. for complete cleansing of glassware were also obtained from Merck Chemicals. Physico-chemical study of surfactants in presence of FLZ was carried out in three different solvent compositions of alcohols i.e. 10, 20, 30% v/vethanol.

Pharmaceutical Ingredients: Fluconazole (FLZ) was received as a gift sample from Meridian Pvt. Ltd. (Solan). Polymer; Carbopol 934/ 940 was purchased from Himedia and triethanolamine was purchased from Merck chemicals for formulation of topical gels. Anionic surfactant; sodium dodecyl sulfate (SDS) and Lecithin was obtained from Merck Chemicals. Surfactant used in the study was of AR grade and purity > 99.0%. Rhodamine B ($C_{26}H_{31}CIN_2O_3$) was purchased from Oxford Laboratory, Calcein (C_{30} H_{26} N_2O_{13}) were purchased from FINAR® chemical Pvt. Ltd. and Neil Red was purchased from Sigma. All dye used in study was of ARgrade.

Pre-formulation Drug Analysis

Determination of melting point

Capillary fusion method was used to determine the melting point of FLZ using Indosati Scientific Lab melting point apparatus. The melting point was determined and compared with the literature value(Hajare et al., 2009).

Determination of absorption maxima

A solution of FLZ (10µg/ml) in methanol was scanned between 200-400 nm, using Shimadzu 1700 spectrophotometer. The scanned λ_{max} was compared with literature value (Hajare et al., 2009).

Determination of solubility

Solubility studies of drug sample were carried out in pure methanol and optimized ratio of phosphate buffer and methanol i.e. 1:1. A certain amount of drug was added to screw capped vials containing 10 ml of solution, in each. The saturated solution then filtered through whatmann filter paper and was analyzed on UV Spectrophotometer at $\lambda_{max}$of each solvent.

Preparation of standard plots

Standard plots of fluconazole were prepared in; Methanol: Phosphate buffer 7.4. FLZ (100 mg) was dissolved in small volume of methanol : phosphate buffer 7.4 (1 : 1) in
100 ml volumetric flask and volume was made up to 100 ml with methanol: phosphate buffer 7.4 (1 : 1) to get a concentration of 1000 µg/ml. From this stock solution, aliquots of 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml and 0.5 ml were withdrawn into a series of 10 ml volumetric flask and volume was made with methanol: phosphate buffer 7.4 (1 : 1) to get a concentration ranging from 10-50 µg/ml. The absorbance of the resulting solutions was then measured at 260 nm using Nano drop spectrophotometer (Thermo Scientific) against parent solvent as areference.

Drug – excipient compatibility studies

While preparing a formulation for the development of final dosage form, it is mandatory to confirm the compatibility between the drug and polymer to be utilized. and to ensure that the drug is not interacting with polymer. Two techniques have been used to determine the interaction of drug with excipients.

Accelerated stability study

Physical mixture of drug and carbopol 940 in the weight ratio 1:1 was prepared. This physical mixture of drug and polymer was filled in vials and kept under accelerated stability conditions (40 ± 2 °C and 75 ± 5 % RH) for 4 weeks in stability chamber and the vials were examined at regular intervals for discoloration, liquefaction and clump formation in the mixture.

Fourier Transform Infra-Red spectral analysis

The Fourier Transform Infra – Red (FTIR) analysis of the drug and polymer were carried out for qualitative compound identification using Agilant technology 630, CARY. The spectra were scanned over wave number range of 4000 cm^{-1} – 400 cm^{-1}. Since FTIR is related to covalent bonds or hydrogen bonds, the spectra provide detailed information about the structural arrangements of molecular compounds. FTIR confirm the functional identity of the drug and to detect the interaction of the drug with excipients.

Formulation of gel

Two approaches were utilized in the study;

1. Dispersion of SDS-aided fluconazole (FLZ) in carbopol 940(with/without *Mallotusphillipinensis*) base topical gel.
2. Dispersion of nano-sized fluconazole (FLZ) - ethosomesin carbopol 934 (with/without *Mallotusphillipinensis*) base topicalgel.

DispersionofSDS-aidedfluconazole(FLZ)incarbopol940(with/without *Mallotusphillipinensis*) base topical gel.

Preparation of Gel (SDS-aided)

Gel base was prepared by dispersing carbopol 940 in distilled water i.e. using the dispersion method. After soaking for 2 hr a measured quantity of carbopol 934 in distilled water and then dispersed with/without *Mallotusphillipinensis*, using a magnetic stirrer in order to obtain a homogeneous gel base of 1% w/w.

Formulation code	**Ethanol (%)**	**Carbopol 940 (%)**	**FLZ (%)**	**SDS (mg)**	***Mallotusphillipinensis* (%)**
F1	30	1	1	69	0.1
F2	30	1	1	78	0.1
F3	30	1	1	97	0.1
F4	30	1	1	69	-
F5	30	1	1	78	-
F6	30	1	1	97	-
F7	30	1	-	-	0.1
F8	30	1	1	-	-

Table 1.1 Compositions of SDS-aided fluconazole (FLZ) gel

Preparation ofethosomes

Ethosomal suspension was prepared by cold method [73], utilizing lecithin (1, 2, 3, and 4%), ethanol (30% v/v), active molecules as described, and water upto 100% (v/v). Drug was dissolved in ethanol, and the mixture of drug and ethanol was added to the phospholipid dispersion in water at 40º C. After mixing for 5 min, the preparation was sonicated at 4º C for three cycles of 5 minutes each with a 5-minute gap in between the cycles using a probe sonicator (diameter 22 mm, 4000rpm; Citizen) to get nanosizedethosomes. Composition of various ethosomal formulations is given in Table1.2

Formulation code	**FLZ (mg)**	**Lecithin (%w/w)**	**Ethanol (%v/v)**	**Propylene glycol (% v/v)**
E1	10	1	30	1
E2	10	2	30	1
E2	10	3	30	1
E4	10	4	30	1

Table 1.2 Composition of various ethosomal formulations.

Evaluation of Ethosome

Vesicle size and zetapotential

The particle size and zeta potential of prepared ethosomes was determined by dynamic light scattering (DLS) (Nano ZS, Malvern, UK). The temperature was kept at 25°C constant and polydispersity index (PDI) was used as a parameter of the size distribution.

Visualization of vesicle by fluorescencemicroscopy

The morphology of the ethosomeswere analyzed using Fluorescent microscope (Olympus). Samples were prepared by loading measured amount of Rhodamine B and Calcein dye into the ethosomes solutions and then analyzed with the microscope. Lipid containing outer bilayer of

ethosomes was visualized by using Niel Reddye.

Determination of fluconazole entrapment efficiency(EE)

Entrapment efficiency (EE) was measured as follows: 2 ml of ethosomal suspension was centrifuged at 13000 rpm in microcentrifuge (Himac CT 15E, Hitachi) for 30min. The supernatent obtained was analyzed in UV spectrophotometer to check the % drug entrapped according to the formula mentioned below [55]:

EE (%) = Measured drug loading/ Theoretical drug loading X 100(2.1)

Preparation of gels (FLZ loadedethosomes)

Gel base was prepared by dispersing carbopol 934 in distilled water i.e. using the dispersion method [74]. After soaking for 2 hr a measured quantity of carbopol 934 in distilled water and then dispersed with/without *Mallotusphillipinensis*, using a magnetic stirrer in order obtain a homogeneous gel base of 1% w/w. Firstly, FLZ- loaded ethosomes suspension was centrifuged (Eltek® MP 400 R) at 2000 rpm for 20 minutes, and the pellets obtained were incorporated into the previously prepared gel base (with or without *Mallotusphilippensis*) to get 1% FLZ in the gel base. then further, triethanolaamine (TEA) was added dropwise to obtain gel mixture. The formulative composition of the ethosomes FLZ gels is summarized in Table 2.3.

Table 1.3 Formulation design for the preparation of various fluconazole (FLZ) ethosomal gels.

Component(% w/w)	**Formulation codes**							
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**	**G8**
Carbopol 934	1	1	1	1	1	1	1	1
Ethosomal FLZ	1	1	1	1	1	1	1	1
Mallotusphilippensis	0.1	0.1	0.1	0.1	-	-	-	-
Triethanolamine	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Double-distilled water	100	100	100	100	100	100	100	100

Evaluation of gels

In vitro drug release study

The diffusion studies of the prepared gels were carried out using Franz diffusion cell with the diameter of cm. For studying the dissolution release profile of SDS aided and FlZ-loaded ethosomal gels. A cellophane membrane was used which were hydrated in pH 7.4 prior to use for 12 hrs before placing them between donor and receptor compartments. The receptor compartment contained 18ml phosphate buffer at pH 7.4, under magnetic stirring. The temperature of the Franz diffusion cells was maintained at 37±0.5°C. Gel sample (1g) was

taken in cellophane membrane and the diffusion studies were carried out at 37 ± 1°C using phosphate buffer (pH 7.4) as the dissolution medium. Samples of 5 ml were withdrawn periodically at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h and each sample was replaced with equal volume of fresh dissolution medium to maintain the sink conditions. Then the samples were analyzed for the drug content by using phosphate buffer as blank spectrophometrically.

Modeling and comparison of diffusion profiles(Jose et al., 2013)

Mathematical modeling, whose development requires the comprehension of all the phenomena affecting drug release kinetics, has a very important value in the process optimization of such formulation. The use of mathematical modeling turns out to be very useful as this approach enables, in the best case, the prediction of release kinetics before the release systems are realized. More often, it allows the measurement of some important physical parameters, such as the drug diffusion coefficient and resorting to model fitting on experimental releasedata.

The data from the *in vitro* study was fitted to the following kinetic models to determine the kinetics of drug release. The suitability of equation in judged on the basis of best fit to the equation using statistical indicators like R^2.

In vitro antifungal activity of gel.

Standardized protocol M27-A2, CLSI was followed to perform the experiment. The inocula were performed after growth (48 h/35 °C) on Sabouraud dextrose agar. The colonies were suspended in 0.85% sterile saline and this suspension was homogenized in a vortex mixer for 15 s; after that, cell density was set in a spectrophotometer and transmittance (λ=530 nm) was adjusted to match standard 0.5 on the McFarland scale (1×10^6 to 5×10^6 cells/ ml). In the sequence, a 1:50 dilution in water was done, followed by a 1:20 dilution in RPMI 1640 medium, resulting in a final concentration of $1.5\pm 1.0\times10^3$ cells/ml.
The micro-dilution technique was performed in polystyrene sterile plates with flat-bottom, disposable, with 96 wells diluted in RPMI 1640 buffered broth. One hundred milliliters of samples were twice more concentrated than the desired final concentrations in the series in wells 1 to 10. Rows 11 and 12 represented the negative (medium) and positive (medium + microorganisms) controls, respectively. To each well micro-dilution plate were added 100 μL of the standardized inoculum. The plates were incubated at 35 °C for 48 h and then 10 mL of 0.5% 2,3,5-triphenyltetrazolium chloride dye was added to all wells, and the plates were re-incubated at 35 °C for 20 min. After this period, minimum inhibitory concentration (MIC) was determined.

Result and Discussion

Conductivity measurements

Conductivity measurement is considered to be one of the best methods for determining the critical micelle concentration (CMC) of surfactant-additive system (Beyaz, Oh, & Reddy, 2004; Sharma et al., 2013). Hence, the value of CMC of SDS was obtained from the plots of the specific conductance (□) versus concentration of SDS (1.00 – 14.08) mmol kg^{-1} containing FLZ (3.3 mmol kg^{-1}) in hydro-ethanol solutions (10%, 20% and 30% v/v) as a

solvent medium. The plot between the specific conductance (□) and SDS concentration yield reverse sigmoidal curves having the two straight lines, corresponding to the monomeric SDS and SDS aggregation zones, respectively; intersection of these two lines usually coincides to get critical micelle concentration (CMC) as shown in**Fig. 3.1**. all the specific conductance values have been provided in *APPENDECIS-I*

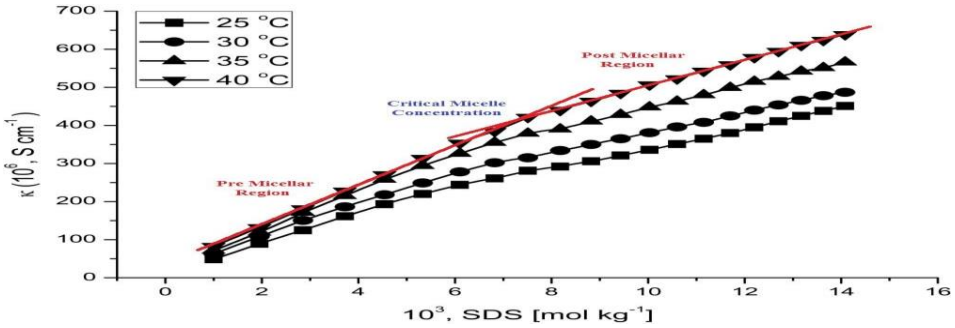
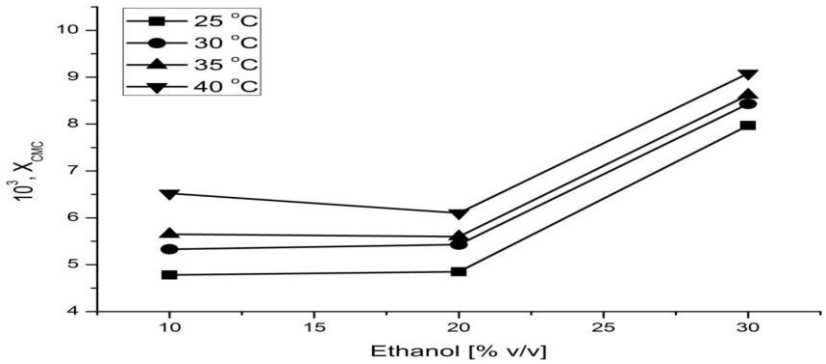


Fig. 3.1 The plot of specific conductance versus SDS concentration in 20% v/v ethanol solution containing 3.3 mmol kg^{-1} FLZ at *T* = 25, 30, 35 and 40°C. The



experimentally determined CMC values of SDS in the presence of FLZ were found to lie in the range of (6.0–9.8) mmol kg^{-1}. The influence of temperature and nature of solvent (ethanol) on the degree of micellizationof SDS was also evaluated and given in **Table 3.1.** It is well observed that in presence of drug at 10% & 20% hydro-ethanol solution, the value of CMC found to be less than that of CMC of SDS in water i.e. ~8 mmol kg^{-1}(Bhardwaj, Chauhan, Sharma, & Sharma, 2014; Marcolongo & Mirenda, 2011), whereas for 30% hydro-ethanol solution, the value CMC was found to be higher. The decrease in the CMC at 10% and 20% is might be credited to the interactions present between SDS and FLZ moieties and moreover due to hydrophobic effect exerted by the alcohol upon the CMC of SDS (Zana, Yiv, Strazielle, & Lianos, 1981).

Table 3.1 CMC, X_{CMC}, α, ΔH°_m,ΔG°_m, and ΔS°_mvalues of different hydro-ethanolic compositions containing 3.3 mmol kg^{-1} FLZ at *T* = 25, 30, 35 and 40 °C.

T ˚C	**CMC (10^3)**	**X_{CMC} (10^3)**	**ln X_{CMC}**	**α**	**ΔH°_m (kJ mol^{-1})**	**ΔG°_m (kJ mol^{-1})**	**ΔS°_m (J mol^{-1} K^{-1})**
10%v/v							

25	6.0	4.78	5.34	0.496	-11.11	-19.89	29.51
30	6.7	5.33	5.23	0.524	-11.27	-19.45	26.99
35	7.1	5.65	5.18	0.528	-11.61	-19.53	25.70
40	8.2	6.52	5.03	0.538	-11.91	-19.14	23.09
20%v/v							
25	5.8	4.85	-5.33	0.505	-11.04	-19.94	29.21
30	6.5	5.43	-5.22	0.508	-11.39	-19.62	27.16
35	6.7	5.60	-5.18	0.524	-11.64	-19.57	25.77
40	7.3	6.10	-5.09	0.532	-11.96	-19.45	23.92
30%v/v							
25	8.6	7.97	-4.83	0.542	-10.76	-17.45	22.43
30	9.1	8.43	-4.78	0.562	-10.98	-17.32	20.92
35	9.3	8.62	-4.75	0.575	-11.24	-17.33	19.79
40	9.8	9.08	-4.70	0.588	-11.50	-17.27	18.43

In the absence of additives, when the concentration of ethanol increases, the CMC of SDS increases, whereas, when FLZ is added a sharp decrease in CMC value of SDS was obtained which might be due to the fluorine group on the FLZ molecule (Zimmermann et al., 2014). Temperature is another critical parameter which widely affects the CMC, as the hydrophobic and hydrophilic head group interactions changes with temperature. So, the values of CMC were converted in X_{CMC}(CMC value expressed in terms of mole fraction) as shown in **Table 3.1**. The dependence of temperature and hydro-ethanol compositions on X_{CMC}values of SDS in presence of FLZ have been shown in **Fig. 3.2** and **Fig. 3.3**. This shows an increase in X_{CMC}values with temperature increment. Interestingly, a marginal change was obtained in X_{CMC}values at lower ethanol concentrations (10 and 20% v/v) where as significant increase was found at 30% ethanol concentration. This trend can be explained on the basis of predominant change caused by the penetration of ethanol molecules into the micelles (Shinoda, 1954).

Fig. 3.2 The plot of X_{cmc}verses temperature (T = 25, 30, 35 and 40 °C) in 10, 20 and 30% v/v hydro-ethanol solutions containing 3.3 mmol kg^{-1} FLZ.

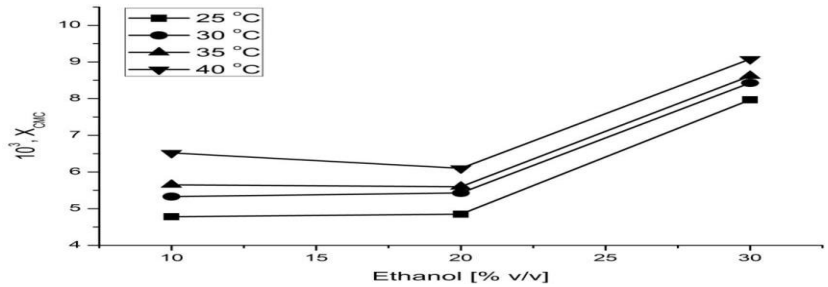


Fig. 3.3 The plot of X_{cmc}verses ethanol % composition (10, 20 and 30% v/v)containing 3.3 mmol kg^{-1} FLZ at T = 25, 30, 35 and 40 °C.

[SDS] mmol kg^{-1}		**10% v/v Ethanol**				**20% v/v Ethanol**				**30% v/v Ethanol**	
	25 °C	30 °C	35 °C	40 °C	25 °C	30 °C	35 °C	40 °C	25 °C	30 °C	40 °C
ρ (kgm^{-3})											
2.0	973.952	971.914	969.669	967.235	962.473	959.915	956.203	953.147	944.823	941.336	934.069
4.0	973.910	971.866	969.614	967.203	962.254	959.860	957.022	953.996	944.049	940.53	933.222
6.0	973.861	971.863	969.608	967.159	962.215	959.655	956.340	953.283	944.412	940.906	933.616
8.0	973.853	971.787	969.519	967.063	962.206	959.344	956.325	953.261	944.209	940.697	933.404
10.0	973.553	971.496	969.181	966.704	961.918	959.242	956.215	953.145	944.000	940.479	933.170
12.0	973.400	971.362	969.112	966.112	961.115	959.109	955.541	952.521	944.183	940.689	933.419
14.0	973.189	971.029	969.097	966.068	960.746	958.935	954.976	951.874	944.818	941.333	934.067
u (ms^{-1})											
2.0	1596.96	1596.15	1594.73	1593.37	1613.18	1604.95	1601.47	1590.67	1586.04	1573.87	1549.07
4.0	1597.06	1596.28	1594.85	1593.45	1613.95	1605.31	1601.69	1591.18	1586.37	1568.46	1543.67
6.0	1597.23	1596.54	1595.08	1593.72	1613.94	1605.65	1602.22	1591.45	1586.54	1571.17	1546.21
8.0	1597.39	1596.71	1595.27	1593.96	1614.44	1606.05	1602.56	1591.79	1586.81	1569.67	1544.95
10.0	1597.63	1596.98	1595.51	1594.22	1615.62	1606.29	1602.83	1591.98	1587.11	1568.22	1543.3
12.0	1597.82	1597.14	1595.75	1594.34	1615.91	1607.44	1603.5	1592.12	1587.3	1567.28	1542.51
14.0	1597.92	1597.46	1595.85	1594.57	1616.53	1607.81	1604.12	1592.63	1587.47	1575.74	1550.79
κ_s (TPa^{-1}) × 10^{-10}											
2.0	4.026	4.038	4.055	4.072	3.992	4.044	4.077	4.146	4.207	4.288	4.461
4.0	4.025	4.038	4.054	4.071	3.989	4.042	4.073	4.140	4.209	4.321	4.496
6.0	4.025	4.036	4.053	4.070	3.989	4.041	4.073	4.141	4.206	4.305	4.480

8.0	4.024	4.036	4.052	4.069	3.987	4.041	4.071	4.140	4.206	4.314	4.488
10.0	4.024	4.036	4.053	4.070	3.982	4.040	4.070	4.139	4.205	4.323	4.499
12.0	4.024	4.035	4.052	4.072	3.984	4.035	4.070	4.141	4.203	4.327	4.502
14.0	4.024	4.035	4.051	4.071	3.983	4.034	4.069	4.141	4.199	4.278	4.451

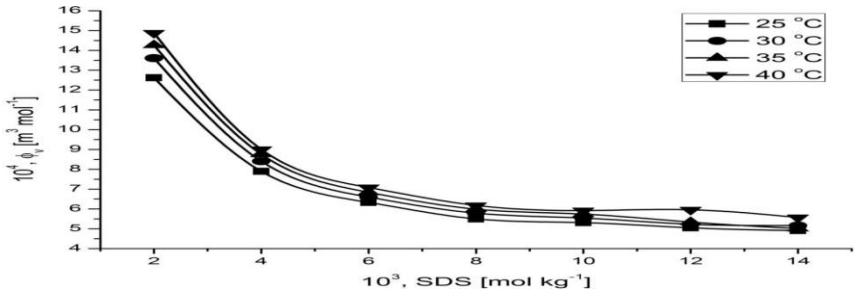
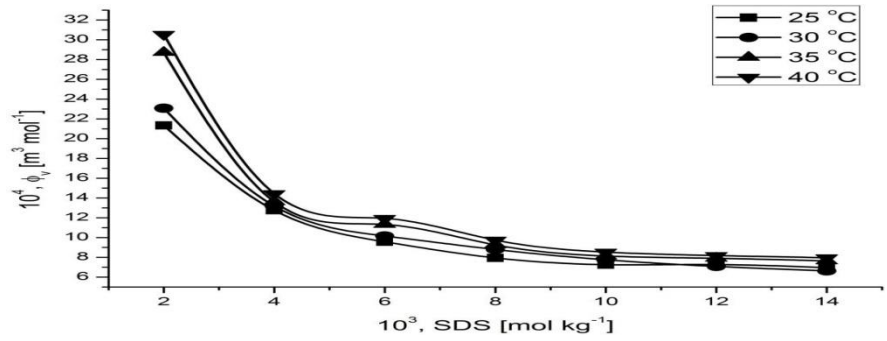


Fig. 3.4 The plot of ϕ_v versus SDS concentration in 10% v/v ethanol solution containing 3.3 mmol kg^{-1} FLZ at *T* = 25, 30, 35 and 40°C.

Fig. 3.5 The plot of ϕ_v versus SDS concentration in 20% v/v ethanol solution containing 3.3 mmol kg^{-1} FLZ at *T* = 25, 30, 35 and 40°C.



Preparation of the standard plot

The standard plot of FLZ were prepared in phosphate buffer (pH 7.4) : methanol [1:1]. 100mg/ 100ml stock solution was prepared and further, dilutions were done to obtained concentration range from 10 - 50 µg/ml. A straight line with correlation coefficient 0.995 was obtained indicating the drug follows Beer's law within the specified concentration range. Thereafter, it was further analyzed by UV spectrometer at 260nm.

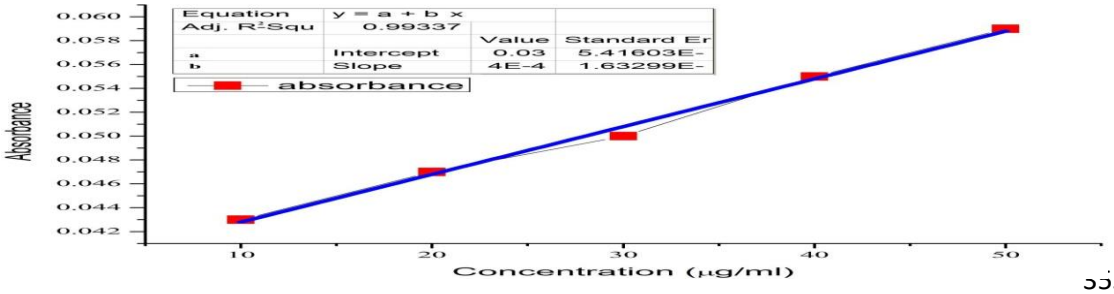


Fig. 3.9 Standard plot of fluconazole

Formulation of gel

Two approaches were utilized in the study;

1. Dispersion of SDS-aided fluconazole (FLZ) (with/without *Mallotusphillipinensis*) in carbopol 940 base topicalgel.
2. Dispersion of nano-sized fluconazole (FLZ) - ethosomes (with/without *Mallotusphillipinensis*) in carbopol 934 base topicalgel.

Preparation of gel (SDS-aided)

The topical gel was prepared by the above citied dispersion method in order to get 1% carbopol 940 base gel. Three concentration of SDS was utilized above the CMC of SDS which was obtained through the physico-chemical interaction studies. Out of three hydroethanolic solutions i.e 10, 20 and 30% ethanol, it was found that 20% hydroethanol solution was suitable for the topical gel formulation. As this composition shows the most feasible and stable system/formulation. Totally eight formulations were prepared, out of which three are with methanolic extract of *Mallotusphillipinensis*in carbpol base, three are without *Mallotusphillipinensis*in carbpol base, one is of *Mallotusphillipinensis*only and last one is of FLZ without SDS and *Mallotusphillipinensis.* The composition of eight formulations are already given in Table 2.1 mentioned in experimental section.

Dispersion of nano-sized fluconazole (FLZ) - ethosomes(with/without *Mallotusphillipinensis*) in carbopol 934 base topical gel.

Preparation of Ethosomes

The aim of present study was to develop the ethosomes at four different concentration of lecithin (1, 2, 3 and 4%) at 30% ethanol concentration for topical delivery of drug. i.e. FLZ with higher skin penetration. The ethosomes was prepared using cold method. It is easy and quick method suitable with simple laboratory setup. Drug delivery can be greatly affected by compositions of carrier systems as used in the present study i.e. ethosomes.

Evaluation of ethosomes

Vesicle size and zetapotential

Vesicle size really influences the topical drug delivery. The vesicle size and zeta potential of ethosomes was measured and summarized in **Table 3.3**. The vesicle size was considerably in nano-size ranging from 223±12.6 to 443±26.1 nm. It is considered that the smaller size is able to penetrate through to deep layer of skin. Present study showed that vesicle sizes of ethosomes are significant smaller for deep skin drug delivery. Zeta potential is the electric potential and thermodynamic stability determinant of the vesicle. Vesicles had zeta potential ranging from - 19.8±0.13 to - 22.4±0.16 mV. Due to the presence of drug or lecithin vesicles might had the negative charge because drug i.e. FLZ carriers negative charge concentration on the N atom and around the carbon atoms of imidazole ring where as lecithin has both the negative and weak positive charge. The polydispersity index (PDI) is measure of homogeneity indicating the

distribution of ethosomal vesicles. The values of PDI, less than 0.3 indicate the homogeneous distribution of vesicles whereas if it is greater than 0.3 demonstrate its heterogeneous nature. It was found that formulations (E1, E2 and E3) were homogeneous in nature whereas formulation E4 had heterogeneous nature. These results indicate that dominant surfactant action of lecithin probably had affected thePDI.

Formulation code	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)
E1	259±15.8	0.25±0.004	-19.8±0.13	52.9±0.03
E2	295±11.8	0.26±0.003	-18.2±0.11	67.4±0.05
E3	223±12.6	0.30±0.006	-22.4±0.16	73.5±0.08
E4	443±26.1	0.43±0.008	-20.3±0.14	78.7±0.12

Table 3.3 Physical characterization of various ethosomes of fluconazole (FLZ).

Determination of fluconazole Entrapment efficiency(EE)

The entrapment efficiency of ethosomal vesicle was obtained by employing the above citied method and formula (given in experimental section). It was found that with increase in concentration of lecithin the entrapment of FLZ increase as shown in **Table 3.3**. The lowest entrapment of FLZ was 52.9±0.03 in 1% lecithin followed by 67.4± 0.05, 73.5±0.08 and 78.7±0.12 % for 2, 3 and 4 % lecithin,respectively.

Visualization of vesicle by fluorescence microscopy

Visualization of vesicles was done by using fluorescent microscope at 10x, 40x and 100x in order to visualize the shapes of ethosomal vesicles. The ethosomal vesicles were prepared by the same method above described i.e. cold method. Neil Red dye was utilized to analyze the outer lipidmembrane of the ethosomeswhere asRhodamine B and Calcein dye was loaded in ethosomal vesicles instead of drug and visualized under the fluorescent microscope. It was found that the ethosomal vesicles are spherical bilayer structure as shown in **Fig. 3.10** (**a, b, c, d, e and f**). The **Fig. 3.10 (a, b** and **c)** are showing the outer lipid membrane of ethosomes where as the **Fig. 3.10 (d, e** and **f)** are showing the inner core of ethosomes.

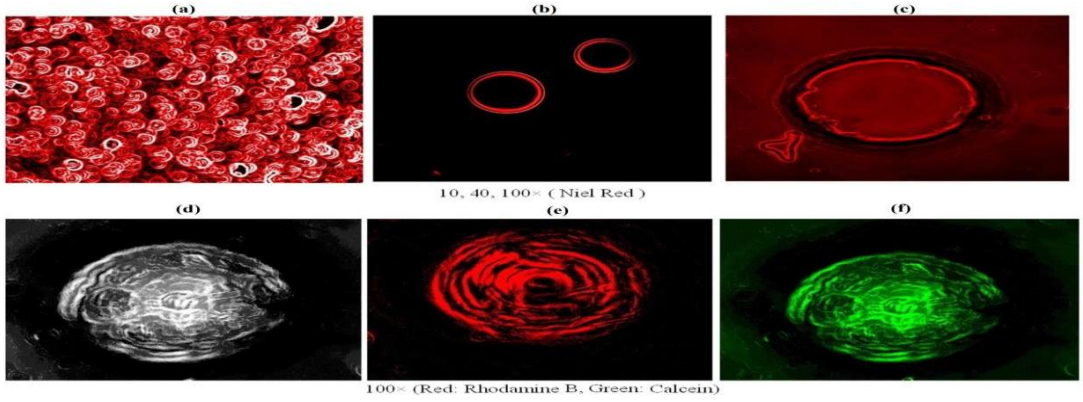
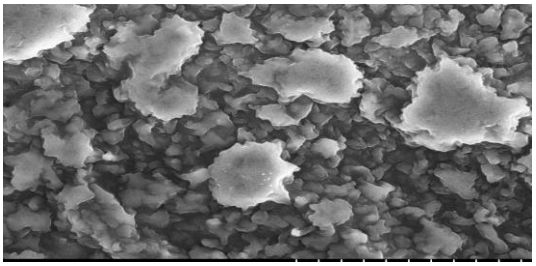
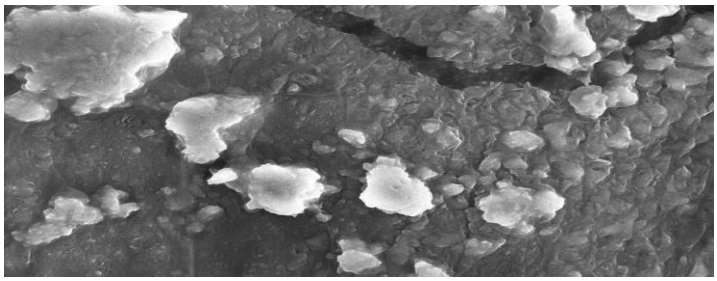


Fig. 3.10 (d, e and **f)**

Scanning Electron



Microscopy (SEM)



In vitro drug release study

In addition, the Korsmeyer-Peppas was also applied to study the drug release mechanism of SDS aided and FLZ loaded ethosomal formulations. The exponent values were found to lie between 0.6247 - 0.6603 and 0.4513 - 0.5695, respectively for both type of formulations. Thus, the *n* values for all formulations are showing the value less than 0.5 indicating that both type of formulations i.e. of SDS aided and FLZ loaded ethosomes are following Fickian diffusion (diffusion controlled release) as shown in **Table 3.6 and3.7**.

Formulation Codes	**Zero order**		**First order**		**Higuchi**		**Peppas**	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
F1	0.9648	8.4450	0.647	0.07290	0.9848	0.0381	0.6382	0.29162
F2	0.9632	7.8987	0.6507	0.07320	0.9307	0.1103	0.6571	0.29552
F3	0.9524	6.9975	0.6428	0.07155	0.9857	0.0457	0.6603	0.30130
F4	0.9604	8.6067	0.6374	0.07234	0.9863	0.0373	0.6247	0.28871

F5	0.9630	8.1332	0.6496	0.07307	0.9865	0.0395	0.6491	0.29409
F6	0.9654	7.6544	0.674	0.07264	0.9860	0.042	0.6627	0.30442
F8	0.9819	4.6337	0.8398	0.07637	0.9363	0.3191	0.8224	0.36001

Table 3.6 Correlation co-efficient (R^2), reaction rate constant (*k)* and diffusion exponent (*n*) of model equations applied to release of FLZ from SDS aided gels.

Formulation Codes	**Zero order**		**First order**		**Higuchi**		**Peppas**	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	*n*
FE1	0.9315	9.767	0.5569	0.06535	0.9962	0.0325	0.4768	0.260921
FE2	0.9335	8.722	0.5542	0.06348	0.9957	0.0365	0.4700	0.266088
FE3	0.9473	7.779	0.6164	0.06717	0.9363	0.1385	0.5695	0.291967
FE4	0.9606	7.745	0.6303	0.06713	0.9363	0.3191	0.5691	0.295267
FE5	0.9283	9.864	0.5405	0.06413	0.9964	0.0322	0.4513	0.254842
FE6	0.9325	8.641	0.5476	0.06261	0.9363	0.3191	0.4557	0.26396
FE7	0.9434	7.860	0.5980	0.06535	0.9363	0.3191	0.5341	0.286105
FE8	0.9593	7.894	0.6199	0.06626	0.9363	0.3191	0.5481	0.291142

Table 3.7 Correlation co-efficient (R^2), reaction rate constant (*k)* and diffusion exponent (*n*) of model equations applied to release ofFLZ from FLZ loaded ethosomal gels.

In-vitro Antifungal Activity

Seven fluconazole resistant and three fluconazole susceptible clinical *C. albicans*isolates were collected from PGIMER, Chandigarh and the formulation code F3 and FE3 was tested against them. The MIC values of FLZ and M.P (*Mallotusphillipinensis*) were found in a range of 16-512 □g/ml and 64-256 □g/ml respectively, as shown in **Table 3.8** and **Table 3.9.** The MIC values of F3 and FE3 topical gel formulation was found in a range of 4-128 □g/ml and 1-128 □g/ml, respectively. Thereafter, the FICI values were determined to investigate the nature of impact provided by the F3 and FE3 gel formulations. In case of formulation F3 out of seven fluconazole resistant clinical *C. albicans*isolates two showed synergistic nature, four showed additive nature and one had not given any affect and out of three fluconazole susceptible clinical *C. albicans*isolates two showed synergistic nature and one showed additive nature as shown in **Table 3.8**. In case of formulation FE3, out of seven fluconazole resistant clinical *C. albicans*six

showed synergistic and one showed additive nature and all three fluconazole susceptible clinical *C. albicans*isolates showed synergistic nature as shown in **Table3.9**.

Table 3.8 *In-vitro* antifungal activity (MIC □g/ml) of F3 gel formulation against *C. albicans*clinical isolates and interactions

S. No.	Clinical Isolates	Type	Species	F3	*M.P.*	FLZ	FICI	Interaction
1	PGI/DML14	FR	*C. albicans*	128	256	>512	0.75	AD
2	PGI/DML34	FR	*C. albicans*	64	256	>512	0.375	SY
3	PGI/DML41	FR	*C. albicans*	16	128	32	0.625	AD
4	PGI/DML54	FR	*C. albicans*	-	>512	>512	-	-
5	PGI/DML94A	FR	*C. albicans*	128	256	>512	0.75	AD
6	PGI/DML106A	FR	*C. albicans*	8.0	128	16	0.562	AD
7	PGI/DML05C	FR	*C. albicans*	8.0	256	64	0.437	SY
8	PGI/DSS103	FS	*C. albicans*	16	256	128	0.187	SY
9	PGI/DSS114	FS	*C. albicans*	4.0	64	256	0.078	SY
10	PGI/DSS123	FS	*C. albicans*	128	256	>512	0.75	AD

Table 3.9 *In-vitro* antifungal activity (MIC □g/ml) of FE3 gel formulation against *C. albicans*clinical isolates and interactions

S. No.	Clinical Isolates	Type	Species	FE3	*M.P.*	FLZ	FICI	Interaction
1	PGI/DML14	FR	*C. albicans*	64	256	>512	0.375	SY
2	PGI/DML34	FR	*C. albicans*	32	256	>512	0.187	SY
3	PGI/DML41	FR	*C. albicans*	16	128	32	0.625	AD
4	PGI/DML54	FR	*C. albicans*	128	>512	>512	0.5	SY
5	PGI/DML94A	FR	*C. albicans*	32	256	>512	0.187	SY
6	PGI/DML106A	FR	*C. albicans*	2.0	128	16	0.141	SY
7	PGI/DML05C	FR	*C. albicans*	4.0	256	64	0.078	SY
8	PGI/DSS103	FS	*C. albicans*	8	256	128	0.078	SY
9	PGI/DSS114	FS	*C. albicans*	1.0	64	256	0.019	SY
10	PGI/DSS123	FS	*C. albicans*	32	256	>512	0.187	SY

Whereas, F3 and FE3: Gel Formulation of SDS aided and FLZ loaded ethosomes, respectively; M.P: *Mallotusphillipinensis*; FLZ: Fluconazole; FICI: Fractional inhibitory concentration index;

MIC: Minimum inhibitory concentration; FR: Fluconazole resistant; FS: Fluconazole susceptible

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