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FORMULATION AND CHARACTERIZATION OF FLUCONAZOLE LOADED OLIVE OIL NANOEMULSIONS

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Abstract:

Present study was carried out to develop and evaluate olive oil based nano-emulsion for transdermal delivery of fluconazole, a bistriazole based antifungal agent with poor water solubility and lipophilicity. Olive oil, a natural non-irritating, non-toxic proposed permeation enhancer, is known to have some antifungal activity as well. Screening of common emulsifiers like Tweens (Tween 20, tween 60, tween 80), Spans (span 60, span 80), brij 35, puronic 127, and poloxamer 188 were done based on solubility of fluconazole in these surfactants followed by their efficiency to emulsify olive oil in water. Co-emulsifiers such as glycols (polyethylene glycol 200, polyethylene glycol 400, propylene glycol), and short chain alcohols (ethanol, propanol, butanol and octanol) were also screened similarly. Tween 80 and butanol were selected as emulsifier and co-emulsifier respectively to formulate nano-emulsion by aqueous titration method. However, separation was observed after 24 hours. Therefore, span 80 was added as an auxiliary emulsifier to improve emulsification efficiency. Finally, a blend of tween 80, span 80 and butanol was optimized as emulsifier (56 % wt/wt) to emulsify 9 % wt/wt of olive oil in 33 % wt/wt water. Pseudo-ternary phase diagram was employed to identify and optimize the components. Optimized formulation based on phase separation and thermokinetic stability was characterized for globule size, size distribution, zeta potential, viscosity, refractive index and pH. Globule size analysis by zetasizer nano ZS was further confirmed by transmission electron microscopy. Permeation flux of fluconazole from optimized formulation through artificial skin was approximately three fold higher than the control. In conclusion, developed olive oil based nano-emulsion of fluconazole demonstrated promising solubility, permeability and stability.

Keywords: *Fluconazole, olive oil, nano-emulsion, transdermal permeation*

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INTRODUCTION:

Conventional drug therapy is the most popular and widely used but is not without its deficiencies and limitations. For most therapies, the plasma and blood levels of the active constituents are not consistent and hence frequent dosing is required. This reduces patient compliance. Moreover, most of the drugs are bio-transformed or reduced to inactive forms by hepatic and other enzymes in the body. This is particularly true for oral formulations. Parenteral therapies overcome this effect, but they are expensive, have a short shelf life and are painful to administer. Topical delivery as an option to oral delivery of drugs has several advantages especially that having extensive first pass metabolism [1]. It is better than painful hypodermic injections that produce hazardous biological waste and causes disease transmission because of needle contaminations [2]. However, a very few number of drugs with low dose, low molecular weight and high octanol-water partition coefficients can be successfully delivered, because of the anatomical structure of the barrier layer of skin [3]. To achieve successful transdermal drug delivery, enhancement of skin permeability is of prime concern [4]. Recently several physical, electrical, chemical and biochemical techniques have been proposed to increase the permeability of the skin. Among these, modification of permeability by chemical method is most widely used owing to economical, simple and rapid [5]. Chemical permeation enhancers either improve the solubility or partition coefficient or increase the diffusion of drugs across the skin. Recently, there have been extensively studies on transdermal delivery [6-9]. Despite of their extensive use in literature, chemical skin penetration enhancers are not clinically useful due to the irritation caused by majority of these chemicals [10].

Oleic acid, an unsaturated fatty acid in olive oil has demonstrated skin penetration potential in various studies [11, 12]. However, there is very limited permeation enhancement study of olive oil on human skin [13]. Olive oil as such may be used as permeation enhancer especially for lipophilic drugs. In one study on the use of olive oil in blood vessel suturing, olive oil was found to be non-irritating when compared to Vaseline Paraffin oil [14]. Recently lipid based nano-sized drug delivery systems such as nano-emulsion and micro-emulsions have appeared as attractive carrier systems for transdermal drug delivery owing to its capability of enhancing transdermal permeation of drugs [15-20]. Nano-emulsions and micro-emulsions are nano-sized stable emulsions prepared by Nano emulsification of dispersed phase using a mixture of emulsifier and co-emulsifier. Micro-emulsions are thermodynamically stable and are prepared spontaneously without application of high energy or shear [21]. Nano-emulsions and micro-emulsions are currently one of the most extensively studied lipid based carriers to deliver problematic drug candidates such as having poor solubility and permeability. Components of nano-emulsions are known to improve both solubility as well as permeability of enclosed drug candidates owing to their amphiphilic nature and solvent actions [22, 23].

In this paper, development and evaluation of olive oil based nano-emulsion of fluconazole; a bistriazole based antifungal agent has been presented. It is less lipophilic and more hydrophilic and therefore it is supposed to have less skin penetration potential as compared to other antifungal agents [24]. Olive oil is used as natural, non-irritating, non-toxic permeation enhancer, substituting the toxic and irritant synthetic chemical permeation enhancers. Moreover, olive oil is known to have antifungal activity that would be an added advantage [25-28].

MATERIALS:

Fluconazole, Tween 20, Tween 60, Tween 80, Span 60, Span 80, brij 35, poloxamer 188, Pluronic 127, Polyethylene glycols, propylene glycol, isopropyl alcohol, n-butanol and olive oil were purchased from Sigma-Aldrich (St Louis, MA, USA).

EXPERIMENTAL:

Solubility of drug in olive oil, emulsifier and co-emulsifier

Excess amount of fluconazole was incubated with 5 ml of olive oil, various emulsifier and co- emulsifier at 37 °C and 100 RPM in biological shaker. Suspensions were filtered after 72 hours and amount of fluconazole in solutions were analyzed by UV spectrophotometer at 260 nm after appropriate dilution. Fluconazole Standard calibration solutions were prepared in range of 10-200 µg/ml.

Screening of emulsifier and co- emulsifier

Based on the results from solubility studies, Tween 80 was chosen as primary emulsifier whereas propylene glycol was chosen as co-emulsifier. However, these combinations resulted in gel formation; therefore, all studied co-emulsifiers were mixed separately with tween 80 to prepare different emulsifier mixture (Emix). Equal volumes of olive oil and water were vortex mixed and titrated with each Emix until a clear nano-emulsion was obtained. Total amount % v/v of Emix was recorded for selection of the components. Butanol was selected as co-emulsifier as minimum amount of Emix was required to form nano-emulsion. However, separation

was observed after 24 hours. Therefore, emulsification efficiency was again tested with different emulsifiers keeping butanol as co-emulsifier. Finally, a blend of tween 80 and span 80 was chosen as emulsifier as it exhibited no sign of separation when observed after 24 hours.

Construction of Pseudo ternary phase diagram

Emix containing equal parts of tween 80 and span 80 as emulsifier blend and butanol as co-emulsifier was vortex mixed with olive oil in different ratio representing increasing amounts of Emix with respect to fixed amount of olive oil (1:1 to 1:9) and decreasing amounts of Emix with respect to increasing amount of olive oil (1:9 to 9:1). Aqueous titrations of these mixtures were carried out with vortex mixing for 30 second after each addition and observations are recorded to construct pseudo ternary phase diagram.

Thermodynamic stability tests

To assess the thermodynamic stability, prepared formulations were subjected to centrifugation and freeze thaw cycles. Centrifugation was done for 30 min at 3500 rpm. Intact formulations were frozen at –20ºC for 24 hours followed by thawing at 25ºC. Thermodynamically stable formulations were further evaluated for different tests.

Size estimation, size distribution and zeta potential

The size of nano-emulsion droplets, its polydispersity and zeta potential were evaluated by zetasizer nano ZS (Malvern instruments, UK) which works on laser diffraction principle that analyzes scattering of light due to droplets in the nano-emulsion. Each formulation was diluted 100 times with double distilled water and analyzed in triplicate.

Estimation of Viscosity

Estimation of viscosity of nano-emulsion was done in triplicate by LVDV-I prime digital viscometer (Brookfield USA) using cup and cone spindle at 50 rpm and 25 ºC.

Refractive Index

Rudolph Refractrometer J 257 (Rudolph, Japan) evaluated refractive index of the optimized formulation in triplicate.

Transmission electron microscopy

The surface morphology of droplets of the optimized nano-emulsion was observed using JEOL electron microscopy (JEM-1011, Peabody, MA, USA) operating at 80 KV. Nano-emulsion was suitably diluted with deionized water (1:100). A drop of diluted micro-emulsion was then directly deposited on the holey film grid and observed under JEOL TEM after drying for 30 sec.

***In Vitro* Skin Permeation Studies**

Permeation studies were performed on fully automated Franz-diffusion cells (912-6 Logan, USA) with a receptor cell capacity of 12 ml. Millipore cellulose nitrate membrane with a pore size 0.45 µm, thickness of 110 µm and effective surface area of 1.77 cm^2 for permeation were utilized in the study. These membranes were soaked in phosphate-buffer (pH 5.8) 24 hours before mounting. Five hundred micro liter of sample containing 2% fluconazole was evenly applied on the membrane. Receptor fluid was continuously stirred at 100 rpm by magnetic bar and experiments were conducted at 32°C using a water circulation system. Five milliliter of sample was withdrawn at 0.5, 1, 1.5, 2, 2.5, 3 and 4 hours and replaced with fresh buffer to maintain sink conditions. Samples were suitably diluted and analyzed by UV spectrophotometer at 260 nm. Total drug crossing the membrane (mg/cm^2) were plotted against time. Steady state flux and diffusion coefficient were also calculated as below.

Permeability coefficient = Slope × Volume in donor solution/Skin Area ---------- (1)

Flux = Permeability coefficient × donor solution drug concentration ---------- (2)

Statistical Analysis

Permeation data has been presented as mean ± standard deviation. Comparison among mean values was carried out using unpaired t-test. The differences were considered as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION:

Solubility study of fluconazole in emulsifiers and co-emulsifiers

Solubility of FCL in various emulsifiers and co-emulsifiers was determined so as to choose suitable emulsifier and co-emulsifier based on maximum solubility. Therefore, tween 80 was selected as suitable emulsifier as it demonstrated maximum solubility. Solubility data has been presented in Fig 1. Similarly, solubility of FCL in various polyethylene glycol and short chain alcohols was determined so as to choose suitable co-emulsifier based on maximum solubility. Therefore, butanol was selected as suitable co-emulsifier as it demonstrated maximum solubility.

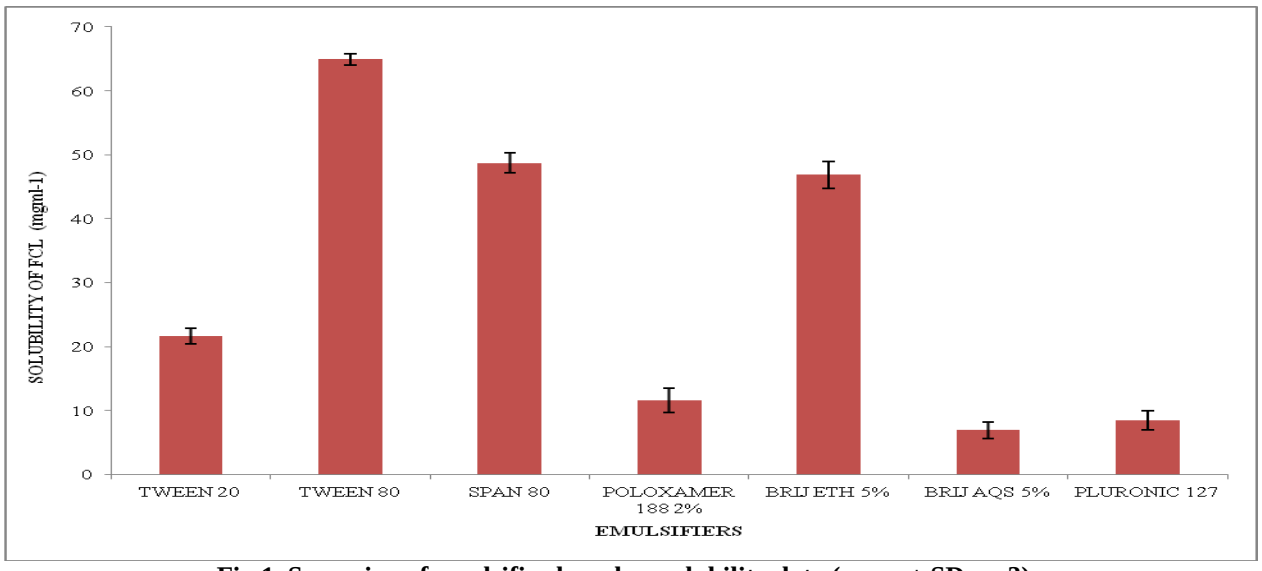

Fig 1. Screening of emulsifier based on solubility data (mean ± SD, n=3).

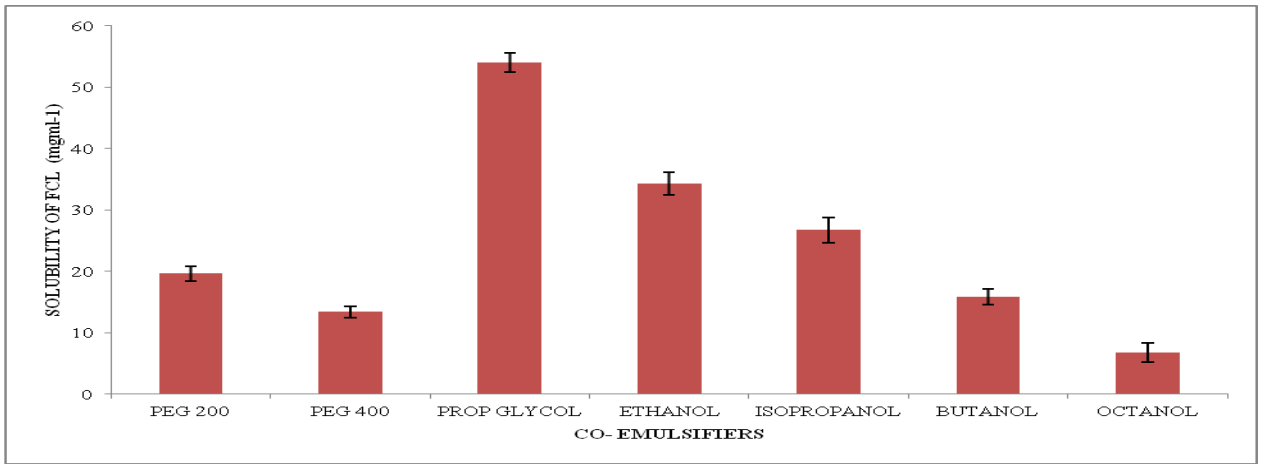

Fig 2. Screening of co-emulsifier based on solubility data (mean ± SD, n=3).

Emulsion efficiency of Emix

Based on solubility data, tween 80 and propylene glycol were chosen as emulsifier and co-emulsifier respectively. An equal part of tween 80 and propylene glycol was mixed with varying amounts of olive oil to prepare different ratio of oil: Emix (from 1:9 to 9:1). These mixtures were then titrated with water and observations are recorded. Aqueous titration of oil: tween 80: propylene glycol, resulted in formation of either emulsion or gel. Therefore, emulsification efficiency of different co-emulsifiers was tested at 1:1 ratio of Emix (co-emulsifier: emulsifier, taking tween 80 as emulsifier in each case). Emulsification efficiency of these Emix was evaluated based on formation of nano-emulsions with minimum amount of Emix consumed in titration of equal parts of water and olive oil. Butanol was chosen as co-emulsifier based on formation of clear nanoemulsion, however, there was some sign of separation after 24 hours. Therefore, different emulsifiers were further tested for emulsification efficiency keeping butanol as co-emulsifier in each case. Emulsion efficiency data has been presented in Table 1. It was observed that none of the emulsifiers alone was capable of forming stable nano-emulsion while emulsifying equal volume of olive oil and water tested as several strengths up to 80% v/v of total mixture. Therefore, span 80 was tested as an auxiliary emulsifier with tween 80. Finally, Emix containing tween 80, span 80 and butanol at 1:1:1 ratio was found to successfully emulsify olive oil and water without application of any force or energy upon simple vortexing for 30 seconds.

Table 1: Effect of Emix on 50: 50 ratios of olive oil & water

Emix composition	Ratio	% v/v of components of nano-emulsions			Observation
		Emix	olive oil	water	
Tween 80:PEG 200	1:1	80	10	10	Gel
Tween 80:PEG 400	1:1	80	10	10	Gel
Tween 80:PG	1:1	80	10	10	Gel
Tween 80:Ethanol	1:1	80	10	10	Emulsion
Tween 80:Isoprapanol	1:1	80	10	10	Emulsion
Tween 80:Butanol	1:1	80	10	10	Separate
Tween 80:Butanol	2:1	80	10	10	Separate
Tween 80:Butanol	3:1	80	10	10	Separate
Tween 80:Butanol	4:1	80	10	10	Separate
Tween 80:Butanol	1:2	80	10	10	Separate
Tween 20:Butanol	1:1	80	10	10	Separate
Tween 60:Butanol	1:1	80	10	10	Separate
Span 80:Butanol	1:1	80	10	10	Separate
Tween 80:Span 80:Butanol	1:1:1	76.2	11.9	11.9	Nano-emulsion

Construction of Pseudo ternary phase diagram

Emix containing tween 80 and span 80 as emulsifier blend butanol as co-emulsifier in equal ratios (1:1:1) was vortex mixed with olive oil in different ratio representing increasing amounts of Emix with respect to fixed amount of olive oil, decreasing amounts of Emix with respect to increasing amount of olive oil, and increasing amounts of Emix with respect to decreasing amount of olive oil as shown in Table 2. Aqueous titrations of these mixtures were carried out with vortex mixing for 30 seconds after each addition and observations are recorded to construct pseudo ternary phase diagram as shown in Fig.3.

Table 2: Observation table of aqueous titration of different ratio olive oil with Emix

Formula-tion code	Ratio of olive oil : Emix		Observation after every addition of 25 µL water					
	Olive oil	Emix	25	25	25	25	25	25
NE1	1	9	EG	EG	E	E	E	E
NE2	2	8	NE	EG	E	E	E	E
NE3	3	7	NEG	EG	E	E	E	E
NE4	4	6	EG	EG	E	E	E	E
NE5	5	5	EG	EG	E	E	E	E
NE6	6	4	EG	EG	E	E	E	E
NE7	7	3	EG	EG	E	E	E	E
NE8	8	2	EG	EG	E	E	E	E
NE9	9	1	EG	EG	E	E	E	E
NE10	1	2	EG	EG	E	E	E	E
NE11	1	3	NEG	EG	E	E	E	E
NE12	1	3.5	NE	EG	E	E	E	E
NE13	1	5	NE	NE	NEG	NEG	NEH	NEH
NE14	1	6	NE	NE	NE	NE	EG	E
NE15	1	7	NE	NE	NE	NE	EG	E
NE16	1	8	NE	NE	NE	NE	EG	E

NE: Clear Nano-emulsion; NEG: Nano-emulsion gel; NEH: Nano-emulsion hazy; EG: Emulsion gel and E: Emulsion.

Based on observation of NE14, NE15 and NE16 from aqueous titration study and Pseudo ternary diagram, few nano-emulsions were prepared as shown in the Table 3. Fluconazole 2% was dissolved in calculated amount of Emix followed by addition and mixing of calculated amount of olive oil. Calculated amount of water is then added drop by drop in above mixture with vortex mixing.

Thermodynamic stability of optimized formulations was conducted by subjecting the formulations to centrifugation and freeze thaw cycles. No separation was observed in any formulations; however, formulation F2 remained the most clear of all tested formulations; therefore, it was chosen for further evaluations such as estimation of size, size distribution (polydispersity index), zeta potential, morphology, viscosity and refractive index etc. The observations of these tested parameters are presented in Table 4. Transmission electron microscope photographs are given in Fig. 4. *In vitro* permeation of olive oil based nano-emulsion of fluconazole was compared with suspension. The cumulative amount of fluconazole permeated per unit area over four hours were plotted on Y axis against the time on X axis as on two separate scales shown in Figure 5. Permeability coefficient and flux of fluconazole from formulation was approximately three fold higher than those observed from fluconazole suspension. Steady state Flux *Js* of fluconazole from simple suspension and olive oil based nano-emulsion formulation F2 was calculated as 0.378 ± 0.072 and 1.16 ± 0.091 $mg.cm^{-2}\ h^{-1}$ respectively.

Table 3: Formulation components of selected nano-emulsions

Formulation code	wt % of drug, oil, emulsifier, co-emulsifier and water					
	FCL	Olive oil	Tween 80	Span 80	Butanol	Water
F1	2	7	20	18	15	38
F2	2	9	21	19	16	33
F3	2	11	22	20	17	28

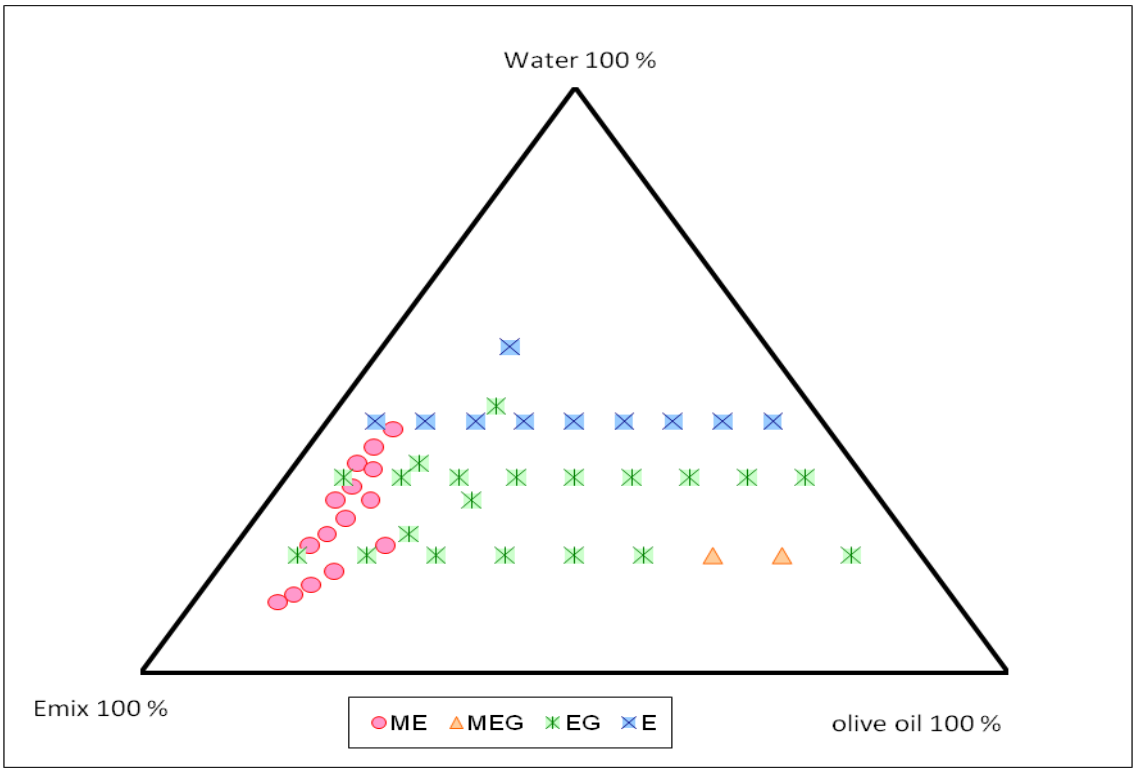

Fig 3. Pseudo ternary phase diagram of Emix (Tween 80: Span 80: Butanol; 1:1:1), Olive oil and water.

Characterization of optimized formulation

Table 4: Characterization of optimized formulation

Parameters tested	Observations
Globule size (nm)	173 ± 15
Poly dispersity index	0.317 ± 0.0583
Zeta potential (mV)	- 5.06 ± 0.3158
Viscosity (cp)	68 ± 4.583
Refractive Index	1.448 ± 0.0008
pH	6.6 ± 0.0208
Permeability coefficient ($cm.h^{-1}$)	0.115 ± 0.0048
Steady state Flux ($mg.cm^{-2}\ h^{-1}$)	1.16 ± 0.091

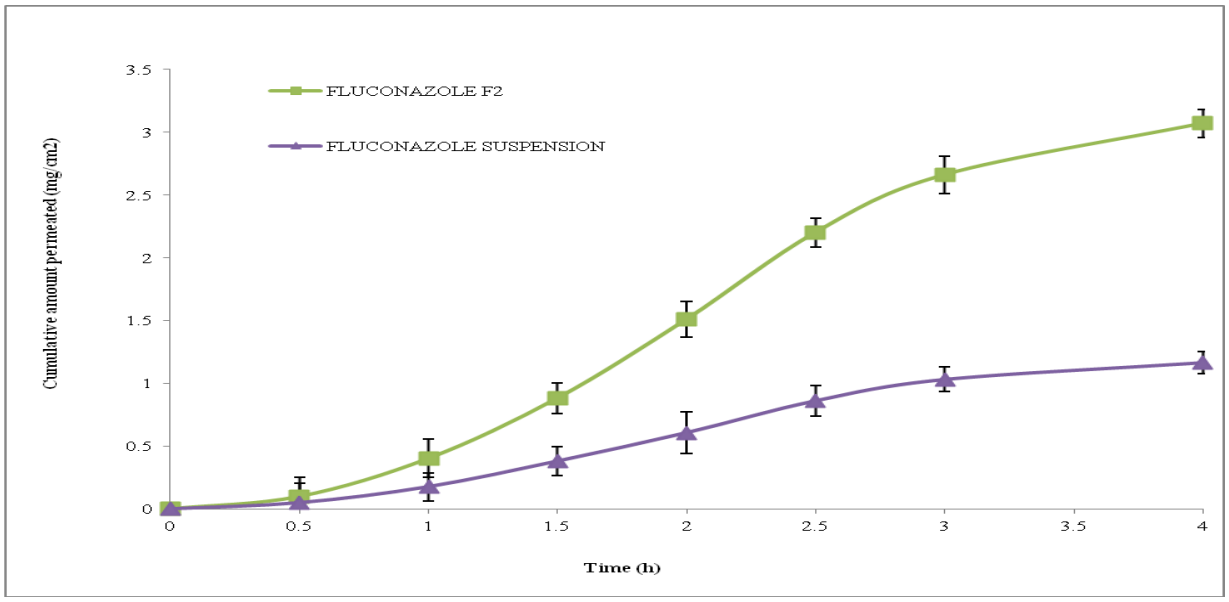

Fig4: Fluconazole permeability plot (mean cumulative amount permeated per hour through artificial membrane ± SD, n=3)

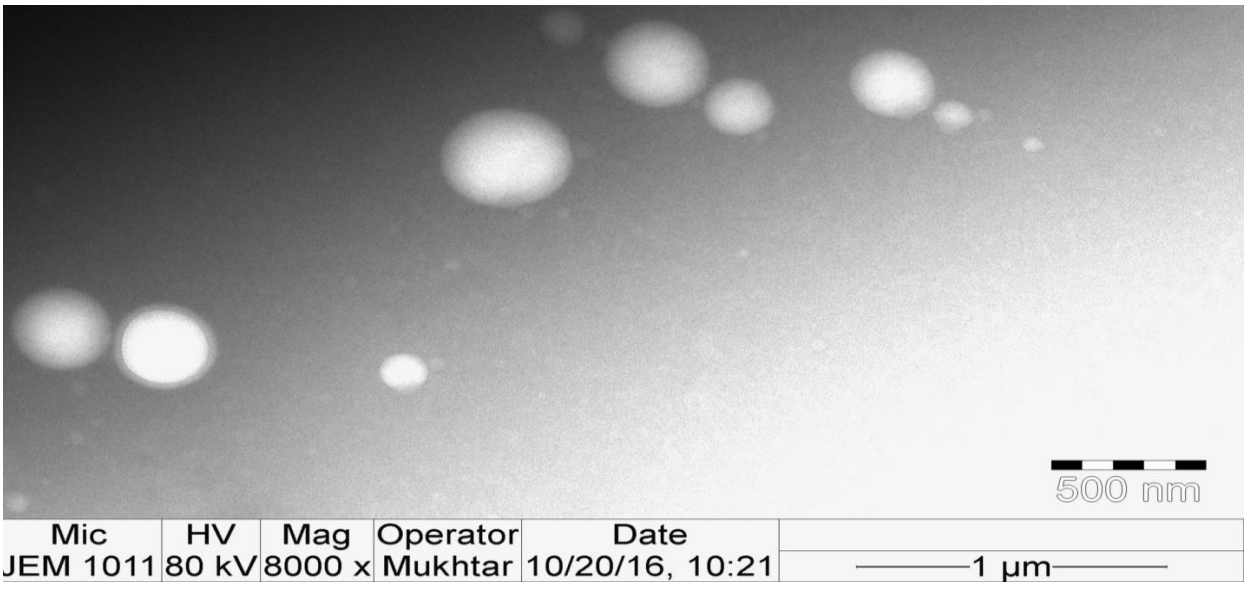

Fig 5: Transmission electron microscopy photograph of olive oil based fluconazole nano- emulsion

Emulsifiers and co-emulsifiers which are generally regarded as safe such as Tweens, Spans, poloxamers, polyethelene glycols and lower chain alcohols were evaluated for emulsification of olive oil in water. As per the available information in the literature, selection and optimization of the emulsifier and co-emulsifier was generally based on solubility data [29-32]. However, in this study, we have employed the emulsification efficiency of emulsifiers and co-emulsifiers for screening purpose as solubility data did not results in formation of nano-emulsion [33, 34]. We have noticed that though tween 80 and propylene glycol had maximum effect on solubility of the fluconazole, their combinations did not result in nano-emulsion. Which could be due to inappropriate hydrophilic lipophilic balance between nano-emulsion components such as olive oil, water, emulsifier and co-emulsifier. Addition of span 80 as an auxiliary emulsifier modified this hydrophilic lipophilic balance and resulted in nano-emulsion by simple mixing with vortex mixer without the need of high shear or high energy by any means.

CONCLUSION:

In this study, olive oil based nano-emulsion were prepared by utilizing energy less aqueous titration method. Optimized formulation based on phase separation and thermokinetic stability were characterized for globule size, size distribution, zeta potential, viscosity, refractive index and pH. Globule size analysis by zetasizer nano ZS was further confirmed by transmission electron microscopy. Permeation flux of fluconazole from optimized formulation through artificial skin was approximately three fold higher than the control. In conclusion, developed olive oil based nano-emulsion of fluconazole demonstrated promising solubility, permeability and stability.

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