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Development, Optimization & Characterization of topical Nanoemulsion Gel of an Antifungal Agent for Effective Treatment of Onychomycosis

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Abstract
Onychomycosis is a fungal infection of the keratinized tissue of the nail plate that is notoriously difficult to diagnose and treat Onychomycosis is a disease involving very difficult treatment mainly because it presents a poor response to available drugs thus treatment is frequently discontinued by patients This disease is a therapeutic challenge determined by nail-substratum anatomical characteristics and due to disease chronic it. The scientist has been using different new delivery system to treat the disease. The new drug delivery system contains nanoemulsion gel iontophoretic system. The other systems are nanoparticles, nanoemulsion, carrier system (liposome, niosome, erythrosome and dendrimers).The nanoemulsion gel is a novel approach to treat the onychomycosis. The onychomycosis is a local candidal infection. The treatment of onychomycosis is quit challenging as it is very difficult to cross nail plate. The selection of antifungal agent is also play a very important role. Clotrimazole shows very potent action on the candida. Clotrimazole is a newer antifungal agent which kills candida very effectively. The molecular weight of clotrimazole is also very low. It is suitable for the topical application. The solubility of the clotrimazole is BCS class 2nd drug. The solubility of the clotrimazolean is increased by nanoemulsion the drug entrapped inside the oil phase.
Keywords: B Nanoemulsion, Clotrimazole

Introduction
The fundamentals of successful formulation are to deliver the active substance at target organ with minimal discomfort and side effects. Topical therapy of Onychomycosis is limited in the[1] infections deep seated, by the nails unique properties, its thickness, and by the ineffective penetration of the deep nail plate by topically applied drugs. In the vascular insufficiency, impaired wound healing, and compromised immunologic status associated with diabetic foot increase the risk of secondary infections in diabetic patients with onychomycosis[2]. Mild onychomycosis sometimes responds to a combination of topical antifungal medication, sometimes applied as special medicinal nail lacquer, and periodic filing of the nail surface.Clotrimazole is an imidazole derivative with a broad spectrum of antimycotic activity.in inhibites the biosynthesis of the sterol ergosterol, an important component of fungal cell membrane.its action leads to increased membrane permeability and apparent disruption of enzyme system bound to membrane[3]. Clotrimazole has low molecular weight that may benefit the drug penetration into the nail plate and it is lipophilic[4].It is lipophilic so by using a suitable carrier drug penetration can be enhanced through the nail barrier. Clotrimazole shows potent activity against *Candida* and non-dermatophytic moulds on topical application. Nanoemulsion wasSolubilisation of poorly water soluble drugs and Potential for sustained release dosage forms. They act as a penetration enhancers which facilitates the drug permeation into deep tissue Onychomycosis because the poor success rate of conventional topical therapy due to low permeability across the nail plate[5]. Thermodynamically as well as kinetically stable isotropic and transparent, liquid dispersions consists of oil, water, surfactant and co surfactant.

Materials and Methods
Materials:
Clotrimazole drug and polymers werereceived as a gift sample from Sun Pharma, Baddi. All the other chemicals used were ofAnalytical grade.

Methods:

Determination of Solubility of Clotrimazole in Oils, Surfactants and Cosurfactants

The most important criterion for screening of components is the solubility of poorly soluble drug in oils, surfactants and co surfactants. The solubility of clotrimazole was determined in different oils viz. oleic acid, isopropyl myristate (IPM), olive oil, triacetin, clove oil, castor oil, safsol, labara, sesame oil and soya bean oil. 2 ml of different oils was taken in small vials and excess amount of the drug was added. The vials were tightly stoppered and were continuously stirred for 72 hours at 37 ± 0.5ºC, and samples were centrifuged at 10,000 rpm for 10 min. The supernatant was separated, filtered and after appropriate dilution with methanol, solubility was determined. Same method was adopted for solubility determination of drug in surfactant and co surfactant.

Optimization of the Formulation

Selection of optimized formulation by using design expert (Box Benkhem method) on the basis of particle size and zeta potential. The box benkhem method is use to optimize the formulation which depends upon the Oil, Smix and Water. The range of Nanoemulsion area was taken by the aqueous titration method. On the basis of aqueous titration method the range of oil, Smix and Water was put in the given range. The design expert software predicts 17 optimized runs. The dependent variables are particle size and zeta potential.

Characterization of Nanoemulsion

Surface morphology

Morphology of the nanoemulsion was studies using TEM (Morgagni 268D SEI, USA) operating at 200 KV and of a 0.18nm capable of point to point resolution. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the nanoemulsion. In order to perform the TEM observations, the diluted nanoemulsion was deposited on the holey film grid and observed after drying.

Droplet size and size distribution

Droplet size was determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion of the particles (using a Zetasizer (1000 HS, Malvern Instruments, U.K). The formulation (0.1 mL) was dispersed in 50 mL of water in a volumetric flask, mixed thoroughly with vigorous shaking and light scattering was monitored at 25°C at a 90° angle.

***Ex-Vivo* Skin Permeation Study**

Ex-vivo permeation studies through rats skin was performed using an automated diffusion cell sampling system (SFDC, LOGAN Inst, Nj USA) (Fig). Epidermal membrane samples were mounted into the diffusion cells (area 0.653 cm^2) equilibrated at 37±0.5ºC for 8-10hrs. Aliquot (500 µl) was withdrawn from the receptor compartment of vertical cell at different time intervals and was analyzed for drug content by UV spectrophotometer. Receiver volume was immediately replenished with the same amount of medium mixture of phosphate buffer (pH 7.4).

Permeation studies:

The receptor cell was filled with the media and 6 mg of clotrimazole in nanoemulsion was applied on the skin surface in the donor compartment. To study the permeation from neat components (neat surfactant, oil), same amount of drug was dissolved in surfactant mixture, oil. The receptor media was maintained at 35 ± 0.5º C & magnetically stirred at 600 rpm. After application of the test formulation on the donor side, 0.5 ml of aliquot was collected from the receiver cell at designated time intervals (viz. 0, 1, 2, 3,4, 5 , 6, 8, 10, 12 and 24 hr) for 24 hr period and replaced immediately with the same volume of fresh media maintained at 35 ± 0.5º C. After appropriate dilutions, the amount of drug in the receptor media was analyzed using uv.

The Histology study of skin by fluorescence microscope[6]

Abdominal skin of Wistar rats were treated with optimized Clotrimazole Nanoemulsion gel in phosphate buffer at PH 7.4. After 24 hrs. Rats were sacrificed and the skin samples treated with fluorescent dye rhodamine and fluorescine dye. The drug containing formulations were applied on the different skin parts. The dye permeates through the skin layer. Different time intervals were taken and then compared with the marketed formulation.

Results and Discussions

Solubility of Clotrimazole in Oils, Surfactants and Cosurfactants

On the basis of above titration large Nanoemulsion area was found with surfactant Tween 80 and with co-surfactant Ethyl alcohol and PEG 400 but Ethyl alcohol acts as penetration enhancer better than PEG 400 and the target is to retain the drug in the skin layer so PEG 400 have been selected. The solubility of clotrimazole in oils, surfactants and co-surfactants shows in figure 1 &2. The finally selected formulation components are given bellow -

Oil Phase: Oleic acid
Surfactant: Tween 80
Co-surfactant: PEG 400

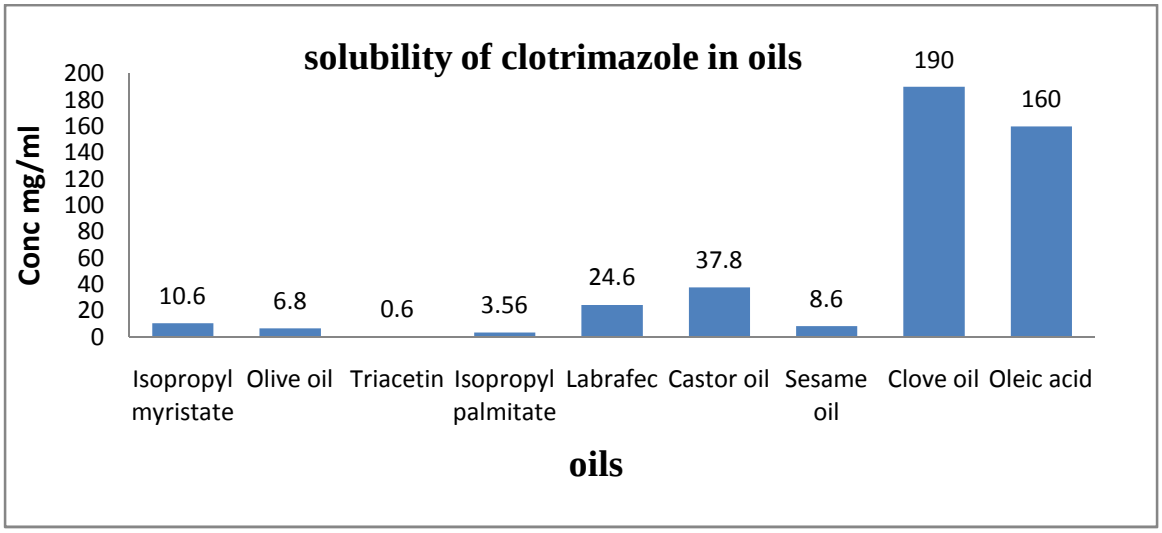

Figure 1.Solubility of clotrimazole in oils.

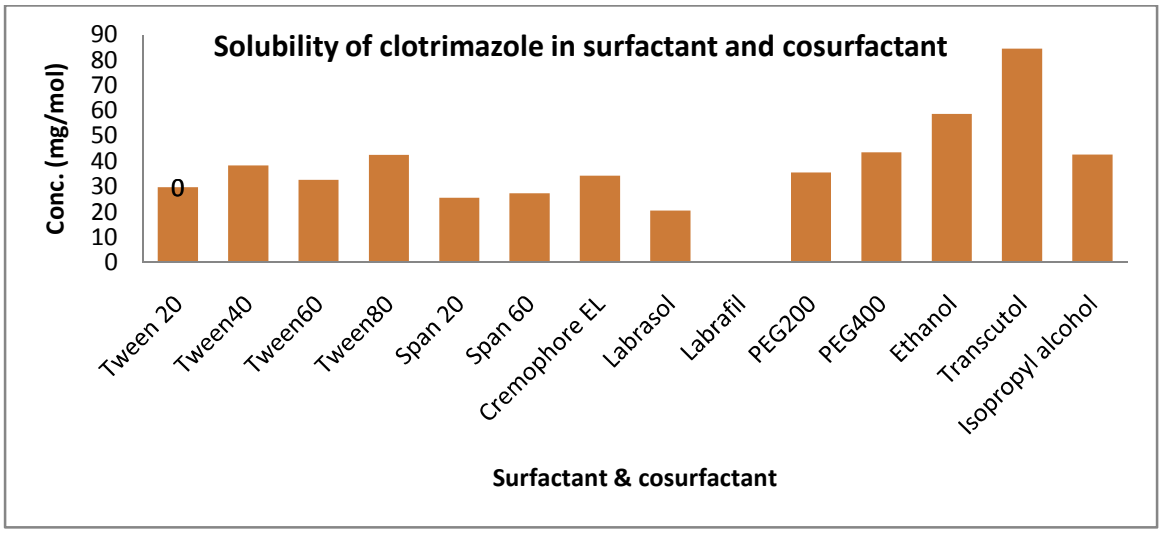

Figure 2.Solubility of clotrimazole in surfactants and co-surfactants.

Optimization of the Formulation

Table 1: The Optimization of Formulation by Box Benkhem Method.

Sample Code	Std	Run	Factor 1 Oil	Factor 2 Smix	Factor 3 Water	Responce1 Particle size(nm)	Responce 2 Zeta potential (mvolt)
Sf 1	1.	7	7	45 (5:1)	32.5	334	-36.8
Sf 2	2.	13	12	45 (4.5:1)	32.5	131.2	-45.2
Sf 3	3.	17	7	60 (4:1)	32.5	11.5	-56
Sf 4	4.	6	12	60 (5:1)	32.5	148.7	-38.9
Sf 5	5.	3	7	52.5 (4:1)	25	139.5	-46.9
Sf 6	6.	15	12	52.5 (4.5:1)	25	172.5	-54.6
Sf 7	7.	12	7	52.5 (5:1)	40	234	-52.3
Sf 8	8.	10	12	52.5 (4.5:1)	40	160.6	- 36.5
Sf 9	9.	9	9.5	45(5:1)	25	295.5	-35.6
Sf 10	10.	4	9.5	60(4.5)	25	81.7	-49.8
Sf 11	11.	5	9.5	45(4:1)	40	277.7	-39
Sf 12	12.	2	9.5	52.5 (4:1)	40	187.5	-52.3
Sf 13	13.	11	9.5	52.5 (4.5:1)	32.5	67	-39.6

Sf 14	14.	1	9.5	52.5 (5:1)	32.5	93.9	-40.4
Sf 15	15.	8	9.5	52.5 (5:1)	32.5	89.7	-41.3
Sf 16	16.	14	9.5	52.5 (4:1)	32.5	77.8	-46.8
Sf 17	17.	16	9.5	52.5 (4:1)	32.5	82.5	-45.6

Characterization of Nanoemulsion

Surface morphology

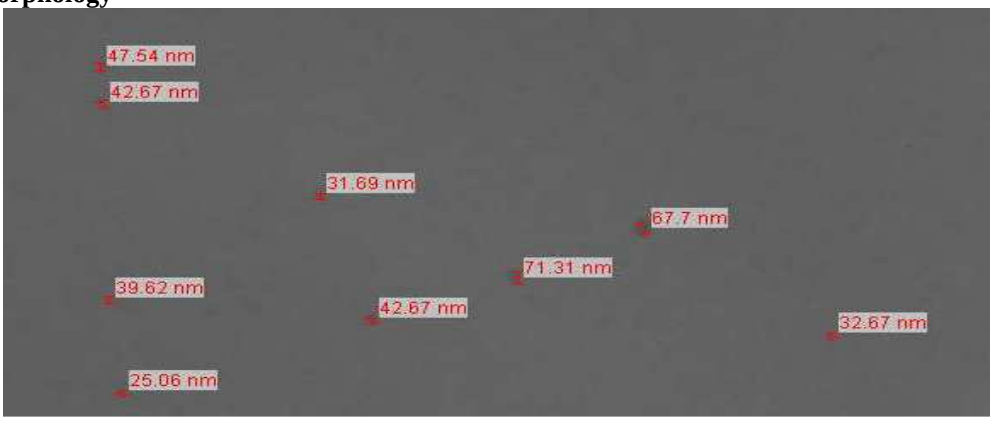

Figure 3.TEM image of drug loaded nanoemulsion f4

Droplet size and size distribution

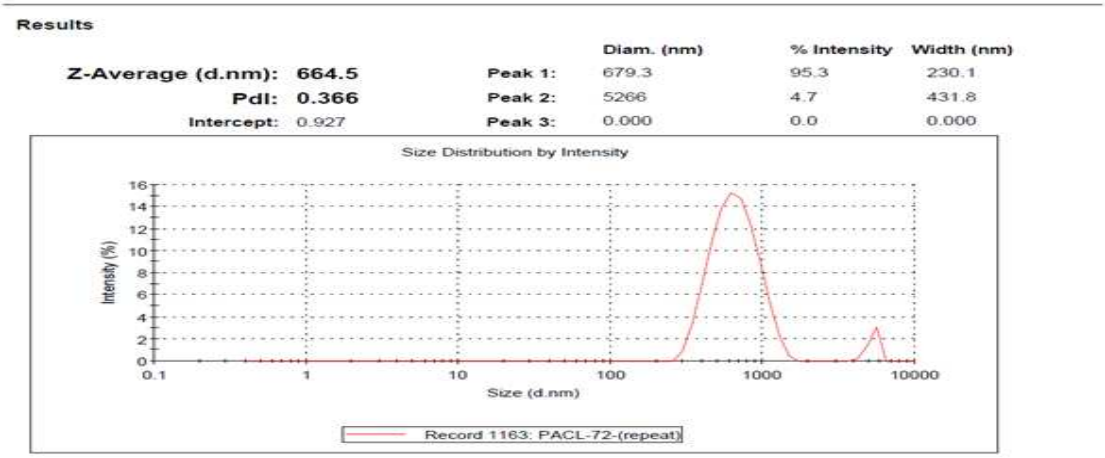

Figure 4.Result of droplet size (nm) vs. intensity (%) of nanoemulsion Sf3

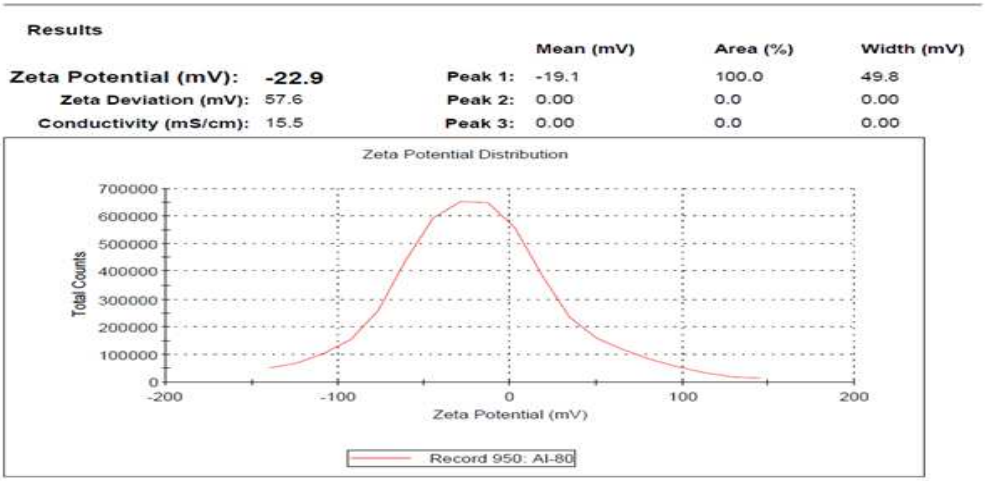

Figure 5.Result of droplet size (nm) vs. intensity (%) of nanoemulsion Sf4

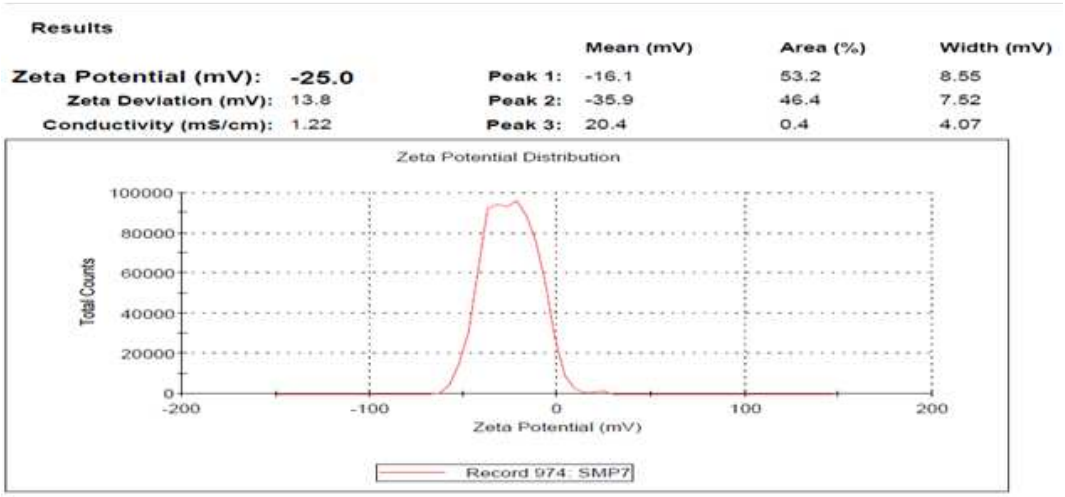

Figure 6.Result of droplet size (nm) vs. intensity (%) of nanoemulsion Sf5

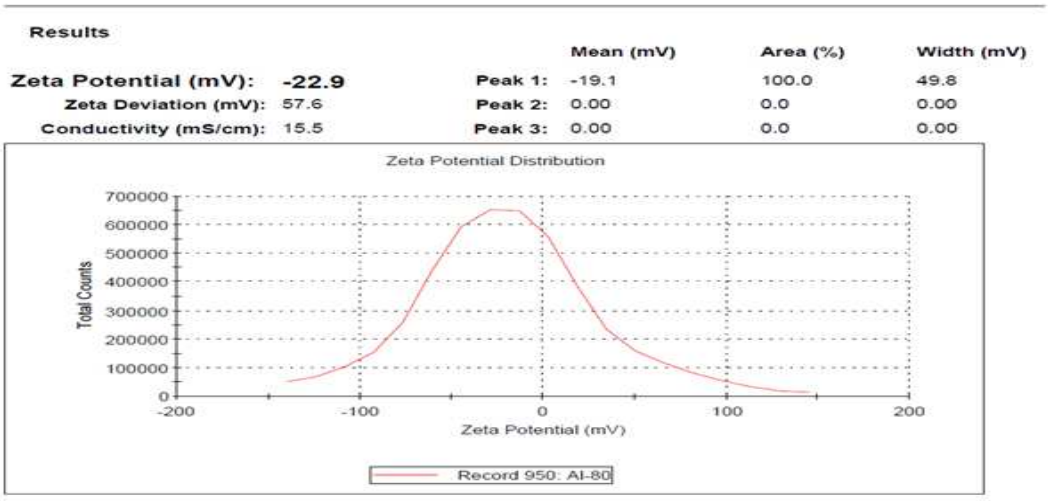

Figure 7.Result of droplet size (nm) vs. intensity (%) of nanoemulsion Sf6

***Ex-Vivo* Skin Permeation Study:**

Table 2: Ex vivo skin permeation of Clotrimazole from nanoemulsion (Sf 1) using Logan Transdermal diffusion cell

S. No.	Time (h)	Abs.	Conc. (µg/ml)	Cumulative amount of drug permeated (µg/cm^2)	Cumulative percentage of drug permeated	Cumulative amount of drug permeated per cm^2	Flux (µg/cm^2/h)	Pb x 10^{-3}
1.	1.	0.026	1.50	150.289	6.0115	200.3854	74.390	2.479
2.	2.	0.064	3.69	369.9422	14.797	493.2563		
3.	3.	0.076	4.393	439.3064	17.572	585.7418		
4.	4.	0.094	5.43	543.35	21.73	724.4701		
5.	6.	0.113	6.531	653.1792	26.127	870.9056		
6.	8.	0.126	7.283	728.3237	29.132	971.0983		
7.	10.	0.145	8.381	838.1503	33.526	1117.534		
8.	12	0.148	8.554	855.4913	34.219	1140.655		
9.	24	0.151	8.728	872.8324	34.913	1163.776		

Table 3: Ex vivo skin permeation of Clotrimazole from nanoemulsion (Sf 4) using Logan Transdermal Diffusion cell

S. No.	Time (h)	Abs.	Conc. (µg/ml)	Cumulative amount of drug permeated (µg/cm^2)	Cumulative percentage of drug permeated	Cumulative amount of drug permeated per cm^2	Flux (µg/cm^2/h)	Pb x 10^{-3}
1.	1.	0.018	1.040	4.161	138.728	138.7283		
2.	2.	0.048	2.774	11.098	369.942	369.9422		
3.	3.	0.067	3.872	15.491	516.377	516.3776		
4.	4.	0.082	4.739	18.959	631.984	631.9846		
5.	6.	0.118	6.820	27.283	909.441	909.4412	73.050	2.435
6.	8.	0.132	7.630	30.520	1017.34	1017.341		
7.	10.	0.139	8.034	32.138	1071.29	1071.291		
8.	12	0.145	8.381	33.526	1117.53	1117.534		
9.	24	0.152	8.786	35.144	1171.48	1171.484		

Table 4: Ex vivo skin permeation of Clotrimazole from nanoemulsion (sf 5) using Logan Transdermal diffusion cell

S. No.	Time (h)	Abs.	Conc. (µg/ml)	Cumulative amount of drug permeated (µg/cm^2)	Cumulative percentage of drug permeated	Cumulative amount of drug permeated per cm^2	Flux (µg/cm^2/h)	Pb x 10^{-3}
1.	1.	0.05	2.890173	289.0173	11.56069	385.3565		
2.	2.	0.075	4.33526	433.526	17.34104	578.0347		
3.	3.	0.096	5.549133	554.9133	22.19653	73s9.8844		
4.	4.	0.102	5.895954	589.5954	23.58382	786.1272	76.233	
5.	6.	121	6994.22	699422	27976.88	932562.6		2.54
6.	8.	0.135	7.803468	780.3468	31.21387	1040.462		
7.	10.	0.145	8.381503	838.1503	33.52601	1117.534		
8.	12	0.15	8.67052	867.052	34.68208	1156.069		
9.	24	0.151	8.728324	872.8324	34.91329	1163.776		

Table 5: Ex vivo skin permeation of Clotrimazole in surfactant (Tween 80) using Logan Transdermal diffusion cell

S. No.	Time (h)	Abs.	Conc. (µg/ml)	Cumulative amount of drug permeated (µg/cm^2)	Cumulative percentage of drug permeated	Cumulative amount of drug permeated per cm^2	Flux (µg/cm^2/h)	Pb x 10^{-3}
1.	1.	0.086	4.971	497.109	16.57	662.81		
2.	2.	0.175	10.115	1011.56	33.71	1348.7		
3.	3.	0.234	13.526	1352.60	45.08	1803.4		
4.	4.	0.285	16.473	1647.39	54.91	2196.5		
5.	6.	0.345	19.942	1994.22	66.47	2658.9	245.95	8.19
6.	8.	0.415	23.988	2398.84	79.96	3198.4		
7.	10.	0.485	28.034	2803.46	93.44	3737.9		
8.	12	0.489	28.265	2826.59	94.21	3768.7		
9.	24	0.494	28.554	2855.49	95.18	3807.3		

Table 6: Ex vivo skin permeation of clotrimazole from Oil solution using Logan transdermal diffusional cell.

S. No.	Time (h)	Abs.	Conc. (µg/ml)	Cumulative amount of drug permeated (µg/cm^2)	Cumulative percentage of drug permeated	Cumulative amount of drug permeated per cm^2	Flux (µg/ cm^2/h)	Pb x 10^{-3}
1.	1.	0.125	7.225	722.54	24.08	963.39		
2.	2.	0.189	10.924	1092.48	36.41	1456.64		
3.	3.	0.279	16.127	1612.71	53.75	2150.28		
4.	4.	0.321	18.554	1855.49	61.84	2473.98		
5.	6.	0.385	22.254	2225.43	74.18	2967.24	257.016	8.5672
6.	8.	0.475	27.456	2745.66	91.52	3660.88		
7.	10.	0.506	29.248	2924.85	97.49	3899.80		
8.	12	0.512	29.595	2959.53	98.65	3946.05		
9.	24	0.516	29.826	2982.65	99.42	3976.87		

Table 7: Ex vivo skin permeation of marketed formulation of Clotrimazole using Logan Transdermal diffusion cell

S. No.	Time (h)	Abs.	Conc. (µg/ml)	Cumulative amount of drug permeated (µg/cm^2)	Cumulative percentage of drug permeated	Cumulative amount of drug permeated per cm^2	Flux (µg/ cm^2/h)	Pb x 10^{-3}
1.	1.	0.275	15.89595	1589.595	52.986	2119.4		
2.	2.	0.462	26.7052	2670.52	89.017	3560.6		
3.	3.	0.495	28.61272	2861.272	95.375	3815.0		
4.	4.	0.502	29.01734	2901.734	96.724	3868.9		
5.	6.	0.506	29.24855	2924.855	97.495	3899.8	257.85	8.59
6.	8.	0.51	29.47977	2947.977	98.265	3930.6		
7.	10.	0.513	29.65318	2965.318	98.843	3953.7		
8.	12	0.516	29.82659	2982.659	99.421	3976.8		
9.	24	0.275	15.89595	1589.595	52.986	2119.4		

Histology study of skin by fluorescence microscope

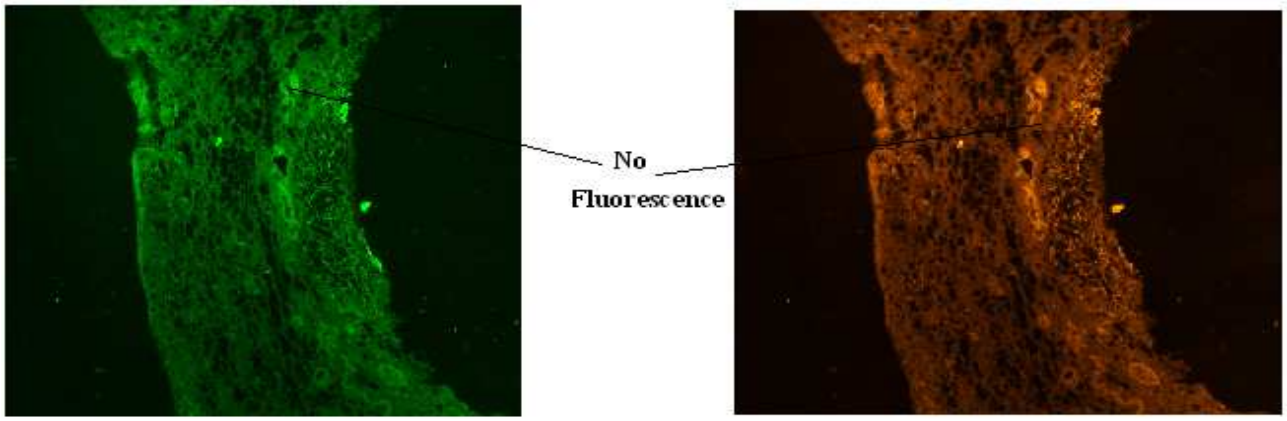

Figure 8A.Histopictographs showing permeation through the skin not showing Fluorescence

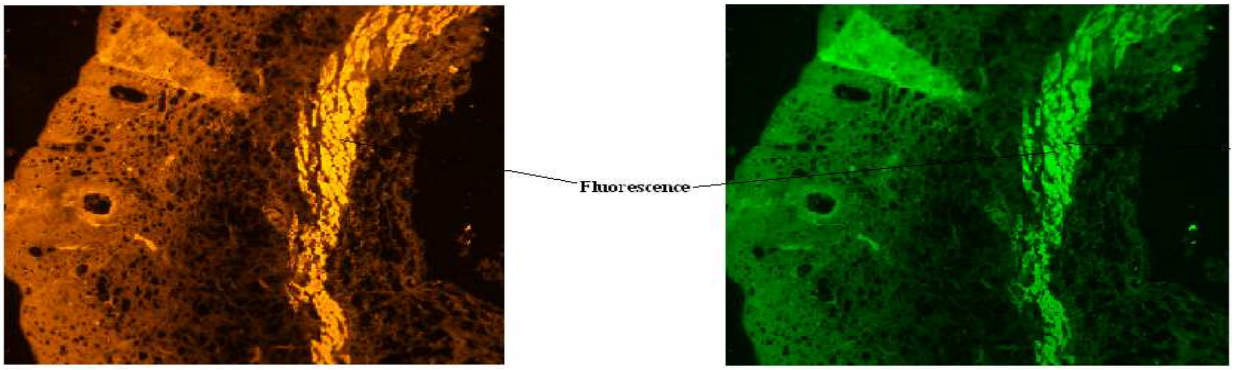

Figure 8B.Histopictographs showing permeation through the skin showing fluorescence

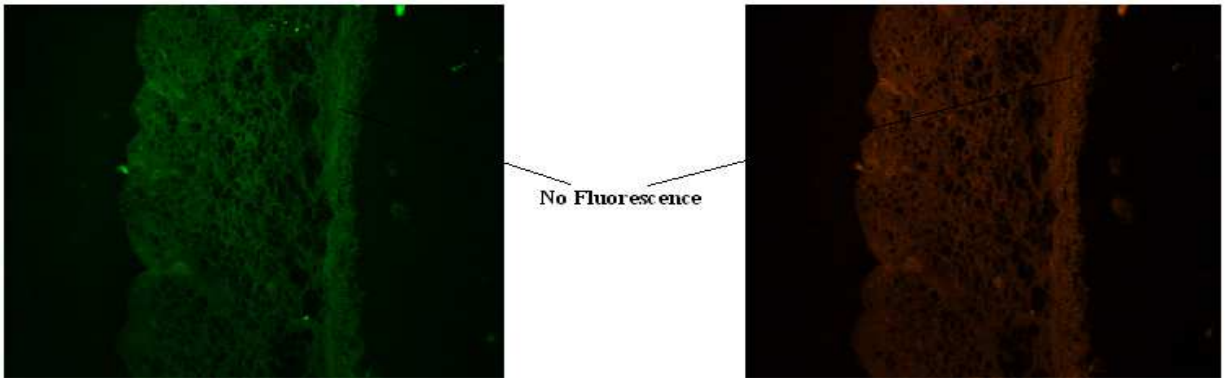

Figure 8C.Histopictographs showing permeation through the skin not showing fluorescence

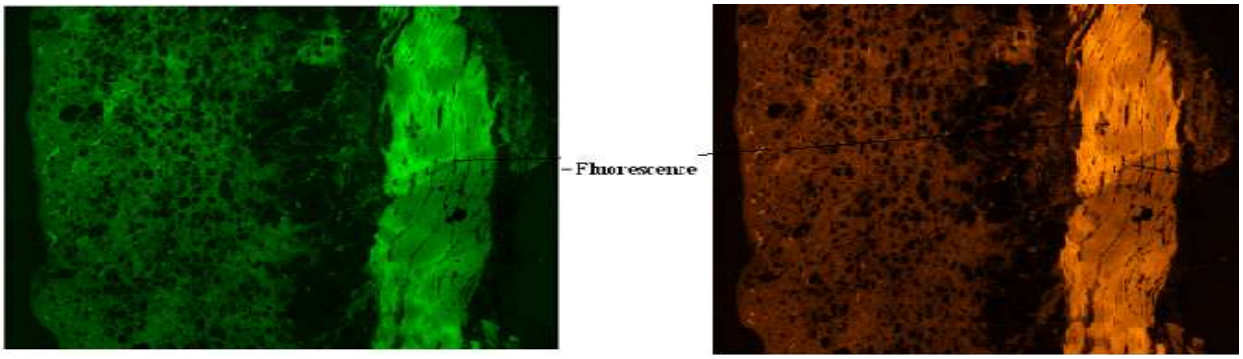

Figure 8f1.Histopictographs showing permeation through the skin showing fluorescence

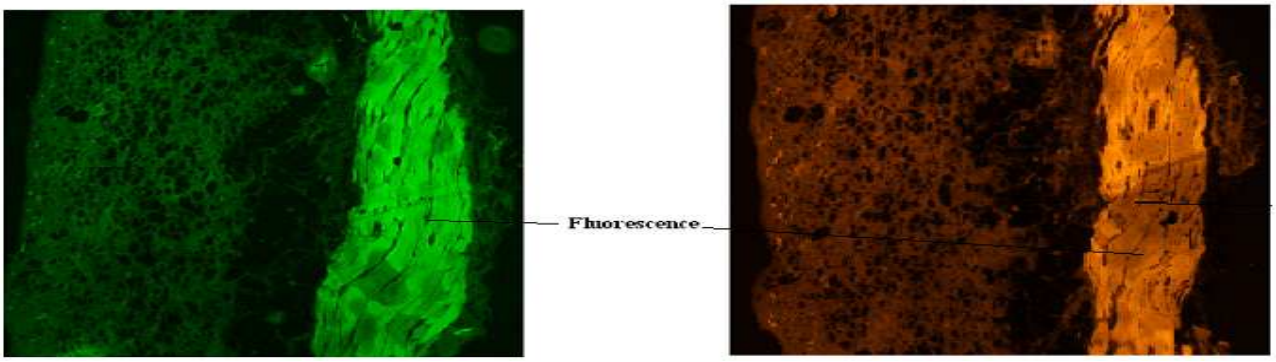

Figure 8f2.Histopictographs showing permeation through the skin showing fluorescence

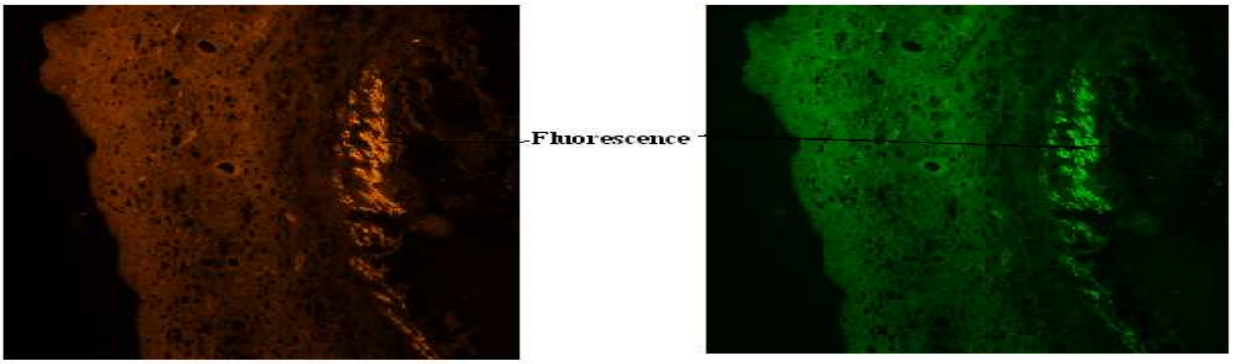

Figure 8f6.Histopictographs showing permeation through the skin showing fluorescence

Summary and Conclusion

Clotrimazole is tetracyclic, classified a topical antifungal agent. The drug is well and absorption is rapid and complete, with maximum plasma concentrations reached after 2 hrs. The bioavailability of clotrimazole is 50% due to first pass metabolism. Oral dose of the drug in adults is 10 mg one day but maximum dose up to 50 mg per day. To reduce or eliminate the presystemic drug metabolism, change the route of drug administration. For the development of nanoemulsion formulation, the solubility of drug in oil phase plays an important role. After performing solubility study in different oils, it was found that clotrimazole exhibited maximum solubility in the clove oil (190mg/ml). but during solubility study it was observed that the oil it was observed that in oil, color of drug was changed it shows physical incompatibility and hence further drug excipient interaction study was performed by, UV spectroscopy and DSC technique. It was inferred from these studies that clotrimazole was incompatible with clove oil and it was dropped out and therefore oleic acid was chosen as the oil phase. The drug solubility is good in oleic acid (160mg/ml).Among the selected surfactant and co-surfactant, pseudo ternary phase diagrams were constructed by phase titration method in order to define the extent and nature of nanoemulsion region and surrounding two and three domains. The construction of pseudo ternary phase diagrams was started using surfactant Tween 80 and co-surfactant PEG 400 in different ratios. It was found that the region of nanoemulsion existence was higher with Tween 80 and PEG 400.

After constructing and analyzing various phase diagrams, olic acid, Tween 80 and PEG 400 were selected as oil phase, surfactant and co-surfactant respectively for further formulation development.Optimized drug loaded nanomulsion formulation (Sf4) was characterized for the various attributes. The nanoemulsion was colloidal dispersions having average droplet size ranging from 25.06 to 67.7nm.An nanoemulsion system composed of clotrimazole, oil, water, surfactant mixture, was prepared using water dilution method it had relatively uniform emulsion droplets,a narrow size distribution, which increases the release rate of the drug in comparison to the other form of formulation like tablet or power ,my challenges for the treatment of onchomycosis is topical route by gels formulation it increases the release rate and solubility and bioavailability. Histopathological studies performed in PBS (pH 7.4), Isopropyl alcohol and blank nonoemulsion. The formulation applied on wistar rat skin. Sample treated with fluorescent dye rhodamine and fluorescine dye .the drug containing formulation were applied on different parts of skins. and checked with an optical microscope.The fig. 8A skin histology shows no permeation through the skin as no fluorescence appears. In the control oleic acid and drug the drug permeate through the skin and it shows fluorescence in the skin layers. The marketed formulation with dye can't cross the skin layer and shows no fluorescence in the skin layer (Fig. 8C). In case of nanoemulsion formulation the drug retained in the skin it was shown in Fig. 8F1 & 8F2 & Fig.8F6. The Nanoemulsion formulation was effective to retain the drug inside the skin which is desired. Very effective for the local treatment of the Onychomycosis. The marketed formulation is not retained in the skin so it will take very long time to treat but the drug is retained so the effect of the drug is prolonged as the concentration of the drug inside the skin layers increased and it raises the cure rate and time of the therapy with fewer side effects.

Conclusion

The selection of antifungal agent is also play a very important role. Clotrimazole shows very potent action on the candida. Clotrimazole is a newer antifungal agent which kills candida very effectively. The molecular weight of clotrimazole is also very low. It is suitable for the topical application. The solubility of the clotrimazole is BCS class 2^{nd} drug. The solubility of the clotrimazole an be increased by nanoemulsion the drug entrapped inside the oil phase.

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