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Research Paper

Cavamax W7 Composite Ethosomal Gel of Clotrimazole for Improved Topical Delivery: Development and Comparison with Ethosomal Gel

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Abstract. The present research work was aimed to formulate clotrimazole encapsulated Cavamax W7 composite ethosomes by injection method for improved delivery across epidermis. 3^2 factorial design was used to design nine formulations (F1-F9) and compared with ethosomal formulations (F10-F12). F9 with vesicle size of 202.8±4.8 nm, highest zeta potential (−83.6±0.96 mV) and %EE of 98.42±0.15 was selected as optimized composite ethosome and F12 as reference ethosomal formulation. As revealed by transmission electron microscopy F9 vesicles were more condensed, uniformly spherical in shape than F12 vesicles. Vesicular stability studies indicated F9 to be more stable as compared to F12. Both F9 and F12 were incorporated in carbopol 934 gel base to get G1–G8 gel formulations and evaluated for *in vitro* skin permeability. Cavamax W7 composite ethosomal optimized gel (G5) showed higher *in vitro* percent cumulative drug permeation (88.53±2.10%) in 8 h and steady state flux (J_{ss}) of 3.39±1.45 μg/cm^2/min against the J_{ss} of 1.57±0.23 μg/cm^2/min for ethosomal gel (G1) and 1.13±0.06 μg/cm^2/min for marketed formulation. The J_{ss} flux of G5 was independent of amount of drug applied/unit area of skin. *In vivo* confocal laser scanning microscopic study of G5 depicted uniform and deeper penetration of rhodamine B (marker) in epidermis from Cavamax W7 composite ethosomal gel in comparison to G1. Finally, G5 demonstrated better ($p<0.05$) antifungal activity against *Candida albicans* and *Aspergillus niger* than G1 thus, signifying that Cavamax W7 composite ethosomes present a superior stable and efficacious vesicular system than ethosomal formulation for topical delivery of clotrimazole.

KEY WORDS: Cavamax W7; clotrimazole; composite ethosomes; drug loading; flux.

INTRODUCTION

Topical drug delivery system is considered to be one of the most relevant routes for treating skin diseases efficaciously. Despite of having the advantages of self administration, patient compliance, and reduction in adverse effects systemically, this system has the limitation of slow diffusion across the stratum corneum (1) and the barrier property of the skin limits the delivery of the drug through the skin (2). Therefore several approaches have been used to weaken this skin barrier and to enhance the administration of the drug through the skin. One of these approaches is the use of vesicle formulations as skin dermal system (3). Of the various vesicular systems investigated, ethosomal vesicles have been found to be capable enough in enhancing permeation of topical agents to the deeper tissues through the stratum corneum (4). Enhanced permeation through these vesicles is not only due to the presence of ethanol but also due to the fact that these vesicles are highly deformable and malleable that allows their better penetration across the skin (5).

Clotrimazole [1 H-imidazole-1-(2-chlorophenyl) diphenyl] is an antifungal drug employed widely in the treatment of various fungal infections such as vaginal yeast infections, oral thrush, ringworm, athlete's foot, and jock itch, in case of both humans as well as other animals (6). It possesses broad antifungal activity against pathogenic yeasts as well as filamentous fungi (7). According to WHO, it is an effective antifungal agent in topical infections, more safer than any other antifungals being listed in its Essential Drugs list currently and is generally recognized as safe (8). One percent solution or cream of clotrimazole is reported to be clinically effective and safe in treating tinea pedis, tinea cruris, tinea corporis, pityriasis versicolor, and cutaneous candidiasis infections, in a double blind, multicentric clinical trial (9). It is also reported to be well tolerated in the treatment of both dermatophytosis and onychomycosis (10). The commercially available cream of clotrimazole has the limitations of lesser skin retention and deposition (11) and poor residence ability at the targeted site (12). In a report by Nagarsenkar and Munot (13), proliposomes of clotrimazole were designed to localize and prolong its residence time with in the skin but the release of the drug was considerably slow from this carrier system.

In order to overcome these limitations, researchers across the globe have put in various efforts for development of superior topical drug delivery system. Liposomes loaded with clotrimazole were formulated with the objective to localize the drug delivery and to improve the solubility and availability of

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the drug at the targeted site that reduced the dose and the systemic side effects associated with it (12). However, these remained confined to the upper layers of the stratum corneum, due to low permeability. Consequently, ethosomes were developed that can efficiently deliver clotrimazole to deep skin strata, localize the drug to the targeted site, and reduce possible side effects associated with the conventional treatment. But the concentration of ethanol used was up to 40% *w/v* (14) that makes the vesicular membrane more permeable and leaky. As concentration of ethanol goes beyond 30% *w/v*, the entrapped drug tends to leak out (15). Additionally, the repeated topical application consisting of high percent of ethanol may affect the skin adversely by causing skin irritation and contact dermatitis (16).

Thus, the project was undertaken to develop superior and safer ethosomal formulation, *i.e.*, Cavamax composite ethosomes that can improve the topical delivery of clotrimazole and at the same time reduce the amount of ethanol so as to reduce the risk of adverse effects associated with the higher concentration of ethanol used in ethosomes. *In vivo* confocal laser scanning microscopic (CLSM) study was done to layout the comparison among control formulation, Cavamax composite ethosomes, and reference ethosomes for improved permeability across the skin.

MATERIALS AND METHODS

Materials

Clotrimazole was obtained as a gift sample from Siemen Laboratories, Gurgaon, India. Soya lecithin, 30% was purchased from Himedia Laboratories, Pvt. Ltd., Mumbai, India. Cavamax W6, W7, and W8 (α-, β- and γ-cyclodextrin, respectively)were gift samples from International Specialty Product Limited, USA and propylene glycol was procured from Sigma-Aldrich Chemie GmbH, Netherlands. Ethanol (95% *v/v*), potassium dihydrogen orthophosphate purified LR, sodium chloride extrapure LR, and polyethylene glycol 400 LR were purchased from SD Fine chemicals Ltd. Mumbai, India. HIMEDIA dialysis membrane 150 and triethanolamine were procured from Qualigens Fine Chemicals, Mumbai, India and iso-propyl myristate and methanol AR were purchased from Qualikems Fine Chemicals, Pvt. Ltd., New Delhi, India. Carbopol 934 LR and rhodamine B were purchased from Central Drug House (P), Ltd., New Delhi, India where as sodium hydroxide pellets LR were procured from RFCL Ltd., New Delhi, India.

Methods

Optimization of Blank Cavamax Composite Ethosomes

Preliminary studies were carried out to optimize the blank composite ethosomes prepared by injection method described later. Preparation of blank composite ethosomes was attempted by using Cavamax W6, W7, and W8, and after selection of the type of Cavamax; the optimum concentration of Cavamax W7 required to prepare composite ethosomes was selected. *In vitro* adsorption study, phase solubility and solubility studies were also done to trace out various factors and their levels factor that can influence ethosomes formulations.

Selection of Type and Optimum Concentration of Cavamax. Blank composite ethosomes of Cavamax W6, W7, and W8 were prepared to identify most appropriate Cavamax for preparation of composite ethosomes. For this, ethosomal suspensions were visualized by photomicrographs taken through optical photomicrograph (Photomicrograph, HICON, Delhi, India) at 100×. Next, blank composite ethosomes were prepared by varying the concentration of Cavamax W7 in the range of 0.1–3.0% *w/v*. Photomicrographs of composite ethosomes were taken and analyzed visually.

In Vitro Adsorption Study. Ten milligrams of clotrimazole was added to 50 ml of distilled water and tenfold weight (100 mg) of soya lecithin was added to it. The mixture was magnetically stirred at room temperature (25°C) for 24 h. Samples (5 ml) were periodically drawn at 0, 2, 6, 18, 20, 22, 24 h and assayed spectrophotometrically at 261 nm using UV Spectrophotometer (Shimadzu, Pharma Spec 1700, Kyoto, Japan) at 261 nm to determine the percent drug adsorbed with respect to time.

Phase Solubility Study. Phase solubility studies were performed at 25°C according to the method reported by Higuchi and Connors (17). An excess amount of drug (10 mg) was added to 10 ml of distilled water containing various concentrations of Cavamax W7 ranging from 0.2–0.8% *w/v* and shaken for 72 h in a water bath shaker at 100 rpm (0.559×*g*). Samples were filtered through nylon filter disk (0.22 μ) and assayed against the blank prepared by using different concentrations of Cavamax W7 in distilled water.

Equilibrium Solubility Study. Ten milligrams of drug was added to 20 ml of distilled water (0.05% *w/v*) in a conical flask and kept for 72 h at 37°C on water bath shaker (HICON, New Delhi, India). Additionally, in six flasks, 10 mg of drug was mixed with Cavamax W7, soya lecithin, ethanol, and propylene glycol (Table I). Samples were withdrawn, filtered through nylon filter disk (0.22 μ) and analyzed spectrophotometrically.

Preparation of Cavamax W7 Composite Ethosomes

Cavamax W7 composite ethosomes of clotrimazole were made by injection method based on 3^2 factorial design. Where 3^2 factorial design is a three-level factorial design as reported by Webb (18), is dependent on two independent variables (factors) and in all, there are nine runs or combinations. In formulating Cavamax W7 composite ethosomes, soya lecithin and Cavamax W7 were two independent variables, both at three different levels (−1, 0, +1). In the design, the lower concentration of soya lecithin, *i.e.*, 0.2% *w/v* has been assigned at −1 level whereas 0.5% *w/v* concentration was assigned the level 0. The concentration at highest level +1 was 1.0% *w/v*. In case of Cavamax W7, concentrations at three levels from lowest to highest (−1, 0, +1) were 0.3% *w/v*, 0.5% *w/v*, and 0.8% *w/v*, respectively. Based on this factorial design, nine Cavamax W7 composite ethosomal formulations were developed. Soya lecithin was dissolved

Table I. Solubility Data of Clotrimazole in Distilled Water in the Presence of Excipients Utilized for Preparation of Cavamax W7 Composite Ethosomes of Clotrimazole

Sample composition	Strength of excipients (% *w/v*)	Solubility (mg/ml)	% Solubility enhancement
D	0.05	0.054	–
D + SL	0.05+0.5	0.206	73.79
D+Cavamax W7	0.05+0.5	0.412	86.89
D + SL + EtOH + PG	0.05+0.5+30+10	1.388	96.11
D+Cavamax W7+EtOH+PG	0.05+0.5+30+10	1.272	95.75
D+SL+Cavamax W7+EtOH+PG	0.05+0.5+0.5+30+10	1.592	96.61

D clotrimazole, *SL* soya lecithin, *EtOH* ethanol, *PG* propylene glycol

in ethanol (30% *w/v*) and to this drug was added as per the composition given in Table II. Propylene glycol was added to the above mixture and the mixture was heated to 30°C±1°C in a water bath. Solution of Cavamax W7 in distilled water heated to 30°C±1°C was injected slowly to the lipid mixture with constant stirring on magnetic stirrer for 20 h while maintaining the system at 30°C±1°C. The suspension of ethosomes was left to cool at room temperature for 30 min and was then subjected to sonication for three cycles of 5 min with rest of 5 min between each cycle at 4,000 rpm (894.0×*g*) using probe sonicator (HICON, New Delhi) to get nanosized ethosomes.

Purification of Cavamax W7 Composite Ethosomes

Cavamax W7 composite ethosomes were purified by dialysis membrane technique to remove the free drug from ethosomal suspension. For this, Hi-media dialysis membrane 150 (MWCO 12–14 kDa) was kept in saline solution for 2 h before dialysis to ensure complete wetting of the membrane. Drug-loaded vesicles were placed in a dialysis bag, that was transferred into 200 ml of phosphate buffer pH 6.8. The receiver medium was stirred with a magnetic stirrer at 500 rpm (13.98×*g*). Five milliliters of sample was withdrawn at appropriate time intervals and replaced with the equal volume of fresh media and analyzed for the amount of free drug spectrophotometrically. Purification time was optimized by applying statistical paired *t* test at 5% level of significance.

Evaluation of Cavamax W7 Composite Ethosomes

Vesicle Size and Zeta Potential Determination

Vesicle size, zeta potential, and polydispersity index were measured by Zetasizer ver. 6.01 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) by diluting one drop of ethosomal and Cavamax W7 composite ethosomal suspension with hydroethanolic solution at 25°C in clear disposable zeta cells. All the measurements were done in triplicate for each sample.

Entrapment Efficiency

Purified vesicular suspension was transferred into centrifuge tube and centrifuged (R-4 C, Remi centrifuge, Vasai, India) for 1 h at 4,000 rpm (894.0×*g*). The sediment was lysed using methanol and filtered through nylon filter disk (0.22 μm). The drug was assayed both in the sediment and supernatant to determine the entrapment efficiency by the following Eq. 1.

$$\%\ \text{Entrapment efficiency} = [W_a - (W_s + W_P)/W_a] \qquad (1)$$

where, W_a = amount of drug added in the system, W_s = amount of drug in the supernatant after centrifugation, W_P = amount of drug in the purification medium.

Table II. 3^2 Factorial Design of Ethosomal and Cavamax W7 Composite Ethosomal Formulations of Clotrimazole

Formulation code	Drug (mg)	Soya Lecithin (% *w/v*)	Cavamax W7 (% *w/v*)	Ethanol (% *w/v*)	Propylene glycol (% *w/v*)
Cavamax W7 ethosomes					
F1	10	0.5 (−)	0.5 (−)	30	10
F2	10	0.5 (−)	0.3 (0)	30	10
F3	10	0.5 (−)	0.8 (+)	30	10
F4	10	0.2 (0)	0.5 (−)	30	10
F5	10	0.2 (0)	0.3 (0)	30	10
F6	10	0.2 (0)	0.8 (+)	30	10
F7	10	1.0 (+)	0.5 (−)	30	10
F8	10	1.0 (+)	0.3 (0)	30	10
F9	10	1.0 (+)	0.8 (+)	30	10
Ethosomes					
F10	10	0.5 (−)	–	30	10
F11	10	0.2 (0)	–	30	10
F12	10	1.0 (+)	–	30	10
Fx[a]	10	0.35	0.4	30	10

[a] Extra design check point formulation

Validation of Experimental Design

The polynomial equation was generated for dependent variable using Design Expert Software version 8.0.5 (Stat-Ease Inc, Minneapolis, Minnesota, USA). An extra design check point formulation (F*x*) was developed by selecting the levels as 0.35% *w*/*v* of soya lecithin and 0.4% *w*/*v* of Cavamax W7. This was used to validate the obtained polynomial equation model. A statistical model consisting of interactive and polynomial terms was used to evaluate the responses. Polynomial and transformed polynomial equations were obtained. Extra design check point formulation was then evaluated for dependent variable, *i.e.*, percent entrapment efficiency to get the experimental value. This value was compared from the predicted value obtained from transformed polynomial equation and evaluated statistically by pooled *t* test at 5% level of significance.

Vesicular Visualization by Transmission Electron Microscopy

Transmission electron microscope (CM 10 Transmission Electron Microscope, Mega View III FW 80 kV, Philips, UK) was used to characterize the optimized Cavamax W7 composite ethosomes and reference ethosomal formulations. For this purpose, one drop of each formulation was placed on a copper grid for 2–3 min and then negatively stained by phosphotungstic acid. The air-dried sample was visualized under transmission electron microscope at 4,500× magnification with accelerating voltage of 80 kV.

Stability Study

Stability of the optimized (F9) and reference (F12) formulation was determined by storing the vesicles at 4°C for 6 months. After appropriate time intervals, samples were withdrawn and evaluated for their mean particle size, zeta potential, and percent entrapment efficiency.

PREPARATION OF GELS

Gels loaded with composite ethosomal vesicles were prepared by dispersion method. Carbopol 934 was soaked in distilled water (1% *w*/*v*) for 2 h to form its aqueous dispersion. To this dispersion, permeation enhancers polyethylene glycol 400 and isopropyl myristate and combinations thereof were added as per the composition (Table III). Optimized Cavamax W7 composite ethosomes and reference ethosomal formulation(s) were centrifuged 2,000 rpm (447.0×*g*) and the pellets were incorporated in Carbopol gel base to get G1–G8. Triethanolamine was added under continuous stirring to the above dispersion. Formulations G1–G4 were designated as ethosomal gels whereas ethosomal optimized gel (G5)–G8 as Cavamax W7 composite ethosomal gels, both categories formulated with or without permeation enhancers.

Evaluation of Gels

pH and Viscosity Measurements

The pH of gels was measured by using Digital pH meter, model 111 E (HICON, New Delhi, India) and viscosity was measured by Brookfield viscometer R/S-CPS (Brookfield Engineering Lab, Inc, USA) using T-spindle S-93 at 20 rpm (22.36×10^{-3} g). The temperature was maintained at 25°C± 1°C.

Determination of Percent Drug Content

Drug content was determined by taking 100 mg of the gel and diluting to 5 ml with methanolic HCl. Vortex shaking (Vortex shaker, HICON, India) was done for 5 min and volume was made up to 10 ml with methanolic HCl and examined spectrophotometrically for drug content.

Animal Studies

All the animal experiments have been conducted in full compliance with the institutional ethical and regulatory principles and as per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care and International Expectations for Animal Care and Use/Ethics committees. The investigations were performed after obtaining approval by the Institutional Animal Ethical Committee of Rajiv Academy for Pharmacy, Mathura, India (IAEC No: IAEC/RAP/3250/2011).

In Vitro Skin Permeability

Three albino rats (Wistar strain), 6–8 weeks old, weighing 120–150 g were sacrificed by spinal cord delocalization. For the *in vitro* permeability study, tested animals were preshaved carefully and the abdominal skin was then separated from the underlying connective tissue with the scalpel, the excised skin was then placed on aluminum foil and the dermal side of the skin was gently teased off for any adhering fat or subcutaneous tissue. Prepared skin was then mounted on Franz diffusion cell (area, 3.14 cm^2) and the test formulation (2 mg/cm^2) was applied on the epidermal side of the skin. One-milliliter sample was withdrawn from the receptor compartment containing 11 ml of the phosphate buffer pH 6.8 maintained at 32°C±1°C, at appropriate time intervals and analyzed spectrophotometrically at 261 nm to determine the cumulative amount of drug permeated across the skin in the receptor medium irrespective of the drug deposited in the skin layers. An equal volume of fresh phosphate buffer pH 6.8 was replaced after each sampling into the compartment. The study was performed in triplicate and average values were calculated. The percent cumulative amount of drug permeated across the skin per square surface area was evaluated and plotted against time to calculate the steady state flux (J_{ss}).

Effect of Concentration of Drug on In Vitro Permeability

Effect of varying concentration of drug on the *in vitro* permeability was determined, by performing the *in vitro* studies using Franz diffusion cell by the method described above. Three different formulations were prepared by varying the strength of the gel as 1, 2, and 5 mg/cm^2. These formulations were applied separately on the epidermal side of the skin and the experiments were conducted as described above.

Table III. Composition of Clotrimazole (CLT) Ethosomes and Cavamax W7 Composite Ethosomes-Loaded Gel Formulations

Ingredient% *w/w*	G1	G2	G3	G4	G5	G6	G7	G8
CLT loaded Cavamax W7 ethosomes (F9)	–	–	–	–	1	1	1	1
CLT loaded ethosomes (F12)	1	1	1	1	–	–	–	–
Carbopol 934	1	1	1	1	1	1	1	1
PEG 400	–	5	–	5	–	5	–	5
Isopropyl myristate	–	–	5	5	–	–	5	5
Triethanolamine	2	2	2	2	2	2	2	2
Double-distilled water q.s.	100	100	100	100	100	100	100	100

Confocal Laser Scanning Microscopic

Depth and mechanism of skin permeation of rhodamine B loaded Cavamax W7 composite ethosomal vesicles were investigated by CLSM. Formulation containing vesicles loaded with fluorescent dye rhodamine B was prepared by adding the dye (marker) to the mixture of soya lecithin in ethanol and propylene glycol. Gels loaded with these vesicles were applied to the dorsal skin of albino rats (Wistar strain) for 8 h. The rats were then sacrificed and skin was excised, washed, and placed on the aluminum foil. Sections of the skin samples were prepared and examined with CLSM (Fluo view FV 1000, Olympus, Japan) at 100×. Optical excitation was carried out with a 488 nm argon laser beam and fluorescence emission was detected above 560 nm for rhodamine B.

Antifungal Activity

Antifungal activity was evaluated by cup plate method against *Candida albicans* and *Aspergillus niger*. Microbial inoculums of tested organisms (0.5% *v/v*) were spread over the nutrient agar media in the separate Petri dishes, three for each fungal strain. After solidifying the agar plate, three cavities of 1 cm diameter and 0.5 cm depth were made. Of the test formulations, 100 mg (Control, reference and Cavamax W7 composite ethosomal formulation) were filled into the cavities. The Petri dishes were left for 1–4 h at room temperature and incubated at 25°C for 24 h. Zone of inhibition around each hole was observed by antibiotic zone reader (HICON, New Delhi, India).

RESULTS AND DISCUSSION

Preliminary Optimization Studies on Blank Cavamax Composite Ethosomes

Blank Cavamax W7 composite ethosomes were prepared by injection method and various preliminary studies were done to optimize the methodology. From the photomicrographs taken, it was observed that Cavamax W6 ethosomal vesicles were non-uniform in size and were undefined structures while Cavamax W8 did not facilitate the vesicle formation. Ethosomes prepared with Cavamax W7 however, were uniform, spherical with defined boundary. It is proposed that the molecules of Cavamax W7 imparted rigidity to the vesicle wall by intercalating with the lipid bilayer or the molecules may be present at the interface of the lipid bilayer and the hydroethanolic core. The hydrophilic outside and hydrophobic inside features are presumed to guide the orientation of Cavamax W7 molecules, both in the lipid bilayer and the interface.

Thus, Cavamax W7 was selected for preparing composite ethosomes. Next, to select its appropriate concentration, composite ethosomes, using increasing concentrations of Cavamax W7 were prepared. It was observed that at 0.5% *w/v* concentration of Cavamax W7, ethosomal vesicles formed were firm, with well-defined boundary and spherical in shape confirming uniformity. At concentration below 0.5% *w/v*, vesicle could not be formed and on increasing the concentration of Cavamax W7 beyond 0.5% *w/v* till 3.0% *w/v*, the size of vesicle increased with loss in uniformity in size. Thus, the optimum concentration of Cavamax W7 was identified to be 0.5% *w/v*. This also guided the selection of levels of Cavamax W7 for 3^2 experimental design.

Optimization of Stirring Time by In Vitro Adsorption Study

Apart from the vesicular bilayer integrity, another important consideration in development of ethosomal systems is its entrapment capability. The drug in a vesicular system is loaded either in the ethanolic milieu or is adsorbed on the phospholipid bilayer depending on drug's characteristic. Clotrimazole being a lipophilic drug (pKa 6.7; 19) will be adsorbed on the phospholipid bilayer and that in turn will be dependent on the processing conditions. Stirring of the reaction mixture is an important consideration that will facilitate contact between the drug and phospholipid. Hence, *in vitro* adsorption studies were carried out. As seen in Fig. 1, the adsorption profile showed an increase in percent drug adsorbed with time and reached plateau level beyond 20 h. As the percent adsorbed at 20 h (20.4%) was nonsignificantly ($p>0.05$) different from the percent drug adsorbed on 24 h (23.1%), 20 h was considered optimum time for stirring to affect maximum drug entrapment. As Cavamax

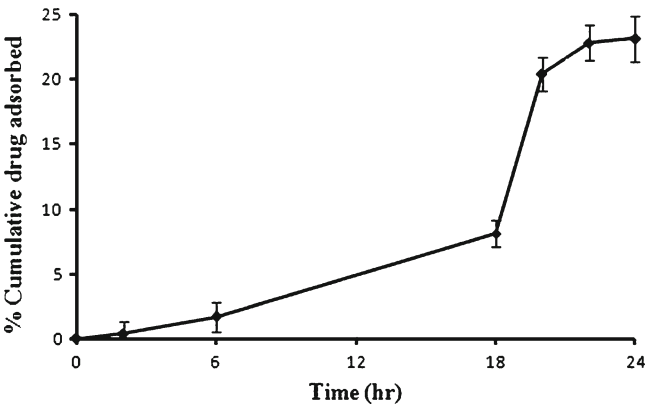

Fig. 1. Adsorption pattern of clotrimazole onto soya lecithin in distilled water for 24 h at 25°C

W7 is soluble in water, *in vitro* adsorption studies cannot be performed, but to analyze the effect of increasing concentration of Cavamax W7, phase solubility studies were done.

Phase Solubility

Phase solubility study was performed to optimize the levels of Cavamax W7 for the experimental design. Phase solubility of clotrimazole with Cavamax W7 indicated increased in its solubility on increased concentration of Cavamax W7 (Fig. 2). The phase solubility curve was biphasic with an initial slope value of 1.98 in the concentration range of 0–0.6% *w/v* of Cavamax W7 followed by steep slope of 261.7 in the 0.6–0.8% *w/v*. The type of phase solubility curve can be categorically stated as A-type with negative deviation from linearity that indicates formation of complexes with limited solubility in the aqueous complexation medium. The phase–solubility profiles do not verify formation of inclusion complexes. They only describe how the increasing concentration of solubilizer influences drug solubility. The change in slope can be correlated either to the change in dielectric constant produced by Cavamax W7 in the aqueous complexation media or modification in the solubility of complex formed due to self-association of Cavamax W7 molecules (20). Based on the phase solubility results, the levels of Cavamax W7 selected were 0.3% *w/v*, 0.5% *w/v*, and 0.8% *w/v* for designing Cavamax W7 composite ethosomes of clotrimazole.

Equilibrium Solubility Study

The solubility of drug in distilled water was found close to its literature value of 0.055 mg/ml (21). In presence of Cavamax W7 and soya lecithin, solubility enhancement was 7.63- and 3.81-fold, respectively. Cavamax W7 improved the solubility of clotrimazole by forming soluble complexes with the drug, enclosing the drug in its hydrophobic cavity (22) whereas soya lecithin increased the solubility due to its surfactant action. The hydrophobic tails and hydrophilic head region in its structure decrease the surface tension in aqueous media and facilitate solubilisation (23). The solvents, ethanol, and propylene glycol displayed a cumulative solubility enhancement effect due to their cosolvent action. Thus, it was concluded that the excipients used in formulating composite ethosomes, present an opportunity for solubility enhancement and hence higher entrapment. Based on these considerations, Cavamax W7 composite ethosomes were designed and compared with reference ethosomal formulations.

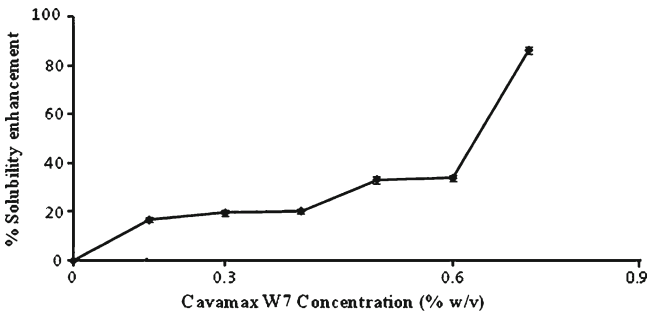

Fig. 2. Phase solubility study profile of clotrimazole in distilled water at increasing concentrations of Cavamax W7

Optimization of Purification Time

Selected formulation (F9) was subjected to purification and the amount of free drug released was observed for 60 min. Initially, the amount of free drug released increased till 10 min and reached plateau level by 20 min (data not shown). Paired *t* test was applied to determine any significant difference in the amount of free drug released at different time intervals. It was observed that there was a significant difference ($p<0.05$) in the amount of free drug released between 10 and 20 min but there was no significant difference ($p>0.05$) in amount of free drug released after 20 and 30 min. Therefore, all the formulations were subjected to purification by dialysis technique for a period of 20 min.

Evaluation of Cavamax W7 Composite Ethosomes and Ethosomes

Determination of Particle Size, Polydispersity Index, and Zeta Potential

Cavamax W7 composite ethosomes and reference ethosomal formulations were evaluated for particle size, polydispersity index, and zeta potential. It was observed that the vesicles of composite ethosomal formulations (F1–F9) varied in size range of 202.8±4.8 and 252.3±6.4 nm where as reference ethosomal vesicles (F10–F12) ranged between 213.7±5.9 and 257.2± 10.5 nm (Table IV). Grossly, the size range was narrower for Cavamax W7 composite ethosomes. The reduction in vesicle size caused by Cavamax W7 can be attributed to the fact that removal of phospholipid from the outer half of the membrane by Cavamax W7, results in an inward bending of the phospholipid layer (22) and consequently smaller vesicles. The vesicle size of Cavamax W7 composite ethosomes, increased with the increase in concentration of both Cavamax W7 and soya lecithin. Availability of higher amount of raw materials probably led to formation of larger-sized vesicles. Similarly, in case of ethosomal formulations, vesicle size was dependent solely on the amount of soya lecithin, thus the size increased as we moved from F10 to F12. The results are consistent with the earlier reports made by Zhaowu *et al.* (24) where the authors reported an increase in vesicular size on increasing phospholipid concentration. Vesicle size is an important parameter that influences the delivery of topical drug delivery system. Vesicle size less than 300 nm is considered to be efficient enough in localizing the drug deep into the skin up to some extent. However, vesicle size of less than 200 nm of ethosomes and liposomes is considered optimum for drug delivery to the skin (25).

The polydispersity index (PDI) dictates the distribution of vesicles as it is measurement of homogeneity. A PDI value of less than 0.3 indicates the homogeneous distribution of particles within the formulation whereas if it is greater than 0.3 exemplify its heterogeneous nature (26). It was found to be minimum for F9 0.113±0.024 among Cavamax W7 composite ethosomal vesicles and 0.320±0.006 (F12) among reference ethosomal formulations showing the homogeneous nature of the formulations. F9 and F12 made with highest levels of soya lecithin displayed least PDI probably due to dominant surfactant action of soya lecithin.

Zeta potential is another parameter responsible for the thermodynamic stability. Cavamax W7 composite ethosomal

Table IV. Pharmacotechnical Properties of Cavamax W7 Composite Ethosomal Vesicles (F1–F9) and Reference Ethosomal Vesicles (F10–F12)

Formulation code	Particle size range (nm)	Polydispersity index	Zeta potential (mV)	Percent entrapment efficiency
F1	209.3±0.0	0.628±0.003	−59.3±0.46	70.91±0.02
F2	212.6±2.9	0.732±0.008	−61.2±0.70	73.37±0.05
F3	220.6±5.6	0.312±0.000	−61.1±0.98	77.07±0.07
F4	211.4±2.3	0.343±0.004	−61/7±0.42	79.95±0.08
F5	234.0±5.9	0.066±0.000	−64.2±0.52	81.05±0.05
F6	252.3±6.4	0.499±0.010	−74.7±0.35	83.87±0.09
F7	232.8±2.5	0.634±0.011	−78.8±0.70	85.80±0.12
F8	220.8±2.1	0.224±0.004	−81.1±0.83	96.95±0.08
F9	202.8±4.8	0.113±0.024	−83.6±0.96	98.42±0.15
F10	213.7±5.9	0.123±0.005	−55.8±1.70	63.32±0.07
F11	235.9±7.1	0.421±0.008	−58.9±0.72	65.90±0.01
F12	257.2±10.5	0.320±0.006	−65.0±0.26	69.88±0.08

vesicles had higher zeta potential −83.6±0.96 mV as compared to reference ethosomal formulations, *i.e.*, −65.0±0.26 mV. The negative charge on the vesicles might be the charge due to the drug or soya lecithin. Clotrimazole carries negative charge concentrated on the N atoms and around the carbon atoms of imidazole ring (26) whereas soya lecithin carries both the negative and weak positive charge (23). Cavamax W7 is electrically neutral (27). So, the net charge on the vesicles was due to the drug and soya lecithin. Analysis of zeta potential values indicates higher magnitude of negative charge on Cavamax W7 composite ethosomal vesicles than ethosomes indicating higher entrapment of drug.

Determination of Entrapment Efficiency

Among all Cavamax W7 composite ethosomes, percent drug entrapment (Table IV) was maximum in formulation F9 (98.42±0.147), where as in case of ethosomal formulations, F12 acquired maximum drug with percent drug entrapment of 69.88±0.08. Thus, on comparing F9 and F12, the percent drug entrapment by Cavamax W7 composite ethosomes was 1.4 times higher than ethosomal vesicle. As clotrimazole is water insoluble, lipophilic in nature, so it can easily be enclosed by Cavamax W7 within its hydrophobic cavity and will be readily entrapped within the ethanolic region of ethosomal vesicle. The presence of Cavamax W7 had a positive impact on percent drug entrapment of the vesicular carrier. On the basis of particle size, zeta potential and percent entrapment efficiency formulation F9 was selected among Cavamax composite ethosomes and formulation F12 was optimized as reference ethosomal formulation for preparation of different gels with or without using permeation enhancers. In all, eight gels were prepared using polyethylene glycol 400 and isopropyl myristate (both included in the Food and Drug Administration Inactive Ingredients Guide) as permeation enhancers.

Validation of Experimental Design

The 3^2 factorial design was validated by Design Expert Software version 8.0.5 (Stat-Ease). A general statistical model can be depicted with respect to the data obtained from the formulations subjected to optimization. The model developed can be characterized by using the polynomial equation representing the respective response data. This can be given as follows

$$\% \text{ Entrapment efficiency} = 83.04 - 9.26x_1 - 1.42x_2 - 4.16x_1^2 + 0.75x_2^2 + 1.28x_1x_1^2 + 2.48x_2x_1^2 - 1.16x_1\,x_2^2 - 1.32x_2x_2^2$$

Whereas the final transformed Eq. 2 obtained by removing all the insignificant values was

$$\% \text{ Entrapment efficiency} = 83.04 - 9.26x_1 - 4.16x_1^2 + 2.48x_2x_1^2 \quad (2)$$

From the above polynomial equations, response surface plot was generated which was then used to predict the response of dependent variables at the intermediate levels of independent variables. From the response surface plot (Fig. 3), it was observed that as the levels of both soya lecithin and Cavamax W7 increased, entrapment efficiency increased. The percent entrapment efficiency of the extra design check point formulation (F_x) was found to be 86.49± 8.69%. Comparison with predicted value of 88.14% obtained from the transformed polynomial equation indicated no

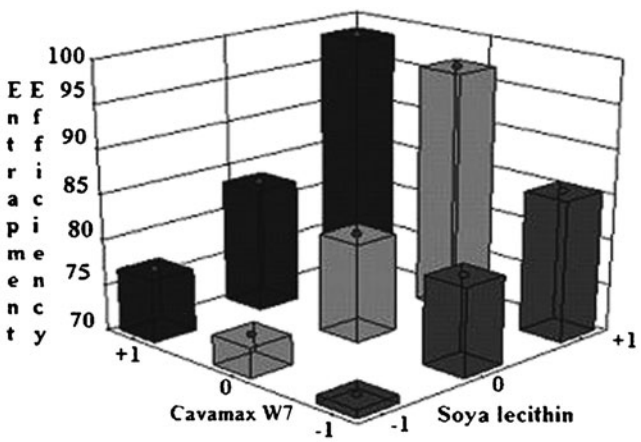

Fig. 3. Response surface plot showing the effect of levels of soya lecithin and Cavamax W7 on entrapment efficiency

significant difference ($p>0.05$) between the two values when checked using pooled t test thus validating the design.

Selection of Optimized Formulation

The optimized formulation of composite ethosomal formulation was identified as F9 on the basis of least vesicle size of 202.8±4.8 nm and highest entrapment efficiency of 98.42±0.15%. Similarly, the ethosomal formulation F12 that was used as reference was selected on the basis of maximum entrapment efficiency of 69.88±0.08%.

Vesicular Characterization by Transmission Electron Microscopy

Vesicular characterization of optimized Cavamax W7 composite ethosomes, F9, and reference ethosomes, F12, loaded with clotrimazole was done by transmission electron microscopy (TEM). TEM images, in both cases, depicted the presence of almost uniform, spherical-shaped vesicles and absence of aggregates. However, Cavamax W7 composite ethosomal vesicles (Fig. 4a) were smaller in size and also the vesicular size distribution was apparently more uniform as compared to reference ethosomal vesicles (Fig. 4b).

Stability Studies

Stability studies were carried out by storing the samples at 4°C to investigate the long-term stability of the vesicles (28) at its storage condition which depicted the storage of ethosomal vesicles at refrigerated temperature (4°C). As at higher temperatures, chemical degradation of lipid bilayers was reported which leads to higher drug loss as well as the defects in membrane packing (29). The stability data of optimized Cavamax W7 composite ethosomal (F9) and reference ethosomal (F12) formulation has been summarized in Table V. As indicated, there was no significant difference in vesicular size, zeta potential, and percent entrapment efficiency of Cavamax W7 composite ethosomal formulation before and after storage. This dictated good stability of Cavamax W7 composite ethosomal formulation upon storage because of the combinatorial stable effect exerted by the presence of both Cavamax W7 and ethanol. Incorporation of beta CD is reported to improve the stability of the vesicles (30) and ethanol provides a net negative charge on the surface of the particles avoiding aggregation of vesicles due to electrostatic repulsion (31).

Evaluation of Cavamax W7 Composite Ethosomal Gel of Clotrimazole

pH, Viscosity, and Drug Content

The gels were transparent with faint odor of raw materials used. The pH of clotrimazole-loaded Cavamax W7 composite ethosomal gel and ethosomal gels were evaluated and the value was found to be in range of 6.8±0.10 and 7.3±0.10 (Table VI). As pH of all the formulations lies between the normal physiological pH range of the skin, *i.e.*, 3.0–9.0 (25), the formulations will be non-irritating to the skin. Higher pH or overly acidic pH of the topical products, alter the pH of the skin, thereby, causing redness, dryness, and irritation. Viscosity of all the gels was found in the range of 38,854.0±5.29 and 52,505.3±5.51 cp. Determination of viscosity of gel matrix, as reported by Mura *et al.* (32), is important as it assists in evaluating the penetration across the skin by controlling the release of the drug into the receptor medium.

Drug content of Cavamax W7 composite ethosomal gel ranged between 97.12±1.7% and 97.89±1.8% whereas in case of reference ethosomal formulations, it was found between 78.68±9.4% and 81.65±9.9% (Table VI) clarifying that the drug was dispersed homogeneously throughout the gels. Drug content of Cavamax W7 composite ethosomes was found to be higher as compared to reference ethosomes due to the obvious reason of presence of Cavamax W7 in composite ethosomes.

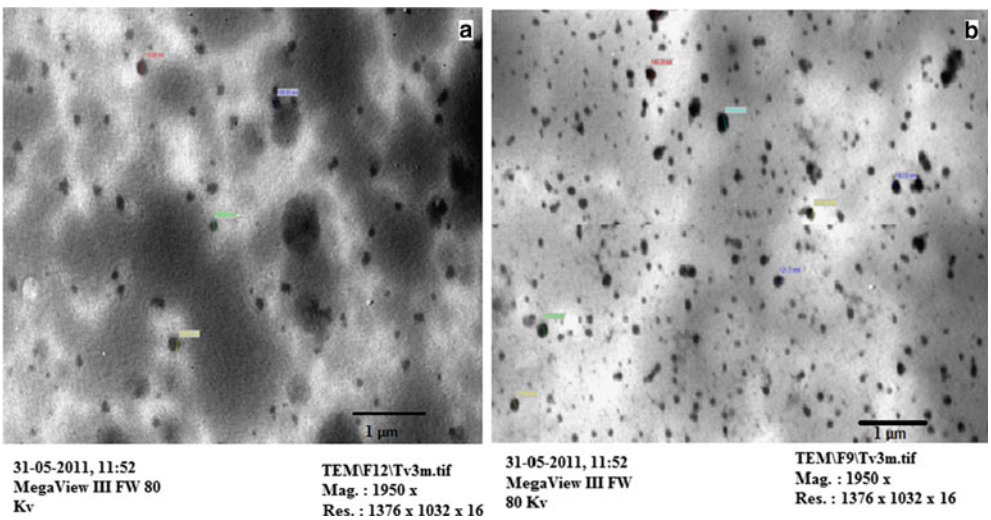

Fig. 4. Transmission electron microscopic image of **a** Cavamax W7 composite ethosomes, F9; **b** reference ethosomes, F12 of clotrimazole

Table V. Stability Study Data of the Optimized Clotrimazole Loaded Cavamax W7 Composite Ethosomes (F9) and Reference Ethosomes (F12)

Formulation code	Time period (months)	Parameters		
		Vesicle size (nm)	Zeta potential (mV)	% Entrapment efficiency
F9	0	202.8±4.8	83.6±0.9	98.42±0.15
	6	227.±13.6	69.8±2.3	85.41±2.80
F12	0	257.2±10.5	65.0±0.2	69.88±0.08
	6	292.2±19.0	54.9±2.4	51.40±2.12

In Vitro Skin Permeability Study: Permeation of Clotrimazole from Cavamax W7 Composite Ethosomal Gel and Reference Ethosomal Gel

In vitro skin permeability studies were done to evaluate the role of Cavamax W7 and ethosomes as a vesicular carrier in enhancing the permeation across the skin. *In vitro* permeability profiles of the formulations in phosphate buffer pH 6.8 Fig. 5a were used to calculate *in vitro* permeability parameters (Table VII). All ethosomal and composite ethosomal formulations were able to reach the target flux of 1.21 μg/cm^2/min irrespective of the presence or absence of permeation enhancers. The target flux was calculated by taking 60% of C_{SS}. The C_{SS} of clotrimazole as reported by Prabagar *et al.* is 1.93 μg/ml (21). It is worth mentioning that the marketed formulation and control gel did not achieve the desired target flux.

The lag time (0.3–0.5 h) of Cavamax W7 composite ethosomal gel was lesser as compared to ethosomal formulations (0.5–0.6 h). Both the lag time values were considerably less than control and marketed formulations. The presence of amphiphilic molecule ethanol in the ethosomes has the ability to penetrate through the biomembrane by causing a reorganization of the phospholipids towards nonlamellar phases. In Cavamax W7 composite ethosomes, in addition to the ethanol effect, Cavamax W7 acts as a permeation enhancer. Thus, it enhances the permeation.

The permeation rate of clotrimazole from G1 to G4 formulations ranged between 23.21±14.08 and 45.31±21.85 μg/cm^2/min$^{0.5}$ whereas for CavamaxW7 (G5–G8) formulations it ranged between 67.75±34.40 and 75.64±25.01 μg/cm^2/min$^{0.5}$ that was higher than marketed and control, formulations. Higher permeation rates from Cavamax W7 composite ethosomal gels was due to higher percent of drug content present in it, which increased the cumulative amount of drug permeated. The permeation rate was further enhanced by the synergistic effect exerted by ethanol and Cavamax W7 in Cavamax W7 composite ethosomes.

In the presence of permeation enhancers, isopropyl myristate (IPM) and PEG 400, in both ethosomal as well as Cavamax W7 composite ethosomal formulations, enhancement in permeation was observed as expected, that is why formulation G8 showed highest percent cumulative drug permeated. IPM is an aliphatic ester and is extensively employed as permeation enhancer in dermatological products. Though, its mode of action is not clear, but, as was described by Mortazavi and Aboofazeli (33) it acts by penetrating between and disrupting the organization of lipid bilayers of stratum corneum whereas polyethylene glycol 400 enhances permeation by decreasing the diffusional resistance of intercellular lipid bilayers. It not only acts by solubilising the lipids but also interfere with the metabolic activities associated with creating an intact barrier (34).

On comparing the permeation profiles with that of control gel profile(reference) using one-way ANOVA significant difference ($p<0.05$) in was observed. Similarity (f_2) and dissimilarity factor (f_1) were also calculated. The f_2 value of all the formulations was less than 50 (Table VII) which implies dissimilarity among permeation profiles. Also, with reference to ethosomal gel (G1), Cavamax W7 composite ethosomal gel permeation profiles (G5–G8) showed higher dissimilarity (f_2 18–28) between two profiles.

Among ethosomal gels (G1–G4), G1 was selected as best formulation as it showed a steady state flux of 1.57±0.23 μg/cm^2/min that followed zero-order release kinetics with minimum lag time of 0.5 h and a release rate of 32.60±8.87 (μg/cm^2/min$^{0.5}$). On the other hand, G5 was selected as optimized gel among Cavamax W7 composite ethosomal gels (G5–G8) as it followed zero-order permeation kinetics, reached the target flux with a steady state flux 3.39±1.45 μg/cm^2/min and lower lag time of 0.3 h. The lag time can be attributed to the sequential events which include release of drug from vesicles, partitioning into the gel structure, traversing the diffusional path length followed by permeation across epidermis (35). The flux achieved by G5 was found to be equivalent to the flux showed by ethosomal gels even with permeation enhancers and was significantly different ($p<0.05$) from ethosomal gel (G1). Patil *et al.* (36) had reported that Cavamax W7 enhanced the permeability by acting as permeation enhancers, thus, assisting in carrying the drug to the biological membrane, *i.e.*, skin. In presence of Cavamax W7, increase in flux is expected, due to the fact that as

Table VI. pH, Viscosity, and Percent Drug Content of Clotrimazole Loaded Ethosomal Gels (G1–G4) and Cavamax W7 Composite Ethosomal Gels (G5–G8)

Formulation code	pH	Viscosity (cp)	Drug content (%)
G1	7.2±0.13	48255.3±5.51	78.68±1.4
G2	7.2±0.06	52505.3±5.51	75.04±2.2
G3	7.2±0.02	40703.3±4.93	79.47±1.4
G4	6.9±0.10	39955.7±6.03	81.65±1.9
G5	7.1±0.16	41707.7±7.09	96.65±1.4
G6	6.8±0.10	42255.3±4.73	97.89±1.8
G7	7.3±0.10	43705.0±5.56	97.12±1.7
G8	6.9±0.06	38540.0±5.29	97.51±2.1

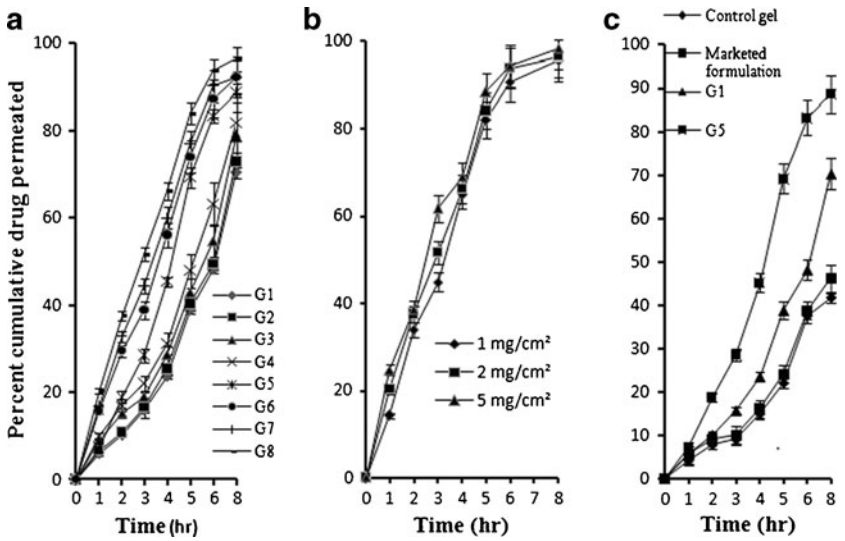

Fig. 5. *In vitro* permeability profile of clotrimazole from **a** different gel formulations in phosphate buffer pH 6.8; **b** depicting effect of drug concentration on percent cumulative drug permeated; **c** comparative *in vitro* permeability profile of clotrimazole loaded Cavamax W7 composite ethosomal gel

the flux is related to the concentration of free drug present and Cavamax can only complex with the drug present in excess of its solubility thus enhancing its flux. Also, the interaction of Cavamax W7 with the skin causing extraction of stratum corneum lipids, thereby, enhancing the skin permeation (37).

Effect of amount of drug per unit area on *in vitro* permeability study (Fig. 5b) was evaluated by calculating the steady state flux at each level of concentration. For every amount/unit area deposited (1, 2, 5 mg/cm^2), the flux was found to increase as the concentration increased. Moving from 1 to 2 mg/cm^2 and finally to 5 mg/cm^2, steady state flux increased from 5.26±1.50, 6.87±1.36, and 7.07±0.65 μg/cm^2/min, respectively. But no significant difference was observed statistically ($p>0.05$). Thus, concluding that the steady state flux was independent of concentration.

Comparison of cumulative drug permeated across the skin and various *in vitro* permeability parameters was done for optimized Cavamax W7 composite ethosomal gel (G5), ethosomal gel (G1), control gel, and marketed formulation (Table VII) showed that Cavamax W7 ethosomal gel (G5) showed maximum cumulative drug permeated (Fig. 5c) and highest steady state flux followed by G1, marketed formulation and control gel which is only a drug suspended in the gelling agent. Significant difference among all the formulations was observed by ANOVA ($p<0.05$, $df=3.8$). The *in vitro* permeation results were further confirmed by CLSM report.

Confocal Laser Scanning Microscopy

CLSM traces the delivery of both hydrophobic and hydrophilic fluorescent probes entrapped in ethosomes. In our investigation, rhodamine B was selected as a fluorescent marker, as it is a lipophilic dye, soluble in ethanol and has been

Table VII. *In Vitro* Permeability Parameters of Clotrimazole Loaded Ethosomal Gels (G1–G4) and Cavamax W7 Composite Ethosomal Gels (G5–G8) Across Rat Skin

Formulation code	% Cumulative drug permeated (8 h)	Flux (μg/cm^2/min)	Permeation rate (μg/cm^2/min^{05})	ER	f_2	Lag time (h)
G1	70.19±2.19	1.57±0.23	32.60±1.87	0.46	46	0.5
G2	72.68±7.51	1.66±0.12	23.21±2.08	0.49	34	0.6
G3	78.57±8.19	3.20±1.55	43.76±1.02	0.94	43	0.6
G4	81.49±5.19	3.55±1.69	45.31±2.85	1.04	40	0.5
G5	88.53±5.10	3.39±1.45	67.75±1.40	1.00	34	0.3
G6	92.19±5.19	5.00±1.44	72.77±2.29	1.47	20	0.4
G7	92.18±4.11	5.67±1.50	75.64±2.01	1.67	19	0.5
G8	96.23±3.22	6.87±1.36	70.21±2.18	2.03	16	0.4
Control gel[a]	41.63±5.13	0.67±0.46	14.02±1.51	0.20	100	0.8
Market formulation	46.09±8.09	1.13±0.06	21.42±0.73	0.33	49	0.7

ER enhancement ratio
[a] Reference formulation for calculation of f_2

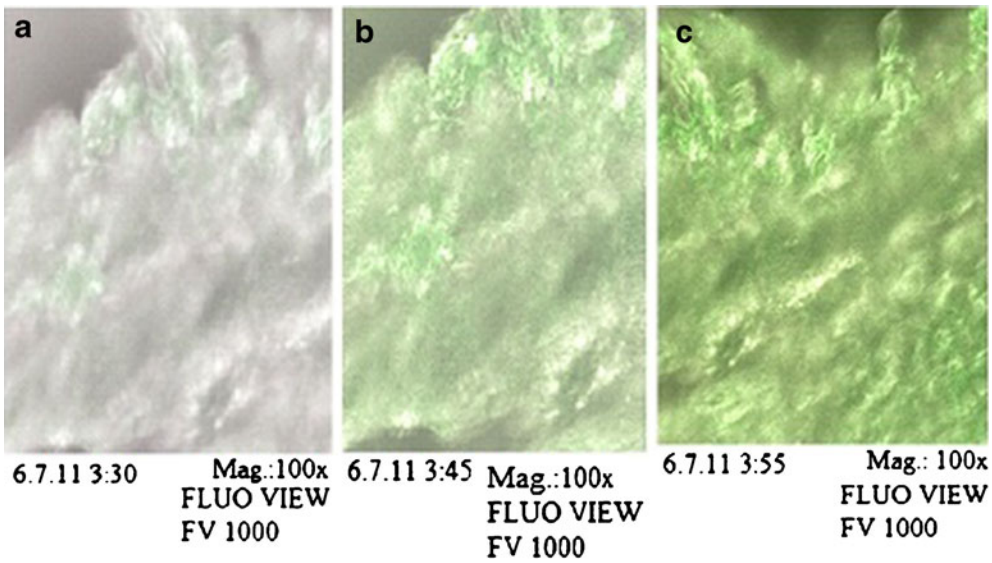

Fig. 6. Photomicrograph of **a** control gel, **b** ethosomal gel, **c** Cavamax W7 composite ethosomal gel through confocal laser scanning microscope at 100×

extensively used in permeability studies (37) and can be easily incorporated within the vesicles. Thus, Cavamax W7 composite ethosomes and reference ethosomes loaded with rhodamine B were prepared. Carbopol gels loaded with these vesicles were then prepared and penetration across the skin was measured by CLSM. Penetration of marker from control gel, reference ethosomal gel and Cavamax ethosomal gel loaded with fluorescent dye rhodamine B was visualized through confocal laser scanning microscope. It was observed that penetration from control gel (Fig. 6a) was confined only to the upper layer of the skin. While in case of ethosomal gel (Fig. 6b), enhanced permeation of rhodamine B was observed deeply into the epidermis. Increased permeation was due to the presence of ethanol that possesses the ability to get interaction with the lipid molecules, resulting in increased stratum corneum fluidity. This in turn enhances inter and intracellular permeability of the ethosomes. Ethanol, inspite of imparting fluidity, also provides flexibility to the ethosomal membrane that enhances the skin permeation across the skin. Aggarwal *et al*. (38) similarly explained the effect of ethanol as a permeation enhancer. Where its fluidizing effect results in disturbance of the skin lipid bilayer organization and this in turn leads to the penetration of soft vesicles through the disorganized stratum corneum lipid bilayer more easily. The permeability across the biomembrane also based on the tolerance and adaptation level in the presence of ethanol. The amphiphilic character of ethanol is related to its ability to partition throughout the membrane by increasing its fluidity. So, the ethosomes permeate very easily inside the deep skin layers, where these get fused with skin lipids and release the drug into the deep layers of skin. However, it was observed that, though the dye penetrated deep into the epidermis, more amount of it was observed to be accumulated at the upper layer. However, in case of Cavamax W7 ethosomal gel (Fig. 6c), penetration of rhodamine B was observed till the last layer (stratum basale) of epidermis and it was uniform throughout the epidermis. Thus, it was found to be superior to control and ethosomal gel in the treatment of deep-seated fungal infections. The results are consistent with the *in vitro* permeability studies.

Antifungal Activity

Antifungal activity was titrated for control gel, reference ethosomal gel (G1) and Cavamax W7 ethosomal gel (G5) against two strains, *i.e.*, *C. albicans* and *A. niger*. Predictively, all the formulations showed antifungal activity against both the strains and the activity was higher for *C. albicans* as compared to *A. niger*. On comparing the zone of inhibition produced for each tested formulation, it was observed that more antifungal activity was exerted by G5 as more value of inhibition zone than control gel (G5>G1>Control gel). Higher drug loaded Cavamax W7 ethosomal gel allowed higher diffusion of drug through the fungal cell membrane, exerting better antifungal activity than ethosomal gel.

CONCLUSION

Cavamax W7 composite ethosomal formulations represented a stable and efficient vesicular carrier for enhanced topical delivery of clotrimazole that exhibited higher entrapment efficiency, and stability than ethosomal vesicles. Steady-state flux of Cavamax W7 composite ethosomes was higher than the reference ethosomes. *In vivo* CLSM study confirmed the uniform and permeation of Cavamax W7 ethosomes deep into the epidermis. Thus, Cavamax W7 composite ethosomes signifies to be a superior ethosomal system for topical drug delivery.

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