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Formulation and evaluation of tizanidine hydrochloride loaded ethosomes for transdermal delivery

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Abstract:

Objective: The present study aims to develop ethosomes loaded with tizanidine hydrochloride and formulated them as a topical gel with an aim to provide sustained delivery of the drug through transdermal route.

Method: Ethosomes were prepared by cold method. The independent variables were concentration of phospholipid and ethanol while entrapment efficiency (Y1) and flux (Y2) were dependent variables. The ethosomes were optimized by Taguchi OA (L9) experimental design using Minitab 18 English software.

Results and Discussion:

Ethosomes provide better flux and reasonable entrapment efficiency. Optimized ethosomal formulation ET6 composed of 3% soya phosphatidyl choline and 40% ethanol with a mean particle size of 83.4 nm, zeta potential of -35 mV and it showed 72.87 % entrapment efficiency and achieved a mean transdermal flux of 32.4 µg/cm^2/hr. Based on SEM studies the vesicles were observed as spherical and unilamellar. *Ex-vivo* permeation studies showed sustained release of the drug from ethosomal formulation. Stability studies were conducted at 4˚C and room temperature, formulations were stable. ET6 formulation was incorporated in 0.9% Carbopol 940 for the preparation of gel and evaluated for drug content, viscosity and *ex-vivo* drug permeation studies. Ethosomal gel showed a flux of 24.9 µg/cm^2/hr. Stability studies of gel showed stable at room temperature with no change in clarity, viscosity and drug content. Skin irritation studies showed that gel was non-irritant to rabbit skin.

Conclusion

Ethosomal formulation of tizanidine hydrochloride is an effective as vesicular system and can efficiently deliver the drugs through transdermal route to treat spasms, cramping and tightness of muscles.

Keywords: Transdermal route; Ethosomes; Cold method; Taguchi design; Entrapment efficiency; Fux;

INTRODUCTION

Transdermal drug delivery system (TDDS) showed promising result in comparison to oral drug delivery system as it eliminates gastrointestinal involvement and first pass metabolism of the drug. The skin acts as a major target as well as a principle obstruction for topical/ transdermal drug delivery. Stratum corneum (SC) permits only the lipophilic drugs having molecular weight < 500 Daltons [1, 2, 3] and acts as a barrier. Several approaches have been attempted to overcome this property of skin, which includes the use of chemical enhancers like surfactants, organic solvents, physical enhancers such as iontophoresis, sonophoresis, microneedles, electroporation etc. and various methods have been assessed to increase permeation and amongst them the best is lipid vesicles can modulate barrier property of SC [4, 5]. Vesicles act as carrier systems, able to transport large molecular weight drugs into the skin or even into the systemic circulation [6].

Conventional liposomes have been generally reported as carriers of drugs with minimal diffusion into deeper tissues, due to their large size and lack of flexibility [7, 8]. After careful research led to the development of a new class of lipid vesicular system that are shows ultraelastic property and which were considered as ethosomes. Touitou et al., (1998) discovered lipid vesicular systems embedding ethanol in relatively with higher concentration [9].

Ethosomes are soft malleable lipid vesicles made of phospholipids and ethanol and water for enhanced delivery of active agents. Ethosomes are composed of phospholipids, but can contain 20-45% ethanol. Ethosomes are commonly prepared by first dissolving the lipids and drug in ethanol, then adding the aqueous component in small quantities with thorough mixing. High ethanol content results in ethosomes being much smaller than liposomes, negating the need for size reduction. Furthermore, ethanol enhances the solubility of more hydrophobic drugs. Ethosomes enhance permeation of drug through skin for transdermal and dermal delivery. Due to high flexibility of ethosomal membranes because of excess ethanol, these are crossed or entered into the skin their self through pores, became much smaller than their actual size. Ethosomes are soft, flexible vesicles and extensive dosage forms can efficiently load higher quantities of drug and permeate in depth to the skin than conventional liposomes [10, 11, 13, 14].

Tizanidine hydrochloride is a drug that is used as a centrally acting skeletal muscle relaxant, is used to treat spasms, cramping, spasms and tightness of muscles. It's particularly useful in treating painful night-time spasms, because its effects last for only 3-6 hours. Tizanidine hydrochloride has a half-life of 2 to 4 hours and undergoes extensive first pass metabolism. Approximately 95% of the orally administered dose undergoes metabolism. It has a low bioavailability of 40 to 44%. Thus, it requires frequent administration. In order to eliminate these problems, transdermal delivery has been chosen as it maintains a constant drug level in plasma. So, formulation of transdermal formulation can increase its duration of action and can reduce side-effects as it is most successful non-oral systemic drug delivery system.

MATERIALS AND METHODS

Materials

Tizanidine hydrochloride was obtained as a gift sample from RA. Chem Pvt. Ltd., Hyderabad. Soya lecithin was obtained as a gift sample from Sonic-Biochem extractions Pvt. Ltd., Indore. Phospholipon 80H & 90H was procured from VAV Lipids Pvt. Ltd., Mumbai, India. Absolute ethanol, propylene glycol, methanol, Triethanolamine were procured from S.D. fine chemicals Ltd., Mumbai, India and Carbopol were procured from Corel Pharma Chem, Ahmedabad. All other chemicals and reagents used were of analytical grade.

Preformulation Studies

Analytical Methodology

UV spectrophotometric method was used to detect the drug for drug content, % encapsulation efficiency, *in-vitro* drug release and *ex-vivo* permeation samples. Analysis was carried out by an UV–Visible double spectrophotometer (Chemito 2600 double beam spectrophotometer). For *in-vitro* in 7.4 pH phosphate buffer with SLS was used.

Drug excipients compatibility studies

Drug excipients compatibility studies by Fourier Transform Infra-Red (FTIR) Spectroscopy to check compatibility of drug and excipients.

Preparation of ethosomes & Preliminary trials

Ethosomes were prepared by cold method. For preliminary screening of ethosomes, different types of phospholipids (Phospholipon 80H, Phospholipon 90H & soya lecithin), different concentrations of phospholipids (2-4%) were used **(Table 1)**. Tizanidine hydrochloride were dissolved in ethanol of different percentages (20-40%) in a covered vessel at room temperature at 700 rpm. Water was heated up to 30 ˚C in a water bath and was slowly added in a fine stream to the lipid mixture under magnetic stirring in a closed vessel. Continue stirring for 5 min and then allowed to cool to room temperature for 30 min. Then, probe sonicated to decrease the size to desired levels and stored in a stoppered glass bottle at 4˚C. The beaker containing phospholipid and drug in ethanol was closed with an aluminum foil to prevent evaporation of ethanol [11, 12, 13]. Prepared formulations were evaluated for entrapment efficiency and ex-vivo permeation studies and were optimized.

Optimization of the ethosomes formulation using Taguchi OA L9 design experiment

Based on the preliminary screening results, soya lecithin showed higher entrapment efficiency, *in-vitro* release profile and better surface morphology. So, ethosomes were prepared with different concentrations of soya lecithin and different percentages of ethanol (ET1 to ET9) and optimized using Taguchi robust design. The formulations were characterized for different parameters.

Taguchi L9 orthogonal array was used for choosing the best and optimized ethosomes formulation using the Minitab 18 English software. Taguchi experimental design was used to study the effect of Soya Lecithin (at three levels of 2 %, 3 % and 4 %) and Ethanol (at three levels of 20 %, 30 %, and 40 %) (**Table 2**). Two factors (independent variables) such as Percentage of soya lecithin and Percentage of ethanol were studied at all the three levels. Entrapment efficiency was taken as the response (dependent variable). The resultant formulations were studied for drug content, *in-vitro* dissolution study, *ex-vivo* studies. **Table 3** indicates Taguchi L^9 OA experimental design runs formula ET1 to ET9 for preparation of ethosomes by cold method**.**

Table 1: Preliminary screening composition of ethosomes prepared with different phospholipids

Formulation Code	Tizanidine hydrochloride (mg)	Phospholipid (% w/v)	Ethanol (% v/v)	Distilled water(% v/v) (upto 10ml)
Phospholipon 80H				
F1	4	2	30	q.s
F2	4	3	30	q.s
F3	4	4	30	q.s
F4	4	2	40	q.s
F5	4	3	40	q.s
F6	4	4	40	q.s
Phospholipon 90H				
F7	4	2	30	q.s
F8	4	3	30	q.s
F9	4	4	30	q.s
F10	4	2	40	q.s
F11	4	3	40	q.s
F12	4	4	40	q.s
Soya lecithin				
F13	4	2	30	q.s
F14	4	3	30	q.s
F15	4	4	30	q.s
F16	4	2	40	q.s
F17	4	3	40	q.s
F18	4	4	40	q.s

Design of experiment for choosing the best and optimization formulation

Table 2: Taguchi L^9 orthogonal array (3^3) design of experiment

Factors (Independent variables)	Level 1	Level 2	Level 3
Factor A	2%	3%	4%
Factor B	20%	30%	40%

Note: Factor A: Percentage of soya lecithin; Factor B: Percentage of ethanol;

Table 3: Taguchi L^9 OA experimental design runs formula for preparation of ethosomes

Material	E T1	E T2	E T3	E T4	E T5	E T6	E T7	E T8	E T9
Tizanidine hydrochloride (mg)	4	4	4	4	4	4	4	4	4
Soya lecithin (% w/v)	2	2	2	3	3	3	4	4	4
Ethanol (% v/v)	20	30	40	20	30	40	20	30	40
Distilled water (% v/v)	Up to 10 ml								

Characterization and evaluation for optimization of ethosomes

Total 9 formulations (ET1 to ET9) of ethosomes were prepared by cold method. The prepared ethosomes were characterized for % entrapment efficiency, vesicle size analysis, surface morphology, *In-Vitro* drug release studies, *ex-vivo* permeation studies, evaluations of ethosomal gel, skin deposition study, skin irritation study and stability study [14,15].

% Entrapment efficiency

Entrapment Efficiency is determined by using an ultracentrifuge. Free tizanidine Hcl was separated from the ethosomes by centrifugation at 15000 rpm at 4˚C for 15 min in two cycles to separate the drug containing ethosomes from unentrapped drug. Whereas the drug loaded ethosomes will settle at the bottom. Then supernatant containing the free drug was diluted and analyzed in UV visible spectrophotometer at 318 nm [14,15]. The % EE calculated by the formula% $\mathbf{E.E} = \frac{\text{Total amount of drug} - \text{amount of drug detected in supernatant}}{\text{Weight of total drug added}} \mathbf{X\ 100}$

***In-vitro* drug release studies**

The permeation of ethosomes was determined by using a Franz diffusion cell. The effective surface area of the cell was 2.0 cm^2 and had a receptor volume of 20 ml. Dialysis membrane was cut and soaked in buffer overnight. The dialysis membrane was mounted between the donor and receptor compartment. Weighed amount of ethosomal formulation was placed on one side of the dialysis membrane. The receptor medium was kept with phosphate buffer saline pH 7.4, with constant heat using a thermostatic hot plate with a magnetic stirrer. The receptor fluid was continuous stirring by Teflon-coated magnetic bead fitted to a magnetic stirrer (Remi). At suitable intervals, 1 ml of aliquot was withdrawn and replaced with fresh media. The samples were analyzed by UV spectrophotometrically [14,15].

***Ex-vivo* studies of optimized formulation**

Ex-vivo studies performed after obtaining the approval of the Institutional Animal Ethical Committee (IAEC) and in accordance with disciplinary principles and guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA). (320/CPCSEA; Dated 03-01-2001 and ID number GPRCP/IAEC/10/18/02/PCE/AE-I-Rats-M-21; G. Pulla Reddy College of Pharmacy, Mehdipatnam, Hyderabad, India). The permeation of ethosomes was determined by using a Franz diffusion cell. The effective surface area of the cell was 2.0 cm^2 and had a receptor volume of 20 ml. The male wistar rat skin was mounted on the receptor compartment with the stratum corneum side upwards into the donor compartment. The donor compartment was applied with the ethosomal formulation. 20 ml of pH 7.4 phosphate buffer saline was used as receptor medium to maintain the sink condition. The receptor compartment was maintained at 37 ˚C and stirred by a magnetic bead (Set up of *Ex-vivo* permeation studies as shown in **Figure 1)**. At appropriate intervals, 1 ml of aliquot was withdrawn and replaced with fresh media. The samples were analyzed by UV spectrophotometrically [14,15]. *Ex-vivo* permeation studies such as % drug release and steady state transdermal flux were estimated for different formulations.

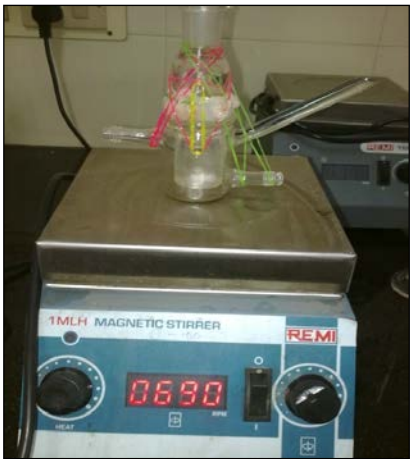

Figure 1: Set up of *Ex-vivo* permeation studies

Calculation of Permeability Parameters

i. Steady state flux (µg/cm²/hr.): Steady state flux (Jss) is defined as the rate of diffusion or transport of a substance through a permeable membrane.

$$SSTF = Q/t \times A$$

Where, SSTF= Steady state transdermal flux (µg/cm2/hr); Q= Amount of permeated drug (µg); t= time (hr); A = area of release membrane (cm^2)

ii. Permeability coefficient (cm/hr.): The permeability coefficient (Kp) was calculated with the following equation:

$$Kp = {}^{Jss}/_{CV}$$

Where, CV is the total donor concentration of the formulation.

b. Calculation of release kinetics for optimized formulation

Various models were tested for explaining the kinetics of drug release. To analyze the mechanism of the drug release rate kinetics of the dosage form, the obtained data was fitted into zero-order, first-order, Higuchi and Korsemeyer-Peppas release model, to study the drug release from the dosage form [16,17,18, 22].

Surface morphology by SEM

The surface morphology (roundness, smoothness and formation of aggregates) of ethosomes was studied by electron microscopy and scanning electron microscopy. The ethosomal formulation was affixed to double sided carbon tape, positioned on an aluminum stub and excess suspension was removed. The stubs were sputter-coated with gold. Electron micrographs were obtained using scanning electron microscopy [14,15, 22].

Vesicle size

The vesicle size was measure by Delsa Nano. The formulation diluted with deionized water before the size measurements [14,15, 22]. The particle sizes were measured at 25˚C.

Zeta potential determination

Zeta potential was determined by measuring the electrophoretic mobility using Malvern Zetasizer Nano ZS 90 (Malvern Instruments, UK). The field strength applied was 20 V. Prior to the measurement, all samples were diluted in distilled water [14,15, 22].

Preparation of ethosomal gel

Gel was prepared by taking 0.9 and 1 % Carbopol 940. Weighed amount of carbopol was dissolved in distilled water under constant stirring. To this, required amount of propylene glycol was added. Triethanolamine was added to achieve required consistency. To this gel base, ethosomal suspension was added such that 1g contains 4% tizanidine hydrochloride. The prepared ethosomal gel was evaluated for clarity, homogeneity, pH, spreadability, viscosity, drug content, extrudability, skin irritation and stability studies [14, 15, 20, 21, 22].

Physicochemical Evaluation of Ethosomal Gel:

Physicochemical evaluations like clarity, homogeneity, pH, spreadability, viscosity, drug content, Extrudability [20, 21, 22] were performed for ethosomal gel.

Clarity

It was determined by visual inspection under black and white background and it was graded as turbid: +, clear: ++, very clear (glossy): +++

Homogeneity

It was determined by visual inspection of gel for the appearance and presence of any aggregates.

Determination of pH

Formulation pH is determined by dispersing 0.5 g of gel in 100 ml of 7.4 phosphate buffer saline. It was checked using digital pH meter at constant temperature. Prior to this, the pH meter was calibrated and then electrode was washed with demineralized water. The electrode was then directly dipped into gel formulation and constant reading as noted.

Spreadability

The spreadability of the gel formulations was determined by measuring the spreading diameter of 1g of the gel between 20X20 cm glass plates for 1min.The mass of the upper plate was standardized at 150g. The spreadability was calculated by using the formula.

Spreadability (S) = m x lt

Where, S = Spreadability (g.cm/sec); m = weight tied to the upper glass slide (g); l = length of the glass slide (cm); t = time taken in seconds (sec)

Determination of viscosity

Viscosity of prepared gels was determined by Brookfield programmable viscometer LVDV-II+PRO. The spindle number 64 was rotated at 50rpm. Samples of the gels could settle over 30 minutes at the temperature 25±1˚C before measurements were taken.

Drug Content

100 mg of gel (equivalent to 0.4mg of drug) was taken and dissolved in 100ml of pH 7.4 saline phosphate buffer. The solution was passed through the Whatman filter paper no.42 and filtered. Appropriate dilutions were done, and the drug content was measured spectrophotometrically at 318 nm.

Extrudability

The extrudability test was carried out by Pfizer hardness tester. A 15 gm of gel was filled in aluminum tube. The plunger was adjusted to hold the tube properly. The pressure of 1kg/cm^2 was applied for 30 sec. The quantity of gel extruded was weighed. The procedure was repeated at three equidistance places of tube. Test was carried out in triplicate.

Skin Irritation Study

Skin irritation study was performed by using control, standard skin irritant, placebo and test which were applied on the left and dorsal surface of rabbit skin and rabbits were examined for 8 hrs and erythema and edema was evaluated and the score was given according to the Primary Dermal Irritation Index classification (PDDI) (PDDI irritancy level: 0.0-Non-irritant; 0.0 to 0.5-Negligible irritant; 0.5 to 2.0- Mild irritant; 2.0 to 5.0-Moderate irritant; 5.0 to 8.0-Severe irritant;) [14, 15, 20, 21, 22].

Stability Studies

The optimized formulation was evaluated for physical stability testing to investigate the leaching of drug from the vesicles. The ethosomal formulation was sealed in 10ml glass vials and stored at refrigeration temperature (4˚C-8˚C) and stored at 30˚C (RT) for two months. The entrapment efficiency of all the samples was determined for every month in the same manner as described previously. Ethosomal gel was evaluated for drug content, viscosity and spreadability [14, 15, 20, 21, 22].

RESULTS & DISCUSSIONS

Analytical Methodology

The analytical method development for tizanidine hydrochloride was performed for the determination of absorption maxima of the drug scanned in phosphate buffer saline pH 7.4 for its absorption maxima and was found that tizanidine hydrochloride exhibited maximum absorption at 318.0 nm.

Drug excipients compatibility studies by FTIR

Tizanidine hydrochloride compatibility with the excipient was studied by FTIR. The FTIR spectra of formulation with ethanol and phospholipid revealed no interaction between the drug and excipient. The FTIR studies from the spectra confirmed the absence of any chemical interaction between the drug and phospholipid. The FTIR spectra are shown in **Figure 2**.

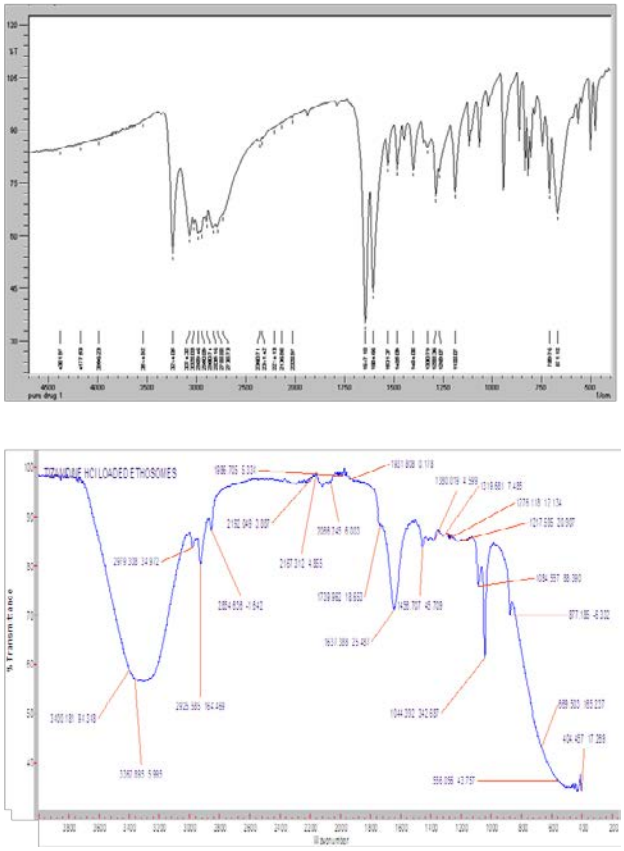

Figure 2: FTIR spectrum of Tizanidine Hcl and FTIR spectrum of optimized formulation

Preliminary screening evaluation of ethosomes

Ethosomes of Tizanidine hydrochloride with different concentrations of phospholipon 80H (F1 to F6), phospholipon 90H (F7 to F12), soya lecithin (2, 3 & 4%) (F13 to F18) and different percentages of ethanol (30 & 40%). Formulations F1 to F18 were evaluated for percentage entrapment efficiency, *in-vitro* drug release showed on **Table 4**.

The entrapment efficiency was found to be an increase in percent drug entrapment with an increase in ethanol concentration. Improvement of aqueous solubility of tizanidine hydrochloride due to co-solvent effect of ethanol could be the possible reason for increased entrapment due to increased ethanol concentration. Ethosomes prepared with phospholipon 80H showed lower entrapment efficiencies. Ethosomes prepared with phospholipon 90H showed lower entrapment efficiencies. The entrapment efficiency increases with increase in the percentage of ethanol and phospholipid due to co-solvent effect of ethanol and increase in the concentration of ethanol leads to lower particle. Ethosomes prepared with soya lecithin showed higher entrapment efficiency.

Table 4: Preliminary screening composition of ethosomes prepared with different phospholipids

Formulation Code	% Entrapment efficiency	*In-vitro* % drug release of ethosomes in 24 hours
F1	30± 0.8	30.67±0.5
F2	40±1.2	36.93±0.5
F3	38±0.91	41.56±0.9
F4	43±1.4	44.78±0.6
F5	50± 0.61	49.89±1.7
F6	46±1.5	32.88±1.9
F7	35±0.32	28.87±1.43
F8	47±0.76	39.76±1.54
F9	50±0.67	36.69±1.98
F10	32±1.43	40.87±1.45
F11	38±1.1	44.51±1.72
F12	45±0.81	35.87±1.55
F13	56±0.61	52.98±1.9
F14	53±0.87	68.34±1.7
F15	65±1.12	58.65±1.3
F16	58±1.21	63.61±0.1
F17	71±0.4	61.34±1.6
F18	62±0.73	57.38±1.23

Note: All values expressed in mean ±SD, n=3

Optimization of proliposomal powder of dolutegravir sodium using Taguchi OA L9 design experiment

Analysis of the results by Taguchi designs

Taguchi L9 orthogonal array was used for choosing the best and optimized ethosomal formulation using the Minitab 18 English software.

Formulations were prepared based on the experimental run's formula from Taguchi design. Total 9 formulations (ET1 to ET9) of ethosomes were prepared by cold method. Ethosomes of Tizanidine hydrochloride with different concentrations of phospholipon 80H, phospholipon 90H,

soya lecithin (2, 3 & 4%) and different percentages of ethanol (30 & 40%). Prepared formulations were evaluated for percentage entrapment efficiency, *in-vitro* drug release, *ex-vivo* permeation studies and vesicle size were done to optimize the formulation. Main effects plot for SN ratios and means a) Concentration of soya lecithin b) Concentration of ethanol are shown in **Figure 3.**

% Entrapment efficiency

Ethosomes prepared with different phospholipids were evaluated for entrapment efficiency using centrifugation. Their % entrapment efficiencies were showed in **Table 5.** Drug entrapment efficiency of a vesicle is an important parameter to evaluate any vesicular preparation. So, the influence of different phospholipid concentration and alcohol percentage drug loading of ethosomes was evaluated. It was found that increased concentration of soya lecithin increased the entrapment efficiency up to 3% upon which there was no significant increase in entrapment efficiency. This may be due to higher encapsulation in lipid bilayer of formulation. Higher alcohol percentages lead to decreased particle size and increased entrapment efficiency up to 40% w/v of ethanol concentration [14, 14, 20, 21].

Table 5: Entrapment efficiency of ethosomes prepared with different concentrations of soya lecithin and ethanol

Code	Entrapment efficiency
ET1	53±0.3
ET2	65±0.5
ET3	66.2±0.2
ET4	68±1.5
ET5	79.5±0.6
ET6	70±1.8
ET7	55±1.8
ET8	64±0.1
ET9	61±1.6

ET1= 2% SPC & 20% ETOH, ET2 = 2% SPC & 30% ETOH, ET3= 2% SPC & 40% ETOH, ET4= 3% SPC & 20% ETOH, ET5= 3% SPC & 30% ETOH, ET6=3% SPC & 40% ETOH, ET7= 4% SPC & 20% ETOH, ET8= 4% SPC & 30% ETOH, ET9=4% SPC & 40% ETOH.

Note: All values expressed in mean ±SD, (n=3)

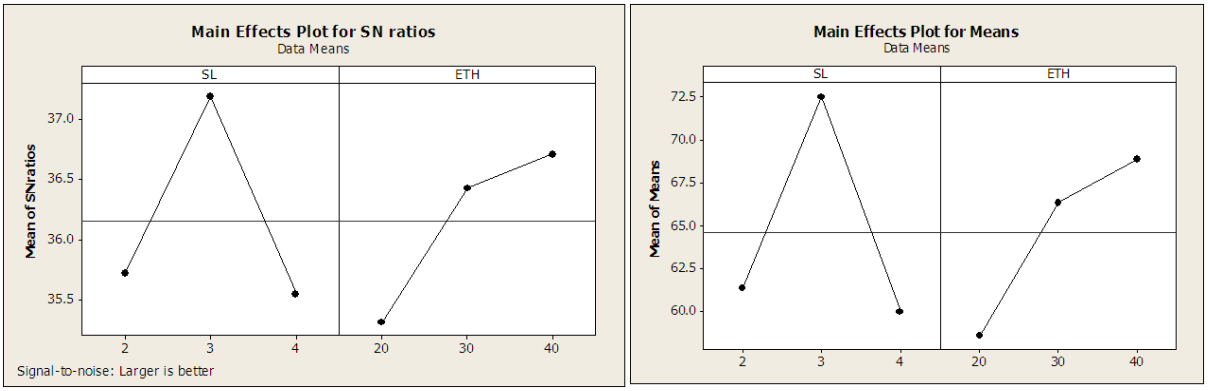

Figure 3: Main effects plot for SN ratios and means a) Concentration of soya lecithin b) Concentration of ethanol

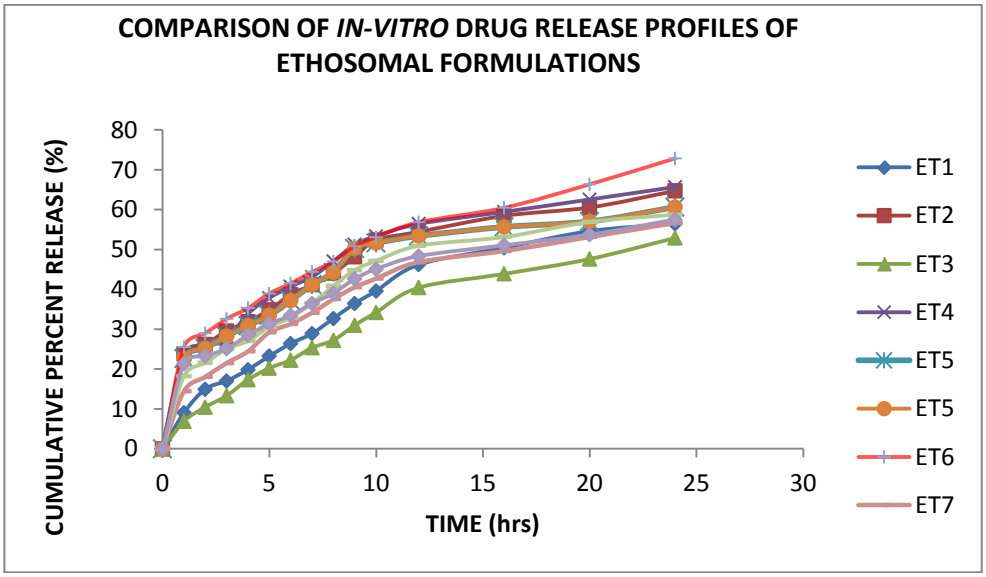

Figure 4: *In-vitro* drug release profile of ethosomes with different concentrations of soya lecithin and ethanol

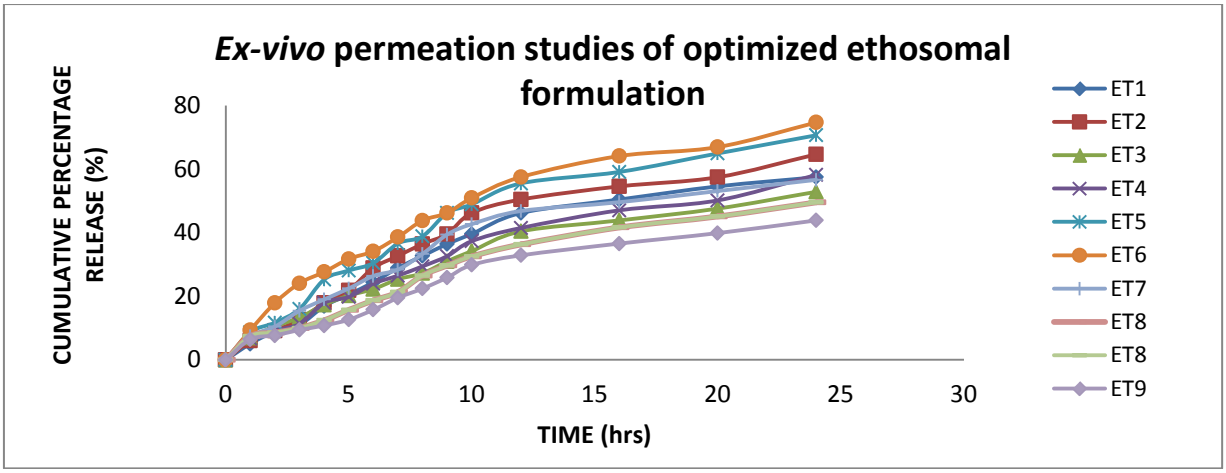

Figure 5:. *Ex-vivo* drug release profile of ethosomes with different concentrations of soya lecithin

Table 6: Permeability coefficient values

Formulation code	Flux: Amount of permeated drug (µg) / Area of release membrane (cm^2) in 24 hours	Permeability coefficient (cm/hr)x 10^{-3}	Q24 (µg/cm^2)	Lag time (hr)
ET1	466.76±1.98	6.2±1.98	466.76±3.98	1.5±0.02
ET2	522.61±5.21	6.825±0.78	522.65±0.78	1.9±0.01
ET3	424.75±2.76	5.525±1.01	424.64±1.01	2.1±0.04
ET4	474.19±1.98	5.925±0.69	474.26±0.69	1.4±0.012
ET5	584.09±2.43	7.85±1.12	584.09±1.12	1.2±0.021
ET6	608.69±1.98	8.1±1.76	608.69±1.76	1.1±0.032
ET7	461.64±1.87	6.3±2.12	461.64±2.12	1.6±0.054
ET8	403.07±1.34	5.15±1.32	403.07±1.32	2±0.087
ET9	358.14±3.76	4.55±1.09	358.14±1.09	2.2±0.032

Note: All values expressed in mean ±SD, (n=2)

***In-vitro* permeation studies using dialysis membrane**

In-vitro permeation studies were conducted using a vertical Franz diffusion cell with an effective surface are of 2.0 cm^2 and a receptor volume of 20 ml. Formulation equivalent to 4 mg of drug was taken in donor compartment. *In-vitro* % release for 24 hours of ethosomes prepared with different concentrations of soya lecithin and ethanol were showed in **Figure 4**.

Ethosomes prepared with 3% soya lecithin and 40% ethanol (ET6) showed an *in-vitro* release profile of 72.87% for 24 hours. There was an enhanced release in this formulation due to an increase in entrapment efficiency mainly due to the presence of ethanol which improves the solubility and there by drug loading and hence entrapment efficiency.

***Ex-vivo* permeation studies using rat skin**

Ex-vivo permeation studies using rat skin was conducted using a vertical Franz diffusion cell with an effective surface are of 2.0 $cm^2$and a receptor volume of 20 ml. Ethosomes prepared with 3% soya lecithin and 40% ethanol showed an *ex-vivo* permeation of 600 µg/cm^2 for 24 hours (**Figure 5**). There was an enhanced release in this formulation due to an increase in entrapment efficiency mainly due to the presence of ethanol which improves the solubility and there by drug loading and entrapment efficiency. As ethanol increases the lipid fluidity by interacting with the skin, it improved the *ex-vivo* permeation of drug through skin [14, 15].

Flux

Mass transport of molecules in a solution or molecular transport across a barrier is normally measured by flux. The flux of a solute is simply defined as the mass or number of molecules moving through a given cross sectional area during a given period of time. It is obtained by plotting a graph between time and cumulative percentage release per 4.91cm^2 area. Slope gives the value of flux and was found to be 32. 4±2.98 µg/min/cm^2 (ET6). Flux calculation showed in **Table 6.**

Permeability coefficient:

The rate of drug permeation through 4.91cm^2 area of the skin per unit time along the concentration gradient gives the value of permeability coefficient (**Table 6**). It is obtained by dividing flux with donor concentration.

$$K_p = J_{ss} / Co$$

J_{ss}=steady state flux (µg/cm^2/min); Co=drug donor concentration (mg/cm^3)

Cumulative release rate (Q24) and Lag time

Cumulative release rate is obtained by dividing cumulative amount permeated through skin data/ area of the patch or exposed inner area of the diffusion cell at 24 hours. Cumulative release rate (Q24) and Lag time showed in **Table 6**.

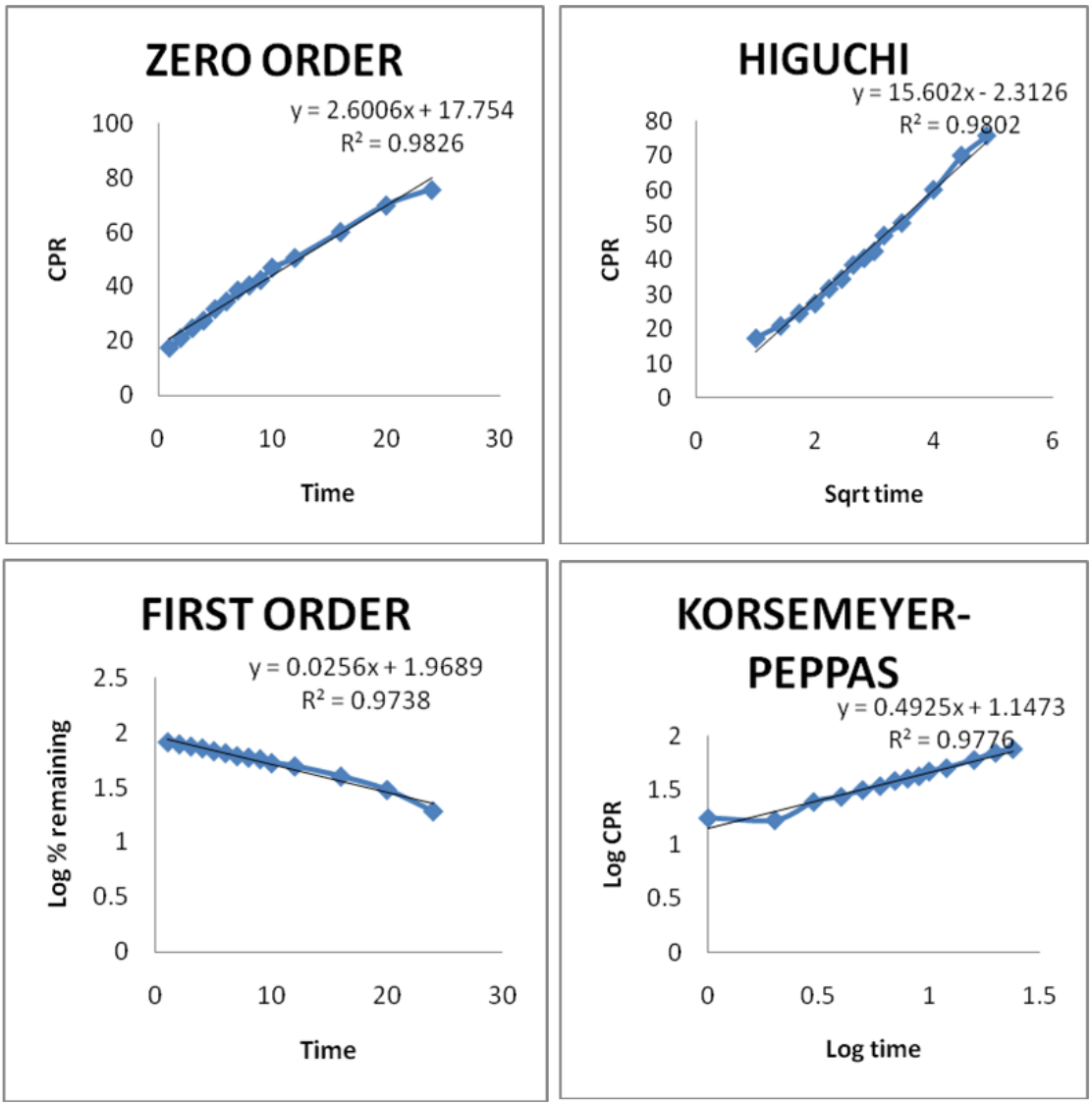

Figure 6: Curve fitting for the optimized formulation

Model dependent kinetics

Model dependent kinetics provides the information related to mechanism of drug release from the dosage form. Four models were plotted based on the data obtained from *ex-vivo* drug diffusion studies and their R^2 values were compared. The model which has the greater R^2 value indicates that the drug follows such mechanism of drug release. Curve fitting for the optimized formulation ET6 showed in **Figure 6.** Based on the R^2 value it was found that the formulation follows Higuchi's release kinetics that is drug release is by diffusion [16, 17, 18].

Surface morphology by SEM

Surface morphology and three dimensional nature of ethosomes was studied through SEM. The optimized formulation was characterized for surface morphology which was analyzed using SEM [14, 22]. SEM photographs showed that surface morphology was spherical as well as the presence of unilamellar vesicular system (**Figure 7**).

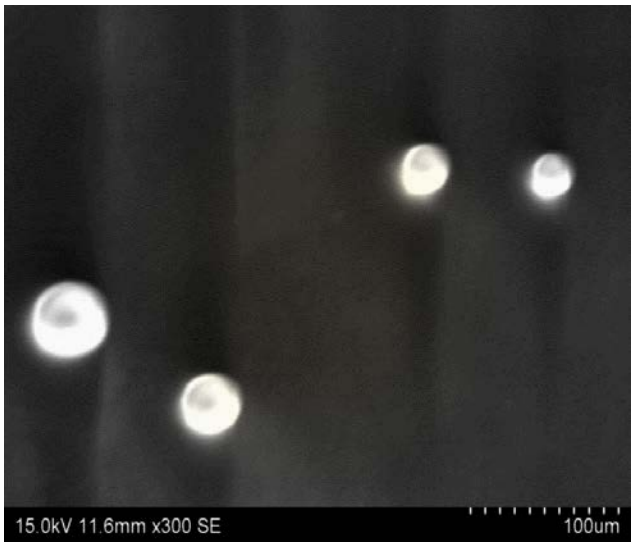

Figure 7: SEM photography of optimized ethosomal formulation (ET6)

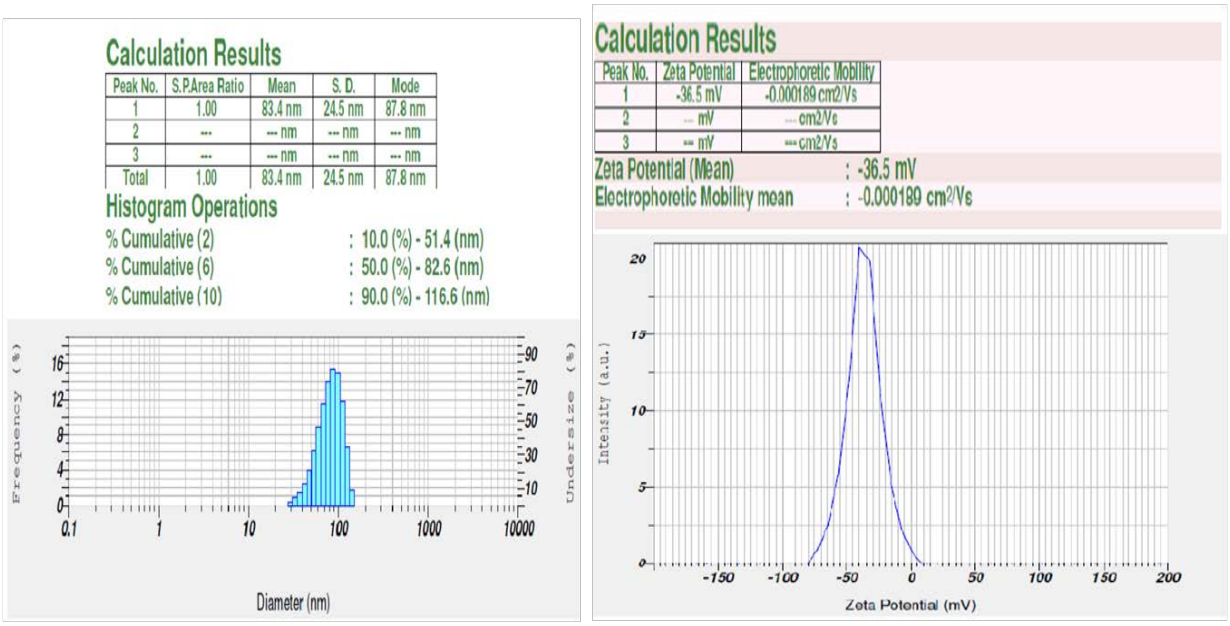

Figure 8: Vesicle size and Zeta potential of optimized ethosomal formulation (ET6)

Table 7: Evaluation of ethosomal gels

Formulation code	Clarity[a]	pH	Spreadability (g.cm/sec)	Extrudability	Homogeniety	Drug content (mg)	Viscosity measurement (cps)
G1	+++	7.2	25.98	+++	Good	3.87	33820
G2	+++	7.0	24.76	+++	Good	3.51	32129

[a] turbid: +, clear: ++, very clear (glossy): +++

Vesicle size analysis

Vesicle size was found to be 83.4 ± 5 nm (**Figure 8**). Increasing concentrations of ethanol decreases the vesicle charge as it imparts a net negative charge & confers the system some degree of steric stabilization that may finally lead to a decrease in the mean particle size.

Zeta Potential

Zeta potential of the optimized formulation for ET6 was found to be -36.5 mV which lies in the range of zeta value for colloidal carriers (**Figure 8**). Zeta potential values which are more positive than +30 mV and more negative than -30 mV are normally considered stable for colloidal dispersion.

Evaluation of ethosomal gel

Physicochemical evaluations like clarity, homogeneity, pH, spreadability, viscosity, drug content, extrudability were performed for ethosomal gel. Ethosomal gel was found to be clear, transparent and homogenous without any lumps. The pH of the gels were found to be in the range of 7.12. The Spreadability results indicated that the gel was found to be 25.98 g.cm/sec, easily spreadable by small amount of shear. The viscosity of gel formulations was found to be in the range of 33820 centipoise. The drug content in the prepared gel reservoirs were found to be in the range of 3.51 to 3.87 mg. This showed that the drug was uniformly distributed in the formulated gels. The extrudability of ethosomal gel was found to be good for both the gels (**Table 7**).

***Ex-vivo* permeation studies**

It was performed by placing gel containing equivalent amount of tizanidine hydrochloride in donor compartment and PBS in receptor compartment using rat skin in a Franz diffusion cell [14] (G1= 0.9 % gel and G2 = 1% gel). *Ex-vivo* permeation studies of ethosomal gels G1 and G2 showed in **Figure 9.** Q24 values of ethosomal gel showed in **Table 8** and permeability coefficient of ethosomal gel showed in **Table 9.**

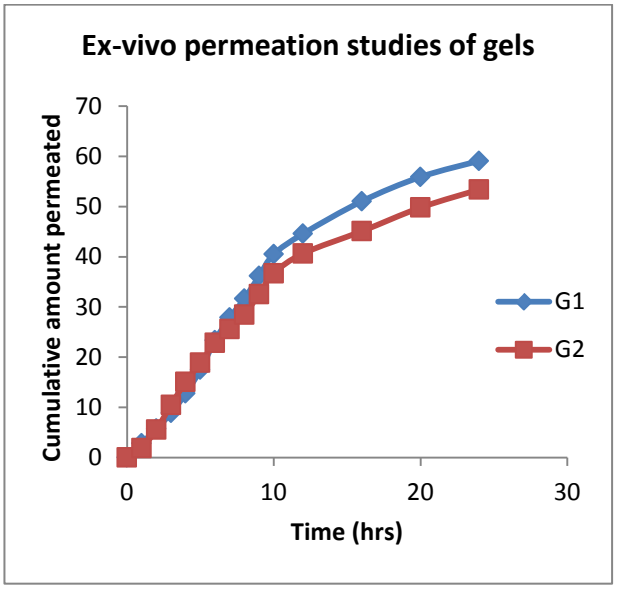

Figure 9: *Ex-vivo* permeation studies of ethosomal gels

Table 8: Q24 values of ethosomal gel

Formulation code	Q24 (µg/cm²)
G1	481±2.32
G2	434±2.52

Note: All values expressed in mean ±SD, (n=3)

Table 9: Permeability coefficient of ethosomal gel

Formulation code	Permeability coefficient (X 10-3 cm/hr)
CNT	5.07±0.02
CHL1	3.78±0.08

Note: All values expressed in mean ±SD, (n=3)

Skin irritation studies

Skin irritation study was performed by using control, standard skin irritant, placebo and test which were applied on the left and dorsal surface of rabbit skin and rabbits were examined for 8 hrs and erythema and edema was evaluated and the score was given according to the Primary Dermal Irritation Index classification (PDDI). This study confirmed that there was no sign of erythema or edema (**Table 10** and **Figure 10**). Erythema was not observed for ethosomal gels indicating that the gel is non-irritant to rabbit skin.

Table 10: Score of skin irritation of ethosomal gel

Test	Score[a]
Erythema	0
Edema	0
Total score of erythema and edema for primary irritation	0

[a]**0.0:**Non-irritant**; 0.0-0.5:** Negligible irritant**; 0.5-2.0:** Mild irritant**; 2.0-5.0:** Moderate irritant**; 5.0-8.0:** Severe irritant

Table 11: Entrapment efficiency of ethosomes during stability studies

Time (months)	At 4˚C (%)	At 30˚C (RT) (%)
0	79.5	79.5
1	79.3	68.2
2	79	60.3

Table 12: Stability studies of 0.9% carbopol containing ethosomal gel

Parameter	Time (months)	At 4˚C	At 30˚C (RT)
Clarity[a]	0	+++	+++
Viscosity (cps)	1	33820	33820
Drug content (mg)	2	3.87	3.71

[a] turbid: +, clear: ++, very clear (glossy): +++

Stability studies

The entrapment efficiency of all the samples was determined for every month in the same manner as described previously (**Table 11** and **Figure 11**). Stability studies of 0.9% carbopol containing ethosomal gel described in **Table 12.** The ethosomal formulation was stable at 4˚C as there was no much change in the entrapment efficiency. The ethosomal gel showed no changes in clarity, viscosity and drug content indicating that the gel system is stable at 4˚C and at room temperature.

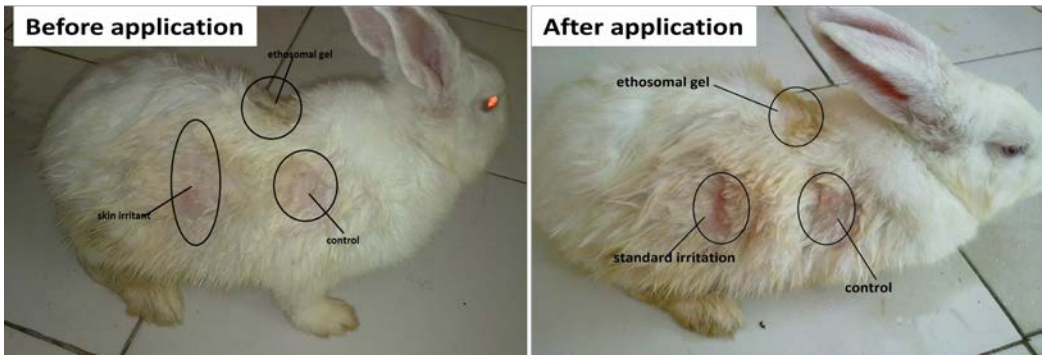

Figure 10: Skin irritation studies- before and after application of ethosomal gel

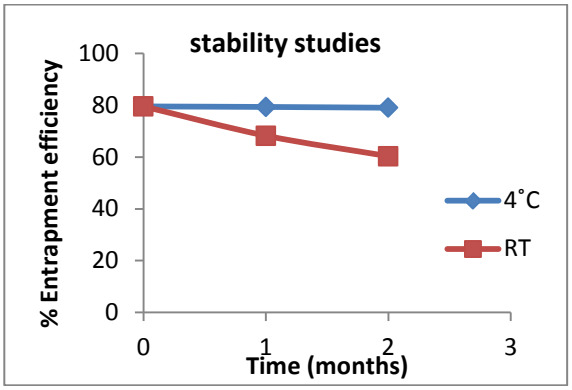

Figure 11: Stability of ethosomal formulation

CONCLUSION

Tizanidine hydrochloride loaded ethosomes were formulated by cold method and optimized using Taguchi design. Results showed that concentration of soya lecithin plays a significant role on ethosomal formulations. Stable unilamellar spherical nanosized vesicles were obtained, indicates that the formulation has good stability. The ethosomes incorporated Carbopol gels indicated that ethosomal formulation was stable at 4 ºC. In conclusion, it was found that ethosomes can efficiently deliver the drugs through transdermal route.

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Conflict Of Interest
The authors declare no conflict of interest.

Abbreviations
None.

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