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SYSTEMATIC DEVELOPMENT OF BINARY ETHOSOMAL NSAID BASED GEL

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ABSTRACT: The present study describes formulation optimization, *in-vitro* evaluation and *ex-vivo* assessment of indomethacin loaded binary ethosomal gel using central composite design. Two formulation factors, concentration of lipid and concentration of ethanol were optimized for their effects on vesicle size (Y_1), zeta potential (Y_2), polydispersity index (Y_3), entrapment efficiency (Y_4) and % cumulative drug release (Y_5). The lipid concentration was found to significantly affect particle size, zeta potential and *in-vitro* drug release and ethanol concentration was found to significantly affect zeta potential while the interaction effects varied with each response. On identification of optimum desirability, an optimized formulation was prepared, characterized and loaded into transdermal gel. The optimized ethosomal formulation showed observed values for Y_1, Y_2, Y_3, Y_4 and Y_5 of 185.5nm, -27mV, 0.237, 87.86% and 45.65% respectively. Ethosomal gels were formulated using carbopol and HPMC and were characterized for pH, spreadability, drug content, viscosity, and % CDR wherein the optimized formulation showed values of 6.85, 31.07 g.cm/sec, 99.87%, 52000cP, and 56.11% respectively. *Ex-vivo* permeation study for the optimized ethosomal gel was conducted and the drug permeation mechanism was identified. Skin irritation studies performed on Wistar Albino rats showed no erythema or oedema. Stability studies at -20 °C and 2-8 °C showed satisfactory results. Comparison of *in-vitro* drug release from ethosomal gel and marketed gel depicted that formulated ethosomal gel improved permeation of drugs. Hence, ethosomal formulation could be considered a suitable drug delivery system especially when loaded into transdermal vehicles with a possible reduction in side effects and improvement in drug permeation.

INTRODUCTION: Indomethacin (IND) is a non-steroidal anti-inflammatory drug (NSAID) and is generally used for the treatment of pain, edema and tissue damage that results due to inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis, *etc.* [1]

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It is often used as the first line of action for treatment of rheumatoid arthritis and ankylosing spondylitis in order to reduce the symptoms and tissue damage until the slow onset of action of DMARDs. Generally, chronic long term therapies of NSAIDs are required to cure the associated symptoms.

IND acts by inhibition of cyclooxygenase that in turn inhibits prostaglandin synthesis, which is necessary for homeostatic maintenance such as platelet aggregation, regulation of blood flow in the kidney and stomach and regulation of gastric acid secretion [1]. Oral administration of IND is often

related to increased risk of adverse effects including mild problems such as dyspepsia as well as chronic problems such as gastric ulcers, perforation, and gastrointestinal bleeding. Therefore, there is greater risk in cases of long term therapies with higher doses that are generally used for the treatment of chronic inflammatory diseases such as rheumatoid arthritis [2]. Oral therapy of NSAIDs is not recommended due to these various mild as well as severe adverse effects and increased risks in long term high doses therapy.

In order to minimize the increased risks of oral administration of NSAIDs, the transdermal route of delivery could prove to be a better alternative. It has several advantages over the oral route wherein the therapeutic agent can directly reach the inflammation site which helps in the reduction of systemic adverse effects as well as in the reduction of the first-pass metabolism. The level of a therapeutic agent in the bloodstream is often lower as compared to oral administration thus helps in minimizing the side effects and improving its safety. Also, possible drug-drug interactions can be avoided by omitting the gastrointestinal tract.

However, the transdermal route of drug delivery has several drawbacks, mainly the stratum corneum barrier that limits the permeability of several exogenous substances as well as therapeutic agents. The achievement of therapeutic effect at the target site depends on the nature of the drug as well as its chemical composition and physical microstructure. Considering the barrier properties of the stratum corneum and the physicochemical properties of the therapeutic agent, several chemicals, as well as mechanical penetration enhancer techniques, have been developed.

Another approach to overcome this problem is the development of novel carrier systems such as liposomes, transferosomes, ethosomes, *etc*. that can enhance the penetration of the therapeutic agents [2].

The present study aims at formulating a binary ethosomal gel of IND for transdermal delivery by a cold method. Ethosomes are novel lipid-based vesicular systems comprising phospholipids and a higher concentration of ethanol which makes them soft and malleable thus improving penetration into the deeper layers of the skin. Also, ethanol gives an add-on effect for improving penetration through stratum corneum by disrupting the lipid bilayer of stratum corneum. These vesicles, in turn, will overcome the problem of the stratum corneum barrier and will help in delivering the therapeutic agent in the deeper layers of the skin.

Ethosomal carriers have the following advantages: [3]

- Increases the efficacy and therapeutic index of the drugs.
- Reduces toxicity of the encapsulated agent.
- Improves permeation through the skin under occlusive as well as non-occlusive conditions.
- Delivery of hydrophilic, lipophilic and amphiphilic agents, as well as large molecules such as proteins and peptides, can be achieved.

Objective of the Study is:

- ✓ To systematically develop and optimize Indomethacin loaded binary ethosomes for transdermal delivery in order to prevent the gastrointestinal side effects caused by oral administration of Indomethacin.
- ✓ To improve penetration of Indomethacin into the deeper layers of the skin by encapsulation in ethosomal vesicles.
- ✓ To incorporate the ethosomal vesicles into a suitable gel base for ease of application.
- ✓ To provide a quick onset of action as required for pain management in rheumatoid arthritis as the ethosomal vesicles penetrate the deeper layers of skin and get accumulated at the inflamed tissues, thus providing faster onset of action. The accumulation of ethosomes in the skin or tissue serves as a drug reservoir, thus providing a gradual release of the drug from the carrier system.
- ✓ To improve patient compliance.
- ✓ To investigate the release of drug from ethosomal gel and compare it with that of commercially available marketed gel.

MATERIALS AND METHODS:

Materials: Indomethacin was a kind gift from Indoco Remedies Pvt. Ltd. Phospholipon 90G was received as a gift sample from Lipoid GmBh. Carbopol 974P was kindly supplied by Lubrizol. Cholesterol AR grade was procured from Molychem. All other reagents and solvents were of analytical grade.

Methods:

Application of Central Composite Design: Based on the information obtained from the literature review, two formulation parameters were selected for their effect on the preparation of indomethacin ethosomal vesicles. These independent variables were; concentration of lipid (A) and concentration of ethanol (B).

TABLE 1: LEVELS OF INDEPENDENT VARIABLES AND STUDIED RESPONSES OF INDOMETHACIN LOADED ETHOSOMAL FORMULATION BY CENTRAL

Independent Variables	Levels		
	High (+1)	Medium (0)	Low (-1)
A (%)	5	3.5	2
B (%)	50	35	20
Studied Responses		**Aim**	
Y_1 (nm)		Minimize	
Y_2 (mV)		Minimize	
Y_3		Minimize	
Y_4 (%)		Maximize	
Y_5 (%)		Maximize	

Composite design

These variables were studied for their effect on particle size (Y_1), zeta potential (Y_2) polydispersity index (PDI) (Y_3), entrapment efficiency (% EE) (Y_4) and % cumulative drug release (% CDR) (Y_5). Design Expert 11.1.2.0 software was used to develop the trials in which two factors three-level central composite design was selected. The levels of independent variables and studied responses are illustrated in **Table 1**.

Preparation of Ethosomes: According to the composition of ethosomes illustrated in **Table 2**, ethosomes were formulated by a cold method as described by Touitou *et al.*, with slight modification. The calculated amount of lipid, cholesterol, and drug was dissolved in a specified quantity of ethanol in a covered vessel by vigorous stirring with the help of a magnetic stirrer. A calculated amount of propylene glycol was added to the above mixture and it was heated to 30 °C in a water bath. The sufficient quantity of distilled water to prepare 30 ml formulation was added at a constant rate of 1 ml/min in a fine stream with constant mixing using a magnetic stirrer at 1500 rpm in a closed vessel. The temperature was maintained at 30 °C during the formulation period and the mixing was continued for additional 5 min.

The formulation was allowed to cool and then subjected to sonication using an ultrasonic processor. Sonication was carried out for 15 min at an amplitude of 40% and having a pulse of 5 sec. The ethosomal suspension was kept overnight at 4 °C for maturation.

TABLE 2: COMPOSITION OF ETHOSOMAL FORMULATIONS

Formulation code	Run	Factor A (%)	Factor B (%)	Drug (mg)	Cholesterol (%)	Propylene Glycol (%)	Distilled water
EF1	2	-1	-1	150	0.1	10	q.s
EF2	6	-1	0	150	0.1	10	q.s
EF3	3	-1	+1	150	0.1	10	q.s
EF4	9	0	-1	150	0.1	10	q.s
EF5	1	0	0	150	0.1	10	q.s
EF6	5	0	+1	150	0.1	10	q.s
EF7	7	+1	-1	150	0.1	10	q.s
EF8	4	+1	0	150	0.1	10	q.s
EFF9	8	+1	+1	150	0.1	10	q.s

Characterization of Ethosomes:

Particle Size, Zeta Potential and PDI: All the ethosomal formulations EF-1 to EF-9 were analyzed for particle size, polydispersity index (PDI) and zeta potential wherein the principle of dynamic light scattering was utilized using Malvern Zetasizer (Sanofi Synthelabo India, Pvt. Ltd., and Manipal College of Pharmaceutical Sciences). The aliquots from each formulation were measured and measurements were done in triplicate [4].

Entrapment Efficiency: Aliquots of 2 ml of each ethosomal formulation were centrifuged using a refrigerated centrifuge at 14000 rpm for 1 h. The

supernatant was separated, suitably diluted and analysed UV-Visible spectrophotometer at 318 nm. The pellets were lysed by treatment with ethanol in order to determine the encapsulated drug by UV-Visible spectrophotometer at 318 nm after suitable dilutions. The % entrapment efficiency was calculated using the following equation [5]:

% Entrapment efficiency = Drug encapsulated in pellets × 100 / Drug encapsulated in pellets + Drug in the supernatant

***In-vitro* Drug Release:** *In-vitro* drug release studies of ethosomal formulations were performed by using the dialysis membrane previously soaked in phosphate buffer pH 7.4 for 12 h before use. Modified Franz diffusion cell was used wherein the dialysis membrane was mounted between the donor and receptor compartments. The receptor compartment comprised 100 ml of phosphate buffer pH 7.4 which was stirred continuously using a magnetic stirrer at 100 rpm and temperature was maintained at 37 ± 1 °C. The donor compartment consisted of 1 ml of ethosomal formulation. Aliquots of 5 ml of samples were withdrawn from the receptor compartment at specified time intervals of 0.5, 1, 2, 4, 6, and 8 h. The samples were analysed by UV-Visible spectrophotometer at 318 nm and % cumulative drug release (% CDR) at the end of 8 h was calculated [6, 7].

Statistical Analysis and Optimisation: The obtained data for particle size, zeta potential, polydispersity index, %entrapment efficiency and % CDR were statistically analysed to identify the significant independent factors affecting each studied response. Also the estimated and quadratic effects among the studied variables were estimated, and the effect was considered significant when p-value was calculated to be < 0.05.

Morphological Study of Ethosomes: The optimised binary ethosomal formulation was visualised by transmission electron microscopy in order to study its morphology [8].

ATR FT-IR Spectroscopy: Infra-red spectrum of ethosomal suspension was scanned from 4000-400 cm^{-1} and the spectra thus obtained was compared with that of pure Indomethacin [9].

Mechanism of *in-vitro* Drug Release: Various models were tested for determining the kinetics of drug release of optimised ethosomal formulation [10]. The obtained data was fitted into various kinetic mathematical models to analyse the mechanism of drug release rate kinetics of the dosage form:

- Zero-order plot (Percentage cumulative drug release *v/s* Time in hours)
- First order plot (Log of percent drug retained *v/s* Time in hours)
- Higuchi plot (Percentage cumulative drug release *v/s* Square root of time)
- Korsmeyer Peppa's plot (Log of percent cumulative drug release *v/s* Log time).

Zero Order Kinetics: Zero order release kinetics refers to the process of constant drug release from a drug delivery system. Zero order release is predicted by following equation:

$$A_t = A_0 - K_0 t$$

Where;
A_t is the drug release at a time 't,'
A_0 is initial drug concentration,
K_0 is the release rate constant.

Release of drug from the dosage form is said to obey zero-order kinetics, when the plot of cumulative percent drug release *v/s* time is linear with slope equal to Ko.

First Order Kinetics: First order kinetics is predicted by the following equation:

$$\text{Log}C = \text{Log}C_0 - K_t / 2.303$$

Where;
C is the amount of drug remained at a time 't',
C_o is the initial concentration of a drug,
K is the first-order rate constant (h^{-1})

If the plot of log % drug retained *v/s* time is linear it indicates that the release follows first order. The constant K can be obtained by multiplying 2.303 with the slope.

Higuchi's Model: Drug release from a matrix by diffusion has been described by Higuchi's classical diffusion equation:

$$Q = D/\gamma \times [2A.\varepsilon.C_s.t]$$

Where;
Q is the amount of drug released at a time 't',
D is the diffusion coefficient of the drug in the matrix, A is total amount of drug in the unit volume of matrix, C_s is the solubility of a drug in a matrix, ε is porosity of matrix, γ is tortuosity of the matrix
t is time in hours.

Since, D, C_s, ε, γ and A are constants, the equation may be simplified into

$$Q = Kt^{1/2}$$

Where; K is Higuchi's constant.

In the Higuchi model, if a plot of percent cumulative drug release *v/s* square root of time is linear, it indicates that the drug is released by diffusion mechanism and the slope is equal to K.

Korsmeyer-Peppa's Model: In Korsmeyer – Peppa's model the data is fitted into the following equation:

$$M_t / M_0 = K.t.n$$

Where;
M_t / M_0 is fraction of drug released at time 't',
K is released constant, t is release time and n is diffusion exponent related to mechanism of release.

$$\text{Log } M_t / M_0 = \text{LogK} + n \text{ Logt}$$

When the data is plotted as log of drug released *v/s* log time if straight line is obtained, slope is equal to n and K can be obtained from the y-intercept.

If 0.89 < n < 1, the release is zero order (case II transport)

If n = 0.5, the release is best explained by fickian diffusion.

If n 0.45 < n = 0.89, the release is through non fickian diffusion.

If n > 1, super case II transport [11].

Stability Studies: To ensure the stability of optimized ethosomal formulation, it was subjected to stability studies at three different storage conditions *viz*. -20 °C, 2-8 °C and ambient conditions (30 ± 2 °C and 65 ± 5% RH) for 3 months. The ethosomal formulation was evaluated for any physical instability that may occur, particle size, zeta potential, PDI and entrapment efficiency.

Formulation of Indomethacin loaded Ethosomal Gels: Ethosomal gels were prepared by dispersion method using HPMC K4M and Carbopol 974P. The polymers of varying concentrations were soaked in distilled water for 2 h and then dispersed homogenously using a magnetic stirrer. Parabens were added as preservatives, and carbomer gel thus obtained was neutralized by the addition of triethanolamine until a transparent get was obtained. The optimized ethosomal suspension containing drug equivalent to 2.5mg/ml was then added to the prepared gel base with continuous stirring to form a homogenous gel [12, 13, 14].

TABLE 3: COMPOSITION OF ETHOSOMAL GELS

Formulation code	G1	G2	G3	G4	G5	G6	G7
Ethosomal Formulation (ml)	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Carbopol 974P (%)	0.5	1	1.5	2	-	-	-
HPMC K4M (%)	-	-	-	-	2	3	4
Methyl Paraben (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Propyl Paraben (%)	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Triethanolamine	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Distilled Water	q.s	q.s	q.s	q.s	q.s	q.s	q.s

Evaluation of Ethosomal Gels:

Organoleptic Characterisation: The ethosomal gels were evaluated for its organoleptic properties such as color homogeneity and presence of any foreign particles.

Determination of pH: Accurately weighed 2.5g of ethosomal gel was dispersed in 25ml of distilled water. The pH was then measured by using digital pH meter.

Spreadability: Spreadability is determined by a wooden block and glass slide apparatus. The time in seconds taken by two slides to slip off from gel which is placed in between the slides under the direction of a certain load is expressed as spreadability. Lesser the time is taken for the separation of two slides, better the spreadability [15].

Spreadability is calculated by using the formula:

$$S = M \times L / T$$

Where,

S = Spreadability,
M = Weight tide to the upper slide,
L = Length of a glass slide,
T = Time taken to separate the slide from each other.

Drug Content: The drug content of ethosomal gels was determined by dissolving 1g of ethosomal gel sample in 10 ml of ethanol. The resultant solution was sonicated for 20 min for the total extraction of the drug. The sample after suitable dilution was analyzed by a UV-Visible spectrophotometer against blank at 318nm [6].

Viscosity: The viscosity of formulated gels was determined by using Brookfield viscometer with spindle S64. Approximately 50 g of ethosomal gel was taken in a glass beaker. The spindle was then rotated at 0.6 rpm and the dial reading was noted [15].

***In-vitro* Drug Release Studies:** *In-vitro* drug release studies of ethosomal gels were performed by using the dialysis membrane previously soaked in phosphate buffer pH 7.4 for 12 h before use. Modified franz diffusion cell was used wherein the dialysis membrane was mounted between the donor and receptor compartments. The receptor compartment consisted of 100 ml of phosphate buffer pH 7.4 which was stirred continuously using a magnetic stirrer at 100 rpm and the temperature was maintained at 37 ± 1 °C. 1g of ethosomal gel was added into the donor compartment. Aliquots of 5 ml of samples were withdrawn from the receptor compartment specified time intervals of 0.5, 1, 2, 4, 6, and 8 hours. The aliquots of samples withdrawn were analyzed by UV-Visible spectrophotometer at 318 nm and % cumulative drug release (% CDR) at the end of 8 h was calculated.

***Ex-vivo* Skin Permeation Studies of Pure Drug, Optimised Ethosomal Formulation and Ethosomal Gel:** *Ex-vivo* skin permeation studies were performed by using goat ear skin. The hair from the skin was shaved off and the skin was rinsed and soaked with phosphate buffer pH 7.4 and was mounted between the donor and receptor compartments of modified Franz diffusion cell. The receptor compartment consisted of 100 ml of phosphate buffer pH 7.4 which was stirred continuously using a magnetic stirrer at 100 rpm and the temperature was maintained at 37 ± 1 °C. The donor compartment consisted of 1g/1ml optimized ethosomal gel / optimized ethosomal formulation. Aliquots of 5 ml of samples were withdrawn from the receptor compartment at specified time intervals of 0.5, 1, 2, 4, 6, and 8 h. The samples withdrawn were analyzed by UV-Visible spectrophotometer at 318 nm and transdermal flux was calculated [16, 17].

$$J_{ss} = Q / t \times A$$

Where;
J_{ss} = Transdermal flux,
Q = Amount of drug traversing he membrane in time t, A = Area of an exposed membrane in cm^2.

Skin Retention Studies: Following permeation studies, the skin tissue washed several times with phosphate buffer pH 7.4, followed by blotting between tissue paper to remove any adhering formulation from the surface. Subsequently, the skin tissue was cut into small pieces and in ethanol for 24 h to extract the drug that was deposited in the skin. After this, the processed skin was sonicated for 20 min using a bath sonicator and centrifuged. The supernatant was separated, filtered if necessary, and analyzed using a UV-Visible spectrophotometer, and the amount of drug was calculated [18].

Skin Irritation Studies: The skin irritation test was conducted according to the method described by Draize *et al.,* by using male Albino Wistar rats. The rats were taken and the abdominal skin of the rat was clipped free of hair 24 h prior to the formulation application. The rats were divided into two groups:

Group I: Positive Control (0.8% Formalin).

Group II: Ethosomal Gel.

Each group was treated with a respected solution and formulation. 0.5g of the test substance was applied to a small area ($4cm^2$) of the skin and covered with a gauze patch, which was left on for an exposure period of 4 h. After which the patch was removed and the treated area of skin was observed for any visible changes such as

erythema/edema at 1, 24, 48 and 72 h post-treatment [9, 19]. Animal ethics committee approval was taken to carry out the test (IAEC/GCP/2018/11).

Stability Studies of Optimised Ethosomal Gel: To ensure the stability of the optimized ethosomal gel, stability studies were performed under two different storage conditions. The optimized ethosomal gel was subjected to different temperature conditions of 2-8 °C and room temperature for 3 months. The ethosomal gel was evaluated for any physical instability that may occur, pH, viscosity, and drug content.

RESULTS AND DISCUSSION:

Particle Size, Zeta Potential, Polydispersity Index, Entrapment Efficiency and *in-vitro* Drug Release: The results of observed values for particle size, zeta potential, polydispersity index, entrapment efficiency, and % CDR are illustrated in table 4. The obtained ethosomes were in the 110.98-593.5nm particle size range, the zeta potential was found to range between -19.9 to -30.1mV, polydispersity index ranged between 0.254 - 0.773, % entrapment efficiency ranged between 55.55 - 91.27% and % CDR was found to range between 31.22 – 54.27%.

Statistical analysis for the effect of factor A and factor B on Y_1, Y_2, Y_3, Y_4, and Y_5 was carried out by multiple regression analysis and analysis of variance using design expert software. Estimated effects, F-ratios, and associated P-values for the studied factors, their interaction and quadratic effects on obtained responses were calculated and data are presented in **Table 4**.

TABLE 4: RESULTS OF RESPONSES $Y_1 – Y_5$

Formulation code	Response 1	Response 2	Response 3	Response 4	Response 5
	Particle Size (Y_1)	Zeta Potential (Y_2)	PDI (Y_3)	Entrapment Efficiency (Y_4)	%CDR (Y_5)
	Nm	mV		%	%
EF1	138.7	-19.9	0.773	82.48	39.35
EF2	110.98	-21.7	0.398	86.78	40.2
EF3	158.39	-23.6	0.471	82.39	39.23
EF4	217.63	-20.3	0.416	90.03	45.62
EF5	212.19	-24.7	0.399	91.27	54.27
EF6	245.25	-28.3	0.406	88.35	45.51
EF7	257.08	-23.5	0.351	73.33	31.81
EF8	413.31	-26.7	0.413	81.5	38.25
EF9	593.5	-30.1	0.254	55.55	31.22

Effect of Concentration of Lipid and Ethanol on Particle Size: On the basis of results of statistical analysis illustrated in **Table 5**, it was concluded that the main effect of A, significantly affected the particle size (Y_1) of ethosomal vesicles. Factor A was seen to have a positive synergistic effect on Y_1 as the coefficient was positive. R-squared and adjusted R-squared statistic values were found to be 0.958 and 0.8887 respectively. The equation of the fitted model was found to be:

$$Y_1 = 209.74 + 142.64(A) + 63.95(B) + 79.18\ (AB) + 53.64\ (A^2) + 22.93\ (B^2)$$

As seen in the 3D surface plot in **Fig. 1**, a significant increase in particle size with an increase in the concentration of lipid was observed. This increase in particle size could be due to the formation of multilamellar vesicles with higher lipid concentrations. Also, an increase in the concentration of ethanol leads to a slight decrease in particle size upto a certain concentration, beyond which further increase in ethanol concentration leads to increase in particle size. This phenomenon could occur probably due to increased fluidity of the vesicles with increasing ethanol concentration.

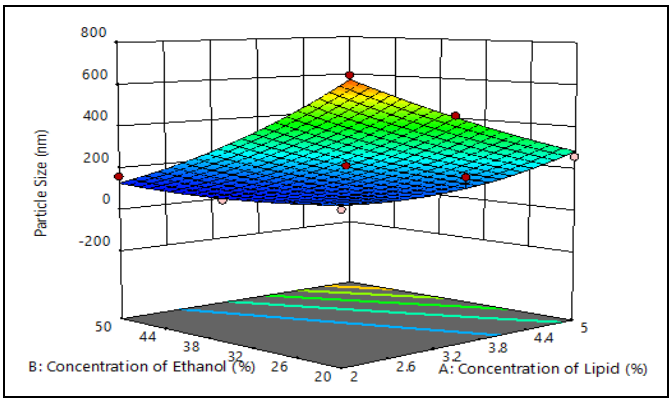

FIG. 1: 3D SURFACE PLOT OF FACTORS AFFECTING PARTICLE SIZE OF ETHOSOMES

Effect of Concentration of Lipid and Ethanol on Zeta Potential: On the basis of results depicted in **Table 5**, it could be concluded that the main effects A and B significantly affected the zeta potential (Y_2) of ethosomal formulations. As the coefficients were found to be negative, the factors A and B had a negative antagonistic effect on Y_2. R-squared and adjusted R-squared statistic values were found to be 0.9716 and 0.9242 respectively and the equation of the fitted model was found to be:

$$Y_2 = -24.49 - 2.52(A) - 3.05(B) - 0.7250(AB) + 0.1833(A^2) + 0.0833(B^2)$$

In the 3D surface plot of factors affecting zeta potential in **Fig. 2**, a significant decrease in zeta potential is seen with increase in concentration of factor A. Similarly, a significant decrease in zeta potential is seen with increase in concentration of factor B. The higher ethanol concentration shifts the vesicular charge from positive to negative because ethanol acts as a negative charge provider for the surface of ethosomes, thereby avoiding aggregation of the vesicles due to electrostatic repulsion.

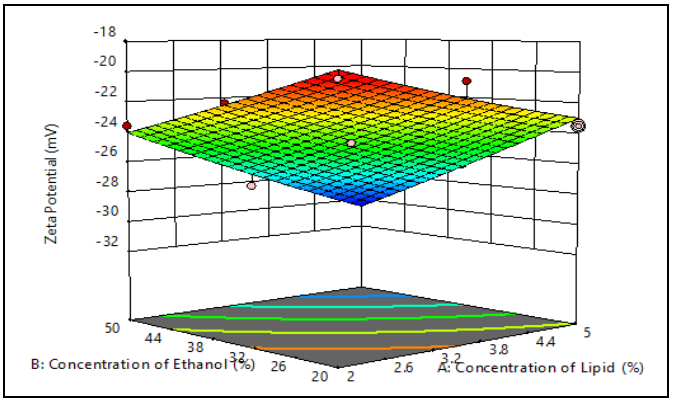

FIG. 2: 3D SURFACE PLOT OF FACTORS AFFECTING ZETA POTENTIAL OF ETHOSOMES.

Effect of Concentration of Lipid and Ethanol on Polydispersity Index: The results depicted in **table 5** show that neither the main effects, nor the interaction and quadratic terms show a significant effect on polydispersity index (Y_3). Factors A and B had antagonistic effects on Y_3 while the interaction and quadratic terms had positive synergistic effects.

R-squared and adjusted R-squared statistic values were found to be 0.6857 and 0.1618 respectively and the equation of the fitted model was found to be:

$$Y_3 = 0.3791 - 0.1040(A) - 0.0682(B) + 0.0513(AB) + 0.0363(A^2) + 0.0418(B^2)$$

As can be seen in **Fig. 3**, an increase in concentration of lipid and ethanol lead to decrease in polydispersity index. This effect was not significant as the P-value > 0.05.

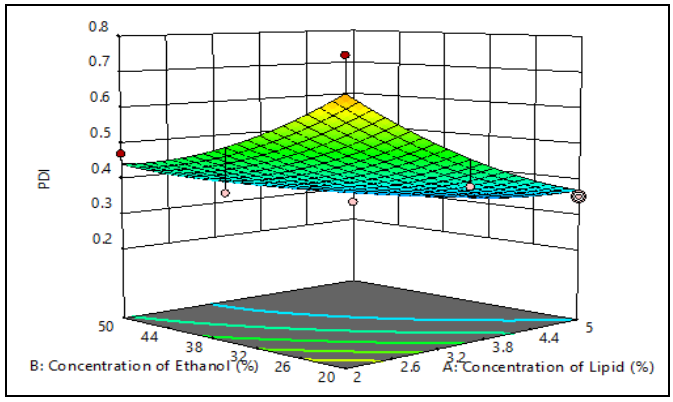

FIG. 3: 3D SURFACE PLOT OF FACTORS AFFECTING POLYDISPERSITY INDEX OF ETHOSOMES

Effect of Concentration of Lipid and Ethanol on Entrapment Efficiency: From the results depicted in **Table 5** it can be seen that neither the main effects nor the interaction and quadratic terms show a significant effect on the entrapment efficiency (Y_4) of the ethosomal formulations. All the factors were seen to have antagonistic effects on Y_4.

$$Y_4 = 95.10 - 6.88(A) - 3.26(B) - 4.42(AB) - 12.88(A^2) - 7.83(B^2)$$

Respectively and the equation of the fitted model was found to be:

$$Y_4 = 95.10 - 6.88(A) - 3.26(B) - 4.42(AB) - 12.88(A^2) - 7.83(B^2)$$

As can be seen in **Fig. 4**, an increase in concentration of factor A upto a certain level increases the entrapment efficiency of ethosomes and on further increase on concentration of factor A has no effect or a decrease in entrapment efficiency is seen. This could be possibly due to increase in vesicular size with increase in factor A concentration. Also, increase in concentration of factor B upto a certain level increases the entrapment efficiency but on further increase in the concentration of factor B, decrease in entrapment efficiency was observed. Initial increase in entrapment efficiency could be due to enhanced solubility of lipophilic and amphiphilic drugs by

ethanol thus increasing the drug loading. While, decrease in entrapment efficiency after a certain concentration of ethanol could be due to solubilisation of phospholipids in ethanol thus making the vesicular membrane more permeable and leaky. However, this effect was not significant.

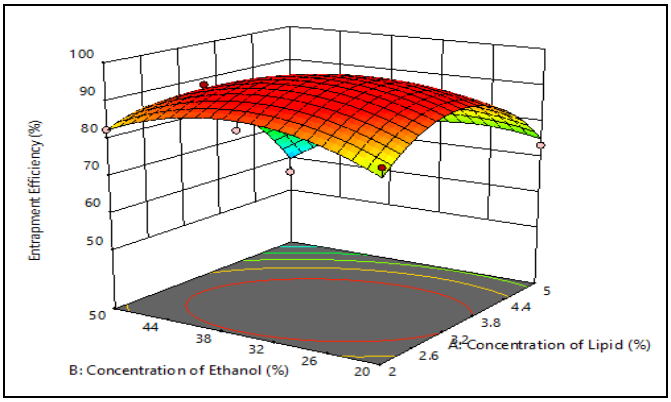

FIG. 4: 3D SURFACE PLOT OF FACTORS AFFECTING ENTRAPMENT EFFICIENCY OF ETHOSOMES

Effect of Concentration of Lipid and Ethanol on % CDR: As illustrated in **Table 5**, it can be seen that the main effects and interaction terms did not have any significant effect on % CDR (Y_5) whereas the quadratic term A^2 was found to have a significant effect on Y_5 of ethosomal formulations. All the factors were found to have negative coefficients thus having antagonistic effects on Y_5. R-squared and adjusted R-squared statistic values were found to be 0.9466 and 0.8576 respectively and the equation of the fitted model was found to be:

$Y_5 = 52.10 – 2.92(A) – 0.1367(B) – 0.1175(AB) – 11.79(A^2) – 5.45(B^2)$

As seen in **Fig. 5**, increase in concentration of factor A lead to increase in the drug release upto a certain concentration, beyond which decrease in % CDR was seen with increase in factor A concentration thus showing a significant quadratic behaviour. The initial increase in % CDR could be due to the smaller vesicle size due to lower concentration of factor A. As the vesicle size increased the permeation of drug through the membrane could have decreased thus decreasing the drug release. Also, increase in concentration of factor B lead to increase in % CDR up to a certain concentration beyond which a slight decrease in drug release was seen. As the concentration of ethanol increased upto a certain concentration, the fluidity of vesicles increased and vesicle size decreased thus improving permeation and drug release. Also ethanol enhanced the solubility of lipophilic Indomethacin thus improving the drug release. Further increase in ethanol concentration lead to formation of leaky vesicles having greater particle size thus reducing permeation and % CDR.

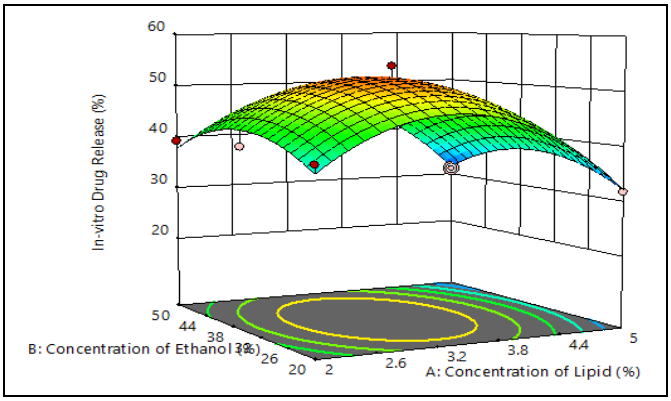

FIG. 5: 3D SURFACE PLOT OF FACTORS AFFECTING % CDR OF ETHOSOMES

TABLE 5: ESTIMATED EFFECTS OF FACTORS, F-RATIO AND P-VALUES FOR RESPONSES Y_1, Y_2, Y_3, Y_4 AND Y_5

Factors		A	B	AB	A^2	B^2
Y_1	Estimated effect	142.64	63.95	79.18	53.64	22.93
	F-ratio	47.10	9.47	9.68	2.22	0.4058
	P-value	0.0063	0.0543	0.0529	0.2330	0.5694
Y_2	Estimated effect	-2.52	-3.05	-0.7250	0.1833	0.0833
	F-ratio	40.59	59.62	2.25	0.0718	0.0148
	P-value	0.0078	0.0045	0.2309	0.8061	0.9108
Y_3	Estimated effect	-0.1040	-0.0682	0.0513	0.0363	0.0418
	F-ratio	3.88	1.67	0.6284	0.1579	0.2093
	P-value	0.1434	0.2871	0.4858	0.7177	0.6784
Y_4	Estimated effect	-6.88	-3.26	-4.42	-12.88	-7.83
	F-ratio	8.15	1.83	2.25	9.52	3.52
	P-value	0.0648	0.2691	0.2309	0.0539	0.1573
Y_5	Estimated effect	-2.92	-0.1367	-0.1175	-11.79	-5.45
	F-ratio	6.98	0.0153	0.0076	38.04	8.13
	P-value	0.075	0.9093	0.9362	0.0086	0.0651

Multiple Response Optimisation and Optimum Desirability:

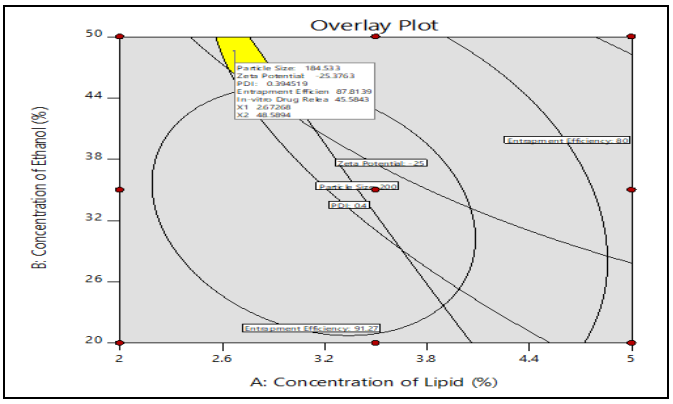

FIG. 6: OVERLAY PLOT SHOWING THE LOCATION OF OPTIMISED BINARY ETHOSOMAL FORMULATION

An optimised formulation that contains the above mentioned optimum levels of studied variables was prepared and characterised as previously stated. The observed values for Y_1, Y_2, Y_3, Y_4 and Y_5 were found to be 185.5nm, -27mV, 0.237, 87.86% and 45.65% respectively. The values were found to be within the tolerance limits hence the applied model was found to be statistically correct.

Morphological Study by Transmission Electron Microscopy: The optimized ethosomal formulation was visualized by transmission electron microscopy wherein the vesicles were found to be almost spherical to slightly elongated in structure as seen in **Fig. 7**.

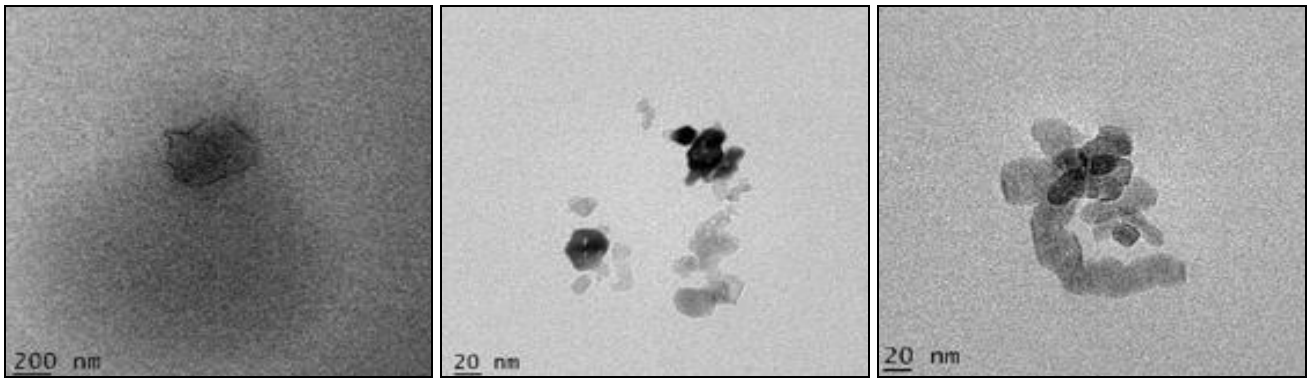

FIG. 7: TRANSMISSION ELECTRON MICROSCOPE IMAGE OF OPTIMISED ETHOSOMAL FORMULATION

ATR-FTIR Spectroscopy: The spectrum of ethosomal formulation showed a broad peak at 3334.92 cm^{-1} which seems to be a particular O-H stretch of ethanol, as ethanol is used as the major formulation solvent. The peak at 2980 was due to C-H aliphatic str. The peaks representing C=O, C=C were found to be of lower intensity which shows that the drug being lipophilic has bound to the phospholipid and cholesterol in the lipid bilayer.

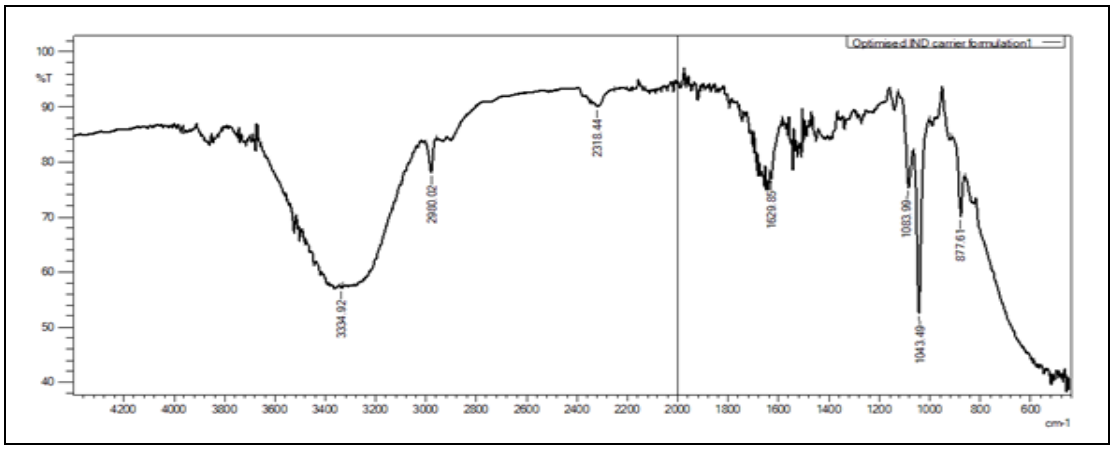

FIG. 8: ATR-FTIR SPECTRUM OF ETHOSOMALDISPERSION

Mechanism of *in-vitro* Drug Release: The *in-vitro* release data of optimized formulation was fitted into 4 kinetic models that are Zero order, First order, Higuchi plot, and Peppa's plot. The data from the *in-vitro* release study of optimized formulation was subjected to linear regression analysis and values for the regression coefficient (R), and release rate constant (K) was determined.

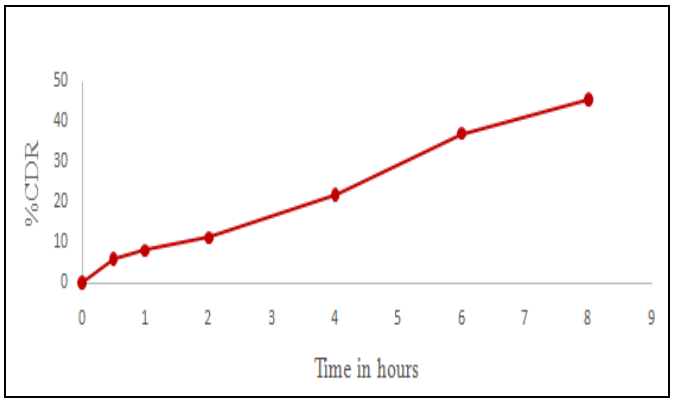

FIG. 9: ZERO ORDER PLOT OF OPTIMIZED FORMULATION

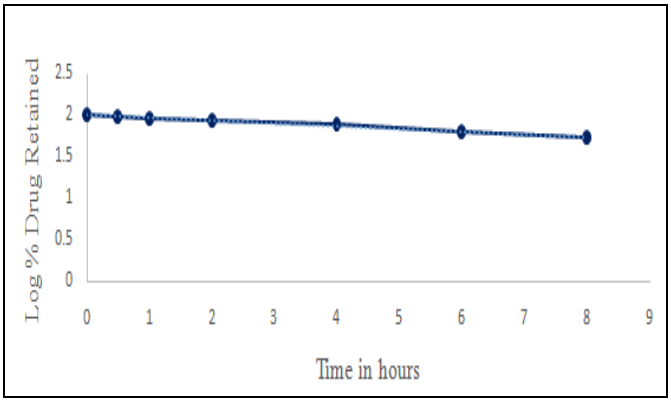

FIG. 10: FIRST ORDER PLOT OF OPTIMISED FORMULATION

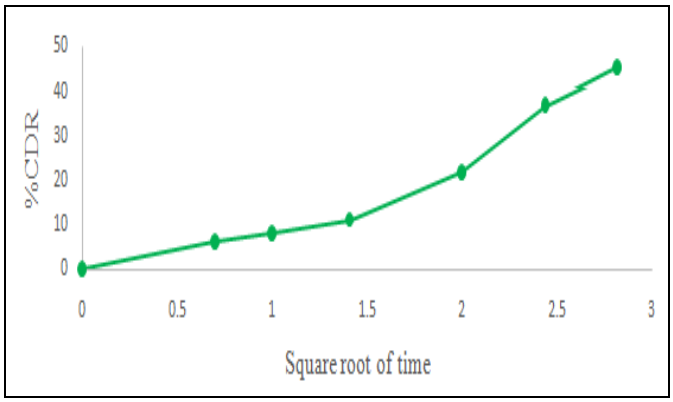

FIG. 11: HIGUCHI PLOT OF OPTIMIZED FORMULATION

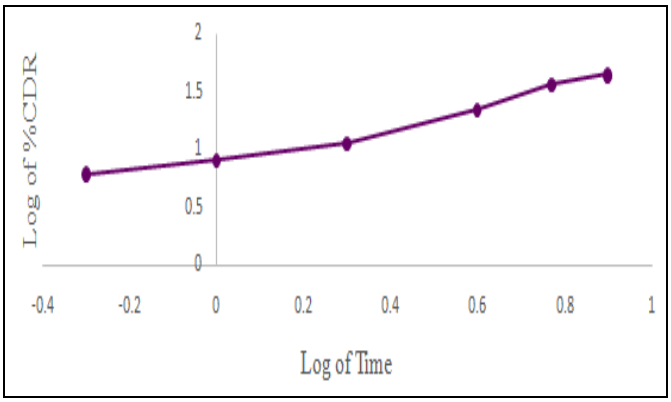

FIG. 12: PEPPA's PLOT OF OPTIMISED FORMULATION

TABLE 6: VALUES OF REGRESSION COEFFICIENTS AND KINETICS FOR OPTIMISED FORMULATION

Formulation code	Zero Order Plot		First Order Plot		Higuchi Plot		Peppa's Plot	
	R^2	K	R^2	K	R^2	K	R^2	N
Optimised Formulation	0.9903	5.6063	0.985	-0.0759	0.9208	16.37	0.9626	0.7536

The zero-order plot of optimized formulation had an R^2 value of 0.9903 which is close to 1 thus showing that *in-vitro* drug release follows zero-order reaction.

The release date was also treated with Higuchi's equation wherein the R^2 value (0.9208) was almost approaching 1 thus showcasing that the *in-vitro* drug release of optimized ethosomal formulation follows the diffusion mechanism.

In order to understand the type of diffusion mechanism, the *in-vitro* drug release data were treated with Korsmeyer Peppa's equation. The R^2 value (0.9626) was found to be close to 1 thus indicating that the release data fitted perfectly in the model. The n value of the optimized formulation was found to be 0.7536 therefore, it can be concluded that the drug release from optimized formulation followed non fickian type of diffusion.

Comparison of Release Profile of Pure Drug and Optimised Formulation: *In-vitro* drug release study of the optimized formulation was compared with ethanolic solution of pure drug in order to observe the effect of ethosomal system on the diffusion of the drug.

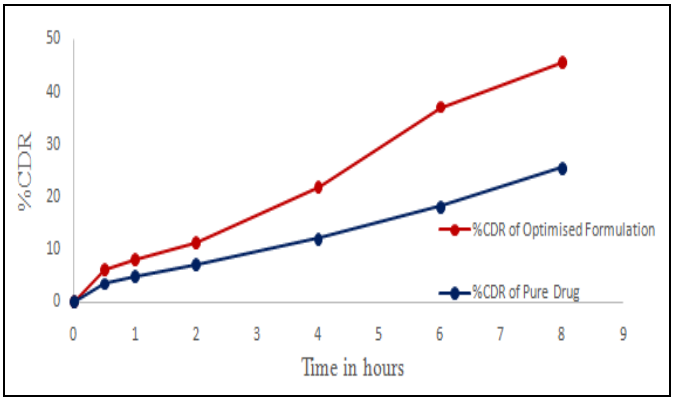

FIG. 13: COMPARISON OF *IN-VITRO* RELEASE PROFILE OF OPTIMISED FORMULATION AND PURE DRUG

On comparison of the two release profiles, it was observed that Indomethacin loaded optimized ethosomal formulation had higher % CDR *i.e.* 45.65% than that of the ethanolic solution pure drug *i.e.* 25.62% at the end of 8 h. Thus, it can be concluded that ethosomal system was successful in improving the solubility as well as permeation of the drug.

Stability Studies of Optimised Ethosomal Formulation: The optimized ethosomal formulation was subjected to stability studies under three different conditions *i.e.*, -20 °C, 2-8 °C and at room temperature. At the end of three months, the ethosomal formulation was found to be physically stable as there was no change in its appearance. The results of particle size, zeta potential, PDI, and % entrapment efficiency as illustrated in **Table 7**. From the tabulated results, it can be concluded that the optimized ethosomal formulation was more stable at temperatures of -20 °C and 2 to 8 °C in comparison to room temperature.

TABLE 7: RESULTS OF STABILITY STUDIES OF OPTIMISED ETHOSOMAL FORMULATION

Temperature	Particle Size (nm)	Zeta Potential (mV)	PDI	%Entrapment Efficiency (%)
-20°C	186.9	-26.72	0.286	87.72
2 to 8°C	187.7	-24.92	0.321	87.22
Room Temperature	191.1	-23.54	0.305	81.11

Evaluation of Ethosomal Gel:

Organoleptic Characterisation: The evaluated ethosomal gels G1-G7 were found to be opaque and white in color. Also, the ethosomal gels were found to be homogenous, smooth and non-gritty and free from any foreign particles.

Determination of pH, Spreadability, Drug Content and Viscosity: The pH of ethosomal gels was determined using digital pH meter and results are illustrated in **Table 8**.

TABLE 8: pH, SPREADABILITY, DRUG CONTENT AND VISCOSITY OF ETHOSOMAL GELS

Formulation Code	pH	Spreadability (g.cm/sec)	Drug Content (%)	Viscosity (cP)
G1	6.82 ± 0.03	39.67	98.01 ± 0.10	26433 ± 3774
G2	6.85 ± 0.011	31.07	99.87 ± 0.18	52000 ± 1637
G3	6.92 ± 0.005	29.70	98.73 ± 0.27	63300 ± 2066
G4	6.89 ± 0.01	26.44	97.17 ± 0.31	87466 ± 665
G5	6.31 ± 0.01	33.70	95.18 ± 1.40	14800 ± 3019
G6	6.02 ± 0.005	22.95	95.36 ± 1.74E^{-14}	23000 ± 2645
G7	6.24 ± 0.04	17.87	93.86 ± 0.10	60333 ± 2516

The results of spreadability are illustrated in **Table 8**. In both cases it was seen that as the concentration of polymer increases the spreadability of the gels decreased, this could be due to increase in viscosity of the gels with increase in polymeric concentrations.

The drug content of ethosomal gels was performed and the results were tabulated in **Table 8**. The drug content of gels G1-G4 containing carbopol was in the range of 97.17% to 99.87% and was found to be satisfactory. Gels G5-G7 containing HPMC were found to have lower drug content in the range of 93.86% to 95.36%. The viscosities of ethosomal gels determined using Brookfield viscometer were tabulated in **Table 8**. Formulations G1-G4 containing carbopol were found to have viscosities ranging from 26433 cP to 87466 cP whereas ethosomal gels G5-G7 containing HPMC were found viscosities ranging from 14800 cP to 60333 cP. Thus, in both the cases viscosities were found to increase with an increase in the polymeric concentrations.

***In-vitro* Drug Release Studies:** From the *in-vitro* release studies plots, it can be observed that the drug release gradually increased over the period of 8 h of study. Also, it was seen that the drug release of ethosomal gel was similar of that of ethosomes thus indicating that the gel formulations act as a vehicle for administration of ethosomal system. Formulations G1 to G4 showed drug release in the range of 42.11 to 56.11% at the end of 8 h while formulations G5-G7 showed drug release in the range of 46.59 to 52.34% at the end of 8 hours. In case of gels made of carbopol there was a decrease in drug release with the increase in the polymeric concentration with G2 as an exception that showed

higher release. Gels made of HPMC showed a linear decrease in drug release with an increase in polymeric concentration which could be again due to an increase in viscosity. Among the 7 batches of ethosomal gels prepared, formulation G2 showed highest drug release.

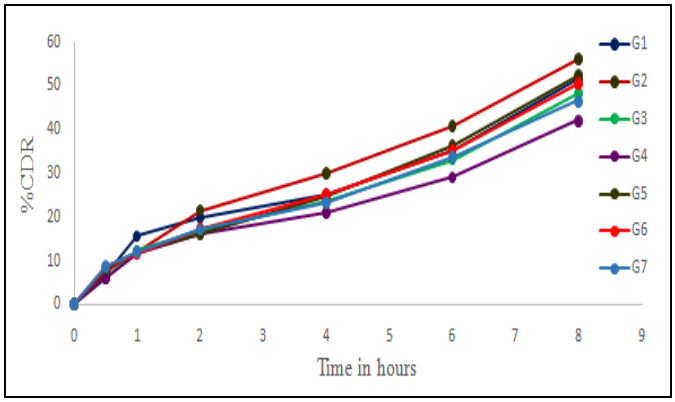

FIG. 14: ZERO ORDER PLOTS FOR G1- G7 ETHOSOMAL GELS

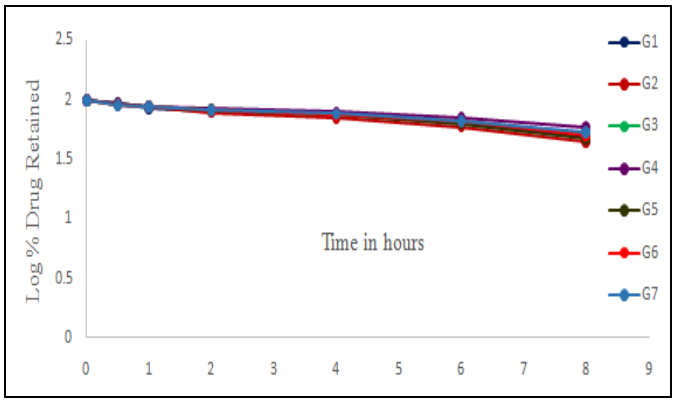

FIG. 15: FIRST ORDER PLOTS FOR G1- G7 ETHOSOMAL GELS

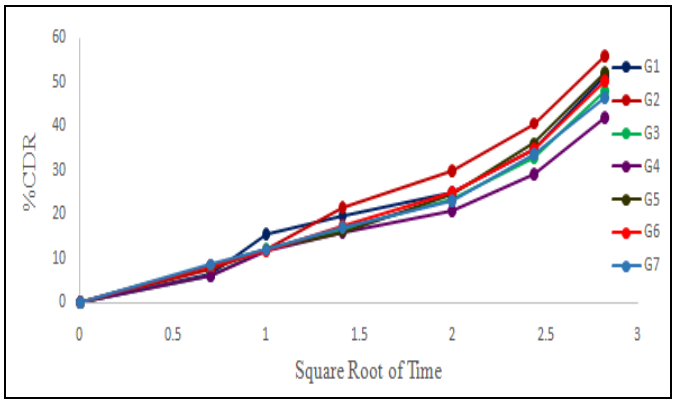

FIG. 16: HIGUCHI PLOTS FOR G1- G7 ETHOSOMAL GELS

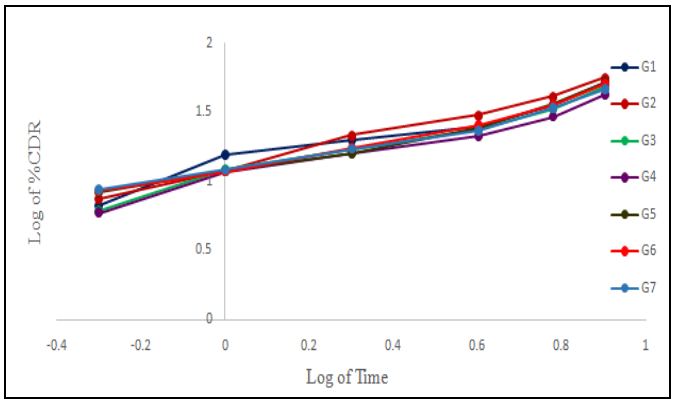

FIG. 17: PEPPA'S PLOTS FOR G1- G7 ETHOSOMAL GELS

TABLE 9: VALUES OF REGRESSION COEFFICIENTS AND KINETICS FOR *IN-VITRO* STUDIES OF ETHOSOMAL GELS

Formulation	Zero Order Plot		First Order Plot		Higuchi Plot		Peppa's Plot	
	R^2	K	R^2	K	R^2	K	R^2	N
G1	0.9536	5.6153	0.9493	-0.0787	0.9394	16.876	0.9451	0.6427
G2	0.9813	6.4566	0.9803	-0.0941	0.9614	19.351	0.9933	0.7018
G3	0.9746	5.3799	0.9673	-0.0730	0.9438	16.031	0.9778	0.6788
G4	0.9652	4.6353	0.9641	-0.0601	0.9491	13.919	0.9709	0.6364
G5	0.98	0.8567	0.9644	-0.0824	0.9318	17.293	0.9686	0.6309
G6	0.9781	5.6053	0.9691	-0.0778	0.9453	16.686	0.9784	0.6178
G7	0.9733	5.1561	0.9725	-0.0695	0.9526	15.446	0.9747	0.5805

The *in-vitro* drug release data of all ethosomal gels was fitted into 4 kinetic models, *i.e.* Zero order, First order, Higuchi plot, and Peppa's plot. The data from the *in-vitro* release study of all formulations were subjected to linear regression analysis and values for regression coefficient (R) and release rate constant (K) was determined. The R^2 values for zero-order plots ranged from 0.9536-0.9813 while that for first-order plots ranged from 0.9493-0.9803.

The R^2 values for zero-order plots were found to be close to 1; hence the release of drug from all the formulations followed zero-order kinetics. Similarly, the data were treated according to Higuchi's diffusion equation. The correlation coefficient of all formulations with Higuchi's equation ranged from 0.9318-0.9614, with the highest value of 0.9614 that is nearly approaching 1. The results indicated that all ethosomal gels exhibited diffusion mechanism of drug release.

To understand the drug release mechanism from the ethosomal gel system, the data was subjected to Peppa's model where the R^2 values revealed that the Peppa's model best fitted in all formulations. The values of 'n' as derived from Peppa's model were found to be in the range of 0.5805-0.7018, therefore, it was concluded that the drug release from ethosomal gels followed anomalous or Non-Fickian diffusion type.

Based on the results of evaluation tests performed on all formulation batches of ethosomal gels, an optimized formulation was selected that gave satisfactory results as compared to others. Formulation G2 was selected as the optimized batch as it showed the highest drug content (99.87 ± 0.18%) and maximum *in-vitro* drug release (56.11%). Also, the optimised ethosomal gel G2 was found to have a pH 6.8 ± 1.11 which is almost close to that of the skin. It was found to have a viscosity of 52000cP and the spreadability was found to be 31.07 g.cm/sec.

Comparison of Release Profile of Optimised Ethosomal Gel and Marketed Formulation: *In-vitro* drug release study of the optimized ethosomal gel was compared with a marketed formulation in order to check the efficiency of the ethosomal gel as compared to the marketed formulation.

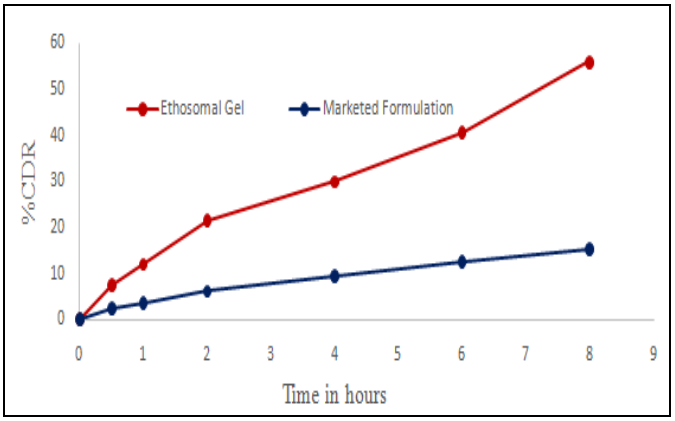

FIG. 18: COMPARISON OF *IN-VITRO* RELEASE PROFILE OF OPTIMISED ETHOSOMAL GEL AND MARKETED FORMULATION

On comparison of the two release profiles it was observed that optimised ethosomal gel had higher % CDR *i.e.* 56.11% than that of the marketed formulation i.e. 15.35% at the end of 8 h. Thus, it can be concluded that ethosomal gel provides a better permeation of the drug than the marketed formulation.

***Ex-vivo* Skin Permeation Studies:** *Ex-vivo* skin permeation studies were carried out by using goat ear skin. After completion of 8 h of study %, CDR was calculated and a plot of % CDR *v/s* Time was constructed. The % CDR at the end of 8 h of ethosomal formulation and ethosomal gel was found to be 51.84% and 58.55% respectively, which was greater than that obtained by *in-vitro* diffusion studies. Thus, ethosomal gel was found to have better permeation through the skin compared to the dialysis membrane. As can be seen in **Table 10**, a transdermal flux of optimized ethosomal formulation and ethosomal gel through the goat ear skin was found to be higher than that of pure drug hence the ethosomal system roved to improve the skin permeability of indomethacin.

TABLE 10: TRANSDERMAL FLUX OF OPTIMISED FORMULATION AND PURE DRUG

Formulation code	Transdermal Flux (mg/cm^{2}h)
Pure Drug	0.61
Optimized Ethosomal Formulation	1.56
Optimised Ethosomal Gel	1.76

Skin Retention Studies: Skin retention studies were performed on the optimised ethosomal gel and the amount of drug retained in the skin was calculated. The amount of drug retained in the skin was found to be 41.467 µg/cm^2. This retained drug in the skin could possibly act as a drug reservoir thus providing a controlled and prolonged release even after the applied formulation is exhausted.

Skin Irritation Studies: Skin irritation study was conducted by using Albino Wistar rats according to the procedure given by Draize *et al*. The rats that were used for skin irritation study of ethosomal gel did not show any signs of allergic reactions, erythema or redness up to 3 days as seen in **Fig. 21**, while the rats treated with positive control showed slight edema as seen in **Fig. 20**. Thus, this study shows that the prepared ethosomal gel is non-irritating and safe for application for transdermal use.

TABLE 11: SKIN IRRITATION STUDY RESPONSES

Duration	Response	
	Positive control	Test formulation
1 h after removal of a patch	0	0
24 h	1	0
48 h	2	0
72 h	3	0

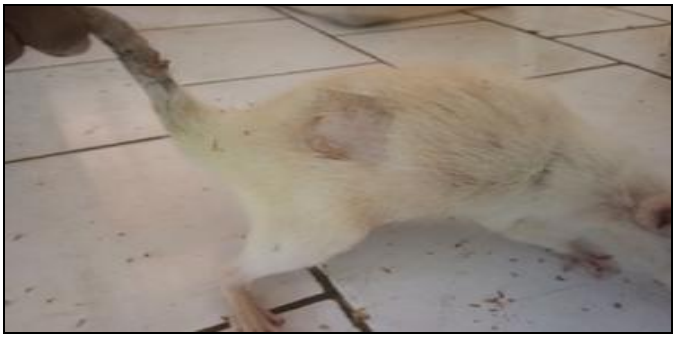

FIG. 19: SHAVED SKIN PATCH ON THE DORSAL SURFACE OF THE RAT

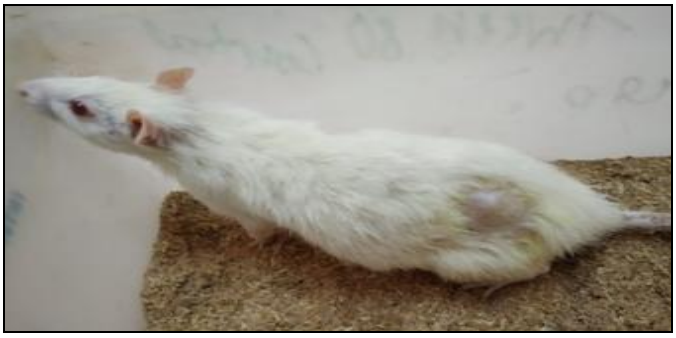

FIG. 20: SLIGHT OEDEMA OBSERVED ON BARE SKIN PATCH OF RAT TREATED WITH 0.8% FORMALIN SOLUTION AFTER 72 h

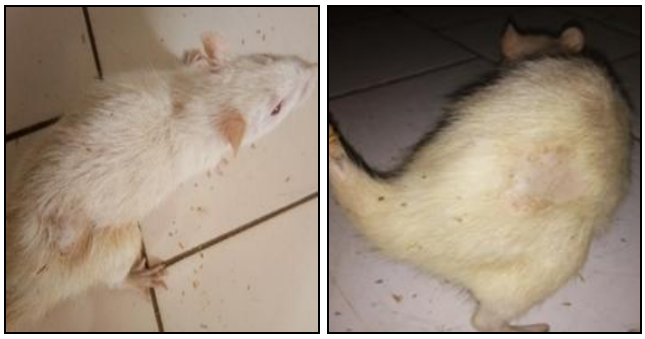

FIG. 21: SKIN OF RAT TREATED WITH ETHOSOMAL GEL AFTER 72 h, NO IRRITATION OBSERVED

Stability Studies of Optimised Ethosomal Gel: The optimised ethosomal gel was subjected to stability studies under two different conditions *i.e.* 2-8 °C and at room temperature. At the end of three months the ethosomal gel was found to be physically stable as there was no change in its appearance and no phase separation was observed. The results of pH, viscosity, and drug content were illustrated in **Table 12**.

TABLE 12: RESULTS OF STABILITY STUDIES OF OPTIMISED ETHOSOMAL GEL

Temperature	pH	Viscosity (cP)	Drug content (%)
2 to 8°C	6.84 ±0.01	51200 ±600	99.15 ±0.18
Room Temperature	6.82 ±0.005	47133 ±1446	93.44 ±0.45

From the results illustrated in **Table 12**, it can be concluded that the ethosomal gel was found to be stable at 2-8 °C.

CONCLUSION: In this research, an attempt was made to improve the permeation of Indomethacin across the transdermal membrane by encapsulating in ethosomes which also help to increase its solubility, produce faster action, provide a prolonged release and also most importantly will reduce the gastrointestinal side effects.

The ethosomal suspensions were found to be white in color and were translucent to opaque in nature. The particle size, zeta potential, and PDI were found to be in the range of 110.98 nm to 593.5 nm, -19.9 mV to -30.1 mV, and 0.254 to 0.773 respectively. The entrapment efficiency and % CDR of ethosomes was found to be in the range of 55.55% to 91.27% and 31.22% to 54.27% respectively. The responses thus obtained were analysed statistically by design expert software and were subjected to ANOVA. The optimized formula was then obtained through the software as a result of the evaluated responses. The optimised ethosomal formulation was then characterised by TEM, ATR-FTIR, particle size, zeta potential, PDI and entrapment efficiency. Drug release kinetics were studied by *in-vitro* and *ex-vivo* drug release studies.

All the results were found to be satisfactory. On comparison of the *in-vitro* release profile of ethosomes with a pure drug, the ethosomal formulation improved the permeability as well as solubility of Indomethacin. The stability studies showed that the ethosomal formulation was quite stable for a period of 3 months and more stable at -20 °C and 2 to 8 °C compared to room temperature.

The optimised ethosomal formulation was incorporated into carbopol and HPMC gel bases of varying ratios. 7 ethosomal gels were formulated and evaluated for several parameters such as appearance, pH, spreadability, drug content and viscosity. Drug release kinetics were studied by *in-vitro* and *ex-vivo* drug release studies. The ethosomal gel formulation was also analysed for e*x-vivo* skin permeation, skin retention and skin irritation studies. The gels were white in color, homogenous and free from grittiness. The results of

pH, spreadability, drug content and viscosity were found to be satisfactory.

The *in-vitro* release studies showed a gradual increase in drug release over a period of 8 h. The ethosomal gels were found to follow zero-order kinetics and the drug release occurred by non fickian diffusion. Based on the results of evaluation formulation G2 was considered as the optimised formulation.

In comparison with the marketed formulation, formulated ethosomal gel was found to improve the permeation of drugs. The transdermal flux of ethosomal formulation as well as ethosomal gel was found to be higher than that of the pure drug thus proving that ethosomes increased the permeation of Indomethacin though the skin. Skin irritation studies proved that the formulated ethosomal gel was safe to use as it did not cause any edema or erythema on an application in rats. The optimised ethosomal gel was subjected to three months' stability studies at 2-8 °C and room temperature and then evaluated for any phase separation, pH, viscosity and drug content. Results showed that formulation G2 was more stable at 2-8 °C than at room temperature.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: Nil

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