Original Article

Development and Evaluation of Azelaic Acid Based Ethosomes for Topical Delivery for the Treatment of Acne

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ABSTRACT

Purpose: To design, develop and evaluate an Azelaic acid encapsulated Ethosomal formulation for acne. **Methods:** Encapsulated ethosomes were prepared by three methods viz. hot method, cold method and thin film hydration method. Central Composite Design was employed for optimisation of ethosomal formulations. Concentrations of phospholipid, cholesterol and ethanol were selected as independent variables and their effect on the dependent variables (Entrapment Efficiency and Drug Diffused) was studied. The optimised vesicular carriers were evaluated for vesicle size, entrapment efficiency, *in-vitro* and *ex-vivo* diffusion studies, anti-microbial activity, skin irritation studies and stability studies as per ICH guidelines. Ethosomal formulations with varying soya phosphatidylcholine, cholesterol and ethanol were prepared. **Results:** Vesicles were spherical, unilamellar with a smooth surface. The optimised formulation showed a vesicle size of 4.25 ± 1.35 μm and entrapment efficiency of 91.86 ± 2.25%. *In-vitro* and *ex-vivo* drug diffusion of the ethosomal gel was compared with a conventional gel and a marketed cream. The developed novel formulation exhibited enhanced anti-acne activity as compared to conventional gel and a marketed cream. **Conclusion:** We can conclude that the ethosomal formulation is an efficient vesicular carrier system for topical delivery.

Key words: Ethosomes, Azelaic acid, Composite, Vesicles, *Propionibacterium acne*, Topical.

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INTRODUCTION

Acne vulgaris, one of the most common skin disorders, is the result of chronic inflammation of sebaceous follicles and is characterized by tender inflammatory papules and nodules mainly scattered on the face, chest, and upper back. An increase in circulating androgens at the onset of puberty stimulates the production of sebum. It is caused by cutaneous micro-organisms such as *Propionibacterium acnes* (*P. acnes*).[1] *P. acnes* itself has been shown to stimulate toll-like receptor 2 (TLR-2) activity, resulting in skin inflammation and comedogenesis. The individual lesions of Acne vulgaris, are of three types: inflamed lesions, non-inflamed lesions, and scars. [2]

Topical Azelaic acid (AzA) is approved for the treatment of acne vulgaris and inflammatory (papulopustular) rosacea. Due to diverse mechanisms of action that correlate with potential therapeutic benefit, AzA has been used to treat several common dermatoses including acne vulgaris, perioral dermatitis, inflammatory rosacea, melasma, and post inflammatory hyperpigmentation.[3]

AzA is a naturally occurring dicarboxylic acid produced by the yeast fungus Pityrosporum ovale. Another beneficial effect of

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this agent is to decrease hyper-pigmentation caused by acne. It helps scavenge reactive oxygen species, reduces expression of kallikrein-5 (KLK-5) and pro-inflammatory cathelicidins like LL-37, as well as inhibits TLR-2. It also inhibits the pigment producing enzyme tyrosinase, may reduce epidermal hyperkeratinization and has comedolytic properties and[4] TLR-2 over-activity plays a role in the pathogenesis of acne. *P. acne* is a gram-positive and propionic acid-producing bacterium that colonizes anaerobically within the hair follicles of the skin.

Drugs used to treat acne include AzA, benzoyl peroxide, clindamycin phosphate, adapalene, erythromycin, tretinoin and isotretinoin.

Limitations of conventional topical formulations include side effects like scaling, erythema, dryness, stinging, irritation, burning, itching, rash, pruritus and sunburn. To reduce side effects, novel carrier-based drug delivery systems are formulated which distribute the topical medicament gradually, reduce the irritancy of drugs and maintain better efficacy. Therefore, penetration is more efficient than non-particulate systems that provide a high local concentration.

Vesicles are an example of novel drug delivery systems which act as drug carriers to deliver entrapped drug molecules into or across the skin. They act as enhancers for the penetration of the individual lipid components into the stratum corneum and subsequently altering the inter-cellular lipid lamellae within this skin layer. Vesicles also serve as a depot for sustained release of dermally active compounds and as a rate limiting membrane barrier for the modulation of systemic absorption, therefore, providing a controlled transdermal delivery system.[5]

Ethosomes are phospholipid-based elastic vesicles containing 20-45% ethanol. Ethanol is a proven permeation enhancer imparting high flexibility to vesicular membrane which permits the elastic vesicles to squeeze themselves through the pores. The mechanism of penetration enhancement with the ethosomal system suggests the intercalation of ethanol into the polar head group environment resulting in increased membrane permeability. Ethosomes are reported to be more stable than liposomes because of the presence of ethanol, which provides a net negative charge on the surface thereby avoiding aggregation of vesicles due to electrostatic repulsion.[6,7] Topically applied ethosomes can increase the residence time of active ingredients in the stratum corneum, epidermis and reduce the systemic absorption of drugs. All these properties allow ethosomes to permeate easily into the deeper layers of the skin and circulation where ethanol causes skin disruption which increases the lipid fluidity that allows enhanced permeation of medicament through the skin. It then fuses with skin lipids to release the medicament into deep skin layers. Ethosomes enhance the permeation of the drug through skin, disperse better, exhibit improved therapeutic efficacy and good storage stability, improved bioavailability and provide protection from toxicity. Thus, the rationale of this work is to enhance the accumulation of a model drug for acne treatment at the site of administration and achieve sustained release.

The Central Composite Design (CCD)

This is the most popular of all second-order designs and the design consists of the following three portions:

1. A complete (or a fraction of) 2 k factorial design whose factors' levels are coded as −1 and 1, is called the factorial portion.
2. An axial portion consisting of 2 k points arranged so that two points are chosen on the axis of each control variable at a distance of a from the design centre (chosen as the point at the origin of the coordinates system).
3. n_0 centre points.[8]

MATERIALS AND METHODS

Materials

AzA was purchased from Otto Chemie pvt. Ltd (Mumbai), Soya phosphatidyl choline from Himedia, Cholesterol from Qualigens, Solvents like methanol, ethanol, and chloroform were procured from SD-Fine. Carbopol 934 was obtained as a gift sample from Lubrizol. Dialysis membrane was purchased from Himedia. Male Albino rats were purchased from Bharat Serum, Thane.

METHODS

Preparation of Ethosomes[9,10]

The methods tried for preparation of ethosomal formulations were Hot Method, Cold Method and Thin film hydration method using rotary evaporator.

Hot Method

A mixture of drug, ethanol and propylene glycol was prepared, heated at 40°C and the mixture was added to the phospholipid dispersion in water at 40°C and mixed for 30 min at 700 rpm.

Cold Method

The phospholipids, drug, and other lipid materials were dissolved in ethanol, in a covered vessel, at room temperature, with vigorous stirring and heated up to 30°C on a water bath. The water was heated to 30°C

in separate vessel, and added to the above mixture and stirred for 30 min in a covered vessel.

Thin film hydration method using rotary evaporator

Phospholipid, cholesterol was taken in round bottom flask containing glass beads and dissolved in chloroform and methanol. The solvents were evaporated at 40°C by applying vacuum for 30-40 min for the film formation. The film was then hydrated by using pH 7.4 phosphate buffer and ethanol for 1 h until a suspension is formed.

Preparation of ethosomal gel

AzA ethosomal vesicles with the best entrapment efficiency result were chosen for gel formulation. Specific amount of Carbopol 934 was added in minimum amount of water and left to swell for an hour. Appropriate amount of formulation incorporated to the swollen carbopol with continuous stirring until homogeneous ethosomal gel was formed. pH of the gel was then adjusted by adding triethanolamine (TEA) and stirred slowly.

Preformulation studies

Preformulation studies conducted include:

Description

AzA was checked for its colour and odour.

Solubility studies

AzA was added to solvents like distilled water, boiling water, methanol, ethanol, chloroform, pH 5.5, 5.6, 6.8, 7.4 Phosphate buffer, pH 6.8 Phosphate buffer + 0.5% Tween 80, pH 6.8 Phosphate buffer + 1% Tween 80 in 1:1 ratio.

FTIR studies

These studies were carried out to determine authenticity of the drug and drug-excipient compatibility using Fourier Transform Infrared Spectrophotometer (IR Affinityl, Shimadzu). KBr pellets were prepared of the active ingredient and the excipients.

Melting point

Melting point of the active ingredient was determined.

Assay Method Development

Potentiometric titration method (IP 2014): 0.1 g of API was dissolved in 10 ml of methanol and 40 ml of water was added. Titration was carried out with 0.1 M sodium hydroxide (NaOH), to determine the end point potentiometrically. Blank titration was carried out.

1 mL of 0.1M NaOH is equivalent to 0.0094115 g of AzA ($C_9H_{16}O_4$).

Optimisation

Central Composite Design (CCD)

Experimental design

A three-factor, three-level Central composite experimental design was constructed using DESIGN EXPERT® SOFTWARE (Version 9.0.0). The independent variables selected were the proportion of phospholipid, proportion of cholesterol and concentration of ethanol. Their corresponding levels and the dependent variables are shown in Table 1.

Table 2 mentions the optimised formula for Ethosomes.

EVALUATION PARAMETERS

Evaluation of Ethosomes

% Drug content

Assay was carried out in triplicates to determine the drug content of a) ethosomal suspension, b) **ethosomal gel** c) conventional gel. The assay was performed using potentiometric titration by the developed method.

pH

pH of all formulations and gel were determined using calibrated pH meter.

Drug Entrapment Efficiency

Entrapment Efficiency was obtained by centrifugation technique. Ethosomal suspension was centrifuged for 2 hrs to obtain supernatant and sediment. The supernatant and sediment were analysed for drug entrapped using potentiometric titration. Entrapment Efficiency was obtained by the indirect method using supernatant and also by the direct method using the sediment.

Entrapment Efficiency was calculated using the formula:

Entrapment Efficiency (%)=(D/DT) x 100%

Where, D=Amount of drug in sediment
DT=Total amount of drug in supernatant and sediment

Optical Microscopy

The ethosomal formulation was evaluated by observing under Motic Instrument under 40x to determine its vesicle size.

Vesicle size determination and Zeta Potential Measurement

Vesicle size was determined by using a computerized inspection system viz. (Malvern Zetasizer). Sample was diluted with distilled water and vesicle size was determined.

Zeta Potential was also determined, which indicates the charge present on the surface of vesicles.

Scanning Electron Microscopy (SEM) Studies

SEM studies were carried out to determine particle size, surface morphology and texture.

Evaluation of Gels

Drug Content

Required amount of gel corresponding to 0.1g of AzA was taken in a beaker and using potentiometric titration the % drug content was obtained.

Viscosity

Viscosity of the prepared ethosomal gel was determined by Brook-field Viscometer at 25°C using spindle 96.

Spreadability

Apparatus to determine spreadability consists of a fixed slide on wooded block to measure the length and an upper slide tied to weighing pan. Specific amount of ethosomal gel and blank carbopol gel was placed individually in between two glass slides and weight was added gradually to the pan. The time (seconds) required to separate the two slides, was taken as a measure of spreadability. It was calculated using formula,

$$S = M. L / T$$

Where, S=spreadability, M=weight tied to upper slide, L=length of glass slide, T=time taken (seconds).

In-vitro drug release

In-vitro drug release studies were performed using Franz diffusion cell. Specific amount of ethosomal gel was applied on the dialysis membrane. The receptor compartment contained (pH 7.4 phosphate buffer). 1mL of aliquot was withdrawn at regular intervals for 12 hrs and was replaced with phosphate buffer pH 7.4. Samples were analysed using potentiometric titration.

Ex-vivo drug release

Freshly excised porcine skin ear was immersed in pH 7.4 phosphate buffer. Ethosomal gel was placed on top of the skin facing the dermal side and studies were carried out using Franz diffusion cell. The receptor compartment contained pH 7.4 phosphate buffer. 1 mL of aliquot was withdrawn at regular intervals for 12 hrs and was replaced with pH 7.4 phosphate buffer. Samples were analysed using potentiometric titration.

Anti-microbial testing

Anti-microbial testing was carried out using the cup and plate method. The nutrient media viz. Reinforced Clostridial Agar was prepared and autoclaved for 15 min at 121°C. Media was poured in the plates and allowed to solidify. A cup was made in the centre of the plate with cork borer and the culture (*P. acne*) was inoculated. Formulation was added in the cup. Prepared plates were placed in anaerobic condition using the anaerobic jar and were incubated at 37°C for 24 hrs. Ethosomal gel, conventional gel, marketed cream and pure drug were compared.

Skin irritation study

Protocol for the study was approved by Institutional Ethics Committee Approval number: CPCSEA/IAEC/BNCP/P-46/2014.

Ethosomal gel was tested for irritation on male albino wistar rats. Dorsal side of rat skin was shaved using hair removal cream and subjected to wash out period. optimised formulation ethosomal gel was applied. Control was used and rats were observed after 24 hrs.

Stability Studies

Ethosomal suspension and gel were subjected to stability studies as per ICH guidelines to obtain 3 months data. Formulations were studied at 5 ± 3°C, 25°C ± 2°C/60 ± 5% RH and 40°C ± 2°C/75 ± 5% RH.

RESULTS

Preformulation studies

Description

AzA was white in colour and odourless.

Solubility studies

The active medicament was found soluble in boiling water, methanol, ethanol, chloroform, pH 7.4 phosphate buffer and was insoluble in pH 6.8 Phosphate buffer + 0.5% Tween 80, pH 6.8 Phosphate buffer + 1% Tween 80, distilled water, pH 5.5, 5.6, 6.8 phosphate buffer.

FTIR studies

The frequencies of AzA were found at C=O stretch (1701 cm^{-1}), Broad –OH (2934.6 cm^{-1}), C-O medium intensity (1301.3 cm^{-1}) and O-H oop (927.5 cm^{-1}) (Figure 1).

Melting point

Melting point of the active ingredient obtained was 106°C ± 2°C. Result obtained was in the reported range (109°C -111°C).

Inter-day and Intra-day precision

% Relative Standard Deviation (%RSD) obtained for inter-day precision was 0.33 and intra-day precision was found to be 0.48.

Optimisation

Central Composite Design

RESPONSES

Entrapment Efficiency (E.E)

Value of "Prob > F" less than 0.0500 indicates model terms are significant.

Equation: % E.E = 88.71 -3.52 *X_1+ 1.14 *X_2 + 3.63 * X_3-2.30 * X_1X_2 -1.07 * X_1X_3 +0.18 * X_2X_3 -12.80* X_1^2 + 3.90 * X_2^2 -17.76 * X_3^2

Figure 2 mentions the ANOVA for response surface quadratic model (%E.E)

Std. Dev.- 5.79, Mean: 75.38, C.V. %: 7.68, PRESS: 1860.68, R-Squared: 0.9113, Adj R-Squared: 0.8314, Pred R-Square: 0.5074, Adeq Precision: 9.553

Drug Diffused (D.D)

Value of "Prob > F" less than 0.0500 indicates model terms are significant.

Equation: % Drug Diffused = 86.78 + 4.4 *X_1 + 0.3 * X_2 -1 * X_3 -0.37 * X_1X_2 -2.37 * X_1X_3 -0.87 * X_2X_3 -9.45* X_1^2 -1.95 * X_2^2 -25.45 * X_3^2

Figure 6 mentions the ANOVA for response surface quadratic model (%D.D)

Std. Dev.- 8.86, Mean: 68.35, C.V. %: 12.96, PRESS: 5542.73, R-Squared: 0.8811, Adj R-Squared: 0.7741, Pred R-Square: 0.1603, Adeq Precision: 7.055

Results of the optimised formulation of Ethosomes

% Drug Content

% Drug content of optimised Ethosomal formulation was found to be 97.3% ± 1.3 and that of Conventional Gel: 95.8%.

pH

The pH of the obtained ethosomal formulation was found to be 5.6. pH obtained was in the range of skin pH i.e 4.5 to 7.6.

Optical Microscopy

Vesicle size obtained was in the range of 4.2-5.1 μm

Entrapment efficiency (E.E)

In case of Hot Method, formulations showed low entrapment efficiency in the range 35%-43%.

In case of Cold Method, the results obtained were not promising due to less entrapment efficiency and clarity, hence it was not chosen for further studies.

Based on the results obtained from hot method and cold method further batches was prepared by thin film hydration method using rotary vacuum evaporator.

Thin film hydration method using rotary evaporator: Entrapment efficiency of the batches tried with the above mentioned ranges of Phosphatidylcholine: Cholesterol, Ethanol and drug were obtained in the range of 55%-88% by direct method. Entrapment efficiency obtained for the optimised formulation was 91% by direct method and 91.24% by indirect method.

Vesicle size determination and Zeta Potential Measurement

Average vesicle size obtained was 514.3 nm and Polydispersity index obtained was 0.08. Zeta Potential was found to be -35.44 mV.

Scanning Electron Microscopy (SEM)

On characterization spherical, unilamellar vesicles with smooth surface were observed under scanning electron microscope (Figure 10).

Results of Ethosomal gel

% Drug Content

% Drug content of Ethosomal gel and Conventional Gel was found to be 98.2% ± 1.2 and 96.8% ± 0.3, respectively.

Viscosity

Viscosity of the formulated ethosomal gel was in the range 59987 to 984 mpas at rpm 0.5 to 100 using Spindle 96.

Spreadability

Spreadability of the prepared ethosomal gel found to be 8.4 g cm/sec and that of Blank gel was found to be 1.14 g cm/sec.

In-vitro and *Ex-vivo* drug release studies

In-vitro drug release studies show an average drug diffusion of 89.6% and *Ex-vivo* drug release studies showed an average drug diffusion of 94.3% in 12 hrs. In comparison, *in-vitro* drug release and e*x-vivo* drug release the % drug diffused of 10% marketed cream was compared with that of ethosomal gel and conventional gel. Conventional gel (105% in 1 hr) and marketed cream (102%) showed faster release as compared to ethosomal gel (Table 3).

The formulated ethosomal gel showed sustained release as compared to conventional gel and marketed cream and i*n-vitro* and e*x-vivo* results were comparable (Figure 11 and Figure 12).

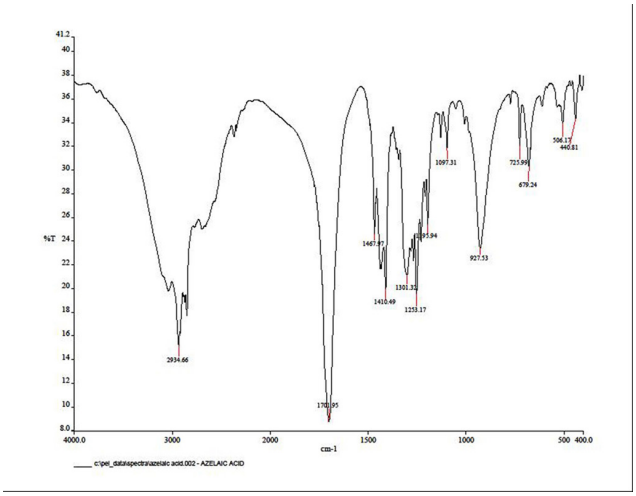

Figure 1: FTIR spectrum of Azelaic acid.

Response 1 Entrapment Effieciency

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	3442.07	9	382.45	11.41	0.0004	significant
A-PC	*123.90*	*1*	*123.90*	*3.70*	*0.0834*	
B-CHOLEST.	*13.00*	*1*	*13.00*	*0.39*	*0.5474*	
C-ETHANOL	*131.62*	*1*	*131.62*	*3.93*	*0.0757*	
AB	*42.32*	*1*	*42.32*	*1.26*	*0.2874*	
AC	*9.24*	*1*	*9.24*	*0.28*	*0.6109*	
BC	*0.24*	*1*	*0.24*	*7.309E-003*	*0.9336*	
A^2	*450.62*	*1*	*450.62*	*13.44*	*0.0043*	
B^2	*41.81*	*1*	*41.81*	*1.25*	*0.2902*	
C^2	*867.49*	*1*	*867.49*	*25.88*	*0.0005*	
Residual	335.19	10	33.52			
Lack of Fit	*328.58*	*5*	*65.72*	*49.72*	*0.0003*	*significant*
Pure Error	*6.61*	*5*	*1.32*			
Cor Total	3777.26	19				

Figure 2: Anova for Response Surface Quadratic model (% Entrapment Efficiency).

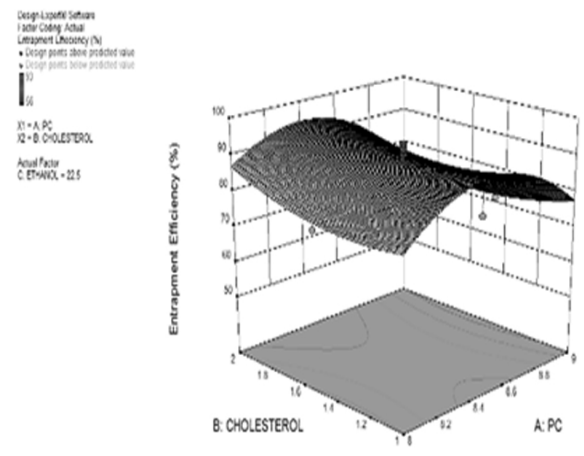

Figure 3: 3D Surface plot showing effect of PC and % Cholesterol on % Entrapment Efficiency.

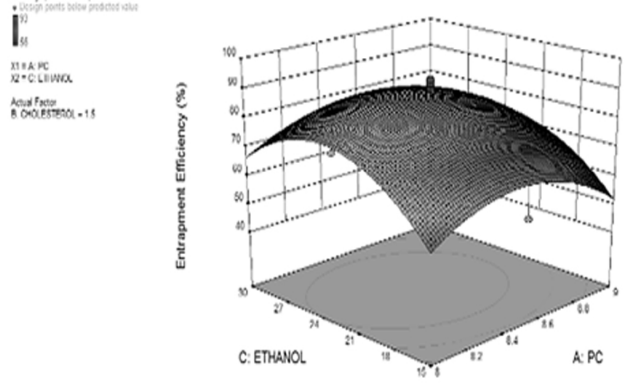

Figure 4: 3D surface plot showing effect of PC and ethanol on %Entrapment Efficiency.

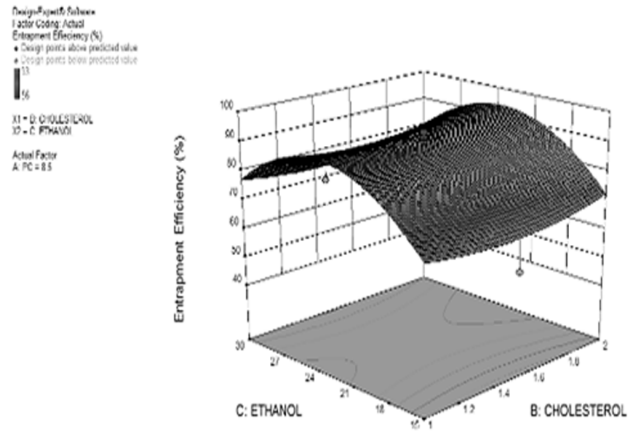

Figure 5: 3D surface plot showing effect of Cholesterol and Ethanol on % Entrapment Efficiency.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	5815.94	9	646.22	8.24	0.0014	significant
A-PC	*193.60*	*1*	*193.60*	*2.47*	*0.1473*	
B-CHOLEST.	*0.90*	*1*	*0.90*	*0.011*	*0.9168*	
C-ETHANOL	*10.00*	*1*	*10.00*	*0.13*	*0.7285*	
AB	*1.13*	*1*	*1.13*	*0.014*	*0.9071*	
AC	*45.12*	*1*	*45.12*	*0.58*	*0.4657*	
BC	*6.13*	*1*	*6.13*	*0.078*	*0.7856*	
A^2	*245.82*	*1*	*245.82*	*3.13*	*0.1071*	
B^2	*10.51*	*1*	*10.51*	*0.13*	*0.7220*	
C^2	*1781.82*	*1*	*1781.82*	*22.71*	*0.0008*	
Residual	784.61	10	78.46			
Lack of Fit	*777.27*	*5*	*155.45*	*105.99*	*< 0.0001*	*significant*
Pure Error	*7.33*	*5*	*1.47*			
Cor Total	6600.55	19				

Figure 6: ANOVA for Response Surface Quadratic model (% Drug Diffused).

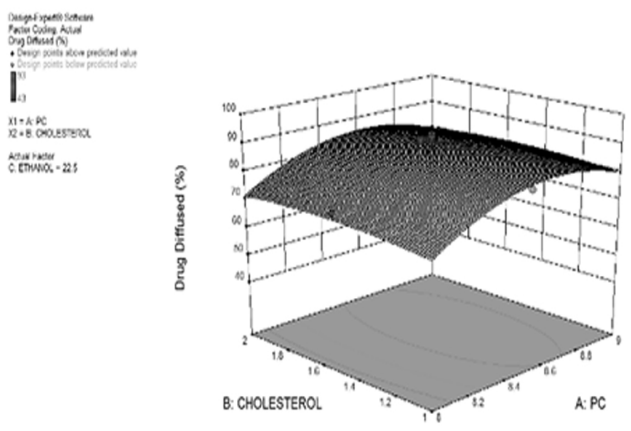

Figure 7: 3D surface plot showing effect of PC and Cholesterol on % Drug Diffused.

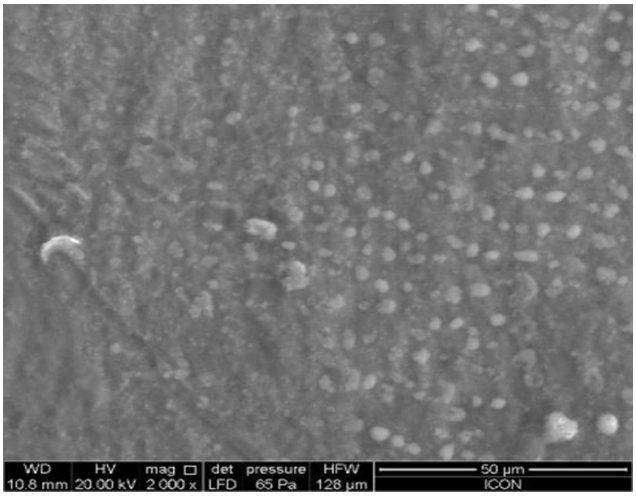

Figure 10: SEM image of Ethosomal Formulation.

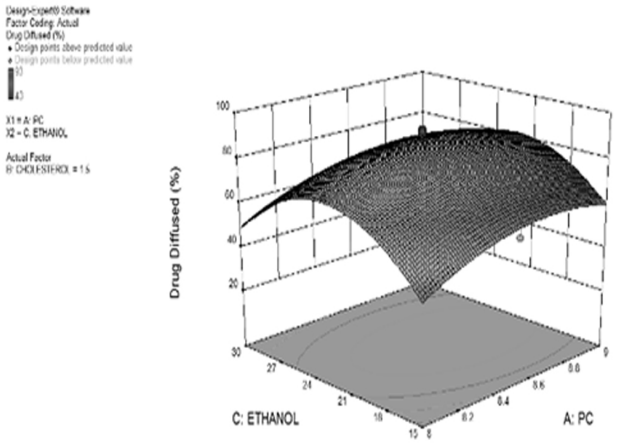

Figure 8: 3D surface plot showing effect of PC and Ethanol on % Drug Diffused.

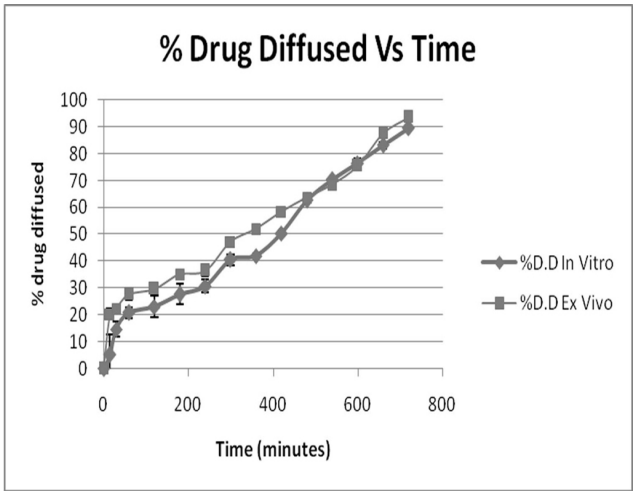

Figure 11: % Drug diffused of Ethosomal gel (*In vitro* and *Ex vivo*).

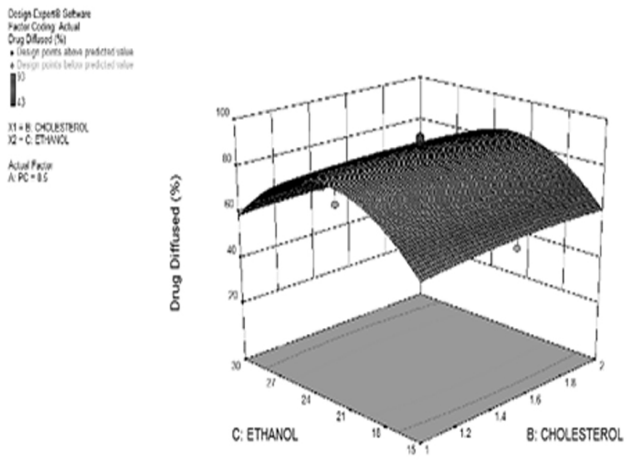

Figure 9: 3D surface plot showing effect of Ethanol and Cholesterol on % Drug Diffused.

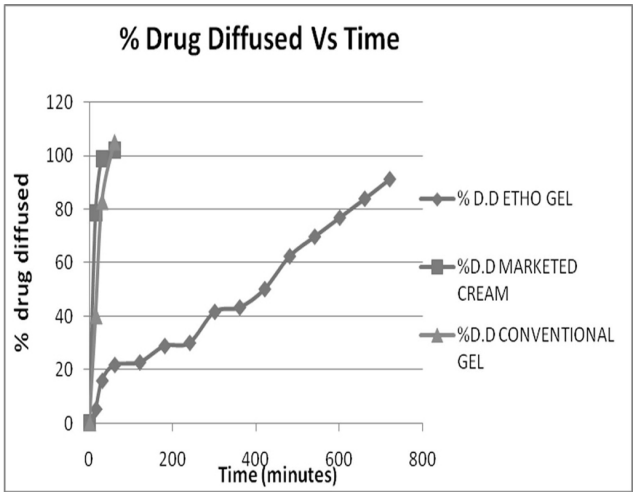

Figure 12: Comparison of ethosomal gel with marketed cream and conventional gel.

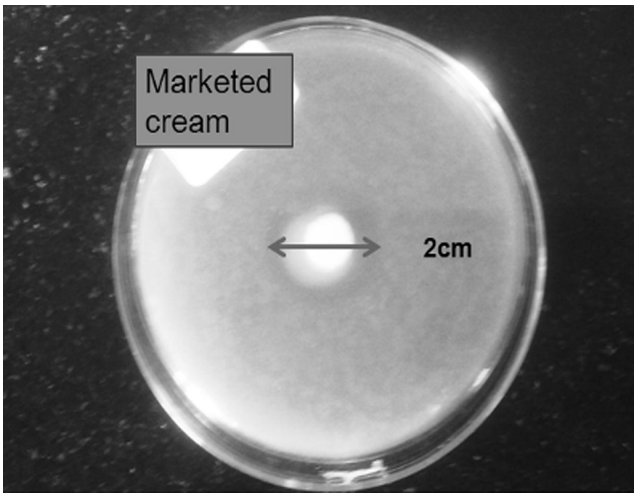

Figure 13: Zone of Inhibition of Marketed cream.

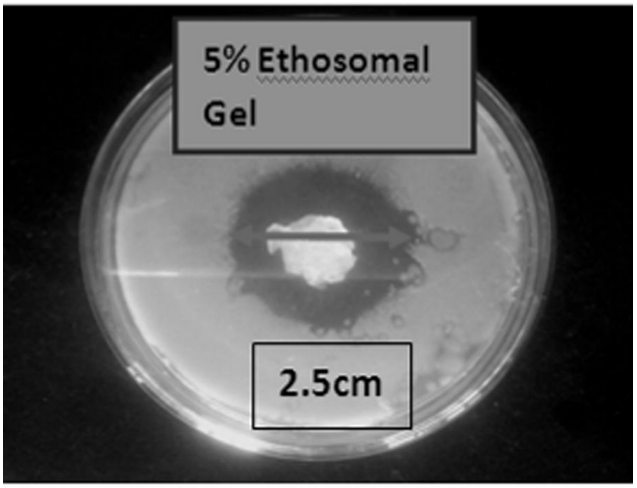

Figure 14: Zone of Inhibition of 5% ethosomal gel.

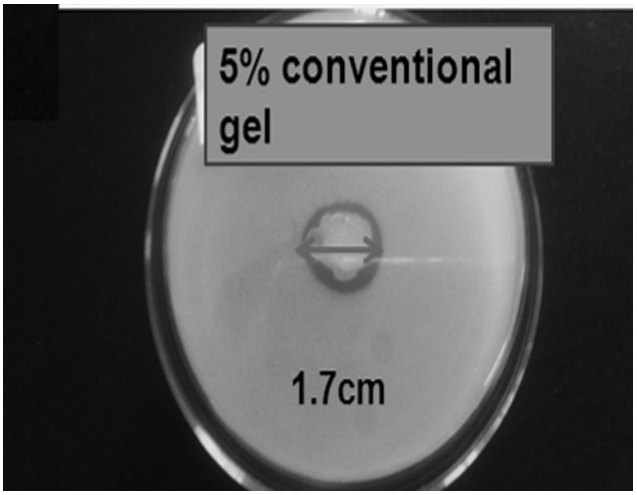

Figure 15: Zone of Inhibition of 5% conventional gel.

Kinetic Modelling

In-vitro drug release profile of ethosomal gel showed that it follows zero order kinetics as its R^2 value was closest to unity i.e 0.9819. *Ex-vivo* drug release profile of ethosomal gel also was found to follow zero order kinetics having R^2 value as 0.9643 which is closest to unity.

Determination of Flux

Diffusion flux (J) measures the amount of substance that will flow through a small area during a small time interval. Flux was obtained from the slope values plotted for amount diffused per unit area against time. Flux of Ethosomal gel was found to be 25.54 mg/cm^2/hr, that of conventional gel was 20.6 mg/cm^2/hr and marketed cream was 16.30 mg/cm^2/hr.

Anti-microbial testing

Zone of inhibition of the formulations were found as follows:

Marketed Cream = 2 cm (Figure 13)

5% Ethosomal Gel = 2.5 cm (Figure 14)

5% Conventional gel = 1.7 cm (Figure 15)

Skin irritation study

Ethosomal gel showed no reddening and inflammation after 24 hrs of skin irritation study.

Stability Studies

Table 4 mentions Stability data of Ethosomal Suspension and Ethosomal Gel.

DISCUSSION

Preformulation Studies

FTIR studies

The observed frequencies were not affected when AzA was combined with phospholipid and cholesterol. From the results obtained it can be concluded that the active ingredient is compatible with the excipients without interaction.

Melting point

Results conclude that AzA is authentic.

Inter-day and Intra-day precision

Inter-day precision and Intra-day precision was found to be less than 2. This indicates that the developed method is precise and reliable to use.

Table 1: Observed response in central composite design for Azelaic acid ethosomal formulation				
Independent Variables			Response (Dependent Variables)	
X1: Phosphatidyl Choline (PC)	X2: Cholesterol	X3: Ethanol %	Drug diffused %	Entrapment Efficiency %
8.5	1.5	30	51	76.68
8.5	2	22.5	73	90
8.5	1.5	22.5	90	93
9	1	30	58	62.5
8	1	30	43	65.6
8.5	1.5	15	58	56
8	2	30	51	72.5
8	1	15	43	60
8.5	1.5	22.5	92	93
8.5	1	22.5	83	86
9	2	30	53	60
8	1.5	22.5	75	77.6
8.5	1.5	22.5	93	92
9	2	15	66	58
8.5	1.5	22.5	91	91.5
8.5	1.5	22.5	92	90
8	2	15	43	66
8.5	1.5	22.5	90	91.2
9	1.5	22.5	66	65
9	1	15	56	61

Table 2 : Optimised formula for Ethosomes	
Ingredients	Quantity taken
Azelaic acid	0.75%
Soya phoshatidyl choline	0.85%
Cholesterol	0.15%
Chloroform	37.5 ml
Methanol	12.5 ml
Ethanol	22.5 ml
pH 7.4 Phosphate Buffer	q.s 100 ml

Optimisation

Central Composite Design

RESPONSES

% Entrapment Efficiency

From Figure 3 and based on the p value of phosphatidylcholine (PC) and cholesterol, PC having lesser p value of 0.0834 than p value of cholesterol i.e 0.5474 which indicates PC has more effect on the %E.E. It was found that with increase in proportion of PC from 8 to 8.5, the %E.E significantly increased from 60% to 93% (Table 1) with lesser proportion of cholesterol. However, a further increase in proportion of PC to 9 led to a significant decrease in %E.E to 58%, this may be due to the leakage of excess drug from the vesicular structure.

From Figure 4 and based on the p value of PC and ethanol, ethanol having lesser p value of 0.0765 than p value of PC i.e 0.0834 which indicates ethanol has more effect on the %E.E. It was found that with increase in ethanol concentration from 15% to 22.5%, the %E.E significantly increased from 56% to 93% with lesser

Table 3: % Drug Diffused of 5% Ethosomal gel (*In-vitro* and *Ex-vivo*), 10% Marketed cream, 5% conventional gel (n=3)

Time (Minutes)	%D.D of 5% Ethosomal gel (*In-vitro*) ± Standard deviation	%D.D of 5% Ethosomal gel (*Ex-vivo*) ± Standard deviation	%D.D marketed cream ± Standard deviation	%D.D conventional gel ± Standard deviation
0	0	0	0	0
15	5.31 ± 0.13	20.25 ± 0.41	78.5 ± 0.52	40 ± 1.3
30	14.73 ± 1.11	22.36 ± 0.45	98.6 ± 1	82.8 ± 0.8
60	20.9 ± 0.57	27.72 ± 0.38	102 ± 0.2	105 ± 0.6
120	23.16 ± 0.49	29.76 ± 0.56	_	_
180	27.73 ± 1.11	35.17 ± 0.71	_	_
240	30.83 ± 1.17	36.53 ± 0.26	_	_
300	40.5 ± 0.96	47.18 ± 1.05	_	_
360	42 ± 0.90	51.97 ± 1	_	_
420	50.5 ± 0.40	58.47 ± 0.81	_	
480	62.5 ± 0.67	63.73 ± 1	_	_
540	70.26 ± 0.67	68.4 ± 0.8	_	_
600	76.43 ± 0.75	75.53 ± 0.53	_	_
660	83.2 ± 1.45	87.52 ± 0.93	_	_
720	89.6 ± 1.09	93.44 ± 0.5	_	_

Table 4: Stability data of Ethosomal Suspension and Ethosomal Gel

Parameters	Initial			After 3 months		
Stability Conditions	5±3°C	25°C±2°C/ 60±5%RH	40°C±2°C/ 75±5% RH	5±3°C	25°C±2°C/ 60±5%RH	40°C±2°C/ 75±5% RH
Ethosomal Suspension						
Vesicle size (µm)	1-4	2-6	3-8	1-6	2-8	Coalescence
% Entrapment Efficiency	76.4	91.86	58	60	88.2	48
Redispersibility	Good	Good	Moderate	Good	Good	Poor
Ethosomal Gel						
Appearance	Pale Yellow, smooth gel	Pale Yellow, smooth gel	Pale Yellow, smooth gel	Pale Yellow, smooth gel	Pale Yellow, smooth gel	Pale Yellow, smooth gel
%Drug Diffused	83.2% in 11hours	89.6% in 12 hours	82.1% in 9 hours	76.5% in 9 hours	81.6% in 11 hours	75.4% in 6 hours
%Drug Content	93%	98.25%	91.5%	94.3%	96.3%	89.8%

proportion of PC. However, a further increase in ethanol concentration to 30% led to a significant decrease in %E.E to 60%, which may be due to the leakage of excess drug from the vesicular structure.

From Figure 5 and based on the p value of cholesterol and ethanol, ethanol having lesser p value of 0.0756 than p value of Cholesterol i.e 0.5474, which indicates ethanol has more effect on the %E.E. It was found that with increase in ethanol concentration from 15% to 22.5%, the %E.E significantly increased from 56% to 93% with lesser proportion of cholesterol. However, a further increase in ethanol concentration to 30% led to a significant decrease in %E.E to 60%, this may be due to the leakage of excess drug from the vesicular structure.

% Drug Diffused

From Figure 7 and based on the p value of PC and cholesterol, PC having lesser p value of 0.1472 than p value of cholesterol that is 0.9168 which indicates PC has more effect on the %D.D. It was found that with increase in proportion of PC from 8 to 8.5, the %D.D

significantly increased from 43% to 93% with lesser proportion of cholesterol. However, a further increase in proportion of PC to 9 led to a significant decrease in %D.D to 53%.

From Figure 8 and based on the p value of PC and ethanol, both exhibit an effect with ethanol having a larger effect on the % D.D. It was found that with increase in ethanol concentration from 15% to 22.5%, the %D.D significantly increased from 43% to 93% with lesser proportion of PC. However, a further increase in ethanol concentration to 30% led to a significant decrease in %D.D to 51%.

From Figure 9 and based on the p value of cholesterol and ethanol, ethanol having lesser p value of 0.7285 than p value of Cholesterol i.e 0.9168, which indicates ethanol has more effect on the %D.D. It was found that with increase in ethanol concentration from 15% to 22.5%, the %D.D significantly increased from 43% to 93% with lesser proportion of cholesterol. However, a further increase in ethanol concentration to 30% led to a significant decrease in % D.D to 51%.

Fitting of the data for observed responses to various models; it was observed that the best-fitted model for the two dependent variables was quadratic model. Higher values of the standard error (SE) for coefficients indicate the quadratic (non-linear) nature of the relationship. A positive value in regression equation for a response represents an effect that favours the optimisation (synergistic effect), while a negative value indicates an inverse relationship (antagonistic effect) between the factor and the response.

From all the above figures, the effect of PC, Cholesterol and ethanol on % Entrapment Efficiency and % Drug Diffused was obtained from the Design Expert software. From the experimental data a maximum of 92% Entrapment efficiency and 91% Drug Diffused was obtained when quantities of phosphatidylcholine, cholesterol, and ethanol were 0.85%, 0.15% and 22.5% respectively.

DISCUSSION OF THE FORMULATION OF ETHOSOMES AND ETHOSOMAL GEL

Vesicle size determination and Zeta Potential Measurement

Results obtained indicate negative charge present on the surface of ethosomes.

Viscosity

Viscosity was found to decrease with increase in rpm indicating a Non-Newtonian flow.

Spreadability

Spreadability of the ethosomal gel was found to be better than the blank gel indicating ethosomal gel is having good spreadability.

In-vitro and *Ex-vivo* Drug Release Studies

The formulated ethosomal gel showed sustained release as compared to conventional gel and marketed cream and i*n-vitro* and e*x-vivo* results were comparable.

Flux of Ethosomal gel was found to be better than conventional gel and marketed cream. Incorporation of drug in ethosomes and presence of ethanol as a permeation enhancer were responsible for enhancement of flux.

Anti-microbial testing

Results of activity tests indicate that the, ethosomal gel of varied strengths viz 5% and 10% showed better zone of inhibition as compared to conventional gel and marketed cream which indicates that the ethosomal gels are more efficient and reliable than conventional gel and marketed cream. Out of 5% and 10% strengths, 5% Ethosomal gel gave more promising results.

Skin irritation study

The ethosomal gel prepared was found to be non-irritant to the skin.

Stability Studies

Ethosomal suspension: Redispersibility and appearance was found to be good when stored at 5°C, sedimentation was observed at 25°C and phase separation was observed at 40°C at the end of 3 months. In case of vesicle size, no drug leakage was observed after 3 months, vesicle size was found to be increased at 5°C and at higher temperature coalescence was observed. There was a marked difference in % Entrapment Efficiency at 5°C and 40°C, whereas formulation at 25°C showed very less difference in % Entrapment Efficiency.

Ethosomal Gel: Appearance was unchanged at the end of 3 months and there was no marked difference in % drug content. In case of % drug content, maximum amount of drug was released in 6 hrs; this may be because of high coalescence due to leakage of drug.

CONCLUSION

Ethosomal formulations of AzA were successfully prepared, optimised and evaluated. Thin film hydration method exhibited good results in terms of % Entrapment efficiency, % Drug diffused. Vesicle size obtained was suitable. Ethosomal gel prepared was superior and efficient as compared to conventional formulations

based on the *in-vitro*, *ex-vivo* release profiles and antimicrobial activity. Also, formulated gels provided sustained release of the medicament. The dosage form prepared was non-irritant to the skin and therefore, can be used successfully as a topical formulation.

AzA vesicular based delivery systems are hence promising in the treatment of acne and vesicular systems can be employed topically in the treatment of various skin disorders.

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CONFLICTS OF INTEREST

No conflict of interest.

ABBREVIATION USED

AZA: Azelaic acid; **P. acne:** Propionibacterium acne; **TLR-2:** Toll-like receptor 2; **KLK-5:** Kallikrein-5; **CCD:** Central composite design; **FTIR:** Fourier Transform Infrared Spectrophotometer; **IP:** Indian Pharmacopoeia; **KBr:** Potassium bromide; **NaOH:** Sodium hydroxide; **SEM:** Scanning electron microscopy; **ICH:** International Conference on Harmonisation; **RH:** Relative humidity; **RSD:** Relative standard deviation; **E.E:** Entrapment efficiency; **D.D:** Drug diffused; **ANOVA:** Analysis of variance; **PC:** Phosphatidylcholine; **TEA:** Triethanolamine; **SE:** Standard error.

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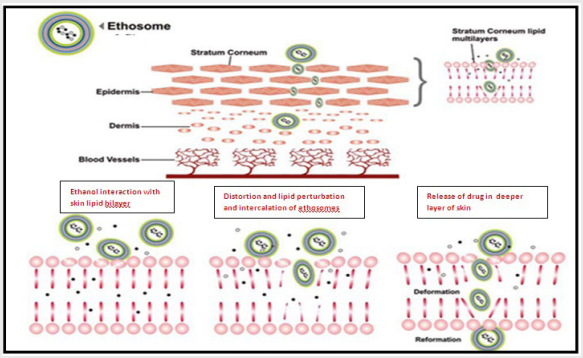
PICTORIAL ABSTRACT



SUMMARY

- Azelaic acid ethosomes were formulated for the treatment of acne.
- Encapsulated ethosomes were prepared by three methods viz. hot method, cold method and thin film hydration method.
- Formulated ethosomes were optimised using central composite design.
- The optimised formulation showed a spherical, unilamellar vesicle of size 4.25 ± 1.35 μm and entrapment efficiency of 91.86 ± 2.25%.
- Optimized formula was used for the formulation of ethosomal gel.
- The developed novel formulation exhibited enhanced anti-acne activity, *in-vitro*, *ex-vivo* release profiles as compared to conventional gel and a marketed cream.
- The developed azelaic acid based ethosomal gel was found to be stable from the 3 months data obtained.
- AzA vesicular based delivery systems are hence promising in the treatment of acne.
- Vesicular systems thus can be employed topically in the treatment of various skin disorders.