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Flurbiprofen-loaded ethanolic liposome particles for biomedical applications

Sarvesh Paliwal[a], Amita Tilak[a], Jaiprakash Sharma[b], Vivek Dave[a,*], Swapnil Sharma[a], Kanika Verma[a], Kajal Tak[a], Kakarla Raghava Reddy[c], Veera Sadhu[d,*]

[a] Department of Pharmacy, Banasthali Vidyapith, Rajasthan, India
[b] Department of Pharmacy, SMS Medical College, Rajasthan, India
[c] School of Chemical and Biomolecular Engineering, The University of Sydney, Sydney, NSW 2006, Australia
[d] School of Physical Sciences, Banasthali Vidyapith, Banasthali, Rajasthan, India

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ABSTRACT

Present study deals with the preparation, characterization and in-vivo evaluation of flurbiprofen loaded ethanolic liposome which provides predetermined and controlled release of drug through a transdermal drug delivery system. Ethanolic liposomes were prepared by using flurbiprofen, phospholipon 90-G, and ethanol in varied concentration ratio. The prepared ethanolic liposomes were optimized and characterized for particle size, zeta potential, polydispersive index and % entrapment efficiency. FTIR study was performed to analyze the interaction between drug and excipient. To study the thermal behavior of the formulation DSC and TGA were carried out. The surface morphology of ethanolic liposome was performed with the help of SEM, TEM, and AFM. In-vitro drug permeation study of the optimized formulation was carried out using the albino rat skin model and peripheral nociceptive activity was evaluated by writhing assay. In addition, formulations were also inspected for stability study for three months at a different temperature. The optimized formulation EF5 exhibited a particle size of 167.2 ± 3.7 nm with a zeta potential of −51.6 ± 0.2 mV and PDI of 0.209. The optimized formulation showed an ideal surface morphology with a maximum % entrapment efficiency i.e. 93.51 ± 2.1. In-vitro permeation study shows a release of 70.23% in 24 h and transdermal flux was found as 238.2 μg/cm^2/h. Writhing assay demonstrate that the optimized formulation decreases the number of writhes and thus shows the peripheral analgesic activity. In stability study, optimized formulation showed maximum stability at 4 °C. These results suggest that transdermal system mediated application of flurbiprofen loaded ethanolic liposome can be considered as an effective way to afford consistent and predictable release of flurbiprofen which could provide beneficial effects in the management of various inflammatory diseases.

1. Introduction

Transdermal drug delivery system offers some key advantages over other routes of drug administration. In particular, when a transdermal patch is applied to the skin it delivers a drug across skin through passive diffusion at a predetermined and controlled rate to attain an optimal therapeutic concentration in a subject (Dave et al., 2010). Phenylalkanoic acid derivative flurbiprofen is a non-steroidal anti-inflammatory drug which acts via cyclo-oxygenase inhibition which in turn inhibits prostaglandin synthesis. Prostaglandin has been considered as a principal marker of inflammation, pain, swelling, fever. Flurbiprofen is widely indicated in the treatment of various pathological conditions like acute and chronic rheumatoid arthritis, osteoarthritis and ankylosing spondylitis.

Ethanolic liposome was first developed and reported by Touitou and her colleagues in 2000 (Touitou et al., 2000). An ethanolic liposome is characterized as a lipid bilayer phospholipid vesicular system primarily composed of phospholipid and excessive ethanol. Bearing high ethanol concentration, they can effectively penetrate across stratum corneum and deeper layers of skin and possess higher transdermal flux as compared to conventional liposome formulation which gets remain confined to upper layer of skin i.e. stratum corneum (Bragagni et al., 2012). Presence of ethanol in the ethanolic liposome interacts with polar head group region of membrane lipids and alters the membrane fluidity and permeability. However, enhanced permeability of ethanolic liposome is also attributed to a collective mechanism between a high concentration of ethanol, phospholipid vesicles, and skin lipids. Ethanolic liposomes have been successfully tailored for enhanced delivery of various therapeutic agents such as ketoprofen, aceclofenac, Testosterone, Cannabidiol, Buspirone hydrochloride, Erythromycin, ibuprofen, Benzocaine,

* Corresponding authors.
E-mail addresses: vivekdave1984@gmail.com (V. Dave), veera.sadhu@gmail.com (V. Sadhu).

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Fluconazole, Finasteride, Lamivudine, 5-aminolevulinic acid etc. as they are soft, spongy and have malleable vesicles. They have been considered as non-invasive, novel innovative drug carrier with attractive features (Dave et al., 2010). The ethanolic liposome may encapsulate every type of drug-like hydrophilic drugs, lipophilic drugs, cationic drugs, proteins, and peptide and can be used as an effective drug carrier system for systemic and topical applications. Ethanolic liposomes have been considered as an improved form of liposome and can be prepared easily without the use of any special instrument. These systems are known to possess key features like better patient compliance, enhanced drug efficacy and also reduces the total cost of treatment (Hua, 2015). In light of these facts in the present study, we have developed an effective transdermal delivery system of flurbiprofen through ethanolic gel preparation and evaluated its efficacy using in-vitro and in-vivo models.

2. Materials and method

Flurbiprofen was received as a gift sample from Lupin Research Park, Pune, India. Phospholipon 90-G was provided as gift sample from Lipoid Germany. Ethanol, chloroform, propylene glycol, isopropyl alcohol, and methanol were purchased from Sigma-Aldrich Chemicals, USA. Carbopol 934 K was received from Himedia laboratory Mumbai, India. All other chemicals used during the experiment were of analytical grade.

2.1. Preparation of flurbiprofen loaded ethanolic liposome particles

Ethanolic liposomes of flurbiprofen were prepared by the method described by Touitou et al. (2000). All ingredients were weighed in different concentration ratio as described in Table 1. Initially, drug and phospholipid were dissolved in ethanol and propylene glycol and the resulting solution was heated in the water bath at a temperature of about 30 °C. Further, the distilled water was added as a fine stream to the solution with continuous stirring on a magnetic stirrer at a speed of 500 rpm in the closed vessels. The prepared solution was kept at a temperature of 4 °C, and sonicated using the probe sonicator (for 3 cycles each of 5 min with 5-min rest between each cycle) (Dave et al., 2010).

2.2. Flurbiprofen loaded ethosomal gel

To prepare the ethosomal gel, carbopol 934 K (0.75% *w/v*) was weighed and immersed in water for an hour then 20 ml of flurbiprofen loaded ethanolic liposome containing 250 mg of flurbiprofen was added to the carbopol gel and the mixture was placed on magnetic stirrer for continuous stirring at a speed of 500 rpm in a closed vessels and kept at a temperature of 30 °C until a homogenous ethosomal gel was achieved. To neutralize the pH, a small amount of triethanolamine was added followed with slow stirring until an ethosomal gel was formed. pH of the formulation was measured by using pH meter (Agrawal et al., 2013; Dave et al., 2017a).

2.3. Particle size, polydispersity index (PDI) and zeta potential

Particle size, polydispersity index, and zeta potential are the significant parameters for flurbiprofen loaded ethosomal system. Particle size and their size distribution were determined using malvern zetasizer nano ZS which is based on the principle of dynamic light scattering. For the size measurement, 1 ml of the ethanolic liposomal suspension was diluted with 10 ml of phosphate buffer saline (PBS). PDI was determined to know the particle size distribution. Zeta potential is the overall charge that the particles acquire in a particular medium and it was measured through Malvern zetasizer nano ZS which is based on principle laser Doppler velocimetry and phase analysis light scattering. Zeta potential above than + 30 mv and –30 mv were considered as more stable formulation (Dave et al., 2017a).

Table 1
Composition of flurbiprofen containing ethonolic liposomes.

Composition %w/w	EF1	EF2	EF3	EF4	EF5	EF6	EF7	EF8	EF9
Drug (Flurbiprofen) mg	250	250	250	250	250	250	250	250	250
Phospholipon 90-G (mg)	100	100	100	200	200	200	300	300	300
Ethanol (%)	30	35	40	30	35	40	30	35	40
Propylene glycol (%)	1	1	1	1	1	1	1	1	1
Water	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

2.4. Surface morphology of vesicles by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM)

Surface morphology of the lyophilized sample was examined with the help of scanning electron microscope SEM (EVO 18, Zeiss, Germany). Prior to analysis, the sample was placed on double-sided carbon adhesive tape with uniform gold sputtering and then it was analyzed at different magnification power (Dave et al., 2017b; Sharma et al., 2017a).

The morphology and size of the prepared ethanolic liposome were also evaluated using transmission electron microscopy (TEM) (AIIMS, Delhi) operated at 200 kV at a magnification of 9000×. In nutshell, 1 ml of the formulation was diluted 10 times with deionized water in Eppendorf tube. Subsequently, a small amount of sample was taken on a coated copper grid and was stained with 2% *w/v* phosphotungstic acid (for 30 s). It was dried and viewed randomly using calibrated microscopic magnification (Sharma et al., 2017b).

Atomic force microscopy was performed using AIST-NT (model no. Smart SPM 1000). Mica slips were used to prepare the AFM slide for ethanolic liposome. In brief, approximately 1 ml of the ethanolic liposomal suspension was dropped on mica slips which resulted in the formation of a thin film with the aid of spin coater dryer machine. The prepared slide was kept under the lens and observed at different magnifications. The images of the ethanolic liposome were captured in AC mode and for capturing of images AISTNT SPM Control software was used (Dave et al., 2017a).

2.5. Attenuated total reflections Fourier transform infrared spectroscopy (ATR-FTIR)

Infrared spectra of Pure drug (flurbiprofen), Phospholipon 90-G, ethanolic liposomes loaded with flurbiprofen, and physical mixture of phospholipon 90-G and flurbiprofen were performed with the help of Bruker EQUINOX 55 spectrophotometer equipped with the liquid nitrogen cooled mercury cadmium telluride detector at a resolution of about 2 cm^{-1}. Diamond was used as an internal reflection element placed at an incidence angle of 45°. The spectra were scanned from all regions i.e. from 4000 cm^{-1} to 400 cm^{-1} and advanced ATR correction was applied to all spectra. Peak fitting was performed with the help of opus software (Shavi et al., 2015).

2.6. Raman spectroscopy

Raman spectroscopy helps to provide the information on vibrational, rotational, and other low- frequency modes in the system. Raman was performed with the help of thermo-scientific instrument (DxRxi), equipped with a software OMINICxi-analysis. The 532 nm laser beam was used to collect the spectra of flurbiprofen loaded ethanolic liposomes formulation, flurbiprofen, phospholipon 90-G and physical mixture with the laser power of 5–100 mW. The spectra were taken in the range of 125–4000 cm^{-1} (Sharma et al., 2017a).

2.7. Differential scanning calorimeter (DSC)

DSC was executed with the help of NETEZCH DSC 204 F1 phoenix differential scanning calorimeter chamber, comprised of the calorimeter, a flow controller, a thermal analyzer, and operating software (NETZSCH Proteus thermal software). The optimized formulation EF4 was placed in an aluminum pan and weighed but the weight of the sample should be > 5 mg and < 25 mg then seal the pan with an aluminum lid with a hydraulic press. Further, the pan was placed in a DSC chamber and heated under the nitrogen flow at a heating rate of 40 °C/min from 25 °C to 350 °C. The scan with temperature on X-axis and heat flow on Y-axis was recorded and plotted. The same procedure was followed for the pure drug (flurbiprofen), phospholipon 90-G and physical mixture of phospholipon 90-G and flurbiprofen.

2.8. Thermogravimetric analysis (TGA)

TGA provides information about weight loss, sublimation, vaporization, absorption, adsorption etc. It was performed with the help of PROTEUS thermal analysis (TGA 400) to study the thermal behavior of drug, polymer and lyophilized formulation. For this study, the weight of empty crucible and crucible with sample were measured. The sample was analyzed in assembly and the thermogravimetric graph was scanned, recorded and plotted with temperature on X-axis and percent weight loss on Y-axis (Yub et al., 2013).

2.9. % entrapment efficiency

The total amount of drug entrapped within the colloidal system was determined by entrapment efficiency. Briefly, a known amount of formulation was taken in Eppendorf and centrifuged at speed of 14,000 rpm at 4 °C for 15 min. Centrifugation was repeated several times until clear supernatant was collected and the concentration of drug was determined using UV/visible spectrophotometer at 272 nm (Ling et al., 2010; Shah et al., 2012).

The percentage of the entrapped drug was calculated by the formula of:

Entrapment Efficiency (%) = (Amount of the drug in the ethosomes /Total amount of drug loaded into the ethosomes) × 100%

2.10. pH and viscosity

pH was measured through a digital pH meter by dipping the glass electrode completely into the ethanolic gel (Dave et al., 2017b). The viscosity of the prepared gel was determined by Brookfield viscometer (model LVDV II Pro) with the help of rotating spindle S 96 at 10, 15 and 20 rpm at room temperature. In brief, the spindle was dipped in the ethanolic gel in a beaker and observed at different intervals with lower, middle and upper case (Dave et al., 2017b).

2.11. Spreadability and extrudability

Spreadability was evaluated by sandwiching ethanolic gel between two glass slides (8 cm). Different weight pulleys were tied to the slides, further the weight and time at which the upper glass slide move was recorded (Panigrahi et al., 2006). The measurements were taken in triplicate and Spreadability was calculated by the formula of:

$$S = M^{*} L/T$$

where S represents the spreadability of the ethanolic gel, M is the weight knotted to the upper slide (g), L shows the distance moved by the slides (cm) and T is the time taken by the upper slide to roll down. The prepared ethanolic liposomal gel was packed in a 20 g of collapsible tube and the tube was pressed. The clamp was added to the tube to prevent rollback and the amount of gel extruded until the pressure degenerated was recorded (Dave et al., 2017b).

2.12. Experimental animals

Wistar albino male rats weighing 150–200 g were housed in cleaned polypropylene cages with free access to feed and water. They were kept under maintained laboratory conditions at temperature 24 ± 0.5 °C and 12/12 dark-light cycles. Entire procedures conducted in the study was in strict accordance to CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines and was approved by Institutional Animal Ethical Committee (Ref. no. BU/3431/16-17).

2.12.1. In-vitro skin permeation study

Male albino Wistar rats (150–200 g) were used in in-vitro skin permeation study. The rats were anaesthetized and hairs were removed from dorsal region skin using hair removal cream (Jaybhaye et al., 2011). After trimming subcutaneous fat and connective tissue the skin was washed using physiological salt solution (PSS) and distilled water and stored in a refrigerator at 4 °C until used. Skin permeation study of flurbiprofen loaded ethanolic liposome was performed by using Franz's diffusion cell. Skin act as a semi-permeable membrane and was attached to the donor compartment and the receptor compartment was filled with phosphate buffer saline. Accurately weighed flurbiprofen loaded ethanolic gel (1 g) was added into the donor compartment of the diffusion cell maintained at a temperature of 37 °C with continuous stirring on a magnetic stirrer at 200 rpm. Then the samples were withdrawn at a predetermined interval i.e., at 1, 2, 3…24, hr respectively and were replaced by the same volume of buffer to maintain sink condition in the receptor compartment. The withdrawn sample was analyzed in UV/vis spectrophotometer. For all ethosomal batches, in-vitro skin permeation studies were accomplished in triplicates and expressed as the Mean ± SD (Dave et al., 2010; Agrawal et al., 2013).

2.13. Data treatment

The flux of the formulated flurbiprofen loaded ethosome was estimated through the Fick's second law of diffusion, in which the total amount of drug (Q_t) appearing in the receptor compartment in time t is expressed as (Shokri et al., 2001).

$$Q_t = AKLC_o\,[(D_t/L^2) - (1/6) - (2/\pi^2) - \varepsilon((1-1)^n/n^2)\,\exp.(D^n 2\pi^2 t/L^2)] \quad (1)$$

where,

A = effective diffusion area,

C_0 = concentration of drug that remains constant in the vehicle,

D = diffusion coefficient,

L = thickness of the skin membrane and.

K = partition coefficient of the drug between the membrane and the vehicle.

At steady state, Eq. (1) can represent as follows:

$$Qt = KLC_O\,[(D_t/L^2) - (1/6)] \quad (2)$$

Thus, the flux (J) was calculated through the slope of the equation to show the steady-state position of the amount of the drug permeated through an area with respect to a given time t. Hence, from Eq. (2), the flux (J) can be expressed as follows:

$$J = \frac{CoKD}{L} = CoKp \quad (3)$$

where, Kp is known as the permeability coefficient.

2.13.1. Acetic acid induced writhing assay

The writhing assay was performed to evaluate peripheral nociceptive activity of the formulation. Rats were divided into different groups

(I–III) each comprising of six rats, group I receives Marketed preparation Brugel, group II receives optimized formulation Etho 4 at a dose of 10 mg/kg and group III receives optimized formulation Etho 4 at a dose of 20 mg/kg (Table 3). In brief, Brugel marketed preparation and test formulation were applied topically 30 min after the administration of the inducing agent (1% acetic acid solution, i.p.). The behavior of animal was consistently monitored for the next 20 min. Being a robust and consistent component of acetic acid-induced nociception, writhing (constriction of abdomen, turning of trunk (twist) and extension of hind legs) was the observed for evaluation of nociceptive response. Number of writhes per animal was counted, immediately after induction (Sowemimoa et al., 2013; Kasture, 2009).

2.14. Stability studies

The major problem with the ethanolic liposome was the stability problem because of leaching and drug accumulation properties in the lipid layers. Stability study was performed to determine the drug retention ability of ethanolic liposome for three months at a different temperature. Two batches of lyophilized ethosomes and ethosomal suspension optimized (formulation EF4) were placed in sealed vials (10 ml) at 4 °C/60 ± 5 RH ($n = 3$) and at 25 °C/60 ± 5 RH respectively. Samples integrity was analyzed at 7, 15, 30, 60 and 90 days of storage.

3. Result and discussion

3.1. Particle size, polydispersity index, and zeta potential

Particle size analysis was carried out based on the principle of Dynamic Light Scattering (DLS) and the results of particle size and PDI were presented in Table 2. Prepared formulations exhibited mean particle size range 167.2 ± 3.7 to 205.4 ± 6.2 with PDI of 0.209 ± 0.03 to 0.446 ± 0.03. Amongst all the formulation EF4 exhibited maximum entrapment efficiency with the desired particle size of 167.2 ± 3.7 nm and PDI of 0.209 ± 0.03. These results were found in agreement with the findings of (Dave et al., 2010). Notably, an increase in the concentration of phospholipon 90-G and ethanol above 200 mg and 30% result in an increase in a particle size with a narrow distribution. It was also observed that an increase or decrease in the lipid ratio and ethanol concentration directly affects the particle size and size distribution of the ethanolic liposome. Being small sized vesicles with an optimal concentration of ethanol formulation EF4 was considered as ideal to afford effective drug permeability across the skin.

Zeta potential helps to determine the stability of the ethanolic liposome. It was ranged between −27.6 ± 0.8 and −51.6 ± 0.2. The results of the zeta potential were shown in Table 2. Formulation EF8 exhibited minimum zeta potential value i.e. –27.6 ± 0.8 whereas formulation EF4 exhibited maximum zeta potential value i.e. –51.6 ± 0.2. It was observed that the particles present in the ethanolic liposomal suspension carry the anionic charge and when the concentration of Phospholipon 90-G and ethanol used in the concentration

Table 3
Experimental design ($n = 6$).

Group	Treatment
I	Brugel gel
II	EF4 (10 mg/kg)
III	EF4 (20 mg/kg)

Table 4
Screening of analgesic activity of selected formulation using acetic acid induced writhing in rats.

S.No.	Treatment	No. of writhes (20 min)
I	Brugel gel	490 ± 14.14
II	EF4 (10 mg/kg)	325 ± 7.07***
III	EF4 (20 mg/kg)	260 ± 14.14***

Table 5
Stability study of optimized formulation EF4.

Ethosomal suspension (EF4)		
Time(days)	Microscopic evaluation	%Entrapment efficiency
At 4 °C /60 ± 5RH(n = 3)		
Initial	Smooth spherical vesicles	93.51 ± 2.1
7	Smooth spherical vesicles	90.8 ± 1.5
15	Smooth spherical vesicles	89.7 ± 0.8
30	Smooth spherical vesicles	86.5 ± 1.7
60	Rough spherical vesicles	82.4 ± 2.7
90	Rough spherical vesicle	79.2 ± 0.6
At 25 °C/60 ± 5RH(n = 3)		
Initial	Smooth spherical vesicles	93.51 ± 2.1
7	Smooth spherical vesicles	87.4 ± 3.6
15	Rough spherical vesicles	84.2 ± 2.0
30	Rough spherical vesicles	76.8 ± 1.3
60	Agglomerate	65.7 ± 1
90	Agglomerate	52.2 ± 2.5

Lyophilized ethosomal suspension (EF4)		
At 4 °C/60 ± 5RH(n = 3)		
Initial	Smooth spherical vesicles	93.51 ± 2.1
7	Smooth spherical vesicles	89.6 ± 0.6
15	Smooth spherical vesicles	85.4 ± 1.5
30	Smooth spherical vesicles	82.8 ± 2.1
60	Rough spherical vesicles	78.7 ± 1.8
90	Rough spherical vesicles	75.2 ± 2.7
At 25 °C/60 ± 5RH(n = 3)		
Initial	Smooth spherical vesicles	93.51 ± 2.1
7	Smooth spherical vesicles	87.4 ± 0.5
15	Rough spherical vesicles	81.5 ± 2.9
30	Rough spherical vesicles	75.1 ± 1.3
60	Rough spherical vesicles	68.7 ± 1.9
90	Agglomerate	63.9 ± 0.8

Table 2
Characterization of flurbiprofen loaded ethonolic liposomes.

Characterization	EF1	EF2	EF3	EF4	EF5	EF6	EF7	EF8	EF9
Vesicle size (nm)	186.4 ± 2.4	175.1 ± 2.9	172.3 ± 4.6	167.2 ± 3.7	179.4 ± 4.4	182.6 ± 5.8	189.4 ± 2.4	195.2 ± 3.5	205.4 ± 6.2
% Entrapment efficiency	81.39 ± 1.4	87.0 ± 0.8	84.64 ± 0.6	93.51 ± 2.1	78.66 ± 0.2	86.32 ± 0.5	83.15 ± 0.2	76.08 ± 1.6	90.22 ± 2.4
PDI	0.248 ± 0.04	0.318 ± 0.02	0.289 ± 0.05	0.209 ± 0.03	0.346 ± 0.08	0.365 ± 0.02	0.381 ± 0.07	0.413 ± 0.06	0.446 ± 0.03
Zeta potential (mV)	−39.0 ± 1.0	−41.6 ± 0.3	−46.4 ± 1.8	−51.6 ± 0.2	−36.4 ± 0.6	−34.8 ± 1.2	−44.3 ± 0.2	−27.6 ± 0.8	−37.4 ± 1.4
J (flux)(g/cm^2/h)	143.5	185.0	184.6	238.2	198.5	196.7	199.1	207.3	219.6
pH	6.8	6.4	5.6	5.1	6.2	5.4	6.6	6.1	5.4
Viscosity (cps)	6543 ± 1.5	7123 ± 2.5	7649 ± 1.8	7472 ± 2.7	8762 ± 0.8	6879 ± 2.1	6348 ± 2.0	6019 ± 2.4	7921 ± 1.6
Spreadability (cm)	3.24 ± 0.2	6.4 ± 0.2	6.3 ± 0.7	7.84 ± 1.2	7.36 ± 0.4	4.9 ± 0.5	6.72 ± 1.2	6.19 ± 0.3	4.6 ± 0.2
Extrudability	++	++	+++	+++	++	++	+++	++	+++

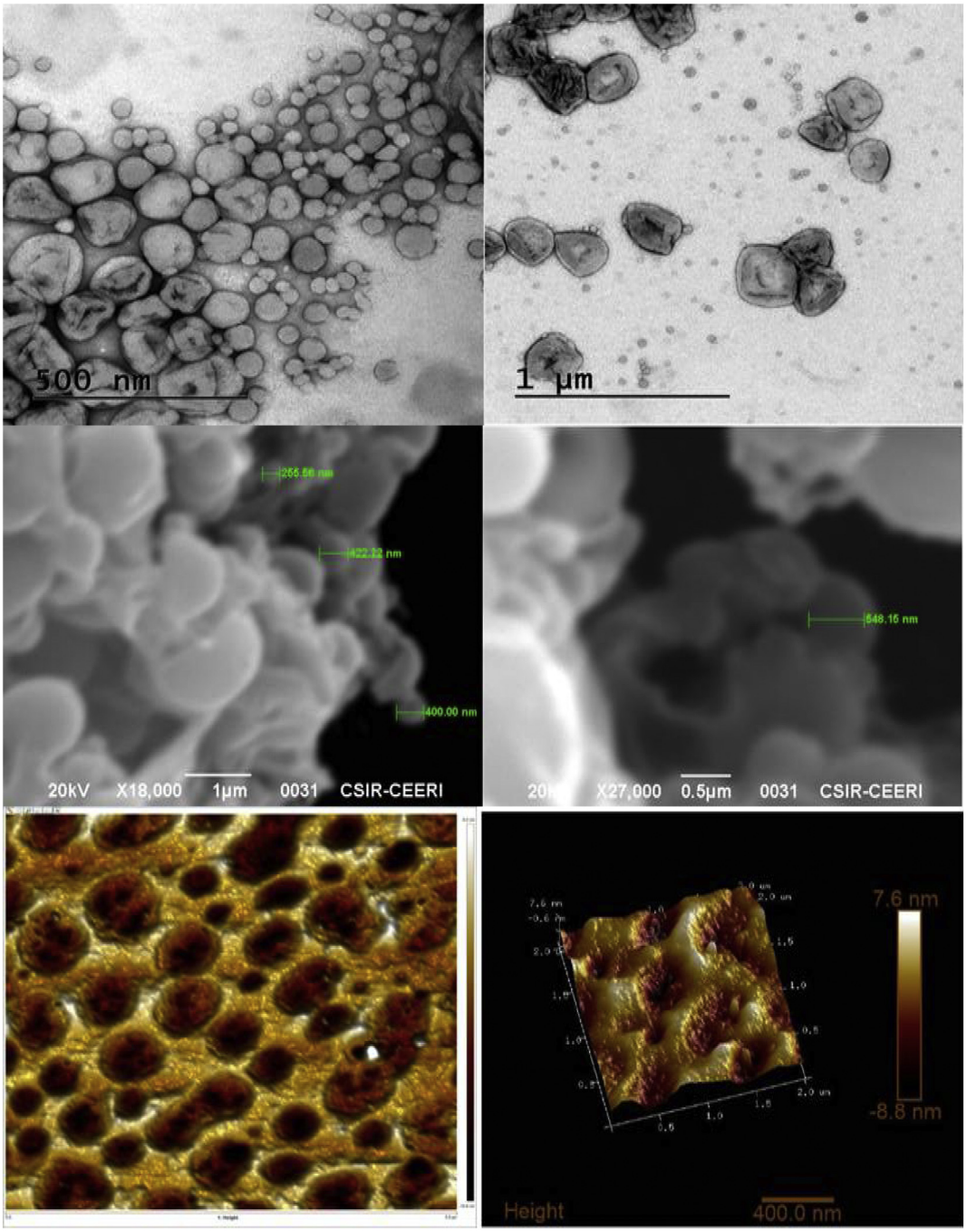

Fig. 1. Surface micrograph of optimized formulation EF 4 shown by [A] TEM, [B] SEM, and [C] AFM.

of 200 mg and 30%, the anionic charge was also increased which resist the agglomeration of the particles present in the suspension and provides more stability to the ethanolic liposomal suspension. According to the results of obtained for different formulations, EF4 showed maximum stability and it can be concluded that the charge carried by vesicles get influenced on varying the concentration of the lipids.

3.2. Morphology of flurbiprofen loaded ethanolic liposome by SEM, TEM, and AFM

SEM images of the flurbiprofen loaded ethanolic liposome were shown in Fig. 1B These images indicated that the ethanolic liposomes were smooth, round and bears spherical structure. Notably, the dense particle in the SEM image indicates the presence of high-density lipids over particles which could offer controlled release of drug from the formulation.

TEM images of optimized formulation EF4 were shown in Fig. 1A. TEM images confirmed the surface morphology and confirmed the spherical shape of particles in the formulation. TEM images indicated the ethanolic liposomes were round, smooth and free from crystalline nature of the drug.

AFM images help to provide the information which was not easily gained by the SEM and TEM images. AFM images were shown in Fig. 1c which further confirmed morphological characteristic of the ethanolic liposome.

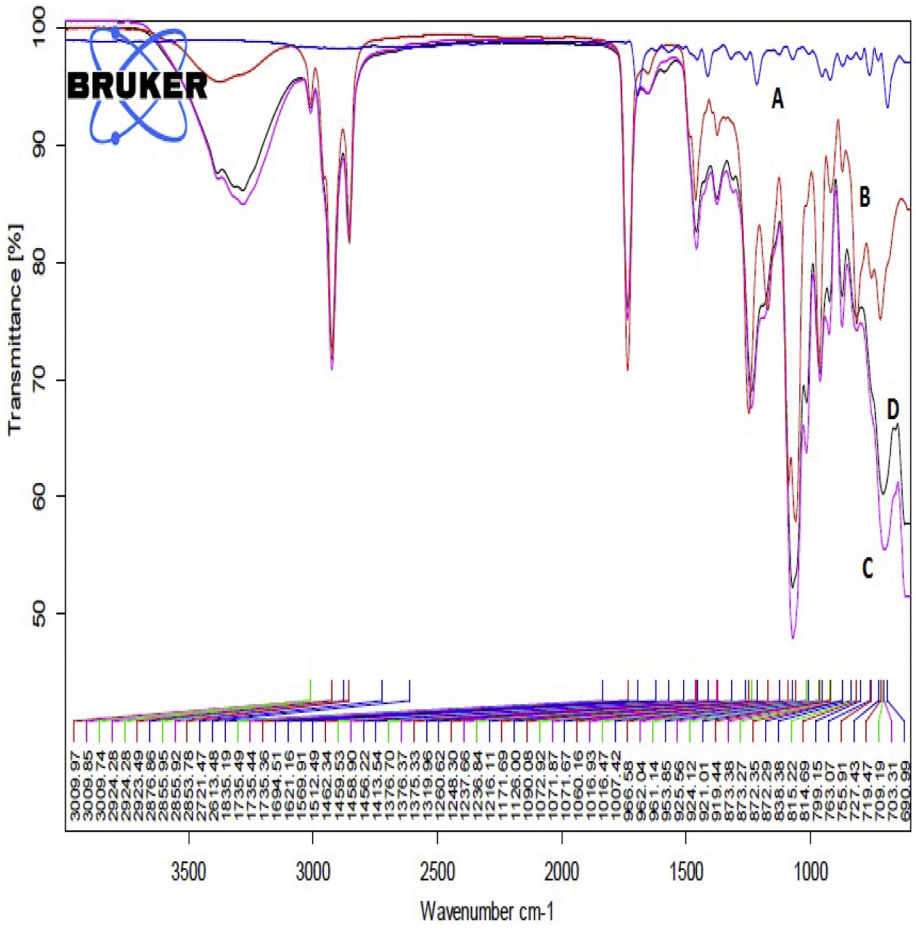

Fig. 2. Overlapped ATR-FTIR data of [A] Flurbiprofen, [B] Phospholipon 90-G, [C] Physical mixture, [D] Optimized formulation EF 4.

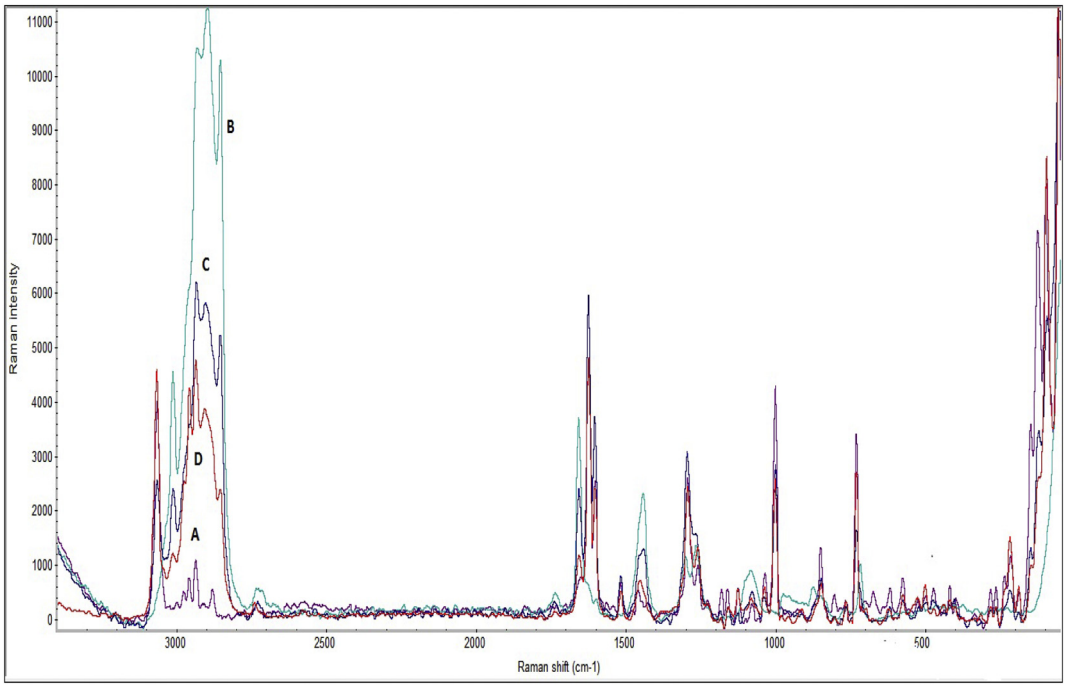

Fig. 3. Overlapped RAMAN spectra of [A] Flurbiprofen, [B] Phospholipon 90-G, [C] Physical mixture, and [D] Optimized formulation EF 4.

3.3. Attenuated total reflection – Fourier transform infrared spectroscopy

The ATR-FTIR spectra of pure drug, phospholipon 90-G and ethanolic liposome loaded with flurbiprofen were depicted in Fig. 2. ATR-FTIR study was performed to examine possible interactions between drug and polymer in formulations. ATR-FTIR spectra of pure drug showed major characteristic peak at 3741.09 Cm^{-1} due to O—H stretching, 2921.58 Cm^{-1} due to = CH and aromatic H stretching, and 2852.38 Cm^{-1} due to CH_2 stretch, 1731.74 Cm^{-1} due to C═O stretching, 1261.16 Cm^{-1} due to O—H bending, 1040.62 and 1070.93 Cm^{-1} due to C—F bending and 836.36 cm^{-1} due to distribution of aromatic protons. Optimized formulation showed peak in a very close range to that of pure flurbiprofen peak i.e. at 2924.28 Cm^{-1}, 2855.95 Cm^{-1}, 1735.49 Cm^{-1}, 1236.84 Cm^{-1}, 1072.92 Cm^{-1}, 873.27 Cm^{-1} respectively. As there was no change in the IR peak of flurbiprofen and optimized formulation, it was concluded that there was no possible interaction between the drug and polymer and can be considered as compatible with each other.

3.4. Raman spectroscopy

Raman spectroscopy gives information about the functional group present in the sample and is dependent on the electric polarizability of the molecule. The drug (flurbiprofen) show major peak at 3063 Cm^{-1} (–OH stretching), 3071 Cm^{-1} (aromatic CH_2 stretching), 1615 Cm^{-1} (–OH bending), 1300 cm^{-1} (CH_3 bending), 1033 cm^{-1} (C—F) while the formulation show distinguishable peak at 3065.84 Cm^{-1}, 2932.83 Cm^{-1}, 1622.77 Cm^{-1}, 1449.86 Cm^{-1}, 1290.41 Cm^{-1}, 1000.74 Cm^{-1}, 846.70 Cm^{-1}, 727.57 $Cm^{-1,}$ 499.14 Cm^{-1}, 215.27 Cm^{-1}. The Phospholipon 90-G showed peak at 3010.40 Cm^{-1}, 2853.41 Cm^{-1}, 1665.40 Cm^{-1}, 1441.11 Cm^{-1}, 1264.61 Cm^{-1}, 1087.18 Cm^{-1}, 715.19 Cm^{-1} and physical mixture show peak at 3062.96 Cm^{-1}, 2932.08 Cm^{-1}, 1622.78 Cm^{-1},1515.39 Cm^{-1}, 1292.99 Cm^{-1}, 999.21 Cm^{-1}, 727.52 Cm^{-1}. The results of Raman study was predicted in Fig. 3. Peak analysis revealed that the peak of optimized formulation was overlapping with a peak of pure drug which in turn indicates the presence of the drug in the ethanolic liposome.

3.5. Differential scanning calorimetry (DSC)

DSC helps in the evaluation of effectiveness and determining the purity of drugs. DSC was also performed to study the thermal transition of drug flurbiprofen and drug-loaded ethanolic liposome. The pure drug showed a sharp endothermic peak at 117 °C whereas phospholipon 90-G exhibited a broad and low-intensity peak at 40.54 °C. The optimized formulation showed a sharp and high-intensity peak at 122.67 °C which was found close to the peak of pure drug flurbiprofen (Fig. 4). The shift of the peak suggests that all the components present in the formulation interact with each other which leads to a decrease in the crystallinity of the drug and increases its amorphous behavior. DSC study confirms the presence of flurbiprofen in the ethanolic liposomes.

3.6. Thermo gravimetric analysis (TGA)

In the present study, TGA was performed to measure the alteration in the mass of flurbiprofen, phospholipon 90-G, physical mixture and optimized formulation (EF4) with respect to temperature change. The % weight loss for flurbiprofen, phospholipon 90-G and physical mixture was found as 200 °C, 97 °C, 187 °C, and 167 °C respectively. Optimized formulation EF4 showed instant weight loss at 80% and became constant till 300 °C. From TGA results it was clearly indicated that the mixture of phospholipon 90-G with the drug improves its stability at a higher temperature. These outcomes also revealed that the excipients have no moisture content or minimal effects on the formulations. Overlayed TGA spectra were shown in Fig. 5.

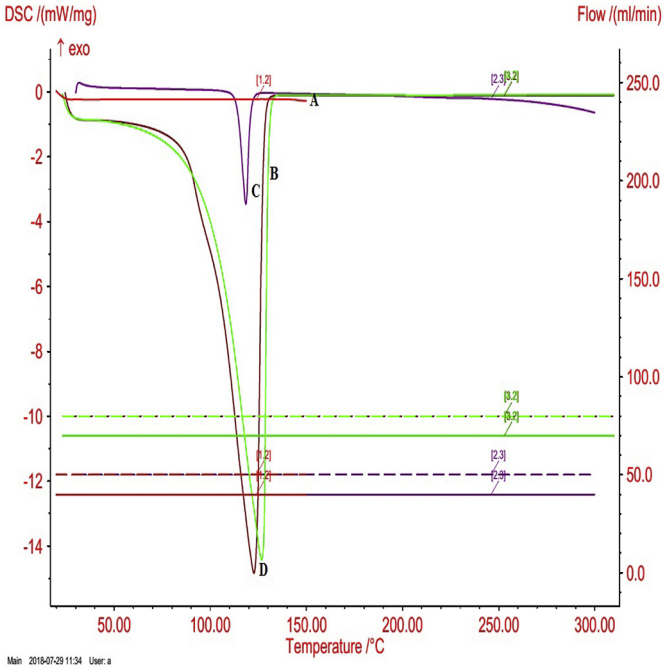

Fig. 4. Overlapped DSC Thermogram of [A] Phospholipon 90-G, [B] Optimized formulation EF 4, [C] Flurbiprofen, and [D] Physical mixture.

3.7. % entrapment efficiency

In percent entrapment efficiency study, it was found that formulation EF 4 exhibited maximum entrapment efficiency i.e. 93.51 ± 2.1 whereas formulation EF 8 exhibited minimum entrapment efficiency i.e. 76.08 ± 1.6. Formulation EF2, EF3, EF6, EF9 showed no significant difference in entrapment efficiency value i.e. 87.00 ± 08, 84.64 ± 0.6, 86.32 ± 0.5, 90.22 ± 2.4 respectively. However a decrease in the entrapment efficiency values were observed in case of formulation EF1, EF5, EF7 i.e. 81.39 ± 1.4, 78.66 ± 0.2, 83.15 ± 0.2 (Dave et al., 2017a; Mandal et al., 2013). From these result it was concluded that entrapment of drug is maximum when ethanol was used in the concentration up to 30% however a marked reduction in entrapment efficiency was observed due to the increased ethanol concentration > 30%, which occurs could be due to leakage of drug from the vesicles as the vesicles gets more permeable due to high concentration of ethanol. In addition, entrapment efficiency was also influenced by a concentration of phospholipon 90-G. In particular, the entrapment efficiency of the drug was maximum when phospholipon 90-G was used in a concentration up to 200 mg whereas a significant reduction in entrapment efficiency was noted above 200 mg of phospholipon 90-G with reduced drug permeability (Table 2 and Fig. 6). Therefore, it was concluded that on varying the concentration of lipid and ethanol directly influences particle size and entrapment efficiency of a formulation. The order for entrapment efficiency was found as EF4 > EF > 9 EF > 2 EF > 6 EF > 3 EF > 7 EF > 1 EF > 5 EF > 8. The formulation EF 4 showed maximum entrapment efficiency bearing average amount of lipid and ethanol i.e. 200 mg and 30%.respectively.

3.8. pH, viscosity, spreadability, and extrudability

The pH, viscosity, and spreadability of all the formulation were ranged between 5.1 and 6.8, 6019 to 8762, and 3.24 to 7.84 and the results were shown in Table 2. Results indicate that the formulations possess optimal pH, viscosity and spreadability with extrudability with good to excellent range and can be considered suitable in dermal therapy. Optimized formulation EF4 showed sufficient viscosity, pH

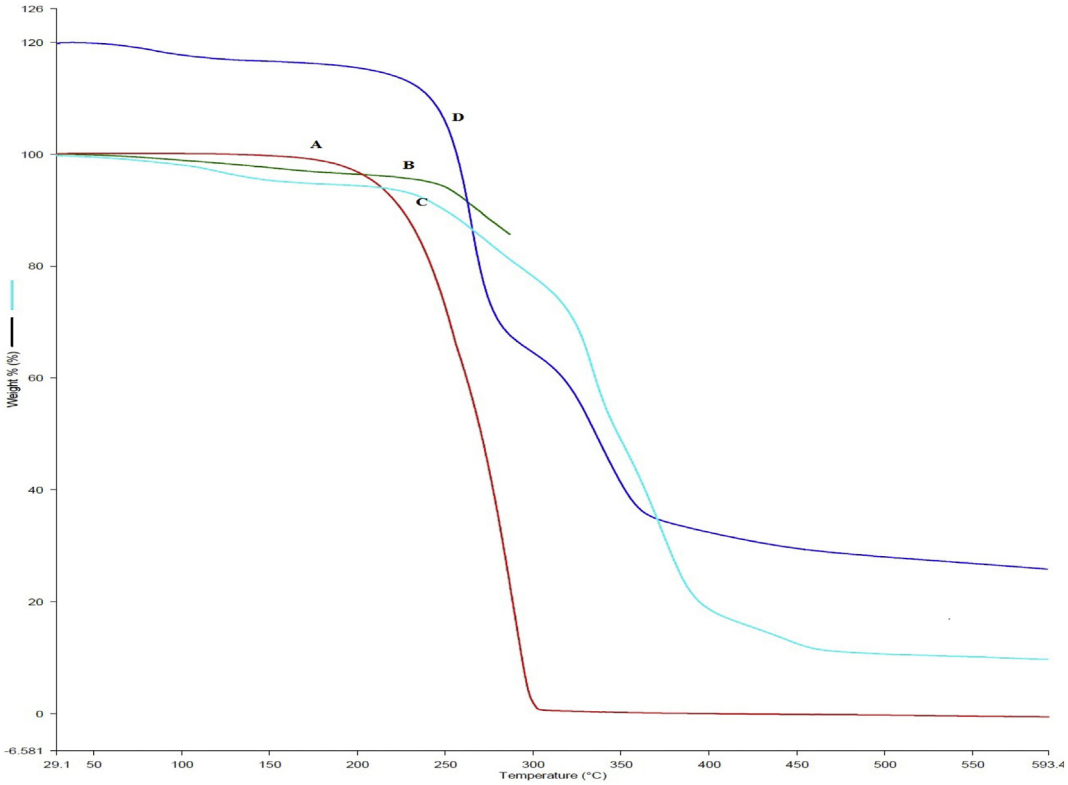

Fig. 5. Overlapped TGA thermograph of [A] Flurbiprofen, [B] Phospholipon 90-G, [C] Optimized formulation EF 4, and [D] Physical mixture.

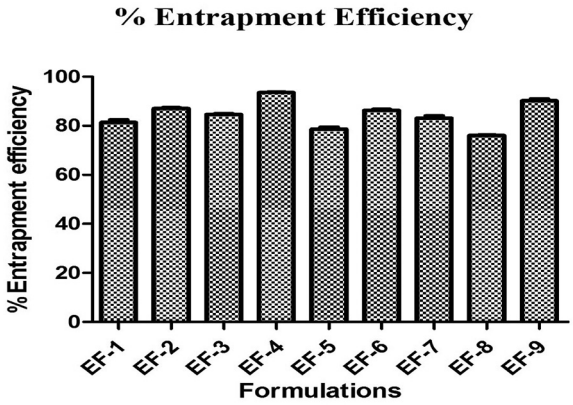

Fig. 6. Percent entrapment efficiency of formulation EF 1–EF 9.

and spreadability and revealed the good gelling property of ethosomal gel for topical application.

3.9. In-vitro skin permeation study

The release profile of all formulation was studied using the intact skin of albino rats. Formulation EF-1 to EF-9 show % cumulative drug release of 45.41 ± 2.04, 48.24 ± 1.67, 34.56 ± 2.48, 70.23 ± 3.54, 58.41 ± 2.29, 43.26 ± 3.48, 61.41 ± 3.15, 56.24 ± 1.47, 33.67 ± 2.56 respectively in 24 h. The results of in-vitro skin permeation study were shown in Fig. 7. Formulation EF-4 displayed maximum release of drug i.e.70.23% which could be due to the higher entrapment of drug in the ethanolic liposome as compared to all other formulation. Initially, all formulation showed a rapid release of the drug but after some time a sustained release was achieved which is due to the presence of lipid bilayer which acts as a rate limiting barrier for the diffusion of the entrapped drug (Puri et al., 2009). In the case of formulation EF-1, EF-2, EF-3 a rapid release of drug was achieved because of the presence of a lower concentration of lipids and lower entrapment of the drug in the formulation. Therefore it was concluded that variation in the concentration of lipid and ethanol may show a drastic variation in the release of drug from the formulation. According to these result, obtained formulation EF-4 was considered optimized and found suitable for in-vivo study.

3.10. Data treatment result

The data obtained from in-vitro skin permeation study were further treated mathematically to obtain the value of flux for all formulations. The flux value for all formulations ranging from EF1 to EF9 lies between 143.5 and 238.2 μg/cm^2/h. Optimized formulation EF4 show maximum flux value i.e. 238.2 μg/cm^2/h. The results of the study were tabulated in Table 2 as well as depicted in Fig. 8. Formulation EF4 exhibited better flux as compared to the other formulations and also provided a better skin deposition and thus considered as an ideal carrier for dermal and transdermal drug delivery. The ethosomal formulation has been reported as non-irritant and can be well tolerated in in-vivo (Dubey et al., 2007; Paolino et al., 2005). Differences in the flux values were indicative of the varying concentration of lipid: ethanol ratio in formulations.

3.11. Acetic acid induced writhing assay

An agent reducing the number of writhes is known to produce analgesia by a peripheral mechanism i.e. inhibition of prostaglandin synthesis (Margaret et al., 2014). The formulations produced a significant ($p < .001$) and dose-dependent attenuation in the number of writhes when compared to Brugel (marketed preparation) as shown in Table 4 and Fig. 9. The mean number of writhes after application of Brugel marketed preparation was 490 ± 14.14. The formulation produced a dose-dependent reduction in the writhes at 10 mg/kg (325 ± 7.07) and 20 mg/kg (260 ± 14.14). Our results indicate that

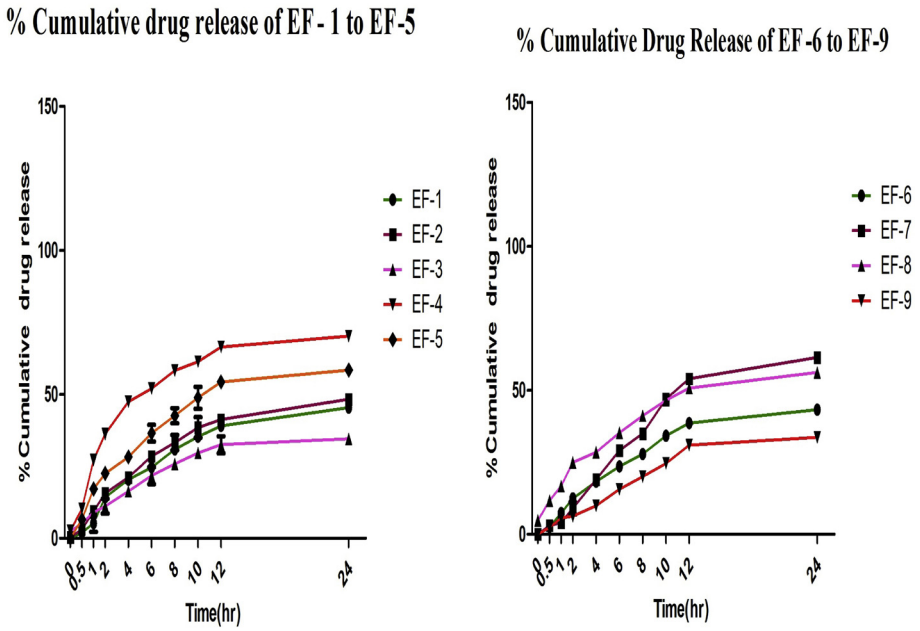

Fig. 7. In-vitro drug release profile of flurbiprofen loaded ethosomal formulations EF 1–EF 9 for 24 h.

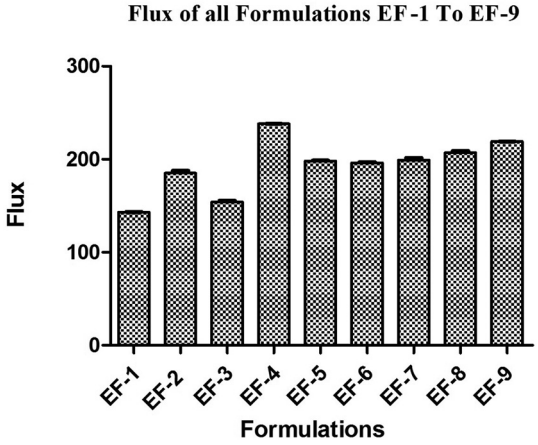

Fig. 8. Flux of the formulation EF 1–EF 9.

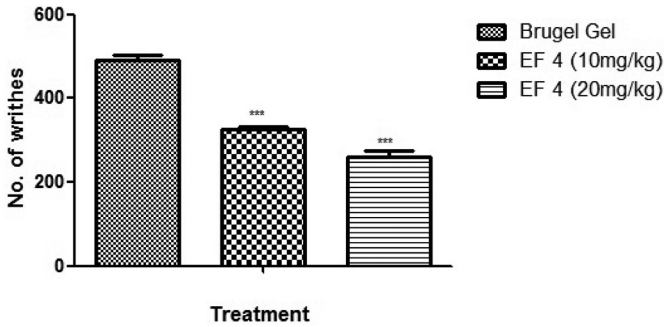

Fig. 9. Effect of ethosomal formulations on the number of writhes in acetic acid-induced inflammation.

topical application of formulation reduced the number of writhes significantly which may be an effect of the peripheral analgesic activity of formulation since it is lowering a number of writhes. Sofidiya M et al. investigated the effect of extract as anti-inflammatory and anti-nociceptive using in-vivo assays in mice. They showed that prostaglandin synthesis portrays a crucial role in inducing acetic acid-mediated peripheral nociception (Margaret et al., 2014).

3.12. Stability studies

Stability study was performed to determine the ability of the ethanolic vesicles to retain the drug. The study was performed for three months at a different temperature. The main problem with the ethanolic liposome was the stability because of leaching and accumulation of the drug in the lipid bilayers. The optimized formulation EF4 was selected to perform a stability study and the results of the study were shown in Table 5. For this study, two batches of the lyophilized sample and ethanolic suspension were used and 10 ml of the sample was placed in sealed vials and placed at a 4 °C/60 ± 5 RH (n = 3) and at 25 °C/60 ± 5 RH. Samples were collected at an interval of 7, 15, 30, 60 and 90 days of storage. The collected lyophilized sample was redispersed in Millipore water and then sonicated for 1–2 min and then subjected for various characterizations like particle size, PDI, zeta potential and entrapment efficiency. The analysis was performed in triplicate so as to obtain a precise result and the results were reported as mean ± SD. From the result, it was observed that lyophilized sample and ethanolic liposomal suspension show a decrease in the % entrapment of the drug when stored at 25 °C/60 ± 5 RH (room temperature) but do not show any significant change in % entrapment efficiency when stored at 4 °C/60 ± 5 RH (n = 3). Therefore, 4 °C/60 ± 5 RH was considered as an optimal condition for storage of formulation EF4.

4. Conclusion

The purpose of the current investigation was to assess the potential of Flurbiprofen loaded ethanolic liposomes particle. Flurbiprofen is hydrophilic drug which limits its permeation into the skin, therefore ethanolic liposome particle appear to be the suitable carrier for the dermal delivery of the flurbiprofen as the drug gets entrapped between the lipophilic layers. The most significant findings of the study were the controlled particle size, high entrapment efficiency and better penetration properties of the vesicles, provides a sustained release of drug with higher stability at 4 °C/60 ± 5 RH. Writhing assay shows that the optimized formulation decreases the number of writhes as compared to the marketed preparation. Thus the overall study concluded that novel vesicular carrier (ethanolic liposome particle) offers a new drug

delivery for the sustained and targeted delivery of flurbiprofen. Based on this approaches, this type of drug delivery system help the industry for development and scaling up a new formulation, further more research work is needed to bring the flurbiprofen loaded ethosomal formulation closer to its clinical realization.

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