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Original Article

Acetyl-11-Keto-β-Boswellic Acid-Loaded Ethosomes as Nanocarriers from a Physicochemical Perspective and *in vivo* Evaluation

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ABSTRACT

Background: Ethosomes are elastic nanovesicles made of phospholipids that contain a high concentration of ethanol. It has shown to improve the skin permeability of many drugs due to interactions between the high ethanol. **Materials and Methods:** The optimization and characterization of 3-Acetyl-11-Keto-β-Boswellic Acid (AKBA) loaded vesicular ethosomes included measurements of particle size, entrapment effectiveness, microscopy using SEM and TEM, and the interaction of the drug and excipients using Fourier transform infrared spectroscopy was carried out. FT-IR studies revealed no interaction between the drug and the excipients. Additionally, *in vitro* drug permeation test utilizing pig ear skin was conducted on ethosomal formulations. E8 (containing 30% alcohol, 2% w/w phospholipid), was chosen for additional skin permeation studies due to its high percentage of drug entrapment (88.43%) and small particle size (129.3±0.75nm). **Results:** The formulation E8 had the greatest amount of drug permeability (73.22%). Furthermore, paw oedema assay using carrageenan induction was used to investigate the *in vivo* evaluation of the produced formulation. The anti-inflammatory efficiency of ethosomal vesicles containing AKBA was compared to that of AKBA-loaded carbopol gel. **Conclusion:** It revealed that ethosomal had higher anti-inflammatory activity than carbopol gel formulation. Our findings suggest that the developed ethosomal system has the potential to deliver AKBA through the skin.

Keywords: Ethosomes, Transdermal flux, Acetyl-11-keto-beta-boswellic acid, Lipid-based vesicles, Permeation enhancers.

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INTRODUCTION

3-O-Acetyl-11-keto-beta-boswellic acid/Acetyl-11-Keto-beta-Boswellic Acid (AKBA) is a pentacyclic triterpene compound derived from the plant *Boswellia serrate* gum resins and is among the most active principles. It is commonly known as Indian Frankincense or salai guggul and is traditionally used as an anti-inflammatory agent. Boswellia species have been demonstrated to be effective against ileitis, ulcerative colitis, hypolipidemia, hypertension, and hepatotoxicity. Boswellia species are reported to have various pharmacological activities like anti-inflammatory activity,[1,2] anti-arthritic activity,[3,4] immunomodulatory activity,[5] ileitis,[6] ulcerative colitis,[7] and anti-cancer.[8] Because of their high lipid solubility and poor permeability, BAs are categorized as BCS Class IV drugs and are known to have poor oral bioavailability; these are the main factors affecting AKBA's capacity to maintain its metabolic stability. It is readily metabolized in phase-1 metabolism once absorbed.[9] To overcome these restrictions, new strategies have been established to enhance bioavailability because of the therapeutic activity of AKBA. Thus, the development of ethosomes containing AKBA might also make an addition to providing new therapy for the treatment of inflammation.

Delivery of the drug to the site of inflammation through the skin may be more effective and can give rapid relief; however, problems arise regarding the penetration of the drug through the skin, a natural barrier that consists of a tight junction of cells in the stratum corneum. To overcome this problem, novel drug delivery systems have been discovered to better deliver the medicines administered through the skin, like the ethosomes (lipid-based vesicular delivery system). Ethosomes are lipid-based carriers and may be denoted as modified liposomes, first reported by Touitou

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et al., 2000. Ethosomes, which are made up of phospholipids, have increased drug penetration across the epidermal barrier due to the high concentration of ethanol. This enhancement is due to ethanol serving as a permeation enhancer, enhancing the fluidity of both the ethosomes lipids and the skin. As a result, ethosomes offer a soft and flexible vesicle that can easily penetrate the skin. They are non-invasive, soft, and flexible vesicles in which an active agent can be incorporated to be delivered through the skin. Ethosomes range in size from a few nanometers to micrometers depending on the preparation methods and application.[10] It can well entrap hydrophilic, lipophilic, and amphiphilic compounds. Active agents can be delivered to the deeper layer of the skin, i.e., to the dermal layer overcoming the skin's natural barriers. It increased the permeability, possibly due to the synergistic mechanism between ethanol, phospholipids, and skin lipids. The lipids' fluidity of both the ethosomes and skin is increased by ethanol, providing a soft flexible vesicle for easy penetration through the skin. The ethosomes will fuse with the skin lipids, then release the drug along the penetration pathway.[11,12] Drugs incorporated in ethosomes, when applied to the skin, can penetrate the deep layer, and reach the nerve fibers, thus providing an alternative non-invasive delivery of the drug.

Inflammation is prevalent all around the world and is mainly treated with synthetic drugs like NSAIDs (Non-steroidal anti-inflammatory drugs) that can inhibit prostaglandins and leukotrienes. NSAIDs are very potent against inflammation, but on long-term administration, they can produce solemn side effects. An alternative potent anti-inflammatory molecule from natural sources has been chosen to avoid such adverseness. BAs obtained from a plant of the Boswellia genus are selective COX-1 inhibitors,[13,14] and act on leukotrienes through an enzyme-directed non-competitive and non-redox reaction, thus inhibiting the 5-lipoxygenase enzyme.[15,16] However, AKBA is known to be highly metabolized with poor oral bioavailability. Thus, formulating it into a topical/transdermal drug delivery system will improve its bioavailability by bypassing the first-pass metabolism. A lipid-based vesicular system like ethosomes will render good permeation of the active molecule through the skin, which may be explained because of the existence of phospholipids and high concentrations of ethanol in their structure.

MATERIALS AND METHODS

Materials

3-acetyl-11-keto-β-Boswellic Acid (AKBA) was obtained from Cayman Chemical, United States, India. Phospholipon-90G, a phospholipid that contained 93% phosphatidylcholine, was kindly donated by Natterman Phospholipid GmbH in Germany. Carbopol 934P, Cellophane membrane grade 110, Triethanolamine and Carrageenan were purchased from Hi Media Laboratories, Mumbai, India. Absolute ethanol was procured from Merck, India. All other materials were of analytical grade.

Preparation of ethosomal formulation

Ethosomal formulations contain a high ethanol content (10-50%) and 0.5-2.0% w/v phospholipon-90G. Traditional dispersion techniques were used to prepare the nanovesicles. Table 1 revealed the ingredients for ethosome preparation. A vessel was specially made with an inlet to add solvents with minimal evaporation. Phospholipid and AKBA were combined in ethanol, and distilled water was gradually added to the lipid drug solution while continuously stirring at 1000 rpm with a magnetic stirrer. After adding the distilled water, the mixture was sonicated for 10 min. The preparation was carried out at room temperature and the storage was done in sealed containers. Empty/blank ethosomes without AKBA were made using a similar technique.

Drug vesicle interaction study

The compatibility of the drug excipients was determined using Fourier-transform infrared (FT-IR) spectroscopy (Shimadzu model 8400). The vesicles' FT-IR spectra were recorded in the range of 4000-400 cm^{-1}, and the resulting spectra were analyzed based on the intensity and shift of vibration bands.

Entrapment Efficiency (%)

The percentage fraction of AKBA present in the prepared formulation is determined by the Entrapment Efficiency (EE). The EE of freshly prepared AKBA-loaded ethosomes was assessed using the ultracentrifugation method. Samples kept overnight at 4°C were centrifuged at 20,000 rpm for 3 hr, and collection of the supernatant was done to measure the entrapment capacity of the prepared ethosomes. A UV-vis spectrophotometer was used to measure the absorbance from the supernatant solution at 250 nm. The following formula was used to determine the EE:

$$EE(\%) = \frac{Initial\ amount\ of\ drug - drug\ in\ the\ supernatant}{Initial\ amount\ of\ drug}\ x\ 100$$

pH and Viscosity

Using a pH meter, the pH of the improved formulation was determined. A Brookfield viscometer was used to measure viscosity.

Determination of Vesicle size and Zeta potential

At 25°C, the Zetasizer (Malvern Zetasizer Nano-ZS90, UK) was used to determine the average vesicle size and Zeta potential (vesicle charges). The investigation was conducted in triplicate and reported.

In vitro drug release

Franz glass diffusion cells mounted with cellophane membrane between the donor and receptor compartments were used in an *in vitro* drug release study. The membrane was hydrated with pH 7.4 PBS for 24 hr before *in vitro* drug release study. Different formulations of AKBA were applied to the membrane's upper side in the donor compartment. The experiments were conducted

at 37°C, with the receptor compartment agitated by a magnetic stirrer at 100 rpm. At predetermined intervals, 1 mL aliquots were taken out of the receptor compartment and replaced with an equal volume of the fresh receptor solution. When analyzing the permeation data, the receiver content's dilution was considered. The sample was taken out of the receptor compartment, and the amount of drug was calculated.[17,18]

AKBA-loaded ethosomal gel

The optimized Ethosomal formulation (E8) was used to prepare a gel-based formulation. After being submerged in water for an hour, carpobol 934 received 20 mL of an ethosomal suspension containing AKBA. After reaching a homogenous ethosomal gel consistency at 30°C, stirring was continued at 700 rpm. Slow stirring was used to add Triethanolamine (TEA) to neutralize the pH until the gel formed. A pH meter was used to determine the pH of the colloidal suspension ethosomal gel formulations.[19]

Visualization of vesicles by Transmission Electron Microscopy (TEM) and Scanning electron microscopy (SEM)

For TEM, the shape and morphology of ethosomal vesicles (E8) were investigated. Before use, the vesicles were negatively stained with 1% aqueous Phosphotungustic Acid (PTA) and visualized using (JEM-2100, 200 kV, Jeol) at an accelerated voltage of 100 kV. The sample was observed under the microscope at 10-100 k fold enlargements.

For SEM, one drop of Ethosome vesicles (E8) was put on a stub and distributed uniformly over the glass. A Sputter-coated (JEOL, Japan) was used to coat the sample with gold, and the coated sample was examined with a scanning electron microscope (JSM-6360, Jeol) with an accelerating voltage of 20 kV.

In vitro Permeation Study

The *in vitro* permeation investigation was conducted using modified Franz diffusion cells with porcine ear skin. The experiment was conducted using phosphate buffer saline (pH 7.4) and 2.5 mg of medication applied to the donor compartment's top side of the skin. Samples were taken from the receptor media every hour and the same amount of new receptor media was introduced to create a sink situation.[20] Using a UV/vis spectrophotometer, the AKBA constant was determined in the samples that had been withdrawn.

Vesicle-skin interaction

Histopathology was used to visualize the topical effect of AKBA ethosomes on skin. The hydroalcoholic solution, Ethosomal gel, and Carbopol gel containing AKBA were given to Group 1, Group 2, and Group 3, respectively. On the rat skins, the formulation was applied before being supported with a parafilm patch then secured by a bandage. After 24 hr, the treated skins from the rats were removed. After that, the treated skins from the rats were removed. Skin specimens were obtained and fixed for 24 hr in 10% formalin. After washing, the skins in warm water were cut vertically into 3-4 m thick sections. Before being examined under light microscopy for histological changes, the paraffin tissue samples were stained using hematoxylin and eosin. Each group of three rats underwent the tests.[21,22]

In vitro anti-inflammatory studies

Protein denaturation method

The standard solutions of 5 mL were made by mixing 0.2 mL of fresh hen's egg albumin, 2.8 mL of phosphate-buffered saline (pH 7.4), and 2 mL of Diclofenac sodium solution in varied doses. The test solutions were made by mixing 5 mL of fresh hen's egg albumin, 2.8 mL of phosphate-buffered saline (pH 7.4), and 2 mL of varied doses of AKBA (50-250 g/mL). 20 mL of AKBA-loaded ethosomes were combined with egg albumin and phosphate buffer (pH 7.4). All reaction mixtures were incubated at 37°C 2°C for 15 min before being heated to 70°C in a water bath for 5 min. All reaction mixtures' absorbance was determined by a spectrophotometer (SHIMADZU UV-1800, Japan) at 660 nm.[23,24] The following is an estimate of the percentage inhibition of protein denaturation:

$$\%\ inhibition = 100\left[\frac{V_t}{V_c} - 1\right]$$

Where V_t=absorbance of the test sample and V_c=absorbance of control.

In vivo anti-inflammatory activity

Animals

30 male Wistar rats, each weighing around 200 g, were used in this investigation. They were fed on typical pellets and allowed unlimited access to water. All the animals were acclimated for a minimum of one week prior to the trial. All methods of experimentation were carried out in compliance with the guidelines

Table 1: Composition table for the experimental formulations.

Composition	Formulation code											
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Ethanol (%)	20	20	20	20	30	30	30	30	40	40	40	40
Phospholipon 90 G (%)	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
Drug (mg)	5	5	5	5	5	5	5	5	5	5	5	5

set forth by the Institutional Animals Ethics Committee (IEAC). The Institutional Animal and Ethics Committee, Department of Pharmaceutical Sciences, Dibrugarh University (Regd. No. 1576/GO/Re/S/11/CPCSEA, Dated: 30/10/2018), Dibrugarh, Assam, India, approved and certified the *in vivo* pharmacodynamic study (Approval no: IAEC/DU/159 dtd. 10/12/2018.).

Carrageenan-Induced Acute Inflammatory Model

The carrageenan-induced rat paw oedema assay was used to assess anti-inflammatory efficacy. For the anti-inflammatory activity against the acute inflammation, animals were divided into five groups. Group I was the Positive control in which no oedema was induced. Oedema was induced in rest of the animal groups that is Group II (Negative control), Group III (Carbopol gel treated group), Group IV (AKBA-loaded ethosomal gel treated group) and Group V (Marketed formulation treated group).

Oedema was induced by subplantar injection of freshly prepared carrageenan solution (1% carrageenan in normal saline) into each rat's right hind paws of all groups except group I. Each therapy was applied to the surface of the hind paw and gently massaged 50 times with the index finger to measure anti-inflammatory activity. Animals in groups III, IV and V were treated with Carbopol gel, AKBA-loaded Ethosomal gel, and marketed formulation respectively 30 min before carrageenan injection.

Paw oedema volume (mL) was assessed soon before the carrageenan injection, at "0 hr," and then at 2, 4, 6, and 8 hr following the injection. The inflammation was measured via quantifying the displaced water by oedema using a digital plethysmometer.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 8 software. For each variable examined, a one-way Analysis of Variance (ANOVA) was performed, followed by Dunnett's test. Results are presented as the mean±standard deviation of triplicate determinations obtained from three independent experiments, and a p value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Ethosomal formulations are composed of 0.5-2.0% w/v phospholipon-90G with a high concentration of ethanol ranging from 10-50%. The nanovesicles were prepared by classical dispersion techniques. Compositions for the preparation of ethosomes are revealed in Table 1. All the ethosomal nanovesicle formulations obtained were turbid, milky, and colloidal. The AKBA ethosomal nanovesicles formulation appeared off-white compared to the blank formulation. Limsuwan *et al.*[25] reported that the addition of low concentration (10% v/v) ethanol was insufficient to solubilized the active ingredients whereas, formulations containing higher concentrations (50% v/v) of ethanol resulted in precipitation due to disruption of the lipid vesicles. In our investigation, we observed a similar outcome where a low concentration of ethanol proved ineffective in solubilizing AKBA, while formulations with higher ethanol concentrations led to precipitation.

Drug vesicle interaction study

Figure 1 presents the FTIR spectra from the individual components (AKBA, Phospholipon-90G, and ethanol), its physical mixture, and drug-loaded vesicles. FTIR spectroscopy found similarity in peaks, proving that drug loading did not impact the ethosomal vesicle system's chemical or structural integrity.

Entrapment Efficiency (%)

The effect of phospholipids and ethanol concentration can directly affect the entrapment efficiency of ethosome formulations. The EE% of the AKBA in ethosome formulations is given in Table 2. The percent Entrapment Efficiency (% EE) of the developed formulations ranged from 82.57±3.46 to 91.67±2.69. EE% was found to increase with increasing ethanol concentration from 20 to 30% v/v (E1-E8) but decreased dramatically at higher ethanol concentrations, owing to the presence of ethanol in the formulation, which increases drug solubility in both the core and membrane.[12,26] However, increasing the concentrations of

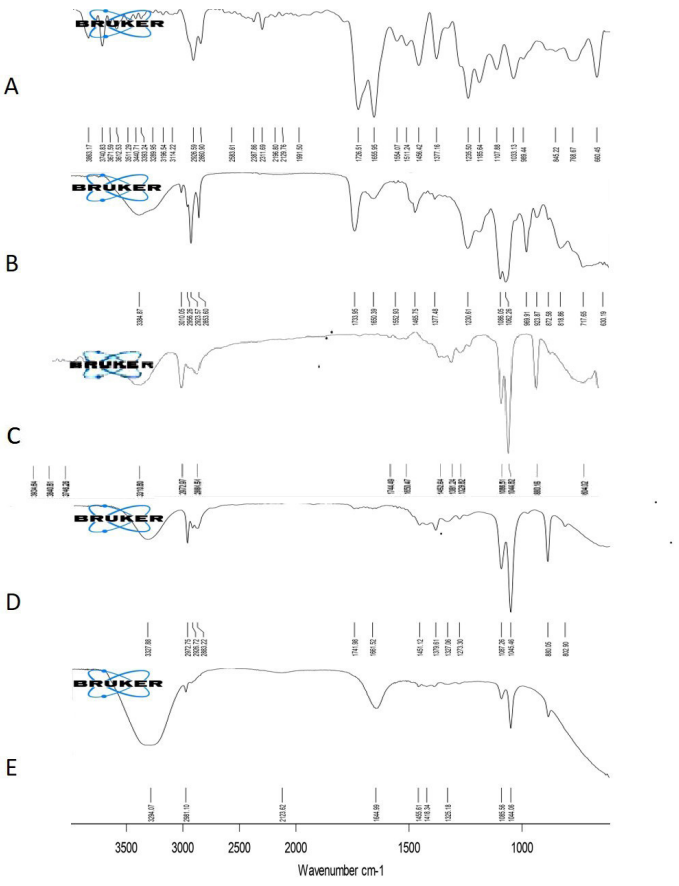

Figure 1: Compatibility study by FTIR spectrum: (A) Acetyl-11-keto-beta-boswellic acid (AKBA) (B) Phospholipon 90 G (C) Ethanol (D) physical mixture of AKBA + Phospholipon 90 G + Ethanol (E) E8 ethosomal formulation.

ethanol concentrations exceeding 40% v/v (E9-E12) resulted in a significant decrease in AKBA entrapment. Bhosale *et al.*[26] similarly reported these findings, which may attribute to the lipid membrane that it is more fluidic and leakier, which makes it more permeable, making the vesicles distorted and decrease in EE%.

pH and Viscosity

The Viscosity of all ethosome was found in the range of 1.72 to 5.82 centipoise (cps). The Viscosity of the optimized Ethosome (E8) was found to be 5.37±0.04 cps. The pH of the optimized formulation (E8) was 5.582±0.04, which is close to the pH of normal skin (pH 4.5-5.5).

Determination of Vesicle size and Zeta potential

The size of the ethosomal nanovesicle was found to be in the range of 79.12 nm to 301.6 nm. Ethosome formulations (E9-E12) with 40% v/v ethanol displayed a vesicle size ranging from 79.12 to 172.4 nm, in contrast to formulations (E1-E4) with 20% v/v ethanol and an equivalent phospholipid concentration (0.5-2% w/w), which exhibited sizes of 249.8 to 301.6 nm. This size difference can be attributed to the elevated phospholipid concentration within the vesicle lipid bilayers. Furthermore, an increase in ethanol concentration led to a decrease in vesicle size. This phenomenon arises from the penetration of ethanol (hydrocarbon chain) molecules into the lipid bilayer, disrupting it and consequently reducing the vesicle size.[27,28]

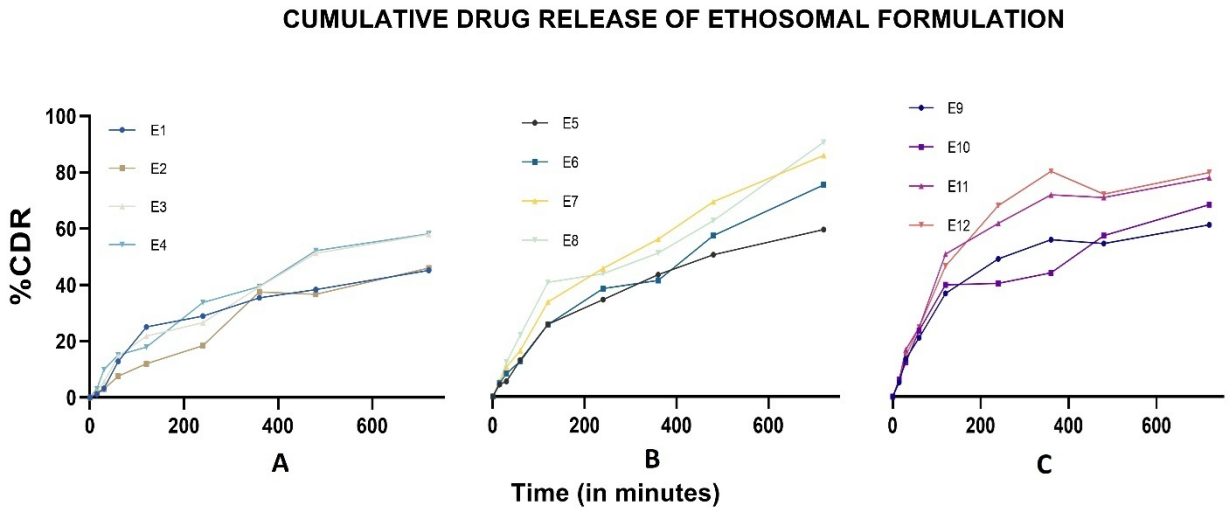

Figure 2: *In vitro* release of AKBA from ethosomes prepared with (A) 20% ethanol and phospholipids (0.5-2%) (B) 30% ethanol and phospholipids (0.5-2%) and (C) 40% ethanol and phospholipids (0.5-2%).

Table 2: Entrapment efficiency, Diameter, zeta potential, Viscosity, and pH of ethosomes formulation (*n*=3, mean value±SD).

Final Formulation Code	Parameters				
	Entrapment Efficiency (%)	Particle size (nm)	Zeta potential (mV)	Viscosity (cp)	pH
E1	89.24±2.78	301.6±6.4	-5.13± 1.17	1.72±0.03	6.672±0.87
E2	90.29±2.40	284.1±4.2	-6.17± 1.88	1.91±0.03	6.0645±0.81
E3	91.16±3.46	253.0±2.5	-6.54± *0.44*	1.97±0.02	5.8185±0.78
E4	91.43±3.11	249.8±4.4	-5.18± 0.94	2.55±0.56	5.582±0.59
E5	85.43±3.27	223.4±6.2	-7.28± 0.66	2.27±0.06	6.7065±0.51
E6	89.10±3.45	187.0±3.5	-5.45± 0.97	2.45±0.02	6.611±0.31
E7	91.52±2.47	179.8±3.4	-8.78± 1.02	4.52±0.06	6.333±0.05
E8	91.67±2.69	145.6±2.4	-8.85± 1.61	5.37±0.04	6.253±0.14
E9	82.57±3.46	169.6±5.1	-6.50± 1.30	2.61±0.08	6.296±0.04
E10	85.91±3.89	172.4±4.1	-6.62± 0.75	3.51±0.38	6.307±0.20
E11	86.29±2.58	88.47±6.2	-5.51± 1.11	4.43±0.65	6.193±0.04
E12	91.09±2.29	79.12±3.9	-4.25± 2.53	5.82±0.29	6.064±0.19

Table 3: Release kinetics for ethosomal formulation.

Formulation code	Zero order	First order	Higuchi model	Korsmeyer Peppas model	
	R^2	R^2	R^2	n	k
E1	0.9837	0.9024	0.8367	0.8329	0.9843
E2	0.9805	0.9490	0.9160	1.6142	1.1435
E3	0.9656	0.9535	0.9060	0.8465	1.1371
E4	0.9829	0.9713	0.9340	0.8764	1.2891
E5	0.9889	0.9687	0.8658	0.8105	1.1251
E6	0.9920	0.9410	0.9251	0.5336	0.3596
E7	0.9945	0.9761	0.9179	0.7494	1.3048
E8	0.9961	0.8734	0.9228	0.6216	0.8913
E9	0.9830	0.9488	0.9423	0.6387	0.9299
E10	0.9902	0.7803	0.9386	0.5983	0.7112
E11	0.9908	0.9549	0.9118	0.5457	0.9830
E12	0.9844	0.9585	0.9259	0.4161	0.7181

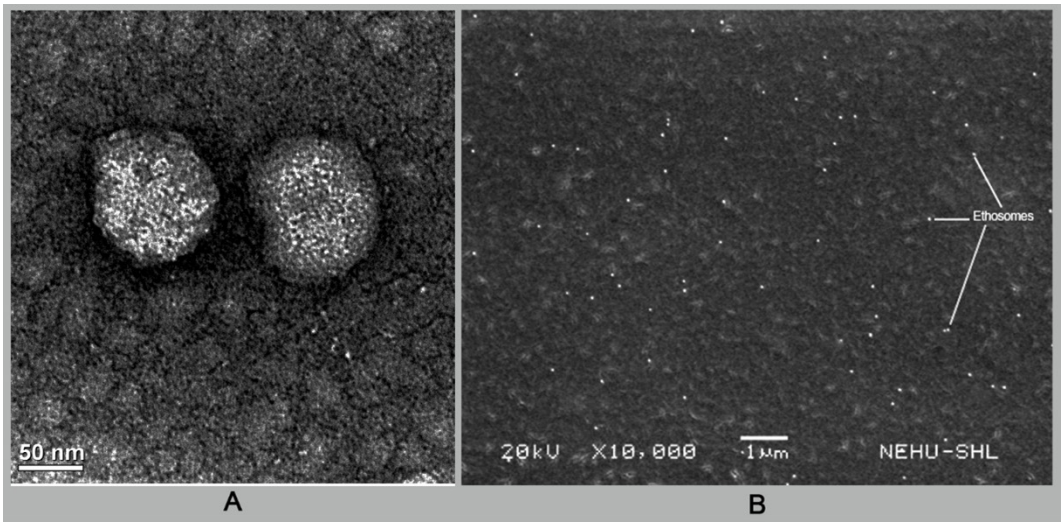

Figure 3: Photomicrograph of the optimal AKBA-loaded ethosome formula as seen by (A) Transmission Electron Microscopy (TEM) and (B) Scanning Electron Microscopy (SEM) images of ethosomes images of ethosomes.

The colloidal system's potential stability can be assessed through the zeta potential. Zeta potential values for the all the ethosomal formulations ranged from -5.13±1.17 to -8.85±1.61 mV, indicating a negative charge. This negative charge is likely due to the presence of ethanol in the system, which is known to induce a negative charge on the vesicle surfaces. Additionally, the interaction between phospholipids in the vesicle bilayer and ethanol molecules may lead to a charge rearrangement on the vesicle's surface, contributing to both the negative charge and steric stabilization.[29]

In vitro drug release

Using Franz diffusion cells and phosphate buffer with a pH of 7.4, AKBA-loaded ethosomal suspensions were subjected to 8 hr *in vitro* drug release studies. Figure 2 displayed the release characteristics of whole ethosomal suspensions. The concentration of phospholipid and ethanol influenced the medication release profile based on the release profile. These were the primary factors affecting size and encapsulation efficiency. From ethosomal formulations E1 to E12, the cumulative percentage of drug release ranged from 46.06% to 88.39%. Every hour, the release of the drug was monitored.

To ascertain the mechanism of drug release, the *in vitro* drug release data of the formulation was subjected to a goodness of fit test by linear regression analysis following zero-order, first-order, Higuchi and Korsmeyer-Peppas, which was reported in Table 3. When the regression coefficient values were compared, it was revealed that the formulation had the highest "r" value, which is 0.9961 (E8), suggesting that the drug release from formulations

was found to follow zero order model of drug release kinetics, which is perfect for dermal delivery since it ensures consistent drug release over a long period of time and improves therapeutic index.

Table 4: Flux and permeability coefficient of formulations.

Formulations	Jss (μg/cm²/hr)	Permeability Coefficient (cm/ʰ)x10⁻³
30 % hydroalcoholic drug solution	50.8±1.71	25.4±1.45
Ethosomes (E8)	84.9±1.11	42.45±1.604
Ethosomal gel	72.8±1.69	36.4±1.106

Visualization of vesicles by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

The prepared ethosomes were found to be flexible, uni-lamellar nano-sized vesicles in TEM images, as shown in Figure 3. The

Table 5: IC_{50} values for inhibition of protein denaturation method.

Sample	IC_{50} (Mean ± SD)
Diclofenac sodium	144.824±0.270
Boswellic acid	131.221±4.672
Ethosomes	162.872±0.328

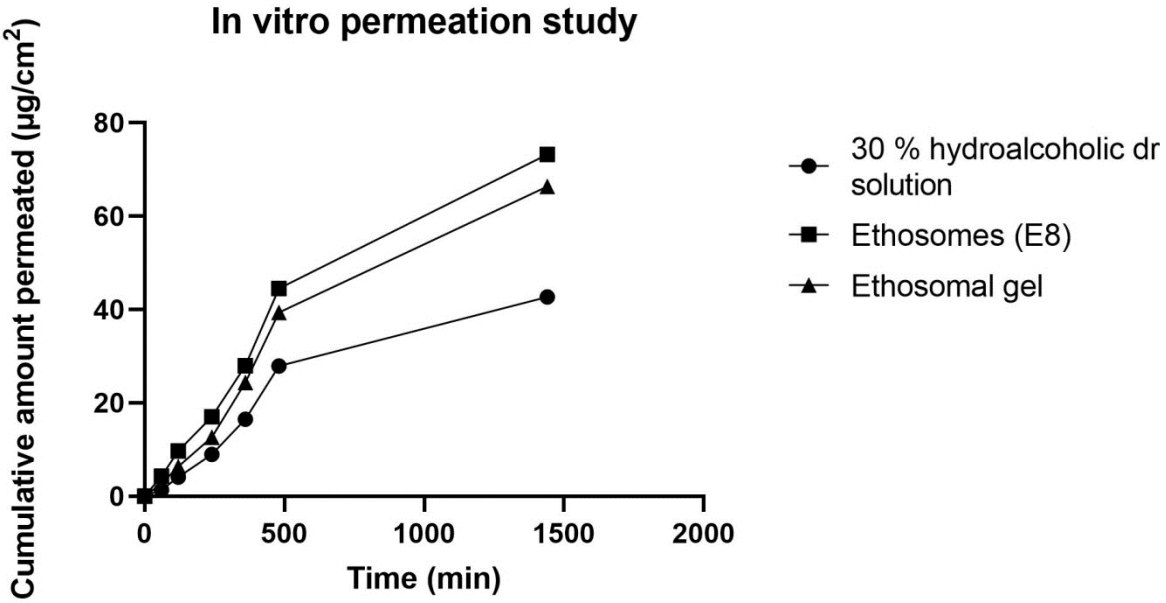

Figure 4: *In vitro* permeation studies of 30% hydroalcoholic drug solution, Ethosomes (E8) and Ethosomal gel.

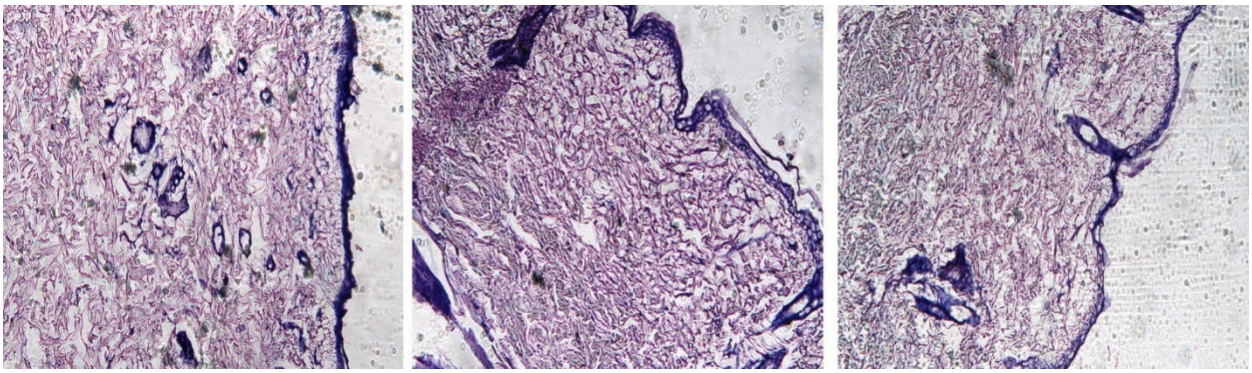

Figure 5: Hematoxylin and eosin-stained skin tissue histopathological image and examination under light microscope. (A): Ethosomal gel, (B): Carbopol gel (C) Hydroalcoholic solution.

images serve to demonstrate the vesicles' spherical nature and to verify the accuracy of the vesicle size measurements. SEM was used for additional research to confirm the morphology of the vesicles because it offers clear, 3D images of the ethosomes (three dimensions). The SEM images confirmed the round and spherical shape of the vesicles with a superior homogeneous population, as shown in Figure 3.

In vitro Permeation Study

The ethosome E8 was chosen for skin permeation investigations after evaluating all the variables, including physiochemical characteristics, release pattern, and entrapment efficiency before and after storage. The amount of AKBA that permeated through the diffusion membrane for the hydroalcoholic solution, ethosomal formulation, and ethosomal gel-based formulation was found to be 42.73%, 73.22%, and 66.39% in 24 hr, respectively. That might be because ethosomes contain ethanol, which gives the vesicles the flexibility they need to penetrate deep epidermal layers more easily. Moreover, because of their hygroscopic nature, phospholipids readily bind with water. Because of this, we consider that these characteristic raises skin humidity, which promotes the maximum hydration of the horny layer.[11,30] Figure 4 shows a plot of the cumulative drug permeation from various formulations as a percentage against the permeation duration in hr (t).

Also, the permeation order from the ethosomal formulation followed the Zero order release. After the cumulative amount of drug permeated per unit area was plotted as a function of time, the steady state transdermal flux was calculated from the linear portion of the curve. The transdermal flux of Acetyl-11-keto-β-boswellic acid through pig ear skin for a hydroalcoholic solution,

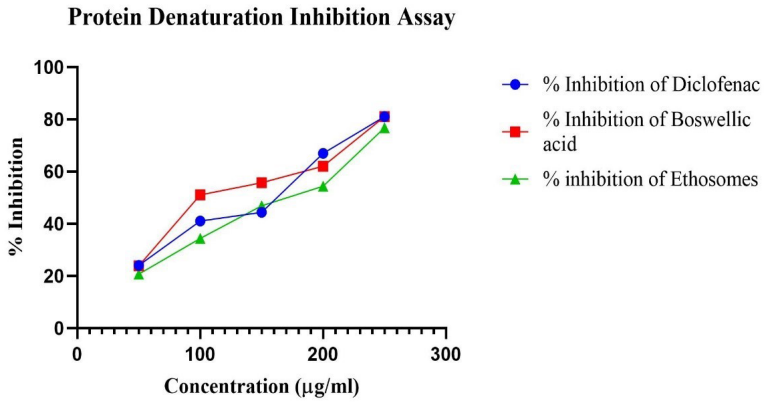

Figure 6: *In vitro* evaluation by (A) Protein Denaturation Assay of diclofenac sodium (Standard), Boswellic acids and ethosomes loaded with Boswellic acids (Test).

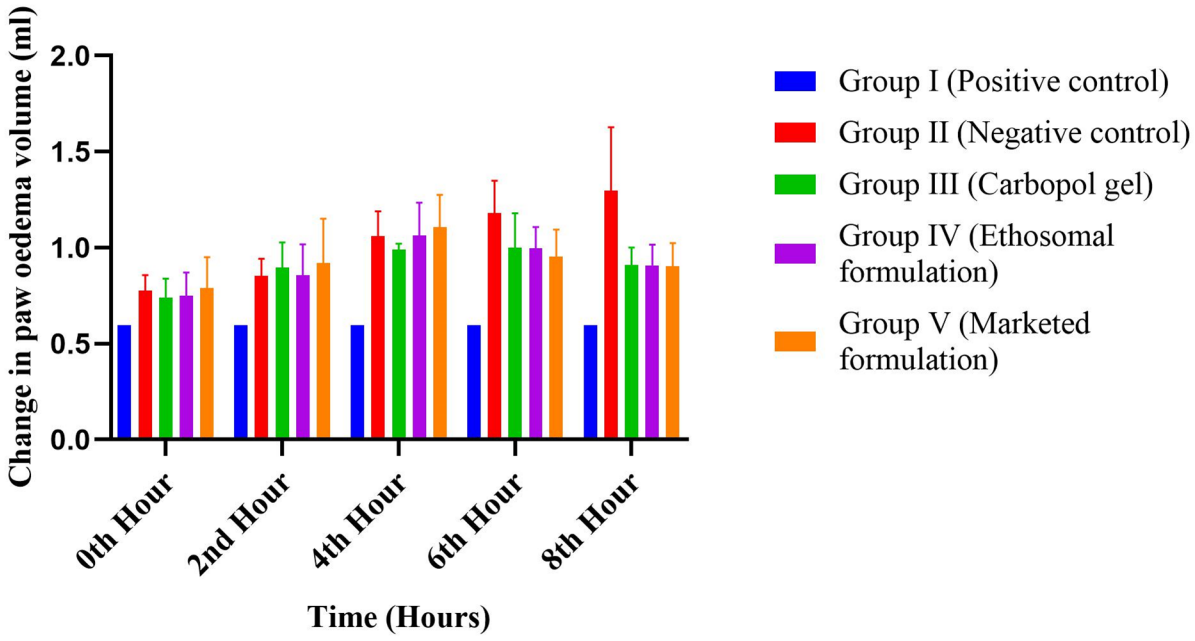

Figure 7: *In vivo* anti-inflammatory evaluation by Carrageenan induced paw oedema.

Table 6: Screening of anti-inflammatory activity of selected formulation in carrageenan induced paw oedema in rats.

Groups	Change in Paw Oedema Volume (mL)				
	0th Hr	2nd Hr	4th Hr	6th Hr	8th Hr
Group I (Positive control)	0.598±0.00	0.598±0.00	0.598±0.00	0.598±0.00	0.598±0.00
Group II (Negative control)	0.777±0.08	0.853±0.09	1.06±0.13	1.18±0.17	1.298±0.33
Group III (Carbopol gel with drug)	0.74±0.10	0.897±0.13	0.99±0.03	1±0.18	0.91±0.09
Group IV (Ethosomal formulation)	0.75±0.12	0.857±0.16	1.065±0.17	0.997±0.11	0.904±0.11
Group V (Marketed formulation)	0.79±0.16	0.92±0.23	1.106±0.17	0.954±0.14	0.904±0.12

Values are mean±S.D. of 6 individual rats; Values are significant at $p<0.05$ over control.

Table 7: Inhibition of paw oedema by selected formulation in carrageenan induced paw oedema in rats.

Groups	% Inhibition of Paw Oedema				
	0th Hr	2nd Hr	4th Hr	6th Hr	8th Hr
Group II (Negative control)	23.04	29.89	43.58	49.32	53.93
Group III (Carbopol gel with drug)	19.19	33.33	39.60	40.20	34.29
Group IV (Ethosomal formulation)	20.27	30.22	43.85	40.02	33.92
Group V (Marketed formulation)	24.30	35.00	45.93	37.32	33.85

Values are % of inhibition over positive control (Group I).

ethosomal formulation, and ethosomal gel-based formulation were observed in Table 4. It was observed that the optimized ethosomal formulation (E8) shows higher transdermal flux when compared to plane hydroalcoholic solution containing drug. The enhanced penetration of acetyl-11-keto-β-boswellic acid from ethosomal formulations compared to hydroalcoholic solution implies a potential synergistic interaction involving ethanol, vesicles, and skin lipids.

Vesicle-skin interaction

A histopathological examination of the treated and control (Carbopol gel) skin was performed using light microscopy to investigate changes in skin morphological features. Micrographs (are shown in Figure 5) of the control and treated samples showed normal skin with distinct epidermal and dermal layers, uniformly layered SC, and other features of normal skin. After 24 hr of ethosome treatment, distinct changes in skin morphological features were observed. The treated section clearly showed a disruption in SC organization, demonstrating the capability of the vesicular carriers to increase penetration. Intercellular vacuoles were discovered in some ethosome-treated skin areas. Lipid vesicles are known to use the follicular transport pathway, and ethosomes vesicles can penetrate the damaged SC bilayers and even make a passageway through the skin. The release of the drug in the deep skin layers and transdermal permeation may therefore result from ethosome fuse with skin lipids and the drug being released at different locations along the penetration.[31,32]

Inhibition of protein denaturation

The current investigation assessed the anti-inflammatory effect of ethosomes derived from AKBA against heat-induced egg albumin denaturation, a known inflammation trigger. Protein denaturation causes inflammation, which causes various inflammatory diseases, including arthritis. When a protein's distinctive three-dimensional structure is subjected to alterations, it denatures the protein. The maximum % of protein denaturation inhibition by ethosomes was 76.80% at a concentration of 250 µg/mL. Diclofenac sodium, a standard anti-inflammatory activity, showed maximum 81% inhibition of protein denaturation at 250 µg/mL concentration. In the study, under linear regression analysis of concentration vs. % inhibition, it was discovered that a significant denaturation reaction was recorded in AKBA-loaded

ethosomes, which is concentration dependent (r^2=0.973) with an IC_{50} value of 162.872 µg/mL. The results were obtained using GraphPad Prism 8 software. The concentration versus % inhibition results are displayed in Figure 6. At the same time, the IC_{50} values for protein denaturation are recorded in Table 5.

AKBA is a phytochemical derived from gum resin extract produced by trees in the genus Boswellia. It is widely recognized for blocking pro-inflammatory enzymes and inhibiting anti-inflammatory activity.[33,34] This study assessed the anti-inflammatory potential of the Boswellic acid-based ethosomal topical gel. This was tested *in vitro* using heat-induced egg albumin denaturation bioassay. This assay's implementation is justified by the denaturation of albumin proteins produces antigens that start type III hypersensitivity reactions and cause inflammation.[35] Therefore, the ability of an agent to prevent the denaturation of the substance shows that it has anti-inflammatory qualities; the greater the degree of inhibition, the greater the potential for anti-inflammatory effects. With an IC_{50} value of 162.872 g/mL, the findings demonstrated that the ethosomes exhibited concentration-dependent anti-inflammatory activity *in vitro*. This discovery is brand-new. As was predicted, the reference medication, diclofenac sodium, likewise exhibited strong and concentration-dependent *in vitro* anti-inflammatory action. Studies show the formulation and anti-inflammatory evaluation of topical nanoemulgel of *Boswellia serrata* and Boswellic acid-loaded proniosomal gel, however *in vitro* studies are not included in the investigation.[36]

In vivo anti-inflammatory evaluation

Carrageenan-induced paw oedema

The goal of the current study is to evaluate the anti-inflammatory effectiveness of an ethosomal formulation based on AKBA in experimental rats. To test the anti-inflammatory efficacy of carrageenan in rats, experimentally produced acute inflammation was used in the current investigation. Rats' right hind paws were sub plantarly injected with 1% Carrageenan to cause acute inflammation. The results showed that sub plantar injection of carrageenan led to time-dependent development of peripheral inflammation. All experimental rats had their rat paw oedema volume recorded every 2 hr (up to 8 hr) following carrageenan administration. Carrageenan injection caused gradual oedema in the hind paw that peaked after 4 hr. As shown in Tables 6 and 7, Figure 7, animals in Group IV reduced the paw volume reduced from 0.997±0.11 to 0.904±0.11 at t=8 hr. In the case of Group I animals paw volume was constant. Group III animals had shown an increase in volume at each hour till the 6th hr and there was a decline in oedema in t=8th hr. The paw oedema of Group V animals was 0.75±0.12 which showed an increase at the end of 4th hr and after that, it decreased to 0.904±0.12 on completion of 8th hr. A one-way ANOVA followed by Dunnett's test produced significant results (p<0.05).

An extremely popular model for evaluating a compound's ability to reduce inflammation is carrageenan-induced paw oedema. Prostaglandins, leukotrienes, histamine, bradykinin, and TNF- are just a few examples of the inflammatory and proinflammatory mediators that are released when carrageenan is administered.[37] Parenterally administered carrageenan typically results in a biphasic reaction, where the first phase lasts for 15 to 45 min and involves the release of mediators including bradykinin, serotonin, and histamine, while the second phase lasts for 60 to 90 min and only involves the production of prostaglandins.[38] In the present investigation, ethosomal formulation based on AKBA exhibited marked anti-inflammatory activity on carrageenan-induced paw oedema in rats. Treatment with gel and ointment formulations of *B. serrata* extract increased the zebra fish's regeneration/wound healing and decreased inflammation. The anti-inflammatory and wound-healing properties of *B. serrata* have been attributed to glycosides present in the plant.[39] Nanoemmigel containing Boswellic acid resulted in 50% inhibition in paw oedema volume at 5 hr as compared to a control group (p<0.05).[40] Proniosomal Gel (PG 1%) containing boswellic acid caused paw oedema to be inhibited by 42.5 0.024% at 5 hr as compared to the control.[41]

CONCLUSION

The study found that ethosomal formulation is a promising vehicle for the transdermal delivery of AKBA. Ethanol and phospholipids were used to load an ethosomal formulation with an AKBA, and Carbopol 934P (a hydrophilic polymer) was used to create formulations based on ethosomal gel. The results of an *in vitro* skin release investigation showed that ethosomal formulations had higher transdermal flux than hydroalcoholic drug solutions. The visualization study's observations also showed that ethosomal formulation altered the skin's normal histology by causing lipid disturbance and expanding the SC's intercellular lipid lamellae gap. The primary factor in the increased skin permeation was the inclusion of ethanol in the composition, which allowed for drug solubilization and the formation of flexible lipid structures that could travel through skin corneocytes, resulting in improved medication skin retention and permeation. This research could provide fresh insight into ongoing work on formulating efficient transdermal delivery systems.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

AKBA: 3-acetyl-11-keto-β-Boswellic acid, **NSAIDs:** Non-steroidal anti-inflammatory drugs, **EE:** Entrapment efficiency, **TEA:** Triethanolamine, **FT-IR:** Fourier transform infrared, **TEM:** Transmission Electron Microscopy, **SEM:** Scanning electron microscopy, **IEAC:** Institutional Animals Ethics Committee.

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