Research Article

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Therapeutic Delivery

Azelaic acid and *Melaleuca alternifolia* essential oil co-loaded vesicular carrier for combinational therapy of acne

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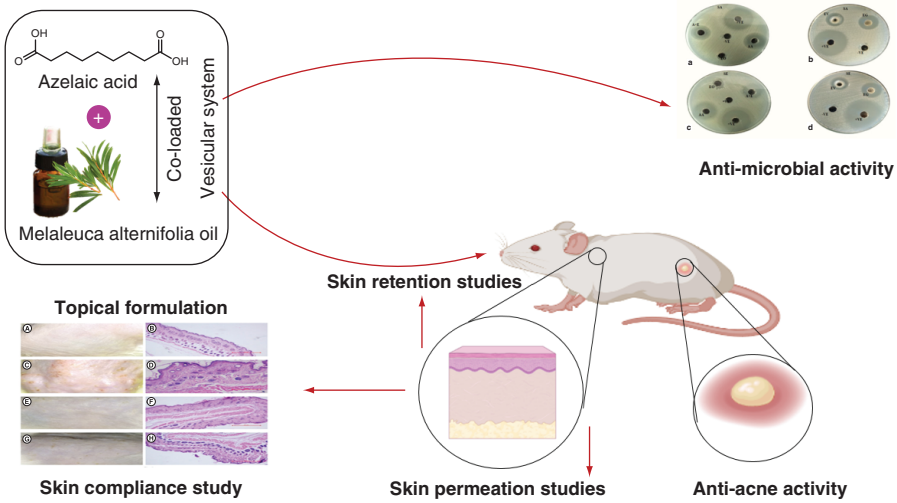
Aim: Azelaic acid (AzA), a comedolytic, antibacterial, anti-inflammatory anti-melanogenic agent, prescribed against acne vulgaris is safe on skin. Its combination with another widely used anti-acne agent, tea tree oil (EO) whose delivery is limited by volatility, instability and lipophilicity constraints was attempted. **Method:** Solvent injection was used to prepare AzA-EO integrated ethosomes. **Result:** Ethosomes were transformed into carbopol hydrogel, which exhibited pseudo-plastic properties with appreciable firmness, work of shear, stickiness and work of adhesion. The hydrogel showed better permeation and retention characteristics vis-a-vis commercial formulation (AzidermTM), when evaluated in Wistar rat skin. Further, ethosome hydrogel composite was better tolerated with no side effects. **Conclusion:** The findings suggests that the aforementioned strategy could be a potential treatment used for acne management.

Graphical abstract:



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Acne vulgaris is a consequence of multiple etiologies such as hyper-seborrhoea, dysregulation of the hormone microenvironment, bacterial colonization and host inflammatory response [1]. It is one of the most common dermatological disease and is associated with various psychiatric comorbidities such as depression, anxiety, social inhibition, body dysmorphia and social ideation, especially due to scarring and pigmentation [2]. Open or closed comedones and pustules, papules, nodules are non-inflammatory and inflammatory manifestations of the condition, respectively [3].

It is desirable to target hair follicles, have increased drug permeation and deposition, improved bioavailability at selected sites in acne and similar conditions [4]. Topical therapy can provide aforementioned benefits when used in conjunct with or as an alternative to systemic therapy. The topical agents commonly prescribed include retinoids (tretinoin, adaplene, tazarotene, isotretinoin and metretinide), antibacterial agents (clindamycin, erythromycin, clarithromycin, azithromycin and nadifloxacin), antiandrogens (Cyproterone acetate) [5]. These drugs are, however, associated with numerous side effects, and some have even developed bacterial resistance over the last decades [6]. Natural AzA can be used topically to treat mild-to-moderate acne.

Azelaic acid (AzA), a dicarboxylic acid analog, is an anti-infective, anti-oxidative, anti-inflammatory, inhibits follicular keratinization and epidermal melanogenesis. It works by inhibiting the expression of proinflammatory cytokines, downregulating cathelicidins, inhibiting ROS production by granulocytes, thereby exerting its anti-inflammatory activity. It reduces the stratum corneum thickness, decreases the number of keratohyalin granules, reduces the amount, distribution of filaggrin in epidermal layers, reduces cell proliferation, is a competitive inhibitor of important mitochondrial respiratory chain enzymes, such as NADH dehydrogenase, succinic dehydrogenase, ubiquinone cytochrome c oxidoreductase. Through all these actions it exerts its anti-keratinizing action. By lowering of bacterial intracellular pH by the drug, pH gradient across the cell membrane ceases to be maintained, causing the bacteria to lose energy, leading to antibacterial activity. Melatonin generation is inhibited by inhibiting tyrosinase like enzymes causing of melanin and melanosomes to degrade [7].

However, AzA has poor aqueous solubility (≈0.24 g/100 g of water at 25°C), and penetrability across the skin. Therefore, it needs to be formulated at higher dose of 10% w/w or 20% w/w to achieve the desired therapeutic effect. This is however limited by dose-dependent side effects like erythema, burning, stinging and tingling, dryness, scaling and peeling and warrants development of a novel strategy for its effective delivery.

Tea tree essential oil (EO) is isolated by steam distillation of follicular leaves from plant *Melaleuca alternifolia*. The antioxidant, anti-inflammatory property, and broad spectrum anti-microbial activity, of EO make it useful as a complementary drug in acne. It has been used for many years in the treatment of acne vulgaris [8,9] and successfully combined with benzoyl peroxide, erythromycin [10], adapalene [11] previously with promising results. However, it is limited by volatility instability, lipophilicity constraints.

Thus, to overcome the limitation of both the bioactives, they can be co-formulated for the management of acne vulgaris. EO ethosomes have been reported as an effective treatment of atopic dermatitis [12]. There is a Korean skin care serum of brand Cos De BAHA that uses a combination of azelaic acid (5%), EO and few other actives. The product is popularly sold as a 'multi-action powerhouse' and promises to clear cystic acne. In the present research the combination of AzA and EO has been encapsulated, to achieve targeted and safe delivery. Although EO could have been quantified, however as no suitable analytical method was found for the same. Hence quantification of EO was not performed. Similar research have used a combination of adaplene and EO before [11]. During the past few years, novel nano-carriers have been reported as effective vehicles for delivering AzA to the skin, for example, via liquid crystal systems [12], liposomes [13], ethosomes [14], proethosomes [15], nanoparticles [16], leciplex, invasomes [17], nanostructured lipid carriers [18], nano emulsion [19] and microemulsion [20]. EO has also been formulated as nanoemulsion [21], liposome [22], ethosome [23], microsponges [24], nanoparticles [25] and microcapsules [26]. The ethanolic vesicle (EV) is a new type of vesicular carrier consisting of phospholipids that are arranged in one or more overlapping bilayers surrounding numerous small pockets of aqueous solution. Biocompatibility, biodegradability and the presence of lipid component have made ethosomes the most rationalized carriers for delivering medicine. In addition having advantages of easy membrane transport, sustained drug release, safeguarded encapsulated bioactives,

enhanced bioavailability, skin penetration through pores smaller than their size, easy applicability in the form of gels and ointments, circumventing toxicity problems, possessing affinity for both hydrophilic and lipophilic drug and easy to scale up manufacturing process makes it a unique carrier for drug delivery.

In this study, the potential for co–administration of AzA and EO was evaluated encapsulated in ethosome, to synthesize a system with synergistic anti-acne properties within carbopol hydrogel, enhance targeted delivery of active drugs into pilosebaceous glands so as to provide prolonged action with negligible adverse effect. Here, we studied the rheological behavior and textural attributes of the hydrogel for its intended topical use. We also demonstrated its antibacterial efficacy against *S. aureus, S. epidermidis* and *P. acnes. Ex vivo* skin permeation and retention of AzA from different systems, were also tested by means of Wistar rat skin and were compared with marketed formulation. The developed optimized ethanolic vesicles formulation of AzA and EO was compared with topical marketed formulation in the testosterone induced acne model in Swiss Albino mice.

Materials & methods

Materials

AzA was purchased from TCI Chemicals Pvt. Ltd., Chennai, India. EO (*Melaleuca alternifolia*) was purchased from Allin Exporters, Noida, India. Phospholipipon 90G (PL-90G) was supplied *ex gratis* by Phospholipid GmbH, Germany. Dimethyl sulfoxide (DMSO), Carbopol 934, PEG 400, Triethanolamine (TEA), Acetonitrile (ACN), Sodium dihydrogen orthophosphate and orthophosphoric acid were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Absolute ethanol was purchased from Changshu Hongsheng Fine Chemical Co. Ltd., Chagshu, China, while sephadex G-50 was purchased from Sigma Aldrich Chemie, GmbH. Nutrient broth (NB), Agar, Brain heart infusion (BHI), Erythromycin and Resazurin Dye were purchased from Himedia Labs Pvt. Ltd., Kolkata, India. The commercial formulation of azelaic acid employed in the study was Aziderm® 10%w/w gel (Micro labs Ltd., Banglore, India) procured from local drug house. Ultrapure water prepared by Micropore ™ was used throughout the study.

For experimentation guidelines were followed as dictated by CPCSEA (Committee for the purpose of control and supervision of experiments on animals). Institutional Animal Ethical Committee of [ANONYMIZED] University approved the experiments (IAEC/SU/09/18). Male Swiss Albino mice and Wistar rats used in the current study were collected from [ANONYMIZED] University's central animal house (H.P).

RP-HPLC instrumentation & chromatographic conditions for AzA analysis

The AzA analysis was carried out on an HPLC system (Agilent Technology 1200 series, Chandigarh, India) equipped with double beam U.V spectrophotometer (Systroni2202, India Pvt. Ltd., Mumbai, India), pressure controlled by prominence pump and operated by EZChrom software. Innoval C18 column (250 × 4.6 mm, particle size 5 µm) was used for the separation. Two different mobile phase solvents for pumps were, (50:50) sodium di-hydrogen orthophosphate (NaH_2PO_4; pH 3.5; 50 mM) Pump A, and ACN for pump B. Mobile phases were filtered through a 0.45 µm nylon membrane filter and ultra-sonicated for 30 min. Samples were filtered through the nylon syringe filter of 0.22 µm pore size, prior to the injection. Sodium di-hydrogen orthophosphate and ACN (50:50) v/v mixture was used as mobile phase in gradient mode. The flow rate was set at 0.6 ml/min. Samples volumes were 10 µl and PDA detector was set at 321 nm. Run time of the samples was 30 min. Analysis was conducted at a detection wavelength of 250 nm. A constant temperature of 25°C was maintained in the column [27].

AzA & EO compatibility studies

Compatibility studies were performed for azelaic and EO using FTIR.

Preparation of AzA & EO loaded ethanolic vesicles

As shown in Table 1, a total of 8 formulations for ethanolic vesicles (EVs) were prepared with phospholipid (PL-90G) concentrations ranging from 10 to 20% w/w. EtOH concentrations ranged from 10 to 40% w/w. All formulations contained 5% w/w. As per the previously published studies 5% azelaic acid and 5% EO concentrations have been used individually for treatment of acne vulgaris [28,29]. AzA and EO. Cold method is the most common way of preparing EVs [30] according to which PL-90G, AzA and EO were dissolved in ethanol to a clear solution. Consequently resultant alcoholic solution was poured into the aqueous phase, it was mixed at 1000 rpm. Stirring of the alcoholic solution continued for five minutes after it had been transferred. The EVs so obtained were properly stored until further use.

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Table 1. Compatibility study of azelaic acid and tea tree oil using FTIR spectrum.

S. No.	Observed peaks of AzA	Functional group of AzA	Observed peaks of EO	Functional group of EO	Observed peaks of AzA + EO	Functional group of AzA/EO
1	1700 cm^{-1}	Dicarboxylic acid	2883 cm^{-1}	-CH cyclic stretch	1700 cm^{-1}	Dicarboxylic acid of AzA
2	2943 cm^{-1}	Hydroxyl group	2965 cm^{-1}	-CH stretch	2965 cm^{-1}	-CH stretch of EO
3	1104 cm^{-1}	(O-C) ether bond in plane	1655 cm^{-1}	-C=C cylic stretch	1104 cm^{-1}	(O-C) ether bond of AzA
4	1201 cm^{-1}	(O-C) ether bond out of plane	3702 cm^{-1}	-OH stretch	3702 cm^{-1}	-OH stretch of EO
5	2853 cm^{-1}	Methylene group	–	–	–	–

AzA: Azelaic acid; EO: Tea tree oil.

Characterization of AzA & EO co-loaded EVs

Vesicular size & size distribution

The Zetasizer (Malvern) was used to measure the mean size and distribution of EV. A quartz cuvette containing distilled water was used for the size measurement, and the scattering angle was 90 degrees. A triplicate of each observation was noted [31].

Zeta potential

Zeta potential for optimized EV formulation was determined using Zetasizer-LS13320 (Delsa™Nano C Beckman coulter). The final result for the sample was the average of three measurements [32].

Percentage entrapment efficiency by ultra-centrifugation method

Ultracentrifugation test was conducted in triplicate to determine the percentage EE (%EE). The EV formulation was centrifuged (REMI CPR 24) at 23,980 ×*g* for 2 h, at 4°C. The drug content of the clear supernatant as well as the vesicular sediment after methanol lysing was determined. The appropriate dilutions were made, and RP-HPLC technique was used to analyze them. Calculation of the% EE was done using the following equation:

$$EE = \frac{(T - C)}{T} \times 100 \quad \text{(Equation 1)}$$

Where, the total amount of drug detected in both the supernatant and sediment is T, and the amount only detected in the supernatant is C [33].

Flexibility index

By means of a vesicle-extrusion device (Eastern Sci. Inc., MD, USA), EV carriers were measured for their membrane flexibility index (FI). In the experiment, vesicular suspensions were extruded through polycarbonate membranes with pores of 0.05 µ sizes. Utilizing the Delsa Nano particle size analyzer (M/s Beckman Coulter Inc., CA, USA), we measured the size and size distribution of EVs before and after extrusion. Equation 2 was then used to calculate FI.

$$FI = \frac{PE - PA}{PA} \quad \text{(Equation 2)}$$

Where P_E and P_A are the values of pre and post-extrusion particle sizes, respectively [34].

Optical microscopy

Phase contrast optical microscope (RXL-5 (B-7692) equipped with a CCD camera was used to measure EV morphology, namely, shape uniformity and lamellarity [33].

Incorporation of drug-loaded EV in the hydrogel

EV incorporated in hydrocolloids facilitates easy application, adhesion and apt drug penetration [34]. To augment topical spreadability, ethosome was further incorporated into 10% by weight of neutralized Carbopol® 934 hydrogel. The final percentage of Carbopol in the gel was found to be 1% w/w.

Characterization of ethosomal gel

Organoleptic properties

In order to assess organoleptic attributes like feel, transparency, tackiness and grittiness, all of the selected EVs were visually examined. Transparency was determined by viewing EV hydrogel against a white light source, and other characteristics were determined by gently rubbing EV hydrogel between thumb and forefinger.

Rheology & texture analysis

The rheogram of the EV gel was constructed by using rotational rheometer (Make-Anton Par, Model-Rheolab QC). An approximate weight of 5 g of gel was placed in sample holders with diameter 9.995 mm and length 14.987 mm. The shear stress value was observed. The relation between shear stress (τ) and shear rate (γ) was determined by power law and Herschel-Bulkey model (Equations 3 and 4).

$$\tau = \tau_o + ky^n \quad \text{(Equation 3)}$$

$$\tau = ky^n \quad \text{(Equation 4)}$$

Where k is consistency index (Pa sec^n), τ_o is yield stress (Pa) and n is power law exponent.

The texture properties of gel was analyzed by Texture analyzer™ (TA.XT plus Texture analyzer M/s Stable Microsystem, Surrey, UK). Sample (15–20 g) was retained into the lower cone. In order to ensure the starting point for each test was the same height, the upper cone probe was calibrated against the lower cone beforehand. The probe reaches a depth of 2 mm above the surface of the sample holder by approaching and then penetrating the sample, as a result, the probe moved 23 mm from its starting point (with test speed of 3.0 mm/s.).

In vitro anti-bacterial activity

The antibacterial activity of AzA, EO, AzA-EO, EV and EV gel formulations were studied against two bacterial strains namely *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (MTCC 3382). Bacterial strains, *S. aureus* and *S. epidermidis* were cultured in nutrient broth (NB) medium, and incubated at 37°C for 24 h.

Antibacterial agar well diffusion assay

Antibacterial activity was done by using agar well diffusion method. In 100 mm sterile petridishes, approximately 25 ml of media was poured in and allowed to solidify. Using sterile coEOn swabs, bacterial culture of 0.2 McFarland Standard 600 nm was evenly spread over the media plates. Different formulations were added into the separate wells punched with the cork borer (6 mm) in the agar. AzA, EO and AzA-EO combination were diluted in 99.9% ethanol so as to have test concentration as 50 mg/ml for AzA, 50 mg/ml for EO, and 50 mg/ml each for AzA-EO combination. EV containing AzA 50 mg/ml and EO 50 mg/ml loaded directly into the wells, while EV gel was first diluted in the DMSO, so as to have the test concentration as 50 mg/ml each for AzA and EO. After the incubation of 24 h at 37°C for *S. aureus* and *S. epidermidis*, zone of inhibition was determined using HiAntibiotic Zone scale-C. The positive control for *S aureus* and *S. epidermidis* was erythromycin (1 mg/ml).

Determination of minimum inhibitory concentration

In broth microdilution assay, minimum inhibitory concentration (MIC) was determined for various bacterial strains with AzA, EO (EO), AzA-EO, EV, and EV gel formulations. MIC is one in which no visible growth of the organisms tested is achieved. (Poomanee *et al.*, 2018). To determine the MIC, 100 µl of each test formulation was taken in a flat-boEOm 96-well micro-titer plates containing 100 µl of NB for *S. aureus* and *S. epidermidis*. Subsequently each well pertaining to every test formulation was serially diluted in ratio of 1:1 upto 12th well. 10 µl of bacterial culture suspension (1×10^8 cells/ml) was added to the each well. On each plate there were also designated wells for controls namely medium control, and test control (AzA, EO, AzA-EO, EV and EV gel alone) without any bacterial inoculums. Control for inoculums viability was also reserved, wherein no test formulation was added. The plates were incubated for 24 h at 37°C for *S. aureus*, and *S. epidermidis*. Resazurin dye (10 µl) was added after incubation, and the color change was observed visually. As growth took place, the color of the growth changed from purple to pink or colorless. An MIC value was calculated by finding the concentration at which color change from purple to pink. The samples were all analyzed in triplicate [35].

Ex vivo skin permeation studies

Studies on *ex vivo* skin permeation were undertaken with Wistar rats (from control groups used in *in vivo* skin compliance study) using a diffusion cell with an effective diffusional area of 2 cm^2. Rat skin, despite its thinness was utilized because it was easy to procure, could be removed fresh before to the skin permeation investigation, had the appropriate viability, enzymatic activity and is often reported to exhibit minimal variability in permeation results [36–39]. Moreover, it is anatomically similar to human skin and is frequently employed in permeation experiments due to its ease of availability, ease of handling, as well as low cost [40]. Although porcine skin would have been an excellent choice (as it has greater structural similarities with human skin), it could not be used due to a lack of availability. By dislocating spine of Wistar rat, they were sacrificed and their dorsal surface hair was removed with electric clippers. Following that, sterile scissors were used to free the harvested skin from adhering adipose tissue. After the tissue had been equilibrated with 0.9% normal saline for 1 h, it was mounted between the donor and receptor compartment of the diffusion assembly having a receptor volume of 15 ml. With a magnetic stirrer, diffusion medium (Ethanol: Phosphate buffer pH 6 (30:70)) was continuously stirred, while an air jacketed thermoregulator maintained the assembly at 32°C. Separate formulations (i.e. AzA-EV, AzA-EV gel and Aziderm®) of 0.5 g were then loaded into each assembly's donor compartment. To maintain the sink condition, 1 ml aliquots were collected from the sampling port and replaced with fresh solution and the sampling was done at 0.5, 1.5, 4, 6, 9, 12 and 24 h. The concentration of AzA in the receptor compartment was analyzed by validated RP-HPLC method and the cumulative amount of AzA permeated was reckoned using Equation 5. The graph between cumulative amount permeated (μg/cm^2) versus time was also plotted [34].

$$Q_n = C_n V_o + \Sigma_{i=1}^{n=1} C_i \times V_i \qquad \text{(Equation 5)}$$

Cn denotes the time at which each sample of the receiver medium is analyzed for drug concentration, C_i is i^{th} sample drug concentration, V_0 and V_i are sample and receiver solution volumes, respectively.

Skin retention studies

The skin mounted on the diffusion cell was carefully removed following the completion of the permeation studies. An analysis of the drug content of the adhered formulation on the skin was conducted. Using ultrapure water, the tissue was washed three-times and then dried on a lint-free coEOn swab. The cleared skin was then macerated in methanol for 24 h and then macerated using tissue homogenizer. Further centrifugation at 7000 rpm was performed, followed by RP-HPLC analysis to quantify the drug from the supernatant [34].

In vivo studies

Skin compliance study

Wistar rats were used for the skin compliance studies. Three group of rats consisted of three animals each. There was an *ad libitum* supply of water and food for each animal in its separate cage. Clippers of 0.1 mm diameter were used to remove hair on the dorsal side of the animals. Using a water-soaked cotton swab, the skin was swabbed three- to four-times. The first group was not treated (control), while the second and third groups were treated with AzA-EV gel and MKT (marketed formulation), respectively. Two weeks, after daily application of the designated treatment or control, the formulation remaining on the skin was removed with a dry cotton swab, then cleaned with a wet cotton swab. A digital camera was used to photograph the skin areas after 1 h of cleaning. Animals were sacrificed, and skin samples were collected and appropriately processed before staining with haematoxylin and eosin. The samples of processed skin were examined microscopically for histopathological changes, if any [32].

Anti acne activity: testosterone-induced acne model

A total of four groups of Swiss Albino mice (male) weighing 20–30 g had three animals in each group. To induce the acne, testosterone (TS) solution (2% ethanolic) was painted to groups 2 to 4 dorsally whereas group 1 acted as control group. Two weeks after acne was induced, AzA formulations (0.1 g per 4 cm^2) were applied once-a-day for 2 weeks continuous. As a control group, group 1 received only normal saline whereas for group 2, no treatment was administered (disease control), while for groups 3 and 4, AzA-EV gel was applied and MKT was used, respectively. There was a careful check for papules or other noticeable changes in the skin. Following treatment, the anti-acne effects of various formulations were determined by measuring decrease in papule density per 4 cm^2 area. The sacrificed animals' skin was harvested after the study concluded. Transverse sections of each specimen were made after fixing it in 10% formalin and embedding it in paraffin. Skin samples were processed and stained with

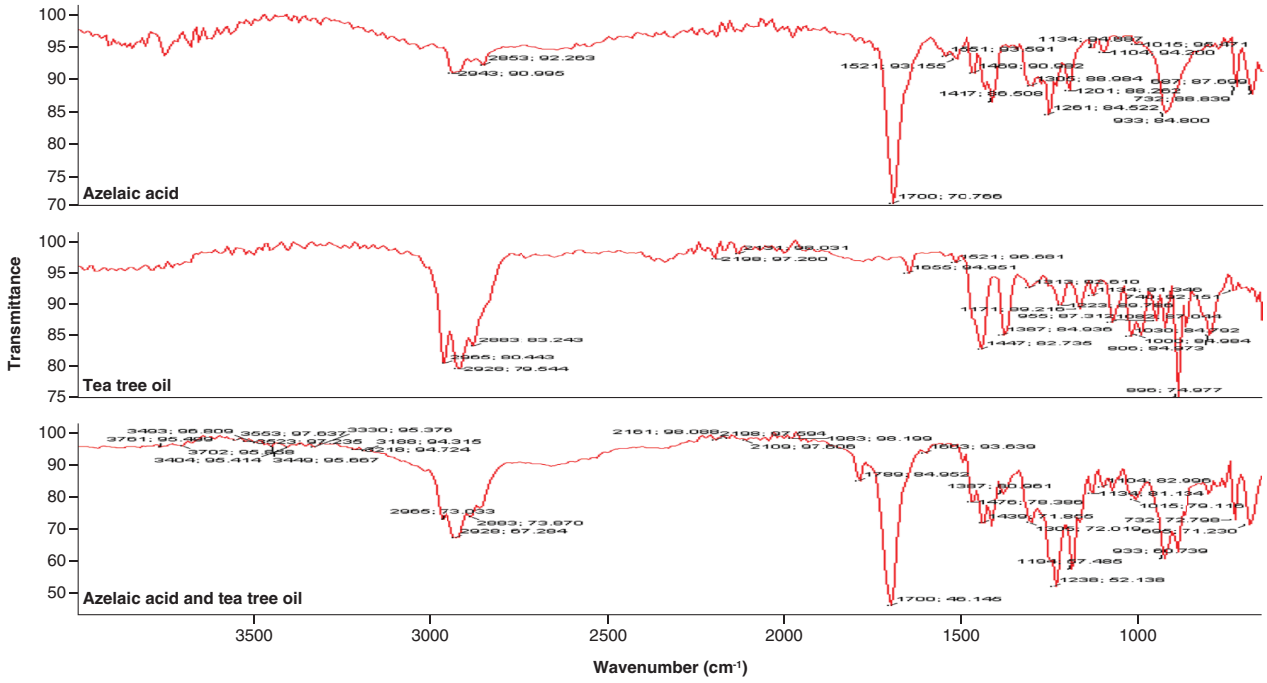

Figure 1. Drug excipients compatibility study. FTIR spectrum of **(A)** azelaic acid, **(B)** tea tree oil and **(C)** azelaic acid and tea tree oil.

haematoxylin and eosin appropriately. Observations were made microscopically for changes in number and size of sebaceous glands in the processed skin samples [41].

Stability studies

One month stability studies were performed for ethosome formulation and hydrogel. Optimized formulations were stored at 4 ± 2°C, 8 ± 2°C and room temperature. Percent drug entrapment was determined at different time intervals (1, 7, 15, 30 days) and noted.

Results & discussions

AzA & EO compatibility studies

In the FTIR of AzA and EO (Figure 1) characteristic bands of AzA and EO were visible (Table 1), indicating no interaction and presence of compatibility between the two compounds (AzA and EO).

Formulation selection & characterization of the EV systems

The various characterization parameters of prepared drug loaded EVs are depicted in Table 2. It was observed that% EE of AzA in EV formulations was high, this could be ascribed to high lipid amount, and solubility of AzA in ethanol, which enabled entrapment of the drug inside vesicles. Varying the PL-90G and ethanol concentration did not influence the% EE appreciably. It is primarily the size of the vesicles that determines the overall effectiveness of a topical drug delivery system. The DLS studies showed that the vesicular size, of the different EV systems, was in nano-metric size range between 312.3 and 789.4 nm. On increasing the concentration of PL from 10 to 20% w/w and ethanol 10 to 30% w/w the vesicle size varied noticeably. At constant PL-90G concentration, in other words, 10%, as ethanol concentration was augmented to 30%, the size of the vesicle augmented considerably. This may be due to high ethanol concentration in the bilayers of the vesicles, which may have reduced the rigidity, and increased the elasticity of the lamellar structure and thus resulted in increased vesicle size. However at 15% PL concentrations, it was noted that increase in ethanol content up to 20% w/w resulted in the decrease in vesicle size, followed by an increase. At higher ethanol concentrations, vesicle size decreases due to the solubilization of phospholipid bilayers. Out of a total of eight combinations, formulations EVO-1, EVO-7 and EVO-8 resulted in formation of clear solution and unstable system, and were devoid of any microscopically visible vesicles. Hence, the

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Table 2. Composition of azelaic acid-tea tree oil-loaded ethanolic vesicle formulations, particle size, polydispersity index and percentage entrapment efficiency of selected ethanolic vesicle formulations.

Sample ID	AzA (mg)	EO (mg)	PL-90G (g)	Ethanol (g)	Water (g)	Particle size (nm)	PDI	%EE
EVO-1	500	500	1	1	7	–	–	–
EVO-2	500	500	1	2	6	399.3	0.821	99.95
EVO-3	500	500	1	3	5	789.4	0.147	99.91
EVO-4	500	500	1.5	3	4.5	447.9	0.595	99.93
EVO-5	500	500	1.5	2	5.5	312.3	0.396	99.86
EVO-6	500	500	1.5	1	6.5	474.5	0.678	99.88
EVO-7	500	500	2	3	4	–	–	–
EVO-8	500	500	1.5	4	3.5	–	–	–

%EE: Percentage entrapment efficiency; AzA: Azelaic acid; EO: Tea tree oil; PDI: Polydispersity index; PL-90G: Phospholipid 90G.

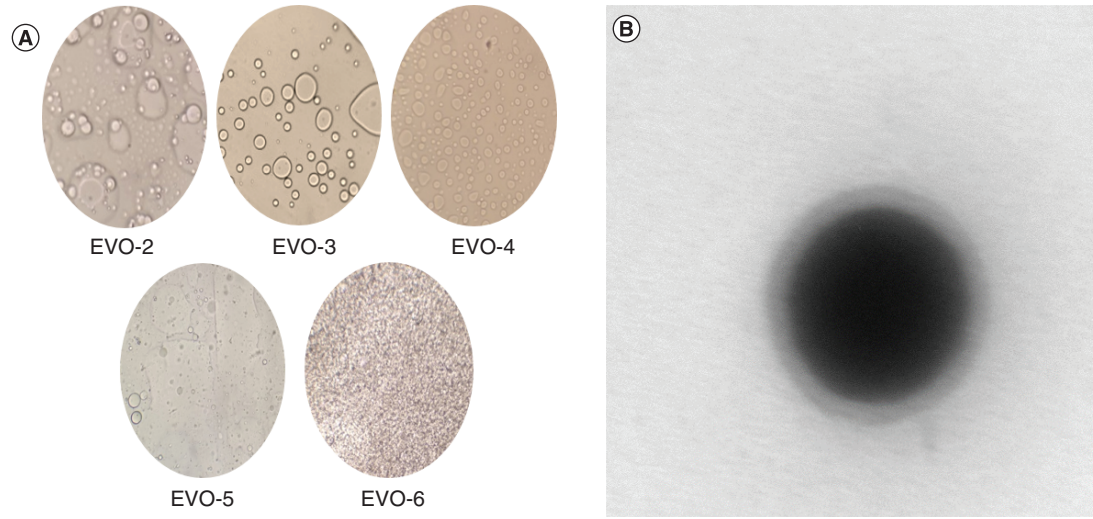

Figure 2. Microscopic evaluation of azelaic acid–ethanolic vesicle formulations. (A) Microphotograph of different azelaic acid entrapped ethanolic vesicle formulations at 100× employing optical microscope **(B)** TEM image of optimized marketed (*Melaleuca alternifolia*) formulation (magnification 200,000×).

concentration ranges of PL and ethanol were selected as 10–15% and 10–30%, respectively, for further studies. The optical microphotographs of different EV formulation are illustrated in Figure 2. All formulations had sphere-like vesicles. The appreciable population of vesicles were without any aggregation or agglomeration, lamellar character of the vesicles could also be visualised. EVO2 and EVO5 exhibited desired size <400 nm and were tested for antibacterial activity, which was found to be 24.6 ± 0.5 mm (EVO2) and 18 ± 1 mm (EVO5). Thus EVO2 was selected for further evaluation as it showed greater zone of inhibition. All the subsequent evaluations were carried out employing this composition of EV. The final EVO2 formulation contained 10% PL and 20% ethanol w/w, with minimum particle size 399.3 nm, polydispersity index = 0.821. Further, the size distribution of AzA-EO co-loaded EVs (Table 2) measured using DLS displayed one narrow peak (Figure 3A), suggesting a relatively homogenous vesicle population. Zeta potential of selected EV dispersion was observed as +1.62 mV (Figure 3B). Even though electrical charge on nanoparticles has insignificant impact on their target potential [42], the positive value of the zeta potential may contribute to the ethosomal system's ability to permeate skin due to its attraction with negatively charged surfaces [43]. Phospholipid 90 G or AzA may have contributed to positive zetapotential value. Low positive zeta-potential values [44] and high positive zeta potential values [45] have been previously reported for ethosomes. The TEM microscopic image (magnification 200,000×) revealed no aggregation. The vesicles were unilamellar, spherical, and nanometric in size, as illustrated in Figure 2B. In order to assess the membrane flexibility of the EVs, the FI was determined using a vesicle extruder (Easter Sci. Inc., MD, USA). This is a critical and specific parameter which enables EVs to be distinguished from conventional vesicular carriers, like liposomes, on the basis

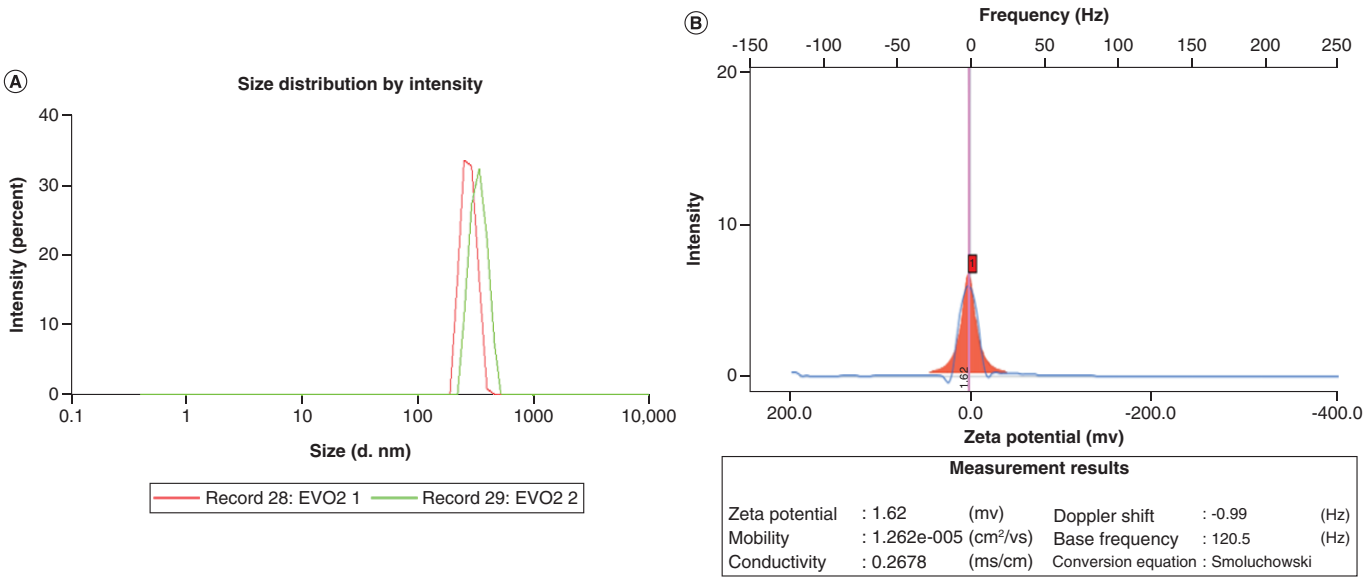

Figure 3. Characterization of optimized azelaic acid–ethanolic vesicles. (A) Particle size distribution of azelaic acid-loaded ethanolic vesicle **(B)** Zeta potential of optimized azelaic acid–ethanolic vesicle dispersion.

Figure 4. Photomicrograph showing the images of azelaic acid and tea tree oil-loaded ethanolic vesicles and ethanolic vesicle gel formulation.

of flexibility and ability to cross the stratum corneum. EVs displayed a FI value of 3.28. This can be explained by the presence of ethanol in the bilayers of ethosomes, which increases their softness and malleability.

Characterization of AzA & EO-loaded EV hydrogels

The AzA-loaded EVs were found to be turbid and white in color (Figure 4). No signs of any kind of phase separation and creaming was observed. The optimized EV hydrogel exhibited smooth feel with no grittiness and tackiness.

Rheological and texture characteristics of optimized AzA-EV gel are shown in Table 3. The rheological profile of the hydrogel, showed moderate viscosity and pseudoplastic behavior, also confirmed by n value less than 1 (Figure 5A). The viscous hydrogel is composed of long-chain polymer molecules that are hooked and entangled at a static or low flow rate. When the shear rate increases, the dispersed polymeric chains are rolled and contracted into a group and hence decrease the apparent viscosity, which leads to the phenomenon of shear thinning [46].

Table 3. Rheological and texture characteristics of azelaic acid and tea tree oil-loaded ethanolic vesicle gel.

Formulation parameter	AzA-EV gel
N	0.332
k (Pa)	17.85
Yield value (Pa)	54.54
Viscosity (Pa-s)	3.43
Firmness (g)	45.572
Work of shear (g-s)	32.483
Stickiness (g)	-37.937
Work of adhesion (g-s)	-13.793

AzA: Azelaic acid; EO: Tea tree oil; N: Power law exponent; k: Consistency index.

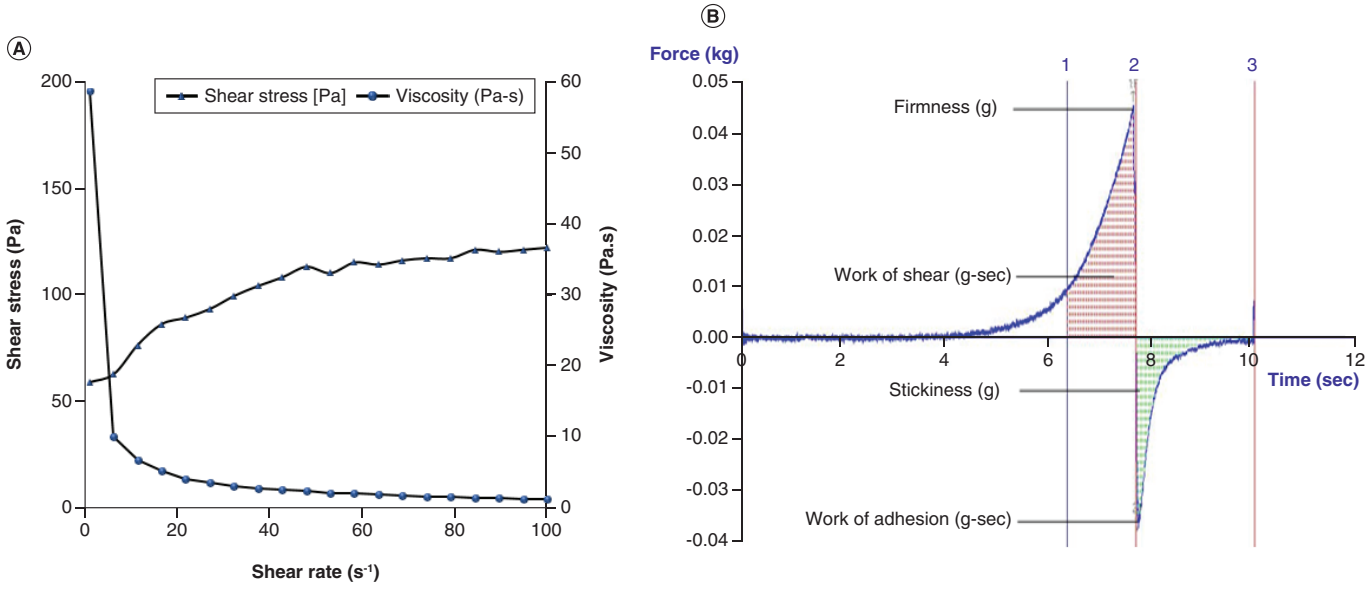

Figure 5. Physical characterization of azelaic acid–ethanolic vesicle gel. (A) For optimal azelaic acid–ethanolic vesicle gel, plots of shear rate versus viscosity and shear rate versus shear stress is shown. **(B)** Presents textural analysis of azelaic acid–ethanolic vesicle gel.

The resulting force-time curve (Figure 5B), textural parameters of cohesiveness (amount of work done during the second compression divided by amount of work done during the first compression), adhesiveness (g*s; negative area of cycle 1 representing work undertaken to remove the compressing probe from the sample), spreadability (work of shear), forward extrusion (firmness), stickiness (area of the negative force curve), were noted.

Gel yield value was moderate (54.54 Pa) which indicates adequate rigidity of the system. Other parameters obtained were good firmness (45.572 g), work of shear (32.483 g-s), work of adhesion (-13.793g-s), and stickiness (-37.937 g). The developed EV hydrogel composite the cohesiveness was adequate, which is necessary to retain the formulation at the application site. These factors predicted the hydrogel's appreciable mechanical strength, easy application, and capability to easily extrude from tubes, characteristics that are essential for its topical application.

In vitro antibacterial activity

Investigation of antibacterial activity of AzA, EO, AzA & EO, EV & EV gel formulations

Table 4 and Figure 6 elucidate diameters of bacterial zones inhibited by multiple test formulations. Ethanol and DMSO, taken as negative control did not show any activity whereas erythromycin employed as +ve control demonstrated excellent antimicrobial activity against the test microorganism. The antibacterial property of AzA was also demonstrated against *S. aureus* and *S. epidermidis*. Surprisingly EO alone did not exhibit any activity against both the test organisms, this could be due to the fact that EO is not affective at such low concentration (50 µg/ml). However in combination with AzA revealed appreciable activity, it may be because the cells become

Table 4. Diameters of bacterial inhibition zones observed using azelaic acid, tea tree oil, azelaic acid and tea tree oil, ethanolic vesicle and ethanolic vesicle gel formulations.

Formulations	Concentration	Volume used	Diameter of inhibition zone, σ ± S.D. (mm); amount of antibacterial tested (mg/ml)	
			ATCC 29213 (*S. aureus*)	MTCC 3382 (*S. epidermidis*)
AzA	50 mg/Ml	80 µl	21.3 ± 0.5	20.6 ± 1.1
EO	50 mg/Ml	80 µl	N D	N D
AzA with EO	50 mg/ml + 50 mg/ml	80 µl	15.3 ± 1.5	16 ± 1
EVO-2	50 mg/ml	80 µl	21.3 ± 1.1	22.3 ± 0.5
EVO-2 gel	50 mg/ml	80 µl	24.6 ± 0.5	23.6 ± 1.5
Erythromycin (+ve)	1 mg/ml	10 µl	23 ± 1.0	29 ± 1.0
Ethanol (-ve)	–	80 µl	ND	ND
DMSO (-ve)	–	80 µl	ND	ND

AzA: Azelaic acid; EO: Tea tree oil; ND: No zone of inhibition detected; the zone of inhibition values are an average from two independent experiments.

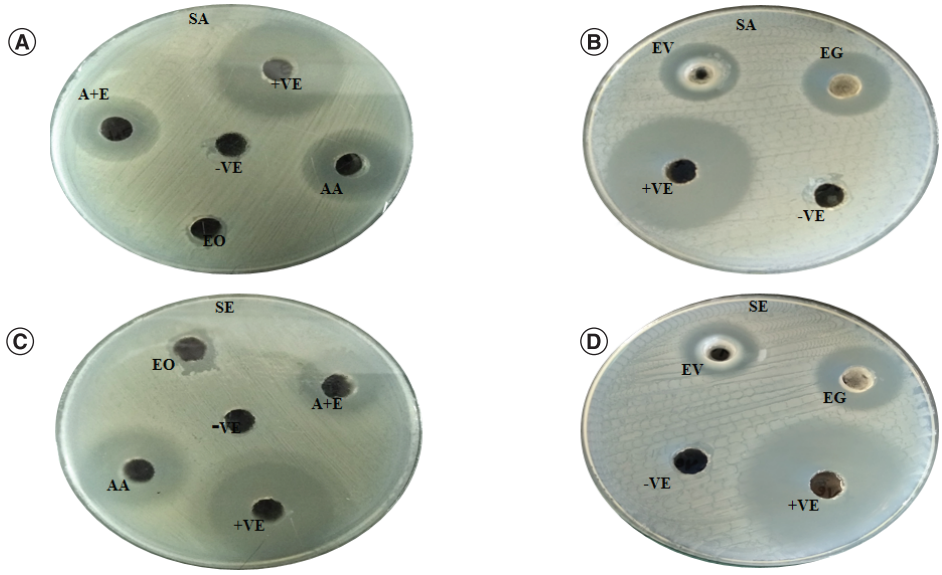

Figure 6. Antimicrobial activity of azelaic acid, tea tree oil, ethanolic vesicle and ethanolic vesicle gel formulations against *S. aureus* and *S. epidermidis*. (A) (SA) AA 50 mg/ml in ethanol, EO 50 mg/ml in ethanol, A + E 50 mg/ml + 50 mg/ml, ethanol (-ve) and +ve 1 mg/ml. **(B)** SA EV – 50 mg/ml, EG – 50 mg/ml in DMSO (-ve) and +ve (1 mg/ml). **(C)** SE AA 50 mg/ml in ethanol, EO 50 mg/ml in ethanol, A + E 50 mg/ml + 50 mg/ml in ethanol, ethanol (-ve) and +ve (1 mg/ml). **(D)** SE EV 50 mg/ml, EG 50 mg/ml in DMSO (-ve) and +ve (1 mg/ml). +ve: Erythromycin; AA: Azelaic acid; A = E: azelaic acid + essential oil; EG: Ethanolic vesicle gel; EO: Essential oil; EV; Ethanolic vesicle; SA: *S. aureus*: SE: *S. epidermidis*.

more susceptible to the bactericidal effects of EO, owing to the keratolytic effect of AzA. On one hand, while the activity of AzA and EO loaded EV was comparable to AzA against *S. aureus*. It was significantly better in case of *S. epidermidis*. Further AzA and EO loaded EV gel demonstrated vis-a-vis all the formulations highest zone of inhibition (≈23 ± 1.0 mm) against both the tested organisms. The antibacterial effects of AzA have been well documented, making AzA a useful material in the treatment of acne and inflammatory skin diseases [47]. In all the microorganisms tested, EV exhibited antibacterial activity by interacting with the microbial cellular membrane or cell wall [48].

Minimum inhibitory concentration of AzA, EO, AzA & EO combination, EV & EV gel formulations against different bacterial strains

The order of minimum inhibitory concentration (MIC) values (Table 4) against *S. aureus* was observed to be as follows: EO ≈ EV hydrogel (3.12 mg/ml)> AzA ≈ AzA with EO (0.76 mg/ml) > EV (0.09 mg/ml) > Erythromycin

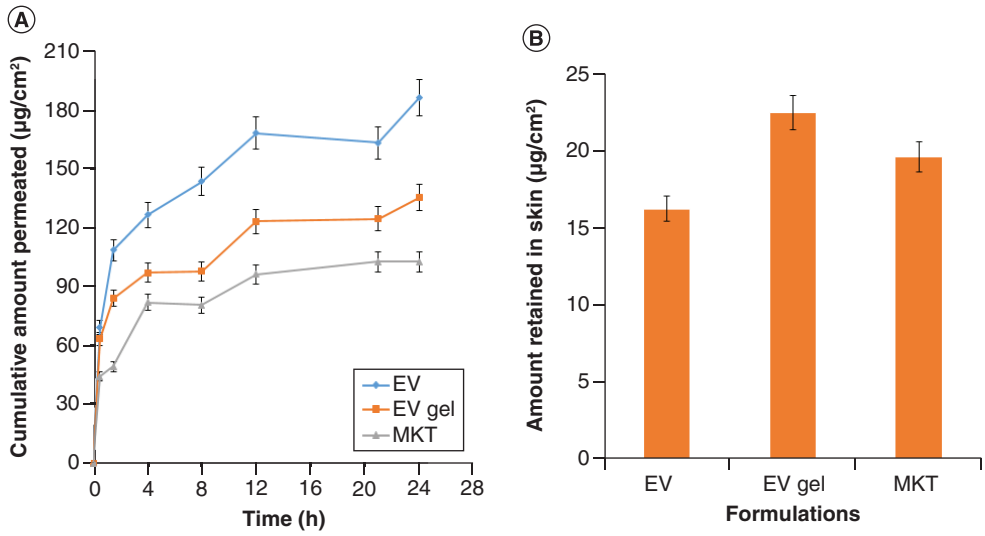

Figure 7. Skin permeation and retention studies of azelaic acid–ethanolic vesicle gel. (A) A comparative representation of *ex vivo* skin permeation profiles of AzA from EVs, EV gels and MKT. **(B)** Plot showing the skin retention values of EV, EV gel and MKT.
AzA: Azelaic acid; EV: Ethanolic vesicle; MKT: Marketed formulation.

(0.02 mg/ml). For *S. epidermidis* MIC values were found to be as follows: EO (6.25 mg/ml) >*EV hydrogel* (1.56 mg/ml) >AzA≈AzA with EO ≈ EV (0.78 mg/ml) >Erythromycin (0.02 mg/ml). However, EO alone does not have a significant antibacterial effect, but AzA loaded EO EV demonstrated significantly lower MICs when compared with EO alone. It is believed that the superior efficacy of EV hydrogel may be attributed to the synergistic effects of the AzA and EO, as well as improved contact with the bacterial cell wall, resulting in an increased contact time and sustained delivery [11].

Ex vivo skin permeation studies

The cumulative amount of AzA, permeated and penetrated (conveyed as cumulative amount permeated (µg/cm^2)) after 24 h, is depicted in Figure 7A. It observed the following order: EV (186 µg/cm^2) >EV hydrogel (135.13 µg/cm^2) >Aziderm® (102.49 µg/cm^2). Ethanol due to its penetration enhancement effect imparts flexibility to EV and hence is responsible for maximum penetration observed in the studies. EVs exert pull and push effects on the intercellular interface of the stratum corneum cells and enhance drug penetration [49]. Push effect is a thermodynamic effect of ethanol evaporation, and pull effect is a result of ethanol fluidizing SC lipids. Additionally in preparation of the EV formulations, the PL-90G and ethanol interact synergistically to increase penetration. However, addition of Carbopol 934 into EV (AzA-EV gel) decreases the permeability of AzA. This could be due to the higher viscosity of hydrogel than that of EV, hindering mobility and preventing permeation of drugs [50].

Ex vivo skin retention studies

Figure 7B illustrates how much AzA was retained in the skin with different formulations. The skin retention observed the following order: EV hydrogel (22.51 µg/cm^2) >Aziderm® (19.64 µg/cm^2) >EV (16.25 µg/cm^2). In comparison to marketed formulations, higher retention can be attributed to the ability of EV hydrogel to create a reservoir within skin layers more efficiently and also to more close contact between the drug and skin lipids thereby increasing drug retention.

In vivo skin compliance study

The images in Figure 8 show skin surface of a Wistar rat exposed to normal saline (Control), EV gel, and MKT gel. Compared with the control-treated group (Figure 8A), the EV-treated group showed little change in the skin histology, however no changes in the dermis were noticed. It was apparent from the histology of the skin sections treated with MKT formulations that there were no marked alterations to the normal tissue, which validates the formulations biocompatibility. Upon application of EV and MKT formulation gel, the SC layer of rat skin

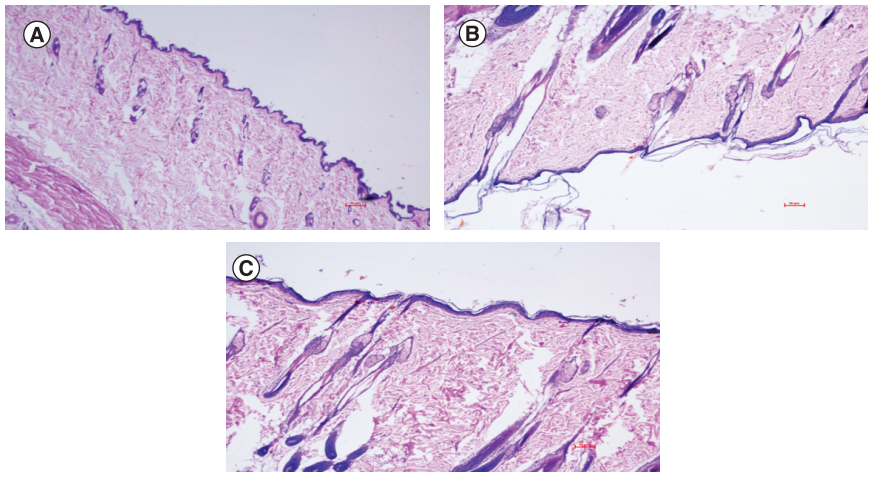

Figure 8. Histological images of rat skin with and without treatment. Photographed sections of skin histology treated with **(A)** control **(B)**, ethanolic vesicles and **(C)** marketed formulations, respectively.

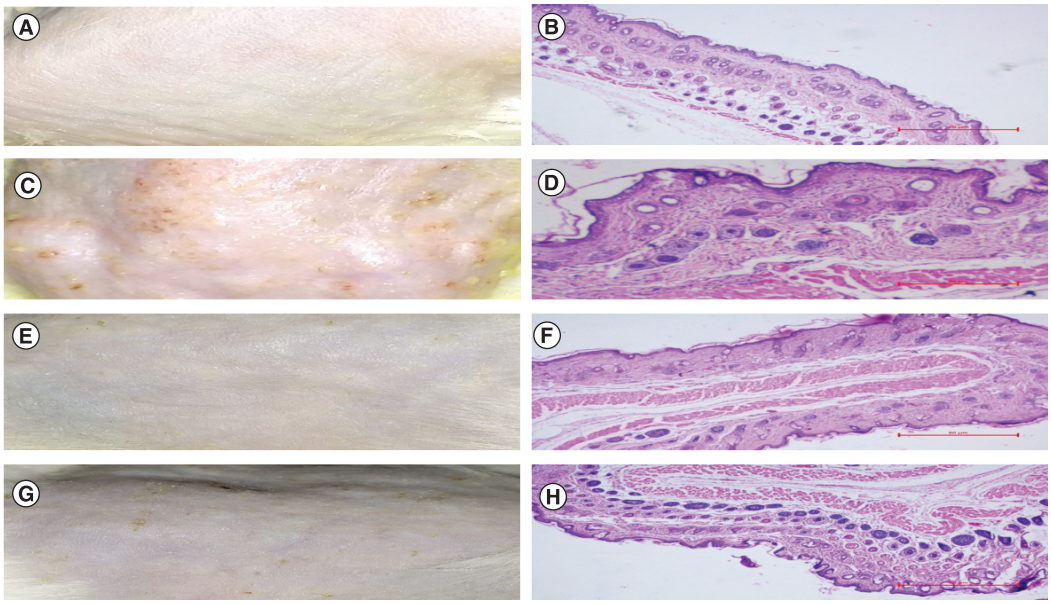

Figure 9. A review of the anti-acne potential of co-loaded azelaic acid–tea tree oil formulations with the testosterone acne model. Presented here are photographs and histology of mouse skin representing **(A & B)** control; **(C & D)** disease control (untreated), **(E & F)** ethanolic vesicle gel and **(G & H)** marketed gel.

became thinner, without an actual change in epidermis or dermis (Figure 8B & C). Encasement of the drug within the biocompatible components *viz.*, phospholipid and surfactant helps with safe delivery of drug. Additionally appending carbopol® 934 in the formulation, causes increased viscosity and development of three-dimensional network structure minimizing the possibility of AzA directly contacting the skin, thus reducing any side effects [51]. Moreover the skin treated with EV hydrogel (Figure 8B) and MKT gel (Figure 8C) shows no sign of inflammation cells attesting to better tolerability of the EV-based formulation. Therefore, the topical drugs delivered via EV systems can be believed as safe.

Anti acne efficacy: testosterone induced acne model

Anti-acne potential of EV gel formulation was compared with MKT gel and untreated group; results are shown in Figure 9. Visual evaluation of animal skin (Figure 9C) showed that TS application caused animals to develop severe acne after 2 weeks and that the acne was alleviated in the animals after treatment with AzA formulations

Table 5. Minimum inhibitory concentration values for azelaic acid, tea tree oil, azelaic acid and tea tree oil combination, ethanolic vesicles and gel formulation against different test organisms.

Formulations	MIC (mg/ml)		
	ATCC 29213 (*S. aureus*)	MTCC 3382 (*S. epidermidis*)	MTCC1951 (*P. acnes*)
AzA	0.78	0.78	3.12
EO	3.12	6.25	6.25
AzA with EO	0.78	0.78	1.56
EV	0.09	0.78	1.56
EV hydrogel	3.12	1.56	0.78
Erythromycin (+ve)	0.02	0.02	–

AzA: Azelaic acid; EO: Tea tree oil; EV: Ethanolic vesicle; MIC: Minimum inhibitory concentration.

Table 6. Stability studies of ethosomal suspension and ethanolic vesicle gel.

Time interval (days)	Entrapment efficiency (%)					
	Ethosomal suspension			Ethanolic vesicle gel		
	4 ± 2°C	8 ± 2°C	Room temperature	4 ± 2°C	8 ± 2°C	Room temperature
1	99.95%	99.95%	99.95%	98.77%	98.77%	98.77%
7	99.87%	99.75%	98.80%	98.62%	98.58%	98.45%
15	99.85%	98.90%	97.68%	98.68%	98.54%	98.40%
30	99.68%	98.66%	97.31%	98.58%	98.43%	98.35%

(Figure 9E & G). Macroscopic changes (visual) in skin and histopathology of animal clearly illustrated superiority of AzA-EO co-loaded EV gel compared with MKT gel. In terms of mean percentage reductions in papule density, following order was observed: MKT gel (63.12 ± 2.2%) <EV Hydrogel (78.80 ± 3.0%). In addition, the EV gel treated group showed no signs of skin peeling and desquamation (indicative of toxicity), reflecting superior tolerability and reduced toxicity compared with marketed MKT gel. In the histological evaluation, sebaceous hyperplasia (i.e., increased number and size of sebaceous gland cells) and follicular hyperkeratosis in pilosbaceous unit (Figure 9D) were observed, which are key indicators of acne development. In contrast, formulation-treated animals showed a significant decrease of lesions, sebaceous gland hyperplasia, and seborrhea (Figure 9F) [41]. AzA's sustained release, its superior skin penetration, combined with its synergistic effect with EO's, could explain the superior therapeutic efficacy and tolerability of EV hydrogel in comparison to MKT formulation.

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Stability studies

Ethosomal formulation showed 2% leakage of drug (from fluidized bilayer of the vesicle) at room temperature. Moreover, hydrogel formulation, remained practically stable at all temperatures (Table 6).

Conclusion

EV and EV hydrogel formulations containing AzA and EO were successfully formulated and tested for efficacy and safety *in vitro*, *ex vivo*, and *in vivo*. EV hydrogel formulations prevents direct contact of skin with the drug, reduce the risk of adverse reactions, and have superior skin permeation and retention characteristics in comparison to commercial formulations. However, EV hydrogel systems showed comparable antibacterial efficacy *in vitro* with the commercial formulation, and showed good zone of inhibition and low MIC values against *S. aureus, S. epidermidis*. In view of the dose-dependent side effects of AzA, the findings are of considerable importance. Additional clinical studies will be needed to be performed to assess the potential of AzA and EO combination in the form of EV hydrogel.

Future perspective

Ethosome hydrogel composite was better tolerated with no side effects. Thus, developed ethosome hydrogel composite of AzA and EO, promises as an efficient and safe drug delivery system for the topical therapy of acne vulgaris.

Summary points

- The optimized AzA-EO ethosome was spherical, lamellar without any aggregation of uniform size (polydispersity index = 0.821), nano-range (~399 nm),% entrapment (~99%) and positive zeta potential (1.62 mV) values.
- *In vitro* antibacterial efficacy in microbial strains such as *Staphylococcus aureus*, Staphylococcus epidermidis was evaluated employing agar well plate diffusion and broth dilution assay. Ethosome exhibited appreciable zone of inhibition (agar plate) and low minimum inhibitory concentration (MIC) (broth dilution) against *S. aureus, S. epidermidis* vis-a-vis AzA.
- In testosterone induced acne model in rats, optimized ethosome exhibited better reduction in the papule density (93.75 ± 1.64%) in comparison to AzidermTM (72.69 ± 4.67%).

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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