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ORIGINAL ARTICLE



Topical creams of piperine loaded lipid nanocarriers for management of atopic dermatitis: development, characterization, and *in vivo* investigation using BALB/c mice model

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ABSTRACT

The aim of the research work was to investigate the efficacy of cream loaded with lipid nanocarriers (ethosomes) of piperine for the management of atopic dermatitis (AD) in comparison to conventional cream. Ethosomes of piperine were formulated with varying concentration of phosphatidylcholine and ethanol; and evaluated for entrapment efficiency (EE), sedimentation behaviour, vesicle size, zeta potential, *in vitro* drug release, and shape. Creams loaded with optimized ethosomal dispersion of piperine were formulated and evaluated for physicochemical parameters, *ex vivo* permeation and drug retention in skin layers. Similarly, conventional creams of piperine in the same concentrations were formulated and evaluated. The optimized ethosomal a conventional cream was evaluated for cytotoxicity using HaCat cell lines and *in vivo* on BALB/c mice model. The EE (%) and vesicle size was $74.30 \pm 3.88\%$ and 318.1 nm, respectively, for optimized ethosomal dispersion. The zeta potential was -32.6 mV and vesicles were spherical in shape. The ethosomal cream showed higher deposition in the epidermis and dermis. The creams were non-cytotoxic to HaCat cell lines. In comparison to the negative control, the ethosomal (0.1%) and conventional (0.125%) cream, both significantly decreased the ear and skin thickness, skin severity; and WBC, granulocytes, and IgE antibodies level in the BALB/c mice model. The efficacy of ethosomal cream was significantly higher than conventional cream as compared to tacrolimus (0.1%). Ethosomal cream of piperine showed good potential for the management of AD in comparison to conventional cream.

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Atopic dermatitis; piperine; ethosomes; BALB/c mice; immunoglobulin E

Introduction

Atopic dermatitis (AD) is a condition with a prolonged reverting highly pruritic inflammatory skin condition (Leung and Bieber 2003, Novak *et al.* 2003, Kumar, Ashawat, *et al.* 2020). Atopy can be described as the hereditary or discrete tendency of sensitization and production of IgE antibodies against environmental stimulators (Schmid-Grendelmeier *et al.* 2001). As per a widely accepted concept, the pathophysiology of AD can be explained by comparative levels of T helper (Th)1/Th2/Th17 cells (Van Bever 2002). Generally AD results due to the relative disturbance in the balance of Th1/Th2 cells, with overall dominance of Th2 cells (Mutius 2000). AD is linked with elevated IgE antibodies in serum and amplified infiltration of immune cells, that is, eosinophils, mast cells, basophils, innate lymphocytes, different types of dendritic cells, and myeloid-derived suppressor cells, B cells, and T cells (Kay 2001).

Corticosteroids have been the mainstay for the long term management of AD. But, its prolonged use can induce severe local and systemic side effects such as skin atrophy, perioral dermatitis, suppression of adrenal function, and Cushing's

syndrome. The calcineurin inhibitors, that is, tacrolimus and pimecrolimus also reported having adverse effects such as nephrotoxicity, neurotoxicity, the disturbed barrier function of skin and skin cancers (Papier and Strowd 2018, Kumar, Ashawat, *et al.* 2020). Nevertheless, the newer options such as biological agents have shown good potential in AD treatment but certain adverse effects such as tumour at the site of injection, infections, and psoriasis-type lesions are generally seen after administration (Simpson *et al.* 2016).

In the traditional system of medicine, that is, Chinese, Korean, Japanese, and Iranian several phytoconstituents or a mixture of phytoconstituents have been reported for the treatment of AD (Kumar, Sharma, *et al.* 2020). A common possible mechanism of action of herbal medicines is by regulating the expression of Th1 and Th2 cell, thus reducing the levels of IgE and eosinophils with improved lesions in atopic skin. Piperine a major alkaloid of black pepper (*piper nigrum*) and long pepper (*piper longum*) has been used traditionally in folk medicines for the treatment of seizures, asthma, and chronic bronchitis. The different pharmacological investigations have reported the anti-inflammatory activity for piperine (Leila *et al.* 2017). Piperine given orally showed inhibitory

action on eosinophils, IgE antibodies, and Th2 cytokines in allergic skin conditions (Jung *et al.* 2015). Forty-seven to sixty-four percent of piperine given orally is subjected to the first-pass metabolism (Bhat and Chandrasekhara 1986). Further, an oral dose of piperine 10–20 mg/Kg body weight for 30 days was reported to have toxic effects on the reproductive system and eye irritation (Priprem *et al.* 2011, Tasleem *et al.* 2014).

The topical delivery system of piperine can have several advantages as compared to other routes of administration in AD viz. local action, improved bioavailability, less dose, and improved patient compliance due to elimination of side effects associated with conventional therapy.

Ethosomes are the phospholipid based flexible and elastic nanocarriers with ethanolic core (20–45% of ethanol) that could infiltrate stratum corneum and supply the drugs locally and deeply in the skin (Touitou *et al.* 2000). Ethanol interacts with the polar heads of phospholipid molecules causing a decrease in the melting point of stratum corneum lipid thereby increased fluidity and permeability. Further, ethanol improves the elasticity and flexibility of vesicles letting them easily pass through the pores much smaller than their own size (Touitou *et al.* 2001, Akhtar *et al.* 2018).

As per the literature reviewed, no work has been reported regarding the topical use of piperine for the treatment of AD. So, in the present research work, ethosomes of piperine were developed, characterized and optimized dispersion was loaded in a base cream. Simultaneously, conventional creams of piperine were also developed in concentrations of piperine similar to ethosomal creams. The creams were characterized for *in vitro* parameters and optimized topical creams were investigated *in vivo* using 2,4-dinitrochlorobenzene (DNCB) induced AD-like skin lesions in BALB/c female mice model. BALB/c mice are the most sensitive immune responder strains and are frequently used in immunological investigations because of their immunocompromised nature as compared to the original albino strain (Kumar, Sharma, *et al.* 2020).

Materials and methods

Materials

Piperine was purchased from Yucca enterprises, Mumbai, India. Phosphatidylcholine (PCH) was purchased from HiMedia, Mumbai, India. IgE antibody (Thermofischer) kit was purchased from Deep distributors, Palampur, India. All other chemicals used were of analytical grades.

Ethics declaration

All the animal experiments were conducted in full compliance with the Institutional ethical and regulatory principles after obtaining approval by the Institutional Animal Ethical Committee of Laureate Institute of Pharmacy, Kathog, H.P, India. (Protocol number CPCSEA/LIPH/2018/06).

Methods

Estimation of piperine

Piperine was quantified by the high-performance liquid chromatography (HPLC) assay method previously reported (Motta *et al.* 2018) using methanol:water (70:30) as mobile phase pumped at 1 ml/min (UPLC Shimadzu, Japan). A 20- μ l injection was eluted in microsorb 5 μ m RP C18 column (4.6 $\times$ 150 mm) at room temperature. The column eluent was monitored at 342 nm using a UV detector (SPDA20). The retention time of piperine was 9.8 min.

Compatibility study with excipients

The compatibility of piperine with excipients was investigated using the TLC technique. A semisolid physical mixture (SPM) of piperine (0.1%w/w) was prepared by phase inversion method using cetyl alcohol, stearic acid, propylene glycol, glycerine, tween 80, span 20, methylparaben, propylparaben, PCH, and ethanol. SPM equivalent to 50 mg of piperine was dissolved in 50 ml of methanol in a volumetric flask and filtered. The filtrate (1 mg/ml) was spotted on a pre-coated TLC plate (Silica GF₂₅₄) against a methanolic solution (1 mg/ml) of pure piperine. Further, the chromatogram was developed in a chromatographic chamber saturated with toluene:ethyl acetate in 70:3 ratio. After sufficient development, the plate was removed from the chamber; the solvent front was marked and observed in a UV cabinet at 254 nm. The R_f values of piperine were compared to determine the compatibility with different excipients.

Formulation and evaluation of ethosomes of piperine

Formulation of ethosomes. The ethosomes of piperine were formulated by the cold method (Touitou *et al.* 2000). The PCH (2–4%) and piperine were dissolved in ethanol (20–40%) maintained at 30 $\pm$ 1 $^{\circ}$ C using a high-speed stirrer (Remi, Mumbai, India) (Table 1). Double distilled water, preheated to 30 $\pm$ 1 $^{\circ}$ C was added at a constant rate of 1 ml/min. Mixing was continued for an additional 5 min and the system was allowed to attain room temperature. The dispersion was sonicated at 4 $^{\circ}$ C for three cycles of 5 min with a rest period of 5 min between cycles using a probe sonicator (Hicon Enterprises, Bengaluru, India).

Evaluation of ethosomes. Entrapment efficiency. The percent entrapment efficiency (EE) was determined by ultracentrifugation method. Ethosomes (1.0 ml) was centrifuged at 12 000 rpm at 4 $^{\circ}$ C for 45 min in a cooling centrifuge (CM 12, Remi, Mumbai, India). The supernatant was separated and analysed by HPLC. Further, sediment was lysed with 1 ml of Triton X100 (0.1%w/v), filtered using 0.45 μ nylon filter (Millipore) and the filtrate was quantitatively analyzed by HPLC. The EE (%) was calculated using the formula:

$$EE (\%) = \left[\left(\frac{Q_s}{Q_t} \right) \times 100 \right] \quad (1)$$

where, Q_t = Total amount of drug added initially Q_s = Amount of drug in the sediment

Table 1. Formulation of ethosomes of piperine.

S.No.	Formulation code	Piperine (%w/v)	PCH (%w/v)	Ethanol (%w/v)	Double distilled water (%w/v)	RPM
1	PE1	0.1	2	20	Q.S to 100%	800
2	PE2	0.1	2	30		
3	PE3	0.1	2	40		
4	PE4	0.1	3	20		
5	PE5	0.1	3	30		
6	PE6	0.1	3	40		
7	PE7	0.1	4	20		
8	PE8	0.1	4	30		
9	PE9	0.1	4	40		

Sedimentation behaviour. The ethosomal dispersions were kept in a refrigerator and observed for sedimentation and nature of sediment at a regular interval of 5 days for 30 days. The sedimentation rate results were reported on the basis of the bottom view of the beakers and redispersion of sediment (Kamboj *et al.* 2014).

Vesicle size distribution and Zeta potential. Vesicle size distribution was determined by differential light scattering technique using Malvern Zetasizer Ver 7.10 (Malvern instruments, Malvern, UK) for formulations with high EE (%) and slow rate of sedimentation (PE1–PE6). The zeta potential of the ethosomal dispersion was determined by electrophoretic mobility (Horiba Scientific Nanoparticle Analyser, SZ 100, Japan).

In vitro drug release. The *in vitro* drug release was determined by placing ethosomal dispersion (1 ml) in dialysis membrane tubing (molecular weight cut off 12 000, HiMedia, Mumbai), both the ends of which were sealed and placed in a beaker containing 100 ml of pH 5.5 phosphate buffer maintained at $37 \pm 1^\circ\text{C}$ (Chourasia *et al.* 2011). The buffer was stirred with a glass rod at 45 min intervals and samples were collected at 2, 4, 6, 8, 10, 12, 18, and 24 h time intervals, replaced with an equal quantity of fresh buffer and analyzed for the drug released using HPLC. The ethosomal dispersions were evaluated in comparison to a hydro-ethanolic (HE) solution of the drug. The optimized formulation was assessed for zero order, first order, Higuchi and Peppas release kinetics; and the mechanism of drug release was determined using the value of diffusional release exponent (n) obtained from Peppas exponential equation (Siepmann and Peppas 2001, Kumar *et al.* 2017).

Visualization of vesicles. The selected ethosomal vesicles were visualised using a transmission electron microscope (FEI, Tecnai G2 20, USA) using 1% phosphotungstic acid as a negative stain (Abdel Messih *et al.* 2017).

Formulation and evaluation of cream loaded with ethosomes of piperine

Formulation of cream loaded with ethosomes of piperine. Ethosomal creams were formulated by adding optimized ethosomes (PE6) equivalent to 0.05% (PEC1), 0.075% (PEC2), 0.1% (PEC3), and 0.125% (PEC4) of piperine in a previously optimized base cream formulated by phase inversion method. The oil phase was prepared by melting cetyl alcohol (2.5%w/w), stearic acid (4%w/w), tween 80 (0.5%w/w) and span 20 (1.5%w/w) at 70°C . The aqueous phase containing propylene glycol (5%w/w), glycerine (5%w/w), methylparaben (0.1%w/w), propylparaben (0.05%w/w) and distilled

water (quantity sufficient upto 100%w/w) was also maintained at 70°C in another beaker. The aqueous phase was mixed with the oil phase with continuous stirring to get a smooth cream. Ethosomes were added carefully when the temperature of the base cream was reduced to 30°C to withstand the temperature (Kaur and Saraf 2011).

Evaluation of cream loaded with ethosomes of piperine.

Psychometric evaluation. The creams were subjected to psychometric evaluation, that is, colour, smoothness, and grittiness.

Determination of pH. Cream ($5 \text{ g} \pm 0.1 \text{ g}$) was added to 45 ml of distilled water in a beaker and heated to 45°C with constant stirring for 15 min on a heating mantle. The solution was filtered and pH was measured using a digital pH metre (Ashawat *et al.* 2008).

Thermal stability. Cream (1 g) was spread on the inner wall of a 100 ml beaker and placed for 48 h in a stability chamber (Thermolab scientific, Mumbai, India) maintained at $45 \pm 1^\circ\text{C}$ and 60–70%RH. After 48 h, the surface was observed for separation of oil phase (Ashawat *et al.* 2008).

Spreadability. Cream (1 g) was placed between two glass plates and pressed to obtain a film of uniform thickness by placing 100 g weight for 5 min. A weight (M) of 50 g was added to the pan and the top plate was pulled with the help of string attached to the pan. The time (T) required for the upper plate to move over the lower plate to cover a distance (L) of 10 cm was noted. The spreadability (S) was calculated using the formula:

$$S = (M \times L)/T \quad (2)$$

The spreadability values of the formulations were compared with a marketed cream Acivir (CIPLA Ltd, Sikkim, India).

Determination of viscosity. Ten-gram sample was taken, placed in covet of LV-1 spindle (Brookfield viscometer DVII) maintained at $25 \pm 1^\circ\text{C}$ and spindle was rotated at 10 rpm.

Determination of acid value and saponification value. The acid value and saponification value was determined as per the method reported in Indian Pharmacopoeia (Ministry of Health and Family Welfare, Government of India 1996).

Determination of drug content. Cream (1 g) was taken in a 100 ml volumetric flask and diluted with ethanol. The flask was kept for 24 h with occasional shaking for complete extraction of the drug. The solution was filtered and the filtrate was analyzed by HPLC.

Formulation and evaluation of conventional piperine cream

Formulation of conventional piperine creams. The different compositions of piperine cream; 0.05% (PC1), 0.075% (PC2), 0.1% (PC3), and 0.125% (PC4) were formulated using a base cream prepared by phase inversion method as similarly in ethosomal creams. Piperine was added during the stirring at 30 °C by dissolving in 0.05 ml of ethanol.

Evaluation of conventional piperine creams. The creams were subjected for psychometric evaluation, pH, thermal stability, spreadability, viscosity, acid value, saponification value, and drug content as per the methods reported previously.

Ex-vivo skin permeation and drug retention study of creams in skin layers

Ex-vivo skin permeation study was performed through dorsal skin of BALB/c mice mounted on glass Franz diffusion cell (Shree Sai Lab Technology, Hoshiarpur, India) assembly (effective surface area of 1.0 cm²) (Verma and Pathak 2012). The dorsal hairs of the mice were trimmed (<2 mm) with an electric razor, and skin was carefully separated from underlying connective tissues with the help of forceps and a scalpel. The skin was washed and mounted on the diffusion cell assembly, keeping the stratum corneum towards the donor compartment and floated on receptor medium for 12 h for equilibration and hydration. 100 mg of the test sample was placed in the donor compartment. The receptor compartment consisted of 10 ml of pH 5.5 phosphate buffer, maintained at 32 ± 0.2 °C under stirring at 10 rpm. The samples were withdrawn at regular time intervals (0.25, 0.5, 1, 2, 3, 4, 5, 6, and 7 h) and replaced immediately with equal volume of fresh receptor medium. The samples were analyzed by HPLC and percent cumulative drug permeation was determined.

After the permeation study, the diffusion cell was dismounted after 24 h; the skin was removed and washed with saline. After washing, the piperine deposited on the skin surface was stripped by a pressure-sensitive adhesive tape. The piperine in the tape represents the deposition in the stratum corneum (Jia *et al.* 2017). Then the skin was chopped using a sharp surgical blade to extract the amount of piperine deposited in the epidermis and dermis. The adhesive tape and chopped skin were soaked separately in 10 ml of ethanol in a volumetric flask. The flasks were kept overnight and then sonicated for complete drug extraction (Kaur *et al.* 2018). The solutions were filtered and filtrates were analyzed by HPLC to estimate the retention (%) of piperine in different skin layers.

In vitro cytotoxicity study on human skin cell lines

In vitro cytotoxicity study of the optimized formulations (PC4 and PEC3) was performed against human keratinocytes HaCat cell line (procured from National centre for cell sciences, Pune, India) by MTT assay (Mitjans and Vinardell 2008). Cell suspension (0.1 ml) containing 10 000 cells was seeded to each well of a 96 well microtiter plate (Nunc and Tarson,

Mumbai, India), and test compositions in the range of 1000 µg/ml to 7.5 µg/ml was added after 24 h seeding. Control cells were incubated with DMSO (solvent). The cellular viability was determined from the treated culture of four wells of each concentration. The absorbance was measured using a microplate reader (Biotek, USA) at 540 nm (Kumar and Kulkarni 2013). The growth inhibition (%) was calculated using the standard formula and the concentration of test substances needed for inhibition (%) of cell growth by 50% (CTC₅₀) values was generated from the dose-response curve. Morphological changes were examined using an inverted microscope (Motic, China).

In vivo evaluation of topical creams

Animals. Female BALB/c mice, 6–8 weeks old were purchased from the animal house facility of CSIR IHBT, Palampur (Himachal Pradesh, India). The mice were housed and used in accordance with the approved protocol.

Induction of atopic dermatitis. After one week of rehabilitation, the mice were divided into five groups ($n=5$ per group): (1) Control (2) Negative control (NC) (DNCB treated group) (3) Positive control (PC) [DNCB + Tacrolimus (0.1%)] (4) Treatment group 1 (T1) [DNCB + conventional cream (0.125%)] (5) Treatment group 2 (T2) (DNCB + ethosomal cream (0.1%)). The dorsal hair was trimmed (~4 cm²) using an electric razor and the remaining hairs were removed using a hair removal cream. 200 µl and 10 µl of 2% DNCB solution (in a 3:1 mixture of acetone and olive oil) were painted on dorsal skin and back of ears, respectively in NC, PC, T1, and T2. After first sensitization, mice were housed without any further treatment for 4 days. The secondary induction (from 5th day) was done by painting 200 µl and 10 µl of 0.5% DNCB solution on dorsal surface and back of ears, respectively once in every 2 days for 21 days (Lin *et al.* 2017, Ku *et al.* 2018). From the 5th day, the PC, T1, and T2 were topically treated on the dorsal surface and ears once daily with 0.5 g of tacrolimus (0.1%), PC4, and PEC3, respectively.

Measurement of ear and dorsal skin thickness. Ear thickness and dorsal skin thickness were measured at 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21st day (before sacrifice) using a digital vernier calliper (Mitutoya, Japan).

Determination of clinical score. Clinical symptoms of mice in each group were examined at 1, 5, 10, 15, and 21st day (before sacrifice) for the degree of severity of erythema, edema, excoriation, and dryness of the dorsal skin surface and was scored as 0 (not visible), 1 (mild), 2 (moderate) and 3 (severe), accordingly. Scoring was performed by three independent observers and the final score was considered as an average of three (Funakushi *et al.* 2011).

Histological examination. Excised dorsal skin and ear pinna of each group were fixed in formalin (10%) solution for 24 h and embedded in paraffin wax. The tissues were cut in 6 µm

thickness and stained with haematoxylin and eosin. The stained sections, using a microscope (Olympus) were observed for infiltration of eosinophils, fibrosis, and thickness of epidermis at 10× magnification.

Serum WBC and granulocytes measurement. Blood samples were collected from each group on the last day before sacrifice by a retro-orbital puncture in a collection tube and processed for WBC and granulocytes estimation using a haematology analyzer (MINDRAY 3600) as per the manufacturer's instructions.

Serum IgE measurement. Serum IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (ThermoFischer scientific) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Biotek, USA).

Determination of body weight. To assess any systemic adverse effects related to the treatment, the bodyweight of the mice in different groups was determined on 1st, 5th, 10th, 15th, and 21st day (before sacrifice) (Ku *et al.* 2018).

Statistical analysis

The data for ear thickness, dorsal skin thickness, dorsal skin severity, and body weight were analyzed by two-way repeated measure ANOVA followed by Tukey test for significance using GraphPad Prism 8.0.2. The data for the determination of WBC, granulocytes, and IgE levels were analyzed by one way ANOVA followed by the Tukey test for significance. The alpha value was set at $p < 0.05$.

Results

Compatibility study with excipients

In the compatibility study by TLC, the R_f of the standard piperine (P) and piperine in SPM was found to be 0.26 and 0.26, respectively (Supplementary figure).

Evaluation of ethosomes of the piperine

Entrapment efficiency

The EE (%) increased with increased PCH and ethanol concentration (Figure 1). The EE (%) of formulation with 4% of PCH was higher than formulations with 3% of PCH, but as the results were analyzed with Student's *t*-test ($p > 0.05$, 95% confidence interval) no significant difference was reported between EE (%).

Sedimentation behaviour

The ethosomal dispersions with 2% and 3% of PCH were considered more stable in comparison to dispersions with 4% of PCH. The ethosomal dispersions with 2% and 3% of PCH exhibited no sedimentation and aggregation for 25 days (Table 2). At the end of the 30th day, sedimentation was

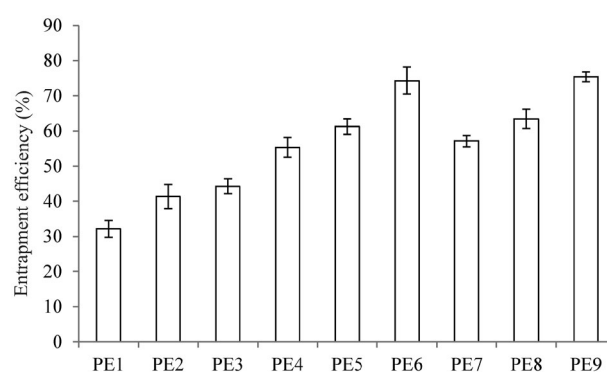


Figure 1. Entrapment efficiency (%) of ethosomes of piperine (PE1-PE9) (mean $\pm$ SD, $n = 3$).

Table 2. Sedimentation behaviour of ethosomes of piperine.

Formulation code	Days						
	0	5	10	15	20	25	30
PE1	—	—	—	—	—	—	+
PE2	—	—	—	—	—	—	+
PE3	—	—	—	—	—	—	+
PE4	—	—	—	—	—	—	+
PE5	—	—	—	—	—	—	+
PE6	—	—	—	—	—	—	+
PE7	—	+	+	++	++	++	++
PE8	+	+	+	++	++	++	++
PE9	+	+	+	++	++	++	++

(—) no sedimentation, (+) sedimentation but redispersible, (++) non-dispersible sediment.

observed but was easily re-dispersible. In the case of formulations with 4% of PCH sedimentation was fast and at the end of the 15th day, the sediment was non-dispersible. The representative figures of ethosomal dispersions for sedimentation behaviour at 0th, 10th, 20th, and 30th day are shown in Figure 2.

Vesicle size distribution and zeta potential

The vesicle size distribution analysis of dispersion with 4% of PCH was not performed due to a higher rate of sedimentation and no significant difference reported in the EE (%) with dispersion having 3% of PCH. The vesicle size increased with increased PCH concentration and decreased with increased ethanol concentration (Table 3). The PDI values of all the ethosomal dispersions were acceptable with uniform vesicle size (Table 3). The zeta potential of dispersions increased with increased ethanol concentration with net negative surface charge (Table 3). The vesicle size distribution curve and zeta potential of PE6 with acceptable EE (%) and vesicle size is shown in Figure 3(a,b), respectively.

In vitro drug release

In the case of HE drug solution, the maximum drug was released within 6 h whereas drug release from the ethosomes was sustained for 24 h (Figure 4(a)). The drug release decreased with the increased concentration of PCH, but the release was to some extent higher with increased ethanol concentration in formulations with equivalent PCH concentration. The drug release from the formulation with 2% of

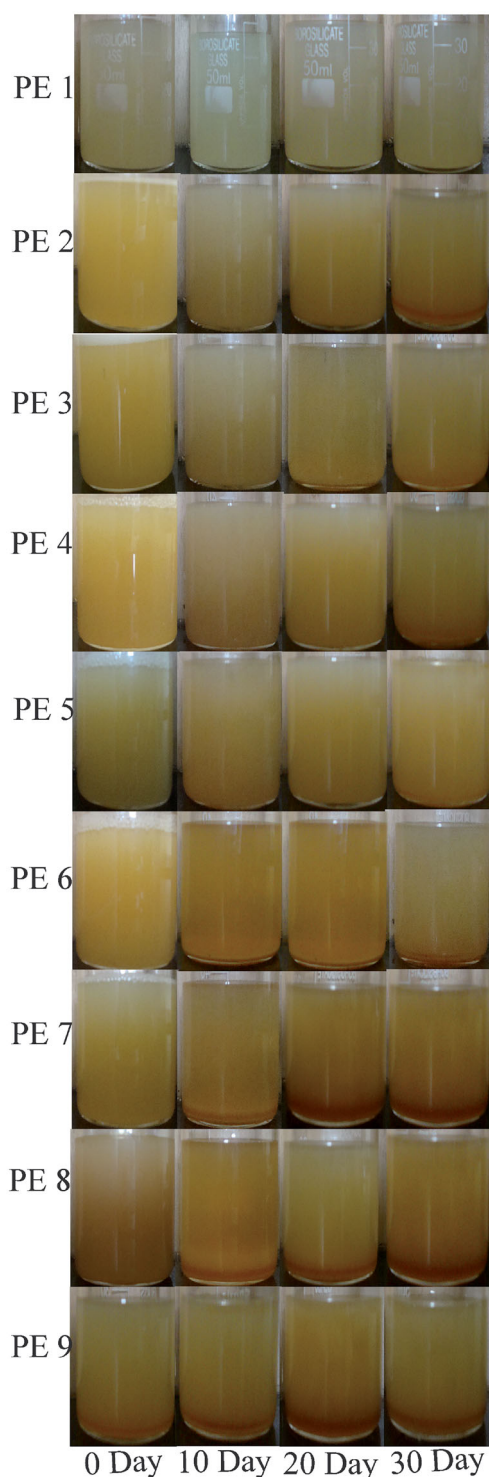


Figure 2. Sedimentation behaviour of ethosomal dispersions of piperine.

Table 3. Vesicle size distribution, PDI and zeta potential of ethosomes of piperine.

Formulation Code	Z-Average (nm)	PDI	Zeta potential (mV)
PE1	614.5	0.655	−29.6
PE2	572.3	0.647	−30.2
PE3	312.7	0.587	−30.7
PE4	1155	0.628	−28.4
PE5	614.1	1.0	−31.9
PE6	318.1	0.432	−32.6

PCH and 20% of ethanol (PE1) was $85.67 \pm 2.53\%$, whereas increasing the amount of alcohol to 40% (PE3) increased the drug release to $95.41 \pm 3.14\%$. Similarly, in formulation with 3% of PCH and 20% of ethanol (PE4) the drug release was $82.34 \pm 1.32\%$, whereas increasing the amount of alcohol to 40% (PE6) increased the release to $91.23 \pm 2.36\%$.

On the basis of EE (%), sedimentation behaviour, Z-average, PDI, and drug release formulation PE6 with 3% of PCH and 40% of ethanol was selected as optimized formulation and further investigated for release kinetics and mechanism. The regression (R^2) values were found to be 0.8625, 0.6437, 0.9722, and 0.7792 for zero order, first order, Higuchi and Peppas model fitting curves. The 'n' exponential value obtained from Peppas model curve fitting was 0.7754. It was seen from the TEM photomicrograph (Figure 4(b)) that vesicles were spherical in shape.

Evaluation of creams loaded with ethosomes of piperine and conventional creams of piperine

The creams of piperine were white in colour, smooth, and free from grittiness. The pH of creams loaded with ethosomes of piperine was acceptable as it was near to the acidic pH of the skin (Table 4). In the case of conventional creams of piperine, an increase in pH was observed with an increased dose of piperine (Table 4). The pH of conventional creams was also acceptable as it was near to the acidic pH of the skin.

During thermal stability studies, no separation of oil phase was observed after 48 h in both types of creams. The viscosity and spreadability value for different creams is shown in Table 4. The spreadability of the creams was satisfactory and acceptable in comparison to marketed cream. The acid value and saponification value for different formulations are also shown in Table 4. The drug content (%) of all the formulations was satisfactory and acceptable (Table 4).

Ex vivo skin permeation and drug retention study of creams in skin layers

The percent cumulative drug permeation was high from the conventional creams of piperine in comparison to creams loaded with ethosomes of piperine (Figure 5(a)). The cumulative amount of drug permeated increased with increased concentration of piperine in ethosomal and conventional creams. The cumulative drug permeation was $23.63 \pm 2.14\%$, $24.10 \pm 1.97\%$, $26.14 \pm 3.01\%$, and $33.27 \pm 2.84\%$ from PEC1, PEC2, PEC3, and PEC4, respectively after 7 h. cumulative drug permeation was $34.29 \pm 2.30\%$, $36.21 \pm 1.34\%$, $41.63 \pm 2.70\%$, and $45.37 \pm 2.31\%$ from PC1, PC2, PC3, and PC4, respectively after 7 h. The retention of piperine in stratum corneum was higher in conventional creams as compared to creams loaded with the ethosomes (Figure 4(b)). Further, the retention of piperine in epidermal and dermal layers of skin was higher with creams loaded with the ethosomes (Figure 5(b)).

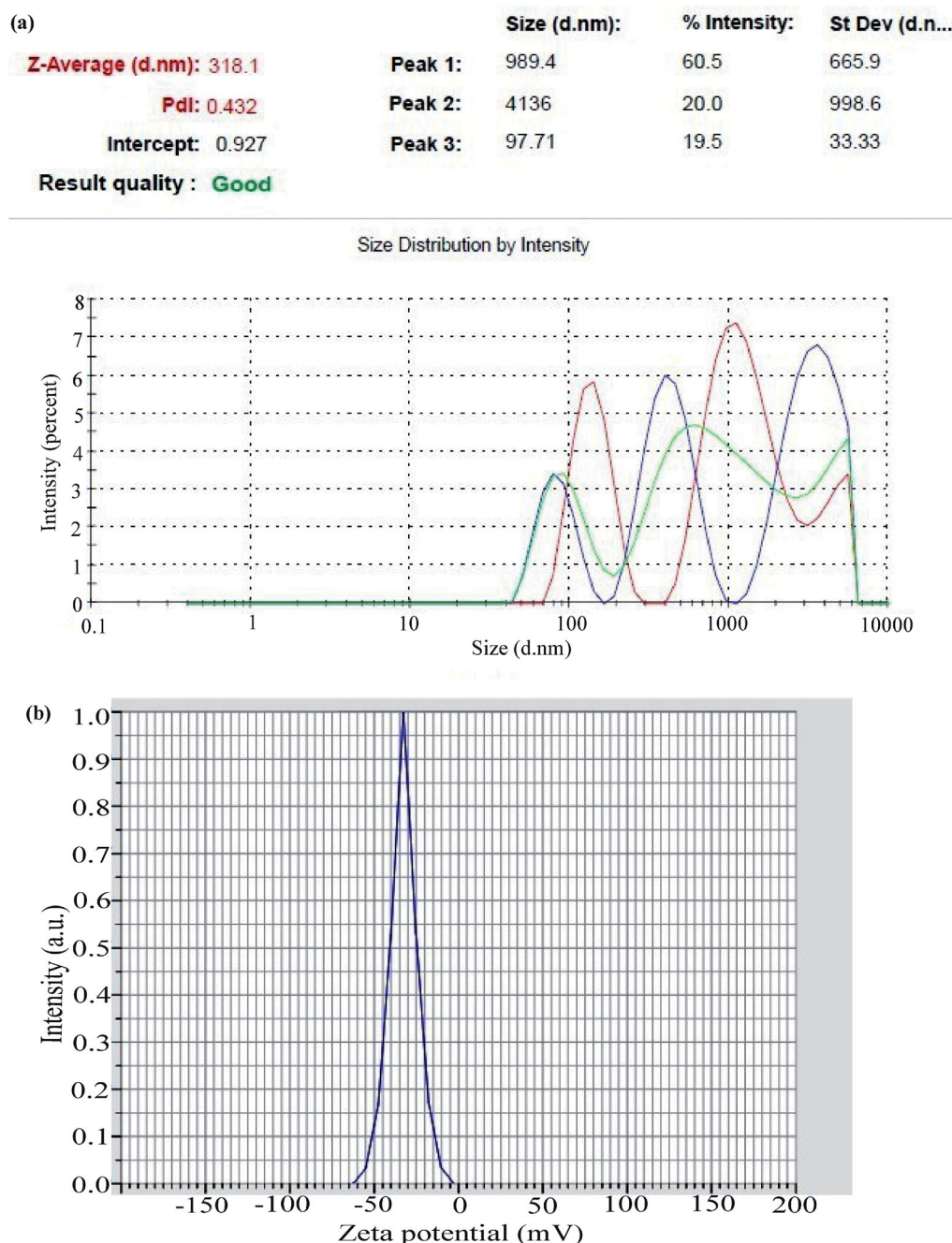


Figure 3. (a) Particle size distribution curve of optimized dispersion PE6, and (b) Zeta potential of optimized dispersion PE6.

In vitro cytotoxicity study on human skin cell lines

The CTC_{50} of PC4 and PEC3 were found to be $529.67 \pm 4.8 \mu\text{g/ml}$ and $623.34 \pm 3.21 \mu\text{g/ml}$, respectively (Figure 6(a)). The representative photomicrographs (Figure 6(b–d)) of morphological changes of cell lines suspension as compared to control also indicated that optimized formulations were non-cytotoxic to human skin cell lines.

In vivo evaluation of topical creams

The treatment within T1 and T2 significantly decreased the ear thickness as compared to NC (Figure 7(a)). No significant

difference was reported between PC, T1, and T2 in decreasing ear thickness on the 21st day. Similarly, a significant decrease in dorsal skin thickness was reported with treatments in comparison to NC (Figure 7(b)). No significant difference was reported between the efficacy of T2 and PC in decreasing dorsal skin thickness. The efficacy of T1 was significantly low as compared to PC in decreasing skin thickness after 21 days.

The clinical manifestations viz. erythema, erosion, and dryness of dorsal skin were low in T1 and T2. The representative figure of dorsal skin is shown in Figure 7(c). There was a significant difference in the average clinical scores given by independent observers (Figure 7(d)) between NC and treatment

groups. At the end of the study, no significant difference was reported between the PC and T1, PC and T2, and T1 and T2.

Histological examination

The specimen of the control group showed normal dorsal skin and ear pinna with stratified squamous epithelium, hair follicles, and sebaceous gland (Figure 8(a)). The histological examination of dorsal skin and ear pinna of NC showed moderate to severe hyperplasia, hyperkeratosis, and inflammatory response (infiltration of eosinophils and neutrophils) in the epidermis and dermis. The level of hyperplasia and hyperkeratosis was mild within treatment groups as compared to NC. The inflammatory response was mild in the PC, T1, and T2 groups as compared to NC in dorsal skin and ear pinna specimens.

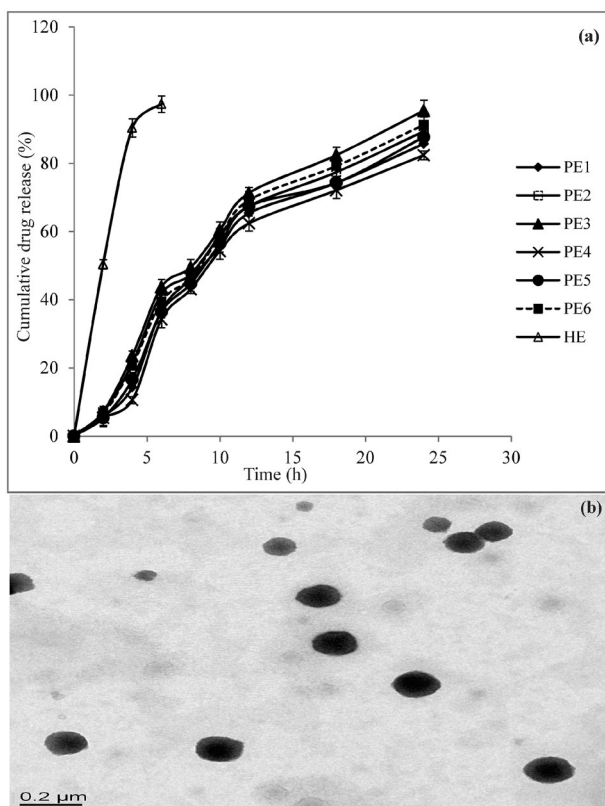


Figure 4. (a) Cumulative drug release (%) of ethosomes dispersions PE1–PE6 (mean $\pm$ SD, $n = 3$), and (b) TEM micrograph of PE6.

Serum WBC, granulocytes, and IgE level

The serum analysis showed a significant increase in the level of WBC (Figure 8(b)) and granulocytes (Figure 8(c)) in NC. In the different treatment groups, WBC and granulocytes level was significantly reduced in comparison to NC. No significant difference was observed between PC and T2 in decreasing WBC levels. But, the efficacy of T2 was significantly higher than T1 in decreasing WBC level after 21 days. Similarly, no significant difference was observed between T1 and T2 as compared to PC in decreasing granulocytes levels. The effect of T2 was significantly higher than T1.

The serum analysis showed a significant increase in IgE level in NC. The IgE level was significantly reduced in treatment groups in comparison to NC (Figure 8(d)). Further, at the end of the study, no significant difference was observed between the PC and T2; and the efficacy of T2 was significantly higher than T1 in reducing IgE level.

Determination of body weight

There was no significant effect observed in the bodyweight of animals in different groups (Figure 8(e)).

Discussion

Formulation and evaluation of ethosomes of piperine

The piperine was compatible with the different excipients of formulations (Wagner and Bladt 2007). Piperine, being a lipophilic drug was entrapped in the phospholipids bilayers, and as the concentration of PCH was increased the number of bilayers also increased and ultimately increased the drug holding capacity of ethosomes. Further, higher EE with increased ethanol concentration was possibly due to increased solubility of piperine in the ethanolic core of ethosomes (Chourasia *et al.* 2011). The sedimentation behaviour with time was investigated to assess the preliminary stability of ethosomal dispersions (Kamboj *et al.* 2014). The vesicles in dispersion should be uniformly dispersed and free from aggregation for sufficient time so that it can be loaded into a base cream.

The decrease vesicle size with increased ethanol concentration was probably due to the modification of net zeta potential of dispersion and confers some steric stabilization (Chourasia *et al.* 2011). The results of zeta potential reinforced the above theory as the negative surface charge of

Table 4. Evaluation parameters for creams loaded with ethosomes of piperine and conventional creams of piperine.

S. No.	Formulations	Parameters					
		pH	Spreadability (g.cm/sec)	Viscosity (cps)	Acid value	Saponification value	Drug content (%)
1	PEC1	5.5 $\pm$ 0.57	10.54 $\pm$ 1.70	2857.14 $\pm$ 32.09	5.47 $\pm$ 0.06	28.86 $\pm$ 3.09	97.36 $\pm$ 5.02
2	PEC2	5.6 $\pm$ 0.11	10.42 $\pm$ 1.15	2848.00 $\pm$ 11.26	5.30 $\pm$ 0.09	27.47 $\pm$ 2.54	98.28 $\pm$ 1.05
3	PEC3	5.6 $\pm$ 0.10	10.92 $\pm$ 1.45	2838.33 $\pm$ 4.04	5.92 $\pm$ 0.16	28.81 $\pm$ 2.31	99.07 $\pm$ 2.09
4	PEC4	5.8 $\pm$ 0.11	11.30 $\pm$ 0.83	2823.00 $\pm$ 16.52	6.05 $\pm$ 0.17	30.56 $\pm$ 1.29	97.16 $\pm$ 0.99
5	PC1	5.5 $\pm$ 0.12	10.01 $\pm$ 1.30	2835.13 $\pm$ 17.49	5.31 $\pm$ 0.41	28.32 $\pm$ 1.12	97.6 $\pm$ 5.85
6	PC2	5.5 $\pm$ 0.53	09.34 $\pm$ 1.14	2861.71 $\pm$ 32.01	5.10 $\pm$ 1.0	27.59 $\pm$ 2.01	97.3 $\pm$ 4.0
7	PC3	5.6 $\pm$ 0.71	10.12 $\pm$ 1.31	2821.11 $\pm$ 16.43	5.82 $\pm$ 0.43	29.17 $\pm$ 0.31	96.6 $\pm$ 5.77
8	PC4	5.7 $\pm$ 0.56	09.62 $\pm$ 1.13	2854.36 $\pm$ 19.30	5.63 $\pm$ 1.03	29.23 $\pm$ 1.24	97.4 $\pm$ 3.09
9	Base cream	5.5 $\pm$ 1.57	10.45 $\pm$ 1.15	2839.62 $\pm$ 26.42	6.13 $\pm$ 1.34	28.95 $\pm$ 2.42	–
10	Marketed cream	–	11.75 $\pm$ 3.41	–	–	–	–

Mean $\pm$ SD ($n = 3$).

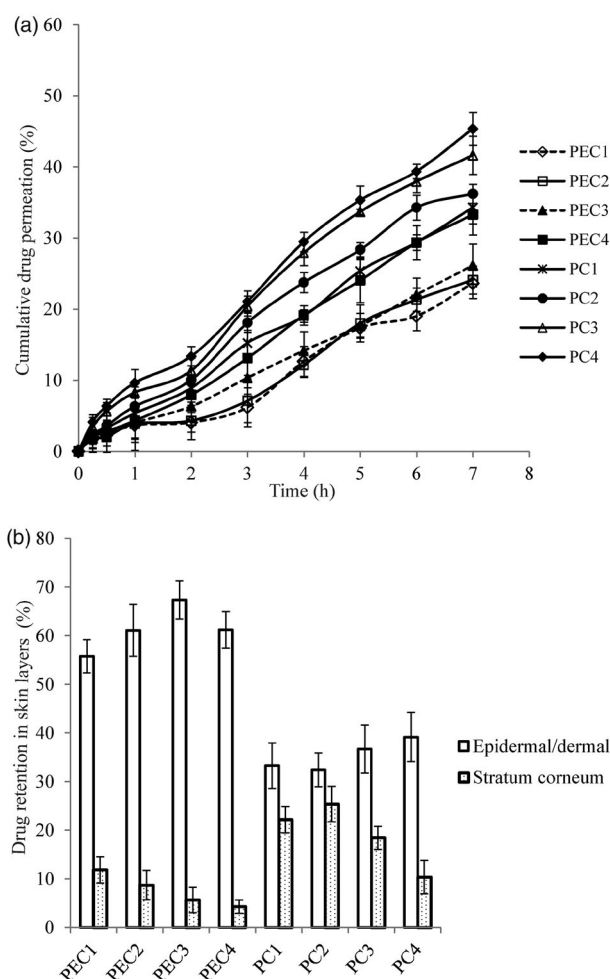


Figure 5. (a) *Ex vivo* cumulative drug permeation of ethosomes loaded and conventional creams of piperine, and (b) retention of piperine in mice skin layers (mean $\pm$ SD, $n = 3$).

the system increased with increased ethanol concentration (Jain *et al.* 2007, Abdulbaqui *et al.* 2016). The PDI is a measure of the width of unimodal vesicle size distribution. A PDI value below 0.7 is acceptable for the vesicular dispersion systems indicating good dispersion homogeneity (Refai *et al.* 2017).

The drug release from the vesicular carriers was regulated by the transfer of molecules from the vesicle to the adjacent aqueous phase and then diffusion through the cellophane membrane to the receptor medium. The release behaviour of HE drug solution and ethosomal dispersions signifies that the drug diffusion from the phospholipid bilayers is the rate-controlling step in the overall drug diffusion to the receptor medium. The increased drug release with increased ethanol concentration might be due to increased fluidity of lipid bilayers (Jain *et al.* 2007, Chourasia *et al.* 2011). The R^2 values of different model fitting curves for PE6 indicated that the best release kinetic model was the Higuchi model and the ' n ' exponential value indicated that the release mechanism was by non-Fickian diffusion (Chourasia *et al.* 2011).

Evaluation of creams loaded with ethosomes of piperine and conventional creams

The pH of atopic skin plays an important role in the maintenance of skin conditions. The pH of atopic skin shifts towards alkaline as the disorder progresses leading to microbial growth and worsening of skin condition. So, a formulation with pH near to normal skin might be able to maintain the pH of the skin towards the acidic side (Panther and Jacob 2015). The observation of the thermal stability study indicated the stability of creams. The spreadability and viscosity influence the acceptability of the formulations

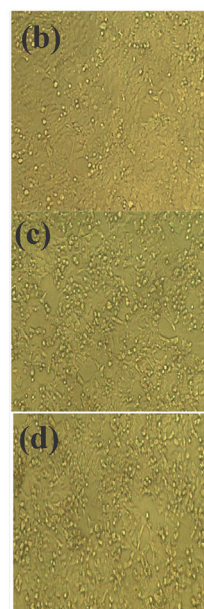
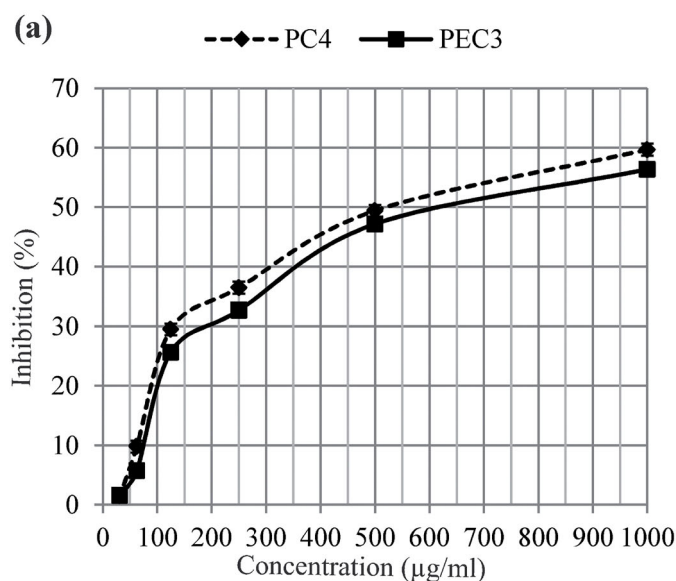


Figure 6. *In vitro* cytotoxicity on HaCat cell lines (a) Inhibition (%) of cell lines by optimised creams (PC4 and PEC3), (b) photomicrograph of control cell lines, (c) photomicrograph of cell lines exposed to 250 µg/ml of PC4, and (d) Photomicrograph of cell lines exposed to 250 µg/ml of PEC3.

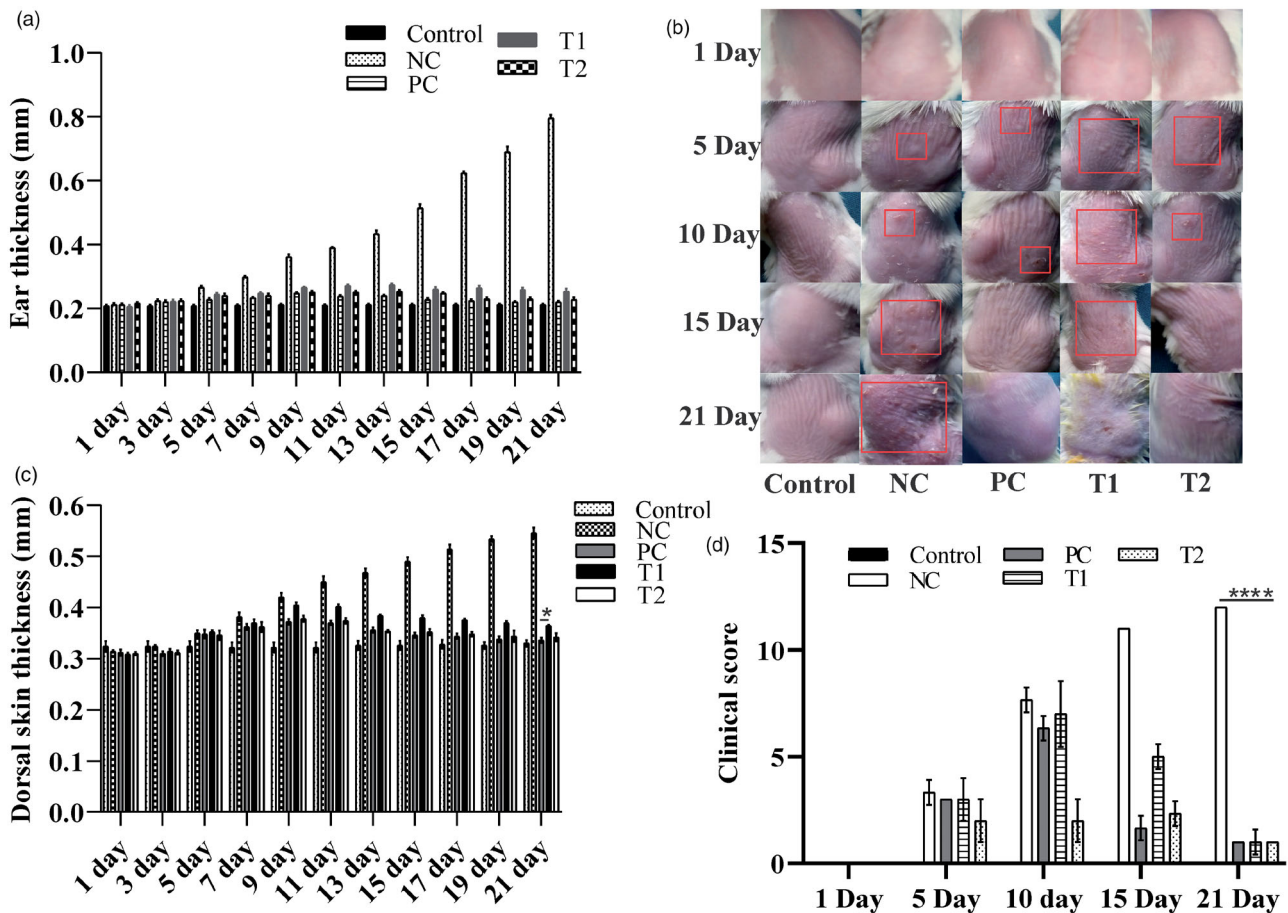


Figure 7. Ear thickness (a), dorsal skin thickness (b), dorsal skin of mice (c), and clinical score of skin severity (d) (mean $\pm$ SME, $n = 5$). NC: negative control; PC: positive control; T1: treatment group 1 with conventional cream; T2: treatment group 2 with ethosomes loaded cream. * $p < 0.05$; **** $p < 0.0001$].

(Ashawat *et al.* 2008). The spreadability of the creams was satisfactory and acceptable in comparison to an established marketed cream.

The acid value is associated with the free fatty acids content. A free acid content higher than 6.5 can lead to skin irritation following application on the skin (Ashawat *et al.* 2008). The acid values of all the cream formulations were acceptable with less potential for skin irritation. The saponification value reflects the presence of free esters, which can influence the formula stability and pH. A saponification value in the range of 10 to 30 is generally acceptable (neither too low nor too high) for the skin formulations (Saraf *et al.* 2010). The saponification values of all the creams were acceptable and satisfactory for formulation stability. No significant difference was observed in the results of viscosity, spreadability, acid, and saponification value of piperine creams and base cream. The drug content of all the formulations was satisfactory and acceptable.

The higher *ex vivo* permeation from the conventional creams in comparison to ethosomal creams of piperine might be due to the sustained release of piperine from the ethosomes in skin layers. The reason for higher deposition of piperine in epidermis and dermis layers with ethosomal creams may be attributed to the enhanced penetrability of

ethosomes as drug carriers through stratum corneum and making the drug available to deeper layers (Azajuddin and Saraf 2010).

The topical formulations are preferred to have the maximum amount of drug retained in the epidermal and dermal layer of the skin (Shah *et al.* 1992). So, PEC3 (0.1%) and PC4 (0.125%), with acceptable psychometric parameters, pH, acid and saponification value; and maximum drug deposition in the epidermal and dermal layers were selected for *in vitro* cytotoxicity and *in vivo* investigations. The CTC₅₀ concentration for the optimized topical creams was higher than 100 μ g/ml, indicating that the creams were non-cytotoxic and can be regarded as biocompatible to skin cells (Gunes *et al.* 2017).

In vivo evaluation of topical creams

Based on the results of *in vitro* and *ex vivo* investigation, PC4 (0.125%) and PEC3 (0.1%) were subjected for *in vivo* evaluations on BALB/c mice model. The ear thickness, dorsal skin thickness, average clinical score of atopic skin; and serum level of WBC, granulocytes, and IgE antibodies are important markers for AD (Choi *et al.* 2011, Ku *et al.* 2018). The

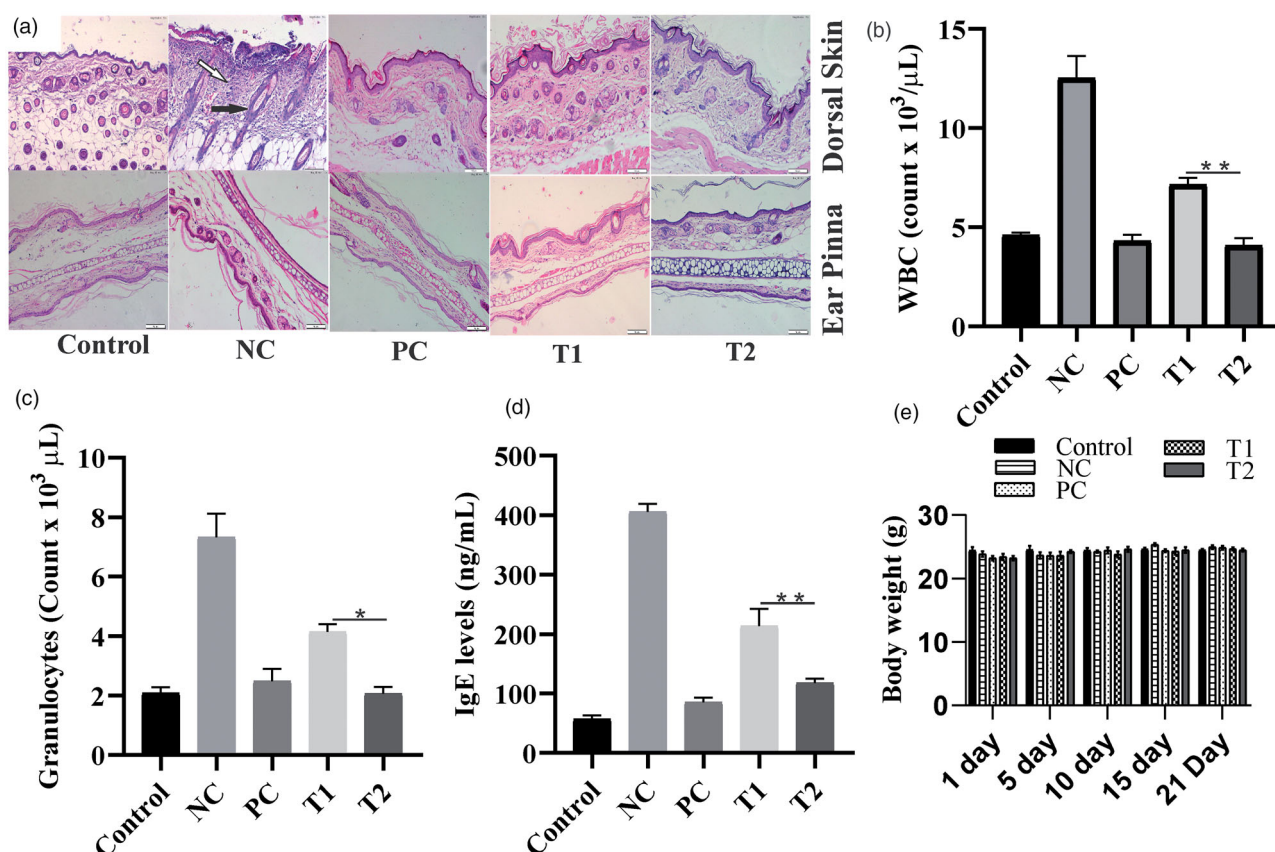


Figure 8. Histogram of dorsal skin and ear pinna. (a) Severe hyperplasia and hyperkeratosis, retes ridges formation (black arrow) and increased infiltration of eosinophils and neutrophils (white arrow) in NC; mild hyperplasia, hyperkeratosis and inflammatory response in PC, T1, and T2 dorsal skin and ear pinna; WBC count (b); granulocyte count (c); serum IgE levels (d); body weight of mice (e) (mean $\pm$ SME, $n = 5$). NC: negative control; PC: positive control; T1: treatment group 1 with conventional cream; T2: treatment group 2 with ethosomes loaded cream. * $p < 0.05$; ** $p < 0.01$].

therapeutic effect of ethosomal cream was significant in reducing the increased level of the above markers in comparison to tacrolimus (0.1%). Further, no significant change in body weight of mice after the study indicated that the treatments do not show any significant systemic side effects (Ku *et al.* 2018). The overall results of *in vivo* evaluation showed that the ethosomal cream with less drug concentration was more efficacious than the conventional cream of piperine in reducing the markers of AD.

Conclusion

The topical formulations are desired to have maximum deposition of drug in skin layers. The ethosomal creams showed high skin penetration and deposition of piperine in the epidermis and dermis as compared to conventional cream. The ethosomal cream (0.1%) and conventional cream (0.125%) of piperine showed efficacy in the treatment of AD in comparison to tacrolimus (0.1%). The efficacy of ethosomal cream with a lesser dose of piperine was higher than the conventional cream in reducing significant markers of AD. The ethosomal cream of piperine can be a possible alternative for the long-term treatment of mild to moderate AD as compared to conventional prescription drugs.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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