Formulation and *in-vitro* characterization of Capsaicin loaded ethosomes

Imtiaz Majeed\(^1\,^2\), Syed Atif Raza\(^2\), Naveed Akhtar\(^3\), Faheem Ahmed Siddiqui\(^1\) and Beenish Iqbal\(^1\)

\(^1\)Faculty of Pharmacy, University of Central Punjab, Lahore, Pakistan
\(^2\)Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan
\(^3\)Faculty of Pharmacy & Alternative Medicine, University of Bahawalpur, Bahawalpur, Pakistan

**Abstract:** The ability of ethosomes to entrap capsaicin was evaluated using four methods of preparation that are; hot method, cold method, classic method and injection method. The ethosomes were prepared, optimized and characterized with the aim to identify a technique best suitable for their formulation. Vesicle shape, size and entrapment efficiency was determined by scanning electron microscopy, dynamic light scattering and ultracentrifugation techniques, respectively. Vesicle sizes varied from an average of 15nm - 400nm depending on the concentrations of phospholipid, ethanol and method of preparation. The formulations demonstrated entrapment efficiency of 29-81% with maximum entrapment obtained in formulations prepared with hot method having high concentration of ethanol. The homogeneity index was measured with Zetasizer that showed formulation prepared with hot method to be more uniform in size distribution having PDI 0.162 while injection method of preparation yielded a moderately broad polydispersity of vesicles (0.276). Physical stability assessment done by storing the selected formulation samples at 4°C and 25°C indicated the refrigerator temperature to be the best for retention of drug in ethosomal vesicles. All formulations kept in refrigerater adequately retained capsaicin during the two months of stability studies while those at ambient temperature noticeably showed leaked drug from vesicles. FTIR analysis showed capsaicin and phospholipid to be compatible with each other with no sign of interaction. DSC studies evidently showed lowering of transition temperature of phospholipid from 327.13°C to 111.63°C in ethosomal formulation due to the presence of ethanol. It was concluded that capsaicin ethosomes can be successfully prepared to employ four different methods and their characterization parameters indicate hot method to be effective for preparation of nano-sized uniform, homogeneous and stable capsaicin ethosomes.

**Keywords:** Ethosomes, capsaicin, Zetasizer, FTIR.

**INTRODUCTION**

Ethosomes are a novel mode of drug delivery targeted to the skin which is an easily accessible route for drug delivery. Touitou was the first to develop this vesicular system and due to the presence of ethanol in their vesicular structure named them as ethosomes (Touitou, 1998). Ethosomes are formulated by ethanol, phospholipid and water and have evidently enhanced topical uptake of numerous drugs (Ainbinder and Touitou, 2005). Earlier lipid constructed preparations like ‘microemulsions’, ‘niosomes’ and ‘liposomes’ has considerably upgraded the skin permeation however their inability to distribute the drug to deep skin strata left room for developing further improved mode of drug delivery (Cevc, 2004). Ethosomes are generally comprised of phospholipids, ethanol and water. Their average size ranges from tens of nanometer to few microns, but the size is dependent on the concentration of phospholipid and ethanol so it can be varied and controlled. Ethosomes act as permeation enhancing carriers, that is, they are a lot better in distributing topical drugs to the skin in manner of amount and range of delivery, compared to liposomes or hydroalcoholic solutions (Ting et al., 2004). Their soft, flexible characteristics and unique properties allow their easier infiltration into deeper skin layers (Fang et al., 2008). The high ethanol content (up to 50%) was proposed to fluidize the ethosomal lipid component and stratum corneum lipid structures, thereby permitting soft malleable ethosomes to penetrate (Ainbinder and Touitou, 2005). Ethanol is proposed to interfere with the polar part of lipid molecules, reducing melting point of stratum corneum lipid and in turn boosting lipid fluidity and cell membrane absorptivity (Touitou et al., 2000). Capsaicin induces “heat sensation” which is ascribed to its binding with Transient receptor potential vanilloid (TRPV1) ion-channel receptors, also called Capsaicin receptors (Ramsey et al., 2006). Hogyes first observed this heating sensation in 1878 when extract of Capsicum was applied on human skin (Toh et al., 1955). Capsaicin has high affinity for TRPV1 and acts as an agonist at these receptors. TRPV1 is a nonselective cation channel favourably expressed on small-diameter sensory neurons (C-fibers and A-δ fibres) (Alawi and Keeble, 2010, Bley et al., 2012). Capsaicin primarily excites the sensory neurons followed by a persisting refractory state known as desensitization where the priorly excited neuron becomes insensitive to diverse stimuli. The persistent

*Corresponding author: e-mail: imtiaz.majeed@ucp.edu.pk

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desensitization is due to depletion of Substance P (Nolano et al., 1999). Capsaicin is classified as a counter-irritant due its ability to produce lasting analgesia on repeated exposure after desensitization. This pharmacological action of capsaicin has been used for pain liberation and US FDA approved an 8% capsaicin dermal patch, which includes synthetic capsaicin 640mcg/cm² marketed as Qutenza for neuropathic pain with postherpetic neuralgia (Qutenza, 2013). Topical capsaicin is generally well tolerated with negligible potential for systemic adverse effects however site-specific burning is experienced by up to 80% of patients (Rains and Bryson, 1995). The local adverse effects produced by topical capsaicin correspond to 'number needed to harm (NNH)' of 2.5 whereas an NNH closer to 10 requires withdrawal of active ingredient. Cough has been reported in 8% of patients using 0.075% of capsaicin cream. TRPV1 receptors existing on sensory airway nerves are crucial in processing of cough reflex. Eye exposure to capsaicin results in burning with tears, pain, conjunctivitis and blepharospasm. Ingestion of large quantities of capsaicin may lead to nausea, vomiting, abdominal pain and burning diarrhea (Hayman and Kam, 2008). Although capsaicin is used topically for temporary relief of pain but our aim is to develop novel drug delivery system that is effective, having fewer side effects, cost effective and improves the treatment.

MATERIALS AND METHODS

The drug capsaicin was of commercial grade obtained from PDH healthcare. Phospholipon 90G was procured from LIPOID Germany. Ethanol (Sigma-Aldrich, Chemie GmBH), cholesterol (VWR International Ltd, West Chester PA), tween 80 (Merck –Schuchardt), polyethylene glycol (USA), methanol (Sigma-Aldrich Chemie GmBH) and propylene glycol (USA) were of analytical grade. Capsaicin loaded ethosomal suspensions were prepared via four different methods which are discussed below.

Hot method

Soya bean lecithin (Phospholipon 90G) was dissolved in distilled water preheated at 40°C in a beaker and placed in water bath maintained at 40°C temperature. In another beaker drug (capsaicin oil) was dissolved in ethanol and then polyethylene glycol and/or propylene glycol were added and heated in a water bath up to 40°C. The organic phase was then added to the aqueous phase with constant stirring. Stirred the subsequent suspension for 30 minutes with hot plate magnetic stirrer at 40°C, vortexed at 1200 rpm for 10 minutes and finally homogenized with the Heidolph homogenizer at 5000rpm for time duration of 10 minutes and cooled to room temperature.

Cold method

Phospholipon 90G and drug (capsaicin oil) were dissolved in ethanol with constant stirring. To this suspension distilled water, preheated at 30°C, was added and stirred vigorously. The resulting suspension was mixed with a magnetic stirrer for additional 10 minutes.

Classic method

In this method, ethosomes were prepared by dissolving phospholipon 90G and capsaicin oil in ethanol with constant stirring and heating the mixture in a water bath at 30°C. To this mixture, distilled water preheated at 30°C was incorporated while stirring. The resultant suspension was homogenized with Heidolph homogenizer at 5000rpm for 10 minutes.

Injection method

To prepare ethosomes by this method, cholesterol was dissolved in ethanol using magnetic stirrer. Afterwards soya bean lecithin, tween 80 and capsaicin oil were added and stirred with magnetic stirrer for 10 minutes in a closed vessel. Propylene glycol was added and stirred for another 5 minutes. Heidolph homogenizer was fitted into the closed vessel containing the above mixture and homogenization at 5000rpm and was continued for 5 minutes. Finally added distilled water by syringe, dropwise into the mixture during homogenization.

Various methods of characterization of ethosomes

Visualization of vesicles with the scanning electron microscope (SEM)

Scanning electron microscopy provides a scan of a sample with focused electron beam, delivering images with facts about the sample’s morphology and topography. SEM was utilized to examine shape and size of particles of the optimized formulations from the trial batches. The SEM produces images by way of sample-electron interaction, that is, a high energy beam of electrons produced by the electron gun is focused on the sample surface by passing through combination of lenses and apertures. The electrons upon interaction with sample produce secondary electrons and various other signals which are collected by detectors and images are formed and displayed on the software attached with it. One drop from formulation was placed on sample holder stub and allowed to dry forming a thin film. The SEM model used was Zeiss LS10, Germany, which had a detector that analyzed sample without any coating. The selected formulations were examined at an accelerating speed of 20kV and a working distance of 10.5mm. All the selected samples were analyzed at three magnifications ranging from 5 KX to 15 KX.

Differential scanning calorimetric study (DSC)

Differential scanning calorimetric analysis of ethosomal vesicles was performed using Sdt Q600 TA instrument. The optimum formulation, Phospholipon 90G and physical mixture of the drug (capsaicin) were run for DSC analysis. The transition temperature (Tm) of phospholipid was evaluated in aluminium pans under constant nitrogen stream. Measurements were performed at a rate of
10°C/min heating in temperature ranging from 21°C - 250°C. Initial sample weight for formulation was about 28 mg.

**Table 1: Composition of capsaicin loaded ethosomes**

<table>
<thead>
<tr>
<th>Method of preparation</th>
<th>Trial code</th>
<th>Drug (capsaicin oil) % v/v</th>
<th>Soya Lecithin % w/v</th>
<th>Ethanol % v/v</th>
<th>PEG 400 % v/v</th>
<th>Propylene glycol % v/v</th>
<th>Tween 80 % v/v</th>
<th>Cholesterol % w/v</th>
<th>Distilled water % v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot method</td>
<td>ET1</td>
<td>0.075%</td>
<td>1%</td>
<td>75%</td>
<td>5%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>20%</td>
</tr>
<tr>
<td>ET2</td>
<td>0.075%</td>
<td>2%</td>
<td>70%</td>
<td>15%</td>
<td>20%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>20%</td>
</tr>
<tr>
<td>ET3</td>
<td>0.075%</td>
<td>3%</td>
<td>65%</td>
<td>15%</td>
<td>20%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>20%</td>
</tr>
<tr>
<td>ET4</td>
<td>0.075%</td>
<td>4%</td>
<td>60%</td>
<td>20%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>20%</td>
</tr>
<tr>
<td>ET5</td>
<td>0.075%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>60%</td>
</tr>
<tr>
<td>ET6</td>
<td>0.050%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>60%</td>
</tr>
<tr>
<td>ET7</td>
<td>0.025%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>60%</td>
</tr>
<tr>
<td>Cold method</td>
<td>ET8</td>
<td>0.075%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>60%</td>
</tr>
<tr>
<td>ET9</td>
<td>0.050%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>60%</td>
</tr>
<tr>
<td>ET10</td>
<td>0.025%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>60%</td>
</tr>
<tr>
<td>Classic method</td>
<td>ET11</td>
<td>0.075%</td>
<td>2%</td>
<td>30%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>70%</td>
</tr>
<tr>
<td>ET12</td>
<td>0.050%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>70%</td>
</tr>
<tr>
<td>ET13</td>
<td>0.025%</td>
<td>2%</td>
<td>30%</td>
<td>10%</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>70%</td>
</tr>
<tr>
<td>Injection method</td>
<td>ET14</td>
<td>0.075%</td>
<td>1%</td>
<td>45%</td>
<td>----</td>
<td>20%</td>
<td>20%</td>
<td>0.4%</td>
<td>15%</td>
</tr>
</tbody>
</table>

**Table 2: Entrapment efficiency of ethosomal vesicles**

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Entrapment efficiency (%EE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET1</td>
<td>81.40%</td>
</tr>
<tr>
<td>ET2</td>
<td>65.00%</td>
</tr>
<tr>
<td>ET3</td>
<td>55.00%</td>
</tr>
<tr>
<td>ET4</td>
<td>36.00%</td>
</tr>
<tr>
<td>ET5</td>
<td>56.08%</td>
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<tr>
<td>ET6</td>
<td>55.00%</td>
</tr>
<tr>
<td>ET7</td>
<td>44.00%</td>
</tr>
<tr>
<td>ET8</td>
<td>59.25%</td>
</tr>
<tr>
<td>ET9</td>
<td>29.00%</td>
</tr>
<tr>
<td>ET10</td>
<td>42.00%</td>
</tr>
<tr>
<td>ET11</td>
<td>60.00%</td>
</tr>
<tr>
<td>ET12</td>
<td>54.00%</td>
</tr>
<tr>
<td>ET13</td>
<td>39.00%</td>
</tr>
<tr>
<td>ET14</td>
<td>79.03%</td>
</tr>
</tbody>
</table>

**The entrapment efficiency of ethosomes**

The extent of capsaicin entangled in the ethosomal vesicles was analyzed after ultracentrifugation. Formulated trial batches were placed in the refrigerator at 4°C overnight and later ultra-centrifuged at 15000 rpm for eight hours. The suspension was separated into sediment and supernatant. The ethosomal pellet settled as sediment. The supernatant and sediment were mixed with methanol down to 10 or 100 degrees of dilution as required and analyzed with UV spectrophotometer at a wavelength of λ= 280nm. The entrapment efficiency (%EE) was calculated using the equation as follows:

\[
\% EE = \frac{Q_t}{Q_s} \times 100
\]

Here \( Q_t \) represents total amount of drug incorporated into the formulation and \( Q_s \) is the quantity of drug observed in the supernatant.

**Fig. 1:** Scanning electron microscope image of capsaicin ethosomes (ET 1) prepared by hot method

**Zeta-potential measurement**

Zeta-potential is the measurement of the degree of electrostatic or charge repulsion/attraction between particles and is a vital parameter of assessing the stability of formulations. Its range highlights the reasons for dispersion, aggregation or flocculation, and can be employed to enhance the stability aspects of formulations like dispersions, emulsions and suspensions. The zeta-potential of selected formulations was measured by dynamic light scattering using Malvern Zetasizer Nano ZS (Malvern, UK) against water as blank.

**Vesicle size and polydispersity index (PDI)**

The size of the ethosomal vesicles was measured as Z-average size or Z-average mean using dynamic light scattering technique. Z-average mean is the optimum...
parameter to quote in reference to quality control assessment of a formulation. The particle size measured using Zetasizer gives the hydrodynamic size of the vesicle (Worldwide, 2011). Polydispersity describes the extent of non-uniformity of a distribution in terms of size of the particles of a formulation. PDI was measured as a mean of determining the uniformity of vesicular suspension (Verma and Fahr, 2004). Polydispersity index was calculated using dynamic light scattering (DLS) technique with Malvern Zetasizer Nano ZS (Malvern, UK) applying cumulants analysis. The polydispersity describes the width of assumed Gaussian distribution identifying the sample as monodisperse, moderately polydisperse or broad polydisperse system (Nobbmann, 2016).

**Scrutiny of drug and excipients interaction**

The compatibility of capsaicin, phospholipid and surfactants together in the formulation was assessed by Infrared spectroscopy. Fourier transform infrared (FTIR) study was carried out using Bruker FTIR (Bruker Analytische Messtechnik GmbH) in the region of 400-4000cm$^{-1}$ using potassium bromide (KBr) pellet method of analysis (Sujitha et al., 2014).

**Physical stability studies**

The formulated ethosomal preparations were tested for their physical stability in terms of physical appearance, vesicle aggregation and drug leaking behaviour from ethosomes during their storage period. The capsaicin ethosomal formulation was placed in glass vials covered with plastic caps and kept at 4-8°C in refrigerator and at ambient temperature. The stability studies were continued for two months. The formulations were checked at regular time intervals; samples from each vial were drawn and analyzed for physical appearance, homogeneity and entrapment efficiency at the end of every second week.

**RESULTS**

**The entrapment efficiency of ethosomes**

The ethosomal formulations prepared by four different methods were analyzed for drug loading capacity via ultracentrifugation technique and later on detected for drug quantity using UV spectrophotometer. The results obtained are shown in table 2. The maximum entrapment was obtained for ET1 formulation, which was prepared by hot method and contained a high concentration of ethanol. One formulation with maximum entrapment efficiency was...
selected from all four methods of preparation and analyzed for SEM, zeta potential measurement and particle size analysis. The selected formulations were ET1, ET8, ET11 and ET14. Also these formulations were retained for stability studies for time duration of two months.

**Visualization of vesicles using Scanning electron microscope (SEM)**

The ethosomal vesicles were subjected to SEM for the analysis of vesicle shape and morphology. Selected samples were visualized under SEM at three magnifications of 5KX, 10KX and 15KX. In SEM image analysis, nano-sized spherical or nearly spherical lipidic vesicular structures were clearly visible. Figs. 1-4 show the SEM generated micrographs for the optimized formulations.

In fig. 1 the SEM micrograph shows homogeneous sized vesicle with a smooth surface. The particle size ranged from 50-300nm.

In fig. 2 the ethosomal batch formulated via cold method resulted in non-homogenous and rough shaped vesicles having a particle size in range of 300-500nm.

In fig. 3 the SEM image for trial ET11, formulated by employing a classic method, showed vesicles of varying size ranging from 97nm -300nm with a mean diameter of 155nm+ 42.3nm.

In fig. 4 the image obtained for trial prepared by injection method revealed ethosomes of diameter ranging from 10-30nm. Clumping of vesicles was visible.

**Physical stability studies**

Formulations kept at 4°C and 25°C temperatures for the observance of physical stability showed following results (as shown in table 3) measured at 0, 4 and 8 weeks of the time interval.

The formulations were pale yellow, colloidal and homogenous when freshly prepared. Only ET1 showed aggregation over a long period of time but the aggregates dispersed on vortexing the formulation. The drug retaining efficiency was effective for formulations stored at 4°C. However as time passed the formulation placed at room temperature showed noticeable drug leakage from the vesicles probably due to the physical degradation of phospholipids at high temperature.

**Zeta-potential measurement and vesicle size and poly-dispersity index**

Zeta-potential measured by DLS technique and the size of the vesicles was calculated as Z-average size as described earlier and poly-dispersity index stated using Zeta sizer Nano ZS showed results in table 4.

The zeta potentials for ET8, ET11 showed good high negative values indicating better stability. The zeta potential value for ET1, however, gave low positive value but the stability was adequate during storage period. This value may be due to the effect of measuring zeta potential against inorganic solvent (water) or human error.

Ethosomal suspension prepared by hot and classic method showed near to narrow distribution of vesicle size. Hot method of preparation resulted in a good average particle size.
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size as compared to cold and classic method of preparation. It might be due to high concentrations of ethanol that is 70% in ET1 as compared to 30% in cold and classic method. Injection method also gave small-sized vesicle but the formulation was heterogeneous.

**Analysis of drug and excipient interaction**

Drug, phospholipon 90G and ethosomal formulation were assessed by Fourier transform infrared spectroscopy for the presence of any chemical interaction among drug and excipients and the following results were obtained as shown in fig. 5, 6.

Characteristic peaks of capsaicin representing N-H stretch (3319 cm\(^{-1}\)), aliphatic C-H stretch (2943 cm\(^{-1}\), 2831 cm\(^{-1}\)), aromatic C-C stretch (1448 cm\(^{-1}\)), C-O stretch (1114 cm\(^{-1}\)) (El Kaaby Ekhlas et al., 2016) are same to the peaks observed in FTIR of drug-loaded ethosomes. The presence of same peaks indicated absence of chemical interaction between drug (capsaicin) and excipients employed in the formulated ethosomal suspension. Also the occurrence of these peaks depicted that capsaicin was entrapped in the ethosomal vesicles.

**Differential scanning calorimetry (DSC)**

The calorimetric results obtained for ET1, phospholipon 90G and physical mixture of capsaicin were plotted into a graph and the curve depicted an endothermic reaction. The fig. 7 shows this effect.

The depression from 54-77°C indicates evaporation and loss of water and ethanol from formulation. A broad asymmetric melting peak was obtained with peak maxima at 111.63°C. While in the scan for DSC of phospholipon 90G the transition occurred at 327.13°C. This difference in transition temperature of phospholipid between pure phospholipon and formulation contained phospholipon occurred due to the presence of ethanol.

**Thermal gravimetric analysis (TGA)**

Analysis performed using Differential scanning calorimeter SDT Q600 also gave data about the thermal gravimetric analysis, a technique of thermal analysis that quantifies the mass of sample over time as the temperature changes. TGA can be employed for assessment of the absolute and relative thermal stability of the given sample. The formulation was analyzed at a heating rate of 10°C/min. The data showed about 5% weight loss at 50°C. Afterwards evaporation and loss of water and ethanol occurred which continued till 111.63°C. The transition of phospholipid occurred at temperature 111.63°C. Weight loss increased rapidly and beyond 75°C multistep weight loss occurred, indicating thermal decomposition. The TGA of capsaicin loaded ethosomal formulation showed curve given in fig. 7. The curve for phospholipon 90G is shown in fig. 8.

The TGA curve for phospholipid showed initiation of decomposition after 275°C and complete decomposition at 390°C.

**DISCUSSION**

Capsaicin is one of the alkaloids naturally occurring in chilli peppers. Its pharmacological action through agonist effect on TRPV1 first stimulates the sensory neurons followed by a refractory state that desensitizes the pain receptors. The desensitization occurs due to depletion of substance P from sensory nerve endings. However, these effects are reversed when capsaicin is discontinued. That is why, topical capsaicin is considered as adjuvant therapy for diabetic neuropathy, post-herpetic neuralgia and osteoarthritis (Rains and Bryson, 1995). Entrapment of capsaicin into ethosomal vesicles can promote enhanced penetration of the drug at the receptor site as it has been studied from the skin invasion studies of ethosomes by scientists. Ethosomes have proven efficiency in penetrating the stratum corneum and enhancing drug transport to the skin attributed to ‘Push effect’ and ‘Pull effect’ of ethanol (Dubey et al., 2007b). In this study, the effect of different preparation methods of ethosomes on the vesicle size and surface morphology, poly-disparsity index, zeta-potential, stability and capsaicin entrapment capability in ethosomes was examined. Four procedures were tested for formulation of ethosomes that are; hot method, cold method, classic method and injection method. No size reduction steps like sonication or filtration were involved.

The ethosomal vesicle size varies from a few nanometers to micrometres and is subjective to the composition of formulation (Verma and Pathak, 2010). The SEM micrographs showed vesicle shape close to spherical and regular in morphology obtained with hot method (fig. 1) while ethosomes prepared through injection method were of smaller size but clumped and varied widely (fig. 4). The cold and classic method of ethosomal formulation resulted in vesicles with larger size ranging up to 400nm as analyzed by ImageJ software (fig. 2, 3 respectively).
This difference in vesicle size may be because ET1 and ET14 contained high ethanol content than ET8 and ET11. A similar pattern of reduction in vesicle size with enhancing ethanol concentration has been reported by Touitou et al. (Dayan and Touitou, 2000) and other researchers (Rakesh and Anoop, 2012). Ethanol considerably reduces the thickness of vesicular membrane most likely to the interpenetration of hydrocarbon chain portion (ethanol) of the vesicles’ phospholipid bilayer. Also ethanol confers a net negative charge to the vesicular system imparting a steric stabilization effect on the vesicles that ultimately reduces the vesicle size (Godin and Touitou, 2004). The poly-dispersity index (PI or PDI) was analyzed as a degree of homogeneity/ uniformity of the vesicular suspension. A minor value of PI (<0.1) designates a homogenous vesicle population, while a higher PI (>0.3) shows increasing heterogeneity (Verma and Fahr, 2004). The Z-average size measured by Malvern Zeta sizer analyzed size of 129.6nm for ET1 (hot method) with a uniformity of 0.162 that can better explain the homogenous appearance of the prepared capsaicin formulation. This can present a sufficient opportunity to capsaicin loaded ethosomes to obtain a better skin infiltration profile since the vesicle size has evident effect on permeation parameters (Verma et al., 2003). ET11 also showed good homogeneity profile of 0.159 but the Z-average size was larger than ET1. Vesicles prepared by cold (ET8) and injection (ET14) method varied with moderately broad vesicular distribution as shown in table 4. The injection method yielded vesicles of small size ranging from 10-30nm but the distribution was heterogeneous.

The charge (zeta-potential) of the ethosomal vesicle is a significant factor that can affect equally the vesicular characteristics like stability, but also skin and vesicle interaction. A high zeta-potential (positive or negative) is effective for physical stability since it helps in avoiding aggregation of vesicles pertaining to electrostatic repulsion (Dubey et al., 2007a). Formulation ET8 showed the highest zeta potential that is why it remained stable for 2 months at both temperatures. ET1 showed an unexpectedly low value of 4.28+4.7 mV potential. The stability of ET1 formulation remained adequate over the period of storage. Therefore this low value of zeta potential may be a consequence of human error or wrong selection of blank solvent.

The vesicles size and entrapment capability of ethosomal carrier are supreme parameters influencing considerations in drug permeation across the skin. The entrapment efficiency of ethosomes was increased with growing concentration of ethanol. Table 1 shows different ethanol concentrations used in the formulation of ethosomes and Table 2 shows the varying effect of ethanol from 30-70% range on the drug entrapment capability of vesicles. With hot method, ET1 showed highest %EE of 81.03% with an ethanol content of 70%. Reducing the quantity of ethanol and increasing phospholipid decreased the amount of drug entrapped in ethosomes (ET1-ET7). The method of preparation, however, did not seem to have widely affected the drug entrapment since both cold and classic method yielded ethosomes with 59.25% and 60% of drug entrapment respectively, keeping all ingredients in same ratio. But classic method of preparation produced vesicles of greater uniformity than the cold method. This may be due to greater shear applied via homogenizer used in classic method.

Results of stability studies shown in table 3 revealed drug leakage from the formulations stored at room temperature. The observed leakage was conceivably due to the chemical degradation of phospholipon (phospholipid) at high temperature, paving the way to weaknesses in membrane packing and reduced drug holding capability.
Also higher ethanol content caused leaking effect on ethosomal membrane (Limsuwan et al., 2017) as it occurred in ET1 formulation at 4°C and 25°C. Therefore, for optimum physical and chemical stability these vesicular formulations should be stored under refrigerator conditions (Rakesh and Anoop, 2012). No aggregation occurred in ET8, ET11 and ET14 formulations under both conditions.

FT-IR studies were conducted to indicate any interaction between drug and excipients. Spectrums of capsaicin, phospholipon 90G and ethosomal formulation indicated same stretching of bonds at respective wave numbers and no sign of interaction occurred that relates to chemical changes in the ethosomal formulation. The prominent peaks for drug (capsaicin) and analyzed formulation were sustained with the theoretical evaluation with reference to the functional groups (Iizhar et al., 2016). The FT-IR spectrums also depicted the entrapment of drug in the ethosomal vesicles. Similarly, DSC thermogram showed the presence of eutectic mixture. The lowering effect of ethanol on the transition temperature of phospholipid was observed via calorimeter and the results are supportive.

**CONCLUSION**

In the following study capsaicin entrapped ethosomes were effectively formulated using phospholipon 90G and ethanol in varying quantities employing four different methods of preparation. The entrapment efficiency, vesicle size and zeta-potential values were found to be mainly dependent on vesicular components and less affected by the method of preparation employed. However, the degree of shear employed in the preparation by different methods affects the uniformity of formulation. All four methods resulted in a formulation with good homogeneity, that is <0.3. The addition of permeation enhancers like tween 80, propylene glycol and polyethylene glycol-400 to selective formulations affected the entrapment efficiency. The FTIR analysis showed the compatibility of the drug with excipients and no peak of chemical interaction was observed among the drug and ingredients. The entrapment efficiency, vesicle size and uniformity of distribution were best obtained with hot method of ethosomal preparation. Only the zeta-potential measurements showed unexpected result. This may be due to human error or choice of nonpolar solvent as blank. All other parameters supported the hot method of preparation of capsaicin ethosomes. This study can contribute as a platform in formulating a better transdermal drug delivery system capable of delivering capsaicin effectively to the target site. Capsaicin ethosomes are stable under refrigerator conditions and their incorporation into gel or cream vehicle can result in effective treatment than the available commercial products.

**REFERENCES**


