NANOEMULSION SYSTEM FOR TRANSDERMAL DELIVERY
OF TAMOXIFEN CITRATE: DESIGN, CHARACTERIZATION, EFFECT
OF PENETRATION ENHANCERS AND IN VIVO STUDIES

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The aim of the present study was to develop and characterize nanoemulsion formulation of tamoxifen citrate (TAM) for transdermal drug delivery system. Various oil-in-water nanoemulsions were prepared by the aqueous titration method. The prepared nanoemulsions were subjected to thermodynamic stability study and characterized for droplet size, transmission electron microscopy (TEM), viscosity studies. FTIR study was subjected to ensure the compatibility among its ingredients. Transdermal permeation of tamoxifen citrate through rat skin was determined by Keshary-Chien diffusion cell. Penetration enhancers effects on the skin permeation of TAM were investigated. Pharmacokinetic and pharmacodynamic parameter was determined on optimized formulations. Mean globule size and viscosity was found to be lowest in A1 formulation. TEM demonstrated spherical particle morphology and FTIR study revealed the compatibility among the ingredients. Significant increase in steady – state flux (Jss) was observed in optimized A1 and DA1 nanoemulsion. Of the various essential oils added, dill oil had the best enhancing ability. In pharmacokinetics study, transdermally applied nanoemulsion increased in bioavailability as compared to oral tablet formulation. In pharmacodynamic study RTV in mice receiving the optimized nanoemulsions was significantly reduced compared to control. Developed nanoemulsions can be used as potential vehicle for enhancement of bioavailability of TAM through transdermal application.

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Keywords: Nanoemulsion; transdermal delivery; penetration enhancers; in vivo studies

1. Introduction

Tamoxifen citrate (TAM), an estrogen receptor antagonist is known to be a drug of choice for hormone sensitive breast cancer and indicated for treatment of estrogen receptor-positive tumors in the premenopausal population (1,2). Tamoxifen is generally administered through oral and parenteral route and undergoes extensive hepatic metabolism after oral administration in humans and usual oral dose of tamoxifen is 10 mg twice a day. The steady - state plasma concentration of 77–274 ng ml has been reported for TAM (3). Despite being quite effective on oral administration, TAM exhibits certain side effects like distaste for food, abdominal cramps, nausea and vomiting. However, its other infrequent side effects include endometrial carcinoma, ocular problems, thromboembolic disorders and acquired drug resistance on long-term therapy (4,5,6). Therefore, developing a therapeutic system to provide a transdermal delivery is beneficial. Transdermal drug delivery may offer an alternative for the delivery of drug because it avoids the
problems of gastrointestinal intolerance, avoids first pass liver metabolism and eliminates the need for intravenous access (7). The recent trend for the enhancement of solubility/bioavailability is the lipid based system such as microemulsions, nanoemulsions, solid dispersions, solid lipid nanoparticle and liposomes etc. This is also the most advanced approach commercially, as formulation scientists increasingly turn to a range of colloids-based solutions to improve drug solubility and bioavailability. One of the most promising techniques for enhancement of transdermal permeation of drugs is the microemulsion or nanoemulsion technique (8,9). Nanoemulsions are thermodynamically stable transparent (translucent) dispersions of oil and water stabilized by an interfacial film of surfactant and cosurfactant molecules having the average droplet size of 10 to 140nm (10-12). Nanoemulsions have been reported to make the plasma concentration profiles and bioavailability of poorly soluble drugs more reproducible (13-17). Many studies have shown that nanoemulsion formulations possess improved transdermal and dermal delivery properties both in vitro (18-30), as well as in vivo (31-35). The high solubilizing capacity of nanoemulsion enables them to increase the solubility of poorly water-soluble drugs. Both increase in solute concentration and the tendency of the drug to favor partitioning into the stratum corneum make nanoemulsion a useful vehicle to enhance transdermal drug permeability (36). It has been revealed by literature review that the studies conducted so far on the TAM nanoemulsion for transdermal drug delivery system restricted to the pharmacokinetic and in vivo efficacy. Thus, the objective of the present study was to develop and characterize nanoemulsion formulation of TAM for transdermal drug delivery system. Another objective of this study was to investigate the effect of penetration enhancers and to increased bioavailability of TAM through transdermal application. Furthermore, for the optimized formulation in vivo studies were also conducted.

2. Materials and methods

Materials
TAM was a gift sample from Biochem Pharmaceutical Ltd (Mumbai, India). Oleic acid, Jojoba oil, Sesame oil, Coconut oil, Castor oil, Dill oil, Lemon oil, Coriander oil was purchased from S.D Fine Chemical(Mumbai, India). Labrafil M 1944CS was gift sample from Gattefosse (France). Cremophore RH 40, Tween 80 was gift sample from Cadila Health Care Ltd (Ahmedabad, India). Ethanol, Butanol, Propanol, Methanol and Formic acid (HPLC) grade were purchased from E-Merck (Mumbai, India). Distilled water was purchased freshly from Chetak Distillery Ltd Rahuri, India. All other chemical and reagent used in the study were of analytical reagent grade.

Methods
Screening of Excipients
The solubility of TAM in various oils (Oleic acid, Jojoba oil, Coconut oil, Sesame oil, Castor oil) surfactants (Cremophore RH 40, Labrafil M 1944CS,Tween-80) and cosurfactants (Ethanol, Butanol, Propanol) was determined by dissolving an excess amount of TAM in 2 mL of each of the selected oils, surfactants and cosurfactants in 5-mL stoppered vials. Excess amount of TAM was added to each 5-mL stoppered vial and mixed using a vortex mixer. The vials were then kept at 37 ± 1.0 °C in an isothermal shaker (Nirmal International, India) for 72 hours to get to equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.45-μm membrane filter. The concentration of TAM was determined in each solution by UV spectrophotometer at 272 nm.

Pseudo-ternary phase diagram
On the basis of the solubility studies, oleic acid was selected as the oil phase. Cremophore RH 40 and ethanol were selected as surfactant and cosurfactant, respectively. Distilled water was used as an aqueous phase. Surfactant and cosurfactant (S_{mix}) were mixed at different mass ratios (1:1, 1:2, 2:1). These ratios were chosen in increasing concentration of surfactant with respect to cosurfactant and increasing concentration of cosurfactant with respect to surfactant for a detailed study of the phase diagrams. For each phase diagram, oil and S_{mix} at a specific ratio was mixed thoroughly at different mass ratios from 1:9 to 9:1 in different glass vials. Nine different
combinations of oil and Smix, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, were made so that maximum ratios were covered for the study to delineate the boundaries of phases precisely formed in the phase diagrams. Pseudo ternary phase diagrams of oil, Smix and aqueous phase were develop using the aqueous titration method (37). Slow titration with aqueous phase was perform for each mass ratio of oil and Smix and visual observations were made for transparent and easily flowable o/w nanoemulsions. The physical state of the nanoemulsion was marked on a pseudo-three-component phase diagram with one axis representing the aqueous phase, the second one representing oil and the third representing a mixture of surfactant and cosurfactant at a fixed mass ratio.

Selection and preparation of nanoemulsion formulation

From phase diagram study, a different formula was selected from the nanoemulsion region so that drug could be incorporated into oil phase. 5 % w/w of TAM, which was kept constant in all the selected formulations were subjected to different thermodynamic stability study.

Thermodynamic stability studies

To overcome the problem of metastable formulation, thermodynamic stability tests were performed. Selected formulations were centrifuged at 3500 rpm for 30 minutes. Those formulations which did not show any phase separations were taken for heating and cooling cycle. Six cycles between refrigerator temperature of 4°C and 45°C for 48 hours were done. The formulations that were stable at these temperature were subjected to the freeze-thaw cycle test. Three freeze-thaw cycles were done for the formulations between -21°C and +25 ºC. The formulations that survived dispersion stability tests were selected for further studies and are shown in Table 1.

Table 1. The composition of selected formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Smix ratio</th>
<th>Oil: Smix ratio</th>
<th>% Wt/Wt of Components in nanoemulsion formulation</th>
<th>Drug %w/w</th>
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</thead>
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<td></td>
<td></td>
<td>Oil</td>
<td>Water</td>
<td>Drug</td>
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<td></td>
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<td>C1</td>
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<td>1:9</td>
<td></td>
<td>6.4</td>
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<td>DA1 (5% Dill oil)</td>
<td>1:1</td>
<td>1:9</td>
<td></td>
<td>5.18</td>
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<tr>
<td>LA1 (5% Lemon oil)</td>
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<tr>
<td>CA1 (5% Coriander oil)</td>
<td>1:1</td>
<td>1:9</td>
<td></td>
<td>5.18</td>
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</tbody>
</table>

Characterization of nanoemulsions

Nanoemulsion droplet size analysis

Droplet size distribution of optimized nanoemulsion was determined by photon correlation spectroscopy, using a Delsa Nano-C (Beckman Coulter Instruments). Light scattering was monitored at 25°C at a scattering angle of 90°. Nanoemulsion was suitably diluted with distilled water and filtered through 0.22 μm membrane filter to eliminate multi scattering phenomena. The diluted sample was then placed in quartz cuvette and subjected to droplet size analysis.

Transmission electron microscopy (TEM)

Morphology and structure of the nanoemulsion were studied using transmission electron microscopy (TEM) (Philips CM-10, USA) operating at 200 kV and capable of point-to-point resolution. To perform the TEM experiments, a drop (50 μL) of the nanoemulsion was suitably diluted with distilled water (1:100), filtered through 0.22-μm filter paper and applied on carbon-coated grid with 2% phosphotungstic acid. It was left for 30 sec for drying purpose. The dried coated grid was taken on a slide and covered with a cover slip for TEM observations.
Viscosity measurement

The viscosity of nanoemulsion was determined using Brookfield cone and plate viscometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) at 25± 0.5º C.

In vitro skin permeation studies

Preparation of rat abdominal skin

The male albino rats were sacrificed by excess chloroform inhalation (Institutional Animals Ethics Committee, reg. no-MESCOP-1211/ac/08/CPCSEA, approved the protocol). The abdominal shaved skin was excised from the animal subcutaneous tissue. The full thickness skin thus prepared was soaked in distilled water at 60°C for 60s, followed by careful removal of epidermis. Skin was dried in desicator at 25%RH and wrapped in aluminum foil and stored at 4 ºC[38].

Procedure

*In vitro* skin permeation studies were performed on a modified Keshary Chien-diffusion cell with an effective diffusional area of 1.76 cm² and 35 mL of receiver chamber capacity, using rat skin. The skin was brought to room temperature and mounted between the donor and receiver compartments of the Keshary-Chien diffusion cell where the stratum corneum side was facing the donor compartment and the dermal side was facing the receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with phosphate buffer saline (PBS) pH 7.4. The receiver fluid was stirred with a magnetic rotor at a speed of 100 rpm and temperature was maintained at 37 ± 1 ºC. The whole PBS was replaced with a fresh one at every 30 min to stabilize the skin. It was found that the receiver fluid showed a negligible peak area after 2.5 h and beyond indicating complete stabilization of the skin. After complete stabilization of the skin, 1 mL nanoemulsion formulation was placed into the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular half intervals, filtered through 0.45-mm membrane filter and analyzed for drug content by UV Spectrophotometer at 272 nm. To improve the skin permeation rate of the TAM, the selected essential oil (Dill oil, Lemon oil, and Coriander oil) were further added to the optimized nanoemulsion formulation, at a concentration of 5% v/v, and their effects on the skin permeation of TAM were evaluated shown in Table 2.

<table>
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<tr>
<th>Formation code</th>
<th>S&lt;sub&gt;mix&lt;/sub&gt; ratio</th>
<th>Oil: S&lt;sub&gt;mix&lt;/sub&gt; ratio</th>
<th>% Wt/Wt of Components in nanoemulsion</th>
<th>Drug %w/w</th>
</tr>
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<tr>
<td>A1(Control)</td>
<td>1:1</td>
<td>1:9</td>
<td>5.18 48.64 46.18 5</td>
<td></td>
</tr>
<tr>
<td>DA1 (5% Dill oil)</td>
<td>1:1</td>
<td>1:9</td>
<td>5.18 48.64 46.18 5</td>
<td></td>
</tr>
<tr>
<td>LA1(5% Lemon oil)</td>
<td>1:1</td>
<td>1:9</td>
<td>5.18 48.64 46.18 5</td>
<td></td>
</tr>
<tr>
<td>CA1(5% Coriander oil)</td>
<td>1:1</td>
<td>1:9</td>
<td>5.18 48.64 46.18 5</td>
<td></td>
</tr>
</tbody>
</table>

Fourier transfer infra red spectroscopy study

To study the compatibility between TAM and excipient study carried out using FTIR. FTIR spectra of all samples of TAM, blank sample without drug and all formulations were recorded on Nicolet Magna 550 (USA) FTIR spectrometer using AgCl plates, in the frequency range 4000-400 cm⁻¹.

Skin irritation test

The authors followed the “Guideline of the institutional Animals Ethics Committee” for this experiment. The hair on the dorsal side of wistar albino rats was removed by clipping one day before the start of the experiment [39]. The rats were divided into four group (n=6).Group I was the control (i.e., without formulation), Group II and III received optimized nanoemulsion formulations and group IV received 0.8% v/v aqueous solution of formalin as a standard irritant
New formulation or new formalin were applied daily for seven days. Finally, the application site was graded according to a visual scoring scale, always by the same investigator.

**Pharmacokinetic study**

Institutional Animals Ethics Committee, reg.no-MESCOP-211/ac/08/CPCSEA, approved the protocol. Male wistar rats were stores under standard laboratory conditions (temperature 25 ±2˚C and relative humidity of 55± 5 %RH). The rats were kept in polypropylene cages (6/cage) with free access to standard laboratory diet. About 15cm² of skin was shaved on the abdominal side of rats in each group except group treated with marketed tablet formulation. They were fasted for the period of 12 h for observations of any unwanted side effects.

The rats were divided into 3 groups, each containing 6 rats. Group I received A1 formulation transdermally, group II received DA1 transdermally and group III received marketed tablet orally. The dose for transdermal and oral was similar. The rat were anaesthetized using light ether anesthesia and blood sample (0.5 ml) were withdrawn from the tail vein of rat at 0( pre-dose), 1, 2, 3, 4, 8 and 12h after administration of formulation oral and transdermal. Blood samples collected at predetermined intervals during the *in vivo* study in microcentrifuge tubes in which 6 mg of EDTA was added as an anticoagulant. The blood collected was mixed with EDTA properly and centrifuged at 5000 rpm for 25 min for separation of plasma. The separated plasma was stored at -21˚C until drug analysis was carried out using high performance liquid chromatographic (HPLC). A Jasco HPLC LC-2000 plus series equipped with PU-2080plus reciprocating intelligent pump, Jasco UV-2075 plus detector, Borwin chromatography software version 1.21 was used. Analysis was performed on a C₁₈ column. The mobile phase consist of 0.1% formic acid in pH 2.5 and added to methanol in the ratio of 40:60 (v/v). The mobile phase was delivered at the flow rate of 1.0 ml/min and detection was performed at 276 nm. The injection volume was 20 µl. The concentration of unknown plasma sample was calculated from calibration curve plotted between peak area.

**Pharmacokinetic data analysis**

The plasma concentration of TAM at different time intervals was subjected to pharmacokinetic (PK) analysis to calculate various parameters like maximum plasma concentration (Cₘₐₓ), time to reach maximum concentration (Tₘₐₓ), area under the plasma concentration-time curve (AUC₀→ₜ ), elimination half life (T₁/₂) and eliminate rate constant (Kₑ).

The values of Cₘₐₓ and Tₘₐₓ were read directly from the arithmetic plot of time and plasma concentration of TAM. The AUC₀→ₜ was calculated by using the linear trapezoidal method. T₁/₂ was determined using the following equation(1):

$$T_{1/2} = \frac{0.693}{K_e}$$

(1)

Kₑ is the elimination rate constant and was determined from the slope of linear portion of graph plotted between logarithm of plasma concentration and time.

The relative bioavailability of TAM after the transdermal administration per the oral administration was calculated using following equation(2)

$$F\% = \frac{AUC \text{ sample}}{AUC \text{ oral}} \times \frac{\text{Dose oral}}{\text{Dose sample}} \times 100$$

(2)

**In-Vivo efficacy anticancer study in xenograft mouse model**

The NOD-scid mouse used for this study, is a recognized model for antitumor testing, because unlike normal mice, they lack a full functional immune system and therefore do not developed response against experiment induced tumor [41]. Study was carried out at Cancer Research Institute in Advanced Centre for Treatment Research and Education in Cancer. Mumbai of Tata Memorial Centre having CPCSEA registration no- 65/1999. Tumor were induced in 5 week old mice by subcutaneous injection of human breast cancer xenograft MCF7 cell in concentration of 3x 10⁶ cell/ml. Mice were monitored every day from the time of tumor cell injection for any appearance of tumor. An electric digital caliper was used to measure the diameter of the tumor. Tumor size was calculated using following formula (3):

$$Tumor \text{ Volume} = (cc) = \left[ \frac{(d₁+d₂)}{2}/3 \right] \times 0.5236/1000$$

(3)
Where \( d \) is diameter.

The tumor volume on a day of measurement/ tumor volume on day 1 is expressed as Relative Tumor Volume (RTV). 18 mice were divided randomly into 6 groups. The treatment groups were control (untreated), A1 and DA1 formulation (0.6mg/kg) transdermal application. Treatment was transdermally applied every 2 days for 23 days.

**Statistical analysis of data**

The results were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test using GraphPad Instat Software (GraphPad Software Inc., CA USA).

### 3. Results

**Screening of Excipients**

The solubilization of TAM was found to be highest in oleic acid (5.26 ± 0.408 mg/ml) as compared to other oils as shown in Figure 1. Hence, oleic acid was selected as the oil phase for the development of optimal formulation. The proper selection of surfactant and cosurfactant combination (\( S_{mix} \)) will contribute to the formulation of nanoemulsion and improving the stability. Although, the concentration of \( S_{mix} \) in the final formulation is smaller, the solubility of drug in \( S_{mix} \) will be additional contribution to the drug loading in nanoemulsion formulation. \( S_{mix} \) (Cremophore RH40: Ethanol) selected in the study had comparatively the highest solubility of drug in respective components. Figure 1 shows that solubility of TAM was highest in cremophore RH40 (11.9 ±1.629 mg/ml) and ethanol (5.09 ± .083 mg/ml) components compared to other surfactant and cosurfactant respectively.

**Pseudo-ternary phase diagram**

Phase diagram were constructed to show the influence of the S/Cos on the area of existence of stable o/w nanoemulsion consisting TAM, oleic acid, cremophore RH 40, ethanol and water (Figure.2). The relationship between the phase behavior of a mixture and its composition can be studied with the aid of a phase diagram [16]. Pseudoernary phase diagrams were constructed separately for each \( S_{mix} \) ratio so that o/w nanoemulsion region could be identified and nanoemulsion formulation could be optimized. In \( S_{mix} \) (1:1), it was observed in the phase diagram that maximum concentration of oil that could be solubilized in the phase diagram was 12.09% using 29.45% of \( S_{mix} \). However, when concentration of ethanol with respect to cremophore RH 40 was increased (1:2), the nanoemulsion area was decreased (Figure.2b) compared to \( S_{mix} \) ratio (1:1).

![Graphical representation of solubility of tamoxifen citrate in selected oily phases](image1.png)

Fig. 1. Graphical representation of solubility of tamoxifen citrate in selected oily phases (jojoba oil, coconut oil, sesame oil, castor oil, oleic acid), surfactant (cremophore RH 40, labrafil M 1944CS, tween 80) and cosurfactant (ethanol, butanol, ethanol: butanol, propanol). Mean ± SD, \( n = 3 \).
As surfactant concentration was increased in the $S_{\text{mix}}(2:1)$ a higher nanoemulsion region was observed (Fig. 2c).

![Diagram](image)

Fig. 2. Pseudo-ternary phase diagrams showing the o/w nanoemulsion regions of oleic acid (oil), cremophore RH 40 (surfactant), ethanol (cosurfactant) at different $S_{\text{mix}}$ ratios: a) $S_{\text{mix}}$ 1:1 b) $S_{\text{mix}}$ 1:2, c), $S_{\text{mix}}$ 2:1.

**Selection of nanoemulsion formulations**

From pseudoternary phase diagrams, the formulations in which the amount of oil phase completely solubilized the drug and visual observations were made for transparent and easily flowable o/w nanoemulsion were selected for the further thermodynamic stability study.

**Thermodynamic stability studies**

The formulations were tested for their physical stability by using centrifugation, heating-cooling cycle and freeze-thaw cycle. Only those formulations which survive thermodynamic stability tests were selected for further study (Table 1).

**Characterization of nanoemulsion**

Nanoemulsions were characterized by a droplet size analysis (Table 3), which shows that mean droplet size of A1 (21nm) and penetration enhancers containing formulation DA1 nanoemulsion having droplet size 25.5 nm was lower compared to other nanoemulsions studied. In Figure 3 it is shown the statistical graph measurement by model distribution. Among the formulations containing $S_{\text{mix}}(1:1)$, the mean droplet size increased as the concentration of oil was increased, and was also increased relatively to same extent as the ratio between surfactant and cosurfactant were varying (i.e 2:1 ,1:2). Addition of essential oils as penetration enhancers has increased the mean droplet size as compared to control A1 formulation. All the formulation had droplets in the nano range, which is very clear from the low polydispersity values shown in Table 3. Polydispersity is the ratio of standard deviation to mean droplet size, so it indicates the uniformity of dispersity, lower the uniformity of the droplet size seen in all formulations. The polydispersity of formulation A1 was lower (0.115) as compared to other formulations. To obtain further information regarding the morphology and structure of nanoemulsions, transmission electron microscopy has been conducted. The TEM analysis revealed that nanoemulsions droplet of all formulations were spherical in shape, discrete with size in nanometer range (<100nm).
Droplet size of A1 and DA1 nanoemulsion were shown in Figure 4A and spherical in shape as shown in Figure 4B, as TEM is capable of point to point resolution. In case of A2 formulation droplet size was greater as compared to other formulations (A1, A3, B1, C1, DA1, LA1, CA1). This observation was consistent with that obtained in the globule size analysis using photon correlation spectroscopy. Viscosity of nanoemulsions was determined and has shown that formulation A1 had the least viscosity (40.17 ± 1.00 cP) compared to other formulations (Table 3). This may be due to the lower oil content.

Table 3. Droplet size, polydispersity, viscosity and flux of tamoxifen citrate nanoemulsions

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Droplet Size (nm)</th>
<th>Polydispersity</th>
<th>Viscosity (cP)</th>
<th>Flux Jss (a) (µg/cm²/h)</th>
</tr>
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<tr>
<td>A1</td>
<td>21.0</td>
<td>0.115</td>
<td>40.17 ± 1.00</td>
<td>97.64±7.997</td>
</tr>
<tr>
<td>A2</td>
<td>117.9</td>
<td>0.245</td>
<td>238.66 ± 1.15</td>
<td>57.77±1.931</td>
</tr>
<tr>
<td>A3</td>
<td>105.5</td>
<td>0.205</td>
<td>245.33 ± 1.52</td>
<td>46.34±3.858</td>
</tr>
<tr>
<td>B1</td>
<td>23.3</td>
<td>0.282</td>
<td>41.66 ± 1.52</td>
<td>91.56±5.871</td>
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<tr>
<td>C1</td>
<td>58.1</td>
<td>0.344</td>
<td>40.66 ± 0.52</td>
<td>61.09±4.7957</td>
</tr>
<tr>
<td>DA1 (5% Dill oil)</td>
<td>25.5</td>
<td>0.306</td>
<td>40.23 ± 1.15</td>
<td>124.96±14.02</td>
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<tr>
<td>LA1 (5% Lemon oil)</td>
<td>27.9</td>
<td>0.296</td>
<td>40.45 ± 1.5</td>
<td>108.25±3.90</td>
</tr>
<tr>
<td>CA1 (5% Coriander)</td>
<td>65.2</td>
<td>0.311</td>
<td>40.66 ± 1.52</td>
<td>106.00±0.53</td>
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\(a\) Mean ± SD, \(n = 3\).

Fig. 3. Photon correlation spectroscopy of nanoemulsion A1 and DA1
In vitro skin permeation studies

In vitro skin permeation experiments were performed using rat abdominal skin showed that permeation was highest in formulation A1 and lowest in A3 formulation (Figure 5). Skin permeation profile of A1 was significantly different when compared with other formulations (p<0.05). Steady state flux (Jss) of A1 formulation was (97.64±7.997 μg/cm²/h) which is significant different (p<0.05) when compared with other formulations as shown in Table 3. To improve the skin permeation rate of TAM from the nanoemulsions, various terpenes containing essential oils were used (dill oil, coriander oil, lemon oil) and were added to nanoemulsion formulation A1. Thus, the addition of permeation enhancers to nanoemulsion formulations caused not much change in their physicochemical properties such as droplet size, viscosity, drug content as shown in Table 3. When essential oils used in concentration of 5%, the droplet sizes of the DA1 (5% dill oil) nanoemulsions were lowest 25.5 nm as compared to lemon oil and coriander oil, but increased to about 65.2 nm in CA1 nanoemulsion containing coriander oil as penetration enhancer. The permeation profiles of nanoemulsion containing essential oil are shown in Figure 6. The most pronounced enhancing effect on the skin permeation of TAM was shown by dill oil due to small droplet and less viscosity. The skin permeation profile of DA1 (5% Dill oil) were significantly different from A1 (control) (p<0.05).

Fig. 4. TEM photograph A and B showing measurement of droplet size less than 100nm and having droplet uniform and spherical in shape of A1 and DA1 nanoemulsion.
Fourier transfer infrared spectroscopy

Drug excipient interaction study is one of the most important parameter, which depicts much information regarding the stability of formulation, drug release from them. The IR spectral analysis of TAM alone showed that the principal peaks were observed at wave numbers 3405.50, 1378.13, 1050.83 cm\(^{-1}\), confirming the purity of the drug. The major peaks of TAM were observed such as aliphatic alcohol O-H stretch (3405.50 cm\(^{-1}\)), phenolic C-O stretch (1378.13 cm\(^{-1}\)), and C-O stretch of ether (1050.83 cm\(^{-1}\)) in all nanoemulsions formulation. These result suggested that there was absence of drug degradation or drug excipient molecular interaction in all formulation.
Skin irritation test
The skin irritation test of optimized nanoemulsion A1 and DA1 resulted in a score of less than 2 (erythema and edema) as shown in Table 4. According to Draize et al, compounds producing scores of 2 or less are considered negative (no skin irritation)(42).

Table 4: Skin irritation study of optimized transdermally citrate nanoemulsion

<table>
<thead>
<tr>
<th>Rat No</th>
<th>Erythema</th>
<th>Edema</th>
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<td>0.83±0.9</td>
<td>1±0.6</td>
<td>0.5±0.5</td>
<td>1.16±0.7</td>
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*Erythema scale: 0 is none, 1 is slight, 2 is well defined, 3 is moderate, 4 is scar formation.
**Edema scale: 0 is none, 1 is slight, 2 is well defined, 3 is moderate and 4 is severe.

Pharmacokinetics study
Plasma concentration of TAM from formulation A1, DA1 and marketed tablet at different time interval was determined by HPLC method. The graph between plasma concentration of TAM and time was plotted for each formulation. Plasma concentration profile of transdermally applied A1 and DA1 nanoemulsion showed greater bioavailability of drug absorption than the oral tablet formulation shown in Table 4. Peak plasma concentration of TAM in A1 and DA1 and oral formulation was 28.92±3.84, 34.25±4.81 and 39.05±6.60 µg/ml respectively. AUC₀→ₜ in formulation A1, DA1 and oral were 118.26±2.8, 141.28±3.2 and 91.91±2.46 µg.h/ml respectively. The Cₘₐₓ, tₘₐₓ and AUC₀→ₜ of A1 and DA1 were significantly different from oral formulation (p<0.05). There were no significant variation in Kₑ and T₁/₂ for A1 and DA1 when compared with oral formulation as shown in Table 5.

Table 5. Pharmacokinetic parameter and Relative bioavailability of tamoxifen citrate from A1, DA1 and marketed tablet.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Cₘₐₓ (µg/ml) ± SD</th>
<th>tₘₐₓ (h)</th>
<th>AUC₀→ₜ (µg.h/ml) ± SD</th>
<th>Kₑ (h⁻¹)</th>
<th>T₁/₂</th>
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<td>A1</td>
<td>28.92±3.84</td>
<td>3</td>
<td>118.26±2.8</td>
<td>0.16</td>
<td>4.29</td>
<td>128</td>
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<tr>
<td>DA1</td>
<td>34.25±4.81</td>
<td>3</td>
<td>141.28±3.2</td>
<td>0.14</td>
<td>4.62</td>
<td>153</td>
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<td>39.05±6.60</td>
<td>2</td>
<td>91.91±2.46</td>
<td>0.16</td>
<td>4.23</td>
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</table>

*p<0.05 when compared with tablet formulation.

In vivo Pharmacodynamic study in xenograft mouse model
To investigate the in vivo efficacy, mice were injected subcutaneously with MCF-7 cancer cell and than treated twice daily by transdermal application for 23 days with A1 and DA1 nanoemulsion formulation (0.6 mg/kg) after growth of the cancer. The tumor size increased significantly in the control group after 23 days in comparison with the initial tumor size. The relative tumor volume after 23 days was significantly smaller in the A1 (51.8) and DA1 (36.4) nanoemulsion group in comparison with those in the control (73.4) as shown in Figure 7. The visual observation and change in tumor size in mice during administration of A1 and DA1
nanoemulsion in comparison with control groups are shown in Figure 7 where skin discoloration and wounds were not observed in treated nanoemulsion groups as compared to control.

**Fig. 7. Relative tumor volume in mice after application of tamoxifen citrate nanoemulsion treatment for 23 days and tumor appearance in mice.**

4. Discussion

Lipophilic drugs are preferably incorporated in o/w nanoemulsions through the surface area of skin, the efficiency of the dosage form applied on the skin depends on the flux of the drug across the skin. Flux of the drug, that a formulator can alter, depends on the formulation components. Oil phase of nanoemulsion, in which the lipophilic drug is solubilized, is an important criteria in the selection of formulation components. The physicochemical properties of TAM suggest that it has good potential for topical drug delivery. TAM has optimum partition coefficient and it suggests that TAM has sufficient lipophilicity to be formulated in to transdermal systems[43].

Nanoemulsion development was initiated by screening different oils, surfactant and cosurfactant ingredients for solubility of TAM. Nanoemulsion regions of existence determined using pseudo ternary phase diagrams. In $S_{mix}$ (1:1), it was observed in the phase diagram that maximum concentration of oil that could be solubilized in the phase diagram was 12.09% using 29.45% of $S_{mix}$. As surfactant concentration was increased in the $S_{mix}$ (2:1) a higher nanoemulsion region was observed. The presence of ethanol decreases the ending stress of interface and makes the interfacial film sufficiently flexible to take up different curvatures required to form nanoemulsions over a wide range (16). Perhaps because of fluidity of the interface, thereby increasing the entropy of the system. There may be greater penetration surfactant monomers [44,45]. However, when concentration of ethanol with respect to cremophore RH 40 was increased (1:2), the nanoemulsion area was decreased (Figure.2b) compared to $S_{mix}$ ratio (1:1). The decrease in nanoemulsion area is possible due to the amount of micelles and consequently decreases the solubilization capacity of nanoemulsion (28). Ethanol is a polar solvent having the tendency to highly incorporate into water, and the relatively low ethanol content in the nanoemulsion system decreases the hydrophilicity of the $S_{mix}$ so the area of o/w nanoemulsion was decreased. Nanoemulsions are thermodynamically and physically stable systems and are formed at a particular concentration of oil, surfactant and water, making them stable to phase separation, creaming or cracking (46,47). These nanoemulsion formulations were than evaluated visually for their physical stability. No sign of phase separation or turbidity were observed for five formulations, indicating good physical stability.

Photon correlation spectroscopy results are in agreement with transmission electron microscopy observations, evidencing that dispersions are characterized by well shaped vesicles.
Furthermore, the results revealed that the presence of TAM does not seem to affect the ultrastructural of nanoemulsion disperse phase. Formulation A1 had the lowest viscosity as compared to other nanoemulsions and may be due to the lower viscosity of oleic acid. Viscosity of all nanoemulsion formulations was very low as expected (44).

In vitro permeation results showed that nanoemulsion system significantly increased transdermal permeation of TAM. Out of all formulations A1 and DA1 showed maximum release when compared with other formulations shown in Table 3. The maximum release in A1 could be due to having the lowest droplet size and lowest viscosity. Moreover, droplet size and viscosity of the nanoemulsion may also affect its efficiency, where the small droplet size and low viscosity of the nanoemulsion make it an excellent carrier for enhancing percutaneous uptake of TAM, since the number of vesicles that can interact on a fixed area of stratum corneum will increase when droplet size and viscosity decrease. To explain the probable mechanism by which nanoemulsions enhance the skin permeation of drugs, the histological and histochemical structure of stratum corneum must be taken into consideration. Drugs permeate stratum corneum through two micro pathways, i.e., intercellular and transcellular pathways. Of these, the intercellular pathway plays a major role in percutaneous uptake of drugs. It is well known that a complex mixture of essentially neutral lipids, which are arranged as a bilayer with their hydrophobic chains facing each others, forms a lipophilic bimolecular leaflet. Most of the lipophilic drugs pass through this region, and it is called a lipid pathway. The polar head group of lipids faces an aqueous region, forming a polar route that hydrophilic drugs generally prefer. A dermally applied nanoemulsion is expected to penetrate the stratum corneum and to exist intact in the whole horny layer, alter both lipid and polar pathways (48). The drug dissolved in the lipid domain of the nanoemulsions can directly penetrate the lipid of the stratum corneum, thereby destabilizing its bilayer structure as found in histopathological examination of nanoemulsion treated skin. These interactions will increase the lipid pathway permeability to drugs. On the other hand, the hydrophilic domain of nanoemulsions can hydrate the stratum corneum to a greater extent and play an important role in percutaneous uptake of drugs. When the aqueous fluid of nanoemulsions enters the polar pathway, it increases the interlamellar volume of the stratum corneum lipid bilayer, resulting in disruption of its interfacial structure. A lipophilic drug like TAM can then permeate more easily through the lipid pathway of stratum corneum. Terpenes have been used to increase the skin permeation of a large number of compounds (34), and have been reported to increase drug diffusivity and partitioning into the stratum corneum due to disruption of the intercellular lipid bilayers. The intensity of their effects depends mainly on the lipophilicities of the drug and vehicle used (35,36).

Skin irritation study was performed to prove the safety of optimized A1 and DA1 nanoemulsions. Score of less than 2 (Erythema and edema) was observed over the period of seven days. The result revealed that the composition of optimized nanoemulsion formulation was safe to be used for transdermal drug delivery. Pharmacokinetics study result revealed that transdermal application can significantly modify pharmacokinetic profile of TAM and also increased bioavailability of TAM in comparison with oral formulation. The formulation A1 and DA1 nanoemulsion were found to enhanced the bioavailability of TAM with reference to the oral tablet formulation. The in vivo outcomes, which have revealed that increased bioavailability of TAM after transdermal application of nanoemulsion was due to the increased skin permeation and avoidance of substantial amount of hepatic first pass metabolism associated with oral administration and also possible explanation of these finding could be due to vary small particle size of nanoemulsions. As reported by others Kotyla et al (49) for instance, demonstrated that the nanoemulsion system increased the bioavailability of transdermally applied delta tocopherol when compared to micron sized emulsion preparation.

In vivo pharmacodynamic study in xenograft mouse model, the efficacy of the nanoemulsion formulation of TAM transdermally applied was significantly greater than control. We have assumed from this result that the nanoemulsion formulations that were applied transdermally at the tumor site, which represents a form of localized delivery has a greater possibility to be taken up by tumor tissue (50,51,52). When a chemotherapeutic agent is delivered by a vehicle like nanoemulsion, its local administration will insure uniform biodistribution (53). There are studies suggesting that particle size can affect the efficacy of tamoxifen, the smaller
particle size the greater the surface to volume ratio and presumably the greater the efficacy (54,55).

5. Conclusion

The percutaneous absorption of TAM from transdermal formulation depends both on the mean size of the organic phase droplets and on the vehicle constituents. In fact, a suitable choice of the components is essential to minimize the irritancy effect and to determine an improvement of the percutaneous permeation of the drug through the stratum corneum. Among essential oils, dill oil increased the skin penetration rate of TAM nanoemulsion. From in vitro and in vivo study, the research outcome in this article suggests that TAM nanoemulsion, potential vehicle for enhancement of bioavailability and used in the treatment of human breast cancer therapy through transdermal application.

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References