# COMPARATIVE STUDY OF TERBINAFINE HYDROCHLORIDE TRANSFERSOME, MENTHOSOME AND ETHOSOME NANOVESICLE FORMULATIONS VIA SKIN PERMEATION AND ANTIFUNGAL EFFICACY

**BY**

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**Abstract**

The purpose of the present research was to compare the skin permeation and study the antifungal efficacy of Terbinafine hydrochloride (TH) transfersome, menthosome and ethosome formulations under non-occlusive conditions. Terbinafine hydrochloride is an antifungal drug for onychomycosis. Poor permeability of its external preparation leads to poor curative effect. Preparation of TH transfersome utilized the mixture component model to determine the optimized desirable formula using different concentrations of nonionic surfactant, Span and Tween ($X_1$ & $X_2$ respectively) were investigated. The results revealed that both $X_1$ and $X_2$ had a pronounced effect on vesicle size ($Y_1$) and entrapment efficiency (EE) of the drug ($Y_2$). The vesicles were prepared and characterized for shape, size, zeta potential and entrapment efficiency. Ex vivo study via Franz diffusion cells was used for the percutaneous absorption studies. The optimum desirable formula of transfersome, menthosome and ethosome formulations were showed vesicle size (78.7, 122.8 and 67.8 nm), zeta potential (-8.28, 8.77 and 11.30 mV) and encapsulation efficiency (92.67, 93.86 and 95.75%), respectively. Ex vivo permeation of the drug-loaded nanovesicle showed more than two to three folds higher permeation rate compared with drug suspension. Deformability verified elasticity of the preparation. Finally, TH nanovesicle formulations improved drug delivery with greater improvement in skin permeation and antifungal activity. Our selected formulae showed potent antifungal effect against Aspergillus niger for the treatment of fingernails or toenails.

**Keywords;** Terbinafine Hydrochloride, Transfersome, Menthosome, Ethosome, Nano-vesicles

## Introduction

Transdermal drug delivery offers many advantages over other routes of administration, including avoidance of first-pass metabolism and targeting of the active ingredient for a local effect (El Maghraby et al., 2008). Unfortunately, the formidable barrier characteristics of stratum corneum present a significant obstacle for most drugs to be delivered into and through it. To overcome the barrier, lipid vesicles have been proposed. Liposomes were first reported as carriers for topical use with lack of permeability of drugs to deeper skin layers (Mezei and Gulasekharam, 1980). Recently, vesicle drug delivery forms like niosomes, transfersomes, menthosome and ethosome

provided several advantages. They are able to enhance drug bioavailability via enhancement of drug permeation and interactions with human skin, as well as prolongation of drug action and reduced administered drug toxicity (Dragicevic-Curic et al., 2010; Kumar et al., 2012; Mahale et al., 2012; van den Bergh et al., 2001)

Transferosomes are the first generation of elastic vesicles, which were first introduced in the early 1990s (Cevc and Blume, 1992). Its main compositions are phospholipids (membrane) and surfactant (membrane softening agent). Transfersomes the most important feature to prepared elastic vesicle caused by the presence of the edge activator. These elastic vesicles are able to squeeze through intercellular regions of the stratum corneum under the influence of the transepidermal water-activity gradient. Phospholipid (PL) hydrophilicity leads to xerophobia (tendency to avoid dry surroundings) thus, for the vesicles to remain maximally swollen on skin surface. The elasticity and flexibility of this vesicle minimizes the possibility of its rupture especially when applied onto the skin (Malakar et al., 2012).

The characters of nano-vesicle affected by edge activator types such as nonionic surfactant (Tween 80 and Span 80) having different HLB values (15 and 4.5 respectively). The vesicle prepared using Tween showed more deformability with less entrapment efficiency, this due to highly flexible and nonbulky hydrocarbon chains. In contrast, the vesicle containing span showed least deformability and high entrapment efficiency. These could be attributed to the highly hydrophobicity that reduced the formation of transient hydrophilic holes, hence, minimizing the amphiphilic property of the bilayers responsible for membrane fluidity. Transfersomes used as a carriers for various drugs such as analgesics, anesthetics, corticosteroids (Cevc et al., 1997; Essa et al., 2004), proteins such as insulin, albumin and sex hormones (Cevc, 2003; Cevc et al., 1995; Planas et al., 1992) and anticancer drugs (Hiruta et al., 2006; Trotta et al., 2004).

While, menthosome it is contain edge activator almost cationic surfactant (cetrimide). An edge activator is often a single-chain surfactant, having a high radius of curvature that destabilizes lipid bilayers of the vesicles and increases deformability of the bilayers (Duangjit et al., 2012). In addition, ethosome is phospholipid-based elastic nanovesicles containing a high content of ethanol (20-45%). Ethosomal systems are much more efficient in delivering substances to the skin in the terms of quantity and depth, than either conventional liposomes or hydroalcoholic solutions.

Terbinafine (TH) is one of the most widely used antifungal drugs clinically (Lee et al., 2008). Onychomycosis is a fungal infection of the fingernails or toenails that causes discoloration, thickening and separation from the nail bed. This disease occurs in 10 % of the general population but is more common in older adults. This higher prevalence in older adults is related to peripheral vascular disease, immunologic disorders or diabetes mellitus (Westerberg and Voyack, 2013). Onychomycosis is mainly caused by dermatophytes of the genus Trichophyton, but occasionally by non-dermatophytic fungi including Aspergillus spp. (Moreno and Arenas, 2010). Aspergillus niger, an opportunistic filamentous fungus, is found to be associated with nail onychomycosis in both toenails and fingernail lesions (Kim et al., 2012). Terbinafine showed higher cure rates, when administered to treat onychomycosis, especially those caused by Aspergillus niger (Gianni and Romano, 2004). TH is an antifungal drug with most frequent adverse effects after oral use are gastrointestinal disturbances such and mild abdominal pain (Sweetman, 2009). Topical administration can avoid its adverse

effects, but the limited solubility of TH, makes its have a quite low bioavailability, especially in topical dosage forms.

Thus, the aim of the present study was to compare the skin permeation of TH as a model drug using different vesicular carriers, viz. transfersome, menthosome and ethosome to improve its antifungal activity. In this study, we adopt transfersome formulations with different ratios of nonionic surfactant (Span 80 and Tween 80 with HLB 4.5 and 15 respectively). First, the terbinafine transfersome formulations were prepared to obtain the optimized desirable formula which made a contribution to deformability and small particle size and high entrapment efficiency of drug obtained. The relationship between formulation factor and response variables was predicted, and the optimal formulation was also optimized using Design Expert®. Finally, comparative study of TH transfersome, menthosome and ethosome nanosize formulations via skin permeation and antifungal activity were studied.

## Materials and Methods

### Materials

Terbinafine hydrochloride was obtained as a gift from Memphes company, cairo, Egypt. Tween 80, Span 80, Chloroform and methanol were obtained from Sigma-Aldrich (St. Louis, MO). L-α-phosphatidylcholine (soy 95%) was purchased from Lipoid Phospholipid GmbH, Germany. Cetremide was obtained from Winlab Chemicals; U.K. Ethyl alcohol was purchased from Algomhorya Company, Cairo, Egypt. Sodium hydroxide and potassium dihydrogen phosphate were purchased from ADWIC Company, Cairo, Egypt. All chemicals and reagents used were of analytical grade.

### Methodology

#### *Development and Optimization of Transfersome Formulations*

Mixture Design was applied approach to identify the best proportion of each component in order to optimize multiple responses simultaneously. Effect of type and proportion of each component (nonionic surfactant) Span-80 ($X_1$) and Tween 80 ($X_2$) were studied. Mixture design containing two components was carried out to optimize transfersome formulations. The response in a mixture experiment usually is described by a polynomial function. This function represents how the components affect the response. The responses measured were particle size ($Y_1$) and entrapment efficiency ($Y_2$). To better study the shape of the response surface, the natural choice for a design would be the one whose points are spread evenly over the whole simplex. A simplex centroid design only includes the centroid points. A simplex centroid design can be used to fit the following model.

$$Y= \beta_1X_1 + \beta_2X_2 + \beta_{1,2} X_1X_2 \qquad \text{Eq. 1}$$

The above model is called special cubic model. Note that the intercept term is not included due to the correlation between all the components (their sum is 100%) (Cornell, 2011).

Y is the measured response associated with each factor-level combination, $X_1$ and $X_2$ are the factors studied, $\beta_1$, $\beta_2$ and $\beta_{1,2}$ are the regression coefficients. The equation enables to study the effect of each factor and their interaction over the considered responses. Mixture design containing three components was carried out to

optimize transfersome formulations (Nutan et al., 2007). Table I illustrates the independent variables and their level used in this study. This optimized formula was prepared and characterized to compare the observed values of $Y_1$ and $Y_2$ with the predicted responses. Preparation and determination of the vesicle size and EE were done. The optimized TH transfersome formulation was compared to TH of menthosome and ethosome formulations.

***Preparation of Terbinafine hydrochloride by different techniques (Transfersome, Menthosome and Ethosome)***

Transfersomes-loaded terbinafine hydrochloride were prepared using lipid film hydration technique previously described (Jain et al., 2003) with little modifications using combination of the edge activators (Span 80 and Tween 80). The drug (10mg), phosphatidylcholine (850 mg) and edge activator (150 mg) were mixed together and dissolved in an organic solvent mixture of chloroform and methanol (1:1 v/v) then placed in a clean, dry round bottom flask. The organic solvent was removed by rotary evaporation under reduced pressure at $40^0$C. The rotor speed was adjusted to 60 rpm. Complete removal of the organic solvent was accomplished after 120 minutes resulting in a homogenously distributed thin film on the wall of the flask. The deposited film was hydrated with a solution of phosphate buffer (pH 6.5) by rotation for 1hr at 45 $^0$C. The vesicular suspension was then sonicated using probe sonicator at 4 $^0$C for 15 min to reduce the size of the vesicles. The preparation of menthosome formula using cationic surface active agent (cetrimide) edge activators at the optimum concentration 10 mg was prepared. The method for preparation of menthosome was nearly the same of the aforementioned method of transfersome formulations; just replace the edge activator nonionic by cationic surfactant (Duangjit et al., 2014). Ethosome was prepared using cold method. The drug (10mg) and phosopholipid (850 mg) were dissolved in ethanol (30%) in covered vessel at room temperature by vigorous stirring. Heat the mixture up to 40 $^0$C in a water bath. In a separate vessel heat the water up to 40 $^0$C and add to the above mixture slowly in a fine stream under constant stirring at 700 rpm, on completion of adding, continue stirring for another 5 min at 40 $^0$C and cool the resultant vesicle suspension at room temperature. Ethosome was subjected to sonication at 4 $^0$C using probe Sonicator for 1 min (Zhang et al., 2012).

**Vesicles characterization**

***Investigation of vesicles size, polydispersity index and zeta potential***

Average vesicles size, the corresponding Polydispersity index and the zeta potential were determined using Malvern Zetasizer (Zetasizer; Malvern Zetasizer, model Nano ZS, U.K). For size measurements, the vesicular suspension was mixed with PBS (pH 6.5). Three measurements with 10 sub-runs were performed for each sample. The data of the size measurements were analyzed by the general purpose mode, and zeta potential measurements

were processed in the monomodal mode (Zhang et al., 2012).

***Entrapment Efficiency of TH formulations***

Indirect method was employed to calculate the amount of TH entrapped in each formula. Samples from the prepared formulae were transferred to eppendorff tubes and refrigerated at -20°C. The frozen samples were centrifuged at 14000 rpm for 30 minutes. The supernatant solutions were assayed for drug content

spectrophotometrically (U.V spectrophotometer; Schimadzu, model UV-1800, Tokyo, Japan) after dilution with phosphate buffer pH 6.5. The entrapment efficiency (EE) of TH was determined spectrophotometrically at 223 nm. The TH content was calculated using the calibration curve. Each sample was analyzed in triplicate. The % EE was calculated from the equation (Zhang et al., 2012).

$$\% \text{ EE } = \frac{\text{Initial drug Concentration} - \text{Concentration of free drug}}{\text{Initial drug Concentration}} \times 100$$

**Physicochemical study of TH nanovesicle.**

Comparative elasticity study of different T-optimize, menthosome and ethosome formulations were carried out by extrusion measurement through a locally fabricated stainless steel pressure filter holder. The vesicles were extruded through polycarbonate filter with (19 mm, 0.1 µm) a pore size of 100 to 120 nm at a constant pressure of 0.17 MPa. The elasticity was measured as a function of time (i.e. the time taken for the extrusion of 10 ml Transfersomal suspension) (Cevc and Gebauer, 2003). The experiments were carried out in triplicates to obtain an average value.

Differential scanning calorimetry (DSC) was performed to investigate the thermal behavior of the drug and the freeze-dried drug-loaded transfersome, menthosome and ethosome formulations.

Fourier transforms infrared (FT-IR) of the drug alone and selected T-optimize, menthosome and ethosome formulations spectra studied in the FT-IR section were obtained in the range 4000–400 $cm^{-1}$ by using (Perkin Elmer Spectrum One, Model 16 PC, Rheinstetten, Germany). Transparent discs were prepared by mixing each sample with KBr. The mixture was compressed and tested.

The TEM pictures of T-optimize desirable formula, menthosome and ethosome nanosize formulae were obtained using TEM microscope (JEM-1230). Samples were negatively stained with a 1% aqueous solution of Phosphotungstic acid. Vesicle suspension samples were dried on a carbon coated grid for staining. After drying, the specimen was viewed under the microscope at 10-100 k-fold enlargements at an accelerating voltage of 100 kV (Ahmed, 2015).

**Ex-vivo skin permeation study**

An accurately quantity amount of TH transfersome, menthosome and ethosome formulations suspension, equivalent to definite amount of TH, were suspended in the Franz diffusion pool (DENG et al., 2011) having the diameter of 2 cm and diffusion area 3.14 $cm^2$. The rat skin was used (All institutional and national guidelines for the care and use of laboratory animals were followed). The isolated skin of the rat with thickness of 0.5 mm was fitted between the supply pool and the reception pool and was placed in the transdermal diffusion apparatus (TK-20 A, Shanghai Kaikai Technology Trade Co., Ltd, China). The reception pool was fulfilled with 50 ml phosphate buffer pH 6.5 with constant stirring at a speed (120 rpm) at 37±1 $^0$C. At predetermined time intervals (0.25, 0.5, 1, 2, 4, 6, 8 and 12 h), 1mL of the reception liquid was withdrawn for analysis and replaced with equal volume of fresh reception solution at the same temperature to maintain a constant volume. After the last sample was collected, the concentration of the drug was determined spectrophotometrically at 223 nm. All experiments were performed in triplicate with standard diviation (±SD). Then the skin was taken off and washed and extract TH that remained in the skin. The supernatant

liquid samples were collected and assayed to know the amount of drug remained on the skin. The release data of TH formulations from the prepared batches were fitted to different kinetic orders or models; zero order, first order, diffusion, and Korsmeyer model to explore the best fit order/model and the exact mechanism of drug release from the TH nano-vesicle size (El-Say, 2016).

**Stability studies**

Stability of the T-optimize, menthosome and ethosome were determined by assaying the vesicles size and entrapment efficiency at time 0, 1, 2 and 3 months by the same aforementioned procedures. Three samples of each of the selected formulae were used and stored at 4 °C temperature (Zhang et al., 2012). All formulae were kept in tightly closed clean umber colored glass container (El Zaafarany et al., 2010). Size change rate (SCR) was calculated from the following equation(Shen et al., 2014)

$$\mathrm{SCR} = \frac{S_3 - S_0}{S_0} \times 100$$

Where $S_3$ is the vesicle size after three months and $S_0$ is the initial vesicle size at the start of experiment. Change are not considered relevant if SCR value is less than 10% (Manconi et al., 2016).

**In vitro antifungal assay of TH formulations**

Pure cultures of two test fungi, one yeast-like Candida albicans (ATCC 10231) and one filamentous Aspergillus niger (ATCC 16404), were used in this study. These fungi were cultured on Sabouraud dextrose agar. The antifungal activities of TH formulations were systematically performed test fungi by agar cup plate diffusion method based on the methodology used by bauer et al., (Bauer et al., 1966) with some modifications. The sterile Sabouraud dextrose agar was poured into sterile Petri plates aseptically and allowed to solidify at room temperature. Petri plates were flooded with 200 µl of the fresh fungal suspension in sterilized saline equivalent to McFarland 0.5 standard solution ($1.5 \times 10^8$ CFU/mL) and uniformly distributed by sterile glass rod, then allowed to dry for 20 minutes with lid in place. In each plate, 3 wells of 6 mm diameter were made with a sterile borer, and the agar plugs were taken out carefully so as not disturb the surrounding medium. Precisely 100 µl of the TH formulations (transfersome, menthosome and ethosome) were added to one cup aseptically and labeled accordingly as well as blank (composed of all components of formulation except TH) and pure TH suspension. After holding the plates at room temperature for 1 h to allow diffusion of test samples into the agar, the plates were incubated at $28^oC$ for 48 hours for fungi. The diameter of the clear zone of inhibition surrounding each well was measured twice at right angles and the average of the two readings was recorded to the nearest mm. The inhibition effects of formulations were compared with that of blank and drug. The zone of inhibition above 7 mm in diameter was taken as positive result.

**Results and discussion**

Terbinafine Hydrochloride is lipophilic and could appropriately permeate through corneous layer into nail plate. Poor permeability of its external preparation leads to poor curative effect. TH is a good candidate for nano-vesicle forms (transfersomes, menthosome and ethosome) to enhance its bioavailability via

transdermal route. This study is aimed to prepare high skin penetration TH vesicle nanosize with high entrapment efficiency (EE) and good stability. The influence of various edge activator (Span and Tween) formulations and processing parameters that affect the vesicle size and EE were investigated. The prepared TH nano-vesicle forms were utilized a simplex centroid design to obtain optimize desirable formula using suitable tool (Statgraphics_plus software) to identify the parameters that are useful for this study.

**Table 1** TH transfersomes independent and dependent variables used in the mixture simplex centroid design.

| | | Level | |
|---|---|---|---|
| | | **Low** | **High** |
| ***Independent variables*** | | | |
| Span 80 | $X_1$ | 0 | 150 |
| Tween 80 | $X_2$ | 0 | 150 |
| | | **Goal of optimization** | |
| ***Dependent variables*** | | | |
| Vesicle size | $Y_1$ | Minimize | |
| Entrapment efficiency | $Y_2$ | Maximize | |

Table 1, illustrates the lower and higher level for the independent studied variables of different nanionic surfactant (Span $X_1$ and Tween $X_2$). The mixtures with different concentration of edge activator (EA) having different HLB values were selected to enhance the drug entrapment efficiency and elasticity of the vesicles size.

The composition of the formulations obtained by the software mixture simplex centroid design showed in Table 2.

**Table 2** Composition of mixture simplex centroid design used for TH transfersome formulations and comparative nano-vasicle formulations.

| Formulae | Drug | Phospholipid | Span 80 | Tween 80 | Cetrimide | Ethanol |
|---|---|---|---|---|---|---|
| $F_1$ | 10.0 | 850 | 150.0 | 0.000 | - | - |
| $F_2$ | 10.0 | 850 | 75.00 | 75.00 | - | - |
| $F_3$ | 10.0 | 850 | 0.000 | 150.0 | - | - |
| $F_4$ | 10.0 | 850 | 75.00 | 75.00 | - | - |
| $F_5$ | 10.0 | 850 | 112.5 | 37.50 | - | - |
| $F_6$ | 10.0 | 850 | 37.50 | 112.5 | - | - |
| **Comparative Nano-vesicle Formulations** | | | | | | |
| T- optimize | 10.0 | 850 | 59.10 | 90.90 | - | - |
| Menthosome | 10.0 | 850 | - | - | 10.0 | - |
| Ethosome | 10.0 | 850 | - | - | - | 35 |

In this study, the effect of the formulation parameters affecting the preparation of TH transfersomes was studied using mixture optimization design. This work is the first study in the development of optimized TH transfersome preparation suitable for transdermal drug delivery. The transfersome formulations were prepared at ratio PL:EA 85:15 as the fixed ratios in all formulations. The edge activator nonionic surfactants with different HLB values (Span and Tween 4.5 and 15 HLB respectively) were studied and develop the effect of nonionic surfactant on transfersome formulations. The desirable and optimized formula via vesicle size and entrapment efficiency was obtained.

**Characterization of the vesicle size, zeta potential, EE and deformability of lipid vesicle**

Transfersome-proposed formulations were characterized via vesicle size, size distribution, zeta potential, EE and deformability of the vesicles. TH is a good choice drug for encapsulation into nano-vesicle forms (transfersomes, menthosome and ethosome) to enhance its permeability power via transdermal route. The results of transfersome formulations were shown in Table 3. The vesicle size ranged from (79.8 to155 nm). Transfersome formulations containing Tween 80 ($F_3$) and Tween combination with different ratios of Span 80 ($F_2$, $F_4$, $F_5$ and $F_6$) showed smaller vesicle size than those containing Span alone ($F_1$). Transfersomes showed narrow particle size distribution as indicated by low Polydispersity index. The zeta potential is the electric potential of the vesicle, including its ionic atmosphere. The zeta potential of transfersome formulations were obtained between (-4.42 to -9.30 mV). The results of entrapment efficiency of TH transfersomes containing different kinds of surfactants are above 85%. By comparing the optimum deformability ratio between lipid and edge activator (85:15%, w/w), it was found that, Tween only ($F_3$) showed the highest deformability (11.7s), the deformability of Tween combination with different ratios with Span 80 ($F_2$, $F_4$, $F_5$ and $F_6$) were smaller than that of transfersomes containing span alone 48.5 s ($F_1$). This could be attributed to the highly flexible and non-bulky hydrocarbon chains of Tween. Span had lower deformability than Tween due to their steroid-like structures which are bulkier than the hydrocarbon chains of Tween (El Zaafarany et al., 2010)

**Table 3** Characterization the vesicle of TH transfersome formulations and comparative of three lipid vesicle types

| Formulae | Vesicle Size (nm) | Zeta Potential (mV) | Polydispersity Index | Entrapment Efficiency (%) | Deformability value (sec) |
|---|---|---|---|---|---|
| $F_1$ | 155.0±6.2 | -4.42±0.2 | 0.322±0.05 | 87.3±2.9 | 48.4±2.6 |
| $F_2$ | 85.60±5.4 | -9.30±0.4 | 0.240±0.03 | 92.2±3.7 | 22.5±2.8 |
| $F_3$ | 79.80±4.6 | -5.12±0.3 | 0.263±0.02 | 82.8±4.6 | 11.7±1.8 |
| $F_4$ | 84.50±5.7 | -8.50±0.5 | 0.280±0.03 | 91.4±6.4 | 23.8±1.9 |
| $F_5$ | 100.0±4.4 | -5.35±0.4 | 0.292±0.04 | 90.5±4.3 | 35.6±3.2 |
| $F_6$ | 83.42±3.8 | -7.88±0.6 | 0.284±0.05 | 85.6±5.2 | 15.5±1.7 |
| **Comparative Nano-vesicle Formulations** | | | | | |
| T-optimize | 80.40±4.6 | -8.28±0.7 | 0.220±0.05 | 93.54±5.3 | 18.5±2.0 |
| Menthosome | 122.8±7.2 | 8.77±0.8 | 0.303±0.04 | 93.86±6.8 | 20.5±2.6 |
| Ethosome | 67.80±5.4 | 11.3±0.5 | 0.248±0.05 | 95.75±6.5 | 13.9±1.5 |

**Parameters studied effect on the vesicle size and entrapment efficiency**

The effect of the studied variables on the vesicle size was investigated for TH transfersome. To illustrate the effect of the studied variables on the vesicle size when two variables are changing, estimated response surface and contours of estimated response surface (Figure1: A1 and A2) were constructed. The equation of the fitted model is:

$$Y_1 = 150.57*X_1 + 83.78*X_2 - 131.15*X_1X_2$$

The above equations were derived by the best-fit method to describe the main effect of process variables ($X_1$ and $X_2$) and their interaction ($X_1X_2$) on the responses $Y_1$. Generally, a positive sign reflects a synergistic effect while a negative sign stands for an antagonistic effect. Analysis of variance (ANOVA) was done for the effect of each factor. One can conclude from the regression Equations and has the most significant synergistic effect (p-value < 0.05) on vesicle size ($Y_1$). The results also revealed that the interaction effect between $X_1X_2$ has an antagonistic effect on vesicle size.

Both surfactants Span ($X_1$) and Tween ($X_2$) affect significantly on the vesicle size of the prepared transfersome at p-values of 0.0087 and 0.0091 respectively, which was also demonstrated in the Estimated Response Surface and contours Estimated Response Surface in (Figure 1: A1 and A2). As the surfactant HLB increased, the vesicle size decreased, which could be related to the affinity of the phospholipid used toward surfactant. The affinity was highest with Span followed by Tween. New finding were reported for the effect of different surfactant combinations on vesicle size and deformability of TH transfersomes. The vascular size was decreased and the elasticity of nanovesicle was enhanced by increasing Tween concentration with low concentration of Span ($F_6$) combination.

To study the effect of the variables on Entrapment efficiency when two variables are changing estimated response surface (Figure1: B1) and contours of estimated response surface (Figure1: B2) were investigated. The equation of the fitted model is: $Y_2 = 87.524*X_1 + 82.02*X_2 + 24.36*X_1X_2$

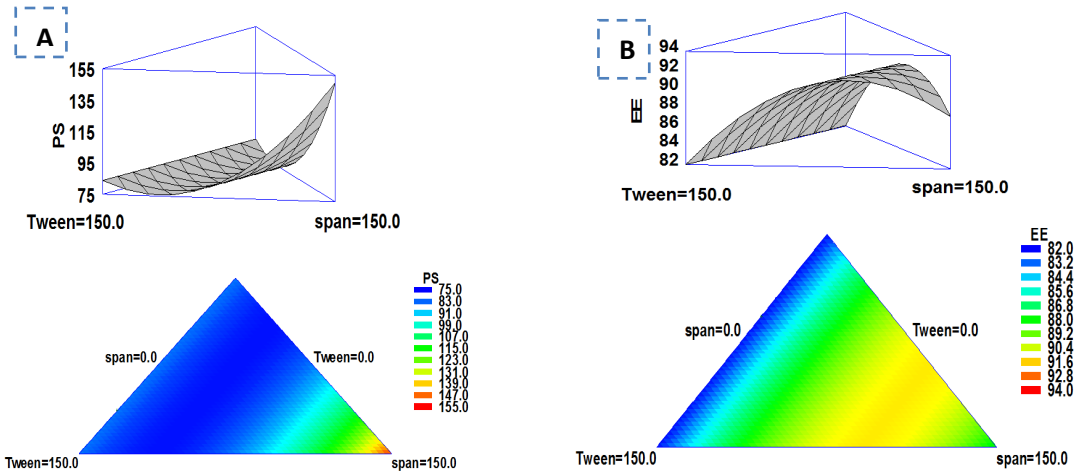

**Fig. 1** Response surface plot estimating and contour estimated response the effect of span ($X_1$),and Tween ($X_2$) on the particle size ($Y_1$ represented in A) and Entrapment efficiency ($Y_2$ represented in B) of TH transfersome formulations.

From equations were derived by the best-fit method to describe the main effect of process variables ($X_1$ and $X_2$) and their interaction ($X_1X_2$) on the responses Y2. One can conclude from the regression Equations and Figures1; B1 and B2 has the most significant synergistic effect (p-value < 0.05) on entrapment efficient ($Y_2$). The results also revealed that the interaction effect between $X_1X_2$ has a synergistic effect on $Y_2$. The combination of two surfactants having different HLB values producing high entrapment efficiency of drug loaded transfersome formulations in comparison to each surfactant alone (Jacob and Kr, 2013). In addition, HLB values of the surfactant affect the entrapment efficiency of the drug loaded nano-vesicle. Since, increasing HLB values decrease the entrapment efficiency of the drug. This finding was in agreement with previous studies demonstrated the effect of surfactant HLB on the entrapment of drug-loaded transfersomes (Jacob and Kr, 2013).

From the composition of the multiple responses the optimized desirable formula was identified. The optimized formula was proposed to contain 59.10 and 90.90 % of $X_1$ and $X_2$, respectively. This optimized formula was prepared and characterized for its vesicle size and EE. The predicted values obtained from optimization were compared to the observed ones in which the residual was calculated and presented in Table 4. The optimized formula where further characterized and compared with other nano-vesicle system (menthosome and ethosome).

**Table 4** Multiple response optimization of Terbinafine transfersome

| Factor | | Low level | High level | Optimum level |
|---|---|---|---|---|
| $X_1$ | | 0 | 150 | 59.10 |
| $X_2$ | | 0 | 150 | 90.90 |
| **Response** | | **Predicted** | **Observed** | **Residual** |
| Vesicle size (nm) | $Y_1$ | 78.75 | 80.40 | 1.65 |
| Entrapment efficiency | $Y_2$ | 92.67 | 93.54 | 0.87 |

**Comparative studies between T-optimize, menthosome and ethosome via vesicle size, zeta potential, EE and deformability of lipid vesicle**

The result of T-optimize, menthosome and ethosome formulations were shown in Table 3. The vesicle size was 80.40, 122.80 and 67.80 nm respectively. The vesicle size of ethosome and T-optimize were smaller than the menthosome formula. In particular, the ethosomal formulation had a much smaller particle size. This may be attributed to the high concentration of ethanol, which could cause a reduction in vesicle size. In addition, the zeta potential of T-optimize, menthosome and ethosome was -8.28, 8.77 and 11.30 respectively. The charge of zeta potential was depending on the types of surfactant used in T-optimize and menthosome while in ethosome attributed to the drug.

The results of entrapment efficiency of TH nanosize formulation were ranged from 93.54 to 95.75. Finally, the deformability values of TH nano-vesicle were showed of T-optimize 18.5 sec, menthosome 20.50 sec and ethosome 13.9 sec. This could be attributed to the vesicle size of TH nanovasicle formulation (Touitou et al., 2000; Zhang et al., 2012). The EE of three formulations (T-optimize, menthosome and ethosome) of lipid vesicles were high. These may be contributed to the weight ratio of PL to surfactant in combination of T-optimize formula. While, the greater distribution of TH loaded in menthosome core was attributed to lipid bilayer containing cetrimide. The ethosome vesicles containing high ethanol concentrations that had high solubility of TH, corresponding to the formation of a phase with interpenetrating hydrocarbon chains, which contributed to improving the EE of the vesicles (Jain et al., 2007).

**Physicochemical characterization of TH nano-vesicle formulations**

The thermal characteristic using DSC of the TH and comparative (T-optimize, menthosome and ethosome) formulations were studied. DSC is a tool used to investigate the crystalline or amorphous nature of the drug within the developed formulations and to elucidate any possible interactions with other ingredients. TH pure drug thermogram showed characteristic thermal peak at reported melting point (198°C) (Kuminek et al., 2013). Thermograms for vesicular systems (T-optimize, menthosome and ethosome) showed disappearance of TH thermal peak which indicates entrapment of the drug into the vesicles and loss of its crystal properties. In addition, thermograms of vesicle systems showed new endothermic peaks which the most probably due to the transition temperature (Tm) of PL.

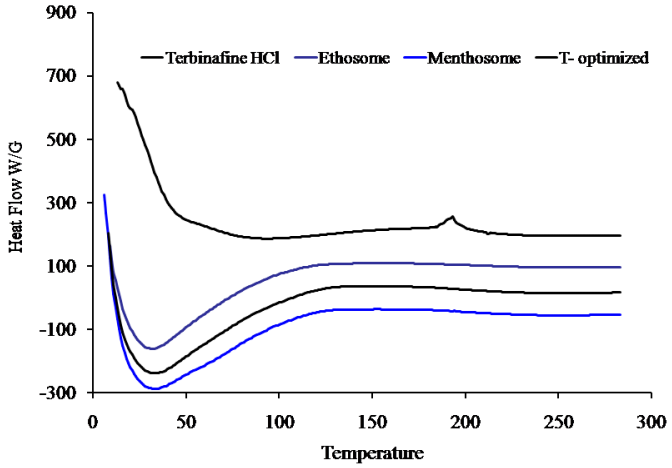


**Fig. 2** DSC thermogram of Terbinafine HCL and drug-loaded transfersome, menthosome and ethosome formulations.

From figure 3, the FTIR spectrum of pure TH showed C≡C stretching bands at 2443.79 $cm^{-1}$, aromatic C=C stretching bands at 1521$cm^{-1}$, aromatic C-H stretching bands at 3041.08 $cm^{-1}$, and C–N bands at 1132.49 and aliphatic C–H stretching bands at 2969 $cm^{-1}$. (Pohle et al., 2001). The lyophilized nano-vesicle TH formulation FTIR spectrum showed slightly change of some bands. Presence of Phospholipid and surfactants used did not produce any major shift in the principal peaks of HT and also the presence of one ingredient did not produce shift in the peaks of other ingredients. This indicates that there was no interaction between drug and the Additives used in the study. Hence FTIR spectral analysis proved the compatibility of the drug and additives

used (Kumar, 2012). The above finding confirms compatibility of the formulation ingredients.

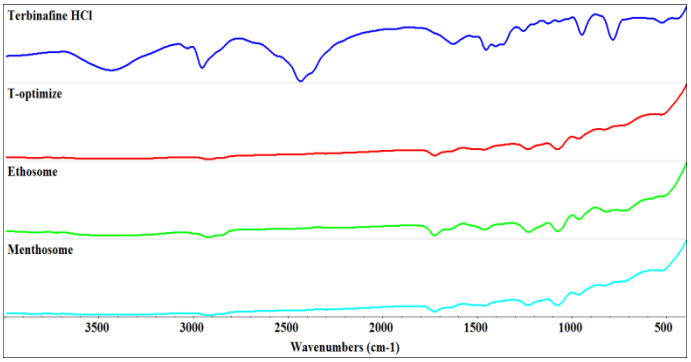


**Fig. 3** IR spectra of Terbinafine HCl and drug-loaded transfersome, menthosome and ethosome formulations.

**Vesicle shape characterization of TH nano-vesicle formulations**

Investigation of morphology for three formulations (transfersome, menthosome and ethosome) using TEM revealed that all types were spherical shape with a few difference in edge of the vesicle surface which attributed to different types of edge activator used (Figure 4).

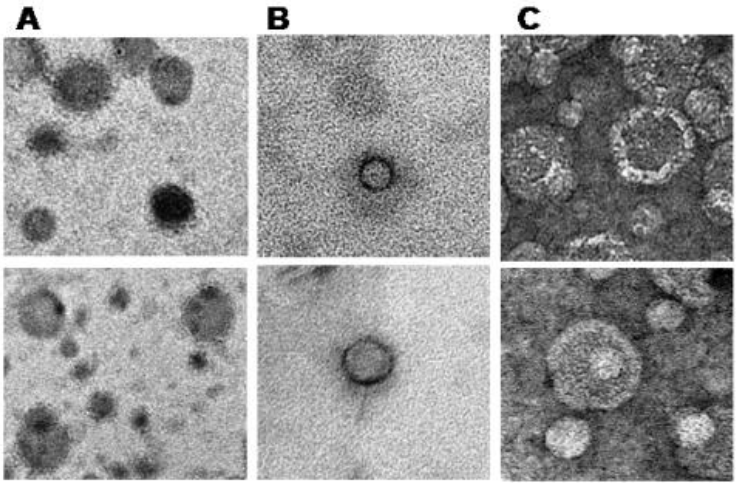


**Fig. 4** TEM pictures of Terbinafine loaded transfersome (A), menthosome (B) and ethosome (C) formulations.

**Ex vivo skin permeation study of TH nano-vesicle formulations**

The Ex vivo permeation of TH loaded lipid vesicles were carried out for different formulations (T-optimize, menthosome and ethosome) and drug suspension. The Ex vivo permeation study of the T-optimize, menthosome, and ethosome showed a considerable difference, more than 3, 3.5 and 4-folds enhancement release respectively when compared with the drug suspension as showed in Figure 5. These nano-vesicle formulations had a marked impact on the ex vivo release of TH in phosphate buffer pH 5.5. TH ethosome nano-size led to a considerably faster release than transfersome and menthosome formulations. In ethosomes, ethanol is a well-known permeation enhancer. The effect of ethanol on vesicle fluidity as well as a dynamic interaction between

ethosomes and the stratum corneum, may contribute to the delivery properties of ethosomes (Dayan and Touitou, 2000). These reasons allow ethosomes to permeate more easily and deeper into skin. While, TH transfersome formulation (T-optimize) was accumulate in skin. Thus, TH release from the vesicles in the stratum corneum is an important step that will affect transdermal flux. In addition, the zeta potential of transfersomes is more negative. This might explain why transfersomes can't effectively penetrate through the skin because the skin has a negative charge (Jain et al., 2007). Finally, the poor drug release resulted in retention of the drug within vesicles in the stratum corneum and elastic vesicles served as a slow release depot system (Honeywell-Nguyen and Bouwstra, 2003). These results are consistent with a previously illustrated by many researchers (Gillet et al., 2011; Yang et al., 2015; Zhang et al., 2012). In general, the mechanism of drug release from the prepared transfersome could be attributed to a diffusion control mechanism as previous investigation (Hathout et al., 2007; Nasr et al., 2008) data not showed.

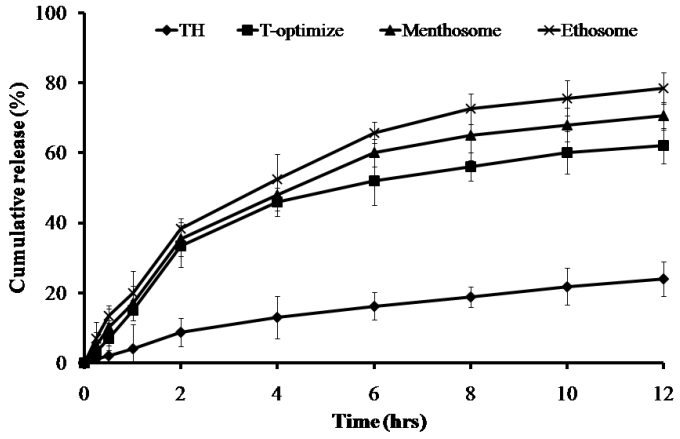


**Fig. 5** Ex vivo Comparison between the release profiles of different T-optimize, menthosome and ethosome formulations and TH suspension via 12 h by Mice Skin, in phosphate buffer pH 5.5.

**Stability of TH lipid vesicles**

In Figure 6 A and B, the nano-vesicle formulations were determined at 0, 1, 2, and 3 months following vesicle preparation. The vesicle size and entrapment efficiency of the three lipid vesicle form showed no significant changes within 3 months at 4 $^{o}$C (El Zaafarany et al., 2010; Naik, 2013). Size change rate (SCR) was also calculated for each vesicle system. SCR less than 10% is reported to be acceptable (Manconi et al., 2012). After three months, TH of nano-vesicle formulations showed SCR of T-optimize, menthosome and ethosome 9.20, 3.25 and 14.7% respectively. Ethosome was reported to be stable at refrigeration temperatures (Chandel et al., 2012). However, this may be due to PL slightly aggregations upon storage at low temperature (Lee and Hong, 2014). This result of drug retained shows that the three lipid vesicle systems are stable under the given conditions.

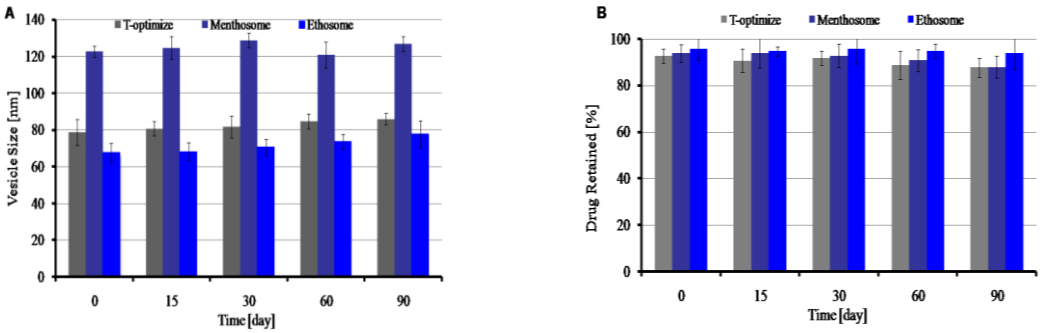


**Fig. 6** Effect of storage stability on the vesicle size (A) and drug retained (B) of T-optimize, menthosome and ethosome formulations.

**In vitro antifungal activity of TH nanovesicular size formulations**

The antifungus activity of the three formulations and drug showed good antifungal activity against the filamentous Aspergillus niger. However, there was no activity against C. albicans. Significantly, the size of zones of inhibition indicated increasing in the antifungal activity of TH transfersome and TH menthosome; however, there was some inhibition of growth in case of TH ethosome formula when compared with drug alone (Table 5 and Figure 7).

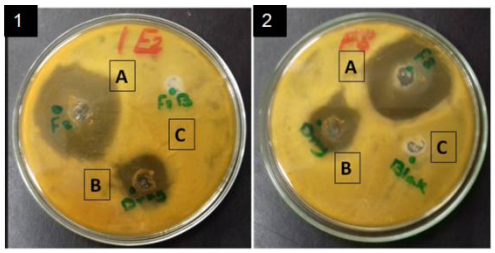


**Fig.7** The antifungal activities of prepared formulations of drug loaded T-optimize (1) and Menthosome (2), against A. niger in comparison to blank and drug alone. (Formula A; Drug B; Blank C).

**Table 5** Antifungal activity profile of drug and three tested formulations by agar well diffusion method

| Fungus | Inhibition Zone | | | | | | |
|---|---|---|---|---|---|---|---|
| | TH | T-optimize | | Menthosome | | Ethosome | |
| | Drug | Blank | Test | Blank | Test | Blank | Test |
| A. niger | 15 | - | 29 | - | 28 | 10 | 30 |
| C. albicans | - | - | - | - | - | - | - |

## Conclusion

From the aforementioned study, we can used the optimization technique with different formulation and processing variables to obtain optimize desirable formula with optimum combination ratio of Span (59.10 %) and Tween (90.90 %) with small vesicle size (80.40 nm) and high EE (93.54 %). The optimum formula was characterized by flexible lipid vesicles which can respond to rapid shape deformation, which enables

them to pass through skin pores and more than 3-folds higher in vitro permeation when compared with the drug suspension. The accumulation of the TH transfersomes in the skin is high with low permeation power rather than menthosome and ethosome. Ethosome improve drug delivery with greater improvement in skin permeation than improvement in skin deposition. Transfersomes have the advantage that high concentrations of drugs can be localized at the site of action, where the goal is to reduce the systemic adsorption and minimize systemic side effects.

**Conflict of interest**

The authors report no conflict of interest in this work.

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# دراسة مقارنة بين تركيبات مختلفة لحويصلات النانولعقارالتربينافين هيدروكلوريد من خلال صياغات تتخلل عبر الجلد وتأثيرها المضاد للفطريات

**للسادة الدكاترة**

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الغرض من هذا البحث دراسة مقارنة بين تركيبات مختلفة لحويصلات النانولعقارالتربينافين هيدروكلوريد من خلال صياغات تتخلل عبر الجلد وتأثيرها المضاد للفطريات. التربينافين هيدروكلوريد هو دواء مضاد للفطريات التى تسيب الاظافر. ونظرا لقلة نفاذية من خلال الاستخدام الخارجى للجلد التى تؤدي إلى ضعف الأثر العلاجى. لذلك تم تحضير عقارالتربينافين هيدروكلوريد فى صورة حويصلات مختلفة وذلك لتحديد الصيغة الأمثل باستخدام تركيزات مختلفة من السطحي غير أيوني النشط (سبان وتوين X1و X2على التوالي ). وكشفت النتائج أن كلا X1و X2كان له تأثير واضح على حجم الحويصلة (Y1)وكفاءة انحباس(Y2) العقار. تم تمييز الحويصلات من خلال الشكل والحجم وإمكانات العامل الايونى وكفاءة انحباس العقار. وقد أظهرت الدراسة أن الصيغ الأمثل للعقارمن خلال التركيبات المختلفة بان حجم الحويصلة (٧٨.٧، ١٢٢.٨ و ٦٧.٨ نانومتر)، وإمكانات العامل الايونى (-٨.٢٨، ٨.٧٧ و 11.30) وكفاءة انحباس العقار (٩٢.٦٧، ٩٣.٨٦ و ٩٥.٧٥٪) على التوالي. وقد أظهرت الدراسة بأن حويصلات النانولعقارالتربينافين هيدروكلوريد من خلال صياغات تتخلل عبر الجلد أكثر من مرتين إلى ثلاثة أضعاف ارتفاع معدل تخلل مقارنة بالعقارالمعلق .وانة قد تحقق من مرونة الحويصلات المحضرة . وأخيرا، قد تبين من التركيبات المختلفة لحويصلات النانولعقارالتربينافين هيدروكلوريد قد تحسن اختراق العقار للجلد بدرجة كبيرة ونشاطة الملحوظ كمضاد للفطريات .وأظهرت لدينا الصيغ المختارة تأثير مضاد فطرى قوي لعلاج فطرأظافر اليدين والقدمين.