

# Development and Optimization of Terpene-Enriched Vesicles (Terpesomes) for Effective Ocular Delivery of Fenticonazole Nitrate: In vitro Characterization and in vivo Assessment

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Rofida Albash<sup>1</sup>  
Abdulaziz Mohsen Al-  
mahallawi<sup>2</sup>  
Mariam Hassan<sup>3</sup>  
Ahmed Adel Alaa-Eldin<sup>4</sup>

<sup>1</sup>Department of Pharmaceutics, College of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology, Giza, Egypt;

<sup>2</sup>Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Cairo University, Cairo, Egypt;

<sup>3</sup>Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt; <sup>4</sup>Department of Pharmaceutics, Faculty of Pharmacy, Fayoum University, Elfayoum, Egypt

**Objective:** The aim of the current study was to load fenticonazole nitrate, a slightly water-soluble antifungal agent, into terpene-enriched phospholipid vesicles (terpesomes) as a potential delivery system for the management of ocular fungal infection.

**Methods:** Thin film hydration method was used to prepare terpesomes according to a 3<sup>2</sup> full factorial design to inspect the effect of several variables on vesicles' features. The investigated factors were terpenes type (X<sub>1</sub>) and terpenes amount (X<sub>2</sub>) while the dependent responses were encapsulation efficiency percent (Y<sub>1</sub>), particle size (Y<sub>2</sub>) and polydispersity index (Y<sub>3</sub>). Design Expert<sup>®</sup> program was used to choose the best achieved formula. The selected terpesomes were further optimized via incorporation of a positive charge inducer (stearylamine) to enhance adhesion to the negatively charged mucus covering the eye surface. The in vivo performance of the optimized fenticonazole nitrate-loaded terpesomes relative to drug suspension was evaluated by measuring the antifungal activity (against *Candida albicans*) retained in the tear's fluid at different time intervals after ocular application in albino rabbits.

**Results:** The optimized terpesomes showed spherical vesicles with entrapment efficiency of 79.02±2.35%, particle size of 287.25±9.55 nm, polydispersity index of 0.46±0.01 and zeta potential of 36.15±1.06 mV. The in vivo study demonstrated significantly higher ocular retention of the optimized fenticonazole nitrate-loaded terpesomes relative to the drug suspension. Moreover, the histopathological studies proved the safety and biocompatibility of the prepared terpesomes.

**Conclusion:** The obtained results verified the potential of the terpesomes for safe and effective ocular delivery of fenticonazole nitrate.

**Keywords:** fenticonazole nitrate, histopathological study, ocular drug delivery, microbiological study, terpesomes

## Introduction

Ocular ailments can range from minor problems, for example conjunctivitis, to disorders that may lead to loss of vision which might influence either the anterior or posterior parts of the eye ball. The various ocular disorders necessitate appropriate drug use and delivery systems according to the affected tissues. The topical administration is considered as a safer option, however it is restricted to the anterior parts of the eye ball. Furthermore, due to the poor retention on corneal surface,

Correspondence: Abdulaziz Mohsen Al-mahallawi  
Faculty of Pharmacy, Cairo University,  
11562, Egypt  
Tel +201008226524  
Email Abdulaziz.mohsen@pharma.cu.edu.  
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a small portion of the instilled dose (1–10%) is absorbed through this barrier and approximately 1% is delivered to the aqueous humor.<sup>1</sup> In general, the ocular bioavailability can be increased if the time for corneal permeability and precorneal drug residence could be increased. However, the main strategy to enhance corneal permeation is achieved by the use of penetration enhancers, which might lead to toxicity of the eye.<sup>2</sup> Hence, several studies were concerned with developing systems with good adhesion characteristics, which could enhance precorneal drug residence time and consequently improve the ocular bioavailability.<sup>3</sup> Lipid vesicles encapsulating antifungal agents represent a potential approach to minimize their toxicity, improve their solubility, and to enhance corneal permeability and ocular bioavailability.<sup>4</sup>

Although fungal eye infections are less frequent than those caused by viruses and bacteria, they are typically more aggressive and may lead to vision loss. In the past, wearing contact lenses and corticosteroid treatment for a long time were considered as common risk factors, however recently the increment in an immunocompromised population, resulting from human immunodeficiency virus (HIV), chemotherapy and surgeries, led to a dramatic rise in the fungal eye infections incidence.

Azoles (compounds based on the imidazole ring) are among the main antifungal therapy groups. On the other hand, the rising resistance to azoles, primarily amongst an immunocompromised population, strongly limits the efficient therapy. Combinatorial delivery of drugs is a promising approach to avoid possible drug resistance. Lately, combining two or more active substances in appropriately designed delivery systems is increasingly used as an approach to enhance the efficacy against fungal infections.<sup>5</sup> One of the antifungal imidazole derivatives is fenticonazole nitrate (FTN). Its mechanism of action includes inhibition of ergosterol synthesis leading to damage to the cell membrane.<sup>6</sup> FTN exhibits fungistatic and fungicidal effects on dermatophytes, yeasts and fungi. It also has activity against Gram positive bacteria.<sup>7</sup>

Terpenes are naturally existing substances derived from essential oils and constituted of several isoprene (C<sub>5</sub>H<sub>8</sub>) units. Recently, they have been used as penetration enhancers.<sup>8</sup> In addition, terpenes could exhibit antifungal and antimicrobial activities owing to their buildup in the hydrocarbon chains of the cell lipid bilayer, which allows the easier transport of essential oils components to inside the cell.<sup>9</sup> This results in leakage of cytoplasm, cell lysis and finally cell death.<sup>10</sup>

Although the use of phospholipid vesicles to improve the ocular delivery of antifungal drugs has been investigated, actually, up to date, no paper has been published ever regarding the use of terpene-enriched phospholipid vesicles (terpesomes (TPs)) enclosing FTN for treating ocular fungal infections. As a consequence, the goal of the current work was to estimate the capability of TPs to improve the ocular delivery of FTN and to assess their safety. To achieve this aim, various variables affecting vesicles' characteristics were investigated via 3<sup>2</sup> full factorial design utilizing the Design Expert<sup>®</sup> program. The terpene type (X<sub>1</sub>) and amount (X<sub>2</sub>) were selected as independent factors, whereas entrapment efficiency percent (EE%; Y<sub>1</sub>), particle size (PS; Y<sub>2</sub>) and polydispersity index (PDI; Y<sub>3</sub>) were chosen as dependent responses. The best achieved formula was further optimized via addition of a positive charge inducer, stearylamine (SA), to improve corneal adhesion through electrostatic interactions with the negatively charged mucin. Finally, in-vivo studies using albino rabbits were conducted to prove the efficacy and safety of the optimized FTN-loaded TPs after ocular application.

## Materials

Fenticonazole nitrate (FTN) was gifted from Andalous Pharmaceutical Co. (Cairo, Egypt). L- $\alpha$  phosphatidylcholine (from egg yolk source) was procured from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Eugenol, fenchone and limonene were purchased from Alfa Aesar (Germany). Stearylamine (SA) was purchased from Fluka Chemical Co. (Germany). Methanol was purchased from El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt).

## Methods

### Preparation of FTN Loaded TPs

TPs were prepared using several terpenes namely; eugenol, fenchone or limonene at various amounts (10, 20 and 30 mg) employing the thin film hydration method (Table 1).<sup>11</sup> Firstly, phospholipid (100 mg), terpene and FTN (10 mg) were placed in a round-bottom flask and dissolved in 10 mL methanol. The organic solution was slowly evaporated under vacuum at 60 °C and 90 rpm using a rotatory evaporator (Rotavapor, Heidolph VV 2000, Burladingen, Germany) so that a thin film was obtained. The formed film was then hydrated using 10 mL of distilled water at 60 °C (which is exceeding the lipid phase transition temperature (T<sub>c</sub>)).<sup>12</sup> To guarantee full film

**Table 1** The Independent and Dependent Variables for the 3<sup>2</sup> Full Factorial Design Used for the Preparation of FTN Loaded TPs

Factors (Independent Variables)	Levels		
X <sub>1</sub> : Terpene type X <sub>2</sub> : Terpene amount (mg)	Eugenol 10	Fenchone 20	Limonene 30
Responses (dependent variables)	Constraints		
Y <sub>1</sub> : EE (%)	Maximize		
Y <sub>2</sub> : PS (nm)	Minimize		
Y <sub>3</sub> : PDI	Minimize		

**Abbreviations:** FTN, fenticonazole nitrate; TPs, terpesomes; EE%, entrapment efficiency percent; PS, particle size; PDI, polydispersity index.

hydration, glass beads were utilized for 45 min. The prepared dispersions were left at 4 °C overnight to get mature vesicles. After that, the dispersions were bath-sonicated (Ultra Sonicator, Model LC 60/H Elma, Germany) for 10 min for additional reduction of PS.

## Characterization of FTN-Loaded TPs

### Determination of Entrapment Efficiency Percentage (EE%)

The vesicular formulation was subjected to centrifugation at 20,000 rpm for 1 h at 4 °C utilizing a cooling centrifuge (Sigma 3K 30, Germany). After that, lysis of the obtained residue was done with methanol and the obtained clear solution was spectrophotometrically analyzed at  $\lambda_{\text{max}}$  252 nm (Shimadzu UV1650 Spectrophotometer, Koyoto, Japan). EE% was calculated from the following equation:<sup>13</sup>

$$EE\% = \left( \frac{\text{Entrapped drug concentration}}{\text{Total drug concentration}} \right) \times 100 \quad (\text{Eq. 1})$$

All measurements were done in triplicate.

### Determination of Particle Size (PS) and Polydispersity Index (PDI)

The PS and PDI of the prepared vesicular formulations were measured after proper dilution with distilled water via a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK).<sup>14</sup> All measurements were conducted in triplicate.

### Experimental Design Construction and Selection of the Best Achieved TPs Formula

The goal of development of pharmaceutical formulations using the “quality by design (QbD)” concept is to recognize the characteristics that are critical for the final product quality. To apply this concept, it is important to identify the critical quality attributes (CQAs), which are defined as the features that must be studied and controlled during the product development to guarantee the final product quality.<sup>15</sup> The particle size (PS) and the entrapment

efficiency (EE%) were the selected CQAs that can significantly affect the quality and efficacy of the developed TPs. Based on literature review<sup>16–19</sup> and preliminary screening experiments (data is not shown), risk assessment was implemented to identify the process parameters that may potentially affect the CQAs of FTN-loaded TPs. In the current study, terpene type (X<sub>1</sub>) and amount (X<sub>2</sub>) were identified as the highest formulation risk factors affecting the CQAs of TPs. The levels of these formulation risk factors were further investigated using a 3<sup>2</sup> full factorial design.

Design expert<sup>®</sup> program version 11 (Stat Ease, Inc., Minneapolis, MN, USA) was utilized for the factorial design analysis. The investigated responses were EE% (Y<sub>1</sub>), PS (Y<sub>2</sub>) and PDI (Y<sub>3</sub>) (Table 1). Afterwards, the best achieved formula selection relied on desirability criterion which permitted the consideration of all responses together, simultaneously. The target was to achieve a formulation with the highest EE% and the least PS and PDI (Table 1). The suggestion with highest desirability solution (near to one) was opted. The selected formulation was subjected to subsequent optimization.

### Optimization of the Selected FTN-Loaded TPs

For optimization of the selected TPs formulation, stearylamine (SA) as positive charge inducer was included in the construction of the TPs vesicular structure. It is worth mentioning that SA (5 mg) was added to methanol before evaporation step during the preparation of the optimized TPs formulation.

### Determination of Zeta Potential (ZP) of the Optimized TPs

The mean values of ZP were determined for the optimized formula before and after SA addition as positive charge inducers using ZetaSizer Nano ZS.<sup>20</sup> Samples were properly diluted with distilled water prior to measurement. The

ZP estimation was performed by monitoring the electrophoretic mobility of the vesicles in an electrical field. The measurements were done in triplicate. Statistical significance was studied using Student's *t*-test adopting SPSS® program 25.0 (SPSS Inc., Chicago, USA).

### Transmission Electron Microscopy (TEM) of the Optimized TPs

The optimized FTN-loaded TPs morphology was determined utilizing transmission electron microscope (Joel JEM 1230, Tokyo, Japan). The prepared formulation was put on a carbon-coated copper grid in the form of a thin film, negatively stained with 1.5% phosphotungstic acid then visualized and photographed.<sup>13</sup>

### In vitro Release of the Optimized TPs

In-vitro drug release was estimated utilizing the United States Pharmacopeia (USP) dissolution apparatus II (Pharma Test, Hainburg, Germany) for 6 hours at 37 °C. Samples of 1 mL (equivalent to 1 mg FTN) from the optimized TPs were placed in plastic cylindrical tubes which have a permeation area of 3.14 cm<sup>2</sup> with one end tightly covered with a cellulose membrane and the other end attached to the shaft of the USP dissolution apparatus instead of the baskets.<sup>11</sup> The receptor medium was 50 mL of phosphate buffer saline solution (pH 7.4) containing 25% ethanol to maintain the sink condition.<sup>16</sup> Aliquots were removed at 1, 2, 3, 4, 5 and 6 hours. Samples were analyzed by UV spectrophotometer at  $\lambda_{\max}$  252 nm. All measurements were performed in triplicate  $\pm$  standard deviation.

### pH Measurement of the Optimized TPs

The pH of the optimized TPs was determined to ensure its safety for ocular use. The measurement was done in triplicate and the value was expressed as the mean  $\pm$  standard deviation.

### Short Term Physical Stability of the Optimized TPs

With the aim of investigating the physical stability of the optimized TPs formulation, it was stored in sealed glass vials at 4–8 °C for 45 days.<sup>21</sup> At the end of the storage period, the formulation was re-evaluated regarding its appearance, entrapment efficiency and particle size. Statistical analysis of the obtained results was performed by Student's *t*-test using SPSS® 25.0 software. Difference at  $p \leq 0.05$  was considered significant.

### Determination of Minimum Inhibitory Concentration (MIC)

The measurement of MIC was conducted utilizing the broth microdilution technique in accordance with the Clinical and Laboratory Standards Institute guidelines.<sup>22</sup> A hundred  $\mu$ L of twofold strength Sabouraud dextrose broth (SDB) was added to every well of a sterile 96-well plate. A hundred  $\mu$ L of each of the tested formulae (FTN suspension and optimized FTN-loaded TPs) was then added to the first well of each row. Two-fold serial dilutions of each of the tested formulae were done from one row to the next till the tenth row (500–0.98  $\mu$ g/mL) (Figure S1 in the supplementary materials). The wells were then inoculated with 10  $\mu$ L of the *Candida albicans* ATCC 60193 suspension (10<sup>7</sup> CFU/mL). One row was made a control for sterility (neither yeast nor tested formula was placed) and another row was used as a control for growth (inoculated with yeast suspension with nothing added to the tested formula). Incubation of the plates was done at 25 $\pm$ 2 °C for 24 hours in aerobic environment. MIC was measured as the lowest concentration having no observable growth. The experiment was repeated three independent times.

### In vivo Studies

#### Animals

Nine adult male albino rabbits, having an average body weight of 2 $\pm$ 0.2 kg, were housed individually (one per cage) at 25 $\pm$ 2 °C, with the 12:12 hours cycle of light and dark. Animals were supplied with the standard commercial food and tap water ad libitum. Initial examination of all eyes was carried out with a hand held slit lamp. Animals having no signs of ocular inflammation were only included in this study. Approval of animal procedures was obtained from the Research Ethics Committee for experimental and clinical studies at the Faculty of Pharmacy, Cairo University, Egypt (Approval no. MIC2672) according to the local and national regulatory standards set for animal care.

#### Susceptibility Testing

Six rabbits were divided randomly into 2 groups (3 rabbits in each group,  $n = 3$ ) where group I received FTN suspension and group II received the FTN optimized TPs. *Candida albicans* ATCC 60193 was used as the test organism. The experiment was performed as described by Basha et al but with slight modifications.<sup>23</sup> Briefly, fifty microliters of each of the tested formulae (FTN suspension and FTN optimized TPs) were inserted within the lower conjunctival sac of the



right eye of the rabbit using a micropipette. In every rabbit, no drug was inserted in the left eye to serve as the control. At specific time intervals (1–12 hours), four sterile filter paper discs (Whatman no. 5, 6 mm in diameter) were wetted by placing the discs under the eyelid of each eye of each rabbit. For each eye (right and left), two discs were put in an Eppendorf tube (1.5 mL) which contains 500  $\mu$ L Sabouraud dextrose broth (SDB) inoculated with 10% v/v yeast suspension ( $10^7$  CFU/mL). The other two discs were put in an Eppendorf tube containing 500  $\mu$ L uninoculated SDB; this was used as a blank during measuring the optical densities. Afterwards, aerobic incubation of all the tubes was carried out at  $25 \pm 2$  °C for 24 hours. After incubation, 200  $\mu$ L of each tube was transferred to a sterile 96-well plate and the optical densities ( $OD_{600nm}$ ) were read on an automated spectrophotometric plate reader (Biotek, Synergy 2, USA) at a single wavelength of 600 nm. The obtained results were displayed as average growth inhibition % (mean  $\pm$  standard deviation).

The growth inhibition % was determined from the following equation:

$$\text{Growth inhibition \%} = \frac{\text{Control(left eye)} - \text{Test(right eye)}}{\text{Control(left eye)}} \times 100$$

$$\text{Growth inhibition \%} = \frac{OD_{600nm} - OD_{600nm}}{OD_{600nm}} \times 100$$

The area under the curve from 1 to 12 h ( $AUC_{(1-12h)}$ ) was determined by the linear trapezoidal method and used to estimate and compare the mean time for the antifungal activity of FTN in the eye tear fluid produced by the optimized FTN-loaded TPs and FTN suspension.

### Histopathologic Evaluation

In this study, three male albino rabbits had been used to evaluate the biocompatibility of the FTN optimized TPs after ocular delivery. One drop of FTN optimized TPs dispersion was instilled in the right eye of every rabbit while the left one was left without treatment (control). The application was done twice daily for a period of one week. Subsequently, the animals were subjected to anesthesia (using ketamine (200 mg/kg) and xylazine (20 mg/kg)) followed by decapitation. The corneas were separated from the eyeball and stored in formalin saline solution (10%v/v) overnight. The samples were dehydrated with alcohol, fixed in melted paraffin and left to harden in the form of cubic masses. Microtome (Leica Microsystems SM2400, Cambridge, UK) was utilized to make skinny slices (~2 mm), which were then deparaffinized and stained by hematoxylin and eosin. Lastly, the pigmented

samples were visually examined with a digital microscope (DMS1000 B; Leica, Cambridge, UK).<sup>24</sup>

## Results and Discussion

### Full Factorial Design Analysis

Design-expert<sup>®</sup> software (Stat-Ease, Inc., Minneapolis, Minnesota, USA) was utilized for the development of TPs according to  $3^2$  full factorial design (Table 1). The software had generated 9 experimental runs for the development of TPs. The selected model was two-factor interaction (2FI). Adequate precision was used to confirm that the model could be utilized for design space navigation. A ratio greater than four is favored which was observed for all responses (Table 2).<sup>25</sup> In addition, the predicted  $R^2$  and the adjusted  $R^2$  values were in a good agreement in all responses. The effects of formulation variables on the investigated responses are illustrated as 2D plots in Figures 1–3 (also illustrated as 3D plots in Figure S2 in the supplementary materials).

### Effect of Formulation Variables on the EE%

The capability of TPs to enclose a considerable quantity of FTN is a milestone for its intended utilization for the management of ocular fungal infection. The EE% of FTN in TPs ranged from  $73.18 \pm 0.31$  to  $89.96 \pm 0.76\%$  (Table 3). The noticeably high FTN entrapment could be explained by the lipophilic nature of the matrices of TPs which in turn assists the encapsulation of a larger quantity of the lipophilic antifungal agent (FTN). Similar results were obtained by Abdelbary et al in a study on the preparation of ultra-deformable bilosomes for the ocular delivery of another antifungal drug belonging to the azoles group (terconazole).<sup>26</sup> In the current study, it was found that the terpene type ( $X_1$ ) significantly ( $p < 0.0001$ ) affected FTN EE% (Figure 1A).

**Table 2** Output Data of the  $3^2$  Full Factorial Analysis of TPs Formulations and Predicted and Observed Values for the Selected TPs (TP2)

Responses	EE%	PS (nm)	PDI
Adequate precision	13.81	51.30	9.36
Adjusted $R^2$	0.89	0.99	0.83
Predicted $R^2$	0.78	0.98	0.64
Significant factors	$X_1, X_2$	$X_1, X_2$	$X_2$
Predicted value of selected formula (TP2)	78.68	285.65	0.49
Observed value of selected formula (TP2)	78.61	283.30	0.50

**Abbreviations:** TPs, terpesomes; EE%, entrapment efficiency percentage; PS, particle size; PDI, polydispersity index.

**Table 3** Experimental Runs, Independent Variables, and Measured Response of the 3<sup>2</sup> Full Factorial Experimental Design of FTN Loaded TPs

Formulation Code	Terpene Type	Terpene Amount (mg)	EE%	PS (nm)	PDI
TP1	Eugenol	10	80.85±0.41	425.80±18.31	0.73±0.12
TP2	Eugenol	20	78.68±0.36	285.30±0.61	0.50±0.004
TP3	Eugenol	30	73.18±0.31	233.30±2.62	0.51±0.008
TP4	Fenchone	10	82.23±0.89	824.80±36.55	0.76±0.10
TP5	Fenchone	20	81.11±0.37	796.70±30.55	0.72±0.10
TP6	Fenchone	30	73.61±3.26	467.20±13.58	0.46±0.04
TP7	Limonene	10	89.96±0.76	1378.00±77.56	0.83±0.02
TP8	Limonene	20	86.71±0.25	793.70±40.56	0.54±0.02
TP9	Limonene	30	81.12±0.78	777.70±23.03	0.46±0.11

**Note:** Data represented as mean ± standard deviation (n = 3).

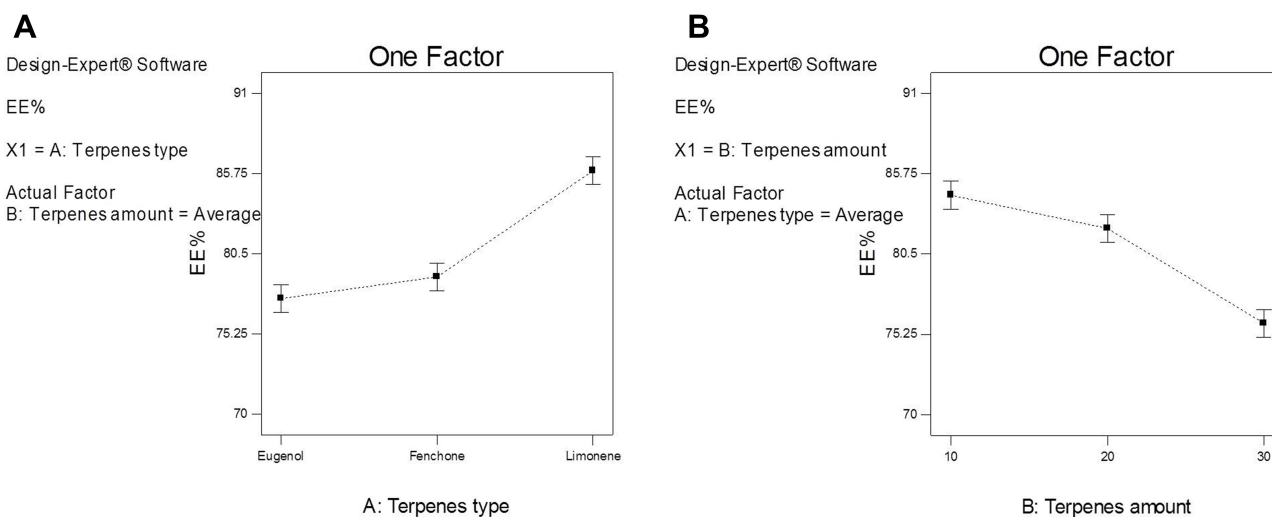
**Abbreviations:** FTN, fenticonazole nitrate; TP, terpesomes; EE%, entrapment efficiency percentage; PS, particle size; PDI, polydispersity index.

The obtained results revealed that amongst the investigated terpenes, limonene enriched TPs showed the highest values of EE% followed by fenchone and lastly eugenol. This could be explained in the light of the lipophilicity of the utilized terpenes. Since FTN is a lipophilic drug, it will be expected that the EE% will increase upon increasing the lipophilic character of the terpene which is incorporated in the vesicles. In the current study, limonene showed the most hydrophobic character compared to other terpenes,<sup>27</sup> as their log *p* values were 2.27, 3.52 and 4.57 for eugenol, fenchone and limonene, respectively.<sup>28–30</sup> Regarding terpene amount (*X*<sub>2</sub>), it was found that increasing the amount of terpene led to a significant decrease in the EE% (*p*<0.0001) (Figure 1B). Dragicevic-Curic et al<sup>31</sup> demonstrated that the addition of terpenes improved the fluidity of the phospholipid around the C<sub>16</sub> atom of the acyl chains of phospholipid that might

comprise the vesicles ability to entrap FTN. Further, increasing the amount of terpenes may result in the formation of pores which might destabilize the lipid bilayers of TPs and decrease the EE% values.<sup>32</sup>

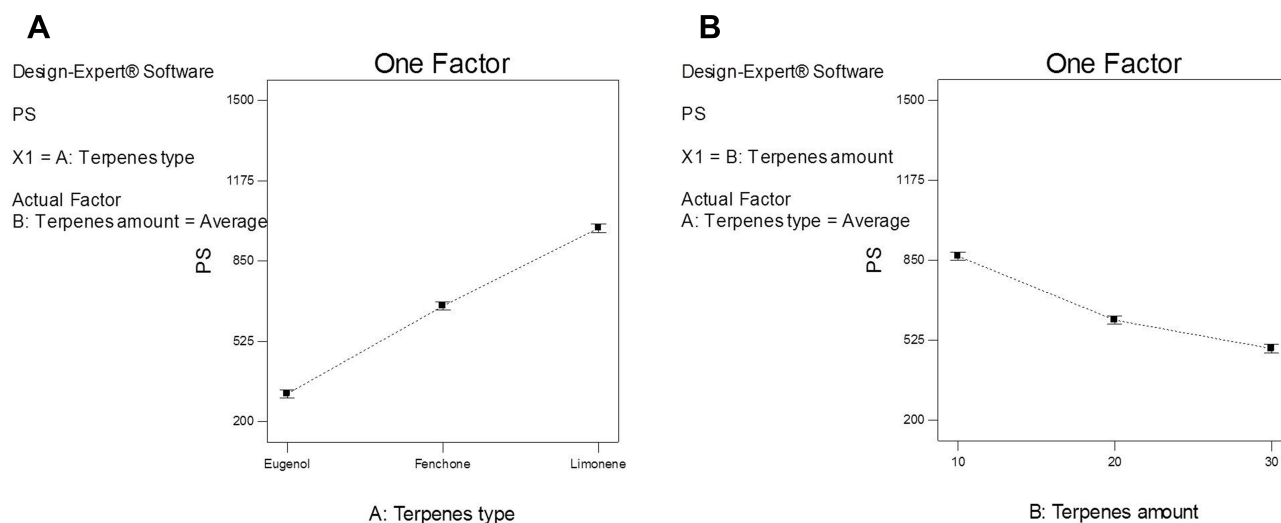
### Effect of Formulation Variables on PS

The particle size (PS) of the fabricated TPs is a key feature to determine their appropriateness for ocular application, since it influences their tolerance and disposition after ocular application.<sup>33</sup> The PS of FTN loaded TPs ranged from 233.30±2.62 to 1378.00±77.56 nm (Table 3). Figure 2A shows that the terpene type (*X*<sub>1</sub>) affected the PS of the prepared TPs significantly (*p*<0.0001), where limonene showed the largest particle size TPs compared to other terpenes. This came in agreement with EE% results as limonene showed the largest EE% values compared to



**Figure 1** The effect of (A) terpene type and (B) terpene amount on EE% of FTN loaded TPs.

**Abbreviations:** EE%, entrapment efficiency percent; FTN, fenticonazole nitrate; TP, terpesomes.



**Figure 2** The effect of (A) terpene type and (B) terpene amount on PS of FTN loaded TPs.

**Abbreviations:** PS, particle size; FTN, fenticonazole nitrate; TP, terpesomes.

fenchone and eugenol. As previously reported by Hathout et al<sup>34</sup> the increase in EE% values could explain the large PS of vesicles. In addition, high drug entrapment might expand the space between the vesicles bilayers due to the insertion of the dug in the hydrophobic regions inside the vesicles so TPs showing the highest EE% will have the largest values of PS.<sup>35</sup> Considering the terpene amount ( $X_2$ ), the mean PS of the formed TPs was significantly decreased ( $p < 0.0001$ ) upon increasing the amount of terpene from 10 mg to 30 mg (Figure 2B). This might be due to the reduction in viscosity and interfacial tension of the internal phase to the dispersion media that might inhibit Ostwald ripening of the formed TPs.<sup>36</sup>

### Effect of Formulation Variables on PDI

Regarding the PDI, a “zero” value points out an entirely homogenous system, while a value of “one” signifies a totally heterogeneous dispersion. The measured PDI values of the prepared formulations ranged from  $0.46 \pm 0.04$  to  $0.83 \pm 0.02$  which implies that they were relatively heterogeneous (Table 3). The obtained relatively high PDI values are frequently obtained in vesicular dispersions prepared by the thin film hydration technique.<sup>37</sup> Similar results were obtained by Al-mahallawi et al where the PDI values of nano-transfersomal ciprofloxacin loaded phospholipid vesicles ranged from 0.26 to 0.61 indicating their relative heterogeneity.<sup>38</sup> The effect of both formulation variables on the PDI of the prepared TPs is demonstrated in Figure 3A and B. Factorial analysis of variance

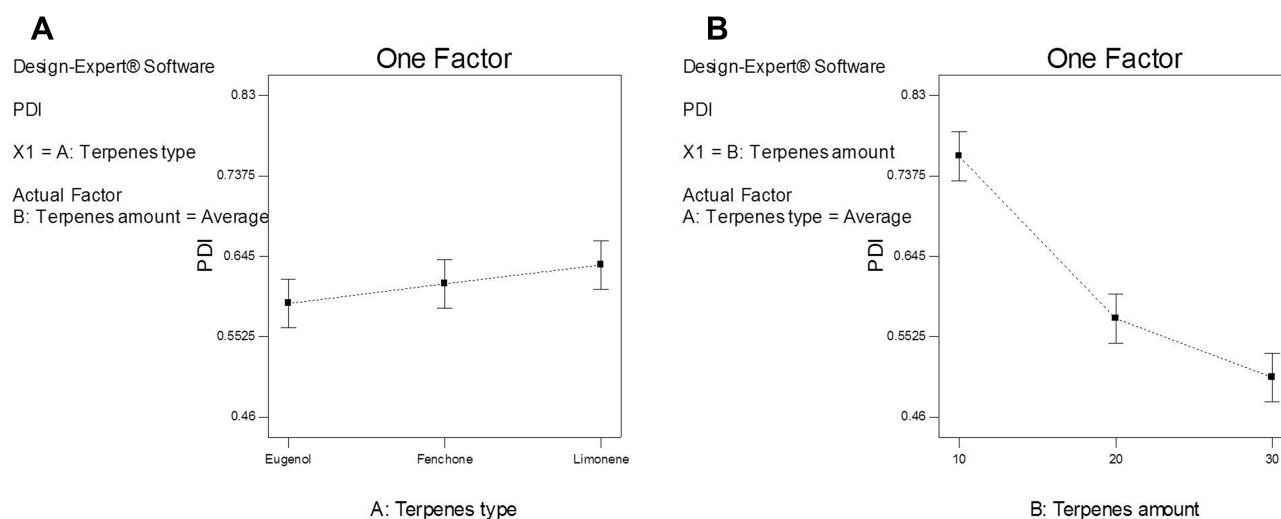
demonstrated that only terpene amount ( $X_2$ ) demonstrated a significant influence on the PDI ( $p < 0.0001$ ). It was obvious that upon increasing the terpene amount, both PS and PDI were found to be small. On the contrary, large PS and PDI values were obtained from a lower amount of terpenes. Comparable results were reported by Llinares et al<sup>39</sup> where the PS was in accordance with PDI values.

### Determination of the Selected TPs Formulation

The desired levels of the independent variables were achieved by numerical analysis using Design Expert® software. The purpose was to attain FTN loaded TPs having the highest EE%, lowest values of PS and PDI. Accordingly, the selected formula was (TP2), which included 20 mg of eugenol in its composition. Therefore, TP2 was selected for further investigations.

### Optimization of the Selected TPs Formula via Incorporation of a Positive Charge Inducer

As previously mentioned, the selected TPs formulation was further optimized by incorporating stearylamine (SA) as a positive charge inducer since the positivity of the TPs would be preferred for the ocular application because it enhances electrostatic interactions with mucin (which is negatively charged), which consequently enhances retention to the corneal surface.<sup>33</sup> In the



**Figure 3** The effect of (A) terpene type and (B) terpene amount on PDI of FTN loaded TPs. **Abbreviations:** PDI, polydispersity index; FTN, fenticonazole nitrate; TP, terpesomes.

current study, the addition of SA did not produce a significant change ( $p>0.05$ ) in the EE%, PS and PDI values upon comparison with formula lacking SA (Table 4). On the other hand, ZP values increased significantly ( $p<0.05$ ) up to  $35.40\pm 1.10$  mV, which is expected to increase the vesicles stability as the chance for vesicles agglomeration will be small for charged particles having a  $ZP \geq 30$  mV (as an absolute value) due to electrostatic repulsion between them.<sup>11</sup> It is worth mentioning that the low positive ZP values before optimization (addition of SA) could be due to the existence of cationic nitrogen atoms in the FTN structure, which imparts a dominating positive charge over the neutral charge of the utilized phospholipid.<sup>8</sup> Figure S3 in the supplementary materials shows the measurement of PS, PDI and ZP (using Zetasizer) of the optimized TPs.

### Transmission Electron Microscopy (TEM)

The assessment of the morphology of the optimized TPs via TEM revealed the spherical shape and the uniform size distribution of the prepared TPs (Figure 4). The PS of the

**Table 4** Characterization Results for the Selected TPs (TP2) and the Optimized TPs

Formula	EE%	PS (nm)	PDI	ZP (mV)
TP2	$78.68\pm 0.36$	$285.30\pm 0.61$	$0.50\pm 0.00$	$5.43\pm 0.19$
Optimized TPs	$79.02\pm 2.35$	$287.25\pm 9.55$	$0.46\pm 0.01$	$35.40\pm 1.10$

**Abbreviations:** TPs, terpesomes; EE%, entrapment efficiency percent; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

TPs measured by Zetasizer was in a good agreement with TEM observations.

### In vitro Release of the Optimized TPs

The in-vitro release profile of FTN from the optimized TPs in comparison with drug suspension is demonstrated in Figure 5. It is obvious from the figure that the release of FTN from the optimized TPs is markedly faster than that from the drug suspension. This could be attributed to the solubilization of FTN by the phospholipid based-vesicular carrier.<sup>40</sup> It is well known that the phospholipid possesses surface active properties which led to improved solubility of the encapsulated poorly water soluble antifungal drug, FTN.<sup>41</sup>

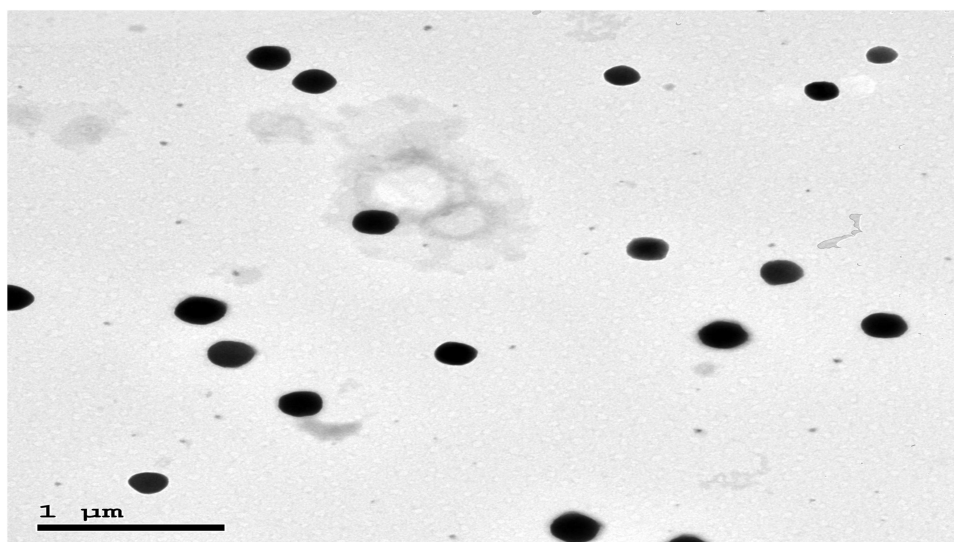
### pH Measurement of the Optimized TPs

The pH value of the optimized formulation was found to be  $7.25\pm 0.04$  implying its suitability for ocular use without causing irritation (pH of lachrymal fluid = 7.4).<sup>42</sup>

### Short Term Physical Stability of the Optimized TPs

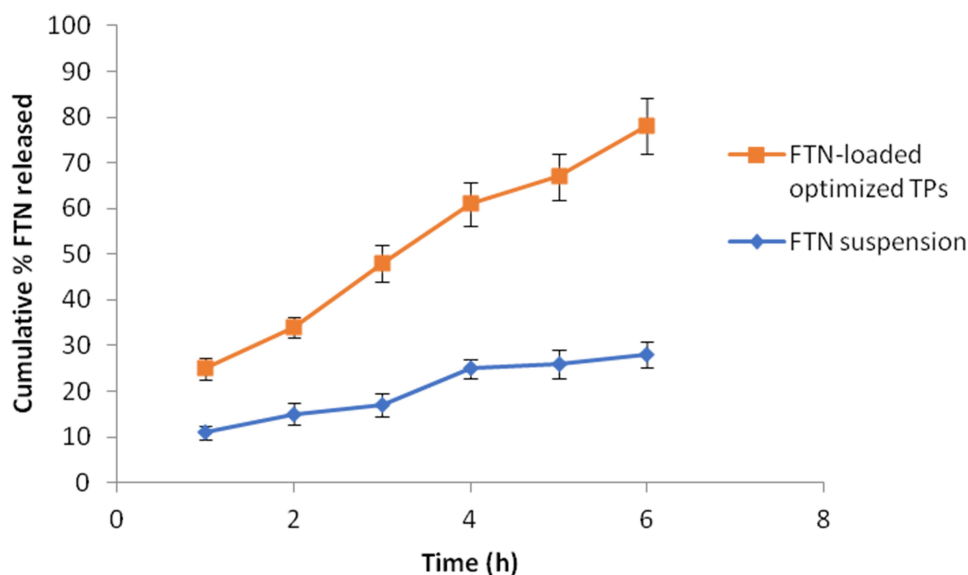
After 45 days of storage at  $4-8^{\circ}\text{C}$ , there was no visible change in the appearance of the optimized FTN-loaded TPs formulation. In addition, EE%, PS and PDI measurements of the stored formula were  $77.91\pm 1.79\%$ ,  $290.40\pm 12.30$  nm and  $0.47\pm 0.05$ , respectively, which showed insignificant variation from the values belonging to a freshly prepared system ( $p>0.05$ ). Therefore, it can be concluded that the storage at the specified conditions did not affect the properties of the optimized FTN-loaded TPs.





**Figure 4** Morphology of the optimized TPs.

**Abbreviations:** FTN, fenticonazole nitrate; TPs, terpesomes.



**Figure 5** In-vitro release profile of FTN from the optimized TPs in comparison with that of drug suspension.

**Abbreviations:** FTN, fenticonazole nitrate; TPs, terpesomes.

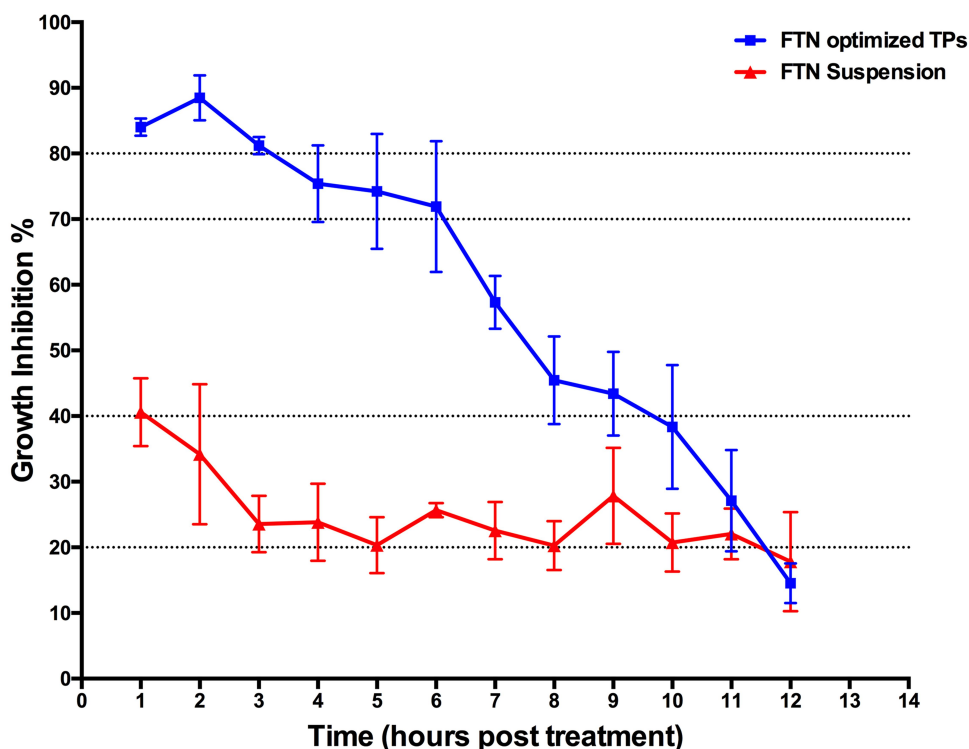
## Determination of Minimum Inhibitory Concentration (MIC)

The antifungal activity of the optimized FTN-loaded TPs and the FTN suspension were evaluated in-vitro using *Candida albicans* ATCC 60193 as the test organism. The MIC of the optimized FTN-loaded TPs was 31.25  $\mu\text{g/mL}$ , while that of the FTN suspension was higher than 500  $\mu\text{g/mL}$ . The optimized FTN-loaded TPs showed higher antifungal activity than that of the FTN suspension which might be due to the lower

particle size and higher zeta potential values. These results conform with previous studies where the PS and ZP significantly influenced the antifungal activity of the tested drugs.<sup>43,44</sup>

## In vivo Studies Susceptibility Testing

The optimized FTN-loaded TPs and the FTN suspension were evaluated in-vivo using *Candida albicans* ATCC 60193 as the test organism. The growth inhibition % of



**Figure 6** In vivo study graphical chart of FTN suspension and FTN-loaded TPs treated groups.  
**Abbreviations:** FTN, fenticonazole nitrate; TPs, terpesomes.

*Candida albicans* was related to the drug's retention time on the eye surface following the topical administration (Figure 6). The inhibition percentage of the FTN-loaded TPs reached the maximum ( $88.50 \pm 3.40\%$ ) two hours post-administration and then decreased gradually. On the other hand, the FTN suspension reached a maximum of  $40.60 \pm 5.20\%$  one-hour post-administration and then showed almost constant level from the third hour until the end of the study period ( $23.60 \pm 4.30\%$  -  $17.80 \pm 7.60\%$  from 3h - 12h). In a significant manner, the growth inhibition percentage of the optimized FTN-loaded TPs was higher than that of the FTN suspension until eight hours post-application (Student's *t*-test,  $p < 0.05$ ). The optimized TPs significantly sustained the antifungal activity of FTN on the ocular surface for a relatively longer time when compared to the FTN suspension with an area under the curve 2.40 folds higher than that of the FTN suspension ( $AUC_{1h-12h} = 652.20$  and  $270.30$ , respectively).

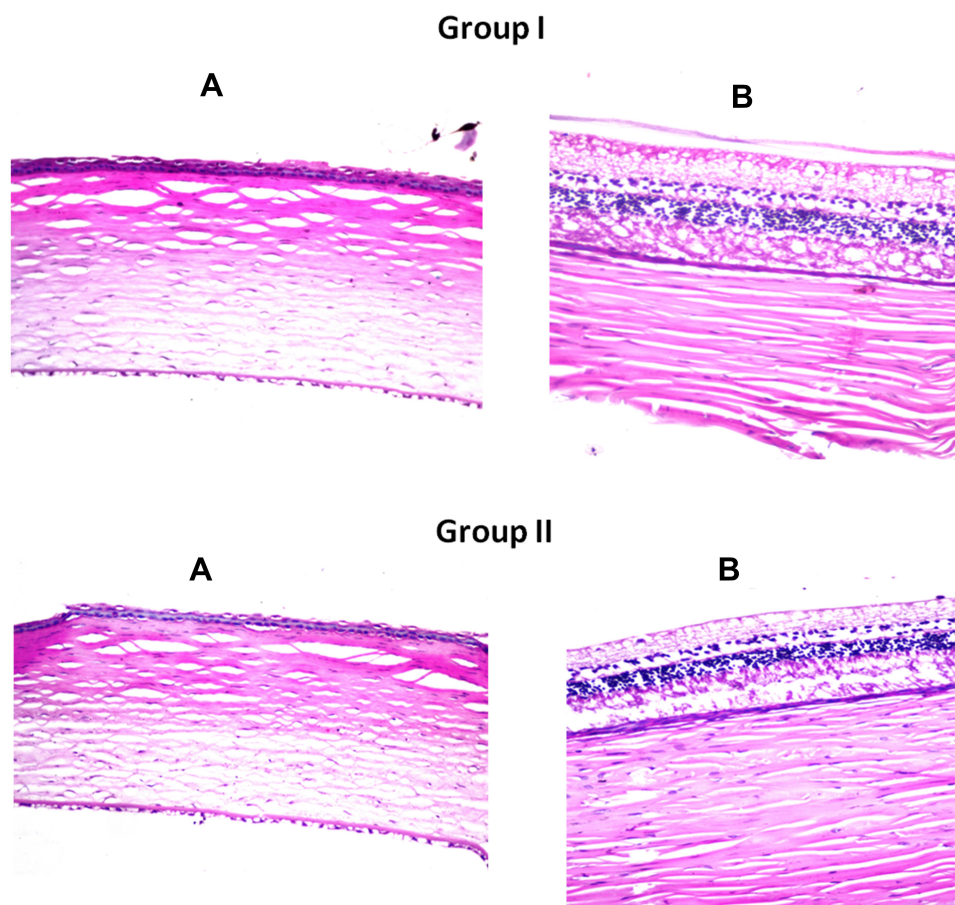
### Histopathological Study

Histopathological evaluation of ocular tissues was performed utilizing male albino rabbits. Microscopic examination of the pigmented pieces of the two groups (group I: untreated rabbit eye and group II: rabbit eye treated with

optimized FTN loaded TPs) showed no histopathological change in the cornea, iris, retina or sclera (Figure 7). The obtained results suggest that the optimized TPs have an acceptable safety level and are not expected to cause ocular irritation in clinical trials according to the fact that there were no clear signs of eye intolerance in the performed in-vivo studies.

### Conclusion

In the current study, fenticonazole nitrate loaded terpesomes were successfully developed in accordance with a  $3^2$  full factorial design. The best achieved terpesomes formulation was further optimized via incorporation of stearylamine (a positive charge inducer) in the vesicular structure to improve corneal adhesion through electrostatic attraction to the negatively charged mucus. The optimized fenticonazole nitrate loaded terpesomes showed small particle size, spherical morphology and reasonable entrapment efficiency. Moreover, it achieved superior in-vivo retention in the external eyes of albino rabbits in comparison to drug suspension. Moreover, the histopathological studies revealed the non-irritant character of the used terpesomes. Consequently, the utilization of terpesomes containing eugenol may be regarded as a valuable drug



**Figure 7** Photomicrographs showing histopathological sections (hematoxylin and eosin stained) of normal untreated rabbit's eye (group I) and rabbit's eye treated with FTN loaded optimized TPs (group II). (A) illustrates histological structure of the cornea and (B) illustrates histological structure of the retina, choroid and sclera.

**Abbreviations:** FTN, fenticonazole nitrate; TPs, terpesomes.

delivery system for enhancing the ocular delivery of fenticonazole nitrate.

## Disclosure

The authors report no conflict of interest.

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