Dapsone-Loaded Invasomes as a Potential Treatment of Acne: Preparation, Characterization, and In Vivo Skin Deposition Assay

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Abstract. Dapsone (DPS) is a unique sulfone with antibiotic and anti-inflammatory activity. Owing to its dual action, DPS has a great potential to treat acne. Topical DPS application is expected to be effective in treatment of mild to moderate acne conditions. Invasomes are novel vesicles composed of phosphatidylcholine, ethanol, and one or mixture of terpenes of enhanced percutaneous permeation. In this study, DPS-loaded invasomes were prepared using the thin film hydration technique. The effect of different terpenes (Limonene, Cineole, Fenchone, and Citral) in different concentrations on the properties of the prepared DPS-loaded invasomes was investigated using a full factorial experimental design, namely, the particle size, drug entrapment, and release efficiency. The optimized formulation was selected for morphological evaluation which showed spherical shaped vesicles. Further solid-state characterization using differential scanning calorimetry and X-ray diffractometry revealed that the drug was dispersed in an amorphous state within the prepared invasomes. Finally, the ability of the prepared DPS-loaded invasomes to deliver DPS through the skin was investigated in vivo using wistar rats. The maximum in vivo skin deposition amount of DPS was found to be 4.11 mcg/cm² for invasomes versus 1.71 mcg/cm² for the drug alcoholic solution, representing about 2.5-fold higher for the invasomes compared to the drug solution. The AUC0–10 calculated for DPS-loaded invasomes was nearly 2-fold greater than that of DPS solution (14.54 and 8.01 mcg.h/cm² for the optimized invasomes and DPS solution, respectively). These results reveal that the skin retention of DPS can be enhanced using invasomes.

KEY WORDS: dapsone; invasomes; terpenes; limonene; ex vivo.

INTRODUCTION

Dapsone (DPS) is a sulfone compound with a unique combination of antibacterial and anti-inflammatory potentials. It is totally absorbed after oral administration and result in 85% bioavailability. DPS is distributed to all organs where it is retained in different body compartments (including skin) up to 3 weeks after therapy termination. It can cross blood-brain barrier as well as placenta and secreted in breast milk (1). DPS is typically used for the treatment of dermatitis herpetiformis and leprosy (2). Owing to its dual action, DPS has a great potential to treat acne which affects 95% of population. Despite its desirable pharmacological activities in terms of combining antibacterial and steroid sparing anti-inflammatory activity, chronic and systemic use of DPS is classically associated with methemoglobinemia and hemolytic anemia (3). These serious side effects of oral DPS therapy render DPS use risky especially in relatively healthy acne population. For treatment of the widespread acne, topical application of DPS had been proposed twice daily to reduce systemic exposure and alleviate associated side effects (4).

Invasomes are novel vesicles with enhanced percutaneous penetration compared to the conventional liposomes. Invasomes are novel elastic phospholipid vesicles composed of phosphatidylcholine, ethanol, and one or mixture of terpenes. Many researchers have already confirmed the capability of terpenes in enhancing percutaneous penetration (5–10). Their permeation-enhancing activity is through the disruption of the stratum corneum lipids, interactions with intracellular proteins, and improvement of partitioning of the drug into the stratum corneum. Ethanol improves the vesicular ability to penetrate the stratum corneum (11–13). In addition, ethanol provides net negative surface charge and prevents vesicle aggregation due to electrostatic repulsion (14,15). A synergistic effect between terpenes and ethanol on
the percutaneous absorption has been significantly observed (14,16,17).

In this study, we investigated the ability of invasomes to increase the topical transport of DPS in order to develop a topical formulation with enhanced DPS skin delivery in order to achieve an effective acne treatment. The effect of different formulation variables on the properties of the prepared DPS-loaded invasomes have been investigated using a full factorial experimental design, namely, particle size, drug entrapment efficiency, and drug release from the prepared invasomes. The optimized formulation was selected for morphological evaluation using transmission electron microscopy and solid-state characterization using differential scanning caloriometry and X-ray diffractometry. Finally, the ability of the prepared DPS-loaded invasomes to deliver DPS through the skin was investigated in vitro and in vivo using wistar rats.

MATERIALS AND METHODS

Materials

DPS (Sigma-Aldrich, Germany), chloroform HPLC grade (Panreac Quimica SA, Barcelona, Spain), ethanol (El-Nasar pharmaceutical CO. Cairo, Egypt), phosphatidylcholine (Lechthin Soya Refined, MP Biomedicals, Germany), limonene, cineole, citral and fenchone (Alfa Aesar., Germany), potassium hydroxide, potassium chloride, sodium dihydrogen phosphate dihydrate and sodium chloride (El-Gomhoria for chemistry industrial, Egypt), dodeca-tungestophosphoric acid extra pure, Laboratory Rassayan, S.D. Fine-Chem Ltd., Boisar, India). Ethyl ether LR (S.d. CHEM LTd pharmaceutical CO. Mumbai, India), Sodium chloride solution 0.9% (Egypt Otsuka Pharmaceutical Co. Cairo, Egypt). All other chemicals were of analytical grade and were used as received.

Methods

Preparation of DPS-Loaded Invasomes

Different DPS-loaded invasomes were prepared using the conventional thin layer hydration technique (17). Accurately weighted amount of phosphatidylcholine (200 mg) was dissolved in 10-mL mixture of methanol/chloroform (2:1, v/v) until a clear solution was obtained. DPS (20 mg) and different concentrations of a terpene (limonene, cineole, citral or fenchone) were added and placed in a clean, dry, 1000-mL round bottom flask and mixed well. The organic solvent was evaporated by rotatory evaporator (Eyela rotavapor, USA) rotating at 120 rpm at 60°C for 15 min to obtain a clear film on the walls of the flask.

The deposited lipid film was then hydrated with 10-mL ethanolic–water mixture (3% v/v) by rotation at 120 rpm for 1 h at 60°C. The resulting nano-dispersion was filtered through a filter paper (pore size 25 μm) to remove any drug crystals (17), then stored at 4°C until use.

Evaluation of the Prepared DPS-Loaded Invasomes

Determination of Entrapment Efficiency. In order to estimate the percentage of DPS entrapped, 1-mL DPS-loaded invasome dispersion was centrifuged at 13,000 rpm at 4°C for 60 min using cooling centrifuge (Hermle labortechnik GmbH, Germany). The residue was separated, dissolved in methanol, and measured spectrophotometrically at 290 nm. DPS EE% was calculated using eq. (1) (18,19):

\[
\text{Entrapment efficiency}\% = \frac{\text{Amount of entrapped drug}}{\text{amount of total drug added}} \times 100
\]

Determination of Particle Size and Zeta Potential of DPS-Loaded Invasomes. The particle size (PS) of the prepared invasomes was measured using the dynamic light-scattering by Malvern Zetasizer (Malvern Instrument Ltd., UK) at temperature of 25 ± 2°C. The zeta potential of a selected DPS-loaded invasomal system was also performed using 90° scattering angle at temperature of 25 ± 2°C. The invasome dispersion was diluted 10-fold distilled water before the measurements to ensure that the light scattering intensity is within the instrument’s sensitivity range. All measurements were performed in triplicate and the mean values obtained were reported.

In Vitro Release Studies of DPS from DPS-Loaded Invasomes. The in vitro release of DPS from different DPS-loaded invasomes was evaluated using the dialysis bag diffusion technique, as previously reported in the literature (20,21). Amount of DPS-loaded invasomes (equivalent to 2 mg DPS) was separated by centrifugation at 13,000 rpm at 4°C for 1 h, then re-dispersed in 1-mL 3% (v/v) ethanolic aqueous solution and placed in a cellulose membrane bag. The dialysis bag was tightly closed at both ends and immersed in a stoppered bottle containing 100-mL phosphate buffer saline (PBS; pH = 7.4) representing the receptor compartment. The bottle was placed in a water bath shaker, stirred at 100 rpm, and maintained at 37 ± 0.5°C. At pre-determined time intervals, 3-mL sample of the receptor compartment was withdrawn and replaced with an equal volume of fresh medium to keep constant volume. The samples were properly diluted and analyzed for the amount of drug released spectrophotometrically at 290 nm. The release experiments were repeated for three times, and results presented are mean values (n = 3 ± SD).

The release efficiency of DPS from different DPS-loaded invasomes was calculated from the area under the release curve at time t. It is expressed as the percentage of the area of the rectangle corresponding to 100% release, for the same total time following eq. (2) (22):

\[
RE = \frac{\int_0^t dt}{y_{100}} \times 100
\]

where RE is release efficiency per, y is the percentage drug released at time t.

Study of the Effect of Different Formulation Parameters on the Properties of the Prepared DPS-Loaded Invasomes Using Full Factorial Design

A complete 4².3¹ factorial design was used to estimate and optimize the influence of different formulation variables.
Dapsone-Loaded Invasomes as a Potential Treatment of Acne

on the properties of DPS-loaded invasomes. In the utilized design, two factors were assessed, one at four levels (X1: type of terpene), and the other at three levels (X2: concentration of terpene), and the experimental trials were performed at all possible 12 combinations. The EE% (Y1), PS (Y2), % DPS released at 2 h (Y3), % DPS released at 24 h (Y4), and % RE (Y5) were designated as dependent variables. Design-Expert® software version 7 (Stat-Ease, Inc., Minneapolis, Minnesota, USA) was used to analyze experimental results followed by analysis of variance (ANOVA) to determine the significance of each factor. Table I shows the design parameters and their levels and Table II shows the experimental runs performed, and the responses measured.

Characterization of Selected DPS-Loaded Invasomes

Transmission Electron Microscopy. Morphological examination of selected DPS-loaded invasomes was carried out using transmission electron microscope (JEOL JEM-1230, Tokyo, Japan). DPS-loaded invasomes were stained using 1% phosphotungstatic acid (12). One drop of DPS-loaded invasomes was placed onto a carbon-coated copper grid placed on a filter paper to absorb the excess of the nanodispersion and allowed to dry to a thin film. Before complete drying of this film on the grid, it was stained with 1% phosphotungstic acid. After dryness, the sample was then examined by the transmission electron microscope with an accelerating voltage of 80 kV. Photographs were taken at suitable magnifications.

Lyophilization of Selected DPS-Loaded Invasomes. Selected DPS-loaded invasomes and the corresponding blank invasomes were lyophilized by freezing the separated DPS-loaded invasomes at −18 °C for 24 h. After that, the frozen solutions were lyophilized in a freeze dryer (Novalyph-NL 500; Savant Instruments Inc., USA) under a temperature of −45°C and vacuum of 7 × 10⁻² mbar for 24 h to get dry powder.

Thermal Analysis of the Selected DPS-Loaded Invasome

Differential Scanning Calorimetry. Thermograms were analyzed using differential scanning calorimeter (Shimadzu Corporation, DSC-60 with thermal analyzer, Shimadzu, Tokyo, Japan). The selected DPS-loaded invasomal system and the corresponding blank invasomes in addition to the individual components, namely PC and DPS, were evaluated by DSC. Samples were analyzed in sealed aluminum pans with pinholes. A heating rate of 5°C/min was employed over a temperature range of 25–250°C. The examination was conducted in nitrogen atmosphere, with nitrogen purging rate of 5 mL/min, for all samples. The temperature was calibrated with indium. Alumina powder was used as the reference material for the DSC runs.

X-Ray Diffraction Study. The selected lyophilized DPS-loaded invasomes and the corresponding blank invasomes in addition to the individual components, namely PC and DPS, were evaluated by XRD (XD-610; Shimadzu Corporation, Kyoto, Japan). The investigated samples were prepared by irradiation with Ni-filtered Cu Ka radiation, at 45 kV and 40 mA. The scanning rate employed was 2/min over a diffraction angle (2θ) range of 3–70.

Ex Vivo Evaluation of Selected DPS-Loaded Invasomes

Full thickness rat skin was prepared following the procedure explained previously by Panchagnula et al. (23). Ex vivo permeation studies across rat skin were carried out using a permeation set-up with receptor compartment filled with PBS (pH 7.4). The skin was stretched over diffusion cells of inner diameter 2.5 cm, with the dermal side in contact with the receptor phase, stirred at 100 rpm, and maintained at 37 ± 0.2°C for 24 h. At the end of the study, the skin was collected, cut into small pieces, and homogenized in 0.5 mL methanol for extraction of DPS. Samples were sonicated for 1 h followed by centrifugation at 4000 rpm to separate the drug extract. The procedures were repeated in triplicate and methanol extract was combined. The volume in each container was accurately completed to 2 mL with methanol and maintained at 4°C. Samples were diluted 1:10 in mobile phase prior injection in the HPLC for determination.

The concentration of DPS was measured using a Jasco HPLC system equipped with a PU-20180 Plus pump and a DG-2090-54 degasser. Chromatographic elution was performed on a SeQuan® ZIC-HILIC column (150 × 4.6 mm, 5 μm)using 50 mM ammonium acetate/methanol (20:80, v/v; pH 7.8) as the mobile phase running at a flow rate set at

<table>
<thead>
<tr>
<th>Factors (independent variables)</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1: Type of terpene</td>
<td>Limonene</td>
</tr>
<tr>
<td>X2: Concentration of terpene</td>
<td>0.5%</td>
</tr>
<tr>
<td>Responses (dependent variables)</td>
<td>Constraints</td>
</tr>
<tr>
<td>Y1: EE (%)</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y2: PS (nm)</td>
<td>Minimize</td>
</tr>
<tr>
<td>Y3: % DPS released after 2 h</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y4: % DPS released after 24 h</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y5: RE% after in vitro-release</td>
<td>Maximize</td>
</tr>
</tbody>
</table>

EE = entrapment efficiency, PS = particle size
N.B: All formulations contained DPS (2 mg/mL), 3.3% w/v ethanol, and 2% w/v P.C.
Animals was performed at 294 nm. The developed method was capped were used as drug pools with an area of 4.906 cm\(^2\) and rated into two groups with 18 animals in each group. Bottle In Vivo Skin Deposition Studies (Publication No. 85-23, revised 1985). and followed the recommendations of the National Institutes of laboraories and in accordance with the Animal proce-
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duced in accordance with the guide for care and use of

water, and libidium. All experimental procedures were con-
ducted in accordance with the guide for care and use of laboratory animals and in accordance with the Animal procedures were performed in accordance with the Ethics Committee and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

In Vivo Skin Deposition Studies

On the experiment day, the rats were randomly sepa-
rated into two groups with 18 animals in each group. Bottle caps were used as drug pools with an area of 4.906 cm\(^2\) and fixed to rat dorsal skin (previously shaved to remove hair with shaving cream 24 h before application of the sample). Half milliliter of the optimized DPS-loaded invasomes (INV\(_2\)) or DPS alcoholic solution (each equivalent to 1 mg/mL DPS) was added dropwise into the drug pool and wait for absorption of the formulation during the anesthesia of the rats using ether to calm them. At the end of the experiment, the rats were sacrificed with mercy.

The groups were treated according to the following pattern:

- Group I: topical application of DPS-loaded invasomes.
- Group II: topical application of DPS solution.

At specified time intervals (1, 2, 4, 6, 8, and 10 h after application of treatments), three animals from each group were sacrificed and the part of the dorsal rat skin contacting the treatment was excised and washed with 10 mL of normal saline on two portions. The excised skin sections were cut into pieces, homogenate, and sonicated in 2 mL methanol for 30 min. The skin was then centrifuged at 4000 rpm at 4 °C, and the concentration of DPS was determined using an accurate, validated HPLC analysis method as previously mentioned. The obtained data were used to determine the skin deposition of DPS from the formulations tested (invasomes and drug solution). ANOVA was performed to find out significant difference among the formulations using SPSS software 19.0.

RESULTS AND DISCUSSION

Analysis of Factorial Design

Factorial designs can simultaneously analyze the joint influence of different variables on the properties of the prepared drug delivery system (24). Table III shows the results of regression analysis of all the measured responses. Results show that for all the studied responses, the predicted \(R^2\) values were in good agreement with adjusted \(R^2\) (Table III). Adequate precision with a ratio greater than 4 (the desirable value) was observed in all responses confirming that the model can be used to navigate the design space (24).

Effect of Formulation Variable Entrapment Efficiency

Drug EE% inside the vesicles is a direct commentary on the ability of the drug to integrate with the lipoidal content to form particles of suitable integrity (17). The EE% of DPS in different invasomes ranged between 49.15 ± 1.34 and 82.25 ± 4.03% (Table II). ANOVA results showed that both independent variables evaluated (X\(_1\) and X\(_2\)) had a significant effect on the EE% (Y\(_1\)) \((p < 0.0001\) and \(p < 0.0001\), respectively).

The type of terpene significantly affected DPS EE% (Fig. 1a). Results show that among the tested terpenes, limonene containing invasomes showed the highest EE% followed by cineole, fenchone, and finally citral. This could be attributed to the lipophilicity of the used terpenes (as manifested by their log \(P\) values log \(P = 4.83, 2.82, 2.13, and

<table>
<thead>
<tr>
<th>Invasive formulations</th>
<th>(X_1): Terpene type</th>
<th>(X_2): Terpene concentration</th>
<th>(Y_1): EE (%)</th>
<th>(Y_2): PS (nm)</th>
<th>(Y_3): DPS released after 2 h* (%)</th>
<th>(Y_4): DPS released after 24 h* (%)</th>
<th>(Y_5): RE%* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INV(_1)</td>
<td>Limonene</td>
<td>0.5%</td>
<td>70.15 ± 1.91</td>
<td>296.80 ± 8.77</td>
<td>58.76 ± 1.96</td>
<td>84.79 ± 2.61</td>
<td>75.99 ± 1.29</td>
</tr>
<tr>
<td>INV(_2)</td>
<td>Limonene</td>
<td>1.5%</td>
<td>78.85 ± 0.21</td>
<td>319.30 ± 9.19</td>
<td>47.88 ± 5.62</td>
<td>67.06 ± 2.49</td>
<td>54.78 ± 1.30</td>
</tr>
<tr>
<td>INV(_3)</td>
<td>Limonene</td>
<td>3%</td>
<td>82.25 ± 4.03</td>
<td>494.50 ± 24.75</td>
<td>34.75 ± 1.18</td>
<td>49.40 ± 1.87</td>
<td>41.08 ± 0.40</td>
</tr>
<tr>
<td>INV(_4)</td>
<td>Cineole</td>
<td>0.5%</td>
<td>66.05 ± 1.48</td>
<td>311.70 ± 10.47</td>
<td>37.42 ± 3.54</td>
<td>59.17 ± 3.98</td>
<td>50.90 ± 0.39</td>
</tr>
<tr>
<td>INV(_5)</td>
<td>Cineole</td>
<td>1.5%</td>
<td>74.85 ± 1.02</td>
<td>329.95 ± 12.94</td>
<td>38.25 ± 2.16</td>
<td>67.20 ± 4.96</td>
<td>48.05 ± 3.08</td>
</tr>
<tr>
<td>INV(_6)</td>
<td>Cineole</td>
<td>3%</td>
<td>78.16 ± 1.45</td>
<td>509.05 ± 14.64</td>
<td>39.25 ± 2.16</td>
<td>70.98 ± 3.12</td>
<td>54.50 ± 2.69</td>
</tr>
<tr>
<td>INV(_7)</td>
<td>Citral</td>
<td>0.5%</td>
<td>49.15 ± 1.34</td>
<td>571.70 ± 22.49</td>
<td>10.40 ± 1.44</td>
<td>21.33 ± 2.43</td>
<td>13.57 ± 2.93</td>
</tr>
<tr>
<td>INV(_8)</td>
<td>Citral</td>
<td>1.5%</td>
<td>56.00 ± 1.41</td>
<td>668.05 ± 63.57</td>
<td>12.23 ± 0.92</td>
<td>29.68 ± 4.18</td>
<td>19.15 ± 1.97</td>
</tr>
<tr>
<td>INV(_9)</td>
<td>Citral</td>
<td>3%</td>
<td>64.15 ± 4.03</td>
<td>760.80 ± 24.89</td>
<td>11.59 ± 0.90</td>
<td>40.80 ± 3.48</td>
<td>27.37 ± 1.53</td>
</tr>
<tr>
<td>INV(_10)</td>
<td>Fenchone</td>
<td>0.5%</td>
<td>68.15 ± 2.62</td>
<td>528.30 ± 32.39</td>
<td>25.18 ± 0.98</td>
<td>40.81 ± 2.19</td>
<td>34.94 ± 0.66</td>
</tr>
<tr>
<td>INV(_11)</td>
<td>Fenchone</td>
<td>1.5%</td>
<td>70.10 ± 2.12</td>
<td>613.40 ± 32.39</td>
<td>24.73 ± 1.26</td>
<td>35.18 ± 4.12</td>
<td>32.68 ± 3.73</td>
</tr>
<tr>
<td>INV(_12)</td>
<td>Fenchone</td>
<td>3%</td>
<td>73.85 ± 0.21</td>
<td>319.30 ± 9.19</td>
<td>47.88 ± 5.62</td>
<td>67.06 ± 2.49</td>
<td>54.78 ± 1.30</td>
</tr>
</tbody>
</table>

0.5 mL/min. The injection volume was 20 μL and detection was performed at 294 nm. The developed method was validated in terms of linearity, accuracy, and precision.
Dapsone-Loaded Invasomes as a Potential Treatment of Acne

Table III. Results of Regression Analysis of the Measure Responses

<table>
<thead>
<tr>
<th>Responses</th>
<th>$R^2$</th>
<th>Adjusted $R^2$</th>
<th>Predicted $R^2$</th>
<th>Adequate precision</th>
<th>Significant factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE%</td>
<td>0.9708</td>
<td>0.9441</td>
<td>0.8833</td>
<td>252.10</td>
<td>$X_1$, $X_2$</td>
</tr>
<tr>
<td>PS</td>
<td>0.9801</td>
<td>0.9618</td>
<td>0.9204</td>
<td>45,613.94</td>
<td>$X_1$, $X_2$</td>
</tr>
<tr>
<td>%DPS released after 2 h</td>
<td>0.9851</td>
<td>0.9715</td>
<td>0.9405</td>
<td>283.98</td>
<td>$X_1$, $X_2$</td>
</tr>
<tr>
<td>%DPS released after 24 h</td>
<td>0.9911</td>
<td>0.9829</td>
<td>0.9642</td>
<td>239.00</td>
<td>$X_1$, $X_2$</td>
</tr>
<tr>
<td>%RE</td>
<td>0.9923</td>
<td>0.9853</td>
<td>0.9693</td>
<td>207.24</td>
<td>$X_1$, $X_2$</td>
</tr>
</tbody>
</table>

2.02 for limonene, cineole, fenchone, and citral, respectively) (25). The higher the lipophilicity of the terpene, the higher solubilization of the lipophilic drug (DPS), and hence the higher EE% owing to an increased space for the drug incorporation (26) in the lipid bilayer resulting in high EE%. Therefore, invasomes prepared with the more lipophilic terpenes (limonene and cineole) produce the higher EE% than less lipophilic terpenes (citral and fenchone). Similar results were reported by Prasanthi D et al., in their study on the preparation of finasteride vesicular invasomal carriers. They reported that limonene and cineole invasomes had higher drug EE% than nerolidol invasomes (27). Also, Lakshmi PK et al. reported that curcumin invasomes prepared using limonene showed higher EE% than invasomes prepared using fenchone (28). Another study by Saffari M et al. (29) reported that invasomes prepared using limonene as a terpene had higher EE% than those prepared using cineole for the antisense oligonucleotide drug.

Results also show that increasing the terpene concentration ($X_2$) leads to an increase in DPS EE% in the prepared DPS-loaded invasomes (Fig. 1a). The EE% of the drug in the prepared invasomes increased upon increasing the concentration of the lipophilic terpene owing to improved solubilization of the lipophilic drug (DPS). The lipophilic terpene was initially dissolved together with the PC during vesicle preparation, within the vesicular bilayer, where the acyl chains of phospholipids provide a favorable environment for the lipophilic terpene and the lipophilic drug. Therefore, the higher the terpene concentration, the more chains resulting in increasing solubilization of the lipophilic drug in bilayer and hence increased EE% (30). Similar results were obtained by Dragevic-curic et al. (14) in their study on the preparation of temoporfin-loaded invasomes. Their results showed that EE% of temoporfin is increased with increasing the amount of terpene (limonene, cineole, and citral) in the prepared invasomes.

Effect of Formulation Variables on the PS

Results of PS of the prepared DPS-loaded invasomes are shown in Table II. All the prepared DPS-loaded invasomes were found to lie in the nano-range, with their PS ranging between 263.10 ± 58.27 and 760.80 ± 24.89 nm and PDI values ranging between 0.348 ± 0.02 and 0.636 ± 0.1. ANOVA results show that both independent variables evaluated had a significant impact on the PS of the prepared invasomes, namely, the type of terpene ($X_1$) and concentration of terpene ($X_2$) had a p value < 0.0001 and < 0.0001, respectively.

As shown in Fig. 1b, the type of terpene significantly affected the PS of the prepared invasomes. The PS of DPS-loaded invasomes showed an inverse relation with the lipophilicity of the used terpenes. DPS-loaded invasomes prepared using citral had the largest PS compared to other invasomes prepared using other terpenes. This may be attributed to the low lipophilicity of citral (log P of citral is 2.02 which is the lowest among the used terpenes). This could have resulted in a high repulsion force between the lipophilic composition of vesicular system and the hydrophilic terpene. Similarly, DPS-loaded invasomes prepared using limonene has the smallest PS due to highest lipophilicty of limonene (log P of limonene is 4.83). Similar results were reported by Saffari M et al. (29) where invasomes prepared using limonene were smaller than those prepared using cineole.

Figure 1b also shows that increasing the concentration of the terpene ($X_2$) had a significant direct impact on the PS of DPS-loaded invasomes, where increasing the concentration of terpenes resulted in larger PS of the prepared invasomes. This is in good agreement with the results of EE%, where increasing the concentration of terpene resulted in a higher entrapment of DPS in the prepared invasomes. This could be attributed to the higher amount of lipophilic drug entrapped inside the prepared invasomes containing higher concentration of terpene. Similar results were obtained by Dragevic-curic et al. (14), in their study on the preparation of temoporfin-loaded invasomes, where they reported that the PS of invasomes increased with increasing the amount of terpenes used in the preparation.

Effect of Formulation Variables on % DPS Released After 2 and 24 h

The release profiles of DPS from different DPS-loaded invasomes are presented in Fig. 2a–d. Results of the ANOVA analysis show that both the type ($X_1$) and concentration of terpene ($X_2$) had a significant impact on the % DPS release after 2 and 24 h.

As presented in Fig. 1c, d, DPS-loaded invasomes prepared using limonene had the highest % DPS released after 2 and 24 h followed by cineole, fenchone, and finally citral. The higher enhancement of limonene may be attributed to its low boiling point. Limonene exhibits the lowest boiling point among the tested terpenes (176°C), followed by cineole (177°C), then fenchone (192°C) and finally citral (229°C). The lower the boiling points of the terpene, the weaker the cohesiveness or self-association of the molecules.

The concentration of terpene ($X_2$) also had a significant effect on this response except in the case of limonene, as increasing concentration of limonene resulted in lowering % DPS-released from the invasomes after 2 and 24 h to a great extent compared to other terpenes.
This could be attributed to the fact that limonene is the most lipophilic among the tested terpenes. Limonene has the highest log $P$ value (4.83) followed by cineole (log $P = 2.82$), fenchone (log $P = 2.13$) and finally citral (log $P = 2.02$) which shows the last value. The high lipophilicity may have retarded the drug release by creating a lipophilic matrix inside the invasomes for the lipophilic drug. However, increasing the concentration of other terpenes tested resulted in increase in % DPS released after 2 h, owing to their lower lipophilicity. Similar results were obtained by Prasanthi P et al. (27) in their study on the evaluation of finasteride invasomes, where invasomes containing 0.5% limonene showed higher release percentage than those containing 1% limonene.

Results in Fig. 1e show that different types of terpenes ($X_1$) had a significant effect on the RE% of DPS from the prepared invasomes. DPS-loaded invasomes prepared using limonene had the highest RE% followed by cineole, fenchone, and finally citral, owing to the effect of their boiling points, as mentioned earlier.

Selection of the Optimized Formulation

The optimum values of the variables were obtained by graphical and numerical analysis using the Design-Expert software in order to obtain DPS-loaded invasomes with the highest EE%, lowest PS, highest % DPS released after 2 and 24 h, and highest RE%. The optimized formulation was found to be INV2 with limonene as the terpene in the concentration of 1.5%, achieving desirability of 0.735. Therefore, INV2 was selected for further examinations.

Characterization of Optimized Formula

Zeta Potential

Zeta potential indicates the degree of repulsion between adjacent similarly charged particles. A high zeta potential reveals stability and ensures that the dispersion will resist aggregation. In general, the dividing line between stable and unstable dispersion is set to higher or lower value of ± 30 mV.
The value of zeta potential of the optimized DPS-loaded invasomes (INV2) was measured to be $-37.5 \pm 0.21$ mV. This high negative charge is attributed to the presence of ethanol which provides net negative surface charge and prevents vesicle aggregation due to electrostatic repulsion (14,16). These results ensure the stability of invasomal dispersion and avoidance of aggregation of the nanovesicles.

Morphological Examination of the Optimized DPS-Loaded Invasomes

Morphological examination of the optimized DPS-loaded invasomes was carried out using transmission electron microscope (TEM). As shown in Fig. 3, the developed invasomes were unilamellar with uniform spherical discreet shape with no fusion. The figure indicates that the diameters of the vesicles observed in the micrographs are close to the values obtained by particle size analysis.

Differential Scanning Calorimetry

DSC is commonly used to evaluate the crystalline or amorphous nature of the drug within the developed formulation and to detect any possible interactions with other ingredients (32). Figure 4 shows the thermograms of the pure DPS, PC, the selected lyophilized DPS-loaded invasomes (INV2), and the corresponding lyophilized blank invasomes.

The DSC scan of pure DPS exhibited a melting endothermic peak at 177.76°C, corresponding to its melting temperature which is closed to that reported previously (34). The endothermic peak of DPS was completely absent from the lyophilized DPS optimized formulation. The melting point of PC in the blank formulation shifted to 155.51, 181.49, and 196.02°C. The melting point of PC in INV2 formulation shifted to 146.84 and 221.42°C. This shift in the melting point and enthalpy values might be associated with lattice defects and is an indication of the formation of amorphous regions in which the drug is located. These results reveal that the drug could be homogeneously dispersed throughout the nanovesicular formulation in the amorphous state (24,35).

Fig. 2. Release profile of DPS from DPS-loaded invasomes prepared using limonene (a), cineole (b), citral (c), fenchone (d)

Fig. 3. TEM photomicrograph of the optimized DPS-loaded invasomal formulation (INV2)
X-Ray Diffraction Study

The integrated intensity (the area under the curves of the XRPD patterns) is the characteristics of given sample (36). The XRD of DPS, PC, the lyophilized optimized formulation (INV2), and the corresponding lyophilized blank invasomes are plotted in Fig. 5. DPS is crystalline in nature with sharp intense peaks at 12-14-16-18-20-22-28 . On contrary, PC and the blank lyophilized invasomes are completely amorphous and showed no distinct diffraction peaks.

The lyophilized optimized formulation (INV2) showed complete absence of the characteristic peaks of DPS in XRD. This could indicate the encapsulation of DPS in the formed nanovesicles in an amorphous form (34). Similar results were previously reported in the literature (24,35).

Ex Vivo Evaluation of the Optimized DPS-Invasomes

For comparing DPS solution and INV2 in terms of skin permeability, DPS was allowed to contact the skin for a period of 24 h. After that, DPS was extracted from skin as described earlier and analyzed by HPLC.

Several columns (RP-C18 and HILIC) and mobile phase blends were tried. Initially, an C18 column was tried as the stationary phase. DPS retention was not satisfactory using this reversed phase column. This may be attributed to the polarity of DPS, a case which will render the assay highly liable to interference from endogenous substances in skin extract. To enhance DPS retention, the pH of the mobile phase was increased to 7.4 in order to reduce DPS polarity. Additionally, the aqueous component of the mobile phase was increased. This chromatographic system resulted into a better DPS retention (5.2 min); however, the high aqueous content led to an increased system pressure. Moreover, on testing the linearity of analytical response, an unexplained elevated intercept was obtained; this may indicate interference from endogenous skin extract components.

Based on the polar nature of DPS, a HILIC column was tried to achieve proper retention and overcome previous RP column shortcomings. A ZIC®HILIC® column was used. Retention was enhanced (8 min) using a mixture of methanol and 50 mM ammonium acetate (80:20, v/v); however, the obtained peak suffered from obvious tailing. This was abolished on adjusting the pH of the mobile phase to 7.8. The detection was performed at 294 nm. DPS determination was carried out against an external calibration curve. The developed method was linear (R² = 0.9998), accurate (= 99 ± 4), and precise (CV < 3) in the range (10–100 μg/mL).

Analyzing the concentration of DPS in the rat skin revealed that the amount of DPS retained in the skin after treatment with DPS solution and INV2 dispersion was equivalent to 120.24 ± 10.7 and 457.56 ± 17.6 μg/mL, respectively, corresponding to ≈ 4-fold enhancement in the invasomes-treated skin.
Statistical analysis revealed a significant difference in the DPS flux between the DPS solution and the optimized invasomes (INV₂) \( (p < 0.05) \). This result could be explained by ability of terpenes to enhance the permeation of the drug across the skin mainly by disrupting highly ordered intercellular packing of the stratum corneum lipids and increasing drug diffusivity \( (37,38) \). Similar results for the penetration studies were obtained by Dragicevic-Curic et al. \( (14) \) who reported that liposomes containing 1% terpenes delivered temoporfin 2.7-fold higher than liposomes.

### In Vivo Evaluation of the Optimized DPS-Loaded Invasomes

The in vivo skin deposition profiles of DPS from the optimized invasome formulation (INV₂) in comparison with DPS ethanolic solution are displayed in Fig. 6. The obtained skin deposition parameters are collectively presented in Table IV. As depicted in Fig. 6, DPS showed higher in vivo skin deposition from the investigated invasomes during the study period compared to DPS solution. The highest in vivo skin deposition amount of DPS was 4.11 mcg/cm² from invasomes versus 1.71 mcg/cm² for the drug alcoholic solution, representing about 2.5-fold higher for the drug-loaded invasomes compared to the drug solution. The AUC\( _{0–10} \) calculated for DPS-loaded invasomes was nearly 2-fold greater than that of DPS solution \( (14.54 \text{ and } 8.01 \text{ mcg.h/cm}^2 \text{ for INV₂ and DPS solution, respectively}) \). Invasomes are known to be efficient nanocarrier systems for delivering hydrophobic drugs through the skin \( (14) \). In addition, incorporation of limonene led to an efficient delivery system, as terpenes act as penetration enhancers through the skin layers \( (39,40) \). Previous literature has shown the importance of the synergistic effect of phospholipids, terpenes, and ethanol \( (14) \). These conclusions are in accordance with results obtained by other authors who proposed that ethanol and phospholipids \( (11–13,41) \) as well as terpenes, phospholipids, and ethanol \( (42) \), applied together, have a synergistic effect on fluidizing the intercellular SC lipids, which results in an enhanced penetration of substances. The precise mechanism of the penetration enhancing ability of invasomes should be further investigated. Similar results were reported by Dragicevic-Curic et al. \( (16) \) who reported similar results for invasome dispersions for delivering the photosensitizer (mTHPC) to the skin.

### CONCLUSION

In this work, invasomes were investigated as a possible carrier for transdermal delivery of DPS. Invasomes were successfully prepared using thin film hydration method. A full factorial design was used to study the effect of varying the terpene type and concentration on the properties of different invasomes prepared and to select the optimal invasome system with reasonable drug EE\%, small PS, together with higher DPS skin retention ability compared to the DPS solution. The existence of DPS in an amorphous state within the structure of invasomes was demonstrated by DSC and XRD studies. The performed ex vivo skin permeation and in vivo skin deposition studies revealed that invasomes enhanced the skin drug deposition. The highest in vivo skin deposition amount of DPS was about 2.5-fold higher for the drug-loaded invasomes compared to the drug solution. Furthermore, the AUC\( _{0–10} \) calculated for DPS-loaded invasomes was nearly 2-fold greater than that of DPS solution. These results clearly demonstrate that the skin deposition parameters for DPS-loaded invasomes and DPS solution are presented in Table IV:

### Table IV. In Vivo Skin Deposition Parameters of DPS After Topical Application of DPS-Loaded Invasomes and DPS Solution

<table>
<thead>
<tr>
<th>Skin deposition parameters</th>
<th>DPS-loaded invasomes (INV₂)</th>
<th>DPS solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} ) (µg/cm²)</td>
<td>4.12</td>
<td>1.71</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>( \text{AUC}_{0–10} ) (µg h/cm²)</td>
<td>14.54</td>
<td>8.01</td>
</tr>
</tbody>
</table>

**Fig. 6.** In vivo skin deposition profiles of DPS after treatment with DPS-loaded invasomes and DPS solution by topical application.
deposition characteristics of DPS can be improved using invasomes.

COMPLIANCE WITH ETHICAL STANDARDS
All experimental procedures were conducted in accordance with the guide for care and use of laboratory animals and in accordance with the Animal procedures were performed in accordance with the Ethics Committee and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Conflict of Interest The authors declare that they have no conflict of interest.

REFERENCES


Dapsone-Loaded Invasomes as a Potential Treatment of Acne


