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Penetration Enhancer Containing Vesicles (PEVs) as Carriers for Enhancing the Dermal Deposition of Thymoqunione

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ABSTRACT

Thymoquinone is the main active ingredient in the black seed oil that is used in traditional medicine for the treatment of various diseases. Although having excellent reported antifungal and antibacterial properties, it has not been thoroughly investigated for topical applications. Here we investigated the potential to enhance its skin deposition by using penetration enhancers containing vesicles (PEVs). Different hydrophobic and hydrophilic penetration enhancers have been investigated including Cineole, Transcutol, Polyethylene glycol and propylene glycol. All the prepared formulae were in the size range between 100 and 300 nm with good particle size homogeneity and negative surface charge. The influence of the penetration enhancer on the entrapment efficiency, *in vitro*drug release and the *exvivo* skin deposition in rat skin, has been studied. It was found that the PEVs prepared with 20% v/v Transcutol or 30% v/v propylene glycol had the most significant enhancing effect on Thymoquinone entrapment and skin retention. Additionally, it was found that all the prepared PEVs enhanced the skin retention of Thymoquinone without any enhancement of its permeation through the skin proposing an excellent carrier for the localized treatment of skin bacterial and fungal infections using Thymoquinone.

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1. INTRODUCTION:

Recently, the use of nanocarriers has been explored extensively for different drug delivery purposes aiming for higher therapeutic efficiency. Indeed, nanocarriers could overcome different limitations in drug delivery applications such as the low solubility and the subsequent low bioavailability, stability problems, difficulty in drug targeting and rapid metabolism.Due to their special characteristics, reactivity and unique interactions with biological systems, their use can significantly improve pharmacokinetics and therapeutic efficiencies of the encapsulated drugs [1].

Among the different drug delivery routes, dermal and transdermal drug delivery has been always considered an attractive strategy for achieving higher drug therapeutic efficiency with minimum systemic side effects. Between the different chemical and physical methods used to enhance the dermal drug delivery is the use of nanocarriers [2]. There is especially a great interest in lipids vesicles as a tool to improve dermal and transdermal drug delivery with a continuous need to improve their performance compared to the conventional liposomes [3]. One of the main ways to improve liposome efficacy in dermal use is to modify the hydrophilic phase mixing water with others solvents such as Propylene glycol (PG) and Polyethylene glycol (PEG) [4]. Another promising alternative is the use of permeation enhancers containing vesicles (PEVs), which proved to be particularly able to enhance *ex vivo* cutaneous drug deposition of various active ingredients [3, 5,6].

Thymoquinone (TQ: 2-methyl-5-isopropyl-1,4-benzoquinone) is the main active ingredient in the volatile oil of *Nigella sativa* seed, which is commonly known as the black seed or black cumin. It constitutes around 27% of the seeds volatile oil composition. It is used and known for centuries as a traditional medicine for treatment of various diseases. It has been known to exhibit various pharmacological effects including anti-neoplastic, anti-oxidant, anti-inflammatory, immunomodulatory as well as anti-diabetic. Interestingly, TQ was found to have excellent properties making it an excellent candidate to be used as a topical drug for the treatment of microbial and fungal skin infections [7]. It has been shown that TQ is an inhibitor of some human pathogens (11 dermatophytes, 8 yeasts, and 11 mold isolates) isolated from skin and nails of patients with fungal infections[8]. Among the other components of the *Nigella sativa* seeds essential oil, TQ proved to possess the highest antifungal activity against dermatophytes and yeasts. TQ especially showed up as an inhibitor of the germination of the dermatophytes [9]. Therefore, and due to the limited number of

available effective antifungal agents with limited toxicity [10] as well as the increased resistance developed against the existing ones [11], there is a great need for new antifungal agents with high efficiency, wide spectrum, and limited toxicity.

Due to the poor aqueous solubility of TQ, its light and heat sensitivity, its bioavailability and successful therapeutic use have been hindered. Its encapsulation into the vesicular system would enhance its stability as well as its skin deposition and bioavailability in the skin tissue for the treatment of various skin conditions. Its anti-oxidant, anti-inflammatory, antibacterial and antifungal properties would be highly improved and higher therapeutic benefits would be achieved. The aim of this work is to encapsulate TQ into PEVs and to test the influence of four different penetration enhancers (PE) (Cineol, Transcutol, Polyethylene Glycol and Propylene Glycol) on the skin retention of TQ. For such a purpose, different formulations were prepared with the different amount of each PE, and were characterized in terms of Entrapment Efficiency (EE%), Particles size, Zeta potential, *In vitro* release, and *ex vivo* skin permeation, and compared to a conventional liposome formulation of TQ.

2. MATERIALS AND METHODS:

2.1. Materials:

Soybean Lecithin (SL) was purchased from Caelo, Germany. Thymoquinone (TQ), Diethylene glycol monoethylether (Transcutol (Trc)), Polyethylene Glycol 400 (PEG), and 1,8-Cineol (Ci) were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Propylene Glycol (PG) was from Fagron GmbH & Co. KG, Germany. Spectra/Por dialysis membrane,12,000–14,000 molecular weight cutoff, was purchased from Spectrum Laboratories Inc., Canada.Rats' skin was kindly provided by the Hospital of Bonn University.All other chemicals were of analytical grade or equivalent purity.

2.2. Preparation of the vesicles:

Vesicles were prepared bythin film hydration method. Briefly, Lecithin(200 mg) and Thymoquinone(15 mg) were dissolved in chloroform and the quantities were selected based on previous preliminary experiments. The lipid–drug mixture solution was dried into a thin film in a round-bottom flask by evaporating the chloroform under vacuum(Rotavapor Büchi RE 120, Germany),with a continuous reduction of pressure for 1hourto ensure total removal of any solvent traces. The film was hydrated for 1 hat room temperature by the gradual

addition of Phosphate Buffer pH=7.4 (PBS) under mechanical stirring. The resulting vesicle suspension was sonicated for three intermittent cycles of 3 minutes in an ice bath for the protection of the drug from any heat generation during the sonication time (Sonoplus Bandelin HD 2200).

Cineol, a hydrophobic penetration enhancer (PE) was added to the lipid-drug mixture, while hydrophilic PEs were added in a different amount in the PBS, as shown in Table 1.

Vesicles were purified from the non-encapsulated drug by exhaustive dialysis against PBS pH=7,4 for 1 hour using dialysis tubing(Spectra/Por® membranes: 12–14 kDa MW)and the vesicles were then stored at 4°C for further investigations.

2.3. Characterization of the vesicles

PEVs suspensions were characterized by measuring their particle size and size distribution in terms of the average volume diameters and polydispersity index by photon correlation spectroscopy using particle size analyzer (Brookhaven Instruments Corporation, New York, USA) at fixed angle of 90° at 25 °C.

Entrapment efficiency of Thymoquinone in the PEVs suspensions was determined directly by disruption of the dialyzed PEVs using methanol. The entrapped thymoquinone was measured in the solution of PEVs by high-performance liquid chromatography (HPLC) at $\lambda_{max}$of 254 nm. Waters 2695 HPLC was used, equipped with a dual λ absorbance detector Waters 2487 using a column LiChrospher 100 RP 18-5µ (Chromatography-Service GmbH). The mobile phase was a mixture of acetonitrile and water (80:20, v/v). The sample volume injected was 10µL. The flow rate was maintained at 1ml/min. Retention time of TQ was 2,3 min and the detection limit was $9{,}88x10^{-5}$ mg/ml, for concentrations in the range 0–0,11 mg/ml a good linearity was obtained ($R^2 = 0.996$). Samples were analyzed in triplicate (n = 3).

2.4. *In vitro* drug release experiments

Drug release from the different PEVs was tested using dialysis technique. Simply 1 ml of the vesicles suspension was placed in a dialysis bag sealed from both ends by plastic clips (regenerated cellulose dialysis tubing of MWCO 12-14 KDa soaked in release medium overnight). Dialysis bags were then immersed in 50 mL of phosphate buffer pH 7.4 at 37°C in a shaking water bath moving at a speed of 100 rpm. Aliquots of 200 µl were withdrawn at

predetermined time intervals (15min, 30min, 1h, 1.5 h, 2h, 4h, 6h, 8h) and the TQ content was measured by HPLC as described in the previous section. All experiments were performed six times.

2.5. *Ex vivo* skin permeation study

The skin obtained from Rats' back was carefully shaved and cleaned with cold water and stored at -20°C until used. Skin permeation study was conducted by cutting the skin into appropriate pieces and mounted with the *Stratum Corneum* (SC) uppermost in Franz cells displaying a permeation area of 3,14 cm^2. 12mL PBS (pH = 7.4) maintained at 37°C was added to the receptor chamber and constantly stirred at 450 rpm with a Teflon-coated magnetic bar. Test formulations (500µL) of each formulation were then placed in the donor chamber. At various time intervals (30min, 1h, 2h, 4h, 6h, 8h), 1 mL samples were withdrawn from the receptor chamber and replaced with fresh thermostated PBS over a period of 8 h. The amount of permeated drug was determined using HPLC in the same way as described in section 2.3. All experiments were performed six times.

2.7. *Ex vivo* skin retention study

At the end of the skin permeation experiments, the skin was removed from the Franz cell and washed thoroughly with PBS to remove retained particles if any from the formulation. The skin was then separated into the three distinctive layers namely Stratum corneum (SC), epidermis (ED) and dermis (D). SC was removed by tape stripping 20consecutive times with adhesive tape Tesa® AG (Hamburg, Germany). Each piece of the adhesive tape was firmly pressed on the skin surface and rapidly pulled off with rapid movement.

ED is separated from the D by peeling with sterile surgical scalpel and forceps. Tape strips, epidermis, and dermis tissue were cut and each placed in a flask with 10mL ethanol then sonicated for 5 minutes, and kept extracting the drug overnight in a shaking water bath at 25°C. Samples of tissue extracts were then centrifuged and the supernatant extract layer was filtered to remove any skin debris. Drug retained in each layer was then determined by measuring the quantity of drug in skin layers extract by HPLC.

2.8. Statistical Analysis:

Differences between groups were investigated statistically using ANOVA on ranks followed by the Tukey test for multiple comparisons. Differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION:

3.1. Preparation and Characterization of TQ-PEVs:

In present work,we have investigated the potential of encapsulating the phytochemical drug Thymoquinone into penetration enhancer containing vesicles (PEVs) as a tool for enhancing its dermal deposition for various therapeutic applications. Thymoquinone has reported anti-inflammatory, antibacterial and antifungal properties against different dermatophytes making it an excellent candidate for the treatment of various skin infections [8]. Treatment of skin infections especially those caused by fungal invasions is always challenging due to different factors including development of resistance to existing therapies, need for long treatment duration and poor penetration and deposition of the highly hydrophobic antifungal agents [12]. The use of conventional liposomes has proved efficient in the enhancement of therapeutic efficacy of econazole and reduced the treatment duration required by increasing the localized drug delivery to the site of action [13]. PEVs are characterized by highly fluidized membranes that easily penetrate into the skin due to the entrapped PEs in their phospholipid bilayer. Moreover, the presence of PEs led to enhancement of the stability of PEVs compared to the conventional liposomes. In addition, they are mainly composed of biocompatible and safe ingredients making them very promising carriers for dermal drug delivery [14]. Therefore, the use of PEVs would be highly encouraged for enhancing the solubility, stability as well as skin deposition and retention of the antifungal agents in the skin for longer times compared to the conventional formulations.

The research was aimed to test the effect of different penetration enhancers commonly used on the particle size of the prepared PEVs, entrapment efficiency, *in vitro* drug release and the *ex vivo* skin deposition of TQ in rat skin.

Conventional liposomes (L-TQ) were successfully prepared using soybean lecithin with a particles size around 118 nm and the entrapment efficiency was around 10%. Good size

homogeneity was confirmed by the Polydispersity index (0.256) and the liposomes carrying a negative surface charge (Table 1).

Table 1: Detailed composition and characterization of the different TQ containing PEVs:

Formula Code	PE conc. (% v/v)	Average Particle diameter ± SD (nm)	Zeta potential (mV)	Polydispersity index	Entrapment efficiency (%)
L-TQ	--	118 ± 1.2	-8.8 ± 2.5	0.256	10.44
	Cineole				
Ci-TQ	0.1	117 ± 1.2	-16.6 ± 6.2	0.316	33.28
	Transcutol				
Trc1-TQ	10	144 ± 0.4	-9.1 ± 3.5	0.248	22.55
Trc2-TQ	20	200 ± 2.0	-4.4 ± 1.68	0.176	23.47
Trc3-TQ	30	252 ± 1.3	-36.2 ± 4.9	0.247	11.96
	PEG 400				
PEG1-TQ	10	172 ± 1.3	-12.6 ±2.8	0.249	13.01
PEG2-TQ	20	186 ± 0.5	-7.1 ± 0.7	0.230	19.94
PEG3-TQ	30	394 ± 4.6	-27.7 ± 3.0	0.285	22.19
	PG				
PG1-TQ	10	156 ± 1.0	-6.1 ± 2.2	0.247	21.02
PG2-TQ	20	203 ± 3.0	-8.1 ± 2.3	0.247	32.11
PG3-TQ	30	242 ± 4.4	-3.2 ± 1.9	0.241	40.43

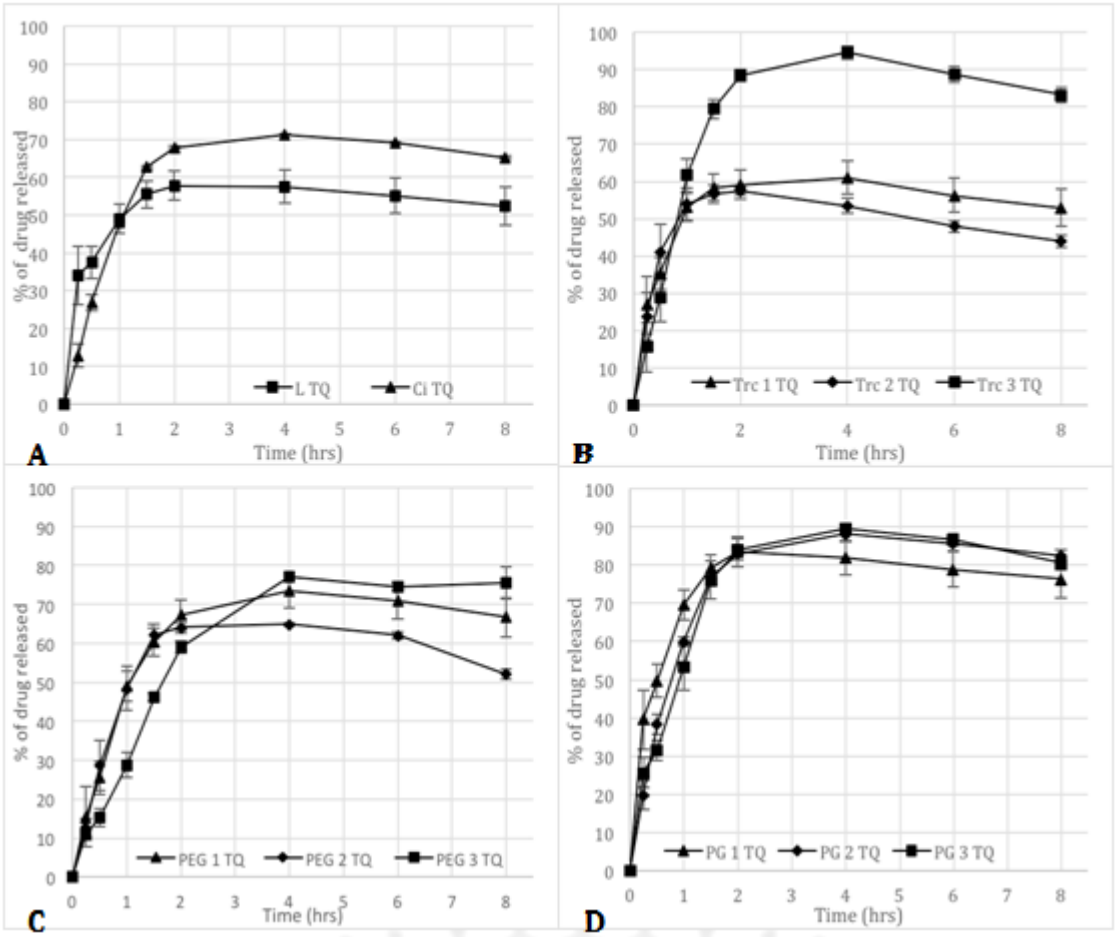
The addition of a lipophilic penetration enhancer was also performed but only with very limited concentration (cineole 0.1%) as higher concentrations or other PEs led to significant size enlargement as well as phase separation and failure in preparing stable vesicles (Oleic acid and higher cineole concentrations; data not shown). However, in the concentration used here, the minor effect was observed on the particle size or surface charge. Apparently due to the very small concentration used and its high lipophilicity leading to its complete impedance into the lipid bilayer with no effect on the vesicle size**.** The entrapment efficiency of TQ was significantly enhanced up to 33.28% with the added lipophilicity to the lipid bilayer allowing for higher TQ encapsulation.

Other hydrophilic PEs have been used namely, Transcutol (Trc), Polyethylene glycol 400 (PEG) and propylene glycol (PG) in the concentrations of 10, 20 and 30% of the aqueous phase. All the prepared formulae were carrying negative surface charge indicating good storage stability potential. It was found that all the prepared PEVs were stable against aggregation or fusion on storage for 1 month due to the negative zeta potential which is most probably owed to the heterogeneous anionic lipid fractions of the used phospholipids [3]. However, the increase in the concentration of the PE added significantly led to an increase in the particles size of the produced PEVs (from 144 to 252, 172 to 394 and 156 to 242 nm in case of using 10 and 30% of the Trc, PEG, and PG respectively). This is explained by the higher viscosity of the hydration medium leading to larger vesicles formation when using the same sonication power during the preparation of the vesicles**.** Entrapment efficiency of TQ has been also significantly enhanced by increasing the PE concentration reaching a maximum of 40% in case of using 30% PG. however, this was not the case for Trc where increasing its concentration from 10 to 20% increased the entrapment of the drug while a further increase to 30%.Trc reduced the entrapment of the drug again to around 11%. Higher entrapment efficiency was obtained for TQ with the addition of the different hydrophilic PEs, acting as co-solvents led to the enhancement of TQ solubility in the aqueous core of the PEVs. This holds true for PG, PEG, and Transcutol. It is to be noted that with the highest concentration of transcutol (30%), the entrapment of TQ has been decreased apparently due to its relative hydrophilic structure that might have rendered the aqueous core too hydrophilic to entrap the TQ. This limited the encapsulation of TQ to the phospholipid bilayer resulting in a comparable entrapment to the conventional liposomes.

3.2. *In vitro* drug release from TQ-PEVs:

The *in vitro* drug release behavior of the prepared vesicular systems was also tested using a dialysis technique. As shown in Figure 1, the drug release was significantly improved by using the different PEs. Slight release enhancement was achieved with cineole due to the higher drug entrapment. The addition of the hydrophilic PEs had a more significant effect on the drug release due to the increased hydrophilicity of the prepared vesicles thus facilitating TQ release into the aqueous buffer.

Figure 1: *In vitro* release of TQ from different PEVs: (A) conventional liposomes and Cineole PEVs, (B) Transcutol PEVs (C) Polyethylene glycol PEVs (D) Propylene glycol PEVs.



3.3. *Ex vivo* skin permeation and retention of TQ from PEVs:

Previous reports indicated the ability of PEVs to enhance the dermal drug delivery due to the synergistic effects of the vesicular system phospholipids and the penetration enhancer [15-19]. Penetration enhancer can increase the fluidity of the stratum corneum, which facilitate drug diffusion and deposition in skin tissue [20].The presence of both stabilizing phospholipids and destabilizing permeation enhancers and their tendency to redistribute in the lipid bilayer make these PEVs much more elastic than the conventional liposomes and enhance their skin permeation and deposition[21].

The *ex vivo* skin penetration of TQ in rat skin has been performed using Franz diffusion cells as described previously. It was quite clear that the permeation of TQ is very poor finding that no TQ was detected in the receptor compartment after the whole experiment duration (8

hours). Only in the case of using Trc in the concentration of 20% a minor quantity of TQ was detected in the receptor compartment after 8 hours (0.2 ±0.08%).It was found that the addition of most of the PEs used significantly improved the skin deposition of TQ in all skin strata(Figure 2). One observation was that both the incorporation of the hydrophobic PE cineole or the highest concentration of the hydrophilic PEs (30%) had always led to significant enhancement of the accumulation of the drug in the stratum corneum compared to other skin layers relative to the conventional liposomes.

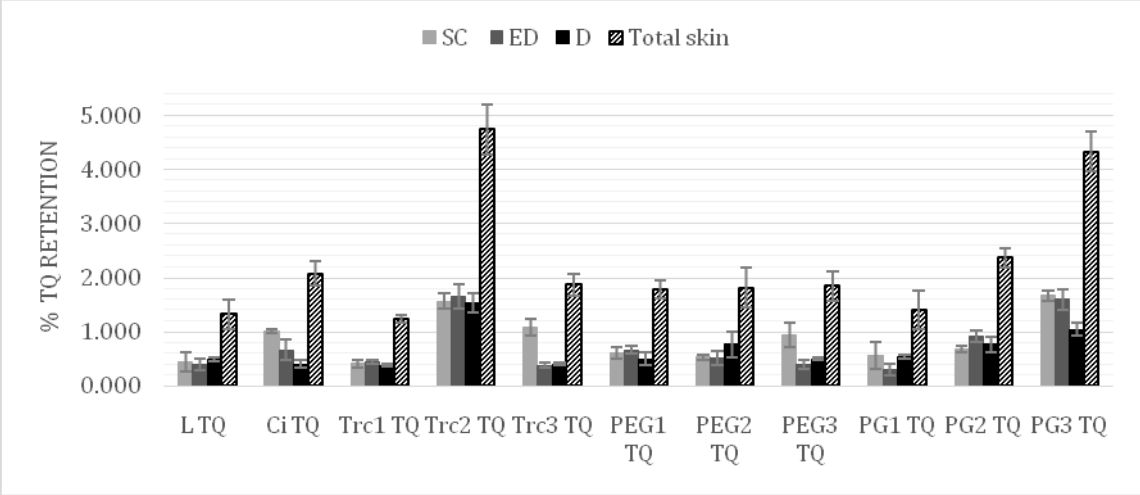


Figure 2: *Ex vivo* skin retention of thymoquinone in different skin layers from the different PEVs.

Regarding Trc-TQ PEVs, the concentration of 10% Trc led to comparable *ex vivo* skin permeation than the conventional liposomes. Transcutol is known to enhance skin permeation by increasing the swelling of the intercellular lipids in the stratum corneum yet without changing the bilayer structure [19].

Transcutol as a hydrophilic PE when used in small concentrations, is mostly dispersed in the aqueous core of the PEVs and is hardly able to interact neither with the lipid bilayer of the vesicles nor with stratum corneum lipid giving rise to similar *ex vivo* permeation results to the conventional liposomes while enhancing the entrapment of the drug due to the increased incorporated portion of the drug in the aqueous core [19]. However, when used at 20%, significant enhancement of skin retention in all skin strata was observed. It was found that the drug delivered to the skin tissues was three times higher than that from conventional liposomes. This formula was the only one able to deliver the drug to the deeper skin strata and therefore, it was the only one in which minor drug was delivered to the receptor

compartment. Similar results indicated the superiority of Trc in enhancing the delivery of diclofenac to the dermis compared to other PEs, which enhanced drug accumulation mainly in the stratum corneum [3]. At higher Trc concentration, and in the presence of the lipophilic drug TQ, transcutol was able to interact with the lipid bilayer and enhancing the deformability of the vesicles significantly with subsequent deeper skin penetration [19]. Apparently, at the highest concentration (30%) the high hydrophilicity of the Trc even enhanced the solubilization of the drug into the external aqueous phase thus minimizing the entrapment efficiency and skin deposition.

Propylene glycol containing liposomes has been proposed as new carrier system with superior entrapment efficiency to hydrophobic drugs due to the solubilizing power of PG [4]. It proved to be superior to conventional liposomes and Ethosomes in the skin delivery of local anesthetic cinchocaine. They have been also investigated for the treatment of skin fungal infections by using miconazole and had higher therapeutic efficiency, entrapment and enhanced skin deposition compared to the conventional liposomes [22]. This has been explained by the synergistic effect of PG on both membrane elasticity of the liposomes and the penetration enhancement through the skin barrier [23].

For maximum therapeutic benefits especially for the antifungal uses of thymoquinone in the treatment of skin fungal infections, the PEVs giving the highest concentrations in the skin would be optimum. Out of all the prepared formulae, Transcutol (20%) and PG (30%) seem to achieve the highest skin deposition of TQ in all skin layers. Given that only in case of Trc 20%,aminor fraction of TQ was detected in the receptor compartment, indicating highest skin penetrability and deposition potential of this formula. This proves that Transcutol could perturb SC lipids highly arranged domain and hence allow the molecules to easily penetrate down the skin layers and deliver the loaded drug [24]. Therefore, this formula and the one containing 30% PG can be recommended as very promising carriers for the topical application of thymoquinone.

CONCLUSION:

This work shows that permeation enhancers containing vesicles are eligible for the use as suitable carriers for Thymoquinone to the skin. *Ex vivo* studies disclosed that the Thymoquinone formulations containing 30% of PG and the one containing 20% of Transcutol are the best for the Thymoquinone local accumulation. However, with the one

containing 20% of Transcutol permit also a skin permeation, therefore, the formulation with 30% of PG for a topical use of Thymoquinone would be preferred. The present therapeutic approach may be promising in the treatment of skin fungal infections.

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