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Approach for Development of Topical Ketoconazole-Loaded Microemulsions and Its Antifungal Activity

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Abstract

Loading ketoconazole in microemulsions can enhance the solubility and permeation of ketoconazole into the skin. The systematic approach as quality by design (QbD) can help for better product and process understanding. This study aimed to develop topical ketoconazole-loaded microemulsions (KME) based on the QbD approach. After risk assessment, the design of experiment was utilized to determine the optimal ratio of water, isopropyl myristate, and surfactant mixture (polysorbate 80 and ethanol), which were chosen as critical material attributes. The particle size and polydispersity index, selected as critical quality attributes, were evaluated as responses. Then, characterizations, *in vitro* permeation, cytotoxicity, and antifungal activity studies were tested. As result, the equations from the D-optimal mixture design model successfully predicted the composition of the optimized formulation and obtained the acceptable design space. The optimized KME was 16.00 % w/w of isopropyl myristate, 73.50 % w/w of surfactant mixture, and 8.50 % w/w of water resulted in a water-in-oil system. The particle size and polydispersity index of KME were 21.1 ± 1.6 nm and 0.329 ± 0.020, respectively. KME showed prolonged skin retention after 24 h and was non-toxic. KME exhibited a broader zone of inhibition and lower inhibition concentration compared to unloaded ketoconazole against dermatophytes, indicating that KME enhanced effectiveness in antifungal activity for skin infection. The results of the KME meeting the quality target indicated a well-designed and suitable microemulsion for topical usage. Therefore, employing QbD in identifying the process and formulation is a promising approach for future development of high-quality topical KME products.

Keywords: Quality by design, Ketoconazole, Microemulsions, Design of experiment, Design space, Optimization, Antifungal

Introduction

Ketoconazole, a well-known antifungal agent, exhibits potent biological activity against various fungi, particularly those causing skin infections. It belongs to the class of azole antifungals and its mechanism of action involves the inhibition of ergosterol synthesis, a key component of fungal cell membranes [1]. With its broad-spectrum activity against dermatophytes, yeasts, and other filamentous fungi, ketoconazole effectively disrupts the growth and reproduction of these fungi, leading to the treatment of the infection [2]. The biological activity of ketoconazole against antifungal pathogens makes it a valuable therapeutic option for the treatment of fungal skin infections. Ketoconazole has been widely used as a topical dosage form. Administering ketoconazole by topical route instead of an oral route can avoid changes in the conversion of other drugs and reduce the risk of liver damage [3]. Ketoconazole belongs to the Biopharmaceutical Classification System (BCS) class II [4]. It is insoluble in water but soluble in an acidic environment. Moreover, it easily decomposes when exposed to light [5]. Despite its therapeutic potential,

ketoconazole faces challenges due to its limited solubility and difficulty in penetrating infected skin [6]. However, nanotechnology, specifically microemulsion-based drug delivery, offers a promising solution.

Nanotechnology has facilitated the advanced drug delivery system development that enhance the effectiveness and efficiency of medications. Nanoparticles can encapsulate drugs, enhance solubility, protect them from degradation and deliver them to specific targets in the body. This targeted drug delivery allows for reduced side effects, lower doses, and increased therapeutic efficacy [7]. Nanomedicines are complex structures made up of different parts that fit together in specific ways. Even small modifications to the method or composition of these structures can have a substantial impact on their interactions and functions [8]. The development of nanomedicine involves a complex and multifaceted process that requires careful attention to various aspects, including chemistry, manufacturing, quality control, as well as economic and regulatory considerations. Hence, it is essential to assess the feasibility of nanomedicine development by gaining a comprehensive understanding of the composition, structure, and preparation process of the initial formulation. This approach ensures in subsequent confirmatory studies and ensures the safety and efficacy of the nanomedicine during human clinical trials and large-scale production processes [9].

Microemulsion-based drug delivery has been introduced to the promising nanotechnology to overcome therapeutic efficacy. Microemulsions are mixture of 2 different liquids that are immiscible and stabilized by surfactants and co-surfactants with sizes ranging from 10 - 100 nm [10,11], Microemulsions can be categorized into 3 types: Oil-in-water (o/w), water-in-oil (w/o), and bicontinuous [12]. They are thermodynamically stable, and the systems can occur spontaneously [12]. The advantages of microemulsions that make them a suitable delivery system for pharmaceutical purposes are their solubility and permeability enhancement, transparency, ease of preparation, and long shelf life [4]. Microemulsion manufacturing processes are often simpler compared to other drug delivery systems. They can be easily scaled up to meet commercial production demands. The formulation and manufacturing methods can be optimized for large-scale production without compromising the quality and performance of the microemulsions [13].

The development and manufacturing of microemulsion-based pharmaceuticals requires careful consideration of formulation optimization, characterization techniques, and regulatory requirements [14]. Adopting a quality by design (QbD) approach, as recommended by the ICH guidelines, a QbD concept is a part of the ICH guideline Q8 (R2) on pharmaceutical development which provides guidance on the pharmaceutical development of drug products and emphasizes the principles of QbD. It focuses on the scientific and risk-based approach to product development and highlights the importance of understanding the product and process to ensure consistent quality and regulatory requirements. [15]. A QbD model recommended by ICH guidelines is anticipated to overcome those issues. With the QbD approach, the quality target product profile (QTPP) is first determined. Next, the variables affecting the formulation based on critical quality attributes (CQAs) assessment, risk assessment of variables affecting quality are evaluated, design of experimentation (DoE), and finally controlling the variables in the range to achieve the desired results [15]. QbD can minimize trial and error and reduce large-scale experiment costs [14]. Importantly, QbD ensures consistent manufacturing processes and product quality that meet patients' needs [15].

The integration of QbD principles in microemulsion development can bridge the research gap between science and regulation by providing a systematic, science-based approach that ensures the quality, safety, and efficacy of microemulsion formulations. Therefore, this study aimed to develop optimized topical ketoconazole-loaded microemulsions for treating dermatophyte infection using QbD approach. In this study, the ratios between the oil phase, water phase, and surfactant and co-surfactant mixture were determined using the D-optimal mixture design model, and their effects on critical quality attributes (CQAs) were assessed. Characterization, *in vitro* drug permeation, cytotoxicity towards keratinocyte cells, and antifungal activity of the optimized topical ketoconazole-loaded microemulsions were performed. By gaining a comprehensive understanding of the compositions and processes involved, we successfully identified microemulsions with desirable therapeutic effects. These findings have implications for future large-scale production, ensuring consistent quality and efficacy of the developed microemulsions.

Materials and methods

Materials

Ketoconazole was purchased from S. Tong Chemical Co. Ltd. (Bangkok, Thailand). Pharmaceutical excipients (isopropyl myristate (IPM), ethanol, and polysorbate 80) were obtained from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck Millipore (Billerica, MA, USA). Human immortalized keratinocyte (HaCaT) cell lines were purchased from Elabscience Biotechnology Inc., (Houston, TX, USA). Media and reagents for cell culture

were purchased from Invitrogen™ and GIBCO-BRL (Grand Island, NY, USA). Media and reagents for microbiological test were obtained from Bacto-Difco Lab Co., Ltd (Detroit, MI, USA) and Merck Millipore (Billerica, MA, USA). Other chemicals, reagents and solvents were AR grades and HPLC grades.

Identification of quality target product profile (QTPP) and critical quality attributes (CQAs)

The QTPP was determined as a prospective summary of ketoconazole-loaded microemulsions (KME) characteristics related to quality, safety, and efficacy. CQAs are derived from the QTPP so that those product characteristics impacting product quality can be studied and controlled.

Risk assessment

An Ishikawa diagram analysis was performed to identify factors that could affect the quality of KME, following the "ICH guideline Q9 on Quality Risk Management". This guideline provides a framework for implementing a systematic approach in the pharmaceutical industry to recognize, evaluate, manage, and communicate risks that may impact product quality throughout the product lifecycle [15]. Failure mode and effect analysis (FMEA) was used as a tool for classifying risks based on severity (S), occurrence (O), and detectability (D). The risk associated with the final product was quantified using the Risk Priority Number (RPN). The calculation of RPN was expressed using the following equation:

$$RPN = S \times O \times D \quad (1)$$

Design of experiment (DoE) and formulation preparation

The design of experiment (DoE) was carried out by the D-optimal mixture design using Design-Expert® software (Version 11.0; Stat-Ease Inc., Minneapolis, MN, USA). IPM, surfactant and co-surfactant were selected according to the high solubility of ketoconazole from previous studies [16,17]. The defined constraints for the mixture variables were as follows: The oil phase (IPM) ranged from 10.00 to 40.00 % w/w, water content ranged from 1.00 to 40.00 % w/w, and the surfactant mixture (Smix) consisting of polysorbate 80 and ethanol in a 1:1 w/w ratio ranged from 48.00 to 80.00 % combined weight. The compositions of all samples were within the range determined for microemulsion formation in a preliminary study. The evaluation parameters were chosen regarding the high-risk priority number.

Microemulsion preparation

KME was prepared as described by Khonkarn *et al.* with some modifications [18]. Ketoconazole powder was dissolved in IPM. Next, Smix and water were added. Then, the mixture was stirred using a magnetic stirrer (500 rpm) until KME was completely transparent. The unloaded microemulsions (ME) were prepared by a similar method without adding ketoconazole, which was used as a negative control for anti-fungal and cytotoxicity studies.

Characterization of KME

Particle size and zeta potential analysis

The particle size and zeta potential of KME were carried out using a Zetasizer Nano ZS series instrument (Malvern Instruments, Worcestershire, UK), following the procedure outlined by Anantaworasakul *et al.* [19]. A dilution of KME at a ratio of 1:100 v/v in deionized water was prepared. The measurements were conducted at a temperature of 25 °C and a light scattering angle of 173 °.

Electrical conductivity measurement

The electrical conductivity of KME was measured using a conductivity meter (D-24 Horiba, Kyoto, Japan) with a 3-in-1 electrode (9625-10D, Horiba, Kyoto, Japan). The electrode was immersed in the KME samples, and the conductivity measurements were performed at a temperature of 25 °C.

pH measurement

The pH of KME was measured using a pH meter (Model Cyberscan 510, Eutech Instruments, Singapore). The pH measurements were performed at 25 °C.

Viscosity measurement

The viscosity of KME was determined using a Brookfield viscometer (DV-II, Brookfield Engineering Labs Inc., Stoughton, MA, USA). The viscosity measurement was conducted at 25 °C.

In vitro permeation study

KME's skin permeation was assessed using Franz diffusion cells, following the methodology described by Nitthikan *et al.* [20]. A transdermal diffusion membrane (Strat-M®, Merck Millipore (Billerica, MA, USA)) was positioned on the upper section of the receiver chamber. The receiver medium consisted of a mixture of phosphate-buffered saline (PBS, pH 7.4) and ethanol in a ratio of 70:30 v/v. The receiver chamber was filled with 13.0 mL of the medium, maintained at a temperature of 37 ± 2 °C, and continuously stirred using a magnetic bar. Each formulation (1.0 g) was introduced into the donor compartment. Subsequently, 1.0 mL of the receiver medium was collected at specific time points: 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h. After 24 h, the membrane was rinsed with 1.0 mL of receiver medium. The washed membrane was then cut into small fragments, immersed in methanol, and subjected to sonication for 15 min. The concentrations of ketoconazole on the membrane, within the membrane, and in the receiver, medium were determined using high-performance liquid chromatography (HPLC).

HPLC analysis

The quantity of ketoconazole was determined using an HPLC method with some modifications based on the previous work [18]. In summary, either ketoconazole or KME was mixed with methanol and then filtered through a 0.22-µm pore size membrane filter from Filtrex (Virginia Beach, VA, USA). The sample was subsequently subjected to analysis using a Hewlett Packard/hp1100 HPLC system, equipped with a UV-visible detector. The HPLC system employed a C18 column (particle size: 5 µm, dimensions: 250×4.6 mm) with an accompanying guard column (Purosphere® STAR, Merck, Darmstadt, Germany). Elution of the components was achieved using a mobile phase consisting of 85 % v/v acetonitrile and 15 % v/v phosphate buffer (pH 4) at a flow rate of 0.7 mL/min. An injection volume of 10 µL was utilized, and the detection was recorded at a wavelength of 254 nm. To establish a standard curve, a ketoconazole standard solution was prepared with concentrations ranging from 30 to 250 µg/mL.

Cytotoxicity study

HaCaT were utilized as test cells in the MTT assay, with modifications based on the methodology described by Nitthikan *et al.* [21]. Firstly, the cells were seeded into the wells of 96-well microtiter plates at a density of 1×10^5 cells/well and incubated at 37 °C with 5 % CO_2 for 24 h. Following the 24 h incubation, different concentrations of KME, corresponding to 6.25 - 100 µg/mL of ketoconazole, were added to the wells and incubated at 37 °C with 5 % CO_2 for 48 h. Subsequently, 100 µL of the supernatant was removed, and 15 µL of a 5 mg/mL MTT dye in PBS was added to each well, followed by a 4 h incubation period. After 4 h, the supernatant was discarded, and 200 µL of dimethyl sulfoxide was added to each well. The samples were thoroughly mixed to dissolve the dye crystals, and the absorbance at 540 nm was measured using a microtiter plate reader. The percentage of cell viability for each tested sample was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (2)$$

where $\text{Abs}_{\text{control}}$ is the absorbance of the control, and $\text{Abs}_{\text{sample}}$ is the absorbance of the test sample.

Antifungal activity

Agar disc diffusion method

The screening test of KMEs was performed according to Khonkarn *et al.* and Clinical and Laboratory Standards Institute (CLSI) M38-A2 protocol with some modifications [18,22]. *Microsporum gypseum* (*M. gypseum*) and *Trichophyton mentagrophytes* (*T. mentagrophytes*) were cultured in potato dextrose agar at 25 °C for 7 - 14 days. This potato dextrose agar can promote conidia in most dermatophyte isolates. The turbidity of each dermatophyte was adjusted to 1 McFarland standard turbidity (3×10^8 CFU/mL) in a 0.85 % sterile normal saline solution. The dermatophytes were added onto the Sabouraud dextrose agar plate which was a selective media used for general isolation of dermatophytes and other types of fungi and distributed uniformly. Next, 20 µL of KME or unloaded ME was added into a 6.0-mm sterilized paper disc. Then, a paper disc sample was placed on the surface of a culture plate and incubated at 30 °C for 4 days. Each test sample was subjected to 3 replicates. After incubation, the mean diameter of the clear zone was measured and recorded.

Minimal inhibitory concentration (MIC) determination

MICs determination was performed by broth microdilution [23,24]. Each *M. gypseum* and *T. mentagrophytes* were cultured in potato dextrose agar at 25 °C for 7 - 14 days. Each dermatophyte was inoculated in 0.85 % sterile normal saline solution and was filtered through a glass wool filter. The number of conidia was counted using a hemocytometer to obtain a concentration equal to 5×10^4 cells/mL. Next, 100 µL of RPMI-1640 culture medium containing 0.165 mol/L MOPS, pH 7.0 was added to the 96-well microtiter plate. Then, a serial 2-fold dilution of KME and 100 µL of *M. gypseum* or *T. mentagrophytes* was added to each well and incubated at 30 °C for 96 h. The MIC was observed as the lowest concentration of KME, resulting in no fungal growth compared to the control.

Minimal fungicidal concentration (MFC) determination

Minimum fungicidal concentrations (MFCs) were determined by spot inoculating [25]. Ten µL from a well in MICs test with the invisible fungal growth result was added onto Sabouraud dextrose agar plates, and the plates were incubated at 35 °C for 72 h. The MFC was observed as the lowest concentration resulting in no growth in the subculture. Fungicidal activity based on the MFC/MIC ratio was assessed. When the MFC/MIC ratio was less than 4, the test substance had fungicidal activity, and when MFC/MIC ratio was higher than 4, the test substance had fungistatic activity [26].

Statistical analysis

The experiments were conducted in triplicate, and the data were presented as the mean ± standard deviation (SD). The comparison of means was analyzed using one-way analysis of variance (ANOVA) with the assistance of SPSS statistical software version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered when the *p*-value was less than 0.05.

Results and discussion

Identification of quality target product profile (QTPP) and critical quality attributes (CQAs)

According to QbD-based formulation development, Firstly, QTTP was the primary step that classified the desired characteristic and performance of KME including the intended route of administration, therapeutic goals, and patient requirements for the final product. Secondly, CQA was selected from QTPP, which product quality attributes need to be monitored and improved during the production process and product life cycle. The classification of QTPP and CQAs is shown in **Table 1**.

Table 1 Quality target product profile and critical quality attributes of ketoconazole-loaded microemulsions.

Target product profile	Target	Justification	CQA
Dosage form	Microemulsion	Increase solubility and permeability of ketoconazole	No
Route of administration	Topical	Delivery of ketoconazole locally to the skin and reduce systemic adverse effects	No
Dosage strength	2 % w/w	The dose is within the range of minimum effective concentration.	No
Appearance	Transparency	Indicate complete loading of ketoconazole in microemulsions	Yes
Particle size	10.0 - 30.0 nm	Enhance permeation through stratum corneum	Yes
Particle size distribution (Polydispersity index; PDI)	< 0.350	For uniform ketoconazole distribution	Yes

Risk assessment

Quality risk management is aimed to minimize the risk of quality, safety, and efficacy of the drug product for the best benefit of the patient. Various material attributes and formulation parameters were identified using the Ishikawa fishbone diagram, as shown in **Figure 1**. The Ishikawa fishbone diagram illustrates the effect of various factors in formulation development that could affect the quality of the finished product. This present study is aimed to develop KME with desirable characteristics as mentioned in QTPP. The components of the microemulsion system, including the oil phase, Smix, and water, were the critical material attributes (CMA). FMEA was used as a risk assessment tool to rank failure methods during

microemulsion production and identify potential critical process parameters. Severity, probability of occurrence, and detectability were categorized for each factor from 1 to 5 (low to high risk), as shown in **Table 2**. Further risk assessments were performed under the FMEA guidelines, as shown in **Table 3**. The arrangement of ratings in various aspects could help to distinguish the risk potential of material attributes or process parameters that impact CQAs, which were particle size and PDI, where a high RPN score of 75 and above was an important attribute or parameter. The assessment excluded transparency because we preliminarily selected the transparent microemulsion system to simplify the optimization process.

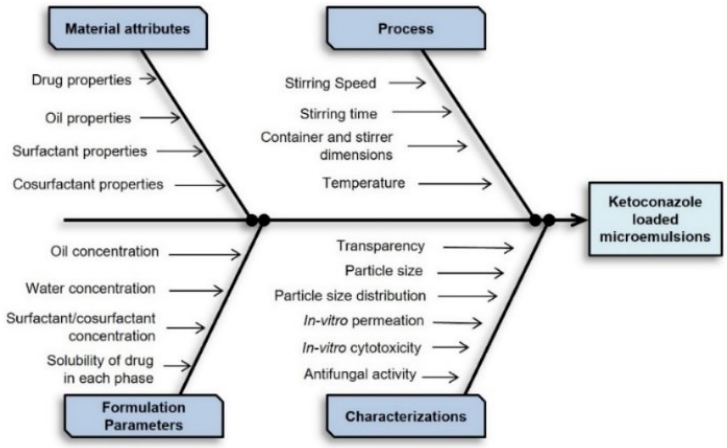

Figure 1 Ishikawa fishbone diagram for ketoconazole-loaded microemulsion development.

Table 2 Descriptions of score values for severity, probability of occurrence, and probability of detectability categories in calculating Risk Priority Number.

Categories	Score		
	1	3	5
Severity (S)	No impact on product quality	Requires action, but the product is recoverable	Impact on product quality and irreparable
Occurrence (O)	Has not occurred	Occurs infrequently	Occurs regularly
Detectability (D)	Easily detectable	Detected, but not promptly	Not detectable within the current operation

Table 3 FMEA of the material attributes and process parameters that impacted to CQAs of ketoconazole-loaded microemulsions.

Cause of failure	S	O	D	RPN
Oil concentration	5	3	5	75
Smix concentration	5	3	5	75
Water concentration	5	3	5	75
Stirring time	3	3	1	9
Stirring rate	3	3	1	9
Container size	3	3	1	9
Temperature	5	3	1	15

Design of experiment (DoE)

The preliminary study was carried out to determine the levels of independent variables: the concentration of IPM, Smix, and water. Based on the experimental data, the lower and upper limits of the 3 independent components were determined. The microemulsion formulation displayed a transparent system with an approximate range of IPM, Smix, and water to levels of 10.00 - 40.00, 48.00 - 80.00 and 1.00 - 40.00 %, respectively, as shown in **Figure 2**. According to FMEA, the impact of the microemulsion component on CQA, which was due to the highest RPN, was investigated using a 16-run D-optimal mixture design, as shown in **Table 4**.

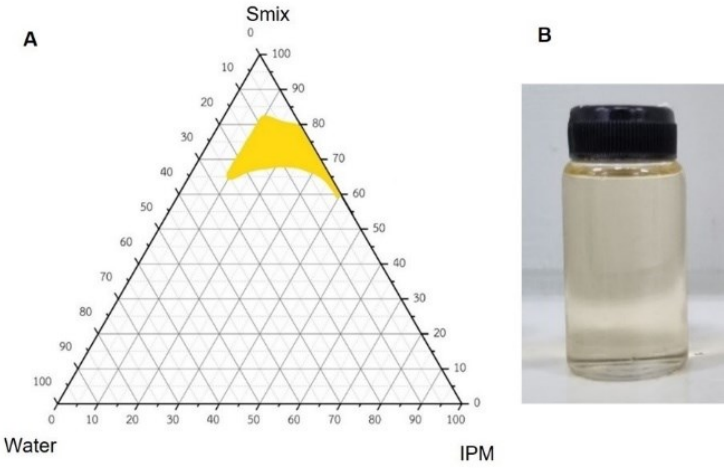

Figure 2 Pseudo-ternary phase diagram of microemulsion region (yellow region) (A) and appearance of microemulsions (B).

Table 4 Experimental data and responses (particle size and particle size distribution) obtained from the D-optimal mixture design.

Run	IPM (%w/w)	Smix (%w/w)	Water (%w/w)	Ketoconazole (%w/w)	Particle size (nm)	PDI
1	10.00	80.00	8.00	2.00	24.8	0.311
2	38.85	58.15	1.00	2.00	276.2	0.355
3	29.00	57.13	11.86	2.00	275.0	0.398
4	40.00	48.00	10.00	2.00	236.2	0.391
5	29.10	67.90	1.00	2.00	182.2	0.325
6	14.79	53.48	29.73	2.00	1,187.1	0.589
7	18.42	66.92	12.66	2.00	142.8	0.346
8	10.00	64.00	24.00	2.00	45.3	0.273
9	40.00	48.00	10.00	2.00	292.3	0.468
10	20.13	76.87	1.00	2.00	141.5	0.366
11	25.38	48.00	24.62	2.00	1,869.9	0.667
12	10.00	64.00	24.00	2.00	17.7	0.288
13	25.38	48.00	24.62	2.00	1,775.3	0.601
14	10.00	80.00	8.00	2.00	16.8	0.262
15	10.00	48.00	40.00	2.00	1,181.4	0.588
16	10.00	48.00	40.00	2.00	882.0	0.512

After the data were analyzed, the 2 types of models for both responses were observed in a reasonable agreement between predicted R^2 and adjusted R^2. The cubic and quadratic models were suggested for response 1 (particle size) and response 2 (PDI), respectively. **Table 5** shows the analysis of variance (ANOVA) of the cubic and quadratic models. For both models, it was observed that the F-value of the model indicated its significance, with a *p*-value less than 0.05. This means that the models were statistically significant and not likely to have occurred by chance alone. In fact, the probability of a large F-value occurring due to random noise was only 0.01 %. Additionally, the lack of fit in both models was found to be insignificant compared to the pure error, suggesting that the models were well-fitted to the data.

Table 5 Analysis of variance (ANOVA) for the D-optimal mixture design of the cubic and quadratic model.

Response	Model	Source	Sum of squares	Df	Mean square	F-value	*p*-value
Particle size	Cubic	Model	5.984E + 06	8	7.479E + 05	99.66	< 0.0001*
		Linear Mixture	3.185E + 06	2	1.592E + 06	212.17	< 0.0001
		AB	6,376.83	1	6,376.83	0.8497	0.3873
		AC	38,661.18	1	38,661.18	5.15	0.0575
		BC	3.326E + 05	1	3.326E + 05	44.32	0.0003
		ABC	8,703.49	1	8,703.49	1.16	0.3172
		AB(A-B)	173.67	1	173.67	0.0231	0.8834
		AC(A-C)	2.326E + 05	1	2.326E + 05	31.00	0.0008
		Residual	52,533.51	7	7,504.79		
		Lack of Fit	1,240.05	2	620.03	0.0604	0.9420
		Pure Error	51,293.45	5	10,258.69		
		Cor Total	6.036E + 06	15			
		R^2	0.9913				
		Adjusted R^2	0.9814				
		Predicted R^2	0.9642				
		Adeq Precision	27.7827				
PDI	Quadratic	Model	0.2351	5	0.0470	18.70	< 0.0001*
		Linear Mixture	0.1629	2	0.0815	32.41	< 0.0001
		AB	0.0028	1	0.0028	1.12	0.3153
		AC	0.0323	1	0.0323	12.86	0.0050
		BC	0.0315	1	0.0315	12.55	0.0053
		Residual	0.0251	10	0.0025		
		Lack of Fit	0.0158	5	0.0032	1.69	0.2895
		Pure Error	0.0093	5	0.0019		
		Cor Total	0.2602	15			
		R^2	0.9034				
		Adjusted R^2	0.8551				
		Predicted R^2	0.7625				
		Adeq Precision	11.3908				

An asterisk (*) denoted that the model term was significant (*p*-value < 0.05)

The adequacy of the models was also evaluated using the concept of adequate precision, which measures the signal-to-noise ratio. A ratio greater than 4 is considered desirable. In this case, both response variables showed high-value ratios of 27.7827 and 11.3908, respectively, indicating an adequate signal in the models. Therefore, these models were effectively used to navigate the design space, providing valuable insights for predicting particle size and PDI based on the factors included in the models. Furthermore, the cubic coefficient and quadratic coefficient were obtained as the following equations:

$$Y_1 = 506.51A^* + 317.18B^* + 1030.95C^* - 988.53AB + 2421.38AC - 2902.14BC^* - 3623.47ABC + 186.76AB(A-B) - 8328.52AC(A-C)^* \quad (3)$$

$$Y_2 = 0.21A^* + 0.35B^* + 0.57C^* + 0.26AB + 0.83AC^* - 0.76BC^* \quad (4)$$

In Eqs. (3) and (4), Y_1 and Y_2 represented particle size and PDI, respectively. A B and C represented the material attributes; IPM, Smix, and water, respectively. An asterisk (*) denoted that the model term was significant (p-value < 0.05).

The obtained equations were utilized to predict the particle size and polydispersity index (PDI) of the system. These equations provided information about the impact of different factors, such as the oil phase, Smix, and water phase of KME, on the particle size and PDI. The significance of each model term was determined based on p-value, with a value less than 0.05 indicating a statistically significant effect. By analyzing the model terms, the analysis helped identify which factors had a significant influence on the particle size and PDI of KME. A positive value indicated a synergistic effect of the independent variable, whereas a negative value indicated an antagonistic effect. The particle size of KME will increase with the increasing IPM, Smix, and water concentrations, and the biggest effect on particle size was water concentration. The interaction between Smix and water (B and C) significantly negatively affected particle size. Besides, increased IPM, Smix, and water concentration also had significant positive effects on PDI, as well as the interaction between IPM and water (A and C) (p-value < 0.05). On the contrary, the interaction between Smix and water (B and C) significantly had a negative effect on PDI (p-value < 0.05). The contour plots and 3-dimensional surface plots of both responses are shown in **Figures 3(A) - 3(B)**.

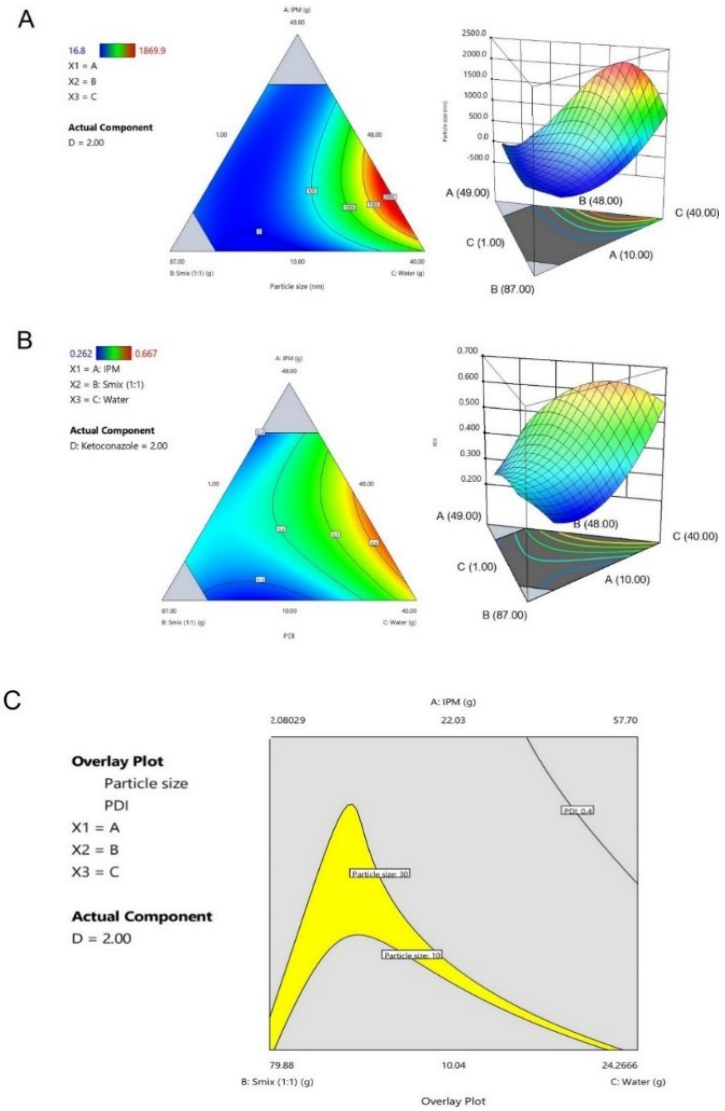

Figure 3 Contour plot and a 3-dimensional surface plot showing the interaction effect between 3 variables for the particle size (A), PDI (B), and the overlay plot with the design space highlighted in yellow (C).

The design space was obtained from the overlay plot of both responses after fixing the constraints of CQAs, as shown in **Figure 3(C)**. Particle size and PDI range were set up between 10 and 30 nm and 0.20 and 0.35 nm, respectively. According to the navigation of the design space, we selected 1 point in the middle of the design space in which microemulsion compositions were 16.00 % w/w of IPM, 73.50 % w/w of Smix, and 8.50 % w/w of water. **Table 6** presents the predicted values and the experimental values of particle size and PDI for the optimized KME obtained under the design space. The verification results indicated that the percentage errors were below 10 %, demonstrating the accuracy and reliability of the predictions made by the design of experiment program. Then, this optimized KME formulation was used for further characterization, *in vitro* permeation, cytotoxicity, and antifungal activity studies.

Table 6 Predicted values, actual values, and error of the prediction for each response of selected KME from the design space.

Parameters	Predicted values	Actual values	Error (%)*
Particle size (nm)	21.0	21.1 ± 1.6	0.47
PDI	0.328	0.329 ± 0.020	0.30

*Error = (Actual value − Predicted value)×100/ Actual value

Characterization of KME

The optimized KME formulation, chosen from the mentioned design space, revealed a uniform transparent liquid with particle size and PDI values that met the specified criteria in the QTTP. The results presented in **Table 7** indicated a low electrical conductivity, suggesting that the optimized KME was the water-in-oil (w/o) type regarding to the value less than 10 µS/cm [27]. The formulation also displayed favorable viscosity, Furthermore, the zeta potential exhibited a negative value, and the pH was within the acceptable range for topical usage.

Table 7 Electrical conductivity, viscosity, zeta potential, and pH of optimized KME.

	Electrical conductivity (µS/cm)	Viscosity (cP)	Zeta potential (mV)	pH
KME	0.67 ± 0.01	51.3 ± 1.2	–11.4 ± 0.3	6.45 ± 0.01

***In vitro* permeation study**

To characterize their behavior, the optimized KME was selected for *in vitro* permeation study. A synthetic transdermal diffusion membrane was used as a substitute for human skin. The receiver medium was the mixture of PBS, pH 7.4, and ethanol (70:30 % v/v) which helped to solubilize ketoconazole and ensured a sink condition. **Figure 4** reveals the percentage of ketoconazole accumulative amount in the receiver chamber after 24 h. The results showed that ketoconazole permeated through the membrane after 30 min and reached 32.35 ± 2.41 % after 24 h. The cumulative amount of ketoconazole permeated per unit membrane surface area (2.46 cm^2) after 24 h was 2,630.46 ± 196.02 µg/cm^2. The cumulative amount of ketoconazole from extracted membrane significantly showed the highest amount of ketoconazole (4,917.31 ± 506.65 µg/cm^2), whereas the cumulative amount of ketoconazole on the membrane significantly showed the lowest amount (770.89 ± 3.98 µg/cm^2) (p-value < 0.05).

Cytotoxicity study

HaCaT was used as a representative cell of human skin cells. The cell viability was evaluated to confirm that KME and its excipients did not cause serious damage to skin cells. Various concentrations of KME (6.25 - 100 µg/mL) and ME (volume equal to KME) were treated with HaCaT for 48 h. The results showed that KME and ME slightly decreased the viability of HaCaT (**Figure 5**). However, the reduction in cell viability for all tested samples did not exceed 30 %. There was no significant difference observed among the samples.

Antifungal activity

Antifungal activity of KME, ketoconazole suspended in culture media (KZ), and ME was evaluated against *T. mentagrophytes* and *M. gypseum*. **Table 8** shows the summarized results of antifungal activity.

The screening test of KME was performed by the agar disc diffusion method. The results demonstrated that KME had a better inhibitory effect on the growth of both dermatophytes than that KZ. The MIC and MFC studies found that the MIC and MFC values of KME against *T. mentagrophytes* and *M. gypseum* were lower than KZ. In comparison, ME did not reveal any effect on antifungal activity testing. The triplicate test yielded the same results, so no standard deviation was reported. MFC/MIC ratios were calculated to predict the activity of the test substance. All MFC/MIC ratios against both dermatophytes were higher than 4, indicating that they were fungistatic.

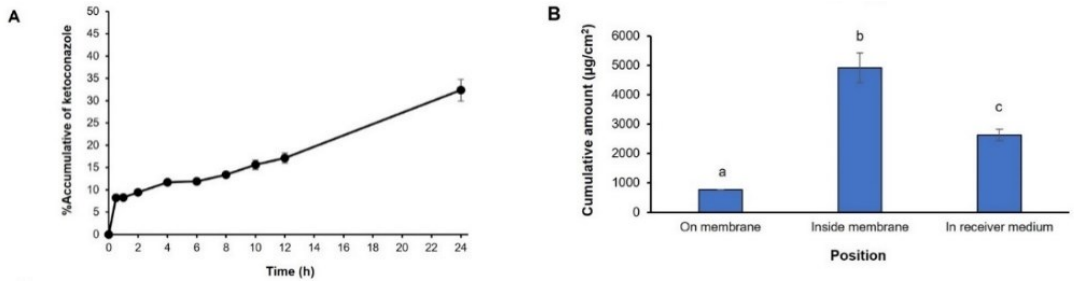

Figure 4 The percentage of the accumulative amount of ketoconazole from KME after 24 h (A) and the cumulative amount of ketoconazole from KME in different positions. Different letters show statistical significance (p-value < 0.05).

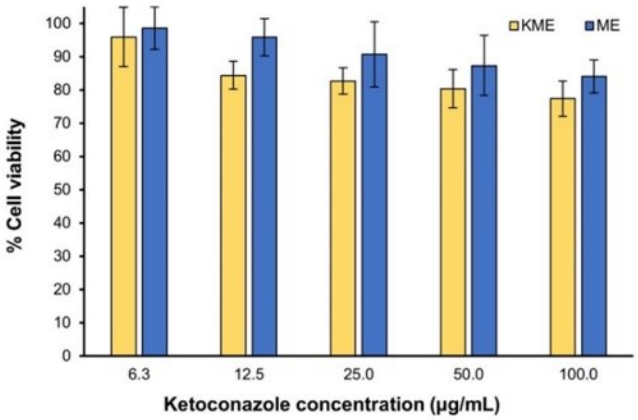

Figure 5 The percentage of cell viability of KME and ME after 48 h.

Table 8 Antifungal activity of ketoconazole loaded microemulsions (KME), ketoconazole suspended in culture media (KZ), and unloaded microemulsions (ME) against *T. mentagrophytes* and *M. gypseum*.

		Inhibition zone (mm)	MIC (µg/mL)	MFC (µg/mL)	MFC/MIC ratio
T. mentagrophytes	KME	39	0.57	14.9	26
	KZ	29	1	32	32
	ME	ND[1]	ND[1]	ND[1]	ND[1]
M. gypseum	KME	29	1.14	14.9	13
	KZ	19	4	32	8
	ME	ND[1]	ND[1]	ND[1]	ND[1]

[1]Not detected

In this present study, we developed the optimized topical KME based on the QbD approach to explore robust microemulsions and understand the attributes that impact particle size and particle size distribution. The QTPP was predominantly identified for characteristics of the desired final product, including dosage form, dose, route of administration, appearance, particle size, and size distributions. Particle size and size distributions (PDI) were justified as CQA because the smaller particle size, the more surface skin contact area, that can deliver ketoconazole to infected skin effectively [28]. After that, the microemulsion formulations were selected through the literature review and preliminary study. IPM, polysorbate 80, and

ethanol were selected as materials for the microemulsion system due to their high solubilization of ketoconazole [16,17]. The concentration of ketoconazole was 2 % w/w as imitated the marketed product. Moreover, IPM and ethanol also have a penetration enhancement property that can synergistically increase ketoconazole penetration to the skin [29-31]. The risk potential using the FMEA model was estimated to identify the attributes and process parameters that most likely impacted CQA. The amount of oil and surfactant was highlighted as high risk because the too high mixing ratio of oil and surfactant can cause large microemulsions [32]. The stirring process, temperature, and container size were considered a low risk due to their high detectability and controllability. Therefore, to reduce the process viability and simplify the optimization study, only CMA was selected for the design of experiment. The process parameters were carried out under the best condition from trial batches. DoE for the optimization data established the relation in the mathematical model. The post-analysis using ANOVA implied the significance of the cubic and quadratic models that can be used for predicting particle size and particle size distribution. The obtained 2 equations defined the relation of CMA (IPM, Smix, and water concentration) for the CQA (particle size and particle size distribution). Our results from the equation showed that each microemulsion component had a significant positive effect on particle size and PDI. The interaction between Smix and water significantly reduced the particle size and PDI. These were in line with the other previous study that reported increasing the oil component in microemulsion resulted in increased particle size [33]. Cui *et al.* also reported that the contents of surfactant, co-surfactant, and oil of self-microemulsifying drug delivery systems significantly affected particle size [34]. Concurring with our study, Nasr *et al.* reported that the interaction of surfactant and cosurfactant, and water also negatively affected the particle size of microemulsions [35]. The increased surfactant and cosurfactant content can reduce the interfacial tension to provide a smaller particle size [36]. Through a screening DoE, a design space was identified in which particle size and PDI depended on KME components. The design space could be suggested as follows: IPM (14 - 19 %), Smix (65 - 80 %), and water (7 - 24 %). The results demonstrated that validated design space could be acceptable for the formulation within the desired quality attributes. Additionally, working within the design space can avoid a regulatory post-approval change process and save time and expenses [37].

After selection from a design space, the other characterization and biological activity evaluations were performed. Each test assessed different aspects of the performance and potential effects of ketoconazole-loaded microemulsions (KME). The optimized KME appeared as a uniform transparent liquid with particle size and PDI values that met the specified criteria, suggesting that the components were thoroughly mixed and uniformly dispersed within the system. Phase behavior was observed by electrical conductivity confirmed that the optimized KME was a w/o microemulsion. This type of microemulsion is characterized by water droplets dispersed within an oil continuous phase [38]. The viscosity observed in the optimized KME formulation aligned with the typical characteristic of microemulsions, which is low viscosity. This characteristic contributed to the ease of handling, spreading, and absorption of microemulsions, making it suitable for topical applications [39]. Additionally, the zeta potential can predict the physical stability of nanoformulations. The repulsive forces between similarly charged particles help prevent aggregation and maintain a homogeneous system [13]. The zeta potential of the optimized KME indicated a net negative charge on the particles in the microemulsion. Even though a zeta potential of less than ± 30 mV was observed in this study, stability could still be maintained, likely supported by the presence of a high amount of non-ionic surfactant (polysorbate 80) that promoted steric stabilization [40]. This resulted in the formation of a protective film around the droplets, preventing their coalescence and ensuring stability [41]. This finding was in line with a previous study conducted by Mehanna *et al.* which demonstrated that non-ionic surfactants could lead to relatively low zeta potential values while providing stability to the formulation [42]. Moreover, the pH of the optimized KME was within the acceptable range of 5 - 7 which was important for topical usage to ensure compatibility with the skin and to minimize potential irritation [43]. The *in vitro* permeation study evaluated the ability of KME to permeate through a membrane, providing insights into its potential for effective drug delivery. The *in vitro* permeation of optimized KME depicted that ketoconazole from KME permeated through the membrane by approximately 30 %, whereas it was retained in the membrane by approximately 60 % after 24 h. Our result was in accordance with a previous study that reported the combination of microemulsions with reduced water content revealed high drug deposition in the skin [35]. It is hypothesized that increasing Smix would reduce the skin hydration and permeation flux, and the small particle size of microemulsions would provide a large surface area for drug penetration [44,45]. Regarding the high skin retention, it is indicated that KME could effectively retain in the skin layer within the area of the target infection site and could have a longer antifungal action. Furthermore, in this present study, the cytotoxicity against human keratinocyte cells (HaCaT) by MTT assay was used as a screening test to predict the *in vitro* safety of KME. The results inferred that KME was safe for topical application. Likewise, the other previous studies also reported that their microemulsion

formulations had low cytotoxicity against human keratinocyte and lymphocyte cells [4,46,47]. These indicated that microemulsions are suitable for topical or transdermal delivery. In addition, antifungal activity testing determines the effectiveness of KME in inhibiting the growth of dermatophytes. It is worth noting that KME possessed a better antifungal activity than KZ against *T. mentagrophytes* and *M. gypseum*. KME exhibited stronger antifungal activity than KZ which concurred with their enhanced solubility and penetration [16,48]. Another study provided evidence that the diffusion and enhanced permeation of the drug from microemulsions allow the drug to last longer on the dermatophyte membrane [49]. Therefore, the obtained results demonstrated alignment with the desired characteristics outlined in the QTPP for the final KME product.

Conclusions

This present study successfully adapted the QbD approach to develop optimized KME for the treatment of dermatophyte infection. Through risk assessment, QTPP, CMA and CQA were identified, respectively. The obtained equations from the D-optimal mixture design model accurately predicted the composition of the optimized KME formulation constituting the design space. The obtained design space ensured that the KME was within an acceptable range of variables. This provided a robust framework for ensuring consistent product quality within the desired specifications. The optimized KME demonstrated a nano-size range, prolonged skin retention, non-toxicity, and enhanced antifungal activity against dermatophytes compared to unloaded ketoconazole. These findings demonstrated consistency with the desired characteristics outlined in QTPP for the final KME as a topical drug delivery system. The Future perspectives involve the application of QbD principles to stability assessment and scale-up studies, enabling the commercialization of the topical KME formulation.

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References

[1] M Ivanov, A Ćirić and D Stojković. Emerging antifungal targets and strategies. *Int. J. Mol. Sci.* 2022; **23**, 2756.
[2] EH Endo, RY Makimori, MV Companhoni, T Ueda-Nakamura, CV Nakamura and BPD Filho. Ketoconazole-loaded poly-(lactic acid) nanoparticles: Characterization and improvement of antifungal efficacy *in vitro* against Candida and dermatophytes. *J. Mycol. Med.* 2020; **30**, 101003.
[3] AK Gupta and DC Lyons. The rise and fall of oral ketoconazole. *J. Cutan. Med. Surg.* 2015; **19**, 352-7.
[4] N Tiwari, A Sivakumar, A Mukherjee and N Chandrasekaran. Enhanced antifungal activity of Ketoconazole using rose oil based novel microemulsion formulation. *J. Drug Deliv. Sci. Tech.* 2018; **47**, 434-44.
[5] N Nema, N Singh and A Pandey. The supramicellar solutions of polymeric surfactant (PEG 400) for the determination of poorly soluble antifungal drug. *Chemistry* 2010; **19**, 93-102.
[6] Q Ma, J Zhang, B Lu, H Lin, R Sarkar, T Wu and X Li. Nanoemulgel for improved topical delivery of desonide: Formulation design and characterization. *AAPS PharmSciTech* 2021; **22**, 163.
[7] AA Halwani. Development of pharmaceutical nanomedicines: From the bench to the market. *Pharmaceutics* 2022; **14**, 106.
[8] AR Kirtane, M Verma, P Karandikar, J Furin, R Langer and G Traverso. Nanotechnology approaches for global infectious diseases. *Nat. Nanotechnol.* 2021; **16**, 369-84.
[9] S Soares, J Sousa, A Pais, C Vitorino. Nanomedicine: Principles, properties, and regulatory issues. *Front. Chem.* 2018; **6**, 360.
[10] T Shukla, N Upmanyu, M Agrawal, S Saraf, S Saraf and A Alexander. Biomedical applications of microemulsion through dermal and transdermal route. *Biomed. Pharmacother.* 2018; **108**, 1477-94.
[11] CM Hsieh, TL Yang, AD Putri and CT Chen. Application of design of experiments in the development of self-microemulsifying drug delivery systems. *Pharmaceuticals* 2023; **16**, 283.
[12] G Tartaro, H Mateos, D Schirone, R Angelico and G Palazzo. Microemulsion microstructure(s): A tutorial review. *Nanomaterials* 2020; **10**, 1657.

[13] EB Souto, A Cano, C Martins-Gomes, TE Coutinho, A Zielińska and AM Silva. Microemulsions and nanoemulsions in skin drug delivery. *Bioengineering* 2022; **9**, 158.
[14] L Mockus, D LeBlond, PK Basu, RB Shah and MA Khan. A QbD case study: Bayesian prediction of lyophilization cycle parameters. *AAPS PharmSciTech* 2011; **12**, 442-8.
[15] ICH Quality Guidelines, Available at: https://www.ich.org/page/quality-guidelines, accessed April 2023.
[16] M Wróblewska, E Szymańska and K Winnicka. The influence of tea tree oil on antifungal activity and pharmaceutical characteristics of Pluronic® F-127 gel formulations with ketoconazole. *Int. J. Mol. Sci.* 2021; **22**, 11326.
[17] BG Prajapati, H Paliwal and PA Shah. *In vitro* characterization of self-emulsifying drug delivery system-based lipsticks loaded with ketoconazole. *Future J. Pharmaceut. Sci.* 2023; **9**, 35.
[18] R Khonkarn, OS Kittipongpatana, V Boasouna and S Okonogi. Enhanced UV protection of ketoconazole using *Hyptis suaveolens* microemulsion. *Pakistan J. Pharmaceut. Sci.* 2018; **31**, 733-9.
[19] P Anantaworasakul, S Anuchapreeda, S Yotsawimonwat, O Naksuriya, S Lekawanvijit, N Tovanabutra, P Anantaworasakul, W Wattanasri, N Buranapreecha and C Ampasavate. Nanomaterial lipid-based carrier for non-invasive capsaicin delivery; manufacturing scale-up and human irritation assessment. *Molecules* 2020; **25**, 5575.
[20] N Nitthikan, P Leelapornpisid, S Natakankitkul, W Chaiyana, M Mueller, H Viernstein and K Kiattisin. Improvement of stability and transdermal delivery of bioactive compounds in green robusta coffee beans extract loaded nanostructured lipid carriers. *J. Nanotechnology* 2018; **2018**, 7865024.
[21] N Nitthikan, P Leelapornpisid, O Naksuriya, N Intasai and K Kiattisin. Potential and alternative bioactive compounds from brown *Agaricus bisporus* mushroom extracts for xerosis treatment. *Sci. Pharm.* 2022; **90**, 59.
[22] Clinical and Laboratory Standards Institute, Available at: https://clsi.org/, accessed June 2023.
[23] FF Veiga, MC Gadelha, MR Da Silva, MI Costa, B Kischkel, LVD Castro-Hoshino, F Sato, ML Baesso, MF Voidaleski, V Vasconcellos-Pontello and VA Vicente. Propolis extract for onychomycosis topical treatment: From bench to clinic. *Front. Microbiol.* 2018; **9**,779.
[24] AE Moskaluk and S VandeWoude. Current topics in dermatophyte classification and clinical diagnosis. *Pathogens* 2022; **11**, 957.
[25] J Roana, N Mandras, D Scalas, P Campagna and V Tullio. Antifungal activity of *Melaleuca alternifolia* essential oil (TTO) and its synergy with itraconazole or ketoconazole against *Trichophyton rubrum*. *Molecules* 2021; **26**, 461.
[26] KC Hazen. Fungicidal versus fungistatic activity of terbinafine and itraconazole: An *in vitro* comparison. *J. Am. Acad. Dermatol.* 1998; **38**, S37-S41.
[27] C Chittasupho, S Ditsri, S Singh, M Kanlayavattanakul, N Duangnin, W Ruksiriwanich and S Athikomkulchai. Ultraviolet radiation protective and anti-inflammatory effects of *Kaempferia galanga* L. rhizome oil and microemulsion: Formulation, characterization, and hydrogel preparation. *Gels* 2022; **8**, 639.
[28] L Zhao, Y Wang, Y Zhai, Z Wang, J Liu and G Zhai. Ropivacaine loaded microemulsion and microemulsion-based gel for transdermal delivery: preparation, optimization, and evaluation. *Int. J. Pharm.* 2014; **477**, 47-56.
[29] A Haq and B Michniak-Kohn. Effects of solvents and penetration enhancers on transdermal delivery of thymoquinone: Permeability and skin deposition study. *Drug Deliv.* 2018; **25**, 1943-9.
[30] TN Engelbrecht, B Demé, B Dobner and RH Neubert. Study of the influence of the penetration enhancer isopropyl myristate on the nanostructure of stratum corneum lipid model membranes using neutron diffraction and deuterium labelling. *Skin Pharmacol. Physiol.* 2012; **25**, 200-7.
[31] LB Lopes. Overcoming the cutaneous barrier with microemulsions. *Pharmaceutics* 2014; **6**, 52-77.
[32] M Herneisey, E Lambert, A Kachel, E Shychuck, JK Drennen III and JM Janjic. Quality by design approach using multiple linear and logistic regression modeling enables microemulsion scale up. *Molecules* 2019; **24**, 2066.
[33] A Akram, A Rasul, MK Waqas, M Irfan, SH Khalid, MN Aamir, G Murtaza, KU Rehman, M Iqbal and BA Khan. Development, characterization and evaluation of *in-vitro* anti-inflammatory activity of ginger extract based microemulsion. *Pakistan J. Pharmaceut. Sci.* 2019; **2**, 1327-32.
[34] J Cui, B Yu, Y Zhao, W Zhu, H Li, H Lou and G Zhai. Enhancement of oral absorption of curcumin by self-microemulsifying drug delivery systems. *Int. J. Pharm.* 2009; **371**, 148-55.
[35] M Nasr, S Abdel-Hamid. Optimizing the dermal accumulation of a tazarotene microemulsion using skin deposition modeling. *Drug Dev. Ind. Pharm.* 2016; **42**, 636-43.

[36] N Aggarwal, S Goindi, R Khurana. Formulation, characterization and evaluation of an optimized microemulsion formulation of griseofulvin for topical application. *Colloid. Surface. B Biointerfaces* 2013; **105**, 158-66.
[37] M von Stosch, R Schenkendorf, G Geldhof, C Varsakelis, M Mariti, S Dessoy, A Vandercammen, A Pysik and M Sanders. Working within the design space: Do our static process characterization methods suffice? *Pharmaceutics* 2020; **12**, 562.
[38] M Gradzielski, M Duvail, PM de Molina, M Simon, Y Talmon and T Zemb. Using microemulsions: Formulation based on knowledge of their mesostructure. *Chem. Rev.* 2021; **121**, 5671-740.
[39] MD Chatzidaki, S Demisli, E Zingkou, PG Liggri, DP Papachristos, G Balatsos, V Karras, F Nallet, A Michaelakis, G Sotiropoulou and SE Zographos. Essential oil-in-water microemulsions for topical application: Structural study, cytotoxic effect and insect repelling activity. *Colloid. Surface. Physicochem. Eng. Aspect.* 2022; **654**, 130159.
[40] VK Rai, N Mishra, KS Yadav and NP Yadav. Nanoemulsion as pharmaceutical carrier for dermal and transdermal drug delivery: Formulation development, stability issues, basic considerations and applications. *J. Contr. Release* 2018; **270**, 203-25.
[41] AA Umar, IB Saaid, AA Sulaimon and RB Pilus. A review of petroleum emulsions and recent progress on water-in-crude oil emulsions stabilized by natural surfactants and solids. *J. Petrol. Sci. Eng.* 2018; **165**, 673-90.
[42] MM Mehanna, KK Abla and HA Elmaradny. Tailored limonene-based nanosized microemulsion: Formulation, physicochemical characterization and *in vivo* skin irritation assessment. *Adv. Pharmaceut. Bull.* 2021; **11**, 274.
[43] ZD Draelos. The science behind skin care: Cleansers. *J. Cosmet. Dermatol.* 2018; **17**, 8-14.
[44] A Hussain, A Samad, S Singh, M Ahsan, M Haque, A Faruk and F Ahmed. Nanoemulsion gel-based topical delivery of an antifungal drug: *In vitro* activity and *in vivo* evaluation. *Drug Deliv.* 2016; **23**, 642-57.
[45] Z Wang, HJ Mu, XM Zhang, PK Ma, SN Lian, FP Zhang, SY Chu, WW Zhang, AP Wang, WY Wang and KX Sun. Lower irritation microemulsion-based rotigotine gel: Formulation optimization and *in vitro* and *in vivo* studies. *Int. J. Nanomedicine* 2015; **10**, 633-44.
[46] Y Zhang, H Hu, Q Jing, Z Wang, Z He, T Wu and NP Feng. Improved biosafety and transdermal delivery of aconitine via diethylene glycol monoethyl ether-mediated microemulsion assisted with microneedles. *Pharmaceutics* 2020; **12**, 163.
[47] X Lv, Z Cong, Z Liu, X Ma, M Xu, Y Tian, X Zhang, B Xu, J Zhang and Z Tang. Improvement of the solubility, photostability, antioxidant activity and UVB photoprotection of trans-resveratrol by essential oil based microemulsions for topical application. *J. Drug Deliv. Sci. Tech.* 2018; **48**, 346-54.
[48] J Che, Z Wu, W Shao, P Guo, Y Lin, W Pan, W Zeng, G Zhang, C Wu and Y Xu. Synergetic skin targeting effect of hydroxypropyl-β-cyclodextrin combined with microemulsion for ketoconazole. *Eur. J. Pharm. Biopharm.* 2015; **93**, 136-48.
[49] VK Rapalli, S Banerjee, S Khan, PN Jha, G Gupta, K Dua, MS Hasnain, AK Nayak, SK Dubey and G Singhvi. QbD-driven formulation development and evaluation of topical hydrogel containing ketoconazole loaded cubosomes. *Mater. Sci. Eng. C Mater. Biol. Appl.* 2021; **119**, 111548.