Enhanced UV protection of ketoconazole using *Hyptis suaveolens* micro emulsion

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Abstract: Ketoconazole is photolabile antifungal drug. Photochemical reactions may decrease its therapeutic effect or induce toxic compounds. The aim of this study was to prepare ketoconazole loaded microemulsion containing *H. suaveolens* oil with antifungal and antioxidant powers in order to obtain effective antifungal formulation. The release study, antifungal activity and photostability test, were then evaluated. The results showed that optimized *Hyptis suaveolens* microemulsion for ketoconazole loading was selected through construction of pseudo-ternary phase diagrams. It consisted of 12.5% *H. suaveolens* oil, 12.5% capryol, 25% tween 80, 25% ethanol and 25% water. Mean globule size was 153 nm, as analyzed by photon correlation spectroscopy. Ketoconazole-loaded *Hyptis suaveolens* microemulsion and *Hyptis suaveolens* microemulsion had antifungal activity against *Candida albican, Microsporum gypseum* and *Trichophyton mentagrophyte*, showing inhibition zone ranged from 28-37 mm and 23-32 mm, respectively. Ketoconazole was released from *Hyptis suaveolens* microemulsion more than 90% within 5 days. In the results of photostability test, ketoconazole-loaded *Hyptis suaveolens* microemulsion gave significantly higher remaining ketoconazole than ketoconazole solution. This study demonstrated that *Hyptis suaveolens* microemulsion could be used to improve the photoprotection of photolabile drug.

Keywords: Ketoconazole, *H. suaveolens*, microemulsion, photoprotection.

INTRODUCTION

Ketoconazole is an effective medicine which is commonly used to treat infection caused by fungus. However, its low aqueous solubility limits its pharmaceutical use (Nema *et al*., 2010). Besides, it has been reported that ketoconazole is unstable under oxidative and hydrolysis stressed conditions (Mhaske and Sahasrabudhe, 2011). Ketoconazole was least stable at low pH and the major degradation partway was specific acid catalysis (Skiba *et al*., 2000). Ketoconazole is photolabile azole antimycotics (Staub *et al*., 2010). The increasing of antioxidant level adversely affected the stability of ketoconazole (Skiba *et al*., 2000).

Hyptis suaveolens (Lamiaceae) is a medium, aromatic annual shrub commonly grown in the tropical and subtropical regions. The essential oil of *H. suaveolens* has been associated with biological and cellular activities, including cytoprotective, hepatoprotective, gastro-protective and neuroprotective proterties (Ghaffari *et al*., 2012; Vera-Arzave *et al*., 2012; Ghaffari *et al*., 2014). Importantly, this essential oil showed antimicrobial activity against *Trichophyton mentagrophytes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It also reported to possess interesting antioxidant potential determined by the DPPH (IC_{50} = 3.72 mg/ml) and ABTS (TEAC=65.02 μM/mg) methods (Nantitanon *et al*., 2007; Ghaffari *et al*., 2014). Because the activity as antimicrobial and counteracting the oxidative stress, the oil of *Hyptis suaveolens* is therefore interesting oil selected in ketoconazole formulation.

Microemulsions are interesting drug delivery systems because of their easy preparation and spontaneous to form stable and transparent nano-sized system. They compose of oil phase, aqueous phase and a combination of surfactant and co-surfactant (Dai *et al*., 2004; Sohoo *et al*., 2014). Moreover, solubility and chemical stability of many compounds could be improved by loading into microemulsions (Spernath and Aserin, 2006). In present study, we formulated ketoconazole incorporated *Hyptis suaveolens* microemulsion. *In vitro* drug release study and antifungal activity were then investigated. Finally, stability assessment of ketoconazole in *Hyptis suaveolens* microemulsion was studied.

MATERIALS AND METHODS

Materials

Hyptis suaveolens was harvested from the North region of Thailand as previous report (Nantitanon *et al*., 2007). Ketoconazole was purchased from S. Tong Chemical Co. Ltd. (Bangkok, Thailand). Sabouaud dextrose agar was obtained from Bacto-Difco Lab Co., Ltd (Detroit, MI, USA). All other chemicals and solvents were of analytical grade.

Sample preparation

The fresh aerial part of *H. suaveolens* was cut into small pieces and put into a round-bottomed flask containing DI

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water. *H. suaveolens* oil was isolated from this sample by hydro-distillation clevenger apparatus. After that, anhydrous sodium sulfate was used to absorb moisture in the isolated oil. The obtained *H. suaveolens* oil was stored in a desiccator at 4 ºC for further studies.

GC-MS analysis

For chemical characterization of *H. suaveolens* oil**,** Hewlett-Packard 6890 gas chromatography and Agilent Technologies HP 5973N mass spectrometry detector (EI Mode, 70 eV) was used. Volatile compounds were separated on HP-5MS column (5% phenyl dimethylsiloxane, length: 30 m, inner diameter: 0.25 mm and film thickness: 0.25 µm) that were operated with temperature program as follows: 3 min at 70°C, 3°C/min up to 188°C, 20°C/min up to 280°C and constant at 280°C for 5 min. The temperature of injector was 260°C whereas the temperature of detector was 280°C. The flow rate of carrier gas (Helium) was 1 ml/min. The compositions of *H. suaveolens* oil were identified and quantified by comparison with reference data of Wiley and NIST library.

Pseudo-ternary phase diagrams

Pseudo-ternary phase diagrams were investigated using water titration method in order to form microemulsions with obtaining appropriate ratio of oil phase, aqueous phase and a combination of surfactant and co-surfactant (Smix). Smix was varied in 2:1, 1:1 and 1:2. Smix and oil were further mixed in ratio of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10. Distilled water was gradually added into each Smix-oil mixture at room temperature. Microemulsion was transparent dispersion. At the end point, the cloudy or opaque mixer was observed. Microemulsion region was constructed in pseudo-ternary phase diagrams using Axio Vision Rel 4.8 software.

Preparation of ketoconazole-loaded H. suaveolens microemulsion

The optimized *H. suaveolens* microemulsion formulation was selected from the result of pseudo-ternary phase diagrams. Ketoconazole powder was dissolved in oil phase. Next, the quantity of Smix and DI water were added, respectively. The mixture was adequately mixed by stirring to form clear ketoconazole loaded *H. suaveolens* microemulsion.

Dynamic light scattering

Malvern Zetasizer consisting of computerized auto-titrate and DLS software (Malvern Instruments Ltd., Malvern, UK) was used to analyze the average globule size of *H. suaveolens* microemulsions and ketoconazole loaded *H. suaveolens* microemulsion. Light scattering was taken at a fixed angle of 173°.

The in vitro release

A volume of 1 ml of a freshly prepared ketoconazole loaded microemulsions was enclosed in a pre-swollen dialysis bag (Cellu Sep®, MWCO: 3,500 Da, Membrane Filtration Products, Inc.). The obtained bag was incubated in 100 ml of release medium at 37°C under gentle stirring at 200 rpm. 50% v/v dioxan of phosphate buffer (pH 7.4) was used as release medium. 5 ml of receiving medium containing the released ketoconazole were withdrawn periodically. After sampling, 5 ml of fresh buffer containing 50% Dioxan was added to maintain the original volume. The concentration of released ketoconazole in the different samples was measured by HPLC at 254 nm UV detector based on a standard curve of ketoconazole.

HPLC analysis

Drug content (ketoconazole) of *H. suaveolens* oil micro-emulsions was diluted with ethanol and filtered using a 0.45-µm pore filter (Filtrex, Virginia Beach, VA, USA). The sample was analyzed using Hewlett Packard/hp1100 HPLC system with a UV visible detector. ZOBRAX SB-C18 column, particle size: 5 µm, 250×4.6 mm (Agilent®), with guard column, was used. A mixture of 85% (v/v) acetonitrile and 15% (v/v) phosphate buffer (pH 4) was the mobile phase at flow rate of 0.7 ml/min. The injection volume was 10 µl and detection was recorded at 254 nm.

In vitro antifungal activity

Microorganisms included in this study were *Candida albican, Microsporum gypseum* and *Trichophyton mentagrophyte*. *In vitro* antifungal activity of ketoconazole *H. suaveolens* microemulsions in comparison with ketoconazole microemulsions and *H. suaveolens* microemulsions was determined by agar disc diffusion method. In brief, *C. Albican* was cultured in Sabouraud dextrose agar at 37°C overnight whereas *M. gypseum* and *T. mentagrophyte* were cultured at 25°C for 3 nights to reach stationary phase of growth. Next, samples (100 g each) were added to agar plates. Then, agar plates containing *C. Albican* were incubated at 37°C for 24 h while agar plates containing dermatophytes were kept at 25°C for 72 h. Three replicates were used for each test sample. After incubation, the mean diameter of clear zone was recorded.

Photostability test

Ketoconazole microemulsion was formulated by mixing 12.5% *H. suaveolens* oil, 12.5% capryol, 25% tween 80 and 25% ethanol. Ketoconazole (0.2%) powder was dissolved into the oil-Smix mixture. Finally, 24.8% of phosphate buffer pH 7.4 was added and the mixture was blended using vortex mixer. On the other hand, 0.2% ketoconazole solution was prepared by dissolving drug powder in 24.8% of phosphate buffer in ethanol. Ketoconazole microemulsion and solution were exposed to UV light (Germicidal lamp GL T5, 15 W). The irradiated samples were collected at 6, 12, 24, 36 and 48 h and assessed for remaining ketoconazole by HPLC method described earlier.

Table 1: Chemical compositions of essential oil from aerial part of *H. suaveolens*

Peak No.	Compound	RT	Composition (%)	Peak No.	Compound	RT	Composition (%)
1	α-Thujene	3.95	0.3	23	β-Cubebene	21.97	0.1
2	Sabinene	5.03	12.77	24	α-Humulene	22.95	1.11
3	β-Pinene	5.10	5.29	25	Aromadendrene	23.22	0.36
4	Myrcene	5.39	0.68	26	Germacrene	24.08	1.28
5	α-Phellandrene	5.79	1.25	27	β-Selinene	24.27	0.75
6	α-Terpinene	5.79	0.41	28	Bicyclogermacrene	24.67	1.40
7	para-Cymene	6.39	0.22	29	δ-Cadinene	25.78	0.21
8	Limonene	6.50	3.31	30	Spathulenol	27.96	0.21
9	1,8-Cineole	6.65	19.47	31	Germacreneβ	30.35	0.25
10	γ-Terpinene	7.42	0.62	32	T-Muurolol	30.85	0.77
11	cis-sabinene hydrate	7.82	0.36	33	Cyclopentanone	32.13	0.14
12	Terpinolene	8.47	9.98	34	Bergamotol	32.30	1.39
13	cis-sabinene hydrate	8.93	0.23	35	Propanamide	41.95	0.13
14	Fenchol	9.48	0.17	36	Rimuene	42.66	2.18
15	2-Cyclohexen-1-ol	9.76	0.11	37	Cyclohexane	43.74	0.23
16	Benzenemethanol	11.90	1.34	38	Benzocyclododecene	43.93	0.39
17	2,6-Dimethyl-1,3,5,7-octatetraene	14.41	0.13	39	Kaur-16-ene	45.09	0.14
18	α-Cubebene	18.75	0.2	40	Sesquirosefuran	46.11	0.12
19	β-Bourbonene	19.81	1.02	41	Benzene	46.20	0.24
20	β-Cubebene	20.42	0.1	42	9-Octadecenamide	46.30	4.36
21	β-Elemene	20.55	0.96	43	4-Epidehydroabietol	46.59	0.63
22	β-Caryophyllene	21.68	14.44	44	others		10.09

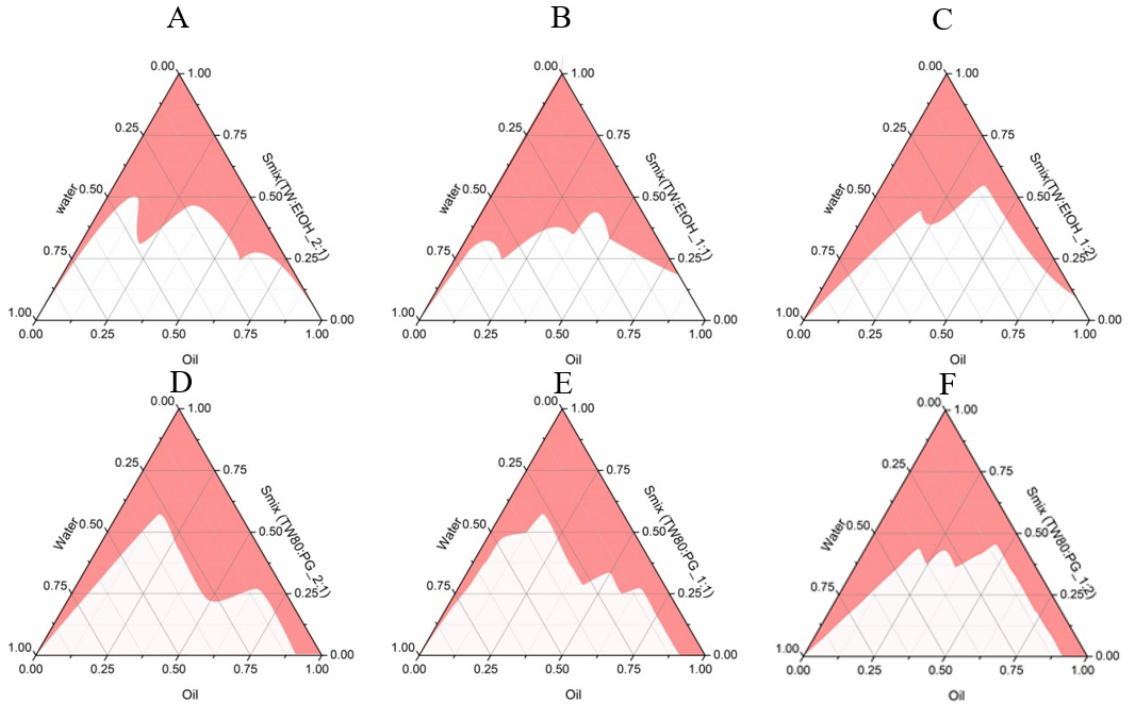

Fig. 1: Pseudo ternary phase diagram of micro emulsionregion (red region) containing water, oil (*H. suaveolens* oil to Capryol in ratio of 2:1) and Smix of Tween 80 to Ethanol in ratio of 2:1 (A); Tween 80 to Ethanol in ratio of 1:1 (B); Tween 80 to Ethanol in ratio of 1:2 (C); Tween 80 to Propylene glycol in ratio of 2:1 (D); Tween 80 to Propylene glycol in ratio of 1:1 (E); Tween 80 to Propylene glycol in ratio of 1:2 (F).

Table 2: Formulation of *H. suaveolens* microemulsion

	Formulation			
	F1	F2	F3	F4
H. suaveolens oil	12.5	12.5	12.5	12.5
Capryol	12.5	12.5	12.5	12.5
Tween 80	25.0	33.3	25.0	16.7
Ethanol	25.0	-	-	-
Propylene glycol	-	16.7	25.0	33.3
Water	25.0	25.0	25.0	25.0

Table 3: The mean globule size, PDI and pH of *H. suaveolens* microemulsion

Formulation	Size (nm)	PDI	pH
F1	152.5±15.7	0.23±0.03	6.84
F2	360.0±3.3	0.25±0.01	6.80
F3	242±17.1	0.30±0.02	6.40
F4	232.0±9.1	0.43±0.03	6.46

Table 4: Inhibition zones of *H. suaveolens* microemulsion, ketoconazole microemulsion, ketoconazole *H. suaveolens* microemulsion against *C. albican*, *M. gypseum* and *T. mentagrophytes*

Microorganisms	Inhibition zone (mm)		
	H. suaveolens Microemulsion	Ketoconazole Microemulsion	Ketoconazole *H. suaveolens* microemulsion
C. albican	22.7±0.4	36.0±0.7	36.7±0.5
M. gypseum	25.9±0.4	30.5±0.5	27.8±0.6
T. mentagrophytes	31.7±0.2	36.9±0.6	35.2±0.7

RESULTS

Essential oil analysis

The essential oil was prepared from aerial part of *H. suaveolens* by hydro-distillation. The results of gas chromatography–mass spectrometry showed that 1,8-cineole (19.47%), β-caryophyllene (14.44%), sabinene (12.77%) and terpinolene (9.98%) were identified as major constituents in *H. suaveolens* essential oil. Other components such as β-pinene, 9-octadecenamide, limonene, rimuene, bergamotol, bicyclogermacrene, benzenemethanol, germacrene, α-phellandrene, α-humulene and β-bourbonene were found in the concentration between 5.29% to 1.02%. The remaining compounds were present at concentrations of less than 1 %, as shown in table 1.

Preparation and characterization of ketoconazole-loaded H. suaveolens oil microemulsion

Fig. 1 shows pseudo-ternary phase diagrams containing various weight ratios of water, oil (*H. suaveolens* oil and Capryol) and Smix (Tween 80 and Ethanol (fig. 1A-C) and Tween 80 and propylene glycol (fig. 1D-F). Red region was represented to microemulsion region whereas white region was referred to the cloudy or opaque region. The result reveals that pseudo-ternary phase diagram B, D, E and F had wide microemulsion region.

The compositions of *H. suaveolens* microemulsion (F1-F4) are shown in table 2. All formulations were transparent with no phase separation after centrifugation at 10,000g for 30 min at 25ºC. Table 3 exhibits the physico-chemical properties of *H. suaveolens* micro-emulsions. The pH values were around 6.40 - 6.84. The mean globule size was 152.5±15.7 to 360.0±33.3 nm. The smallest size was found from F1 and the biggest size was found from F2. In addition, F1 presented the lowest polydispersity indices (0.23±0.03). F1 was therefore selected to load ketoconazole.

Release study

Optimized *H. suaveolens* microemulsion (F1) was selected for *in vitro* release study in 50% v/v dioxan of phosphate buffer (pH 7.4) at 37ºC for 5 days. Result of release study is present in fig. 2. Ketoconazole started to release from *H. suaveolens* oil microemulsions after 1 h of incubation. The dialysis membrane probably delays the permeation of ketoconazole in the beginning of release experiment (Khonkarn *et al*., 2012). The loaded ketoconazole was released from *H. suaveolens* oil micro-emulsions at 37.7±7.6% in the first 24h. Ketoconazole was then subsequently released from *H. suaveolens* microemulsions reaching 94.4±0.7% at day 5.

In vitro antifungal activity

Table 4 is the summarized results of antifungal activity tests using well diffusion method. *H. suaveolens* micro-

emulsion present a measurable antifungal activity against *T. mentagrophytes, M. gymsium* and *C. albican* with inhibition zones of 31.7±0.2, 25.9±0.4 and 22.7±0.4 mm, respectively. Ketoconazole-loaded *H. suaveolens* micro-emulsion had bigger inhibition zone (27.8±0.6 - 36.7±0.5 mm) than *H. suaveolens* microemulsion, however, its inhibition zone was not different from ketoconazole-loaded microemulsion (30.5±0.5 to 36.9±0.6 mm).

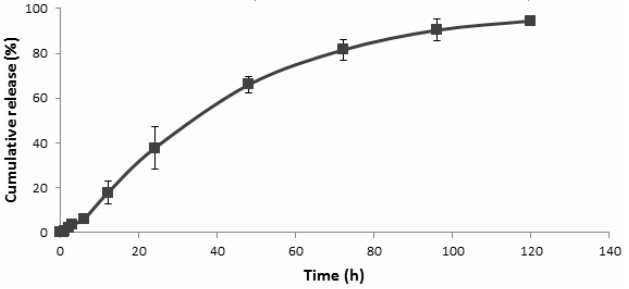

Fig. 2: Release of ketoconazole from *H. suaveolens* oil microemulsion by dialysis membrane, pH 7.4, 37ºC

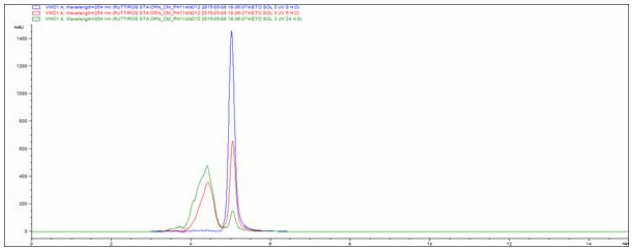

Fig. 3: HPLC chromatogram of the ketoconazole solution subjected to UV light (30 W) at 0 h (blue line), 6 h (red line) and 24 h (green line)

Photostability test

The result of HPLC chromatogram (fig. 3) clearly shows that ketoconazole solution was degraded at 6 h after exposure to UV light. The remaining ketoconazole gradually decreased in time (52.3±8.7% at 6h to 3.2±1.5% at 48 h) as shown in fig. 3 and 4. Interestingly, *H. suaveolens* oil microemulstion delayed photo-degradation of ketoconazole. Ketoconazole-loaded *H. suaveolens* oil microemulstion was significantly degraded at 24h of light exposure (67.0±1.3% of remaining ketoconazole) and remaining ketoconazole at 48 h was only 32.7±6.2%.

DISCUSSION

The variation of essential oil chemical composition may be related to geographical and environmental factors (Nantitanon *et al*., 2007). The extracted essential oil was then standardized by gas chromatography–mass spectrometry and 1,8-cineole, β-caryophyllene, sabinene as well as terpinolene were main compositions of tested *H. suaveolens* oil. Based on the antifungal and antioxidant activity *H. suaveolens* oil (Nantitanon *et al*., 2007) as well as the solubility study of ketoconazole (Nema *et al*., 2010), capryol and *H. suaveolens* oil were chosen as the oil carrier while tween 80, propylene glycol or ethanol was selected as Smix phase.

The effect of various weight ratio of surfactant/co-surfactant on the *H. suaveolens* microemulsion was determined by construction of pseudo-ternary phase diagram. The required amount of compositions to formulate *H. suaveolens* microemulsion of further study was then selected from pseudo-ternary phase diagrams B, D, E and F because of their wide microemulsion regions. The successful formulation of ketoconazole-loaded *H. suaveolens* microemulsion was composed of ketoconazole (1%), *H. suaveolens* oil (12.5%), Capryol (12.5%), Tween 80 (25%), Ethanol (25%) and water (24%). The incorporation of ketoconazole did not have considerable influence on the globule size of the microemulsion. The pH of ketoconazole-loaded *H. suaveolens* microemulsion was 6.85, which was close to pH of stable ketoconazole (Skiba *et al*., 2000). Ketoconazole was gradually released from *H. suaveolens* oil microemulsions within 120 h (>90%) without the initial burst of release ketoconazole.

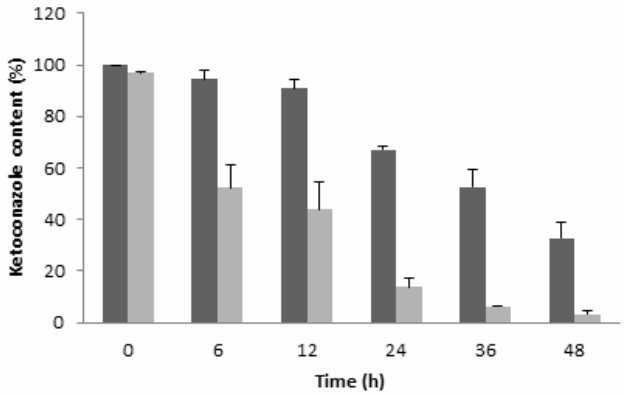

Fig. 4: Photo-stability of ketoconazole in solution (▒) and *H. suaveolens* micro emulstion (■)

It has been reported that the antifungal activity of *H. Suaveolens* oil (20% ethanolic solution) was higher than 4% phenol, but the same as 6% boric acid, 5% salicylic acid or 2% benzoic acid. Besides, 10% *H. suaveolens* oil demonstrated antifungal activity against *T. mentagrophytes* with inhibition zone of 17.2 mm (Nantitanon *et al*., 2007). In comparison with previous report, microemulsion containing 12.5% *H. suaveolens* oil of this study had higher inhibition zone than 10% *H. suaveolens* oil against *T. mentagrophytes*. Furthermore, ketoconazole-loaded *H. suaveolens* microemulsion exhibited more potent antifungal activity against all tested organisms than *H. suaveolens* microemulsion but it had similar activity as ketoconazole loaded microemulsion (not containing *H. suaveolens* oil). This is considered that synergistic effect between *H. suaveolens* oil and ketoconazole was not observed.

It has been reported that oxidative and hydrolysis were major pathways of ketoconazole degradation. Ketoconazole in aqueous media was specific acid catalysis and optimal pH value for physical and chemical stability of ketoconazole formulation was pH 7 (Skiba *et al*., 2000). In photostability testing, phosphate buffer pH 7.4 was then used to formulate ketoconazole-loaded micro emulsion and solution to control the pH of final formulation around 7.

The main degradation products of photodegradation of ketoconazole has been reported (Staub *et al*., 2010). Photochemical reactions are complex. The major reaction is the generated ROS by UV radiation during storage and administration, contributing to drug photodecomposition leading to a loss of potency of drug or producing harmful photo degradation products (Nguyen *et al*., 2013; Badea *et al.,* 2015). It clearly indicated that *H. suaveolens* micro-emulstion significantly improved photostability of ketoconazole. This benefit action maybe from arise antioxidant activity of *H. suaveolens*. Beside, 1,8-cineole exhibited a protective effect under oxidative stress by free radical scavenging activity, inhibition of lipid peroxidation, enhancement of expression of enzymatic antioxidant system and induction of Nrf2 nuclear translocation (Lima *et al*., 2014; Porres-Martínez *et al*., 2015). β-caryophyllene, a highly effective chain-breaking antioxidant agent, exerted antioxidative capacity as evidenced by scavenging activity against reactive oxygen species and reduction in the lipid and protein oxidation levels (Alvarez-González *et al*., 2014; Vinholes 2014; Calleja 2013). Moreover, *Piper nigrum* L. essential oil containing β-caryophyllene, limonene, sabinene, β-pinene, 3-carene, α-pinene, and copaene, also present antioxidative potential (Bagheri *et al*., 2014). Consequently, 1,8-cineole and β-caryophyllene may contribute to the antioxidant activity of *H. suaveolens* essential oil. Besides, its antifungal activity, *H. suaveolens* oil also has benefit on photostability improvement of antifungal drug formulation by against the oxidative stress.

CONCLUSION

Ketoconazole was successfully loaded into *H. suaveolens* microemulsion with mean globule size less than 200 nm and subsequently released from *H. suaveolens* micro-emulsions within day 5. Ketoconazole-loaded *H. suaveolens* microemulsion showed higher antifungal activity against *Candida albican, Microsporum gypseum* and *Trichophyton mentagrophyte* than *H. suaveolens* microemulsion. However, it had similar antifungal activity as ketoconazole-loaded microemulsion. *H. suaveolens* microemulsion significantly improved UV protection of ketoconazole, which corresponded to antioxidant activity of *H. suaveolens* oil.

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