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Original Article

Development and efficacy of tryptophol-containing emulgel for reducing subcutaneous fungal nodules from *Scedosporium apiospermum* eumycetoma

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Abstract

Background and purpose: Subcutaneous infections caused by *Scedosporium apiospermum* present as chronic eumycetomatous manifestations in both immunocompromised and immunocompetent individuals. Serious adverse effects/toxicities from the long-term use of antifungal drugs and antifungal resistance have been reported in patients with *S. apiospermum* infections. The present study aimed to determine the anti-*S. apiospermum* activities of fungal quorum sensing molecule known as tryptophol (TOH) and to develop a TOH-containing emulgel for treating *S. apiospermum* eumycetoma.

Experimental approach: Anti-*S. apiospermum* activities of TOH were determined and compared with voriconazole. Effects of TOH on *S. apiospermum* biofilm formation and human foreskin fibroblast (HFF)-1 cell cytotoxicity were determined. Moreover, TOH-containing emulgel was developed and physical properties, *in vitro*, and *in vivo* antifungal activities against *S. apiospermum* eumycetoma were evaluated.

Findings/Results: The minimal concentration of TOH at 100 µM exhibited anti-*S. apiospermum* activities by reducing growth rate, germination rate, and biofilm formation with less cytotoxicity to HFF-1 cells than voriconazole. Further study on the development of an emulgel revealed that TOH-containing emulgel exhibited excellent physical properties including homogeneity, consistency, and stability. Treatment by TOH-containing emulgel significantly reduced subcutaneous mass in a mouse model of *S. apiospermum* eumycetoma. The histopathological assessment showed marked improvement after 14 days of TOH-containing emulgel treatment.

Conclusion and implications: TOH could be used as an anti-fungal agent against *S. apiospermum* infections. A novel and stable TOH-containing emulgel was developed with excellent anti-*S. apiospermum* activities suggesting the utilization of TOH-containing emulgel as an innovative therapeutic approach in the treatment of *S. apiospermum* eumycetoma.

Keywords: Emulgel; Eumycetoma; Quorum sensing molecule, *Scedosporium apiospermum*; Tryptophol.

INTRODUCTION

Scedosporium fungi are increasingly recognized as the causes of opportunistic fungal infections but also play an important role as primary pathogens (1). *S. apiospermum* is an emerging opportunistic filamentous fungus that can be isolated from impacted environments caused by human activities including urban areas, manure-enriched or polluted soils, and sewage water (2). Traumatic localized infections of *S. apiospermum* can occur both in immunocompromised and immunocompetent individuals and are mostly developed from mycetomatous and chronic progressive subcutaneous infections to systemic or disseminated infections (3,4).

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Traumatic localized infections of *S. apiospermum* are mostly manifested as mycetomatous with 70-80% occurring in the skin of the hands or feet known as *S. apiospermum* eumycetoma (5). The pathology of the disease is developed as granulomatous inflammation penetrating the deep dermis and the subcutaneous tissue (6). Moreover, *S. apiospermum* eumycetoma is endemic among tropical and subtropical regions as well as in Thailand (4,7,8). However, this pathogenic fungus is notorious for its nature of therapy-refractory and is highly resistant to several antifungal agents (9). Thus, there is an urgent need to develop new treatment strategies for treating the therapeutic challenge of *Scedosporium* infections (10).

Several potential antifungal drugs have been extensively investigated over the past 30 years (11). Currently, treatments are limited to three classes of antifungal drugs i.e., polyenes, azoles, and echinocandins (12). Although the drugs effectiveness against drug-resistant-pathogenic fungi have been proven, complications of treatment and adverse outcomes due to the extensive treatment periods and the potentially toxic drugs used have been noted (13). Thus, alternative natural compounds exhibiting antifungal activity have been introduced. Among candidates, naturally occurring indole-based compounds are of great interest due to their properties responsible for cell-cell communication as well as auto-antibiotic actions known as fungal quorum sensing molecules (14-16). Lesser-known but just interesting is tryptophol (TOH), an indole-3-ethanol metabolite that can be found in terrestrial fungi (17). TOH has been found to affect fungal morphogenesis and induced fungal cell apoptosis providing antifungal potential against different fungal species (18). We previously reported that TOH exhibited antifungal activities against pathogenic *Candida albicans in vitro* (19). However, the utilization of TOH for treating *S. apiospermum* eumycetoma is poorly determined.

TOH is soluble in organic solvents such as ethanol (17). However, repeated topical application of ethanol-based-TOH as an antifungal treatment may adversely affect the skin and cause skin irritation and contact dermatitis (20). To improve topical TOH delivery, an alternative drug delivery system is required. Gellified emulsions also known as emulgels are mainly used to formulate hydrophobic drugs topically, which can be formed by the addition of a gelling agent into the emulsion phase (21). The formulated emulgel can entrap active drugs and slowly release-slowly absorb through the skin. Moreover, an emulgel provides good stability, is easily removable, greaseless, soothing, and non-staining than other available topical preparation such as creams or ointments (22). Thus, the current study aimed to determine the antifungal effects of TOH against *S. apiospermum* infection and to design and develop a TOH-containing emulgel. The physical properties, *in vitro*, and *in vivo* antifungal activity against *S. apiospermum* eumycetoma were also evaluated.

MATERIALS AND METHODS

Fungal strain and growth condition

S. apiospermum CBS 117410 used in this study was kindly provided by Dr. Ana Alastruey-Izquierdo (Servicio de Micología, Instituto de Salud. Carlos III, Madrid, Spain) (23). The conidial suspension of *S. apiospermum* CBS 117410 was prepared by suspending 5-day-old culture on Sabouraud dextrose agar (SDA; Oxoid Ltd., Hampshire, UK) in sterile 0.9% saline to 1×10^5 conidia/mL and incubated at 37 °C.

Antifungal susceptibility

TOH ($C_{10}H_{11}NO$) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The working solution of TOH was prepared by diluting 1 M stock solution to 1000 mM (v/v) with absolute ethanol (EtOH). Antifungal activity of TOH against *S. apiospermum* CBS 117410 was evaluated using the agar spot assay according to Kitisin *et al.* (7). Ten µL of *S. apiospermum* CBS 117410 conidia (concentration about 1×10^5 conidia/mL) were spotted on the surface of SDA plates containing either 10, 100, or 1000 µM of TOH or voriconazole (VOZ; Sigma-Aldrich Inc., St. Louis, MO, USA). The plates were incubated aerobically at 25 °C. Absolute EtOH

and RPMI 1640 culture medium (Sigma-Aldrich Inc., St. Louis, MO, USA) at 1% (v/v) were used as the diluent controls for TOH and VOZ, respectively. The colony diameter (mm) was measured every day for 7 days. Un-inoculated SDA plate was used as a negative control.

Conidial germination

The inhibitory effect of TOH on *S. apiospermum* CBS 117410 germination was determined according to Rollin-Pinheiro *et al.* (24). The fungal conidia (1×10^5/mL) were freshly added into a microplate containing RPMI 1640 medium and incubated at 37 °C for 12 h. Then, 500 µL of TOH or VOZ at 10, 100, or 1000 µM was added to each well and incubated for 24 h. A total of 500 cells were counted under an optical microscope, and the length of conidial germination was quantified by ImageJ (National Institutes of Health, Bethesda, MD, USA).

Biofilm formation

S. apiospermum is able to form biofilms on the surface of sterile polystyrene microplates (96-well) or glass-bottom Petri dishes (25). Biofilm formation of *S. apiospermum* CBS 117410 was performed by seeding 100 µL of conidial suspension (1×10^5/mL) to each well of the microplate and incubating at 37 °C for 3 h (adherence phase). As conidia adhered, the supernatant containing non-adherent cells was discarded and fresh RPMI 1640 supplemented with 2% glucose and 20% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was added to each well and incubated for 24 h. A well containing only the growth medium was used as a negative control.

The biofilms were washed three times with sterile phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells. Then, 200 µL of TOH or VOZ at 10, 100, or 1000 µM was added to each well and incubated for 24 h. Absolute EtOH and RPMI 1640 at 1% (v/v) were used as diluent controls. The inhibitory activity of TOH on *S. apiospermum* CBS 117410 biofilm formation was determined using two different approaches including crystal violet and XTT-reduction assays.

Crystal violet assay

Crystal violet assay was used to determine overall biomass including cells and the extracellular matrix of the biofilms by binding to negatively charged cell surface molecules and polysaccharides matrix of the fungi (25). After washing the biofilms with sterile PBS, the remaining biofilm in each well was fixed with methanol for 20 min and stained with crystal violet (0.02%) for 30 min. The solution was removed, and the biofilms were washed twice with sterile PBS. The impregnated crystal violet was dissolved using a 30% acetic acid solution for 10 min. One hundred µL of dissolved solution in each well was collected and transferred into a new 96-well flat-bottom microplate and measured the absorbance at 590 nm using a spectrophotometer (Sunrise Tecan, Grödig, Austria).

XTT assay

XTT assay was used to determine the metabolic activity of the cells inside the biofilms indicating cell viability and density (26). An orange formazan was converted from a yellow XTT dye by mitochondrial enzymes of the healthy cells. After washing the biofilms with sterile PBS, the remaining biofilm in each well was stained with 100 µL of an XTT:menadione solution (0.5 mg/mL:1 µM) as described previously (26). The microplate was incubated in the dark at 37 °C for 2 h. The dissolved solution was transferred into a new microplate and measured the absorbance at 490 nm using a spectrophotometer (Sunrise Tecan, Grödig, Austria).

In vitro human foreskin fibroblast cell cytotoxicity

The human foreskin fibroblasts (HFF-1) cell line was obtained from American Type Culture Collection (ATCC®, Manassas, VA; catalog #SCRC-1041™). HFF-1 cells were routinely maintained in Dulbecco's modified eagle medium (DMEM; Gibco® by Life Invitrogen™, Carlsbad, USA) supplemented with 10% FBS and 100 units/mL of penicillin-streptomycin (Gibco® by Life Invitrogen™, Carlsbad, USA) and incubated under humidified conditions with 5% CO_2 at 37 °C. Measurement of the cytotoxicity of TOH was carried out using

two different assays, which are 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) activities.

MTT assay

HFF-1 cells were seeded at a concentration of 1×10^5 cells/mL onto a 96-well microplate and allowed to incubate overnight. Once reached 80-90% confluence, HFF-1 cells were washed with sterile PSB and treated with 100 µL of TOH or VOZ at 10, 100, or 1000 µM. The plate was incubated for 24 h. Absolute EtOH and RPMI 1640 at 1% (v/v) were used as diluent controls. HFF-1 cells incubated with culture medium alone were used as negative controls (100% viable). After 24 h incubation, the supernatant was aspirated from each well and HFF-1 cells were washed three times with sterile PBS. Then, 10 µL of MTT solution (Sigma-Aldrich Inc., St. Louis, MO, USA) was added and incubated at 37 °C for 2 h (27). After carefully removing the supernatant, formazan was dissolved with 100 µL of 2% NP-40 (Sigma-Aldrich Inc., St. Louis, MO, USA). The absorbance was detected at 540 nm with a reference filter of 620 nm using a spectrophotometer (Sunrise Tecan, Grödig, Austria). The percentage of cell viability was calculated using the following equation:

$$Cell\ viability\ (\%) \frac{\text{Sample OD – background control OD}}{\text{Control OD – background OD}} \times 100 \quad (1)$$

LDH assay

LDH values were determined using the CytoTox 96® non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions (28). Briefly, TOH and VOZ treatments on HFF-1 cells were prepared similar to the MTT assay. After 24 h incubation, 50 µL aliquots of cell supernatants were mixed with 50 µL of CytoTox 96® reagent in fresh 96-well flat clear bottom plates and incubated at room temperature in the dark for 30 min. Fifty µL of stop solution was then added and the absorbance was read at 490 nm using a spectrophotometer (Sunrise Tecan, Grödig, Austria). Untreated HFF-1 cells were used as negative controls, and the positive controls were provided in the assay kit. The percentage of cell cytotoxicity was calculated using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental OD}}{\text{Maximum release OD}} \times 100 \quad (2)$$

Formulation of TOH-containing emulgel

Emulgel formula was developed according to Chen *et al.* (29) and Shahin *et al.* (30) as shown in Table 1. Briefly, emulgel base was prepared by a constant mechanical stirring of the gel-based SEPIGEL 305. The concentration of TOH that exhibited the highest antifungal activities against *S. apiospermum* CBS 117410 infection *in vitro* with the lowest cell cytotoxicity was chosen as an active ingredient. Then, TOH was slowly added under agitation to the emulgel base. The emulgel-base formulation was prepared with absolute EtOH and was used as a control emulgel.

Physical and biological properties of the developed TOH-containing emulgel including visual appearance, viscosity, spreadability, stability, pH, TOH content, *in vitro* and *in vivo* antifungal activity, and fungal identification of skin samples were determined. All experiments were performed in triplicate.

Table 1. Composition of optimized TOH-containing emulgel formula

Components	Emulgel base	TOH-containing emulgel
Deionized water	q.s. to 100% w/w	q.s. to 100% w/w
SEPIGEL 305	10% w/w	10% w/w
Triethanolamine	q.s. to pH 5.5	q.s. to pH 5.5
1% EtOH as TOH diluent	10% w/w	-
TOH (active ingredient)	-	10% w/w
Tween™ 20	1% w/w	1% w/w
Glycerin	2% w/w	2% w/w
Propylene glycol	2% w/w	2% w/w
Allantoin	1% w/w	1% w/w
Phenoxyethanol	0.2% w/w	0.2% w/w

TOH; Tryptophol; SEPIGEL 305, polyacrylamide and C13-14 isoparaffin and laureth-7; q.s, quantity sufficient to adjust the pH to 5.5.

Visual inspection

For visual appearance, the prepared emulgel formulae were examined for their physical appearances including color, homogeneity, and phase separation (30).

Viscosity

The viscosity of prepared emulgel formulae was determined using a Brookfield viscometer (Brookfield, Model programmable DV2, USA) (30). Briefly, about 0.5 g of each formula was placed into the beaker and left for equilibrium (30 min) at 25 °C. Measurements with the spindle were made at a moderate speed of 100 rpm for 10 min. The viscosity reading was recorded.

Spreadability

The spreadability of each emulgel formula was measured using two glass slides and a wooden block, which provided the basis for the slip and drag characteristics of the emulgel (30). About 1 g of each prepared emulgel was placed onto the ground fixed glass plate and covered with another glass plate. Weight (20 g) was then placed on the top of this slide. The time (s) required to separate the two plates was noted. The spreadability was calculated using the following equation:

$$S = \frac{M \times L}{T} \quad (3)$$

where S stands for spreadability, M, for weight tied to the upper slide; L is the length of glass slides; T, the time taken to separate the slides completely from each other.

Stability

The prepared emulgel formulae were tested for stability using centrifugation and temperature cycle tests (30). For the centrifugation test, 10 g of each prepared emulgel formula was subjected to centrifugation at 9000 rpm for 30 min. The stability of emulgel after cooling-heating and freeze-thaw was determined using a temperature cycle test. Briefly, 2 g of each prepared emulgel formula was subjected to 10 temperature cycles, each cycle was 24 h, starting at -4 °C (8 h) and 40 °C (16 h). Satisfactory physical stability was evaluated at the end of these tests.

pH

The pH of all the formulated emulgels was measured by a digital pH meter (31).

TOH content of emulgel

To determine the TOH content in the emulgel, 1 g of the formulated emulgel was diluted with 100 mL EtOH. The diluted solution was filtered through a 0.45 µm Millipore™ filter and scanned at 278 nm spectrophotometrically. Standard TOH solution in each concentration of a calibration curve was prepared by dilution of the TOH stock solution with an absolute EtOH. The amount of TOH in the emulgel was determined from the standard plot of TOH (31).

In vitro antifungal activity of TOH-containing emulgel

Agar well diffusion method was adopted for evaluating the antifungal activity of the TOH-containing emulgel as described previously (32). Briefly, *S. apiospermum* CBS 117410 conidia (1×10^5/mL) was poured onto SDA plates. Sterilize cork borer was used to make holes of 6 mm and formulated emulgels were aseptically applied. Commercial VOZ cream (100 µM) was used as a positive control. Inhibition zones were measured.

In vivo antifungal activity of TOH-containing emulgel

Female BALB/c mice (aged 8-week-old, body weight: 20-25 g) were purchased from Nomura Siam International Co, Ltd., Thailand, and further divided into 2 groups, 5 each; including uninfected mice and *S. apiospermum*-infected mice. The disseminated scedosporiosis mouse model was produced and validated as described previously (33). Briefly, all mice were infected intraperitoneally with a single dose of 200 mg/kg cyclophosphamide to induce a neutropenic condition. On the following day, the dorsal hairs of the mice were shaved and the skin was sterilized with 70% EtOH. Mice were subcutaneously inoculated with 1×10^7 *S. apiospermum* CBS 117410 conidia per mouse. Eumycetoma was characterized as subcutaneous nodules with ulcer formation.

The lesion was seen on day 5 post-inoculation and considered day 0 of treatment. Then, the TOH-containing emulgel and the commercial VOZ cream (100 μM) were topically applied once a day from day 1 to 21. Dissected skin of *S. apiospermum* induced eumycetoma in mice after treatment with TOH-containing emulgel and their controls were collected on day 14 and day 21 of treatments and fixed with 10% neutral buffer formalin for 48 h. The wounded tissues were dehydrated and embedded in paraffin wax. Sections of skin samples were further stained with hematoxylin and eosin (H&E) or Gomori methenamine silver (GMS) and examined under a light microscope. Changes in histologic disease activity of the tissues were semi-quantified by H-score (multiplication of the hyphae distribution of the positive area 0-100% as scored with 0 = absent, 1, 2, and 3 = mild, moderate, and severe, respectively) (33).

All animal experiments were conducted in accordance with the Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015), Thailand. Experiments on mice were approved by Institutional Animal Care and Use Committee (IACUC) of Khon Kaen University, Thailand (approval number: IACUC-KKU-44/63, reference number: 660201.2.11/271 (29)).

Statistical analysis

Experimental data were analyzed using GraphPad Prism, version 6.05, (GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicate. Data were expressed by mean ± SD. Significant differences at $P < 0.05$ were calculated using an independent *t*-test or analyses of variance (ANOVA) followed by Tukey's multiple comparison test.

RESULTS

Antifungal susceptibility

Table 2 showed the colonies diameters of *S. apiospermum* CBS 117410 after 3 days of incubation with TOH and standard antifungal drug VOZ by agar spot assay. SDA media containing either 1% EtOH (v/v) or 1% RPMI 1640 (v/v) were utilized as diluent controls for TOH or VOZ, respectively. The colony diameters of untreated *S. apiospermum* CBS 117410 were increased from day 3 to day 7 in a time-dependent manner similar to diluent control groups (Table 2). VOZ treatments at the concentration ranging from 10 μM to 1000 μM remarkably lessened the growth rate of *S. apiospermum* CBS 117410, especially at moderate (100 μM) and high concentrations (1000 μM). Thus, the minimal effective concentration of VOZ at 100 μM was used for further subsequent analyses.

An increase in *S. apiospermum* CBS 117410 colony diameter was observed from day 3 to day 5 in the similar manner to the untreated or diluent control groups (Table 2). Treatments of 1% EtOH did not reduce the growth rate of *S. apiospermum* CBS 117410 from day 3 to day 7 when compared to the untreated controls ($P > 0.05$). However, treatments of TOH at concentration ranging from 10 μM to 1000 μM remarkably lessened the growth rate of *S. apiospermum* CBS 117410 in a concentration-dependent manner ($P< 0.05$). Interestingly, there was no growth of *S. apiospermum* CBS 117410 at any time point in the 100 μM and 1000 μM of TOH treated groups ($P < 0.05$). Therefore, the minimal effective concentration of TOH at 100 μM was used for further subsequent analyses.

Table 2. Colony diameters of TOH-treated *Scedosporium apiospermum* CBS 117410 at 25 °C.

Culture condition	Colony diameter in mm (mean ± SD)		
	Day 3	Day 5	Day 7
Untreated control	11.3 ± 1.65	20.6 ± 1.98	29.6 ± 1.95
1% EtOH	9.9 ± 1.56	17.7 ± 1.09	29.1 ± 1.65
1% RPMI 1640	13.2 ± 1.01	20.4 ± 2.15	29.8 ± 3.00
TOH 10 μM	2.9 ± 0.44[a]	7.3 ± 1.85[a]	10.6 ± 1.97[a]
TOH 100 μM	ng[b]	ng[b]	ng[b]
TOH 1000 μM	ng[b]	ng[b]	ng[b]
VOZ 10 μM	5.1 ± 0.98[a]	8.9 ± 2.04[a]	13.6 ± 2.90[a]
VOZ 100 μM	ng[b]	ng[b]	ng[b]
VOZ 1000 μM	ng[b]	ng[b]	ng[b]

TOH, Tryptophol; VOZ, voriconazole; ng, no growth; [a–b]$P < 0.05$ Indicates significant differences between experimental conditions by two-way ANOVA

Conidial germination

Germination of fresh *S. apiospermum* CBS 117410 conidia was lessened after treating with TOH or VOZ at 24 h of incubation in a concentration-dependent manner (Table 3). EtOH at 1% (v/v) did no effect on germ tube formation. TOH or VOZ at 10 μM didn't affect germ tube formation. Whereas the greatest effects on germ tube inhibition were observed in the highest concentrations (1000 μM) of TOH and VOZ ($P < 0.05$). However, treatments with TOH at moderate to high concentrations of 100 to 1000 μM significantly reduced the germ tube length of *S. apiospermum* CBS 117410 than VOZ. Thus, TOH at 100 μM was the minimal effective concentration for controlling *S. apiospermum* germination *in vitro*.

Biofilm formation

Treatments with TOH remarkably reduced the fungal total biofilm biomass and metabolic activity rates of *S. apiospermum* CBS 117410 when compared to the VOZ-treated groups in a concentration-dependent manner as determined using crystal violet and XTT reduction assays, respectively (Table 4). EtOH at 1% (v/v) had no effect. Moreover, treatments of TOH at 100 to 1000 μM significantly reduced the amount of biomass produced and metabolic activity rates than VOZ treatments ($P < 0.05$). Thus, TOH at 100 μM was the minimal effective concentration for controlling *S. apiospermum* biofilm formation *in vitro*.

In vitro human foreskin fibroblast cell cytotoxicity

After 24 h incubation, the HFF-1 cell viability did not show a significant change after treating with 10 and 100 μM of TOH as determined by MTT assay (Fig. 1A). However, HFF-1 cell viability (85 ± 3.30%) was significantly decreased at 1000 μM TOH concentration when compared to controls. Treatment with VOZ at 100 and 1000 μM significantly decreased HFF-1 cell viability compared to untreated controls (Fig. 1B). In addition, HFF-1 cell viability was decreased to lower than 80% when treated with VOZ at 1000 μM. The results from the LDH assay were correlated to the MTT data after 24 h generation times. The percentages of cell cytotoxicity in TOH and VOZ-treated groups were increased in a concentration-dependent manner when compared to controls in all treatments (Fig. 1C and D). However, TOH treatments significantly exhibited less cytotoxic effect on HFF-1 cells than VOZ treatments. Collectively, the results suggested that TOH at 100 μM was the minimal effective concentration for controlling *S. apiospermum* CBS 117410 infections without compromising risks to human skin *in vitro* and thus were selected for further emulgel development.

Formulation of TOH-containing emulgel

An emulgel that has 100 μM of TOH as an active ingredient was successfully developed. The parameters of TOH-containing emulgel and control emulgel were presented in Table 5.

Table 3. Germ tube length of TOH-treated *Scedosporium apiospermum* CBS 117410 after 24 h incubation at 37 °C.

Culture condition	Germ tube length in μm (mean ± SD)		
	10 μM	100 μM	1000 μM
1% EtOH	30.98 ± 1.04		
1% RPMI 1640	35.39 ± 3.67		
TOH	29.78 ± 2.84	11.96 ± 1.33[b]	2.09 ± 0.03[b]
VOZ	29.96 ± 2.95	19.64 ± 3.03[a]	10.03 ± 1.77[a]

TOH, Tryptophol; VOZ, voriconazole; [a–b]$P < 0.05$ Indicates significant differences between experimental conditions by two-way ANOVA

Table 4. Biofilm formation of TOH-treated *Scedosporium apiospermum* CBS 117410 after 24 h incubation at 37 °C.

Culture condition	Crystal violet staining (mean ± SD) at $OD_{590\ nm}$			XTT assay (mean ± SD) at $OD_{490\ nm}$		
	10 μM	100 μM	1000 μM	10 μM	100 μM	1000 μM
1% EtOH	7.55 ± 0.26			0.79 ± 0.08		
1% RPMI 1640	8.33 ± 0.44			0.93 ± 0.07		
TOH	3.16 ± 0.12[b]	1.43 ± 0.01[b]	0.74 ± 0.09[b]	0.49 ± 0.01[a]	0.11 ± 0.02[b]	< 0.01 ± 0.002[b]
VOZ	3.46 ± 0.05[a]	1.84 ± 0.17[a]	1.13 ± 0.11[a]	0.52 ± 0.03[a]	0.29 ± 0.02[a]	0.15 ± 0.01[a]

TOH, Tryptophol; VOZ, voriconazole; [a–b]$P < 0.05$ Indicates significant differences between experimental conditions by two-way ANOVA.

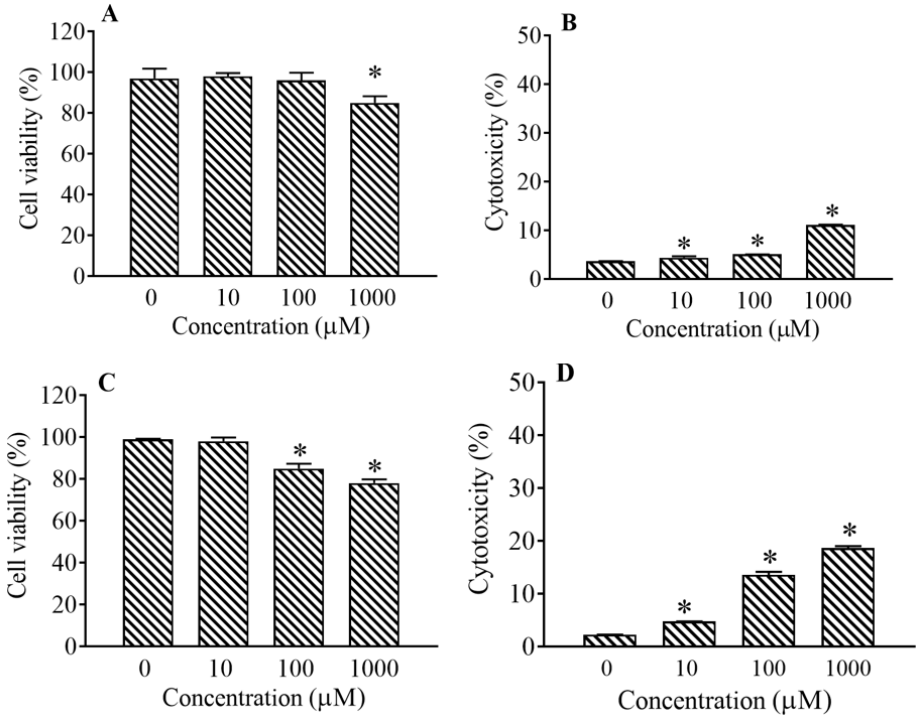

Fig. 1. Cell viability and cytotoxicity of (A and B) TOH- and (C and D) VOZ-treated human foreskin fibroblasts (HFF-1) cells at 37 °C as determined by MTT and LDH assays, respectively. Percentages of cell viability and cytotoxicity were shown as mean ± SD. $^{*}P < 0.05$ indicates significant differences compared with the control group (concentration 0). TOH, Tryptophol; VOZ, voriconazole.

Table 5. Physical properties of the TOH-containing emulgel.

Characteristics	Emulgel base	TOH-containing emulgel
Color	White and translucent	White and translucent
Homogeneity	Excellent	Excellent
Consistency	Excellent	Excellent
Phase separation	None	None
Viscosity (cp)	2,350	2,401
Spread ability (gm.cm/sec)	25.00 ± 0.10	26.00 ± 0.50
Centrifugation test	Excellent	Excellent
Cooling-heating and freeze-thaw cycle test	Excellent	Excellent
pH	5.40 ± 0.10	5.53 ± 0.01
% TOH content (OD_{278nm})	None	99.98 %

TOH, Tryptophol.

Upon investigation, the prepared TOH-containing emulgel appeared as white, translucent, homogenous of semisolid consistency, and non-sticky skin feel. Moreover, there was no phase separation reported both in TOH-containing and control emulgels (Fig. 2A). The viscosity of the TOH-containing emulgel was 2401 cps, whereas the control emulgel was 2350 cps. The spreadability of the TOH-containing emulgel was 26.00 ± 0.50 g.cm/s., whereas the control emulgel was 25.00 ± 0.10 g.cm/s. The effects of accelerated stabilities including centrifugation and temperature cycle test were determined. The results showed no color change or signs of instability involving phase separation or inconsistency increases were observed (Fig. 2B and C and Table 5). The pH of TOH-containing and control emulgels were 5.53 ± 0.01 and 5.40 ± 0.10, respectively. Moreover, the TOH content of the formulated TOH-containing emulgel was 99.98 %.

In vitro antifungal activity of TOH-containing emulgel

The antifungal activity of TOH-containing emulgel against *S. apiospermum* CBS 117410 using the agar diffusion method was

investigated. From the results, the control emulgel base did not show any antifungal activity (no zone of inhibition) against *S. apiospermum* CBS 117410. However, TOH-containing emulgel exhibited antifungal activity against *S. apiospermum* CBS 117410 with a mean zone of inhibition (23.80 ± 1.09 mm), which was similar to the mean zone of inhibition exhibited by the commercial antifungal drug VOZ (19.50 ± 3.16 mm, Table 6). Moreover, the antifungal activity of TOH-containing emulgel was similar to TOH solution (100 µM) suggesting that the emulgel base formation does not negatively interfere with the antifungal medication of TOH.

In vivo antifungal activity of TOH-containing emulgel

After day one post-inoculation of *S. apiospermum* CBS 117410 into the subcutaneous layer of the skin, experimental mice showed the erythematous patch in the inoculated areas. Subcutaneous mass with few sinuses and few white grains were observed in the infected mice on day 3 post-inoculation. On day 5 post-inoculation, the eumycetoma was developed as hard consistency subcutaneous mass with multiple sinuses, white grains, and central necrosis (Fig. 3A), which indicated as day 0 for applying topical tested emulgels. After 7 days and 14 days of experimental treatments, the photographs of eumycetoma at the back of mice were taken at a distance of 10 cm from the lesion site using a digital camera as shown in Fig. 3B. There was an insignificant difference in the mass size between experimental groups at the first day of the treatments (Fig. 3C). Treatments with TOH-containing emulgel and commercial VOZ cream significantly reduced the mass size of *S. apiospermum* eumycetoma than the emulgel base treated groups after 7 days of treatments. Interestingly, treatment of TOH-containing emulgel significantly reduced the mass size than commercial VOZ cream after 14 days and 21 days of treatments (Fig. 3B and C).

Histopathological evaluation

Mice that inoculated with *S. apiospermum* CBS 117410 into the subcutaneous layer of the skin and received topical emulgel base treatment for 14 days exhibited histological pathology of eumycetoma. The H&E stained wound sections revealed a large mass area of fungal infection surrounded by mononuclear infiltrates (Fig. 4A). Moreover, GMS stained wound sections revealed a high density of fungal hyphae within the lesion area. *S. apiospermum* CBS 117410-infected mice that received topical TOH-containing emulgel treatment for 14 days exhibited significantly lower damage of fungal infection than commercial VOZ cream and emulgel-base treatments (Fig. 4A and C).

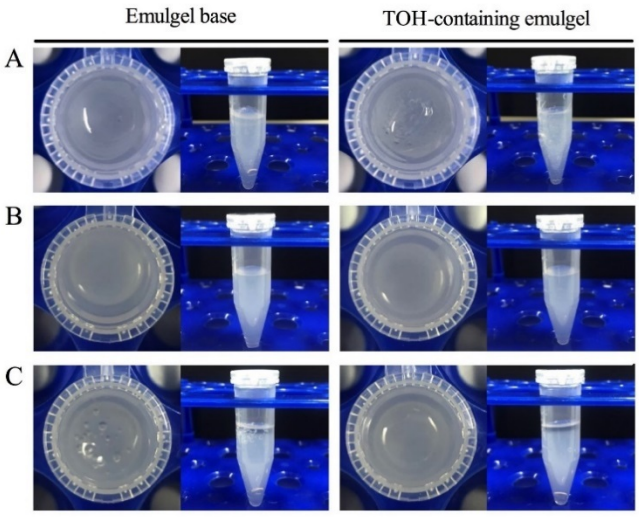

Fig. 2. Visual appearances of emulgel base and TOH-containing emulgel after (A) formulation, (B) centrifugation test, and (C) cooling-heating and freeze-thaw cycle test. TOH, Tryptophol.

Table 6. Diameter of the inhibition zone of TOH-containing emulgel-treated *Scedosporium apiospermum* CBS 117410 at 72 h post-incubation, 25 °C.

Culture condition	**Diameter of the inhibition zone in mm (mean ± SD)**
Emulgel base	ND
TOH-containing emulgel (100 µM)	23.80 ± 1.09
TOH solution (100 µM)	21.10 ± 2.64
Commercial VOZ cream (100 µM)	19.50 ± 3.16

TOH, Tryptophol; VOZ, voriconazole; ND, not detectable.

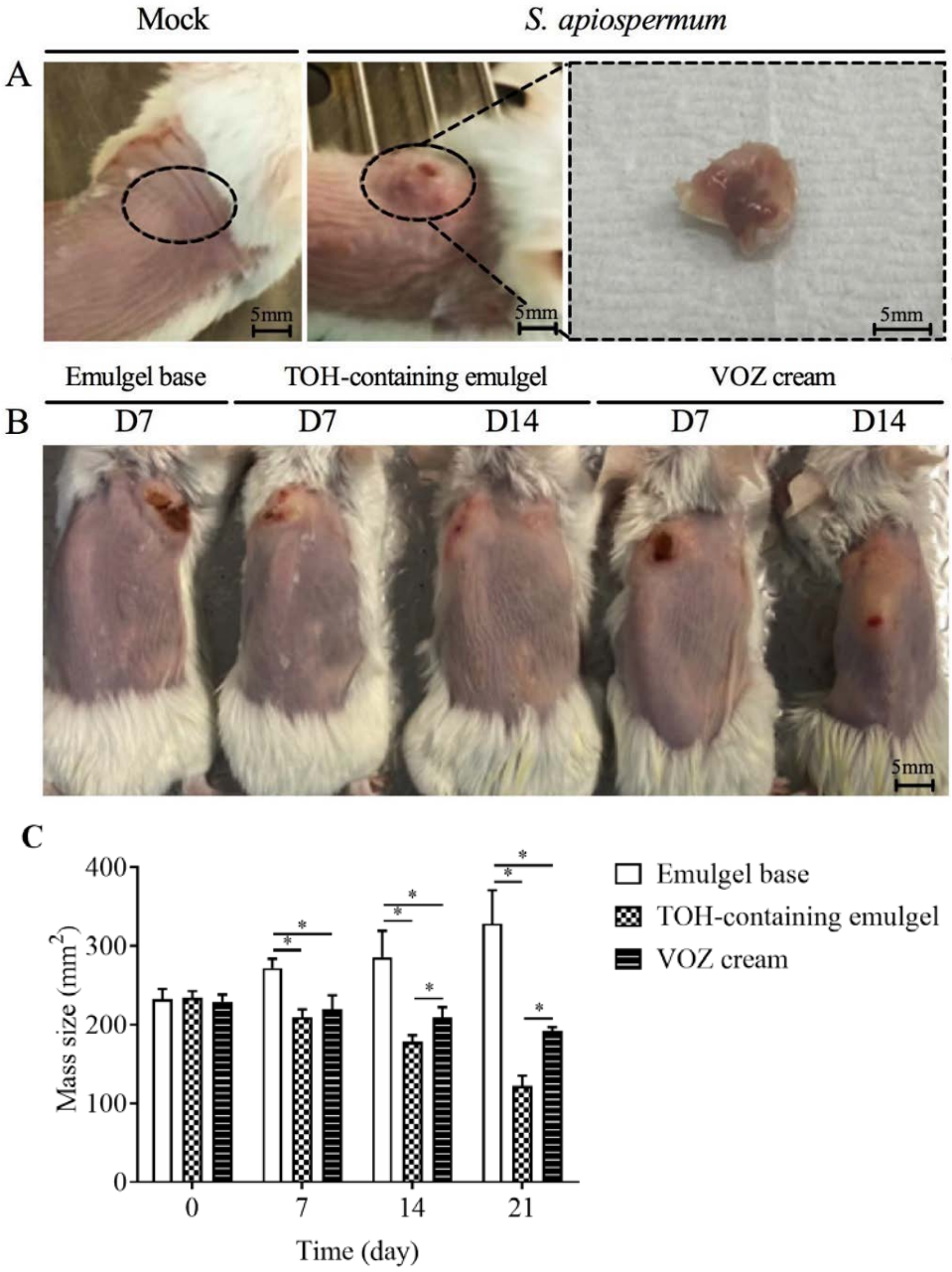

Fig. 3. *In vivo* antifungal activity of TOH-containing emulgel. (A) Formation of eumycetoma from *Scedosporium apiospermum* CBS 117410 after 5 days inoculation; (B) macroscopic eumycetoma in mice that received topical emulgel base, TOH-containing emulgel, or commercial VOZ cream. The representative photographs indicated the time course of eumycetoma after applying the treatments in mice on days 0, 7, and 14; (C) graphical presentation of eumycetoma area in mice that received topical emulgel base, TOH-containing emulgel, or commercial VOZ cream. Values are represented as the mean ± SD of five animals per group. **P* < 0.05 Indicates statistically significant differences between experimental conditions by two-way ANOVA. TOH, Tryptophol; VOZ, voriconazole.

Reduction in eumycetoma pathology with a lessened amount of fungal hyphae was significantly observed on day 21 of topical TOH-containing emulgel treatment when compared to commercial VOZ cream and emulgel-base treatments (Fig. 4B). The lesion area of TOH-containing emulgel-treated group showed skin scab and skin reepithelization but not in other experimental conditions (Fig. 4B). Moreover, tissue inflammation and density of fungal hyphae within the lesion area was significantly decreased in topical TOH-containing emulgel treated group than other groups (Fig. 4B and C).

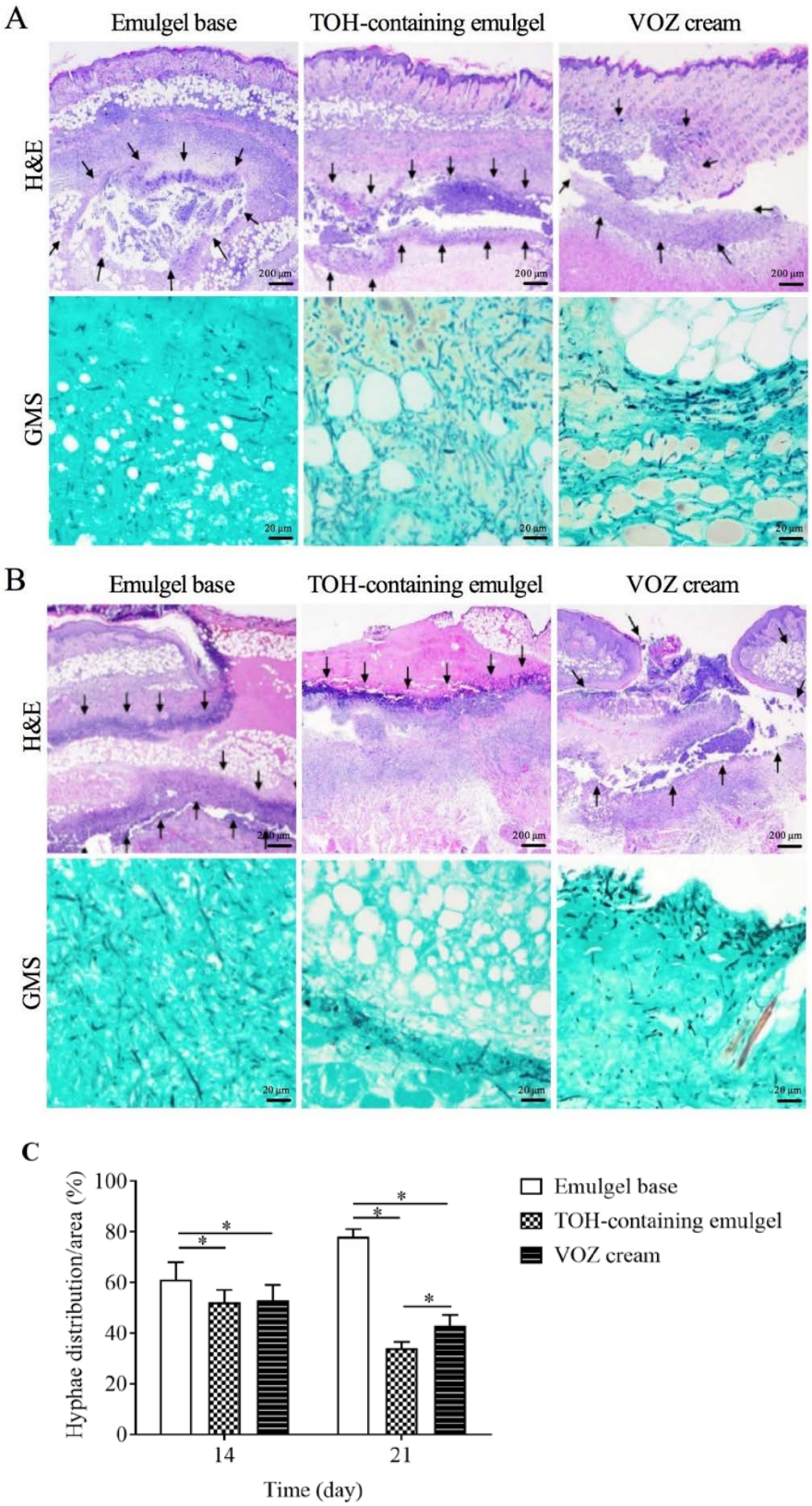

Fig. 4. Histopathological micrographs of *Scedosporium apiospermum* CBS 117410 eumycetoma lesions in mice after topical application with TOH-containing emulgel. After topical treatments of emulgel base, TOH-containing emulgel, or commercial VOZ cream for (A) 14 days or (B) 21 days on the eumycetoma lesions in mice, skin lesions were stained with H&E and GMS. Arrows indicated the eumycetoma area. Scale bar = 200 μm (for H&E) and 20 μm (for GMS); (C) percentages of hyphae distribution per area were determined after 14 days or 21 days of treatments. Values represent the mean ± SD of five animals per group. **P* < 0.05 Indicates statistically significant differences in each experimental condition by two-way ANOVA. TOH, Tryptophol; VOZ, voriconazole; H&E, hematoxylin, and eosin; GMS; Gomori methenamine silver.

DISCUSSION

Scedosporium species especially *S. apiospermum* have been found in clinical settings as well as in human-impacted areas including urban parks, agricultural soils, and polluted water (34). Opportunistic infections of *S. apiospermum* are increasingly reported worldwide both in immunocompromised and immunocompetent individuals (3). Disseminated disease with involvement of the lungs, brain, and skin infections is commonly reported and related to trauma or near-drowning events (35). Moreover, traumatic localized infections in both immunocompromised and immunocompetent individuals are mostly manifested as eumycetoma, a chronic progressive subcutaneous infection that penetrates muscles and bones (36). Successful treatments of *Scedosporium* spp. are largely dependent on accurate and prompt diagnosis and effective medications (37). However, current surgical intervention or commercially available antifungal treatments may not be adequate enough to treat *Scedosporium* infections due to their intrinsic antifungal resistance and poor penetration of antifungal agents from debridement (38). Thus, this study was designed to develop and evaluate the effectiveness of alternative topical applications to treat *S. apiospermum* eumycetoma using a TOH-containing emulgel.

In the present study, we found that treatment of TOH lessened the growth of *S. apiospermum* CBS 117410 in a concentration-dependent manner. In consistent with our previous reports, we found that a moderate concentration (100 μM) of TOH remarkably lessened the growth of *S. apiospermum* (7). Although the drug of choice for *S. apiospermum* infections is VOZ (39), we found that TOH appeared to be as effective as VOZ. In the current study, an inhibitory effect of TOH on *S. apiospermum* CBS 117410 germination was investigated as a reduction of germination rate might be important for controlling its biofilm formation. As expected, TOH at 100 μM was able to decrease the germ tube length and effectively controlled *S. apiospermum* biofilm formation more than VOZ. Therefore, our study showed that treatment of TOH is able could be utilized as an alternative therapeutic potential for controlling the growth of *S. apiospermum* CBS 117410. Moreover, TOH treatments exhibited lesser cytotoxic effects on the human foreskin fibroblast (HFF-1) cells than VOZ treatments. The TOH belongs to a class of 3-substituted indole compounds featuring a 2-hydroxyalkyl side chain (14). The previous study has suggested that TOH can disrupt fungal morphogenesis and induce fungal cell apoptosis, which can be used as an antifungal agent against different fungal species (17). Therefore, our present study demonstrated that TOH can be utilized as a promising antifungal agent against *S. apiospermum* CBS 117410 infections without compromising risks to human skin and was selected for further emulgel development. Further study is required to investigate the molecular mechanisms of TOH on antifungal activity against cutaneous pathogenic fungi.

TOH at 100 μM was successfully incorporated into emulgel base containing SEPIGEL 305 as a gelling agent. SEPIGEL 305 is composed of polyacrylamide, polyoxyethylene 7 lauryl ether, and isoparaffin, which acted as a gelling agent, a non-ionic emulsifier, and fatty oil, respectively (40). Thus, the addition of SEPIGEL 305 increases viscosity, a degree of cooling power, and optimal dermo-cosmetic qualities. Triethanolamine was added to neutralize the formulae to the pH range of 5.5-6.5 by uncoiling the polymer chains and forming the gel structure (30). This pH range caused the TOH-containing emulgel to be compromised with the pH of the skin (pH 5) (30). Propylene glycol acts as a humectant to the emulgel, which helps to increase the spreadability as well as the aesthetic feeling of the product (41). Our study showed that TOH-containing emulgel exhibited an excellent level in terms of homogeneity, consistency, stability, and a high percentage of TOH content. We further demonstrated that TOH-containing emulgel exhibited antifungal activity against *S. apiospermum* CBS 117410 *in vitro*. Thus, our study suggested that the developed TOH-containing emulgel formulation was optimal for use as topical drug delivery *in vivo*.

Infections of *Scedosporium* spp. into the subcutaneous layer of the skin cause a chronic progressive granulomatous infection known as eumycetoma (6). In the present study, we successfully developed a eumycetoma mice model from *S. apiospermum* CBS 117410 subcutaneous infections. The pathological findings in the established mouse model in this study were similar to evidence in infected patients as characterized by the formation of grains containing aggregates of fungal hyphae (42). VOZ is a new triazole antifungal agent that exhibits a broad spectrum of antifungal activity against *Aspergillus* spp., *Cryptococcus* spp., and *Candida* spp., as well as fluconazole-resistant groups (43). VOZ has been reported to be effective in most patients, including children against *Scedosporium* spp. (44). Although there were reports of successful treatment with VOZ for *S. apiospermum* eumycetoma, however, uptake of VOZ caused several side effects (36,37,39). The most common side effect of VOZ has altered color discrimination, blurred vision, and photophobia have been noted (45). Several adverse effects including hepatitis, photohypersensitivity, and pseudoporphyria have also been reported (39,46). Therefore, new antifungal agents are needed for the treatment of *S. apiospermum* infections.

Treatment of TOH-containing emulgel on eumycetoma mice model from *S. apiospermum* CBS 117410 infections showed positive antifungal activity in the control of eumycetoma mass and improved mouse skin lesions than commercial VOZ cream. Thus, our study suggested that TOH may help to promote tissue remodeling and significant inhibition of *S. apiospermum* CBS 117410 infection. Further studies are required to reveal the molecular mechanisms of immunological reaction during TOH-containing emulgel treatment. We previously reported that treatments of TOH reduced the *C. albicans* pathogenicity by increased cellular apoptosis (19). In this report, we demonstrated that treatments of TOH remarkably elevate the apoptosis pathway in *C. albicans* by transcriptional upregulation of caspase recruitment domain-containing protein *(CARD)-9* and *Noxa* and downregulation of *Bcl-2*. In general, the activation of apoptosis is positively regulated by the activation of CARD-9 and Bcl-10 through the nuclear factor-kappa B stress pathway (47). Moreover, oxidative stress-induced apoptosis is coordinately regulated by anti-apoptotic Bcl-2 protein and Noxa (a pro-apoptotic protein of Bcl-2) (48). Taken together, it is possible that TOH-containing emulgel can promote cellular apoptosis and lessen the virulence of *S. apiospermum* eumycetoma similar to the pathogenic *C. albicans*. Therefore, the collective results from the present study purpose that TOH-containing emulgel can be used for treating *S. apiospermum* eumycetoma, which provides an alternative way to utilize fungal quorum sensing molecules with potential applications for combating fungal infections. We plan to measure user satisfaction with TOH-containing emulgel for future registration as a topical antifungal drug. Nevertheless, we plan to deepen our study into the molecular mechanisms of TOH-containing emulgel-mediated pathogenicity attenuation of *S. apiospermum* and other cutaneous pathogenic fungi in the future.

CONCLUSION

A promising *in vitro* anti-*S. apiospermum* assay proved TOH to be highly effective. Treatment of TOH at 100 µM exhibited anti-*S. apiospermum* activities by reducing growth rate, germination rate, biofilm formation, and less cytotoxicity to HFF-1 cells than VOZ. TOH-containing emulgel was developed. Formulation of TOH-containing emulgel revealed an excellent physical property including homogeneity, consistency, and stability. Treatment of TOH-containing emulgel significantly ameliorated subcutaneous mass in *S. apiospermum* eumycetoma mice and improved skin histological architecture after 14 days of treatment. Therefore, our study demonstrated an innovative approach for efficient treatment against *S. apiospermum* eumycetoma using TOH-containing emulgel topical application. Further experimental and clinical trials are required to confirm the findings of the present study.

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Conflicts of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

P. Sukphopetch designed and supervised the project; T. Kitisin, W. Muangkaew, S. Ampawong, and N. Thitipramote performed the experiments, analyzed, and interpreted the data; N. Sansurin provisioned the experimented animals. T. Kitisin contributed to the writing of the manuscript and revision; S. Ampawong and N. Thitipramote were involved in planning and supervising the work. The final version of the manuscript was approved by all authors.

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