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Original Article

Combined Effect of Heat, Essential Oils and Salt on the Fungicidal Activity against *Trichophyton mentagrophytes* in Foot Bath

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Abstract

This work was originally undertaken to determine the effective conditions of essential oils against *Trichophyton mentagrophytes* in vitro for the treatment of tinea pedis in a foot bath. Agar blocks implanted with *T. mentagrophytes* were immersed in 0.1% aqueous agar containing two-fold dilutions of essential oils with or without sodium chloride at 27°C, 37°C and 42°C for 10 and 20 min. The number of surviving mycelia on the agar blocks was determined from the standard curves of the colony diameter and original inocula of the conidia. At the same time, the thermal effect on the cellular morphology was examined using SEM. Most fungal mycelia (99.7%) were killed after treatment at 42°C for 20 min without essential oil. The fungicidal activity of essential oils was markedly enhanced by treating at 42°C for 20 min as compared with that at 27°C, showing 1/4〜1/32-fold reduction of minimum fungicidal concentration (MFC to kill 99.99%). The order of the fungicidal activity of 11 essential oils was oregano, thyme thymol, cinnamon bark＞lemongrass＞clove, palmarose, peppermint, lavender＞geranium Bourbon, tea tree＞ thyme geraniol oils. MFCs were further reduced to 1/2〜1/8 by the addition of 10% sodium chloride. The salt effect was explained, at least partly, by an increase in mycelial adsorption of antifungal constituents in the presence of sodium chloride. Considerable hyphal damage was done at 27°C by the essential oils, but no further alteration in morphology of the hyphae treated at 42°C with or without oil was observed by SEM. The inhibitory effect of heat and oils was also observed against mycelia of *T. rubrum* and conidia of *T. mentagrophytes*. Thermotherapy combined with essential oils and salt would be promising to treat tinea pedis in a foot bath.

Key words: foot bath, essential oil, *Trichophyton mentagrophytes*, heat stress, salt stress

Introduction

Tinea pedis is one of the most prevalent superficial infections, and is usually treated by topical or oral administration of an antifungal agent. However, there are frequent relapses. Essential oils such as tea tree and eucalyptus oils have been used alone or in combination with antifungal agents for treatment of interdigital tinea pedis and onychomycosis[1-4].

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Foot baths are frequently used in aromatherapy, spa therapy and balneotherapy for treating various skin disorders[5, 6], for relaxation to improve sleeping[7], and to control infection in cattle[8, 9]. A foot bath containing essential oils has been recommended for the treatment of tinea pedis[10, 11], although it is not accepted as a well-established treatment modality in dermatology. Bathing of the foot is done by immersing the infected foot in warm and moving water containing essential oil, but the mechanical, thermal and biochemical effects involved have not been elucidated.

The purpose of this work was to determine experimentally the effective conditions of eleven essential oils against *Trichophyton mentagrophytes* that is one of the major dermatophytes causing tinea pedis. Treatment conditions, especially the thermal and salt effects of a foot bath using a variety of essential oils were the main target in this study.

Materials and Methods

Fungal strain

T. mentagrophytes TIMM 2789 is a stock culture of Teikyo University Institute of Medical Mycology. The strain is highly pathogenic to guinea pigs and human beings, and its microconidia have been well reproduced by repeated experiments. *T. rubrum* TIMM 5497 was a clinical strain freshly isolated.

Essential oils and chemicals

Essential oils used were obtained from Sanoflore Laboratoire, France via Hyperplants Co., Tokyo, unless otherwise stated. Oregano, thyme geraniol, thyme thymol and cinnamon bark oils were supplied by Pranarom International S.A. Belgium via Kenso Igakusha, Ltd., Yamanashi. The major components were determined by gas chromatography/mass spectrometry and are listed in Table 1. The analytical conditions were reported earlier[12]. Clotrimazole was from Sigma, USA.

Counting of *Trichophyton* cells

Number of the filamentous cells was calculated from the colony size according to Majima *et al.*[13] with slight modification. A part (100 μl) of the microconidia suspension (1×10^8/ml) of *T. mentagrophytes* or *T. rubrum* was spread uniformly on a sterile petri plate (8.5 cm in diameter) containing 15 ml of melted Difco™ Sabouraud dextrose agar and 1.5% Bacto™ agar. The dishes were incubated at 27°C for 2 days for *T. mentagrophytes* and 3 days for *T. rubrum*, during which time most conidia had germinated to give short hyphae. Agar plates on which mycelia had grown uniformly were cut out using a cork borer to obtain agar blocks (7 mm in diameter, 4 mm in depth) with hyphae on the surface.

After treatment in a water bath containing essential oils and salt at an optional temperature, three agar blocks were placed on 1.5% Sabouraud dextrose agar with the mycelial side on top in a No.1 square plate ($230 \times 80 \times 15.5$ mm, Eiken Kizai, Tokyo). The agar plates were incubated at 27°C for 5 days for *T. mentagrophytes*, and the diameter of colony formed was measured using slide calipers; the average colony size of three was obtained. The number of surviving cells was calculated from the standard curve obtained from the correlation between diameter of the colony after incubation and the number of conidia implanted originally on the agar blocks at 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2/dish. A linear relationship was observed between number of conidia and the colony diameter in the range of $10^4 \sim 10^6$/dish. Since the growth of *T. rubrum* was slow, the number of surviving cells was determined after incubation for 7, 8, and 9 days. The average value is listed in Tables 2 and 4. The minimum detection concentration was 10^3 inoculum/dish.

Determination of the thermal stability of *T. mentagrophytes* hyphae and fungicidal activity of essential oils in a water bath

A DMSO solution of essential oils at 640, 160, 40 mg/ml was prepared, and parts (25 or 12.5 μl) were diluted with 0.1% aqueous agar to obtain test samples with oil concentrations of 0.32, 0.16, 0.08, 0.04, 0.02, 0.01 and 0.005%. For more concentrated oil suspensions, a net oil

Table 1. Major constituents and composition of 12 essential oils used

Essential oil	Major constituents (composition)
Cinnamon bark	cinnamaldehyde (54.1%)
Clove	eugenol (86.5%)
Geranium "Bourbon"	citronellol (24.4%), citronellyl formate (10.1%)
Lavender	linalyl acetate (36.6%), linalool (30.1%)
Lemongrass	geranial (36.6%), neral (26.5%)
Oregano	carvacrol (26.0%), thymol (22.5%)
Palmarosa	geraniol (83.5%)
Patchouli	patchoulol (42.3%), δ-guaiene (14.2%), α-guaiene (10.4%)
Peppermint	menthol (44.9%), p-menthone (13.0%)
Tea tree	terpinen-4-ol (42.5%), γ-terpinene (23.0%), α-terpinene (10.0%)
Thyme geraniol	geranyl acetate (51.7%), geraniol (29.8%)
Thyme thymol	p-cymene (41.0%), thymol (42.0%)

(256, 128, 64 or 32 mg) was suspended vigorously in 0.1% aqueous agar to obtain oil suspensions with concentrations of 5.12, 2.56, 1.25 and 0.64%. A DMSO solution of clotrimazole (1 mg/m*l*) was diluted with 0.1% aqueous agar to give suspensions of 0.008, 0.004, 0.002, 0.001, and 0.0005%. The maximum concentration of DMSO was 0.5%.

Agar blocks implanted with 10^7 conidia/dish of *T. mentagrophytes* or *T. rubrum* were immersed in 5 m*l* of the sterile 0.1% aqueous agar, the 10% sodium chloride-0.1% aqueous agar or a test solution containing essential oil of different concentrations in a glass tube. The temperature of aqueous media was maintained at 27°C, 37°C, 42°C and 47°C in a water bath (Thermominder Mini-80, Taiyo Kagaku Kogyo, Ltd., Tokyo) before immersion of the agar blocks. Heating of the blocks was continued for 10, 20, and some for 15, 30 and 60 min with gentle shaking. After treatment, the agar blocks were washed with sterile saline (10 m*l*) at room temperature, and excess saline was removed by placing them on a filter paper. The number of surviving cells on the agar blocks was determined by the standard curve. The minimum fungicidal concentration (MFC) was defined as the minimum oil concentration that reduced cells to 10^3/dish or less from the original 10^7/dish; that is, 99.99% or more killing of the original inoculum. The experiment was repeated thrice, and the reproducibility of the MFC was confirmed.

Determination of the thermal stability of conidia of *T. mentagrophytes* in the presence or absence of the oils

An aqueous suspension (5 m*l*) of the conidia (10^4/m*l*) in 0.1% agar containing 0.05% DMSO in a glass tube was heated at 27°C for 20 min, and at 42°C for 20 min and 40 min. Similarly, an aqueous conidia suspension (5 m*l*) in 0.02% and 0.2% oregano oil or 0.02% and 0.2% patchouli oil containing 0.05% DMSO was heated. A part (200 μl) of the conidia suspension or its 10-fold and 100-fold diluents was spread uniformly in a petri dish (8.5 cm in diameter) containing 15 m*l* of Sabouraud dextrose agar. The dishes were incubated at 27°C for 5 and 7 days, and the number of colonies was counted visually.

Preparation of dried mycelia of *T. mentagrophytes* TIMM 2789

Aqueous suspension (100 μl) of microconidia (7.2×10^8 conidia/m*l*) was added to 200 m*l* of Sabouraud dextrose broth consisting of 1% Bacto peptone and 2% glucose. The fungal broth was cultured at 30°C for 1 week under shaking at 150 rpm. The mycelia grown were collected by centrifugation, washed three times with distilled water, and suspended in 200 m*l* of distilled water to sonicate with a gel sonicator (Tosho Denki GS-02, Cosmobio Ltd., Tokyo) for 5×3 min for mycelial dispersion. The mycelia were again washed with distilled water, and lyophilized at room temperature for 3 days using a freeze-drying apparatus (Model 4K, Edwards, UK). The yield from 3 flasks was 1.89 g as a dried power.

Determination of adsorption of oil constituents on mycelia of *T. mentagrophytes*

A part of the dried mycelia (50 mg) was suspended at 42°C in 0.1% aqueous agar or 10% sodium chloride-0.1% aqueous agar (5 m*l*) containing MFC of three essential oils, that is, 0.16% tea tree, 0.08% lavender and 0.02% thyme thymol. After shaking at 42°C for 20 min, the mycelia treated were collected by centrifugation at 3,500 rpm for 10 min. Extra water remaining in the precipitated mycelia was removed by filtration through Whatman No. 1 glass filter membrane, followed by washing with distilled water twice (each time using 0.5 m*l*). Removal of extra water was vitally important, since the constituents absorbed on the mycelia could not be extracted with ethyl acetate in the presence of extra water. The mycelia were extracted with ethyl acetate (5 m*l*), and the extract was dried over anhydrous sodium sulfate, and subjected to GC analysis according to the procedure already reported[12]. The measurement was conducted three times, and the adsorption was expressed by μg of oil components per g of the mycelia.

Scanning electron microscopy (SEM)

Agar blocks treated at 27°C or 42°C for 20 min with or without oil as well as untreated control were fixed for 2～16 h in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C. After being washed with the buffer, specimens were post-fixed for 1～16 h with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C. Samples were dehydrated in graded acetone and dried by a CO_2 critical-point dryer (Hitachi Critical Point Dryer HCP-2). The dried samples were finally coated with osmium in an osmium plasma coater (Nippon Laser & Electronics Lab.), and examined using a JEOL JSM-6700 F scanning electron microscope operated at 10 kV.

Results

Effect of temperature and treatment time on the viability of *T. mentagrophytes* in 0.1% aqueous agar with or without 10% sodium chloride

Table 2 shows surviving cells of *T. mentagrophytes* starting from 10^7/dish of conidia upon treatment at 27°C, 37°C, 42°C and 47°C. The viable cells remained almost intact (45～84%) in 0.1% aqueous agar at 27°C for 10 and 20 min, and at 37°C for 10 and 20 min, and slightly decreased at 42°C for 10 min (19%). However, when the treatment time at 42°C was extended to 20 min, a marked decrease in number of viable cells occurred, showing a surviving percentage of 0.29%. Further extension of treatment time to 30 and 60 min caused the killing of more mycelia. Treatment at 42°C for 15 min showed a moderate reduction of the viable cells, and with treatment at 47°C for 15 and 20 min almost all of the mycelia were killed.

When the agar blocks were immersed in 0.1% aqueous agar containing 10% sodium chloride, a slight decrease in number of viable cells was observed at 27°C and 37°C (5.3～7.2%), compared with that in 0.1% aqueous agar as shown in Table 2. Further treatment of the agar blocks in the presence of 10% sodium chloride did not cause significant killing at 42°C for 10 min, but marked killing occurred at 42°C for 20 min (surviving percentage of 0.35%), similar to that in 0.1% aqueous agar. Extension of the treatment time to 30 min caused greater killing of cells.

Anti-*Trichophyton* activity of tea tree oil and thyme thymol oil at various temperatures and treatment times in 0.1% aqueous agar with or without 10% sodium chloride

Table 3 shows the MFC values of tea tree and thyme thymol oils under various treatment conditions. In the case of tea tree oil, the MFC value at 27°C (5.12%) was slightly decreased to 2.56% at 37°C for 10 and 20 min, and at 42°C for 10 min. However, a marked decrease occurred at 42°C for 20 min, showing the MFC of 0.16% (1/30 that at 27°C). Extension of the treatment time at 42°C for 30 min caused greater reduction of MFC (0.08%).

The MFC value of tea tree oil was further reduced when 10% sodium chloride was added. It was 2.56% at 27°C for 10 min, which was gradually reduced to 1.28% and 0.64% when the treatment period and temperature were increased. Again, a marked decrease of the MFC (0.04%) occurred at 42°C for 20 min. Addition of sodium chloride reduced the MFC to 1/4 that in 0.1% aqueous agar, but no further reduction was observed when the treatment time was extended to 30 min.

Time course of the MFC of thyme thymol oil was similar to that of tea tree oil. The MFC

Table 2. Effect of temperature on the viable cells of *T. mentagrophytes* in 0.1% aqueous agar with or without 10% sodium chloride

	Surviving mycelia (%)[a)]						
	27°C		37°C		42°C		
Medium	10 min	20 min	10 min	20 min	10 min	15 min	20 min
0.1% agar	78±23	84±11	64±11	45±10	19±10	1.7±1.6	0.69±0.16
10% NaCl-0.1% agar	7.2±2.1	5.3±1.9	7.2±3.6	5.4±2.0	5.1±2.1		0.75±0.10

	42°C		47°C		
Medium	30 min	60 min	10 min	15 min	20 min
0.1% agar	0.44±0.09	0.18±0.08	0.05±0.03	<0.001	<0.001
10% NaCl-0.1% agar	0.39±0.06				

a) Surviving mycelia (%) = viable cells/initial inoculum ×100 ± standard deviation determined by 3 separate experiments.

Table 3. Effect of temperature and treatment time on the fungicidal activity of tea tree and thyme thymol oils in 0.1% aqueous agar with or without 10% sodium chloride against *T. mentagrophytes*

		Minimum fungicidal concentration (MFC, %)							
		27°C			37°C		42°C		
Essential oil	medium	10 min	20 min	24 h	10 min	20 min	10 min	20 min	30 min
Tea tree	0.1% agar	5.12	5.12	0.08	2.56	2.56	2.56	0.16	0.08
Tea tree	10% NaCl-0.1% agar	2.56	1.28		1.28	0.64	0.16	0.04	0.04
Thyme thymol	0.1% agar	0.16	0.16	0.02	0.16	0.08	0.08	0.02	0.02
Thyme thymol	10% NaCl-0.1% agar	0.08	0.08		0.08	0.08	0.08	0.01	0.01

value (0.16%) was maintained constant at 27°C for 20 min, and at 37°C for 10 min. Then, it was reduced to 0.08% at 37°C for 20 min, and at 42°C for 10 min. However, the MFC was markedly reduced to 0.02% (1/8 that at 27°C) at 42°C for 20 min. Addition of 10% sodium chloride caused further reduction to 0.01% (1/2 that in 0.1% aqueous agar).

Effect of temperature and essential oils on the viability of *T. rubrum* and *T. mentagrophytes* in 0.1% aqueous agar

Table 4 shows the surviving mycelia or conidia of *T. rubrum* and *T. mentagrophytes* heated at 42°C with or without essential oils. Oregano and patchouli oils were selected as representatives of monoterpene and sesquiterpene oils, respectively. Similar to *T. mentagrophytes*, the mycelia of *T. rubrum* was found to be heat-labile, and more than 99% of the mycelia were killed after heating at 42°C for 20 min. Killing was augmented by treating at 42°C for 40 min or at 45°C for 10 min, and was almost complete by addition of 0.2% oregano and patchouli oils. Killing of the mycelia occurred even at 27°C by the addition of 0.2% oils, and was accelerated by heating at 42°C. Unexpectedly, the conidia of *T. mentagrophytes* were heat-labile, and more than 90% of the conidia did not germinate after heating at 42°C for 20 min and 40 min. Notably, the conidia were very sensitive to the essential oils, and germination of the conidia was significantly suppressed by oregano and patchouli oils at 27°C as well as at 42°C.

Table 4. Effect of temperature and essential oils on the viability of *T. rubrum* and *T. mentagrophytes* in 0.1% aqueous agar

		Surviving mycelia or conidia (%)[a)]			
Fungus	Medium	27°C 20 min	42°C 20 min	42°C 40 min	45°C 10 min
T. rubrum	0.1% Agar	115±31	0.74±0.33	0.066±0.021	0.054±0.020
(mycelia)	0.02% Oregano	48±13	0.078±0.054		
	0.2% Oregano	0.018±0.009	<0.01		
	0.02% Patchouli	7.5±0.31	0.064±0.034		
	0.2% Patchouli	0.01±0.009	<0.01		
T. mentagrophytes	0.1% Agar	110±22	0.91±0.16	0.06±0.041	0.021±0.010
(mycelia)	0.02% Oregano	47±33	0.06±0.023		
	0.2% Oregano	0.0043±0.0029	<0.001		
	0.02% Patchouli	83±14	0.80±0.16		
	0.2% Patchouli	0.10±0.043	0.009±0.0043		
T. mentagrophytes	0.1% Agar	107±8.3	5.6±1.7	2.6±1.7	
(conidia)	0.02% Oregano	5.6±2.5	0.33±0.47		
	0.2% Oregano	<0.1	<0.1		
	0.02% Patchouli	9±1.6	0.33±0.47		
	0.2% Patchouli	0.67±0.47	<0.1		

a) Surviving mycelia or conidia (%) = viable cells or conidia/initial inoculum × 100 ± standard deviation determined by three separate experiments.

Table 5. MFC values of eleven essential oils and clotrimazole when treated at 27°C and at 42°C for 20 min in 0.1% aqueous agar with or without 10% sodium chloride against *T. mentagrophytes*

	Minimum fungicidal concentration (MFC, %)		
	42°C, 20 min		27°C, 20 min
Essential oil	0.1% agar	10% NaCl-0.1% agar	0.1% agar
Oregano	0.02	0.01	0.16
Thyme thymol	0.02	0.01	0.16
Cinnamon bark	0.02	0.01	0.32
Lemongrass	0.04	0.02	0.16
Clove	0.08	0.04	0.32
Palmarosa	0.08	0.04	2.56
Peppermint	0.08	0.04	2.56
Lavender	0.08	0.04	2.56
Geranium "Bourbon"	0.16	0.02	2.56
Tea tree	0.16	0.04	2.56
Thyme geraniol	0.32	0.04	5.12
Clotrimazole	0.0002	0.0002	0.0008

Anti-*Trichophyton* activity of oregano, cinnamon bark, lemongrass, clove, palmarosa, peppermint, lavender, geranium "Bourbon", and thyme geraniol at 42°C for 20 min in 0.1% aqueous agar with or without 10% sodium chloride

Table 5 shows the MFC values of 11 essential oils after treatment at 42°C for 20 min with or without 10% sodium chloride. In all of the oils examined, the MFC values were reduced markedly when treated at 42°C for 20 min as compared with the MFC at 27°C. Reduction of the MFC accompanied by the change of temperature from 27°C to 42°C was 1/32 for palmarosa and peppermint and lavender, 1/16 for cinnamon bark, geranium "Bourbon" and thyme geraniol, 1/8 for oregano, and 1/4 for lemongrass and clove oils. Similar to tea tree and thyme thymol oils, addition of 10% sodium chloride caused further reduction of the MFC values in the range of 1/2～1/8. Clotrimazole used as a positive reference also showed the thermal effect, causing a 1/4 reduction of MFC from 0.0008% (27°C) to 0.0002% (42°C), but no effect of sodium chloride was noted.

Effect of sodium chloride on adsorption of oil constituents to the mycelia of *T. mentagrophytes*

Table 6 shows the adsorption of oil constituents of tea tree, lavender and thyme thymol oils on the mycelia of *T. mentagrophytes* after contact at 42°C for 20 min in 0.1% aqueous agar and 10% sodium chloride-0.1% aqueous agar. The concentrations of the essential oils used were the respective MFC. In the case of tea tree oil, adsorption of α- and γ-terpinenes that bound highly on mycelia remained unchanged in both 0.1% aqueous agar and 10% sodium chloride-0.1% aqueous agar, but terpinen-4-ol was 3.9 fold increased in 10% sodium chloride as compared with that in water. Of two constituents of lavender oil, linalyl acetate showed high adsorption, but little change in 0.1% agar with or without 10% sodium chloride, while adsorption of linalool was increased 2.9 times by the addition of sodium chloride. In the same way, highly adsorbed p-cymene in thyme thymol oil was little affected, but poorly adsorbed thymol was doubled by the addition of sodium chloride.

Effect of heat and essential oils on the hyphal morphology of *T. mentagrophytes*

Fig. 1 shows the SEM images of the hyphae treated for 20 min at 27°C and 42°C in a water bath. Most of the hyphae treated at 27°C (1a) were normal with a smooth cell wall. The morphology of hyphae treated at 42°C (1b) was similar to that at 27°C, except for partial roughening of the cell surface. The cells surviving after treatment were 95% in (1a) and 0.18% in (1b). The morphology of the hyphae was not changed after heating at 42°C for 30 min (figure not shown) inspite of increased killing.

Fig. 2 shows the SEM images of the hyphae treated for 20 min with 0.2% thyme thymol oil at 27°C and 42°C. Many hyphae treated at 27°C (2a) were furrowed, disrupted with vesicles on the surface. The hyphae treated at 42°C (2b) showed a furrowed and rough surface, but were less disrupted than those cells at 27°C, although surviving cells were markedly reduced in 2b (<0.001%) compared with 2a (2.6%). The hyphae treated with 0.02% thyme thymol oil showed similar morphological change to that treated with 0.2% oil (figure not shown).

Fig. 3 shows the SEM images of the hyphae treated for 20 min with 1.6% tea tree oil at 27°C and 42°C. Those treated at 27°C (3a) were flattened, furrowed and rough on the surface, which was covered with an abundance of extruded materials. Dissolution of the cell wall and vesicles of cell membrane were also

Table 6. Adsorption of oil constituents on the mycelia of *T. mentagrophytes* when treated at 42°C for 20 min in 0.1% aqueous agar and 10% sodium chloride-0.1% aqueous agar

			Adsorption (μg/mg)		
Essential oil	concentration	medium	α-terpinene	γ-terpinene	terpinen-4-ol
Tea tree	0.16%	0.1% agar	4.7±0.3	13±0.5	2.8±0.6
	0.16%	10% NaCl-0.1% agar	4.8±0.4	12±0.6	11±0.4
Essential oil	concentration	medium	linalool	linalyl acetate	
Lavender	0.08%	0.1% agar	1.9±0.6	13±2.2	
	0.08%	10% NaCl-0.1% agar	5.6±0.3	11±0.4	
Essential oil	concentration	medium	p-cymene	thymol	
Thyme thymol	0.02%	0.1% agar	2.9±0.3	0.6±0.08	
	0.02%	10% NaCl-0.1% agar	2.9±0.7	1.4±0.3	

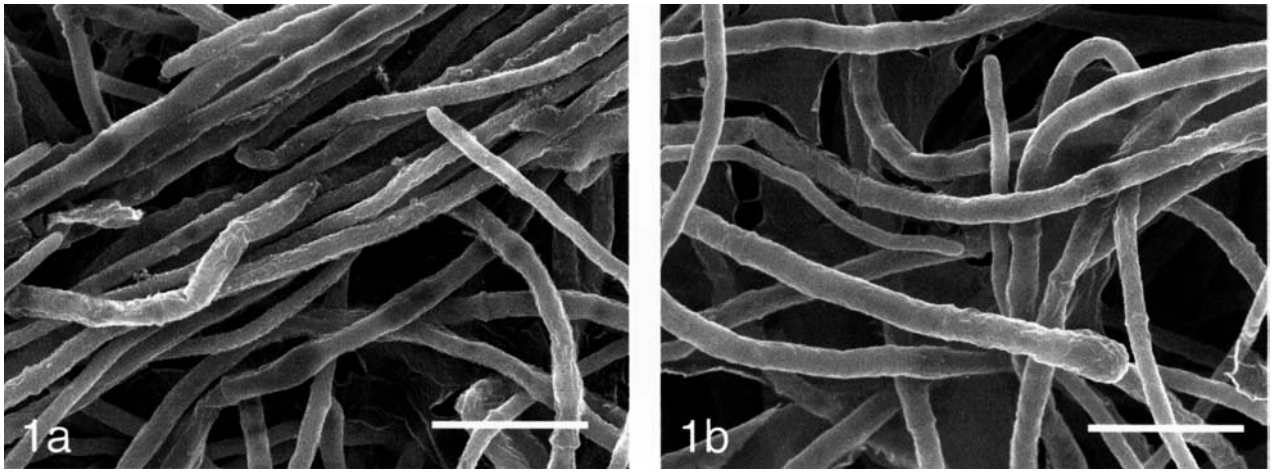

Fig. 1. SEM images of hyphae of *T. mentagrophytes* TIMM 2789 treated at 27°C for 20 min (1a) and at 42°C for 20 min (1b), showing little alteration on the surface structure. A bar indicates 10 μm.

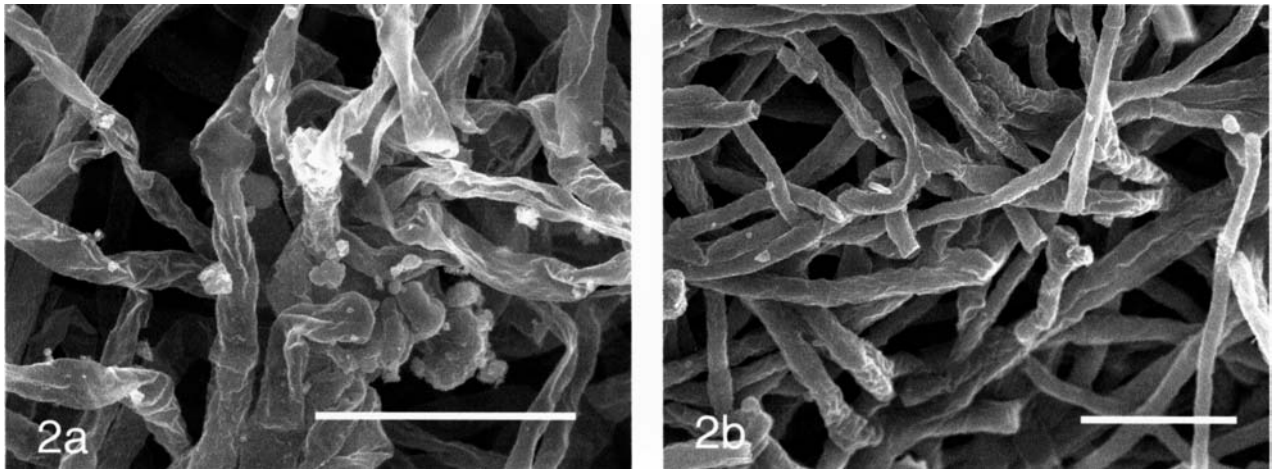

Fig. 2. SEM images of the hyphae treated with 0.2% thyme thymol oil at 27°C for 20 min (2a) and at 42°C for 20 min (2b), showing furrowed and disrupted hyphae with vesicles on the surface (2a) and furrowed hyphae with less disruption (2b). A bar indicates 10 μm.

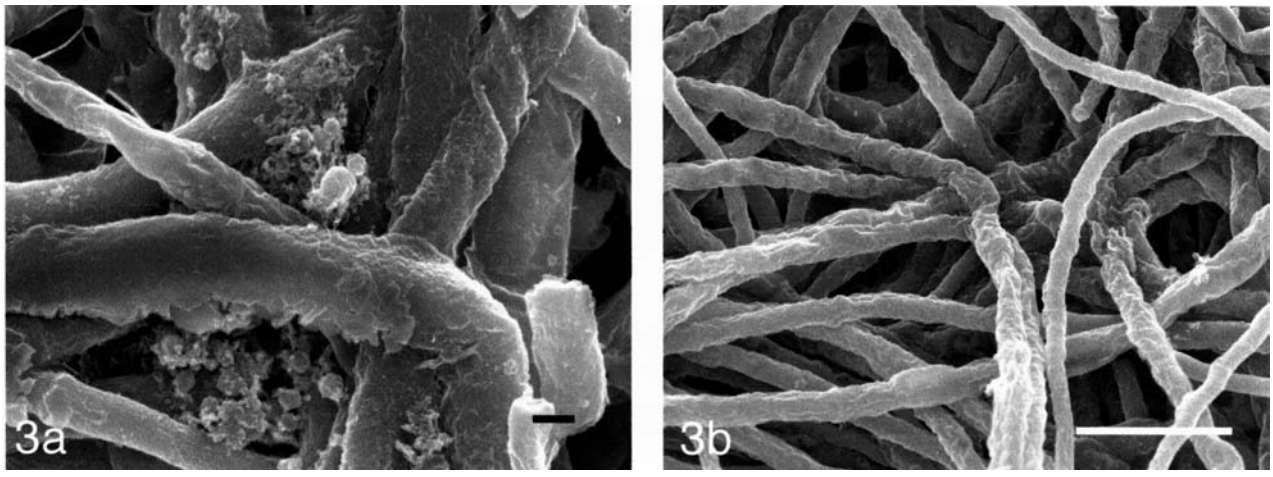

Fig. 3. SEM images of hyphae treated with 1.6 % tea tree oil at 27°C for 20 min (3a) and at 42°C for 20 min (3b), showing dissolution of cell wall and abundant vesicles of membrane (3a) and furrowed hyphae with less dissolution (3b). A bar indicates 1 μm for 3a, and 10 μm for 3b..

observed. The hyphae treated at 42°C (3b) were flattened, furrowed and attached to each other, with a few vesicles on the surface, but the morphological change was mild compared with that at 27°C. Surviving cells were 1.4% in (3a) and <0.001% in (3b). Similar morphological damage was observed on the hyphae treated with 0.16% tea tree oil at 27°C and 42°C for 20

min (figures not shown). Thus, no effect of heat treatment at 42°C with or without oils was observed on the hyphal morphology.

Discussion

The importance of a dispersing agent on determination of the MIC values of essential oils is well known. Use of a surface-active agent such as Tween 80 and organic solvent resulted in a reduction of the bioactivity[14, 15]. In a preliminary experiment, the effect of dispersing agents on the antifungal activity of tea tree and thyme thymol oils was examined using 0.1% agar, 0.1% sodium hyauronate, 0.1% sodium alginate, 0.1% Alcasealan (acidic polysaccharide), 0.1% Tween 80, 0.5% Tween 80 and 0.5% ethanol. The results showed no significant difference in the MFC value, except for Alcasealan. Although the latter produced the most homogeneous suspension, it caused a 50% reduction in bioactivity (data not shown). Since essential oils were dispersed fairly well in 0.1% aqueous agar, agar was used as a dispersing agent in this study. The use of agar was also recommended for a broth dilution assay of essential oils[16].

A foot bath is different from bathing of the whole body in the treatment conditions, and relatively high temperature (42°C) and short immersion time (10 min) are recommended in a foot bath[17]. Appropriate temperature for the growth of *Trichophyton* sp. was 27°C, but it was revealed in this study that *T. mentagrophytes* and *T. rubrum* were thermo-labile at 42°C for 20 min, and more than 99% of the original mycelia were killed at 42°C for 20 min. The thermal effect was more marked at 45°C, where 10 min bathing was enough to kill 99.9% of the mycelia. Similar killing was observed in the presence of 10% sodium chloride, that is, a high rate of killing occurred only after heating at 42°C for 20 min. This means that the salt effect did not work under the conditions used, in contrast to the marked thermal effect. The inhibitory effect of heat and oils was observed not only on the mycelia of *T. mentagrophytes* but also on the conidia. As compared with the mycelia, the conidia appeared to be more resistant to heat, but more susceptible to the action of essential oils (Table 4).

The thermal effect contributed greatly to the enhanced antifungal activity of essential oils, since the MFC of tea tree and thyme thymol oils were reduced markedly only after treatment at 42°C for 20 min (Table 3). It should be noted that the MFC of tea tree at 27°C was 5.12% after 20 min but 0.08% after 24 h treatment, showing that the activity was time-dependent. But, when the temperature was raised to 42°C, the treatment time could be shortened to 20 min to obtain a comparable effect to that at 27°C for 24 h. The same was true for thyme thymol, lavender (MFC at 27°C for 24 h, 0.08%) and lemongrass (MFC at 27°C for 24 h, 0.01%). In other words, treatment time could be saved by raising temperature.

The morphological effect of essential oil on the hyphae of dermatophytes has not been reported so far. Thyme thymol oil treated at 27°C for 20 min caused marked damage to the hyphae, showing destruction of the cell wall and formation of vesicles of membrane. More drastic damage was caused by tea tree oil, although dosages of both oils were the respective MFD and 10 MFD at 42°C. Apparently, the morphological change was caused by disturbance of the metabolic process that was working at 27°C. Cellular damage at 42°C caused by essential oils was comparable to or less than that at 27°C, and no heat effect was recognized on the morphology, although the viable cell count indicated that more hyphae were killed at a high temperature of 42°C than at 27°C. These results suggested that the heat effect did not reflect on the hyphal morphology.

It is known that microorganisms including *T. rubrum*[18] acquire thermotolerance by producing heat shock proteins with various roles such as chaperone activity, ribosomal stability, etc. However, the heat-stressed hyphae of *T. mentagrophytes* in our study were killed with little structural alteration. It was probable that the cell viability was lost by rapid accumulation of denatured or aggregated proteins, thereby freezing the hyphae as they were without a biochemical response to resist the heat stress[19]. The fungicidal activity of essential oils was markedly affected by the growth phase of the hyphae, showing four-fold increase in MFC values when the young hyphae 2 days after germination were changed to old hyphae at 5 days (data not shown). On the other hand, the young and old hyphae were killed at a comparable rate by heat treatment, suggesting that the metabolic change was not related to the thermal killing (data not shown). The heat fragility of *T. mentagrophytes* and *T. rubrum* was in sharp contrast to the heat-stable *Aspergillus* and *Candida* sp., which were grown at 50°C. In this connection, it was noted that oxidative stress induced by heat played a major role in low eucaryotes such as *Aspergillus niger*[20]. *Trichophyton* sp. was rich in mitochondria which

was a target of oxidative stress[21]. Heat-stressed cells have been reported to exhibit increased sensitivity to low levels of chemicals including essential oils[22]. The enhanced effect of essential oils on the heat-treated yeasts was recognized by adding the oils in the recovery medium after treatment at 44-54°C, and inaction of the repair system in heat-injured cells was suggested. In our study, simultaneous actions of heat stress and oil stress were applied to the filamentous cells. It was probable that heat damaged cells became sensitive to the action of oil, suggesting the combined effect of heat and oil.

Bathing in very salty water has been utilized as an the alternative medicine for the treatment of various dermatological conditions[5, 6]. Kurita and Koike[23] reported the synergistic antifungal effect of essential oil constituents and sodium chloride. For the antifungal evaluation, they tested 7-10% sodium chloride and essential oil constituents in agar medium at 27°C for 20 days. The salt effect was slightly observed in our study at 27°C, 37°C and 42°C for 10 min (Table 2). Heat damaged cells were reported to become sensitive to salt stress[24]. But in our study, no synergistic effect due to heat-stress or salt-stress was observed, since the cells surviving after heating at 42°C for 20 min were not different between 0.1% aqueous agar and 10% sodium chloride-0.1% aqueous agar (Table 2). However, the salt effect was shown in the presence of the essential oils at 42°C, causing 1/2～1/4 reduction of the MFC of the oils by 10% NaCl (Tables 3 and 5). The salt effect could be ascribed, at least partly, to the increment in adsorption of alcohol and phenol constituents in the oils, because these constituents possessing high antifungal activity[25] were increased 2～4 times in the salt solution, while adsorption of hydrocarbon and ester constituents that showed little or weak activity were little affected by sodium chloride (Table 6).

The thermal effect was also observed with clotrimazole, an antifungal drug used in clinics, showing a four-fold increase in activity as judged from MFC, but no salt effect was observed. It was of interest to see that successful thermotherapy was reported for subcutaneous infections due to *Alternaria alternata*[26], for chromomycosis[27] and sporotrichosis[28]. The growth of pathogenic *Sporothrix schenkii* was suppressed at 42°C. In clinical trials, local hyperthemia between 40°C and 45°C was maintained. Hyperthemia provided a potent therapeutic tool for cancer treatment[29]. By immersing the tumor-bearing foot at 41.5°C for 30 min in a water bath, chemotherapeutic agents showed enhanced cytotoxicity against selected tumor cells as compared with that at 37°C[30].

In conclusion, *T. mentagrophytes* and *T. rubrum* were shown to be thermo-labile when treated at 42°C for 20 min. Although the thermal effect played a dominant role in killing the fungal cells, eleven essential oils could kill >95% of the viable cells remaining after heat treatment, when combined with heat and salt stress. Although the combined effect of heat, oils and salt must be proven for multiple clinical isolates of dermatophytes and *in vivo*, the fundamental study in this paper paves the way to thermotherapy combined with essential oils or antifungal agents as a new promising modality for treating tinea pedis in a foot bath.

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