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Synergism in Antifungal Activity against *Candida* and *Trichophyton* Species in Combination with the Essential Oil of *Coriandrum sativum* L. and Antibiotics

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Abstract – To determine whether the essential oil from *Coriandrum sativum* and its main component, linalool, exhibit antifungal activity, we employed a broth dilution assay and disk diffusion test using common pathogenic *Candida* and *Trichophyton* species. Both coriander oil and linalool significantly inhibited growth of the tested fungi, with minimal inhibitory concentrations (MICs) in the range of 0.03~2 mg/ml. Furthermore, in a checkerboard titer test, both the oil fraction and linalool exhibited synergism when combined with ketoconazole, with resultant FICIs ranging from 0.06 to 0.53. Notably, hyphal formation in *C. albicans* cells was obviously inhibited by *C. sativum* essential oil in this experiment.
Keywords – *Coriandrum sativum,* essential oil, linalool, *Candida, Trichophyton,* ketoconazole

Introduction

Coriandrum sativum L. (Coriander, family Umbelliferae), a strong smelling, annual herb, is generally used in three forms; young leaves, dried ripe fruits (commonly termed coriander seeds) and oil. The young fresh leaves and seeds are used as a garnish and a spice, respectively. The fragrant leaf-part of the plant is favored as a traditional condiment for various preserved foods, especially in Korea. The oil is used mainly as a flavoring agent in pharmaceutical preparations and as a fragrance in cosmetics. In folk medicine, especially in Europe, coriander seeds are widely used in aromatic, carminative, stomachic and antispasmodic preparations and also in laxatives to prevent stomach griping. Coriander oil is effective in alleviating rheumatism and arthritis pain, as well as muscle spasms, and is also used to treat colds and flu (Said *et al.*, 1996).

Essential oils are one of the most promising groups of natural compounds from which new prototypic antifungal agents may be developed, even though these oils appear to have relatively mild activities against human pathogenic fungi compared to commercial, synthetic, antifungal drugs (Garg and Dengre, 1998; Bidlack *et al.*, 2000). Consequently, the possibility that essential oils may exhibit synergistic effects with synthetic antifungal drugs has been tested in several studies (Hammer *et al.*, 2000; Giordani *et al.*, 2001; Shin and Kang, 2003).

Here, to specifically determine whether stable and safe antifungal agents could be developed from natural products (daily foodstuffs in particular), the antifungal activity of essential oil from *C. sativum* was evaluated against common pathogenic fungi. Three *Candida* fungi and six *Trichophyton* species were employed and antifungal activities were investigated by micro broth dilution and disk diffusion methods. In addition, we determined the synergistic effects of coriander oil or its main component, linalool, in combination with ketoconazole using checkerboard microtiter tests. Inhibition of hyphae induction in *Candida albicans* exposed to coriander oil was also investigated.

Experimental

Sample preparation – The essential oil from *Coriandrum sativum* (flower) was purchased from Primavera Life (Germany).

Fungal strains – Three *Candida* species, *C. albicans* KCCM 11282, *C. utilis* KCCM 11356, *C. tropicalis* KCCM 12578 and six *Trichophyton* species, *T. tonsurans* KCCM 11866, *T. mentagrophytes* KCCM 11950, *T. rubrum* KCCM 60443, *T. shoenleinii* KCCM 60447, *T. erinacei* KCCM 60441 and *T. soudanes* KCCM 60448, were obtained from the Korean Culture Center of Microorganisms (KCCM). They were cultured in yeast and malt extract broth for 48h at 37 °C. The turbidity of

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the cell suspensions was measured at 600 nm and adjusted with medium to match the 0.5 McFarland standard (10^5~10^6 CFU/ml).

GC-MS analysis – GC-MS analysis was carried out using a Hewlett Packard 5973-6890 system equipped with a HP-5MS or HP-INNOWAX capillary column.

The oven was programmed with an initial temperature of 50 °C and heated at a rate of 2 °C/min until it reached 170 °C.

Isolation of linalool – 2.0 g of coriander oil was subjected to column chromatography over a silica gel, and eluted with the solvent, toluene-ethyl acetate (93 : 7). The fractions were combined based on their TLC pattern to yield sub-fractions designated A1-A5. Sub-fraction A3 was further purified by column chromatography over a silica gel, and eluted with the same solvent as the previous process to give 137 mg of linalool. Its UV, MS, ^{1}H-NMR and ^{13}C-NMR data were identical to the authentic standard sample (Sigma, USA).

Determination of MIC – The MICs of the antifungal agents against the various fungi were determined by broth micro-dilution method, as described by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). Essential oil of *Coriandrum sativum* and linalool were serially diluted with ethanol to obtain 0.0125~16 mg/ml solutions and 10ul/ml of Tween 80 was added to each solution. After shaking, 100-ul aliquots of these solutions were added to wells of a 96-well microtiter plate. Ketoconazole was similarly diluted in DMSO to generate a series of concentrations ranging from 128 to 0.25 ug/ml per testing well. A 100-ul suspension of each organism was adjusted to 10^4~10^5 CFU/ml, and then added to the individual wells (10^3~10^4 CFU/well) and cultivated at 25 °C. The MIC was defined as the lowest concentration that completely inhibited visible fungal growth after 72 h of incubation. Each organism was also cultured with a blank solution containing Tween 80, ethanol and DMSO at concentrations equivalent to those in the test solutions to verify that these vehicles did not affect fungal growth. The tests were performed in triplicate.

Disk diffusion assay – Fungal broth culture aliquots were added to Sabouraud's dextrose agar medium and uniformly distributed. Sterile paper discs (8 mm; Advantec, Dublin, CA, USA) were impregnated with 50 ul of 12.5% (v/v, 6.25 mg) or 25%(v/v, 12.5 mg) solutions of each agent (prepared in ethanol), and after alcohol evaporation, discs were placed on the culture plates. The width of the zone of inhibition (mm) was measured from the boundary of the disc after cultivation at 25 °C for 72 h. Values are given as mean ± S.D. of tests performed in triplicate.

Checkerboard microtitre test – Ten serial, two-fold dilutions of *C. sativum* essential oil, linalool and ketoconazole were prepared with the same solvents used in the MIC tests. 5-ul aliquots of each dilution were added to the wells of a 96-well plate in the vertical orientation and 5-ul aliquots of each ketoconazole dilution were added in the horizontal orientation so that the plate contained various concentration combinations of the two compounds. A 100-ul suspension of each fungal strain (10^3~10^4 CFU/well) was added to individual wells and the plate was incubated at 25 °C for 72 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of essential oil/linalool and ketoconazole divided by the MIC of essential oil/linalool or ketoconazole alone. The FIC index was calculated by adding both FICs and interpreted as a synergistic effect when it was < 0.5, as additive or indifferent when it was > 0.5 to 2.0, and as antagonistic when it was > 2.0 (White *et al.*, 1996). An isobologram was constructed from the checkerboard data to illustrate antifungal synergism between coriander oil/linalool and ketoconazole.

Hyphal induction – *Candida albicans* KCCM 11282 cells were induced to form hyphae in medium 199 (Zhang *et al.*, 2006). The medium was pre-warmed to 37 °C. Cells from a 48-h stationary phase culture were transferred to 5 ml of 1 × M199 to obtain a final concentration of 3×10^6 cells/ml. Individual *C. sativum* essential oil and linalool solutions were added to the growth medium to achieve final concentrations of 0, 1 and 2 mg/ml, following which the cultures were incubated for 6 h at 37 °C, 5% CO_2. Hyphal formation was observed with an inverted phase contrast microscope at a magnification of 400X. Ketoconazole (16 ug/ml) was used as a positive control.

Results and Discussion

The known composition of *C. sativum* essential oil is summarized in Table 1. Twenty-two components have been identified to date, and these account for 97.35% of the oil. The predominant component of this oil is linalool (55.97%) while camphor 7.01 (%), geranyl acetate 6.57 (%), α-pinene 6.54 (%) and γ-terpinene 5.38 (%) are the remaining major components.

Both *C. sativum* essential oil and linalool were analyzed for potential antifungal activity using nine pathogenic fungi. The MIC test results are presented in Table 2. The oil showed a strong inhibitory effect against all fungi investigated with minimum inhibitory concentrations (MIC) of 0.03~2 mg/ml, depending on the fungi

tested. The lowest MIC (0.03 mg/ml) was obtained against *Trichophyton shoenleinii* KCCM 60447 and *Trichophyton soudanes* KCCM 60448.

Table 1. Composition of the essential oil from *C. sativum* analyzed by GC-MS

Compounds	RI		Peak Area (%)
	HP-5	HP-IW	
α-thujene	929	910	0.11
α-pinene	935	906	6.54
camphene	948	954	1.51
sabinene	975	1035	0.31
β-pinene	977	1011	0.69
β-myrcene	998	1123	0.83
α-terpinene	1019	1149	0.10
ρ-cymene	1026	1257	3.53
limonene	1030	1195	3.10
γ-terpinene	1060	1235	5.38
cis-linalool oxide	1073	1434	0.39
α-terpinolene	1088	1267	0.75
trans-linalool oxide	1089	1461	0.34
linalool	1113	1557	55.97
camphor	1144	1491	7.01
epoxylinalol	1172	1733	0.11
terpinen-4-ol	1178	1592	0.21
á-terpineol	1193	1689	0.86
geraniol	1265	1848	2.65
myrtenyl acetate	1332	1674	0.29
geranyl acetate	1395	1756	6.57
β-caryophyllene	1419	1576	0.13
In total			97.35

[*] Compounds are listed in their order of their elution on the HP-5MS column.
[a] GC retention indices (RI) were calculated against C_9 to C_{24} *n*-alkanes on a HP-5MS column.
[b] GC retention indices (RI) were calculated against C_9 to C_{24} *n*-alkanes on a HP-INNOWAX column.

Linalool also showed high antifungal activity with MICs in the range 0.25~2 mg/ml. It was most potent against the *Trichophyton* species, with the MIC of 0.25 mg/ml.

As shown in Table 3, the disc diffusion test results were generally consistent with data from the MIC tests. In particular they confirmed that the oil is most potent against *Trichophyton* spp. The disc diffusion tests also revealed that the antifungal activities of coriander oil and linalool are dose-dependent.

We proposed that by identifying synergism between *C. sativum* essential oil/linalool and ketoconazole it might be possible to use natural products to enhance the efficacy of antifungal drugs. To test this hypothesis, checkerboard microtiter tests were performed in which coriander oil or linalool were combined with ketoconazole and the results were used to construct an isobologram. The FIC and FICI calculations from these checkerboard tests (Table 4) demonstrate significant synergism between ketoconazole and both *C. sativum* oil and linalool with FICI values ranging from 0.06~0.31 against the tested fungi. Furthermore, the isobolograms depicted in Fig. 1. were constructed using the concentrations which produced the greatest fungal inhibition in the checkerboard titer tests. The curve patterns deviating to the left indicate that the effective concentrations were significantly lowered by combination and that the extent of this lowering could not be accounted for by simple additive reduction (Davidson and Parish, 1989). Therefore, coriander oil might be useful in antifungal therapy in combination with ketoconazole, especially against *T. mentagrophytes* KCCM 11950.

Hyphae are an important factor in fungal virulence. For example, it is through hyphae that *C. albicans* invades human tissues (Cutler, 1991). By observing the hyphal

Table 2. MICs and MFCs of *C. sativum* essential oil as estimated by the micro-broth dilution method

Organisms	*C. sativum* (mg/ml)		Linalool (mg/ml)		Ketoconazole (ug/ml)	
	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	2.00	4.00	1.00	1.00	16.00	16.00
C. utilis	2.00	2.00	2.00	2.00	1.00	1.00
C. tropicalis	2.00	2.00	2.00	2.00	0.50	1.00
T. tonsurans	0.06	0.13	0.25	0.50	16.00	32.00
T. mentagrophytes	0.50	0.50	0.25	0.50	16.00	32.00
T. rubrum	0.13	0.50	0.25	0.50	16.00	16.00
T. schoenleinii	0.03	0.25	0.25	0.50	8.00	8.00
T. erinacei	0.06	0.50	0.25	0.50	16.00	16.00
T. soudanense	0.03	0.06	0.25	0.50	16.00	16.00

Table 3. Growth inhibition (mm) by *C. sativum* essential oil on saboraud dextrose agar plates

Sample	Fungi					
	Ca[a]	*Ct*[b]	*Tt*[c]	*Tsh*[d]	*Te*[e]	*Tso*[f]
C. sativum						
I	1.00 ± 1.41	0.50 ± 0.71	9.50 ± 0.71	20.50 ± 2.12	24.50 ± 0.71	18.50 ± 7.78
II	3.50 ± 0.71	5.50 ± 0.71	> 39	> 39	> 39	35.00 ± 5.66
Linalool						
I	1.50 ± 0.71	0.25 ± 0.35	> 39	7.00 ± 1.41	2.50 ± 0.71	4.00 ± 1.41
II	11.50 ± 2.12	6.00 ± 1.41	> 39	> 39	> 39	> 39
Ketoconazole						
III	4.00 ± 0.00	5.50 ± 0.35	13.20 ± 0.58	20.10 ± 0.00	11.60 ± 0.29	19.30 ± 0.29
IV	9.60 ± 2.08	11.50 ± 2.12	22.70 ± 0.29	33.50 ± 0.57	27.80 ± 0.58	32.00 ± 1.41

I, 6.25 mg/ml; II, 12.50 mg/ml; III, 50 ug/ml; IV, 100 ug/ml.
[a] *Candida albicans* KCCM 11282; [b] *Candida tropicalis* KCCM 12578; [c] *Trichophyton tonsurans* KCCM 11866; [d] *Trichophyton shoenleinii* KCCM 60447; [e] *Trichophyton erinacei* KCCM 60441; [f] *Trichophyton soudanes* KCCM 60448.

Table 4. FIC and FICI for combinations of ketoconazole and *C. sativum* oil or linalool

Sample	*Ca*[a]		*Cu*[b]		*Tm*[c]		*Tsh*[d]		*Tso*[e]	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
C. sativum oil	0.13	0.19	0.03	0.06	0.25	0.31	0.25	0.31	0.25	0.28
Ketoconazole	0.06		0.03		0.06		0.06		0.03	
Linalool	0.06	0.12	0.50	0.53	0.13	0.38	0.03	0.06	0.25	0.38
Ketoconazole	0.06		0.03		0.25		0.03		0.13	

[a] *Candida albicans* KCCM 11282; [b] *Candida utilis* KCCM 11356; [c] *Trichophyton mentagrophytes* KCCM 11950; [d] *Trichophyton shoenleinii* KCCM 60447; [e] *Trichophyton soudanes* KCCM 60448.
FIC (Fractional inhibitory concentration) = MIC of sample of the most effective combination / MIC of sample alone;
FICI (FIC index) = FIC of sample + FIC of ketoconazole.

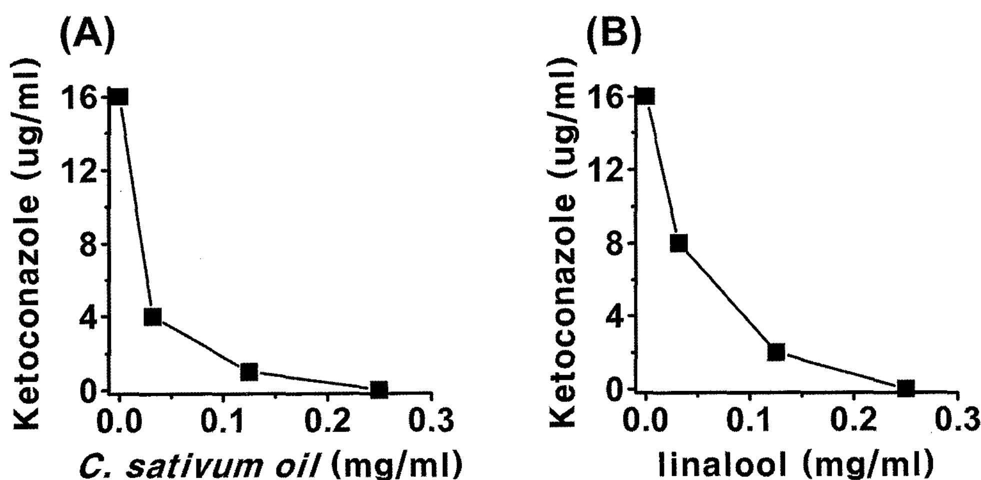

Fig. 1. Isobolograms indicating synergism of the *C. sativum* essential oil (mg/ml) or linalool in combination with ketoconazole (μg/ml) against *T. mentagrophytes*.

induction phase of *C. albicans* via phase contrast microscopy, we found that hyphal formation was clearly inhibited by both *C. sativum* essential oil and linalool (Fig. 2).

While the mode of antifungal action of essential oils has not been clarified, it is thought that, similar to ketoconazole, their inhibitory mechanism may involve disrupting the permeability of the cell membrane (Cox *et*

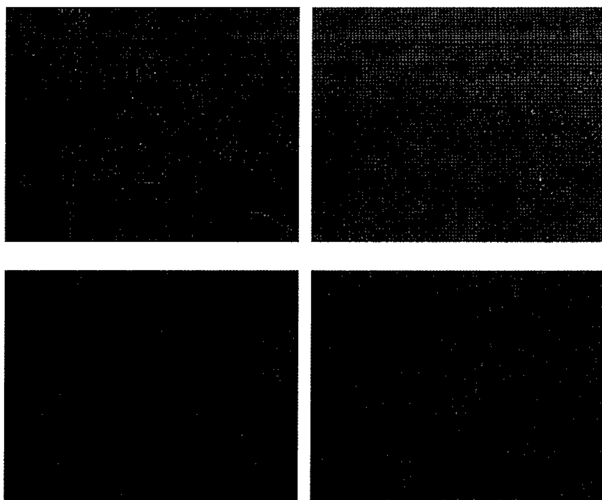

Fig. 2. Inhibition of hyphal formation of *Candida albicans* cells by *C. sativum* essential oil and linalool. A: control; B: *C. sativum* essential oil (1 mg/ml), C: linalool (0.5 mg/ml), D: ketoconazole (16 ug/ml).

al., 2000).

In conclusion, in the treatment of infections caused by *Candida* and *Trichophyton* species combining ketoconazole with either *C. sativum* essential oil or linalool may reduce the minimum effective dose of ketoconazole required and, thus, minimize its side effects. In addition, the therapeutic use of essential oils may provide a solution for the rapid development of fungal resistance and drug-drug interactions that are problematic with commonly used antifungal therapeutics. Further studies on the activities of essential oils against drug-resistant fungi and bacteria, investigations of their mechanisms of action and *in vivo* experiments are now required to assess their potential for chemotherapeutic application.

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