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Combined Effects of the Essential Oil from *Eucalyptus globulus* with Ketoconazole against *Candida* and *Trichophyton* Species

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Abstract – We have evaluated the combined antifungal effects of the essential oil from *Eucalyptus globulus* or its main component 1,8-cineole with ketoconazole. Checkerboard microtiter tests were used to analyze their effects against three *Candida* and six *Trichophyton* species. The susceptibility of the *Trichophyton* species to *E. globulus* essential oil differed distinctly. The fractional inhibitory concentration indices (FICIs) against the tested *Candida* species ranged between 0.09 and 0.38 for ketoconazole combined with *E. globulus* essential oil or 1,8-cineole, indicating significant synergism between ketoconazole and the oil samples. Similar experiments using *Trichophyton* species resulted in FICIs between 0.28 and 0.63, indicating relatively weaker combined effects than those observed with *Candida* species. Thus, the data reported here show that the anti-*Candida* effects of ketoconazole can be significantly improved in the presence of *E. globulus* essential oil or 1, 8-cineole.
Keywords - *Eucalyptus globulus,* essential oil, 1,8-cineol, *Candida, Trichophyton* species, ketoconazole, combination effects

Introduction

Candida albicans is the most frequently occurring opportunistic fungal infection and the predominant cause of candidosis (Baron *et al.*, 1994; Coleman *et al.*, 1998; Witek-Janusek *et.al.*, 1998; Groll and Walsh, 2001; Singh, 2001; Sobel *et al.*, 2001). *Trichophyton* is a fungal species that causes superficial mycoses, commonly known as tinea infections, in humans and other animals (Patra, 2002). Ketoconazole is an antifungal drug that is commonly used for the treatment of both superficial and deep infections caused by *Candida* and *Trichophyton* fungi (Zhang *et al.*, 2006).

Eucalyptus globulus Labill. (Myrtaceae) is a rapidly growing tree that is native to Australia and is cultivated in subtropical areas of the world (Bisset, 1994). Essential oil from the leaves of this plant is usually comprised of over 50 % of the monoterpene oxide 1, 8-cineol. The essential oil has been used in various remedies for the treatment of bronchitis and throat inflammation, and is also widely used in cough drops. The oil and 1,8-cineol have been reported to exhibit antiseptic and antifungal activities (Duke *et al.*, 2002; Shin, 2002; Shin and Lim, 2004); however, their usage is predominantly limited to complementary therapies because their activity is much milder than common antibiotics used in the clinic.

Combination therapy using essential oils and antibiotics is a promising approach that may allow a reduction in the effective dose of the antibiotic due to synergism with the essential oil; thus, reducing unpleasant side effects (Keele *et al.*, 2003; Shin and Kim, 2004; Shin and Pyun, 2004).

In order to develop a combination therapy for the treatment of infections by *Candida* and *Trichophyton* species we combined the essential oil from *E. globules* with the antibiotic ketoconazole. We analyzed the essential oil by gas chromatography-mass spectrometry (GC-MS) and isolated its main components by silica gel column chromatography. The antifungal activity of the oil fraction and its main components was estimated using the broth dilution method. Checkerboard microtiter tests were performed to determine the combined effect of the essential oil components and the antibiotics; FIC and FICIs were calculated for the three *Candida* and six *Trichophyton* species used in this study.

Materials and Methods

Analysis of the essential oil fraction – *E. globulus* oil (Primavera Life, Germany) was analyzed by GC-MS on a Hewlett-Packard 6890 GC and Hewlett-Packard 5973 MSD apparatus using HP-5MS and HP-Innowax capillary

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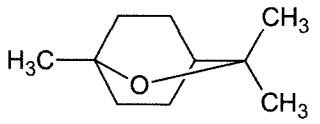
columns (Shin and Lim, 2007). The injector was adjusted to 260 °C and the oven temperature for the two columns was regulated as follows. For the HP-5MS column the temperature was initiated at 50 °C for 5 min, increased by 3 °C/min to 125 °C, 1 °C/min to 145 °C, 3 °C/min to 280 °C, and sustained at 280 °C for 10 min. For the HP-Innowax column the temperature was initiated at 50 °C, increased by 3 °C/min to 150 °C, 3 °C/min to 250 °C, and sustained at 250 °C for 10 min.

The essential oil fraction (2 g) was subjected to silica gel column chromatography and eluted with toluene-ethyl acetate (93 : 7). Five fractions were obtained, and of these, fraction 3 was subjected to rechromatography using the same conditions as described above, to give 1,8-cineole (302 mg). The spectral data of the isolated compounds were compared with those of their corresponding standards.

1,8-Cineole – Yellowish oil. EI-MS: *m/z*: 154 $[M]^+$, 139, 111, 108, 93, 64, 61, 71, 43 (100%); IR ν_{max} (KBr) cm^{-1}: 2967, 1465, 1375, 1214, 1079, 985; ^{1}H-NMR ($CDCl_3$) δ: 2.02 (2H, m, H-3a, H-5a), 1.66 (2H, m, H-2a, H-6a), 1.50 (2H, m, H-3b, H-5b), 1,41 (1H, m, H-4), 1.23 (6H, s, H-9, H-10), 1.05 (3H, s, H-7); ^{13}C-NMR ($CDCl_3$) δ: 73.6 (C-4), 69.7 (C-8), 33.0 (C-3), 31.5 (C-1, C-5), 28.9 (C-9, C-10), 27.6 (C-7), 22.9 (C-2, C-4).



1,8-Cineole

Fungal strains – *C. albicans* KCCM 11282, *C. utilis* KCCM 11356, *C. tropicalis* KCCM 12578, *T. erinacei* KCCM 60411, *T. mentagrophytes* KCCM 11950, *T. rubrum* ATCC 6345, *T. schoenleinii* KCCM 60477, *T. soudanense* KCCM 60448, and *T. tonsurans* KCCM 11866, were obtained from the Korean Culture Center of Microorganisms (KCCM). These strains were cultured in yeast and malt extract broth (YM, Difco, USA) for 48 h at 25 °C. The turbidity of the cell suspension was measured at 600 nm and adjusted with medium to equal the 0.5 McFarland standard (10^5 - 10^6 colony forming units (CFU)/mL).

Determination of the minimal inhibitory concentration (MIC) and the minimal Fungicidal concentration (MFC) – The essential oil samples were serially diluted with YM broth to obtain solutions ranging from 0.062 to 8 mg/mL; 100 μL aliquots of these solutions plus 10 μL of Tween 80 were added to the wells of 96-well microtiter plates. A 90 μL suspension of each *Candida* or *Trichophyton* species adjusted to 10^5 - 10^6 CFU/mL was added to individual wells, and the plates were cultivated at 25 °C. The MIC was defined as the lowest concentration of essential oil in which more than 50% of the visible fungal growth was inhibited after 72 h. In order to evaluate the MFC, a 50 μL aliquot from each cultured well was added to 150 μL of new medium and cultured for 7 days. Each organism was also cultured with a blank solution containing Tween 80 at concentrations equivalent to those in the test solutions in order to demonstrate that this vehicle did not affect fungal growth.

Checkerboard titer test – The effects of the essential oil compounds combined with antibiotics were evaluated using checkerboard titer tests. Ten *Eucalyptus* oil solutions were prepared by two-fold serial dilution with culture medium containing Tween 80, and eight two-fold serial dilutions of ketoconazole were prepared with dimethyl sulfoxide (DMSO) using the same solvent concentrations as those used in the MIC tests. A 96-well microtiter plate was oriented to provide 10 columns and 8 rows. To each column, 50 μL aliquots of diluted *Eucalyptus* oil were added, using one dilution concentration for each column of the plate. To each row, 10 μL aliquots of diluted ketoconazole were added, using one dilution for each row of the plate. Thus, the 96-wells contained various concentration combinations of the two compounds. Each well was then inoculated with 100 μL (approximately 5×10^4 CFU/well) of fungal suspension and cultivated at 25 °C. Fractional inhibitory concentrations (FICs) were calculated by dividing the combined MICs of *Eucalyptus* oil and ketoconazole on the basis of the individual MICs of the respective samples. Similar experiments were performed using 1,8-cineole instead of *Eucalyptus* oil. FICIs ≤ 0.5 were defined as being indicative of a synergistic antifungal effect; those > 0.5 and ≤ 2.0 were considered to be additive or indifferent; and those > 2.0 as were defined as antagonistic (White *et al.*, 1996). An isobologram was constructed from the checkerboard data to depict the synergism of *Eucalyptus* oil with ketoconazole against *Trichophyton* and *Candida* species. Similar experiments were also established for 1,8-cineole. DMSO and Tween 80 were used at concentrations equivalent to those used in the test solutions to demonstrate that these vehicles did not affect fungal growth (Shin and Lim, 2007).

Results and Discussion

Superficial and deep *Candida* or *Trichophyton* infections

Table 1. Constituents of the essential oil from *E. globulus* by GC-MS

Compounds	RI		Peak Area (%)
	HP-5[a]	HP-IW[b]	
α-Pinene	935	906	2.41
Camphene	948	954	0.03
β-Pinene	977	1012	0.44
Myrcene	999	1126	0.14
ρ-Cymene	1027	1258	6.89
1,8-Cineol	1038	1211	81.38
γ-Terpinene	1060	1235	0.07
Linalool oxide	1074	1434	0.19
α-Pinene oxide	1095	1353	0.74
trans-ρ-2,8-Methadien-1-ol	1120	1621	0.20
cis-Limonene oxide	1133	1431	0.63
trans-Pinocarveol	1136	1642	0.90
trans-Limonene oxide	1138	1443	0.28
Verbenol	1145	1668	0.37
Pinocarvone	1162	1544	0.13
α-Terpineol	1168	1664	2.27
4-Terpineol	1178	1591	0.51
ρ-Methylacetophenone	1185	1751	0.17
ρ-Cymen-8-ol	1188	1843	0.16
Myrtenal	1197	1604	0.13
Verbenon	1210	1675	0.30
Carveol	1223	1828	0.52
Carvone	1247	1710	0.89
Total			99.86

* Compounds are listed in the 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.

are frequently treated by the oral administration of ketoconazole; however, the toxicity and unpleasant side effects of this antibiotic such as nausea, abdominal pain, and itching, limit its therapeutic use. In this study, the essential oil from *E. globulus* and its main component, 1,8-cineol were evaluated for their efficacy as antifungal agents against three *Candia* and six *Trichophyton* species.

GC-MS analysis was used to determine the composition of *E. globulus* oil (Table 1). The predominant component of the oil was identified as 1,8-cineole; this monoterpene oxide accounted for 81.3% of the total essential oil components. *p*-Cymene (6.89%), α-pinene (2.41%), and α-terpineol (2.27%) were also identified as less abundant components. The large proportional contribution of 1,8-cineole suggests that this compound could be significant to the activity of the total oil fraction. In many cases, the composition of the essential oil varies depending on the plant. For example, the content of 1,8-cineole in Eucalyptus oil exhibits remarkable fluctuation depending on the developmental stage of the leaves.

As listed in Table 2, the susceptibility of *Candida* (250~4000 μg/mL) and *Trichophyton* (31~500 μg/mL) fungi to *E. globulus* essential oil or 1,8-cineol alone differed distinctly depending upon the species; however, they resulted in similar MICs (8~16 μg/mL) when they were treated with ketoconazole alone. Among the tested *Candida* species, *C. albicans* exhibited a relatively higher MIC than the other two *Candida* species when it was treated with *E. globulus* oil or 1,8-cineol alone. The MICs of the *Trichophyton* species were in agreement with the previously reported data (Shin and Lim, 2004). Following the determination of the MICs, checkerboard microtiter tests were used to assess the combined effects of the essential oil and ketoconazole against three *Candida* and six *Trichophyton* species. The fractional inhibitory concentration indices (FICIs) against the tested *Candida* species ranged between 0.09 and 0.38 for ketoconazole combined with *E. globulus* essential oil or 1,8-cineole, indicating significant synergism between ketoconazole and the oil samples. Similar experiments using *Trichophyton* species resulted in FICIs between 0.28 and 0.63 indicating relatively weaker combined effects than those against *Candida* species. Thus, the data reported in this study show that the anti-*Candida* effects of ketoconazole can be significantly improved by the use of *E. globulus* essential oil or 1, 8-cineole.

An isobologram plot for ketoconazole combined with the *E. globulus* oil fraction or 1,8-cineol against *Candida* species revealed a curve that distinctly deviated to the left (Fig. 1), confirming the presence of synergistic anti-fungal activity (Davidson and Parish, 1989).

In conclusion, these data indicate that components of the essential oil fraction from *E. globules* or 1,8-cineole may be useful agents in the treatment of *Candida* and *Trichophyton* infections. In particular, the synergistic anti-*Candida* effects of the *E. globulus* essential oil or 1,8-cineol with ketoconazole could facilitate the use of lower concentrations of this antibiotic; thus, minimizing its potential side effects. This approach could also provide alternative therapies to overcome the current limitations of the use of ketoconazole for the treatment of fungal infections. Further studies will be required to evaluate the clinical viability of these potential therapies.

Table 2. FICs (fractional inhibitory concentrations) and FICIs (FIC indices) for combinations of ketoconazole and *E. globulus* oil or 1,8-cineol against *Candida* and *Trichophyton* spp

Sample	*C. albicans*			*C. tropicalis*			*C. utilis*		
	MICc/MICo	FIC	FICI	MICc/MICo	FIC	FICI	MICc/MICo	FIC	FICI
E. globules oil	125/4000	0.03	0.09	250/2000	0.13	0.38	250/2000	0.13	0.19
Ketoconazole	1/16	0.06		2/16.	0.25		1/16	0.06	
1,8-Cineol	62.5/2000	0.03	0.09	250/2000	0.13	0.16	250/2000	0.13	0.19
Ketoconazole	1/16	0.06		0.5/16	0.03		1/16	0.06	
Sample	*T. erinacei*			*T. mentagrophytes*			*T. rubrum*		
	MICc/MICo	FIC	FICI	MICc/MICo	FIC	FICI	MICc/MICo	FIC	FICI
E. globulus oil	62.5/125	0.50	0.53	15.5/31.3	0.50	0.63	15.5/62.5	0.25	0.28
Ketoconazole	0.5/16	0.03		2/16	0.13		0.5/16	0.03	
1,8-Cineol	125/250	0.50	0.53	62.5/125	0.50	0.53	31.3/62.5	0.50	0.53
Ketoconazole	0.5/16	0.03		0.5/16	0.03		0.5/16	0.03	
Sample	*T. schoenleinii*			*T. soudanense*			*T. tonsurans*		
	MICc/MICo	FIC	FICI	MICc/MICo	FIC	FICI	MICc/MICo	FIC	FICI
E. globulus oil	62.5/125	0.50	0.56	62.5/250	0.25	0.28	62.5/125	0.50	0.52
Ketoconazole	0.5/8	0.06		0.5/16	0.03		0.25/16	0.02	
1,8-Cineol	250/500	0.50	0.56	250/500	0.50	0.56	250/500	0.50	0.52
Ketoconazole	0.5/8	0.06		1/16	0.06		0.25/16	0.02	

MICo = MIC (μg/mL) of one sample alone; MICc = MIC of the most effective sample combination; FIC = MICc/MICo; FICI = FIC of oil sample + FIC of ketoconazole

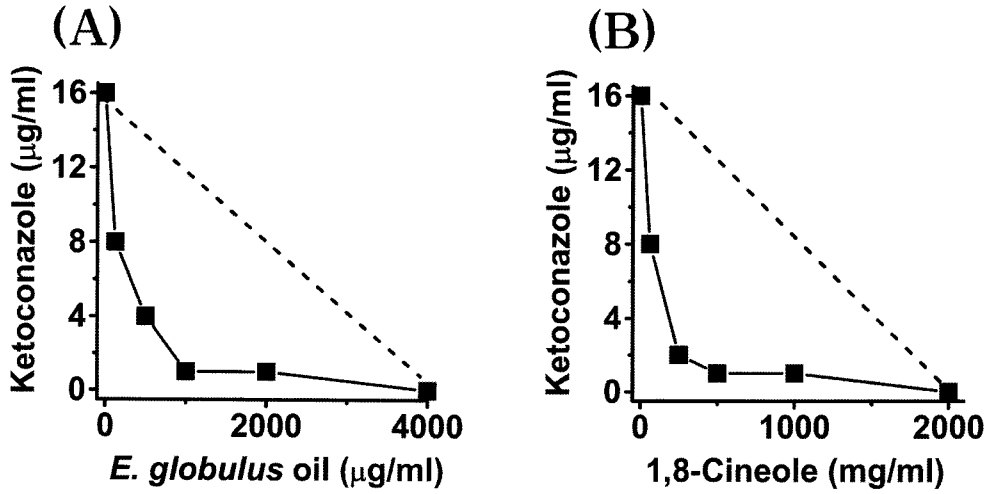


Fig. 1. Isobolograms of ketoconazole combined with *E. globulus* oil (A), or its main component, 1,8-cineolel (B) against *C. albicans*. The curves were constructed by plotting the concentration of the well that exhibited the most advantageous combination of the oil sample and ketocoanzole on the checkerboard titer tests compared to the control representing the sum of the effects of the two samples independently.

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