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Antifungal Activities of Essential Oils from *Glehnia littoralis* Alone and in Combination with Ketoconazole

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Abstract – The antifungal activities of essential oils from the leaves of *Glehnia littoralis,* which is cultivated in Korea, were evaluated against pathogenic *Trichophyton* species by the broth dilution method and the disk diffusion test. Additionally, the effects of the oils together with ketoconazole were tested by the checkerboard titer test. The essential oil fraction and its main components showed significant inhibition of the tested *Trichophyton* fungi, with minimal inhibitor concentrations (MICs) in the range of 16-32 mg/ml. The results suggest that activities of this oil are based mainly on the contents of α-pinene (22.17%), the next prominent component of the oil fraction, while the first main components β-pinene (57.83%) have relatively mild activity. The MICs of α-pinene and β-pinene were 1-4 mg/ml and 4-32 mg/ml, respectively. Additionally the *Glehnia* oil fraction and its main components as well, exhibited significant synergism with ketoconazole against *Trichophyton rubrum.*
Keywords – *Glehnia littoralis,* essential oils, *Trichophyton,* ketoconazole, synergism

Introduction

Many volatile oils are potent sources of natural antifungal chemicals even though they have relatively mild activities compared to the present synthetic therapeutics (Bidlack *et al.,* et al., 2000; Casssella *et al.,* 2002; Cimanga *et al.*, 2002; Giamperi, *et al.,* 2002; Shin and Kang, 2003). Especially, they are advocated as one of the important sources of new natural antifungal agents for treatment of various dermatomycosis (Hammer *et al.,* 2000; Giordani *et al.,* 2001; Harris, 2002; Shin, 2004).

Glehnia littoralis, one of the important traditional medicines, which has been used as for the treatment of various respiratory disease and pruritus by various dermal infections as well. As active compounds of the roots of this plant furanocoumarins, polyines, and essential oils have been reported. (Matsuura *et al.,* 1996; Seo, 1977; McCutcheon *et al.,* 1997, McMichael, 2001; Miyazawa *et al.,* 2001a; Miyazawa *et al.,* 2001b). The raw leaves of this plant also are eaten as a seasoned vegetable and for protection of the palsy.

In this study the composition of essential oils in the leaves of *G littoralis* was analyzed by gas chromatography-mass spectrometry and the antifungal activities of essential oils were evaluated against five *Trichophyton* species. Additionally, the synergistic effects of the oils in combination with ketoconazole were tested by the checkerboard titer test.

Trichophyton is a fungal species that causes superficial mycoses commonly known as tinea infections in various areas in humans and other animals (Inouye *et al.,* 2001; Patra *et al.,* 2002; Shin, 2003). Ketoconazole is one of the commonly used antifungal drugs administered orally for the treatment of both superficial and deep infections caused by *Trichophyton.* However, the unpleasant side effects of this drug include nausea, abdominal pain and itching, and its toxicity limits its therapeutic use in many cases. Moreover, the therapeutic response may be slow, and thus inappropriate for treatment of patients with severe or rapidly progressive mycoses. In many cases, the efficacy of antifungal therapeutics is poor in immuno-suppressed patients and in the treatment of meningitis (Groll *et al.*, 2001; Eggimann *et al.*, 2003).

Materials and Methods

Analysis of essential oils from *G littoralis* – The essential oils were obtained by steam distillation for five hours in a simultaneous steam distillation-extraction apparatus (SDE) from the leaves of *G littoralis* cultivated in Jeju-Do and harvested in April. The essential oil fractions were analyzed by the Hewlett-Packard 6890 GC and the Hewlett-Packard 5973 MSD apparatus (Agilent

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5973 network mass selective detector, 280°C) with a Ultra 2 (5% phenylmethylsiloxane, 50 m×200 μm×0.11 μm) fused silica capillary column. The injector was adjusted to 250°C and the oven temperature was constructed as follows: Initial temperature: 60°C for 5 min, 2°C/min up to 230°C, and then 30 min at 180°C

Strains – *Trichophyton tonsurans* ATCC *10217, T. rubrum* ATCC 6345, *T. schoenlienii* KCCM 60447, *T. erinacei* KCCM 60441 and *T. soudanense* KCCM 60448, were subdivided from the Korean Culture Center of Microorganisms (KCCM) and 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 match the 0.5 McFarland standard (10^5-10^6 colony forming units (CFU)/ml).

Standards-α – pinene (98%), β,-pinene (99%), and ketoconazole (98.8%) were purchased from Sigma Chemical Co.

Determination of minimal inhibitory concentration (MIC) – Samples were serially diluted with YM broth to obtain solutions with 0.09 mg/ml to 25.00 mg/ml essential oil; 10 μl of Tween 80 was added to each solution. After shaking, 100 μl aliquots of the essential oil solutions were added to the wells of 96-well microtiter plates. A 100 μl suspension of each of the fungal species was adjusted to 10^4-10^5 CFU, and then added to individual wells and cultivated at 25°C. The MIC was defined as the lowest concentration that inhibited more than 50% of visible fungal growth after 72 h. Each organism was also cultured with a blank solution containing Tween 80 at concentrations equivalent to those in the test solutions.

Disk diffusion assay – Fungal broth culture aliquots were added to Sabouraud dextrose agar medium and uniformly distributed. Sterile paper disks (8 mm, Advantec, Toyo Roxhi Kaisha) were impregnated with 50 μl of ethanol solutions of the oils (6.25 to 25 mg/disk) and ketoconazole (20-100 μg/disk), and after alcohol evaporation the disks were placed on the culture plates. The diameter of the zone of inhibition (mm) around the disk was measured after cultivation at 25°C for 72 h. The values shown are the means of tests performed in triplicate.

Checkerboard titer test – To evaluate the combined effects of the essential oil compounds, ten serial two-fold dilutions of α-pinene, β-pinene or the essential oil fraction of *G. littoralis* with the culture medium containing Tween 80 and eight serial two-fold dilutions of ketoconazole with DMSO (dimethyl sulfoxide) were prepared using the same solvents as in the MIC tests. Aliquots of 50 μl of each *G. littoralis* oil dilution were added to the wells of a 96-well plate in a vertical orientation, and 10 μl aliquots of each ketoconazole dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. Following this, each well was inoculated with 100 μl (approximately 5×10^4 CFU/well) of one of the *Trichophyton* fungal suspensions and cultivated at 25°C. Fractional inhibitory concentrations (FICs) were calculated by dividing the MICs of the oil and ketoconazole combinations by the MICs of the oil or ketoconazole alone. The FIC index, obtained by adding both FICs, was interpreted as indicating a synergistic effect when it was ≤ 0.5, as additive or indifferent when it was >0.5 and ≤ 2.0, and as antagonistic when it was >2.0 (Davidson *et al.*, 1989; White *et al.*, 1996). An

Table 1. Identified compounds in the essential oil fraction of *G. littoralis*

Compounds	RI *	Peak area (%)
α-pinene	936	22.17
camphene	942	0.76
β-pinene	968	57.83
myrcene	998	2.95
α-terpinene	1013	0.07
β-phellandrene	1026	4.87
benzene acetaldehyde	1035	0.07
γ-terpinene	1047	0.56
α-terpinolene	1074	0.26
α-terpineol	1185	0.78
(−)-bornyl acetate	1280	0.56
trans-pinocarveyl acetate	1293	0.05
δ-elemene	1331	0.24
α-copaene	1369	0.30
β-bourbonene	1377	0.03
β-cubebene	1383	0.13
β-elemene	1387	0.30
β-caryophyllene	1413	0.56
α-humulene	1445	0.06
d-germacrene	1478	3.34
α-copaene	1481	2.16
bicyclo germacrene	1491	0.12
β-elemene	1499	0.08
δ-cadinene	1501	0.28
β-germacrene	1548	0.24
2-nonylphenol	1551	0.09
oplopenone	1622	0.07
t-cadinol	1646	0.08
α-bisabolol	1680	0.06
In total		99.07

**RI* : GC retention indices calculated against C_9 to C_{24} *n*-alkanes on a Ultra 2 capillary column.

isobologram was constructed from the checkerboard data to depict the synergism of α-pinene, β-pinene or the essential oil fraction of *G. littoralis* with ketoconazole against *Trichophyton* species. The solvents DMSO and Tween 80 were used at concentrations equivalent to those in the test solutions to certify that these vehicles did not affect fungal growth.

Results and Discussion

From the fresh leaves of *G. littoralis*, 0.34% (w/w) of essential oil was obtained by steam distillation with an SDE apparatus and extraction with ether. The components of the essential oil fraction, identified by GC and GC-MS analyses, are listed in Table 1. By Wiley 275 Library search with the results of GC-MS and by GC with standards, 31 compounds were identified in the essential oil fraction of the *G. littoralis* leaves. The oil compositions were generally not consistent with previously reported compositions (Miyazawa *et al.*, 2001). In our experiments, this oil was characterized by high contents of non-oxygenated hydrocarbons (97.31%). The predominantly contained compound of this oils was β-pinene (57.83%) and α-pinene (22.27%) which accounted for about 90% of the oil. The contents of these compounds were much lower in the previous reports experimented with the same species cultivated in Japan. This discrepancy might be related to the different status (especially dryness) of the plant material for the steam distillation as well as the culture condition according to the climate or the soil.

As demonstrated in Table 2, the *Glehnia* oil and its main components, α-pinene and β-pinene, showed significant inhibition against the five tested *Trichophyton* fungi with MICs in the range of 1-32 mg/ml in the broth dilution test. Among the tested samples, α-pinene exhibited the highest activity while both of other two samples, β-pinene and the total essential oil fraction of *G. littoralis* showed similarly relatively mild inhibition against all of the tested fungi. Generally, there were no remarkable differences between the activities of the tested oils with respect to the species of *Trichophyton*. however, the oil showed slightly higher activity against *T. tonsurans*, *T. rubrum* and *T. soudanense* with a two-fold or more lower MIC compared to the MIC against *T. schoenlenii* or *T. erinacei*.

Growth inhibition by the oils on agar plates is presented in Table 3. Activities were mostly dose-dependent against all of the tested fungi. The tested samples inhibited the growth of the five *Trichophyton* fungi in a trend similar to the results of MIC assay

Table 2. MICs of the essential oil fraction (EOF) from *G. littoralis*, α-pinene, β-pinene, and ketoconazole against *Trichophyton* species as estimated by the broth microdilution method

Fungi	α-pinene*	β-pinene*	EOF*	Ket**
Trichophyton tonsurans	1	8	16	16
T. rubrum	1	8	16	16
T. schoenlienii	2	16	16	16
T. erinacei	4	32	32	32
T. soudanense	1	8	16	8

Values are the means from the triplicated experiments.
*MICs (mg/ ml) of α-,and β-pinene.
EOF : MICs (mg /ml) of essential oil fraction from *G. littoralis*,
**MICs (μg/ ml) of ketoconazole.

Table 3. Growth inhibition (mm)* of *Trichophyton* spp. on Sabouraud agar plates

Sample	Fungi	*Tto*	*Tru*	*Tsc*	*Ter*	*Tso*
G. littoralis oil	I	1	8	11	2	3
	II	0	3	6	1	1
α-pinene	I	25	15	23	19	21
	II	12	5	11	10	7
β-pinene	I	1	10	12	2	3
	II	0	4	7	1	1
ketoconazole	III	15	4	7	3	3
	IV	4	3	7	3	2

*Values are the means from the triplicated experiments. The width (mm) of growth inhibition of fungi was measured from the boundary of discs.
Tto: *T. tonsurans*, ***Tru***: *T. rubrum*, ***Tsc***: *T. schoenleinii*
Ter: *T. erinacei*, ***Tso***: *T. soudanense*
I: 25 mg/disk, **II:** 12.5 mg/disk, **III**: 50 μg/disk, **IV**: 25 μg/disk

Additionally, when calculated by the checkerboard titer test, *Glehnia* leaf oil, as well as α-pinene and β-pinene, exhibited significant synergism with ketoconazole especially against *T. rubrum*, resulting in fractional inhibitory concentration indices (FICI) of between 0.16 and 1.00 which indicated the MICs of ketoconazole were significantly lowered by the combination with oil. Furthermore, the results of the checkerboard titer test depicted in the isobologram in Fig. 1 confirm the synergism between *G. littoralis* oil and ketoconazole, as the combination of both compounds produced a curve that was to the left of the line (dotted) of the added effects of the two compounds employed separately. Thus, our results fulfill the criterion that defines synergism as "the activity observed when a combination is greater than the sum of the effects observed with the two agents independently" (White *et al.*, 1996). Therefore, in spite of their relatively mild activity, the essential oil from the

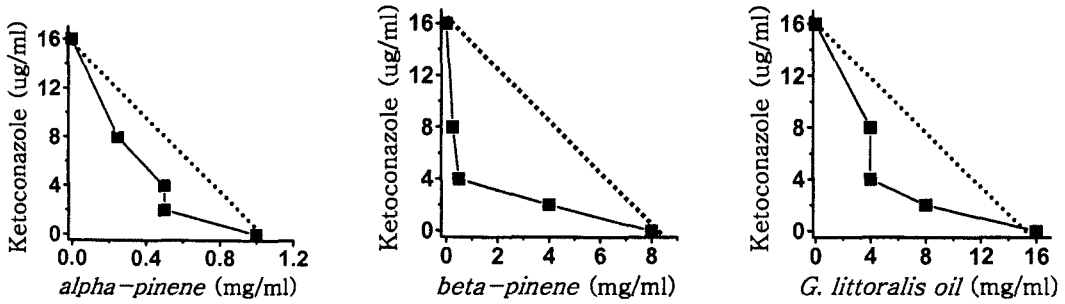

Fig. 1. Isobolograms indicating synergism of the essential oils (mg/ml) from (A) α-pinene, (B) β-pinene, or (C) the essential fraction from the leaves of *G. littoralis* in combination with ketoconazole against *T. rubrum*.. The curves were constructed by plotting with the concentrations in the wells which showed the most advantageous combination of the oil sample and ketoconazole on checkerboard titer tests and compared with the control (additive line: dotted) which describes the sum of the effects with two samples independently.

leaves from *G. littoralis* might be useful in antifungal therapy in combination with ketoconazole, especially against *T. rubrum*. However, further *in vivo* experiments are necessary to assess the potential for therapeutic application.

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