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Essential Oil Compounds from *Agastache rugosa* as Antifungal Agents Against *Trichophyton* Species

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The antifungal activities of the essential oil from *Agastache rugosa* and its main component, estragole, combined with ketoconazole, one of the azole antibiotics commonly used to treat infections caused by *Trichophyton* species, were evaluated in this study. The combined effects were measured by the checkerboard microtiter and the disk diffusion tests, against *T. erinacei, T. mentagrophytes, T. rubrum, T. schoenleinii* and *T. soudanense*. Susceptibility of the five *Trichophyton* species to the oil alone, or ketoconazole alone, differed distinctly. The fractional inhibitory concentration indices (FICI) of ketoconazole combined with estragole or *A. rugosa* essential oil, against the tested *Trichophyton* species, were between 0.05 and 0.27, indicating synergistic effects. These drug combinations exhibited the most significant synergism against *T. mentagrophytes,* with FICIs of 0.05 and 0.09 for estragole and the essential oil fraction from *A. rugosa,* respectively. Isobolograms based on the data from checkerboard titer tests also indicated significant synergism between ketoconazole and the *Agastache* oil fraction or estragole, against the *Trichophyton* species evaluated. *Trichophyton* susceptibility to ketoconazole was significantly improved by combination with the *Agastache rugosa* oil fraction or its main component, estragole.

Key words: *Trichophyton, Agastache rugosa*, Essential oil, Estragole, Ketoconazole, Synergism

INTRODUCTION

Essential oils are natural compounds which show great promise as a new prototype from which antifungal agents may be developed (Yoon *et al.*, 1994; Bidlack *et al.*, 2000; Faleiro *et al.*, 2003; Shin, 2003). *Agastache rugosa* O. Kuntze (Labiatae), a perennial herb ubiquitous in the fields of Korea, has been used as a wild vegetable and an herbal drug in traditional therapies. In a previous report (Shin and Kang, 2003), the essential oils of *Agastache rugosa* and estragole, the main component of this oil, showed significant synergism with ketoconazole against *Blastoschizomyces capitatus*.

Trichophyton species are pathogenic fungi that cause superficial mycoses, commonly known as tinea infections, in various tissues in humans and other animals. The anti-*Trichophyton* activity of various herbal essential oils has been examined in previous reports (Hammer *et al.*, 1998; Shahi *et al.*, 1999; Inouye *et al.*, 2001; Patra *et al.*, 2002). However, the essential oils had much weaker activity than the commonly used synthetic anti-*Trichophyton* drugs, including ketoconazole, which is associated with other problems in its therapeutic application.

In this study an evaluation was performed by the broth dilution method and disk diffusion test, on the antifungal activity of the essential oil from *Agastache rugosa* and its main component estragole, which comprises 49.42% of the oil (Shin and Kang, 2003), against five *Trichophyton* species.

Based on these results, checkerboard micro titer tests were performed and isobolograms were constructed, to determine the combined effect of *Agastache* essential oils and ketoconazole, in order to develop more effective and safer anti-*Trichophyton* therapy.

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MATERIALS AND METHODS

Preparation of the samples

The essential oil fraction of *A. rugosa* Kuntze was

obtained by steam distillation, using a simultaneous steam distillation-extraction apparatus, from the above ground parts of plants cultivated in the herbal garden of Duksung Women's University. Estragole was isolated from the essential oil fraction with silica gel 60 (0.063-0.100 mm) and LH-20 column chromatography and identified by comparison with the spectral data of the standard compound (Sigma, USA). Ketoconazole was obtained from Sigma-Aldrich Korea Ltd (Seoul, Korea).

Fungal strains

Five *Trichophyton* species were obtained from the Korean Culture Center of Microorganisms (KCCM). *T. erinacei* KCCM 60411, *T. mentagrophytes* KCCM 11950, *T. rubrum* ATCC 6345, *T. schoenleinii* KCCM 60477 and *T. soudanense* KCCM 60448 11866 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 match the 0.5 McFarland standard (10^5-10^6 colony forming units (CFU)/mL).

Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC)

Samples were serially diluted with YM broth to obtain solutions with 0.09 to 25.00 mg/mL essential oil; 10 μL of Tween 80, an emulsifier, 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 five *Trichophyton* 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. For evaluation of MFC, 50 uL aliquots from each cultured well were added to 150 uL of new medium and cultured for 168 h. Each organism was also cultured with a blank solution containing Tween 80, at concentrations equivalent to those in the test solutions, to certify that these vehicles did not affect fungal growth.

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). 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 and SDs of tests performed in triplicate. The effect of the combination was measured from estragole alone (25 mg/disk), the mixture of estragole (25 mg/disk) and ketoconazole (50 μg/disk), and ketoconazole alone (50 μg/disk).

Checkerboard titer test

To evaluate the combined effects of the essential oil compounds, ten serial two-fold dilutions of estragole or the essential oil fraction of *A. rugosa* 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 *A. rugosa* 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 estragole and ketoconazole combinations by the MICs of estragole 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 (White *et al.,* 1996). An isobologram was constructed from the checkerboard data to depict the synergism of estragole or *A. rugosa* oil 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

In this study, the essential oil from *A. rugosa* was evaluated for use as an antifungal agent against *Trichophyton* species.

The MICs and MFCs of the *A. rugosa* oil fraction, estragole and ketoconazole against the *Trichophyton* species, tested by the broth dilution assay, are listed in Table I. Both estragole and the *A. rugosa* oil fraction showed significant activity against all five species of *Trichophyton.* The MICs ranged from 0.39-3.12 mg/mL, indicating notably different susceptibility among the species. The MICs of the oil fraction were generally higher than the MICs of estragole. This finding is consistent with a previous report by Shin and Kang (2003), indicating that the activity of the oil fraction is due largely to estragole, which makes up half of the oil fraction, while the other constituents have a relatively mild activity. The MFCs of the oils against the fungi tested were more than twice the MICs. Ketoconazole had a much higher activity than either estragole or *A. rugosa* oil, with MICs ranging between 3.12 and 12.5 μg/mL. However, the MFC/MIC ratio was much higher for ketoconazole than for the *A.rugosa* oils, indicating it is an

Table I. MICs (minimal inhibiting concentrations) and MFCs (minimal fungicidal concentrations) of estragole, *A. rugosa* essential oil and ketoconazole against *Trichophyton* species

Fungi \ Sample	Estragole (mg/mL)		*A. rugosa* oil (mg/mL)		Ketoconazole (μg/mL)	
	MIC	MFC	MIC	MFC	MIC	MFC
T. erinacei	0.39	1.56	0.78	12.50	12.50	>25.00
T. mentagrophytes	1.56	3.12	3.12	50.00	6.25	>25.00
T. rubrum	1.56	3.12	1.56	25.00	6.25	>25.00
T. schoenleinii	0.78	1.56	1.56	12.50	6.25	>25.00
T. soudanense	0.78	6.25	1.56	25.00	3.12	>25.00

effective fungistatic but a poor fungicidal agent. No relationship was detected between the results from the oils and ketoconazole alone, which may be due to the different antifungal mechanisms of these two groups of agents (Sugar *et al.*, 1987; Nidiry, 1998; Giordani *et al.*, 2001). Tween 80 and DMSO, at levels equivalent to those in the test compound solutions, did not affect the growth of the fungi investigated.

The anti-*Trichophyton* activities measured by the disk diffusion test are demonstrated in Table II. The data demonstrates the significant activity of the *A. rugosa* oil fraction and its main component estragole, as shown previously by the broth dilution method. Generally, estragole was more potent at inhibiting fungal growth than the oil fraction. The antifungal activities against the *Trichophyton* species differed but were not correlated with the MICs. The fungal inhibition of estragole and the total oil fraction differed significantly and the inhibition was dose-dependent.

Checkerboard titer tests were performed subsequently to calculate the combined effects between these oils and ketoconazole. As shown in Table III, the FICIs for ketoconazole combined with estragole or *A. rugosa* essential oil, against all of the tested *Trichophyton* species, were between 0.05 and 0.27, indicating strong synergism between the compounds. The synergism was confirmed again by the plotted curves deviating distinctly to the left (Davidson and Parish, 1989) as depicted in Fig. 1 and Fig. 2.

Table III. FIC and FIC indices (FICI), in combination with ketoconazole (Ke) and estragole (ES) or the essential oil fraction (Ar) of *A. rugosa*, against *Trichophyton* species

Fungi \ Sample		Es-Ke		Ar-Ke	
T. erinacei	FIC	0.12	0.01	0.24	0.03
	FICI	0.13		0.27	
T. mentagrophytes	FIC	0.02	0.03	0.06	0.03
	FICI	0.05		0.09	
T. rubrum	FIC	0.05	0.06	0.05	0.06
	FICI	0.11		0.11	
T. schoenleinii	FIC	0.24	0.03	0.12	0.03
	FICI	0.27		0.15	
T. soudanense	FIC	0.10	0.06	0.05	0.05
	FICI	0.16		0.10	

*FIC: Fractional inhibitory concentration
FICI = FIC of estragole + FIC of ketoconazole

Among the tested *Trichophyton* species, the notable control of *T. mentagrophytes* exhibited the remarkable synergism between estragole with ketoconazole, with an FICI of 0.05. Based on this result, a disk diffusion test with the mixture of estragole and ketoconazole against *T. mentagrophytes* was constructed, to demonstrate the combined effects on agar plates. As expected, the inhibited fungal zone increased dramatically in response to the mixture of estragole (25 mg/disk) and ketoconazole (50 μg/disk), compared to the zones resulting from these

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

Sample \ Fungi		*T.erinacei*	*T. mentagrophytes*	*T. rubrum*	*T. schoenleinii*	*T. soudanense*
A. rugosa oil	I	4.6 ± 0.57	3.6 ± 0.57	4.0 ± 1.00	5.0 ± 1.00	7.6 ± 0.57
	II	2.6 ± 0.57	2.3 ± 0.57	2.3 ± 0.57	1.6 ± 0.57	4.0 ± 0.00
	III	1.3 ± 0.57	1.0 ± 0.00	1.3 ± 0.57	1.0 ± 0.00	2.3 ± 0.57
Estragole	I	15.0 ± 0.00	12.0 ± 1.00	8.0 ± 1.52	11.3 ± 1.52	10.6 ± 1.52
	II	7.3 ± 1.52	7.0 ± 1.00	5.3 ± 0.57	5.0 ± 1.00	7.3 ± 0.57
	III	4.3 ± 1.15	2.6 ± 0.57	3.3 ± 0.57	2.3 ± 0.57	4.3 ± 0.57
Ketoconazole	IV	7.6 ± 1.52	7.6 ± 0.57	11.3 ± 1.52	8.0 ± 1.00	18.6 ± 2.08
	V	5.3 ± 0.57	4.3 ± 1.15	4.6 ± 1.52	3.6 ± 0.57	12.3 ± 1.52
	VI	3.6 ± 1.15	3.3 ± 1.52	2.3 ± 0.57	2.0 ± 0.00	6.6 ± 1.52

*The values are the means ± SD of triplicate data.
: 25 mg/disk, II: 12.5 mg/disk, III: 6.25 mg/disk, IV: 100 μg/disk, V: 50 μg/disk, VI: 20 μg/disk

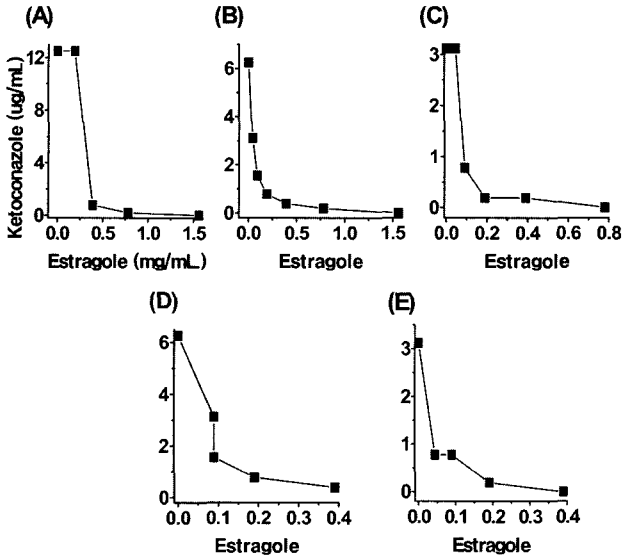

Fig. 1. Isobolograms revealing the synergistic effects of estragole (mg/mL) in combination with ketoconazole (μg/mL), on growth inhibition of *T. erinacei* (A), *T. mentagrophytes* (B), *T. rubrum* (C), *T. schoenleinii* (D), and *T. soudanense* (E). The curves were depicted with the concentrations of ketoconazole and estragole, in the wells which showed the most advantageous combination for fungal inhibition in checkerboard micro titer tests.

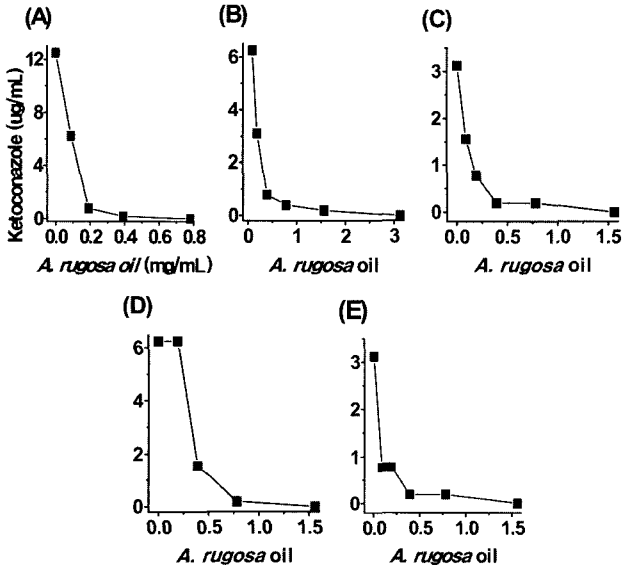

Fig. 2. Isobolograms revealing the synergistic effects of the essential oil fraction (mg/mL) from *A. rugosa* in combination with ketoconazole (μg/mL), on growth inhibition of *T. erinacei* (A), *T. mentagrophytes* (B), *T. rubrum* (C), *T. schoenleinii* (D), and *T. soudanense* (E). The curves were depicted with the concentrations of ketoconazole and the essential oil fraction of *A. rugosa* , in the wells which showed the most advantageous combination for the fungal inhibition in checkerboard micro titer tests.

compounds alone (Fig. 3).

In summary, it is concluded that estragole and the

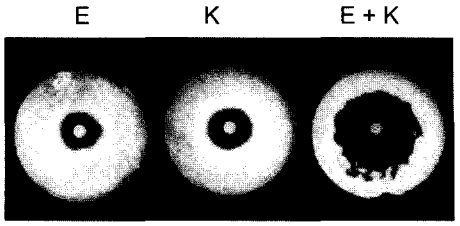

Fig. 3. Growth inhibition zones of *T. mentagrophytes* by disk diffusion test on Sabouraud agar plates. E; estragole alone (25mg/disk), K; ketoconazole alone (50 μg/disk), E+K; ketoconazole with estragole (25 mg/disk and 50 μg/disk, respectively).

essential oil fraction from *A. rugosa* might be useful anti-*Trichophyton* agents, especially in combination with ketoconazole. However, further studies will be required to determine the therapeutic applicability.

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REFERENCES

Bidlack, W. R., Omaye, S. T., Meskin, M. S., and Topham, D., Phytochemicals as bioactive agents. Technomic Publishing Company, Lancaster, pp. 106-110 (2000).

Davidson, P. M. and Parish, M. E., Methods for testing the efficacy of food antimicrobials. *Food Technol.*, 43, 148-155 (1989).

Faleiro, M. L., Miguel, M. G., Ladeiro, F., Venâncio, F., Tavares, R., Brito, J. C., Figueiredo, A. C., Barroso, J. G., and Pedro, L. G., Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Lett. Appl. Microb.*, 36, 35-40 (2003).

Giordani, R., Trebaux, J., Masi, M., and Regli, P., Enhanced antifungal activity of ketoconazole by *Euphorbia characias* latex against *Trichophyton albicans*. *J. Ethnopharmacol.*, 78, 1-5 (2001).

Hammer, K., Carson, C. F., and Riley, T. V., *In-vitro* activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Trichophyton* spp. *J. Antimicrob. Chemother.*, 42, 591-595 (1998).

Inouye, S., Uchida, K., and Yamaguchi, H., *In vitro* and *in vivo* anti-*Trichophyton* activity of essential oils by vapor contact. *Mycoses*, 44, 99-107 (2001).

Nidiry, E. S., Structure-fungitoxicity relationships of the monoterpenoids of the essential oils of Peppermint (*Mentha piperita*) and scented geranium (*Pelargonium graveolens*). *J. Essent. Oil Res.*, 10, 628-632 (1998).

Patra, M., Shahi, S. K., Midgely, G., and Dikshit, A., Utilization of essential oil as natural antifungal against nail-infective fungi.

Flavour Frag. J., 17, 91-94 (2002).

Shahi, S. K., Shukla, A. C., Bajaj, A. K., Medgely, G., and Dikshit, A., Broad spectrum antimycotic drug for the control of fungal infection in human beings. *Curr. Sci.*, 76, 836-839 (1999).

Shin, S. and Kang, C. A., Antifungal activity of the essential oil of *Agastache rugosa* Kuntze and its synergism with ketoconazole. *Lett. Appl. Microbiol.*, 36, 111-115 (2003).

Shin, S., Anti-*Aspergillus* activities of plant essential oils and their combination effects with ketoconazole or amphotericin B. *Arch. Pharm, Res.*, 26, 389-393 (2003).

Sugar, A. M., Alsip, S. G., Galgiani, J. N., Graybill, J. R., Dismukes, W. E., Claud, G. A., Craven, P. C., and Stevens, D. A., Pharmacology and toxicity of high-dose ketoconazole. *Antimicrob. Agents Chemother.*, 31, 1874-1878 (1987).

White, R. L., Burgess, D. S., Manduru, M., and Bosso, J. A., Comparison of three different in vitro methods of detecting synergy: timekill, checkerboard, and E test. *Antimicrob. Agents Chemother.*, 40, 1914-1918 (1996).

Yoon, S. Y., Eo, S. K., Lee, D. K., and Han S. S., Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics. *Arch. Pharm. Res.*, 6, 438-442 (1994).