Letters in Applied Microbiology 2003, **36**, 111–115

Antifungal activity of the essential oil of *Agastache rugosa* Kuntze and its synergism with ketoconazole

S. Shin and C.-A. Kang

College of Pharmacy, Duksung Women's University, Ssangmoondong 419, Dobongku, Seoul, Korea

2002/247: received 29 July 2002, revised 13 November 2002 and accepted 21 November 2002

ABSTRACT

S. SHIN AND C.-A. KANG. 2003.

Aims: To evaluate the fungitoxic activity of the essential oil of *Agastache rugosa* alone and to determine its combination effect with ketoconazole against *Blastoschizomyces capitatus*.

Methods and Results: The antifungal activities of the essential oil of *A. rugosa* and its main constituent estragole were investigated using the broth microdilution, disk diffusion methods and checkerboard microtitre assay. Both estragole and the essential oil exhibited strong activities against the tested fungi and showed synergism with ketoconazole against *B. capitatus*.

Conclusions: Both estragole and the essential oil of *A. rugosa* have significant growth-inhibiting activities against *B. capitatus* showing strong synergistic effect with ketoconazole.

Significance and Impact of the Study: The essential oil of *A. rugosa*, combined with ketoconazole, may be particularly useful against *B. capitatus*, a rare pathogenic fungus documented to cause severe and fatal mycoses in immunocompromised patients.

Keywords: *Agastache rugosa*, antifungal activity, *Blastoschizomyces capitatus*, essential oil, ketoconazole, synergism.

INTRODUCTION

Higher plants with activity against human pathogenic fungi are of interest because present antifungal therapeutics is often toxic (Sugar *et al.* 1987), induce problematic drug–drug interactions (Von Moltke *et al.* 1996) and become non-effective when resistance develops (Metzger and Hoffmann 1997). Given these problems, the development of natural antifungal agents is an attractive objective (Hammer *et al.* 1998; Giordani *et al.* 2001).

Essential oils from plants are a promising source for novel natural antifungal drugs, although their activity against human pathogenic fungi is generally milder than commercial synthetic antifungal drugs (Garg and Siddiqui 1992; Gundidza 1993; Shin and Kang 2001). Combining natural products with synthetic drugs to improve efficacy has been investigated; *Euphorbia characias* latex enhances the antifungal activity of ketoconazole (Giordani *et al.* 2001). Moreover, santolina oil exhibits synergistic effect with clotrimazole against *Candida albicans* (Suresh *et al.* 1997) and anethole, combined with miconazole and amphotericin B, has synergistic activity (Lee and Kim 1999). *Agastache rugosa* (Labiatae), a perennial herb ubiquitous in Korean fields, has been used as a wild vegetable and herbal drug for the treatment of anorexia, vomiting and other intestinal disorders (Ahn and Yang 1991; Wilson *et al.* 1992; Svoboda *et al.* 1995). This plant is increasingly cultivated in Korea to satisfy the rising demand for its essential oil by aroma therapists and herbalists. Here we extend these findings by assessing the antifungal activity of the essential oil of *A. rugosa* and estragole, the main component of this oil, against 10 fungi or yeasts responsible for severe human mycoses. We also examined the synergistic effect of combining the essential oil of *A. rugosa* and estragole with ketoconazole, an azole antifungal drug.

Correspondence to: S. Shin, College of Pharmacy, Duksung Women's University, Ssangmoondong 419, Dobongku, Seoul, 132-714, Korea (e-mail: swshin@duksung.ac.kr).

MATERIALS AND METHODS

Sample preparation for testing antifungal activities and fungal strains

The essential oil of *A. rugosa* Kuntze was obtained by steam distillation using a simultaneous steam distillation–extraction apparatus. The above ground parts of plants cultivated in the herbal garden of Duksung Women's University were used. Voucher specimens have been deposited at the herbarium of Duksung Women's University (No. LABA3). Estragole (A29208) and ketoconazole (K1003) were purchased from Sigma-Aldrich Korea Ltd (Seoul, Korea). The fungal organisms were provided by the Korean Culture Center of Microorganisms (KCCM). *Aspergillus niger* KCCM 11239, *A. flavus* KCCM 11453, *Trichoderma viride* KCCM 11246, *C. albicans* KCCM11282, *C. utilis* KCCM11356, *C. tropicalis* KCCM12578, *Cryptococcus neoformans* KCCM 50564, *Trichosporon mucoides* KCCM50570, *Trichophyton tonsurans* KCCM 11866 and *B. capitatus* KCCM50270 were cultured in yeast and malt extract (YM) broth or malt extract liquid medium for 48 h at 25 °C. The turbidity of the cell suspension, measured at 600 nm, was adjusted with medium to match the 0·5 McFarland standard.

Gas chromatography–mass spectrometry of the essential oil

The composition of the essential oil of *A. rugosa* was analyzed by gas chromatography–mass spectrometry (GC–MS) on a Hewlett–Packard 6890 GC and Hewlett–Packard 5973 MSD apparatus (Agilent, Palo Alto, CA, USA) using an HP-5 capillary column (30 m × 250 μm × 0·25 μm). The temperature program consisted of an initial temperature of 70 °C (isothermal, 5 min) that was first increased by 3 °C min^{-1} to 180 °C and then to 270 °C by 20 °C min^{-1}. The final temperature was 270 °C with a hold time of 10 min.

Minimal inhibitory concentration

The minimal inhibitory concentrations (MICs) of the antifungal agents against the various fungi were determined by the broth microdilution method (Marchetti *et al.* 2000). The Agastache oil and estragole were serially diluted with 10% dimethyl sulfoxide (DMSO) and added with 10 μl of Tween 80 to prepare the solutions, which contained 160–0·625 mg ml^{-1} of oils, respectively. Ketoconazole was similarly diluted in DMSO to generate a series of concentrations ranging from 100 to 0·78 μg ml^{-1} per testing well. After shaking, 100 μl of the antifungal agent solutions were added to the wells of 96-well plates. The suspension of each organism was adjusted to 10^4–10^5 CFU ml^{-1} and then added to the individual wells at 100 μl (*ca.* 5×10^3 CFU per well) and cultivated at 24–28 °C. The MIC was defined as the lowest concentration that completely inhibited visible fungal growth in the wells after 72 h of incubation. Each organism was also cultured with a control solution containing Tween 80 and DMSO at levels equivalent to those in the test compound solutions to certify that they did not affect fungal growth. The tests were performed in triplicate to confirm the values.

Disk diffusion assay

Fungal broth culture aliquots adjusted to *ca.* 5×10^4 CFU ml^{-1} were added to Sabouraud dextrose agar medium and distributed uniformly. Sterile paper disks (8 mm; Advantec, Toyo Roshi Kaisha, Tokyo, Japan) were impregnated with 50 μl of 25% (v/v, 12·5 mg) or 50% (v/v, 25·0 mg) ethanol and antifungal solution and placed on the culture plates after removing ethanol by evaporation. The diameter of the zone of inhibition (mm) around the disk was measured after cultivation at 24–28 °C for 2 days. The values shown (Table 2) are the means of tests performed in triplicate.

Checkerboard titer test and construction of isobolograms

Eight serial twofold dilutions of estragole and ketoconazole were prepared with the same solvents used in the MIC tests. Fifty-microlitre aliquots of each estragole dilution were added to the wells of 96-well plates 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. A 100-μl suspension of *B. capitatus* (*ca.* 5×10^3 CFU per well) was added to each well and cultured for 3 days. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of estragole and ketoconazole divided by the MIC of estragole or ketoconazole alone. The FIC index was calculated by adding both FICs and was interpreted as a synergistic effect when it was $\leq$0·5, as additive or indifferent when it was >0·5–2·0 and as antagonistic when it was >2·0 (Davidson *et al.* 1989). An isobologram was constructed on the basis of the checkerboard experiment to depict the synergism of estragole with ketoconazole against *B. capitatus*. A checkerboard experiment was also performed to determine the effect of combining the essential oil fraction of *A. rugosa* with ketoconazole.

RESULTS

Composition of the essential oil of *Agastache rugosa*

Estragole (49·42%) was the most prominent compound in *A. rugosa* oil, as previously reported. The hydrocarbons

limonene (12·52%) and β-caryophyllene (6·49%) were the next most prevalent components (Dung *et al.* 1996). As hydrocarbons generally are biologically less active than oxygenated compounds, we did not evaluate their antifungal activity.

Estragole and *Agastache rugosa* oil MICs

As shown in Table 1, estragole and the essential oil of *A. rugosa* showed antifungal activities against all the fungi. Estragole showed the strongest activity against *T. viride*, *C. albicans*, *C. neoformans* and *B. capitatus*, with MICs of 2·5 mg ml^{-1}. The fungicidal activity against *A. niger*, *A. flavus*, *C. utilis*, *C. tropicalis* and *T. mucoides* (MICs of 5·0 mg ml^{-1}) was moderate and relatively poor against *T. tonsurans* (MIC of 10 mg ml^{-1}). The MICs of the essential oil of *A. rugosa* were generally lower than those of estragole for most of the fungi. This finding suggests that the activity of the oil fraction is based mostly on estragole, which makes up half of the oil fraction, while the other constituents have relatively mild activity. Ketoconazole had much higher activity than either estragole or *A. rugosa* oil, with MICs ranging between 12·5 and 25·0 μg ml^{-1}.

Tween 80 and DMSO at levels equivalent to those in the test compound solutions did not affect the growth of the fungi investigated.

Disk diffusion assay

The fungi were also tested for susceptibility to the compounds using the disk diffusion assay (Table 2). Estragole and *A. rugosa* oil significantly and dose-dependently inhibited the growth of all the fungi in a trend similar to the inhibition measured by the MIC assay. Both estragole and *A. rugosa* oil were particularly active against *A. flavus* and *B. capitatus*. They produced zones of inhibition of 4–9 mm in diameter.

Table 1 Minimal inhibitory concentrations (MICs) of estragole, the essential oil fraction of *Agastache rugosa* and ketoconazole against 10 known human pathogenic fungi as estimated by the broth microdilution method

Fungi	Est*	AO†	Ket‡
Aspergillus niger	5·0	5·0	25·0
Aspergillus flavus	5·0	10·0	12·5
Trichoderma viride	2·5	5·0	25·0
Candida albicans	2·5	5·0	12·5
Candida utilis	5·0	5·0	12·5
Candida tropicalis	5·0	5·0	12·5
Cryptococcus neoformans	2·5	10·0	25·0
Trichosporon mucoides	5·0	5·0	12·5
Trichophyton tonsurans	10·0	10·0	25·0
Blastoschizomyces capitatus	2·5	5·0	25·0

*MICs (mg ml^{-1}) of estragole.
†MICs (mg ml^{-1}) of essential oil fraction of *A. rugosa*.
‡MICs (μg ml^{-1}) of ketoconazole.

Table 2 Antifungal activity of estragole and the essential oil fraction of *Agastache rugosa* as estimated by the disk diffusion method

	Diameter of inhibited zone (mm)			
	Estragole (mg)		AO (mg)*	
Fungi	12·5	25	12·5	25
Aspergillus niger	4	6	4	7
Aspergillus flavus	5	9	4	7
Trichoderma viride	3	7	2	5
Candida albicans	2	3	1	2
Candida utilis	2	4	1	3
Candida tropicalis	3	6	2	5
Cryptococcus neoformans	4	5	2	3
Trichosporon mucoides	5	7	2	4
Trichophyton tonsurans	2	4	1	2
Blastoschizomyces capitatus	5	9	4	8

*Essential oil fraction of *A. rugosa*.

Activity of oils combined with ketoconazole against *B. capitatus*

To test the activity of the combined agents, we used *B. capitatus* in both the MIC test and the disk diffusion assay because of its high susceptibility to estragole and *A. rugosa*. We assessed various combined concentrations of estragole and ketoconazole using the checkerboard titre assay. Ketoconazole, combined with estragole, caused a remarkable decrease in the MICs compared with each compound alone. An isobologram, constructed with data from the checkerboard titre assay, depicts the ketoconazole–estragole synergism in the curved deviation to the left (Fig. 1a). The essential oil of *A. rugosa* showed a similar synergistic effect with ketoconazole, producing an FIC index of 0·19 in another checkerboard titre assay. The isobologram of this test is shown in Fig. 1b.

DISCUSSION

These results indicate that the antifungal activity of *A. rugosa* oil is predominantly due to estragole, as was shown with basil oil (Lachowicz *et al.* 1998; Wan *et al.* 1998). Eugenol (Oger *et al.* 1994) and chavichol (Aurore *et al.* 1998) are two other components of *A. rugosa* oil reported to have strong antimicrobial properties and may

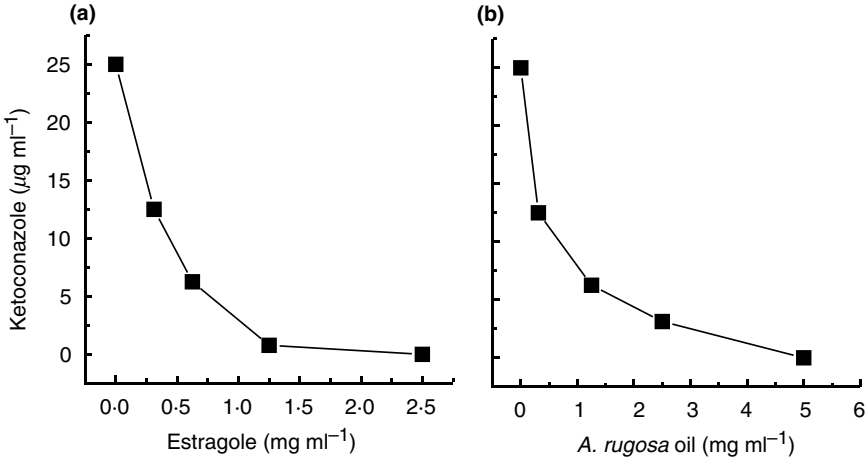

Fig. 1 Isobologram revealing the synergistic effect of combining (a) estragole or (b) *Agastche rugosa* oil with ketoconazole in inhibiting *Blastoschyzomyces capitatus* growth

contribute to the antifungal activity of the oil, although they are present in very low concentrations (1·0 and 0·9%, respectively).

The essential oil of *A. rugosa* showed lower fungistatic activity against most of the fungi, as measured by the broth dilution and disk diffusion methods, than estragole. Therefore, the activity of the oil fraction appears to be due mostly to estragole, which comprises half of the oil fraction, while the other components appear to have relatively mild activity.

The synergistic effects of the essential oil constituents of *A. rugosa* have not been investigated previously. These results show that the combination of ketoconazole and estragole remarkably reduces the MICs of each compound. Thus, the MIC of ketoconazole alone against *B. capitatus* was lowered from 25 to 0·78 μg ml^{-1} when estragole was added at a concentration of 1·25 mg ml^{-1}. The MIC of estragole also decreased from 2·5 to <0·31 mg ml^{-1} when combined with 12·5 μg ml^{-1} ketoconazole. The FIC index of 0·16, calculated from these results, indicates strong synergistic antifungal activities between ketoconazole and estragole.

Thus, estragole and the essential oil of *A. rugosa* may be useful in the clinical application of ketoconazole. This kind of cocktail drug therapy may be particularly useful against *B. capitatus*, a rare pathogenic fungus documented to cause severe and fatal mycoses in immunocompromised patients (Groll and Walsh 2001). However, additional research is required to assess the practical value of the therapeutic application.

ACKNOWLEDGEMENT

This study was supported by a grant (No. M19904010001-01G0501-00110, 1999–2001) from the Ministry of Science and Technology of Korea.

REFERENCES

Ahn, B. and Yang, C.-B. (1991) Volatile flavor components of Bangah herb. *Korean Journal of Food Science and Technology* **23**, 582–586.

Aurore, G.S., Abaul, J. and Bourgeois, P. (1998) Antibacterial and antifungal activities of the essential oils of *Pimenta racemosa* var. *racemosa* P. Miller (J.W. Morre) (Myrtaceae). *The Journal of Essential oil Research* **10**, 161–164.

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

Dung, N.X., Cu, L.D., Thai, N.H., Moi, L.D., Hac, L.V. and Leclereq, P.A. (1996) Constituents of the leaf and flower oils of *Agastache rugosa* O. Kuntze from Vietnam. *The Journal of Essential oil Research* **8**, 135–138.

Garg, S.C. and Siddiqui, N. (1992) Antifungal activity of some essential oil isolates. *Pharmazie* **47**, 467–468.

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

Groll, A.H. and Walsh, T.J. (2001) Uncommon opportunistic fungi: new nosocomial threats. *Clinical Microbiology and Infection* **2**, 8–24.

Gundidza, M. (1993) Antimicrobial activity of essential oil from *Schinus molle*. *The Central African Journal of Medicine* **39**, 231–234.

Hammer, K., Carson, C.F. and Riley, T.V. (1998) In-vitro activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida* spp. *Journal of Antimicrobial Chemotherapy* **42**, 591–595.

Lachowicz, K.J., Jones, G.P., Briggs, D.R., Bienvenu, F.E., Wan, J., Wilcock, A. and Coventry, M.J. (1998) The synergistic preservative effects of the essential oils of sweet basil (*Ocimum bassillicum* L.) against acid-tolerant food microflora. *Letters in Applied Microbiology* **26**, 209–214.

Lee, S.H. and Kim, C.J. (1999) Selective combination effect of anethole to antifungal activities of miconazole and amphotericin B. *Yakhak Hoeji*, **43**, 228–232.

Marchetti, O., Moreillon, P., Glauser, M.P., Bille, J. and Sanglard, D. (2000) Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **44**, 2373–2381.

Metzger, S. and Hoffmann, H. (1997) Fluconazole-resistant *Candida* species from HIV-infected patients with recurrent Candida stomatitis: crossresistance to itraconazole and ketoconazole. *Mycoses* **40**, 56–63.

Oger, J.M., Richomme, P., Guinaudeau, H., Bouchara, J.P. and Fournet, A. (1994) *Aniba canelilla* (H.B.K.) Mez essential oil: analysis of chemical constituents, fungistatic properties. *The Journal of Essential oil Research* **6**, 493–497.

Shin, S.W. and Kang, C.A. (2001) Studies on composition and antifungal activities of essential oils from cultivars of *Brassica juncea* L. *Korean Journal of Pharmacognosy* **32**, 140–144.

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. (1987) Pharmacology and toxicity of high-dose ketoconazole. *Antimicrobial Agents and Chemotherapy* **31**, 1874–1878.

Suresh, B. S., Dhanaraj, S.A., Elango, Sriram, K. and Chinnaswamy, K. (1997) Anticandidal activity of *Santolina chamaecyparissus* volatile oil. *Journal of Ethnopharmacology* **55**, 151–159.

Svoboda, K.P., Gough, J., Hampson, J. and Galambosi, B. (1995) Analysis of essential oils of some *Agastache* species grown in Scotland from various seed sources. *Flavour and Fragrance Journal* **10**, 139–145.

Von Moltke, L.L., Greenblatt, D.J., Harmatz, J.S., Duan, S.X., Harrel, L.M., Crotreau-Bibbo, M.M., Pritchard, G.A., Wright, C.E. and Shader, R.I. (1996) Triazolam biotransformation by human liver microsomes in vitro: Effects of metabolic inhibitors and clinical conformation of a predicted interaction with ketoconazole. *Journal of Pharmacology and Experimental Therapeutics* **276**, 370–379.

Wan, J., Wilcock, A. and Coventry, M.J. (1998) The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas florescens*. *Journal of Applied Microbiology* **84**, 152–158.

Wilson, L.A., Senechal, N.P. and Widrlechner, M.P. (1992) Headspace analysis of the volatile oils of *Agastache*. *Journal of Agricultural and Food Chemistry* **40**, 1362–1366.