Natural Product Sciences
13(3) : 258-262 (2007)

In vivo Anti-fungal Activity of the Essential Oil Fraction from *Thymus* Species and *in vitro* Synergism with Clotrimazole

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Abstract – The antifungal activity of the essential oil fraction from *Thymus magus*, and its major component thymol, against *Candida albicans* was investigated *in vitro* and *in vivo*. The combined effects of the oils and clotrimazole, a commonly used antifungal drug for treatment of external candidiasis, were evaluated in this study. In experimental vaginal candidiasis the essential oil fraction of *T. magnus* resulted in relatively milder inhibition of fungal growth following the inoculation of test mice compared to clotrimazole. However, new fungal growth was not detected up to 12 days after cessation of treatment. In contrast, in a similar experiment using clotrimazole, *C. albicans* was detected in the 12th day post-treatment with the sample. This result indicates that *T. magnus* oil could be a promising drug to control vaginal candidiasis. In checkerboard titer tests, the combination of clotrimazole with the essential oil fraction of *T. magus* or *T. quinquecostatus* resulted in significant synergism, with FIC indices between 0.14 and 0.27 against *C. albicans*, while clotrimazole combined with thymol, the major component of these oils, produced only an additive effect, with FIC indices ranging between 0.50 and 1.00. Thus, the prominent synergistic effects of clotrimazole combined with *T. magus* essential oil indicate that these compounds may be an effective treatment for *C. albicans* infections.
Keywords – *Thymus. magnus, T. quinquecostatus,* essential oil, clotrimazole, *in vivo*, *Candida albicans*, synergism

Introduction

The *Thymus* species are well-known sources of antimicrobial essential oils and vary tremendously in composition, depending on the plant source (Zarzuelo and Crespo, 2003). *T. magnus* and *T. quinquecostatus* (Labiatae) are native to Korea and are used as diaphoretics and carminatives in traditional medicine (Kim *et al*., 1994). The essential oil fractions of these plants contain particularly high percentages of thymol between 39.8 and 41.7%, and exhibit significant *in vitro* activities against various fungi (Shin and Kim, 2004).

Resistance to azole antibiotics including clotrimazole, especially among *Candida* species, has been the subject of intense investigation over the past few years (Canuto and Rodero, 2002). As a consequence of the AIDS epidemic, there has been a rising increase in mucosal infections caused by *Candida* species that are associated with a rapid emergence of resistance to azoles (Kontoyiannis and Lewis, 2002). *C. albicans* is the most common cause of *Candida* related mucosal diseases. The increasing incidence of antibiotic resistant strains are a serious problem, especially in immuno-compromised patients (Zang *et al*., 2006). Combination therapy is one of the promising strategies to overcome these resistant infections by minimizing the effective dose of each drug (Metzger and Hoffmann, 1997; Lee and Kim, 1999; Marchetti *et al.,* 2000; Keele *et al.,* 2001; Keele *et al*., 2003; Shin and Pyun, 2004; Lim and Shin, 2005).

In this study, the antifungal activity of the essential oil fraction from *T. magus* against *C. albicans* was investigated *in vivo*. Moreover, the *in vitro* effects of the oils in *T. magus* and *T. quinquecostatus* in combination with clotrimazole, a commonly used antifungal drug for treatment of external candidasis, were evaluated against *C. albicans* using a checkerboard microtiter assay. The aim of our work is to eventually develop a therapeutic mixture combining the essential oil in *Thymus* species in Korea with clotrimazole for treatment of candidiasis, which can cause severe mucocutaneous, cutaneous, and systemic symptoms, particularly from opportunistic infections (Groll and Walsh, 2001; Singh, 2001).

Experimental

Test samples – Essential oils were obtained by steam

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distillation of cultivated *T. magnus* and *T. quinquecostatus*, which were harvested in July from the herbal garden of Duksung Women's University. The flowers and leaves (1 : 1) of *T. magnus* and *T. quinquecostatus* were extracted for 5 h in a simultaneous steam distillation-extraction apparatus. Voucher specimens were deposited at the herbarium of Duksung Women's University (No. LABT2 and No. LABT1). The oil fraction compositions were analyzed using a previously described method (Shin and Kim, 2004). Thymol (mp. 232°C) was isolated from the essential oil fraction of *T. magnus* by silicagel column chromatography using toluene-ethylacetate (93 : 7) as the mobile phase. Thymol was re-crystallized in methanol. Clotrimazole (C-6019) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Culture of *Candida* species – The fung were provided by the Korean Culture Center of Microorganisms. *C. albicans* ATCC 10231 was cultured in yeast and malt extract broth or malt extract liquid medium for 48 hours at 27°C.

Effects on experimental vaginal candidiasis in mice – Swiss albino female mice with a body weight of 60-80 g were used in this study. The animals were kept in fully air-conditioned animal-rooms maintained at 23-25°C. Vaginal infection with *C. albicans* was conducted as previously reported by Suresh *et al*. (1997). The mice were brought to a pseudo-estrous stage by subcutaneously injecting 0.2 ml of 2.5 mg/mL of estradiol propionate for 4 days. The fungal infection was induced by vaginal inoculation of 10^6 - 10^7 cells of *C. albicans* in 0.1 ml of sterile saline containing Tween80 (2%) on the 5th day. The infected mice were randomized into three groups. A group of six female mice was treated with a 4% oil fraction of *T. magnus* mixed with polyethylene glycol 200 at 24 h post-inoculation of the fungal suspension. This procedure was repeated twice a day for 18 days. On the 3rd, 6th, 9th, 12th, 15th, 18th and 30th days after treatment, the vaginal epithelium was swabbed and checked for fungal growth. The swabs were suspended aseptically in 10 mL of normal saline containing 0.2 mL of Tween80. Serial dilutions (10-fold) of this suspension were prepared and each dilution was plated on SDA (Saboraud's dextrose agar) containing 0.05 mg/mL chloramphenicol and incubated for 24 h at 25°C. The number of colony-forming units per mL (cfu/mL) was then scored to assess the intensity of the vaginal infection. Clotrimazole (2% solution) was used as a positive control and a solvent control (PEG-200) was also maintained throughout the experiment.

Determination of the minimal inhibitory concentration (MIC) and checkerboard titer tests – The essential oil fraction of *T. magnus* was serially diluted with malt extract liquid medium containing Tween80 (2%) and vortexed to prepare suspensions which contained from 160 to 0.625 mg/mL of the oil fraction. Clotrimazole was similarly diluted in DMSO to generate a series of concentrations ranging from 25.60 to 0.20 mg/mL. Aliquots (50 μL) of each oil dilution were added to the wells of 96-well plates in a vertical orientation and 10 μl aliquots of each clotrimazole dilution were added in a horizontal orientation so that the plate contained various combinations of concentrations of the two compounds. 140 μL suspension of *Candida* spp. adjusted to 10^4 - 10^5 CFU/mL was added to each well, for a final volume of 200 μL in each well. The fungal suspensions with the drug combinations were cultured at 25°C. The MIC (minimal inhibitory concentration) was defined as the lowest concentration that completely inhibited visible fungal growth after 72 h. Fractional inhibitory concentrations (FICs) were calculated by dividing the MIC of the combined *Thymus* oil and clotrimazole treatment by the MIC of *Thymus* oil or clotrimazole alone and then adding both FICs to generate the FIC index. The FIC index was interpreted as representing a synergistic effect when it was ≤ 0.5, no effect or an additive effect when it was < 0.5 to 2.0, and an antagonistic effect when it was >2.0 (White et al., 1996). Similar experiments were carried out with purified thymo. Fungi were also cultured with a control solution containing Tween80 and DMSO at levels equivalent to those in the test compound solutions to verify that these compounds alone did not affect fungal growth. Reported data are the means from triplicate experiments.

Results and Discussion

Candidal vaginitis is predominantly caused by strains of *Candida albicans* (90%) (Sobel *et al*., 2001), and remains a common problem in healthy women, especially, in immuno-compromised patients (Zang *et al*., 2006). Resistance of *Candida* species to azole compounds is the most prevalent type of antifungal resistance. This clinical problem arises in two settings: chronic refractory or recurring mucosal candidosis in AIDS patients, and acute or subacute bloodstream candidosis in immuno-suppressed or critically ill patients. In contrast, recurring vaginal candidosis in women without predisposing disorders is rarely associated with resistant *Candida* spp. With the goal of developing new highly active therapeutic agents

Table 1. *In vivo* activity of *Thymus magnus* oil against *C. albicans* in experimental vaginal candidiasis in mice

Group	Colony forming units/mL							
	3rd	6th	9th	12th	16th	18th	30th day	
Control (PEG200)	6.9×10^6	2.2×10^6	7.5×10^5	9.5×10^5	2.3×10^6	5.1×10^6	8.3×10^6	No activity
Clotrimaxole (2%)	3.8×10^4	1.9×10^4	6.9×10^3	1.6×10^3	5.9×10^2	0	2.2×10^3	Active
T. magnus oil (4%)	7.1×10^4	6.7×10^4	3.4×10^4	3.2×10^4	1.8×10^3	0	0	Active

Animals used: Swiss albino female mice. *Candida* strain used: *Candida albicans* KCCM 11282. Strength of inoculum: 10^7-10^8 cells/mL. Positive control: clotrimazole, 2%.
Route of administration: intra-vaginal. Incubation temperature: 37°C. Drug treatment regimen: twice daily.

Table 2. FICs and FIC indices of clotrimazole in combination with two preparations of *Thymus* essential oils or thymol against *C. albicans*

	T. magnus-Clotrimazole		*T. quinque*.-Clotrimazole		Thymol-Clotrimazole	
	T. magnus (mg/mL)	Clotrimazole (μg/mL)	T. quinque. (mg/mL)	Clotrimazole (μg/mL)	Thymol (mg/mL)	Clotrimazole (μg/mL)
MICo	2.00	128	4.00	128	0.50	1.28
MICc	0.50	2.00	0.50	2.00	0.25	64.00
FIC	0.25	0.016	0.125	0.016	0.50	0.50
FICI	0.27		0.14		1.00	

The essential oil fraction of *T. magnus* and thymol were serially diluted with malt extract liquid medium containing Tween80 (2%) to prepare suspensions which contained from 4 to 0.031 mg/ml of the oil fraction in each well of 96 well plates. Clotrimazole was similarly diluted in DMSO to generate a series of concentrations ranging from 128 to 2 μg/mL in each well.
FIC = Fractional inhibitory concentration; FICI = FIC index
MICo = MIC of one sample alone; MICc = MIC of the most effective sample combination; FIC = MICc/MICo; FICI = FIC of each sample + FIC of clotrimazole.

against mucosal infections of *Candida* species, we established an in vivo experimental system to examine the effects of the essential oils from *T. magnus*, a representative native plant source of thymol in Korea, in mice with induced vaginal candidiasis, based on our previous in vitro studies (Shin and Kim, 2004).

As demonstrated in Table 1, all mice were infected when assessed 3 days after inoculation with an average CFU of 8.9×10^6 *C. albicans*. A statistical analysis of the data was performed at the 5% probability level. Both *Thymus magnus* oil and clotrimazole, the positive control, exhibited significant activity against candidal growth in the vagina. Over 18 days of treatment no fungal growth was found. Up to 12 days after treatment, the CFU of the clotrimazole treated group was significantly lower than the *T. magnus* oil treated group. However, when tested at 30 days after fungal inoculation (12 days after the last drug treatment), the *Thymus* oil treated group showed no more candidal growth, while in the clotrimazole treated group fungal growth was again apparent. Thus, these results indicate that *T. magnus* may have more prolonged therapeutic effects than clotrimazole.

The FIC indices calculated from the results of the checkerboard titer assays, which were used to assess the effects of various combinations of clotrimazole with thymol, or with the essential oil fraction of *T. magnus* are listed in Table 2. The MIC results of the *Thymus* oils alone were consistent with the values in our previous report (Shin and Kim, 2004). Combining clotrimazole with *Thymus* oils from either species examined here (*T. magnus* or *T. quinquecostatus*) caused a significant decrease in the MIC of each compound against *C. albicans,* compared to their individual MIC values. For example, the MIC of clotrimazole alone against *C. albicans* was lowered from 128.00 μg/ml to 2.00 μg/mL in the presence of *T. magnus* oil at 1.00 mg/mL. The MIC of *T. magnus* oil alone also decreased from 2.00 mg/ml to 0.50 mg/mL when it was combined with 64 μg/mL clotrimazole. Although *T. magnus* oil showed a higher activity than *T. quinqecostitus* oil when tested with oil alone, when clotrimazole was combined with *T. quinqecostitus* oil a significant combination effect was measured. The FIC indices of *T. quinqecostitus* oil and *T. magnus* oil calculated from the checkerboard titer test were 0.14 and 0.27, respectively, which indicates the synergistic effect of *T. quinqecostitus* oil with clotrimazole was stronger that the combination of *T. magnus* oil and clotirmazole. When thymol was used instead of *T.*

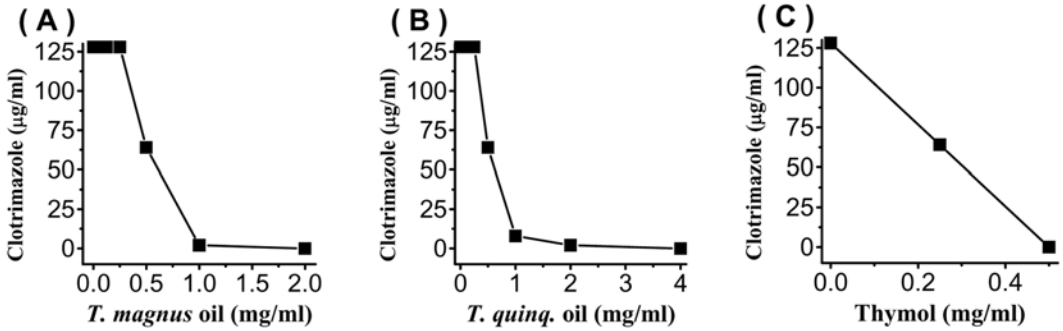

Fig. 1. Isobolograms of *Thymus* essential oils in combination with clotrimazole against *C. albicans*.
The curves were constructed by plotting the concentrations in the wells which showed the most efficacious combination of the essential oil and clotrimazole on checkerboard titer tests.

magnus oil in similar experiments, the effects were additive, giving an FIC index of 1.00. These results may indicate that other components in the essential oil fraction also contribute to the perturbation of lipids in the fungal membrane and increase susceptibility to clotrimazole (Heimark *et al*., 2002). Furthermore, the isobolograms depicted in Fig. 1. were constructed using concentrations which produced the greatest fungal inhibition in the checkerboard titer tests. The patterns depicted with MICs of the essential oil fractions of the tested *Thymus* species in combination with clotrimazole indicate that the effective concentrations were significantly lowered by combination therapy (Fig. 1A and B). The extent of reduction could not be accounted for by a simple additive effect, as indicated by Fig. 1C, in which thymol was used in combination with clotrimazole instead of the oil fraction (Davidson and Parish, 1989).

Thus, we have demonstrated here that the essential oil fraction of *T. magnus* oil may be more useful than the single compound thymol in clinical situations that require the use of clotrimazole. Such combination therapy of essential oils and synthetic antibiotics may be particularly useful against *Candida* species, particularly *C. albicans*, a pathogenic fungus, which is one of the predominant causes of candidal vaginitis in immuno-compromised patients. However, further investigations are required to assess the value of these natural antifungal agents for therapeutic usage.

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(Accepted September 10, 2007)