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In Vitro Inhibitory Activities of Essential Oils from Two Korean *Thymus species* against Antibiotic-Resistant Pathogens

Seungwon Shin and Ji Hyun Kim

College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea

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The *in vitro* inhibitory activities of essential oils from *Thymus magnus* and *T. quinquecostatus* as well as their main constituents were evaluated against susceptible and resistant species of *Streptococcus pneumoniae, Staphylococcus aureus, Salmonella enteritidis,* and *Salmonella typhimurium*. Notably, the essential oil fraction of *T. magnus* and its main components displayed significant inhibitory action against both antibiotic-susceptible and resistant strains of *S. pneumoniae, S. aureus,* and *S. typhimurium* with minimum inhibitory concentrations (MICs) ranging from 0.125 to 8 mg/mL. The differential MIC values imply that the oil fraction and its main components exhibit distinct patterns of activity against the tested bacterial species. Moreover, the disk diffusion test revealed that the inhibitory activities of oil fraction and components were dose-dependent. Data from the checkerboard titer test confirmed synergism between the antibiotic, norfloxacin, and *T. magnus* oil or thymol, particularly against the resistant strains of *S. aureus*.

Key words: *Thymus magnus, T. quinquecostatus,* Essential oils, Resistant bacteria

INTRODUCTION

The rapid increase in the development and variability in antimicrobial resistance is one of the greatest challenges, which threatens the future of human race. Furthermore, increased cases of therapy and application have accelerated the resistance of microorganisms against specific antibiotics, and in many cases, towards multiple drugs (Oluwatuyi *et al.*, 2004).

Plant essential oils have been reported to be prominent natural antimicrobial agents (Bidlack *et al.,* 2000; Giampri *et al.*, 2002; Shin, 2004). The *Thymus* species are well-known sources of antimicrobial essential oils and vary tremendously in composition, depending on the plant source. *T. magnus* (Labiatae), which is native of Korea, contains especially high percentages of thymol. These plants are used as diaphoretics and carminatives in traditional medicine (Kim *et al.*, 1994). A previous report by our group stated that the oil fraction and main components of *T. magnus* exhibit significant inhibitory activities against various fungi (Shin and Kim, 2004).

Staphylococcus aureus is the most common pathogen amongst the *Staphylococci* species. The rapid spread of bacteria, which are resistant to new antibiotics, especially oxacillin and methicillin has become a serious problem in therapy. In many cases, the pathogen develops multi-drug resistance, which strictly limits the treatment options (Chang *et al.*, 1995; Dillard *et al.,* 1996; Ito *et al.,* 2003). Additionally, the incidence of resistance to quinolones such as norfloxacin, has increased steadily over time.

The emerging resistance of strains causing respiratory infections, especially community-acquired pneumonia, is a serious problem worldwide. In particular, the treatment of *Streptococcus pneumonia* infection is currently hampered by increasing antibiotic resistance (Esposito and Principi, 2002).

Salmonella species is one of the common pathogenic bacterial groups, which causes food-borne diseases. The emergence of resistant strains has increased progressively, particularly due to the consumption of processed food and agricultural products which remain in contact with antibiotics (Logue *et al.,* 2003).

In our present study, the *in vitro* inhibitory activities of essential oils from *T. magnus* as well as their main constituents have been evaluated against antibiotic-resistant strains of *S. pneumoniae, S. aureus, Salmonella enteritidis,* and *S. typhimurium*. Moreover, we have also determined the capacity of the oils to modulate the resistance of *S.*

Correspondence to: Seungwon Shin, College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea
Tel: 82-2-901-8384, Fax: 82-2-901-8386
E-mail: swshin@duksung.ac.kr

aureus to norfloxacin, one of the quinolone antibiotics commonly used for the treatment of various *Staphylococcus* infections.

MATERIALS AND METHODS

Samples tested for antibacterial activities

Essential oils were obtained by steam distillation of flowers and leaves (1:1) from cultivated *T. quinquecostatus* and *T. magnus* (harvested in July from the herbal garden of Duksung Women's University) for five hours in a simultaneous steam distillation-extraction apparatus. The compositions of oil fraction were analyzed by following a previously described method (Shin and Kim, 2004). Thymol (112097, 98%), carvacrol (22051), oxacillin (O1002; oxacillin sodium salt monohydrate) and norfloxacin (N9890) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Kanamycin (sulfate salt) and streptomycin (sulfate salt) injections produced by Donga and Chong Kun Dang Pharmaceutical Company (Korea) were used as antibiotic controls.

Strains

Clinically isolated antibiotic-susceptible and resistant strains of *S. pneumoniae* (n=5), *S. aureus* (n=3), *S. enteritidis* (n=3), and *S. typhimurium* (n=3) were obtained from the Culture Collection of Antibiotic Resistant Microbes (CCARM) and the Korean Culture Center of Microoganisms (KCCM), and subcultured on Mueller Hinton II agar plates with 5% sheep blood or trypticase soy agar plates.

MIC (Minimum inhibitory concentration) test

MIC values of the oils were determined by following the broth microdilution method (Shin, 2004). A range of two-fold dilution (160-0.125 mg/mL) of essential oils in a medium containing 2% Tween-80 was prepared. The oil suspensions (100 μL) were added to 96-well plates. Antibiotics were similarly diluted in DMSO to generate a series of concentration, ranging from 100 to 0.78 mg/mL per well. The turbidity of the bacterial suspensions was measured at 600 nm, and adjusted with medium to match the 0.5 McFarland standard (10^5-10^6 colony forming units/mL). Subsequently, 100 μL of bacterial culture was inoculated into each well, and the plates were incubated at 36°C for 24 h. MIC values were determined in duplicate and re-examined when appropriate. Each of the bacterial species was additionally cultured in a blank solution containing Tween-80 at concentrations equivalent to those in test solutions to verify that these vehicles had no significant effect on the bacterial growth.

Disk diffusion test

Bacterial broth cultures of *S. aureus* ATCC 29213 (susceptible to norfloxacin, oxacillin, and erythromycin), *S. aureus* CCARM 3511 (resistant against norfloxacin, oxacillin, and erythromycin) and *S. aureus* CCARM 3523 (resistant against norfloxacin, oxacillin, and erythromycin) were added to Müller-Hinton I agar plates, and distributed uniformly. Sterile paper discs (8 mm) were wetted with 50 ml of each essential oil fraction of *Thymus magnus* and *T. quinquecostatus* as well as their main constituents, placed on plates, and incubated at 36°C for 24 h. After incubation, the diameters of the inhibitory zones (mm) around the disks were measured.

Checkerboard titer tests and construction of isobolograms

For checkerboard titer tests, 50 μL aliquots of each oil dilution were added to the wells of 96-well plates in a vertical orientation, and 10 μL aliquots of norfloxacin dilutions were added in a horizontal orientation, so that the plate contained various concentration combinations of the two compounds. A 100 μL suspension of three *S. aureus* strains was added to each well, and cultured at 36°C for 24 h. An isobologram was constructed on the basis of the checkerboard experiment to depict the synergistic activity of thymol with norfloxacin against the tested bacteria.

The concentrations of oil and norfloxacin of the tested wells, which inhibited the growth of bacteria in combination with the lowest concentration of both the samples, were plotted as x and y axes values, respectively, and curves were constructed from the plots. A combination of *Thymus* oil and norfloxacin produced a curve that was bent to the left, which confirms synergistic activity between the two compounds (Davidson and Parish, 1989; Shin and Lim, 2004).

RESULTS AND DISCUSSION

To develop a new strategy in dealing with the current situation of the rapid increase in antibiotic-resistant pathogens, we have examined the activities of the essential oils of *T. quinquecostatus* and *T. magnus* (Korean plant resources), against antibiotic-susceptible and resistant strains of four species of pathogenic bacteria.

The results of the MIC (minimum inhibitory concentration) tests are listed in Table I. The oil fraction and its main components, thymol and carvacrol, displayed distinct patterns of activity against the species tested, as exemplified by the differential MIC values. The essential oil fractions of the two *Thymus species* and their main components exhibited the highest inhibitory activities against the antibiotic-susceptible and resistant strains of *S. pneumoniae*, with MIC values ranging between 0.25 to 1 mg/mL. No remarkable differences were evident between

Table I. Minimum inhibitory concentrations of oils against antibiotics susceptible and resistant strains of *S. aureus*, *S. pneumoniae*, *S. enteritidis*, and *S. typhimurium*

Sample (mg/mL)	*S. aureus*			*S. pneumoniae*					*S. enteritidis*			*S. typhimurium*		
	Sa13	*Sa11*	*Sa23*	*Sp19*	*Sp09*	*Sp10*	*Sp15*	*Sp59*	*Se21*	*Se10*	*Se11*	*St62*	*St07*	*St09*
T. quinquecostatus	4.00	8.00	2.00	0.25	0.50	0.50	0.50	0.125	8.00	>16.00	8.00	2.00	4.00	8.00
T. magnus	4.00	8.00	4.00	0.50	1.00	1.00	1.00	0.25	16.00	>16.00	8.00	2.00	8.00	8.00
Thymol	4.00	4.00	2.00	0.50	1.00	1.00	1.00	0.125	>16.00	>16.00	>16.00	1.00	4.00	8.00
Carvacrol	2.00	4.00	2.00	0.25	1.00	1.00	1.00	0.125	>16.00	>16.00	>16.00	1.00	4.00	4.00
Norfloxacin ($\times 10^{-3}$)	2.00	512.0	128.00	4.00	4.00	2.00	>64.00	8.00	>64.00	>64.00	>64.00	>64.00	>64.00	>64.00
Oxacillin ($\times 10^{-3}$)	4.00	16.00	16.00	4.00	>16.00	>16.00	>16.00	4.00	2.00	4.00	1.00	8.00	8.00	>512.00
Erythromycin ($\times 10^{-6}$)	0.25	>64.00	16.00	0.50	>1024	>1024	>1024	>1024	2.00	512.00	512.00	32.00	64.00	512.00

The values are the means from the triplicate experiments.

Sa13: *S. aureus* ATCC29213, Sa11: *S. aureus* CCARM3511, Sa23: *S. aureus* CCARM3523, Sp19: *S. pneumoniae* KCCM 49619, Sp09: *S. pneumoniae* CCARM 4009, Sp10: *S. pneumoniae* CCARM 4010, Sp15: *S. pneumoniae* CCARM 4015, Sp59: *S. pneumoniae* CCARM 4059, Se21: *S. enteritidis* KCCM12201, Se10: *S. enteritidis* CCARM8010, Se11: *S. enteritidis* CCARM8011, St62: *S. typhimurium* KCCM11862, St07: *S. typhimurium* CCARM 8007, St09: *S. typhimurium* CCARM 8009.

the susceptible and resistant strains. Additionally, the oils had significant inhibitory activity against *S. aureus* and *S. typhimurium*, with MIC values ranging between 1 to 8 mg/mL. Amongst all the tested species, the oils exhibited relatively weak inhibition against the three strains of *S. enteritidis*.

S. aureus, the resistant strains of which provide the most serious challenge to the present era, was selected for further tests. The strains exhibited differences in the MIC values of norfloxacin, which displayed the most significant combination effects with *Thymus* oil in a checkerboard titer test. As can be seen from Table II, the inhibition results were mostly consistent with data from the MIC tests. *S. aureus* CCARM 3523 was more significantly inhibited by the tested oils than the other two strains as can be seen by the higher MIC values. Under these experimental conditions, MICs of the oils in Table II were ca. 1000-10000 fold higher than the MIC values of antibiotics. However, the relatively higher MICs of oils render them as an valid alternative for the treatment of antibiotic resistant bacterial infections when compared to the clinically impractical concentrations of antibiotics required to obtain the same effect. The disk diffusion test revealed that the activities were dose- dependent.

Table II. Antibacterial activities against *S. aureus* strains as estimated by the disk diffusion tests

Sample		Sa13	Sa11	Sa23
T. quinquecostatus	I	6.7 ± 0.58	6.7 ± 0.58	13.3 ± 0.58
	II	3.0 ± 0.00	3.3 ± 0.58	5.7 ± 1.15
T. magnus	I	6.7 ± 0.58	6.0 ± 0.00	14.7 ± 1.53
	II	2.3 ± 0.58	2.3 ± 0.58	6.7 ± 1.15
Thymol	I	13.7 ± 1.53	14.7 ± 0.58	20.7 ± 1.53
	II	6.0 ± 1.00	6.3 ± 0.58	13.3 ± 1.53
Carvacrol	I	11.7 ± 0.58	13.0 ± 1.00	16.3 ± 3.51
	II	3.3 ± 0.58	3.0 ± 0.00	8.0 ± 1.73
Norfloxacin	III	12.0 ± 1.00	0.0 ± 0.00	0.7 ± 0.58
	IV	9.0 ± 1.73	0.0 ± 0.00	0.0 ± 0.00
Oxacillin	III	14.7 ± 0.58	11.3 ± 0.58	12.0 ± 1.00
	IV	12.7 ± 0.58	9.0 ± 1.00	10.3 ± 0.58
Erythromycin	III	12.0 ± 1.00	1.3 ± 1.15	2.7 ± 0.58
	IV	10.3 ± 2.08	1.0 ± 1.00	2.0 ± 0.00

The values are the means ± SD of triplicate data. The width (mm) of growth inhibition of bacteria was measured from the outline of the disks wetted with sample.

I: 2 mg/disk, II: 1 mg/disk, III: 0.1 mg/disk, IV: 0.05 mg/disk.

Sa13: S. aureus ATCC29213, *Sa11: S. aureus* CCARM3511, *Sa23: S. aureus* CCARM3523

The results of the checkerboard titer test (depicted in Figs. 1-3) confirmed a synergistic action between norfloxacin and *Thymus* oils or thymol. As illustrated in Fig. 1, the MIC values of norfloxacin against *S. aureus* ATCC 29213 (a norfloxacin-susceptible strain) decreased from 2.0 to 0.25 μg/mL when combined with *T. quinqecostatus* oil, and exhibited an obvious bend in the curve to the left, implying synergism between the tested samples. Similarly, synergism was identified in a parallel experiment carried out with thymol, the prominent component of *Thymus* oils. The curves in Fig. 2 demonstrate synergism of the samples against *S. aureus* CCARM 3511 (a norfloxacin-resistant strain), which was more significant than the one against *S. aureus* ATCC 29213. Specifically, the MIC value of norfloxacin was altered from 512 to 32 μg/mL. In a similar experiment with *S. aureus* CCARM 3523, synergism was additionally observed. However, the pattern of the curves was slightly different from that of *S. aureus* CCARM 3511, indicating a

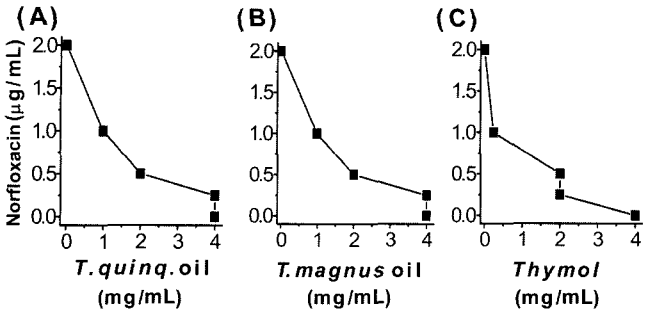

Fig. 1. Isobolograms of *Thymus* essential oils in combination with norfloxacin against *S. aureus* ATCC 29213 (susceptible strain to norfloxacin)

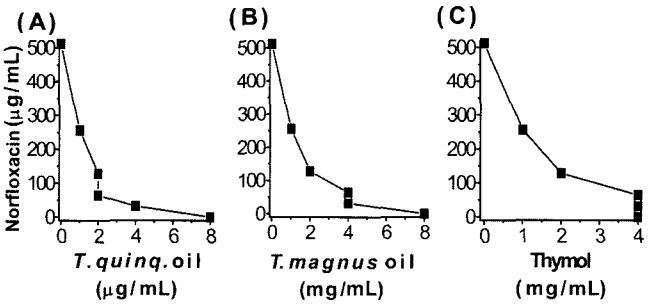

Fig. 2. Isobolograms of *Thymus* essential oils in combination with norfloxacin against *S. aureus* CCARM 3511 (resistant strain to norfloxacin)

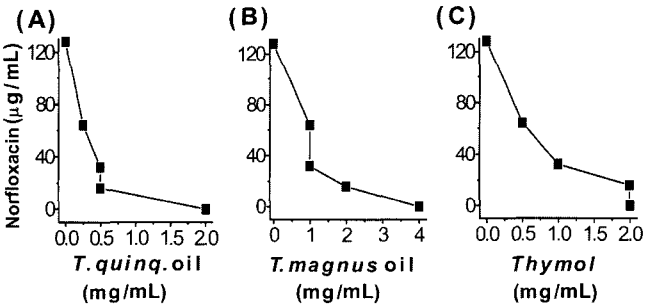

Fig. 3. Isobolograms of essential oils in combination with norfloxacin against *S.aureus* CCARM 3523 (resistant strain to norfloxacin)

relatively lower rate of synergism.

In general, the mechanism of quinolone resistance is related to the target modification, and efflux or decreased permeability (Dougherty *et al.,* 2001). Although the essential oils possess weaker activity when compared with the present antibiotics, recent studies suggest that some modulation can be done against quinolone resistance by changing the properties of the cell wall or cell membrane of the organism.

In conclusion, we establish that the antibiotic-resistant strains of *Staphylococcus aureus, Streptococcus pneumoniae,* and *S. typhimurium* are inhibited by essential oils of the two *Thymus* species. Moreover, the resistance of *Staphylococcus aureus* strains to norfloxacin can be modulated by *Thymus* oils. However, further studies are required to determine the mechanism of antibiotic activity of these oils.

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