Arch Pharm Res Vol 28, No 7, 765-769, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

In Vitro Effects of Essential Oils from *Ostericum koreanum* against Antibiotic-Resistant *Salmonella* spp.

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(Received May 25, 2004)

The essential oil fraction of *Ostericum koreanum* was analyzed by GC-MS. Inhibiting activities of this oil and its main components were tested by the broth dilution assay and disk diffusion test against one antibiotic-susceptible and two resistant strains of *Salmonella enteritidis* and *S. typhimurium,* respectively. The GC-MS analysis revealed thirty-four compounds; the main components were α-pinene (41.12%), ρ-cresol (17.99%) and 4-methylacetophenone (7.90%). The essential oil of *O. koreanum* and its main components were significantly effective against the tested antibiotic-susceptible strains as well as against the resistant strains of the two *Salmonella* species, with MICs (minimum inhibitory concentrations) ranging from 2 mg/mL to 16 mg/mL. The anti-*Salmonella* effects of the oils were dose-dependent on Müller-Hinton agar plates in this experiment. Additionally, checkerboard titer test results demonstrated significant combined effects of streptomycin and *O. koreanum* oil or cresol, one of the main components of this oil, against the two streptomycin resistant strains of *S. typhimurium*, with FICIs ranging from 0.12 to 0.37.

Key words: *Ostericum koreanum,* Essential oils, ρ-Cresol, *Salmonella enteritidis, S. typhimurium,* Antibiotic-resistant, Synergism, Streptomycin

INTRODUCTION

Ostericum koreanum (Umbelliferae) is a perennial herb used in traditional Korean medicines for treatment of the common cold and for relief of rheumatic pains or headaches; the herb imparts a pungent and warm sensation (Chi and Kim, 1993; Kwon *et al.,* 2000; Choi *et al.*, 2004). A rich source of essential oil, *Ostericum koreanum* is widely distributed in the wild and cultivated in Korea.

Plant essential oils have been developed into novel, natural antimicrobial agents (MacCutcheon *et al.,* 1997; Bidlack *et al.,* 2000; Shin, 2004a). However, many essential oils are distinctly less potent against microbes than antibiotics developed from microbiological sources or synthetic drugs (Matsuura *et al.,* 1996; Shin and Lim, 2004; Shin, 2004b). For this reason, microbially derived or synthetic antibiotics are most commonly used as therapeutics in treatment of diseases.

However, the rapid increase in the development and variety of antimicrobial resistance is problematic and warrants a serious new treatment strategy (Groll and Walsh, 2001; Karlowsky and Sahm, 2002). The increased number of illnesses treated medically and the excessive use of antibiotics have accelerated the resistance of microorganisms against specific, and in many cases multiple, antibiotics (Oluwatuyi *et al.*, 2004). Human and animal cases of infection with antibiotic-resistant organisms are on the rise due to the consumption of processed or unprocessed foods contaminated with antibiotic-resistant organisms treated routinely with veterinary antimicrobials (Logue *et al.,* 2003; Mastroeni and Sheppard, 2004). *Salmonella* species comprise one of the common pathogenic bacterial groups which cause food-borne diseases (Zhao *et al.,* 2003; Schlegelova, *et al.,* 2004; Fluit, 2005; Khaschabi and Schöpf, 2005).

The emergence of resistant bacterial strains has increased in all regions of the world. Among the *Salmonella* species, *S. enteritis* and *S. typhimurium* are especially important causes of secondary infections in humans transmitted by infected domestic animals (Low *et al.,* 1996; Chung and Beuchat, 1999; Humphrei, 2001; Alban *et al.*, 2002).

We have evaluated the *in vitro* inhibitory effects of the essential oil fraction from *O. koreanum* as well as its main

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constituents against antibiotic-susceptible and antibiotic-resistant strains of *S. enteritidis*, and *S. typhimurium* in order to develop novel, natural antibiotics against resistant pathogenic *Salmonella.*

MATERIALS AND METHODS

Analysis of essential oils from *O. koreanum*

The essential oils were obtained by steam distillation for 5 h in a simultaneous steam distillation-extraction apparatus (SDE) from the leaves of *O. koreanum* cultivated in Chungcheongbuk-do, Jecheon. The essential oil fractions were analyzed using the Hewlett-Packard 6890 GC and the Hewlett-Packard 5973 MSD apparatus (Agilent 5973 network mass selective detector, 280°C) with an 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 as follows: initial temperature: 60°C, and then 2°C/min up to 220°C.

Strains

S. enteritidis KCCM12201, *S. enteritidis* CCARM8010, *S. enteritidis* CCARM8011, *S. typhimurium* KCCM11862, *S. typhimurium* CCARM 8007, and *S. typhimurium* CCARM 8009 were subdivided from the Korean Culture Center of Microorganisms (KCCM) and Culture Collection of Antibiotic Microbes (CCARM). The organisms were subcultured in Müller-Hinton Broth (YM, Difco, U.S.A.) for 28 h at 37°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%), ρ-cresol (99%) and oxacillin (sodium monohydrate; 99%) were purchased from Sigma Chemical Co. Kanamycin (sulfate salt) and streptomycin (sulfate salt) injections produced by Donga and Chong Kun Dang Pharmaceutical Company (Korea) were used as antibiotic controls.

Determination of minimal inhibitory concentration (MIC)

MIC values of the oils were determined using the broth microdilution method (Shin, 2004). A range of two-fold dilutions (160-0.125 mg/mL) of essential oils in 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 concentrations, ranging from 100 to 0.78 μg/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). Next, 100 μL of bacterial culture was inoculated into each well, and plates were incubated at 37°C for 24 h. MIC values were determined in duplicate and re-examined where appropriate. Each organism was additionally cultured with a blank solution containing Tween-80 and DMSO concentrations equivalent to those in test solutions to verify that these vehicles did not affect growth.

Disk diffusion assay

Bacterial broth cultures of *S. enteritidis* KCCM 12201, *S. enteritidis* CCARM 8010, *S. enteritidis* CCARM 8011, *S. typhimurium* KCCM 11862, *S. typhimurium* CCARM 8007, and *S. typhimurium* CCARM 8009 were added to Müller-Hinton I agar plates, and distributed uniformly. Sterile paper discs (8 mm) were wetted with 50 μL of each essential oil fraction of *O. koreanum* as well as the main constituent and antibiotics, placed on plates, and cultivated at 37°C for 24 h. The diameters of the inhibited zones (mm) around the disks were measured.

Checkerboard titer test

Eight serial two-fold dilutions of *O. koreanum* oil and streptomycin or kanamycin were prepared with the same solvents used in the MIC tests. Fifty microliter aliquots of each oil dilution were added to the wells of 96-well plates in a vertical orientation, and 10 μL aliquots of each antibiotic dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. A 100 μL suspension of each *S. typhimurium* strain (10^4 CFU/well) was added to each well and cultured at 37°C for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of *O. koreanum* oil and antibiotics divided by the MIC of *O. koreanum* oil or antibiotics alone. The FIC index (FICI) was calculated by adding both FICs and was interpreted as a synergistic effect when it was ≤0.5, as additive or indifferent when it was >0.5 to 2.0, and as antagonistic when it was >2.0. A checkerboard experiment was also performed to determine the effect of combining ρ-cresol with antibiotics (White *et al.*, 1996).

RESULTS AND DISCUSSION

From the dried roots (3 kg) of *O. koreanum,* 7.2 g of essential oil fraction (0.24%) 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 I. Thirty-four compounds were identified in the essential oil fraction of *O. koreanum* based on a Wiley 275 Library search, GC standards and the results of GC-MS.

The main components of this oil were α-pinene (41.12 %), ρ-cresol (17.99%) and 4-methylacetophenone (7.90%)

Table I. Compounds identified in the essential oil fraction of *O. koreanum*

Compounds	RI	Area (%)
α-pinene	928	41.12
camphene	940	1.89
berbenne	947	0.07
artemisia triene	963	0.07
β-phellandrene	992	0.08
β-pinene	969	1.42
myrcene	998	0.81
α-phellandrene	1022	0.3
sabinene	1023	7.60
γ-terpinene	1026	1.76
cis-ocimene	1038	0.07
β-myrcene	1052	0.08
1-methyl benzene	1061	0.22
p-cresol	1073	17.99
terpinolene	1077	1.62
α-campholene aldehyde	1117	0.08
1-terpineol	1133	0.23
α-citral	1175	0.07
p-cymen-8-ol	1188	1.98
cis-piperitol	1231	1.28
z-citral	1241	0.05
4-methyl-1,4-heptadiene	1250	1.25
4-hydroxy-2-methylacetophenone	1295	7.90
germacrene-D	1372	0.07
α-cadinene	1518	0.18
β-bisabolene	1528	0.08
cis-ocimene	1535	1.26
trans-β-ocimene	1563	0.06
δ-carene	1587	0.09
valencene	1603	0.07
β-cubebene	1680	1.18
β-eudesmol	1682	1.21
elemol	1697	1.10
α-bisabolol	1720	2.05
In total		95.29

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

in our experiments. The oil compositions were partially inconsistent with previously reported results (Chi and Kim, 1993; Choi *et al.*, 2004). The previous report identified much lower concentrations of these compounds extracted from the same species cultivated in Japan. This discrepancy might be related to the different status (especially dryness) of the plant material used in steam distillation as well as the culture conditions such as climate or soil type (Miyazawa *et al.*, 2003; Choi *et al.*, 2004).

To evaluate the effects of the essential oil of *O. koreanum* and its main components, α-pinene, and ρ-cresol, against antibiotic-resistant and -susceptible *Salmonella* strains (Table II), the broth dilution method and disk diffusion tests were established.

The MIC test results are demonstrated in Table III. The *O. koreanum* oil fraction, α-pinene, and p-cresol displayed distinct patterns of activity against the species tested, as demonstrated by the differential MIC values. The *O. koreanum* oil and main components exhibited significant inhibitory activities against the antibiotic-susceptible and -resistant strains of both *S. enteritidis* and *S. typhimurium*, with MIC values ranging from 2 mg/mL to 16 mg/mL. No remarkable differences were evident between oxacillin, kanamycin and streptomycin against the susceptible and resistant strains. The total oil fraction showed relatively milder activity than α-pinene or ρ-cresol. Among the tested oils, ρ-cresol, one of the representative components of this oil, revealed the highest inhibitory effects, with MICs of 2 mg/mL to 4 mg/mL.

Table II. Susceptibility of the tested *Salmonella* strains to antibiotics

Strains	Antibiotics		
	oxacillin	kanamycin	streptomycin
S. enteritidis			
KCCM12201	resistant	susceptible	susceptible
CCARM8010	resistant	susceptible	resistant
CCARM8011	resistant	susceptible	resistant
S. typhimurium			
KCCM11862	resistant	susceptible	resistant
CCARM 8007	resistant	susceptible	resistant
CCARM 8009	resistant	resistant	resistant

Table III. MICs against antibiotics-susceptible and -resistant strains of *Salmonella* species

Sample	*S. enteritidis*			*S. typhimurium*		
	Se21	Se10	Se11	St62	St07	St09
O. koreanum oil*	16	8	8	4	8	8
α-Pinene*	8	8	8	8	16	16
ρ-Cresol	4	2	2	2	4	2
Oxacillin**	>64	>64	>64	>64	>64	>64
Kanamycin**	2	4	1	8	8	>64
Streptomycin **	2	>64	>64	32	64	>64

The values are the means from the triplicate experiments
*mg/mL.
** μg/mL.
Se21: *S. enteritidis* KCCM 12201, *Se10*: *S. enteritidis* CCARM 8010, *Se11*: *S. enteritidis* CCARM 8011, *St62*: *S. typhimurium* KCCM 11862, *St07*: *S. typhimurium* CCARM 8007, *St09*: *S. typhimurium* CCARM 8009.

Table IV. Inhibition on Müller Hinton agar plates against antibiotics-susceptible and -resistant strains *of Salmonella* species

Sample		*S. enteritidis*			*S. typhimurium*		
		Se21	Se10	Se11	St62	St07	St09
O. koreanum	I	6.1 ± 0.28	6.1 ± 0.28	6.1 ± 0.28	7.3 ± 0.47	5.3 ± 0.47	6.0 ± 0.81
	II	3.7 ± 0.25	4.4 ± 0.92	4.5 ± 0.00	5.16 ± 0.28	3.5 ± 0.00	5.4 ± 0.00
α-Pinene*	I	5.3 ± 0.47	7.0 ± 2.16	13.1 ± 1.54	16.0 ± 5.29	11.6 ± 0.57	13.6 ± 0.57
	II	3.3 ± 0.47	5.7 ± 0.58	10.8 ± 0.28	13.6 ± 0.57	8.1 ± 0.28	11.8 ± 0.28
ρ-Cresol*	I	15.5 ± 1.32	11.8 ± 0.28	14.6 ± 1.15	13.6 ± 0.57	13.3 ± 1.53	15.3 ± 0.47
	II	11.6 ± 0.57	9.3 ± 0.58	11.7 ± 0.58	9.3 ± 0.58	10.8 ± 0.28	11.7 ± 0.58
Kanamycin**	III	11.0 ± 1.00	8.0 ± 1.73	9.7 ± 1.53	11.7 ± 0.58	8.7 ± 2.13	0.0 ± 0.00
	IV	9.0 ± 0.00	6.0 ± 1.00	8.3 ± 1.15	9.3 ± 0.58	7.3 ± 2.08	0.0 ± 0.00
Streptomycin **	III	9.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	8.0 ± 1.00	6.3 ± 1.53	0.0 ± 0.00
	IV	7.3 ± 0.58	0.0 ± 0.00	0.0 ± 0.00	6.3 ± 0.58	5.3 ± 0.58	0.0 ± 0.00

The values are the means±SD from the triplicate experiments.
I; 25mg/disk, II; 12.5mg/disk, III; 100μg/disk, IV; 50μg/disk, *mg/mL, **μg/mL
Se21: *S. enteritidis* KCCM 12201, *Se10*: *S. enteritidis* CCARM 8010, *Se11*: *S. enteritidis* CCARM 8011, *St62*: *S. typhimurium* KCCM 11862, *St07*: *S. typhimurium* CCARM 8007, *St09*: *S. typhimurium* CCARM 8009.

Table V. FICs (fractional inhibitory concentration)s and FIC indices (FICI) in combination with antibiotics and ρ-cresol, or the essential oil fraction of *O. koreanum* against *S. typhimurium* strains

S. typhimurium \ Sample		OK[a]– ST[b]		CR[c]– ST		OK-KA[d]		CR-KA	
KCCM 11862	FIC	0.06	0.12	0.25	0.03	0.25	0.06	0.06	0.25
	FICI	0.18		0.28		0.31		0.31	
CCARM 8007	FIC	0.12	0.25	0.25	0.06	0.06	0.06	0.25	0.12
	FICI	0.37		0.31		0.12		0.37	

[a]*O. koreanum*, [b]Streptomycin, [c]ρ-Cresol, [d]Kanamycin.

$$\text{FIC of oil} = \frac{\text{MIC of oil in combination with antibiotics}}{\text{MIC of oil alone}}$$

$$\text{FIC of antibiotics} = \frac{\text{MIC of antibiotics in combination with oil}}{\text{MIC of antibiotics alone}}$$

FICI = FIC of oil + FIC of antibiotics

As shown in Table IV, the fractional inhibition results were mostly consistent with data from the MIC tests. Against the strains of *S. typhimurium,* the MIC for α-pinene was 4- to 8-fold higher than the MIC for ρ-cresol, while the fractional inhibitory test results against these strains were not so dramatically different, as determined by inhibited diameters on Müller-Hinton agar plates. Discrepant results from different test methods have often been reported. Therefore, the two different methods should be compared closely to accurately evaluate activity and differential susceptibility of the strains, as in this study (Davidson *et al.*, 1989). The disk diffusion test revealed that the activities were dose-dependent.

The checkerboard microtiter tests were performed to explore the possible combined efficacy of *O. koreanum* oil with antibiotics against resistant *Salmonella*. The *S. typhimurium* KCCM 11862 and *S. typhimurium* CCARM 8007 resistant strains, with high yet distinct streptomycin and kanamycin MICs, were chosen for this test. Table V demonstrates a significant synergism between antibiotics and the *O. koreanum* volatile oil fraction or ρ-cresol. The FIC and FICI results calculated from the checkerboard microtiter tests are listed in Table V. The significant combination effects of streptomycin and *O. koreanum* oil were confirmed in this test again with FICIs ranging from 0.12 to 0.37 against two *Salmonella* strains evaluated. Streptomycin combined with ρ-cresol resulted in additive effects, with FICIs ranging from 0.28 to 0.37.

In conclusion, we confirmed significant activity of the essential oils in *O. koreanum* against antibiotic-resistant and -susceptible *S. enteritidis* and *S. typhimurium,* which are common causes of food-borne illness in humans and

animals. In addition, we confirmed the combination of *O. koreanum* oil and antibiotics was significantly more potent against the bacterial strains. These results may be useful for development of a new strategy, i.e., a therapeutic essential oil, to treat illness caused by antibiotic resistant *Salmonella*. However, additional *in vivo* experiments are necessary to assess the potential for therapeutic application.

ACKNOWLEDGEMENT

This work was supported by Korea Research Foundation Grant (KRF-2004-041-E00386).

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