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Screening and Isolation of Antibiotic Resistance Inhibitors from Herb Materials IV- Resistance Inhibitors from *Anetheum graveolens* and *Acorus gramineus*

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The hexane fractions from methanolic extracts of *Anetheum graveolens* L. (Umbelliferae) and *Acorus gramineus* Soland. (Araceae) revealed potent inhibitory activities against the resistance of multi-drug resistant *Staphylococcus aureus* SA2 when combined with ampicillin (Am) or chloramphenicol (Cm). As active principles, carvone and the liquid mixture containing carvone from *Anetheum graveolens* L. and a liquid mixture mainly consisting of benzoic acid phenylmethyl ester (benzyl benzoate) from *Acorus gramineus* Soland. were identified. They showed resistance inhibition at the level of 20~50 μg/ml when combined with 100 or 50 μg/ml of Am or Cm, respectively.

Key words : *Anetheum graveolens, Acorus gramineus,* Carvone, Benzyl benzoate, Antibiotics resistance inhibition, *Staphylococcus aureus* SA2

INTRODUCTION

Activities of numerous antibiotics are easily proved ineffective in the resistance acquired from the microorganism. In order to maintain the effectiveness, the reduction of resistance is thought to be more valuable than to select newer and stronger ones. An alternative approach has been made possible by the discovery of compounds which inhibit bacterial β-lactamases (Brown *et al.*, 1976; Umezawa *et al.*, 1973) such as clavulanic acid (Reading and Cole, 1977; Neu and Fu, 1978; Paisley and Washington, 1978; Ball *et al.*, 1980), sulbactam and tazobactam (Arnoff *et al.*, 1984; Jacobs *et al.*, 1986; Retsema *et al.*, 1986; Kuck *et al.*, 1989). The authors have reported some attempts to find potent resistant inhibitors or reducers from plant sources (Kim *et al.*, 1995; Park *et al.*, 1997; Lee *et al.*, 1998). As the achievement of such attempts, we found that the hexane fractions of the extracts of *Anetheum graveolens* L. and *Acorus gramineus* Soland. have inhibitory activities on the resistance of *Staphylococcus aureus* SA2 when combined with ampicillin (Am) or chloramphenicol (Cm). The strain of *S. aureus* SA2 is a multiple resistant strain with four plasmids and resistant to 10 usual antibiotics (Kang and Moon, 1990; Kim *et al.*, 1992; Lee and Moon, 1993) including Am and Cm. As a continuation of the study, we examined the liquid mixtures which were isolated from the hexane fraction of the herb materials with column chromatography, on the reduction of resistances of *S. aureus* SA2 to Am and Cm.

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EXPERIMENTAL METHODS

Bacterial strain

The multi-drug resistant *S. aureus* SA2 isolated from hospitalized patient in the Pusan area was cultivated and controlled to maintain resistances as follows (Kim, *et al.*, 1995). The bacterial strain was cultivated in tryptic soy broth (TSB) with 50 and 100 μg/ml of Cm and Am, respectively, for sustaining the resistance and suppression of other bacterial strain at 37°C for 12 hrs(Kang and Moon, 1990).

Herb materials

Anetheum graveolens L. (Umbelliferae) and *Acorus gramineus* Soland. (Araceae) used in the study were purchased from a local market and identified (Kim, *et al.*, 1995).

Fractionation and chromatography

From the herb materials, methanolic extracts were obtained through the usual methods (Kim *et al.*, 1995).

Hexane fractions, which were prepared by the partition with hexane from the methanolic extracts, were applied to silica gel column chromatography (solvent system: petroleum benzene-chloroform=10-1) to produce several subfractions of different TLC (solvent system: petroleum benzene-ethyl acetate=10-1; color developing agents: vanillin-sulfuric acid) patterns. Hexane fraction of *Anetheum graveolens* gave nine subfractions, of which the effective subfractions (AH's 3~6) were chromatographed to give two active components. Also the hexane fraction of *Acorus gramineus* was also applied to the silica gel column chromatography to give 16 different subfractions. The effective subfractions (ACH's 5~10) were subjected to flash column chromatography in the same manner.

Determination of *in vitro* resistance inhibitory effect

Resistant inhibitory effect was determined according to the method of the National Committee for Clinical Laboratory Standards (U. S. A., 1990). Each sample of the various doses dissolved in absolute ethanol and the 50 and 100 μg/ml of Cm and Am, respectively, were added to 5 ml of TSB medium with 10^5 cells of *S. aureus* SA2. The mixture was vortexed thoroughly and then incubated at 37°C for 24 hrs. After incubation, the turbidity of the incubate resulted from the growth of the microorganism was measured spectrophotometrically. Effects of the samples were expressed as minimal inhibitory concentration (MIC) of samples themselves and minimal resistant inhibitory concentration (MRIC) of sample-antibiotic combination as described previously (Kim, *et al.*, 1995).

Apparatus

Chemical characteristics of the isolates were analyzed by GC/MS within the databases such as the Shimadzu QP-100 GC/MS system with Public/NIST Library and the VG TriolIA VG Biotech with a lab-base system including Sadtler and NBS Mass Spectral Data. For ^{1}H- and ^{13}C-NMR spectra, Bruker AM-300 system was used.

RESULTS AND DISCUSSION

Resistance inhibitory effects of the components of *Anetheum graveolens* when combined with Cm and Am

The silica gel column chromatography of the hexane fraction of *Anetheum graveolens* L. produced nine subfractions (AH's 1~9) of the different TLC pattern. The results in Table I show that AH 4, which was shown in a brownish spot on TLC, had the most potent inhibitory activity at the level of 50 μg/ml when combined with 50 μg/ml of Cm. It was also more effective when combined with Cm than with Am. Each

Table I. Inhibitory effects and minimal resistance inhibitory concentration (MRIC) of subfractions from hexane fraction of *Anetheum graveolens* when combined with Cm and Am indicated by the growth of *S. aureus* SA2

Subfractions	Combined with Cm[a]		Combined with Am[b]	
	Growth %[c]	MRIC (μg/ml)	Growth %[d]	MRIC (μg/ml)
AH 1	100	>50	100	>100
AH 2	50	>50	80	>100
AH 3*	2	>50	0	100
AH 4*	0	50	0	100
AH 5#	10	>50	10	>100
AH 6#	50	>50	50	>100
AH 7	100	>50	80	>100
AH 8	100	>50	100	>100
AH 9	100	>50	100	>100

Concentrations: [c]Sample, 50 μg/ml and [d]Sample, 100 μg/ml; [a]Cm, 50 μg/ml and [b]Am, 100 μg/ml.
*Brownish and #pinky spot on TLC (petroleum benzene-ethyl acetate=10-1; color developing agents: vanilin-sulfuric acid).

50 μg/ml of AH's 5 and 6, which were shown in a pinkish spot on TLC with the same *Rf* value as the brownish one, revealed comparatively weaker activities.

So AH's 3~6 were mixed together and subjected to column chromatography again to present two liquid compounds containing pinkish (compound 1) and brownish spots on TLC. As the compound 1 was identified as carvone by spectral studies, activities were compared with authentic *d*- and *l*-carvones. The MRIC's of the two components were lower when compared with the MIC's of them (Table II). These results imply that the components have no antibiotic activities but rather, they potentiate the action of antibiotics or reduce resistance of *S. aureus* SA2. The brownish (on TLC) liquid had higher resistant inhibitory activity than the pinkish (on TLC) liquid. They were also shown to be more effective when combined with Cm than with Am.

Resistance inhibitory effects of the components of *Acorus gramineus* when combined with Cm

Sixteen subfractions (ACH's 1~16) with different TLC

Table II. Minimal inhibitory concentration (MIC) and minimal resistance inhibitory concentration (MRIC) of the components isolated from *Anetheum graveolens* and reference compounds when combined with Cm and Am indicated by the growth of *S. aureus* SA2

Treated sample	MIC of sample alone	MRIC when combined with	
		Cm[a]	Am[b]
Mixture(AH's 3 and 4)	>1000	50	100
Compound 1(carvone, AH 6)	600	100	300
d-Carvone(Sigma)	600	100	400
l-Carvone(Sigma)	600	100	400

Concentrations: [a]Cm, 50 μg/ml and [b]Am, 100 μg/ml.

Table III. Inhibitory effects and minimal resisrance inhibitory concentration (MRIC) of the subfractions from hexane fraction of *Acorus gramineus* when combined with Cm[a] indicated by the growth of *S. aureus* SA2

Subfractions[b]	Growth %	MRIC (μg/ml)	Subfractions[b]	Growth %	MRIC (μg/ml)
ACH 1	100	>50	ACH 9	0	50
ACH 2	100	>50	ACH 10	0	50
ACH 3	82	>50	ACH 11	46	>50
ACH 4	15	>50	ACH 12	79	>50
ACH 5	2	50	ACH 13	90	>50
ACH 6	0	50	ACH 14	100	>50
ACH 7	0	50	ACH 15	100	>50
ACH 8	0	50	ACH 16	100	>50

Concentrations: [a]Cm, 50 μg/ml and [b]sample, 50 μg/ml.

Table IV. Minimal inhibitory effects (MIC) and minimal resistance inhibitory concentration (MRIC) of the components isolated from ACH's 5~10 and benzyl benzoate when combined with Cm indicated by the growth of *S. aureus* SA2

Treated sample	MIC of sample alone	MRIC when combined with Cm[a]
Mixture (ACH's 5~10)	>2000	50
Benzyl benzoate	>2000	20

Concentrations: [a]Cm, 50 μg/ml.

pattern from hexane fracton of Acori graminei Rhizoma were obtained by the same column chromatography. The growth percentage at 50 μg/ml of the subfractions are shown in Table III.

ACH's 5~10 had potent resistant inhibitory activity, so they were pooled and subjected to column chromatography again to isolate liquid mixture, which appeared as pinkish and bluish spots on TLC by vanillin-sulphuric acid. The mixtures were identified as benzoic acid phenylmethyl ester (benzyl benzoate) including three compounds by spectral studies. Activity of the mixtures were compared with that of benzyl benzoate purchased from Sigma (Table IV), which was found that the MIC of benzyl benzoate was considerably lower than it's MRIC. Benzyl benzoate, which is usually used as a scabicide and pediculicide, showed no effectiveness in the growth of multi-drug resistant *S. aureus* SA2, but when combined with 50 μg/ml of Cm, benzyl benzoate showed potent resistant inhibitory effect on the strain at the level of 20 μg/ml.

Structures of active principles

One of the liquid principles obtained from AH 6 from *Anetheum graveolens* was revealed as a single pinkish spot on TLC (vanillin-sulfuric acid). By the studies on GC/EI-MS with the databases, it identified the mixture mainly consisting of carvone [2-methyl-5-(1-methylethenyl)-2-cyclohexanone, $C_{10}H_{14}O$, (*R*)-form]. The other mixture (brownish spot on TLC) was found to consist of carvone [(*R*)- and (*S*)-form] and 4,7-dimethoxy-5-(2-propenyl)-1,3-benzodioxazole, which is the isomer of apiole (Tables V) by GC-Mass spectral

Table V. GC-Mass* spectral data of mixture 1 of AH's 3 and 4 isolated from *Anetheum graveolens* (brownish spot on TLC)

Constituents	GC retention time (mins)	Electron impact mass fragnentation (*m/z*)
2-Methyl-5-(1-methylethenyl)-cyclohexanone, $C_{10}H_{16}O$= 152.0	3.58 and 3.66	152.0 (M); 95.0; 67.0 (base); 41.0; 39.0
Carvone[2-Methyl-5-(1-methyl-ethenyl)-2-cyclohexene-1-one, $C_{10}H_{14}O$=150.0)	4.02	150.0 (M); 82.0 (base); 54.0; 39.0
4,7-Dimethoxy-5-(2-propenyl)-1,3-benzodioxazole(isomer of apiole, $C_{12}H_{14}O_4$=222.0)	8.78	222.0 (M); 207; 177.0; 149.0; 121.0; 106.0; 77.0; 53.0

*Shimadzu QP-100 GC/MS system and Public/NIST Library were used.

patterns.

Repeated chromatographies on former mixtures (pinkish on TLC) revealed pure carvone which were identified by spectral characteristics (Table VI).

A liquid component from eluates (ACH's 5~10) from *Acorus gramineus*, which were the pinkish and bluish spots on TLC, were found the mixture of benzoic acid phenylmethyl ester (benzyl benzoate), 1-phenyl-3-methyl-4-[4-(diethylamino)phenylimino]2-pyrazoline-5-one and an unidentified component (molecular weights; 222 and 353) (Table VII) through a spectral study

Table VI. Spectral data of isolated liquid compound 1 (carvone)

IR (cm^{-1})*	1H-NMR (δ)†	^{13}C-NMR (δ)†
751 (cyclohexenyl)	1.78 (6H, *d*, 2×CH_3)	C-1, >C=O (199.0)
900	2.25~2.75 (6H, m,	C-2, >C= (146.2)
1670(C=O)	cyclohexenone)	C-3, -CH< (135.0)
3010~2900 (methyl	4.70~4.8 (2H, *d*, -CH_2-)	C-4, -CH_2- (15.2)
ethenyl)	6.75~6.78 (1H, m, =CH-)	C-5, -CH< (30.8)
		C-6, -CH_2- (20.1)
		C-1', >C= (144.1)
		C-2', =CH_2 (110.1)
		CH_3×2 (42~42.6)

*Sample was coated to the surface of KBr disc.
†300 MHz and †75 MHz, $CDCl_3$, Bruker AM-300 spectrometer.

Table VII. GC-Mass* spectral data of mixture isolated from *Acorus gramineus* (pinkish and bluish spots on TLC)

Constituents	GC retention time (mins)	Mass fragnentation (m/z)
Unidentified 1 (mol.wt.=222)	6.43	222 (M^+); 193 (M-ethyl); 192; 121; 120 (base peak); 107; 84;
Benzoic acid phenylmethyl ester (Benzyl benzoate, m.w.=212)	7.55	212 (M^+); 194; 167; 105 (base peak); 91; 77;
1-Phenyl-3-methyl-4-[4-(diethyl-amino) phenylimino] 2-pyrazoline-5-one	11.12	336 (M^+); 335; 334 (Base);319; 265; 250; 157; 91; 29
Unidentified 2 (Mol. wt.=353)	13.36	353(M^+); 250; 235; 234; 233; 232; 217; 204; 117 (Base); 115;

*VG TriolIA, VG Biotech and Lab-Base system including Sadtler and NBS Mass Spectral Data.

using GC/MS within the databases.

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