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Screening and Isolation of Antibiotic Resistance Inhibitors from Herb Materials. I.—Resistance Inhibition of 21 Korean Plants

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Abstract—*Staphylococcus aureus* SA2, which was isolated from patient, is resistant to 10 usual antibiotics. The methanolic extracts of 21 well-known herb materials were combined with 10 antibiotics and applied to check inhibitory effects on the resistance of *S. aureus* SA2. The hexane fractions from methanolic extracts of Acori graminei Rhizoma and Anethi Fructus had most potent activity to inhibit the resistance of the bacteria when combined with ampicilin or chloramphenicol.

Keywords—*Staphylococcus aureus* SA2 · herb drugs · resistance inhibition

Staphylococcus aureus is one of the most abundant and persistent infectious microorganism (Baird-Parker, 1972 and Sheagren, 1984). As like many other bacteria they also easily get resistance to newly developed antibiotics. So it seems to be meaningful to find a method to reduce the resistance of bacteria to antibiotics. The strain of *Staphylococcus aureus* SA2, which was isolated from hospitalized patient, is a multiple resistant strain with four plasmids and is resistant to 10 usual antibiotics (Kang and Moon, 1990; Kim *et al.*, 1992; Lee and Moon, 1993; Lee and Moon, 1995) such as ampicillin (Am), chloramphenicol (Cm), clindamycin (Cl), erythromycin (Em), gentamicin (Gm), kanamycin (Km), methicillin (Mc), streptomycin (Sm), tetracycline (Tc) and tobramycin (Tm). Augmentin is a good example to reduce the resistance of microorganism to antibiotics. It was prepared from clavulanic acid(Neu and Fu, 1978), a β-lactamase inhibitor, and amoxillin which is ineffective to antibiotic-resistant microorganism.

To develop the antibiotic resistance inhibitors from plants we have screened 21 well-known herb materials (Lee, 1976; Woo *et al.*, 1979; Perry, 1980) on resistance inhibitory effect on 10 antibiotics.

Experimental

Bacterial strain - *Staphylococcus aureus* SA2, which was isolated from hospitalized patient in the Pusan area, was cultivated in our laboratory. The minimal inhibitory concentration (MIC) of 10 usual antibiotics to the strain is higher than the level of 50 μg/ml.

Plant material - The selected 21 herbs were purchased from a herb market in Pusan and identified pharmacognostically.

Extraction and fractionation - The chopped or powdered plant materials were refluxed with 95% methanol for three hrs five times and the extracts obtained were concentrated *in vacuo*. The eight methanolic extracts, which have high resistance inhibitory effect, were suspended in water and were successively partitioned with hexane, chloroform, ethyl acetate and butanol to take corresponding fractions for further studies.

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Table 1. Summary of the effects of each herb extract combined with various antibiotics on the growth of antibiotic-resistant microorganism (*Staphylococcus aureus* SA2) in solid medium.

Sample[a]	% Growth on Combination with antibiotics[b]										
	Control	Am*	Cm	Cl*	Em	Gm	Km*	Mc	Sm	Tc	Tm
Control(Vehicle only)	100	100	100	100	100	100	100	100	100	100	100
Acori graminei Rhizoma	100	30	<1	5	<1	50	20	50	50	50	100
Ailanthi Radicis Cortex	100	100	<1	90	<5	100	100	100	100	100	100
Ampelopsis Radix	100	100	10	100	10	100	100	100	50	100	100
Anethi Fructus	100	<1	<1	10	<1	100	50	80	100	50	100
Asteris Radix	100	100	7	50	50	100	100	100	100	5	100
Bletillae Rhizoma	100	100	100	50	50	100	100	100	100	100	100
Codonopsis pilosulae Radix	100	100	15	100	80	100	100	100	100	100	100
Dictamni Radicis Cortex	100	100	10	100	20	100	100	100	100	100	100
Ecliptae Herba	100	80	<1	100	<5	50	100	100	80	100	100
Eriobotryae Folium	100	100	100	100	70	100	100	100	100	100	100
Euphorbiae Radix	100	100	100	100	100	100	100	100	100	100	100
Euphorbiae kansui Radix	100	100	<1	90	20	100	30	20	10	50	50
Malvae Semen	100	<1	100	5	100	100	5	80	50	10	100
Remotiflorae Radix	100	100	70	100	100	100	100	100	100	100	100
Sanguisorbae Radix	100	100	80	100	100	100	100	100	100	100	100
Stemonae Radix	100	100	100	100	100	100	100	100	100	100	100
Synurus deltoides, aerial	100	100	<1	50	80	100	100	100	100	100	100
Terminaliae Fructus	100	100	15	100	10	100	100	100	100	100	100
Trapae Fructus	100	100	<1	5	70	100	100	100	100	100	100
Trigonellae Semen	100	100	80	100	100	100	100	100	100	100	100
Xanthii Fructus	100	100	100	90	100	100	100	100	100	100	100

[a]Concentration of sample: 200 μg/ml
[b]Concentration of antibiotics: 50 μg/ml was used with the exception of Am* (20 μg/ml), Cl* (300 μg/ml) and Km* (100 μg/ml)

The hexane fractions of Anethi Fructus and Acori graminei Rhizoma were applied to silica gel column chromatography (solvent system: petroleum benzene-chloroform = 10-1) to produce nine and eight eluates of different TLC patterns, respectively.

Sample preparation - Completely dried methanolic extract, each fraction and each eluate were dissolved in 200 μl of absolute ethanol, and were added to tryptic soy broth (TSB) medium.

Determination of resistance inhibitory effect *in vitro* - The bacteria strain was cultivated in TSB with antibiotics at 37°C for 12 hrs. Each sample and antibiotic were added to 5ml of tryptic soy agar(TSA, TSB + 1.5% agar) which was warmed at 50°C with an inoculum size of 10^5 cells for total volume. The medium was mixed thoroughly and then incubated at 37°C for 24 hrs.

The resistance inhibitory effect was represented as minimal resistance inhibitory concentration (MRIC), which means the lowest concentration that inhibits growth of the strain completely. The concentrations of antibiotics used in the study are approximately half dose of minimal inhibitory concentration (MIC) of antibiotics on growth.

Table 2. Summary of minimal resistance inhibitory concentration (MRIC) of each fraction of selected herbs combined with some antibiotics indicated by the growth of antibiotic-resistant microorganism (*Staphylococcus aureus* SA2) in solid medium.

Herb materials	Fractions*	MRIC (μg/ml) on Combination with			
		Am	Cm	Em	Km
Acori graminei Rhizoma	MeOH		100	>200	
	Hex		20	100	
	BuOH		100		
Ailanthi Cortex Radicis	MeOH		100	>200	
	Hex		200	200	
Anethi Fructus	MeOH	200	100	200	
	Hex	100	50	200	
	Chlrf		100		
Ecliptae Herba	MeOH	200		>200	
	Hex	200		200	
Euphorbiae kansui Radix	MeOH		200		
	Hex		200		
Malvae Semen	MeOH		200	>200	
	Hex		200	200	
Synurus deltoides, aerial	MeOH		25		
	Hex		10		
Trapae Fructus	MeOH		200		
	Chlrf		100		

*Types of Extract and fractions: MeOH, methanolic extract and Hex., hexane fr.; Chlrf., chloroform fraction; EtOAc, ethyl acetate fraction and BuOH, butanol fraction of methanolic extract, respectively.

Table 3. Inhibitory effects and minimal resistance inhibitory concentrations(MRIC) of eluates of hexane fraction of Anethi Fructus combined with chloramphenicol and ampicillin indicated by the growth of *Staphylococcus aureus* SA2.

Eluate No.	Combined with Cm		Combined with Am	
	Growth %[a]	MRIC (μg/ml)	Growth %[b]	MRIC (μg/ml)
AH 1	100	>20	100	>50
AH 2	50	>20	80	>50
AH 3	2	>20	50	>50
AH 4	0	20	10	>50
AH 5	10	>20	0	50
AH 6	50	>20	0	<50
AH 7	100	>20	80	>50
AH 8	100	>20	100	>50
AH 9	100	>20	100	>50
Anethole[c]	100	50	0	50

[a]Each sample was added 50 μg/ml
[b]Each sample was added 100 μg/ml
[c]MIC: 50 μg/ml.

Table 4. Inhibitory effects and minimal resistant inhibitory concentrations (MRIC) of eluates of hexane faction of Acori graminei Rhizoma combined with chloramphenicol indicated by the growth of *Staphylococcus aureus* SA2.

Eluate No.	Growth %[a]	MRIC (μg/ml)	Eluate No.	Growth %[a]	MRIC (μg/ml)
AH 1	100	>5	AH 5	–	–
AH 2	100	>5	AH 6	80	>5
AH 3	0	5	AH 7	100	>5
AH 4	0	5	AH 8	100	>5

[a]Each sample was added 5 μg/ml

Results and Discussion

Resistance inhibitory effects of herb materials were shown in Table I. Sample itself (Control), whithout the combination of antibiotics, allowed full growth of microorganism at the level of 200 μg/ml. At those conditions methanolic extracts of Acori graminei Rhizoma, Ecliptae Herba, Euphorbiae kansui Rhizoma, Asteris Rhizoma, Ailanthi Radicis Cortex, Anethi Fructus, aerial parts of *Synurus deltoides*, Trapae Fructus and Malvae Semen had significant inhibitory effects on the resistance of *S. aureus* SA2 when combined with several antibiotics particularly with chloramphenicol. They inhibited the growth of antibiotic-resistant bacteria at lower than 1% levels. Some kinds of methanolic extracts also showed resistance inhibitory effect when combined with Am, Cm, Em or Km.

The MRIC values of eight methanolic extracts and their fractions, which have high resistance inhibitory effects, were summarized in Table II. The hexane and chloroform fractions generally revealed high resistance inhibitory effect. The most potent effect was shown in the hexane fraction of *Synurus deltoides*, which is an edible plant('Chwinamool') in the country, when combined with Cm. Methanolic extracts and their fractions of Acori graminei Rhizoma and Anethi Fructus also showed considerable inhibitory effects. So further studies were focussed on two herb drugs Acori and Anethi.

The silica gel column chromatography of hexane fraction of Anethi Fructus produced nine eluates (AH1~AH9) of different TLC pattern. The results in Table III showed that eluate 4 (AH 4) had potent inhibitory activity at the level of 50 μg/ml when combined with chloramphenicol. So eluate 4 was subjected to column chromatography again to afford two liquid components. Eluates 5 and 6 (AH5, 6) had potent inhibitory activity at the level of 100 μg/ml when combined with ampicillin. Eluates 4~6 showed different TLC pattern from anethole (MRIC's, 5 μl/ml), the representative constituent of the herb. So eluates 5 and 6 were pooled together and were subjected to column chromatography again to afford liquid components. The structures of all isolate will be elucidated and reported later.

Eight eluates with different TLC pattern from hexane fracton of Acori graminei Rhizoma were obtained by the same column chromatographical method. The growth % and MRIC values of the eight eluates were shown in Table IV. Eluates 3, 4 and 5 (AH 3, 4 and 5) had potent inhibitory activity at the level of 5 μg/ml. The TLC pattern of the eluates are quite different from asarone, a major constituents.

Conclusion

Eight methanolic extracts of herbal drugs such as Acori graminei Rhizoma, Ecliptae Herba, Euphorbiae Rhizoma, Asteris Rhizoma, Ailanthi Radicis Cortex, Trapae

Fructus, Malvae Semen, Anethi Fructus and edible plant 'chwinamool' *Synurus deltoides* had significant inhibitory effects on the resistance of *S. aureus* SA2 when combined 10 usual antibiotics. They inhibited the growth of antibiotic-resistant bacteria at lower than 1% levels. Especially the column chromatographical eluates obtained from hexane fraction of Anethi Fructus and Acori graminei Rhizoma, when combined with chloramphenicol respectively, showed significant inhibitory effects at the level of 5 μg/ml.

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