Natural Product Sciences
6(1) : 36~39 (2000)

Screening and Isolation of Antibiotics Resistance Inhibitors from Herb Materials. V. – Resistance Inhibition by Acorenone from *Acorus gramineus* Solander

Hyekyung Kim, Kyung Ho Moon and Chung Kyu Lee*

College of Pharmacy, Kyungsung University, Pusan 608-736, Korea

Abstract – Acorenone, a diterpene isolated from *Acorus gramineus*, showed strong resistance inhibitory activity against multi-drug resistant microorganisms such as *Staphylococcus aureus* SA2, which has resistance to 10 usual antibiotics including chloramphenicol (Cm). at the level of 5 μg/ml when combined with 50 μg/ml of Cm. Bacterial resistance to Cm is due to the presence in resistant bacteria of an enzyme, chloramphenicol acetyltransferase (CAT), which catalyses the acetyl-CoA dependent acetylation of the antibiotic at C-3 hydroxyl group. To elucidate the mechanism of resistant inhibitory effect, the acorenone which had the strongest resistant inhibitory activity, was investigated on the CAT assay. As the result, the combination of Cm and acorenone showed the strongest inhibitory activity on CAT as noncompetitive and dose dependent manner.

Key words – Acorenone, antibiotics resistance inhibition, CAT inhibitor

Introduction

Since the introduction of penicillin, many compounds have been developed as antimicrobial agents or antibiotics, but newly investigated potent antimicrobial agent soon loose its activity (Shanson, 1981). One of the main reasons disappearing the effectiveness is due to the development of resistance to antibiotics by microorganism (Jacoby and Archer, 1991). In order to maintain the effectiveness of antibiotics, the reduction of resistance also is thought to be valuable as well as developing newer and stronger antibiotics. To find the new method for reduction of antibiotic resistance, several herb materials were tested as resistance inhibitors. According to the results of preliminary studies (Kim, *et al.*, 1995; Park, *et al.*, 1997), some essential oil components isolated from *Acorus gramineus* Solander (Araceae), when combined with antibiotics, showed potent inhibitory activities against multi-drug resistant microorganisms such as *Staphylococcus aureus* SA2 (Kim, *et al.*, 1998), which has resistances to 10 usual antibiotics including chloramphenicol (Cm). As active principle acorenone was identifiedand showed resistant inhibitory activity at the level of 5 μg/ml when combined with 50 μg/ml of Cm.

*Author for correspondence.

Experimental

Reagents – Tryptic soy broth (TSB) and agar were purchased from Difco Co., antibiotics, buffers and chloramphenicol acetyltransferase (CAT) were purchased from Sigma Co. Supplies for TLC were purchased from E. Merck.

Microbial strain – The strain *S. aureus* SA2 used in the studies was collected from hospitalized patient in Pusan area and cultivated in the medium containing 10 antibiotics at the laboratory of our university (Kang and Moon, 1990; Kim, *et al.*, 1992; Lee and Moon, 1993). The used concentrations of antibiotics are near or below the half dose of minimal inhibitory concentration of the bacterial growth.

Resistance inhibitory effect – The bacterial strain was cultivated in TSB with 50 μg/ml of antibiotics for sustaining the resistance and suppression of other bacterial strain at 37°C for 12 hrs. Each sample and antibiotics were added to 5 ml of TSB medium with an inoculum size of 10^5 cells of *S. aureus* SA2. The inoculated medium was incubated at 37°C for 24 hrs. After incubation, turbidity of incubate due to the growth of microorganism was measured spectrophotometrically. The resistance inhibitory effect was expressed as minimal resistance inhibitory concentration, MRIC, in μg/ml of medium.

CAT activity – CAT activity was determined by

the method of Seed and Sheen (1988) based on the amount of acetyl-CoA (Ellman, 1959).

Instrumental analysis – UV spectrum was taken with Shimadzu UV-1601 spectrometer, FT-IR spectrum with MAGMA-IR 560 spectrometer, GC-MS spectra with Shimadzu GA-17 spectrometer, and ^{1}H- and ^{13}C-NMR spectra with Brucker-AM 200 spectrometer (200 MHz, $CDCl_3$), respectively.

Results and Discussion

The effects on growth at 50 μg/ml of the subfractions are as shown in Table 1. ACH's 5~10 had potent resistance inhibitory activity, so they were pooled and subjected to column chromatography again to isolate liquid mixture, which appeared as pink and bluish spots on TLC by vanillin-sulphuric acid. The GC/MS spectra of the liquid were found as mixture of constituent I~X (Konstantin and Egon, 1983; Egon and Kostantin, 1983).

Table 1. Inhibitory effects and minimal resistance inhibitory concentration (MRIC) of the subfractions from hexane fraction of *Acorus gramineus* when combined with cm[1)] against *S. aureus* SA2

Sub-fractions[2)]	Growth %	MRIC (μg/ml)	Sub-fractions[2)]	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: [1)]chloramphenicol, 50 μg/ml and [2)]sample, 50 μg/ml.

The GC/MS spectra of the constituents were showed the same MS fragmentation pattern with the molecular ion peak at 220 *m/z*. The database of MS spectrum showed same fragmentation pattern of acorenone [(1S,4S,5S)-1-isopropyl-4,8-dimethyl-spiro [4,5]dec-8-en-7-one], a sesquiterpene, which were already isolated from *A. calamus* by Rascher and Wolf (1977). The compound was confirmed by IR and NMR spectra and GC-MS data which were well agreed with reference data (Konstantin and Egon, 1983; Egon and Kostantin, 1983).

Effect of acorenone on CAT – As acorenone was found to be the most potent resistance inhibitor the mechanism of action was studied. The bacterial resistance to Cm is due to the presence of the enzyme CAT which catalyzes the acetyl-CoA dependent acetylation of the antibiotic at C-3 hydroxyl group(Shaw, 1984; Shaw and Richmond, 1983) as shown in Fig. 1. Assay for CAT activity resulted the combination of Cm and acorenone acted non-competitively and dose dependent manner with CAT. As

Table 2. GC-Mass* spectral data of isolated mixture of Acori graminei Rhyzoma

Compounds	GC Retention Time (min)	Mass Fragmentation (*m/z*)*
I	5.2	220; 205; 192; 177; 163; 150; 140; 123; 109; 93; 81
II	5.3	220; 205; 192; 177; 163; 150; 140; 123; 109; 95; 81
III	5.5	220; 205; 191; 177; 163; 149; 135; 121; 107; 91; 81
IV	5.9	220; 205; 192; 177; 164; 147; 135; 121; 108; 93; 81
V	6.2	220; 205; 192; 177; 163; 153; 140; 121; 107; 97; 79
VI	6.4	220; 205; 192; 177; 165; 149; 135; 121; 107; 95; 81
VII	6.71	220; 202; 187; 177; 159; 147; 131; 121; 107; 95; 81
VIII	6.77	220; 207; 193; 179; 166; 151; 138; 124; 111; 95; 81
IX	6.9	220; 205; 191; 177; 164; 150; 135; 123; 109; 93; 82
X	7.0	220; 205; 191; 177; 164; 150; 135; 121; 109; 93; 82

*Obtained with Shimadzu GA-17 spectrometer

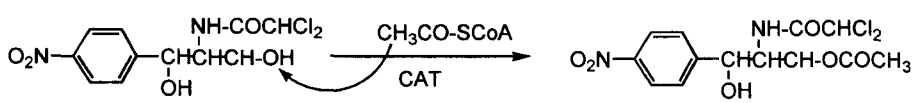

Fig. 1. Acetylation of chloramphenicol by CAT.

Table 3. Activities of acorenone and chloramphenicol* on various antibiotics-resistant strain.

Strains	Cm MIC (μg/ml)	Acorenone MIC (μg/ml)	Cm* + Acorenone MRIC (μg/ml)
S. aureus RN4220 (non-resistant)	4	1024	–
S. aureus pKH7 (resistant to Cm only)	256	1024	2
S. aureus SA2	256	1024	4

*Concentration: 50 μg/ml

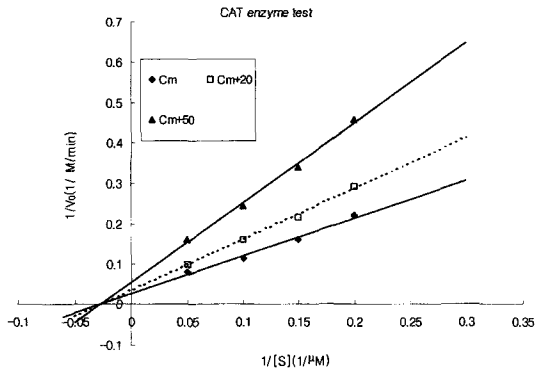

Fig. 2. Effects of acorenone on the CAT activity.

shown in double reciprocal plot (Fig. 2), V_{max} value was increased as 19, 30 and 39 according to the increase of acorenone concentration as 0, 20 and 50 μg/ml. But K_m value was constant in 37.

Table 3 shows the confirmative activities of acorenone on different strain of staphylococci. The combination of Cm and acorenone showed potent resistance inhibitory activity to *S. aureus* pKH7, which is resistant to Cm only at MRIC level of 2 μg/ml and 4 μg/ml with *S. aureus* SA2, which is resistant to 10 antibiotics including Cm. Table 4 shows growth inhibition of the strains at the fixed dose of Cm and acorenone. In this test the inhibition level of acorenone was 4 μg/ml.

Table 4. Growth inhibition of the various strains at the fixed dose of Cm and acorenone.

Strains	Cm[1]	Acorenone[2]	Cm[1] + Acorenone[2]
S. aureus RN4220	–	+++	–
S. aureus pKH7	+++	+++	–
S. aureus SA2	+++	+++	–

Concentration: [1]64 and [2]4 μg/ml

Conclusion

The resistance inhibitory effect of acorenone, which had the strongest resistant inhibitory activity, was concerned with the CAT enzyme as non- competitive and dose dependent manner. The effective dose was less than 5 μg/ml when combined with 50 μg/ml of Cm.

Acknowledgement

This research was supported by Kyungsung University Research Grant in 1998.

References

Egon, S. and Kostantin, K., Extraction of thermolabile compounds with super-critical gases-12 extraction of β-asaron free Calamus Rhizoma, *J. Med. Plant Res.*, **47**, 751-778 (1983).

Ellman, G. L., Tissue sulfhydryl groups, *Arch. Biochem. Biophys.* **82**, 70-77 (1959).

Jacoby, G. A. and Archer, G. L., New mechanism of bacterial resistance to antimicrobial agents, *New Eng. J. Med.*, **Feb. 28**, 601-612 (1991).

Kang, J. S. and Moon K. H., Antibiotic resistance in *Staphylococcus aureus* isolated in Pusan, *Yakhak-Hoeji*, **34**, 122-125 (1990).

Kim, H. K., Moon, K. H. Ryu S. Y., Moon D. C. and Lee, C. K., Screening and isolation of antibiotic resistance inhibitors from herb materials. IV. - Resistance inhibitors from *Anethum graveolens* and *Acorus graminus*, *Arch. Pharm. Res.*, **21**(1), 62-66 (1998).

Kim, H. K., Park, S. W., Park, J. N., Moon, K. H. and Lee, C. K., Screening and isolation of antibiotic resistance inhibitors from herb materials. I. - Resistance inhibition of 21 Korean plants, *Natural Product Sciences* (Korea), **1**(1), 50-54 (1995).

Kim, K. H., Lee, D. W. and Moon, K. H., Characteriza-

tion of antibiotic resistant plasmid of *Staphylococcus aureus*, *Yakhak-Hoeji*, **36**, 486-490 (1992).

Konstantin, K. and Egon, S., Composition of essential oil from β-asaron free *Calamus*, *J. Med. Plant Res.*, **47**, 71-74 (1983).

Lee, D. W. and Moon, K. H., Characterization of chloramphenicol resistant plasmid of multidrug-resistant *Staphylococcus aureus*, *Yakhak-Hoeji*, **37**, 621-624 (1993).

Park, J. N., Kim, H. K., Moon, K. H. and Lee, C. K., Screening and isolation of antibiotics resistance inhibitors from herb materials. II.-effects of Chwinamool' (*Aster scaber*), *Kor. J. Pharmacogn.*, **28**(3), 162-165 (1997).

Rascher, W. and Wolf, H., (±)-Acorenone and (±)-Acorenone-B by configurational inversion at the spirochirality center, *Tetrahedron*, **33**, 575-577 (1977).

Seed, B. and Sheen, J. Y., A simple phase-extraction assay for chloram-phenicol acyltransferase activity. *Gene*, **67**, 271-277 (1988).

Shanson, D. C., Antibiotic-resistant *Staphylococcus aureus*, *J. Hosp. Infect.*, **2**, 11-36 (1981).

Shaw, W. V., Bacterial resistance to chloramphenicol, *Brit. Mol. Bull.*, **40**(1), 36-41 (1984).

Shaw, W. V. and Richmond M. H., Chloramphenicol acetyl transferase: *Enzymology and Mollecular Biology. CRC Critical Reviews in Biochemistry*, **14**, 1-46 (1983).

(Accepted February 17, 2000)