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In vitro activity of garlic oil and four diallyl sulphides against antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

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The *in vitro* antibacterial activities of garlic oil and four diallyl sulphides naturally occurring in this oil were studied against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (total 237 clinical isolates). Garlic oil at 4 × MIC could reduce original inoculum to ≤2 $\log_{10}$ in both *P. aeruginosa* and *K. pneumoniae* within 8 h. The MIC values of four diallyl sulphides against these two pathogens followed the order diallyl monosulphide > diallyl disulphide > diallyl trisulphide (DAT) > diallyl tetrasulphide (DATS) ($P < 0.05$). Most interactions of ceftazidime, gentamicin, imipenem and meropenem with DAT or DATS, determined according to the fractional inhibitory concentration index, showed synergic or additive effects. These results suggest that garlic oil, DAT and DATS may have potential for the prevention or treatment of nosocomial, antibiotic-resistant bacterial infections.

Introduction

Pseudomonas aeruginosa and *Klebsiella pneumoniae* are common nosocomial pathogens in Taiwan.[1–3] Because these bacterial pathogens are multiply resistant to many antibiotics such as ceftazidime and gentamicin, the infections caused by them not only require expensive antibiotic treatment but also increase the morbidity and mortality in hospitalized patients.[3,4] In order to control these infections, there is a need for the development of agents with marked antibacterial activity, greater sensitivity and less toxicity.

The antimicrobial activity and other medical benefits of garlic oil have been widely recognized.[5–8] The potential for clinical benefit from the use of garlic oil as an antimicrobial agent has been suggested by recent studies.[5,6] Chemical analysis of garlic oil showed that 54.5% of the total sulphides was the sum of diallyl monosulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide (DAT) and diallyl tetrasulphide (DATS).[9] Because the commercial sources of DAS and DADS are less expensive and easily obtained, the antimicrobial activities of these two sulphide compounds have been focused upon in many studies.[7,8,10] Although DAT and DATS represented 26.6% of the total sulphides found in garlic oil, little attention has been paid to them so far with respect to their antimicrobial activity.

The inhibitory effect of DAS, DADS, DAT and DATS against *Helicobacter pylori* has been observed in a previous report,[5] the authors of which indicated that the bactericidal activity of these sulphides correlated with the number of sulphur atoms contained within them. On the other hand, it has also been indicated that the bactericidal effects of DAS and DADS against *K. pneumoniae* are due to them inhibiting the activity of arylamine *N*-acetyltransferase, an enzyme found in this pathogen.[11] Thus, it is reasonable to examine the antibacterial activity of these sulphides against other medically important bacterial pathogens such as *P. aeruginosa.*

This study aimed to examine and compare the inhibitory activity of garlic oil and its four diallyl sulphides against two bacterial pathogens, *P. aeruginosa* and *K. pneumoniae*. The interaction of sulphide agents with antibiotics was also investigated. The results were expected to be beneficial for the development of new therapeutic agents.

Materials and methods

Garlic oil preparation

Garlic bulbs (*Allium sativum* L.) were purchased directly from farms. The method of Ravid & Putievsky[12] was

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followed to prepare the garlic oil. Fresh plant materials were steam distilled for 3 h in a 100 L direct steam pilot plant apparatus. The recovered oil (2.2–4.3 g oil/kg garlic bulb) was stored at –80°C before use.

Diallyl sulphide preparation

DAS (purity 97%) and crude DADS (purity 80%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). DADS was purified further by fractional distillation to reach a final purity of >98%, which was examined by high performance liquid chromatography (HPLC). DAT and DATS were obtained by fractional distillation from crude DADS. The identification of DAT and DATS was confirmed by ^{1}H-NMR spectroscopy ($CDCl_3$, 300 MHz) and corresponded with the published data of Sparnins *et al*.[13] The prepared standards were stored at –80°C before use. The concentrations of DAS, DADS, DAT and DATS in the prepared garlic oil were quantified by the HPLC method of Lawson *et al*.,[9] and were found to be 1.8 ± 0.3, 26.7 ± 1.2, 17.2 ± 0.8 and 7.4 ± 0.5%, respectively; close to those of Lawson's original study.

Bacterial strains and medium

P. aeruginosa and *K. pneumoniae* were isolated from infected patients in Chungshan Hospital (Taichung, Taiwan). The total numbers of clinical isolates of *P. aeruginosa* and *K. pneumoniae* in this study were 123 and 114, respectively. All isolates were identified by Vitek (Vitek AMS; bioMérieux Vitek, Inc., Hazelwood, MO, USA) and API 20E (API-bioMérieux, La Balme Les Grottes, France). Antibiotic resistance profiles were determined by using discs with antibiotics placed on the surface of nutrient agar plates seeded with the test organism. Inhibition zones were measured after 24 h incubation at 37°C. Interpretation of resistance was based on the National Committee for Clinical Laboratory Standards (NCCLS) criteria. The antibiotics used were meropenem, ceftazidime, imipenem and gentamicin. The discs with antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA). All cultures were routinely maintained on nutrient agar (Difco, Detroit, MI, USA) at 25°C until used.

MIC determination

Garlic oil and prepared standards of DAS, DADS, DAT and DATS were used for antibacterial tests, and their MICs determined using both antibiotic-susceptible and -resistant *P. aeruginosa* and *K. pneumoniae*. Microdilution MICs were determined with strains grown in cation-adjusted Mueller–Hinton broth according to NCCLS guidelines.[14] The agent concentrations ranged from 128 to 0.125 mg/L. All incubations were at 37°C. Garlic oil, DAT and DATS had lower MICs (Table I), and were therefore used for the following two experiments.

Time–kill study of garlic oil

Twelve *P. aeruginosa* and 14 *K. pneumoniae* clinical isolates, which were multiply resistant to ceftazidime, gentamicin, imipenem and meropenem, were used in this study. *In vitro* kill of garlic oil against 12 multiply antibiotic-resistant *P. aeruginosa* or 12 *K. pneumoniae* was monitored in 10 mL volumes over 24 h at 37°C, after inoculation with actively growing cultures in cation-adjusted Mueller–Hinton broth without agitation. Aliquots (100 μL) were cultured on solid medium at intervals for determination of cfu/mL, and viable counts were read after 24 h incubation. The limit of detection was 20 cfu/mL.

Interaction of DAT or DATS with antibiotics

The interactive relationships between DAT or DATS and the four antibiotics against both antibiotic-susceptible and -resistant *P. aeruginosa* and *K. pneumoniae* were evaluated by the chequerboard method recommended by the NCCLS.[15] Aliquots (100 μL) of each agent at 10 times the targeted final concentration were used. Agent–agent interactions were classified as synergic, additive or less-than-additive based on the fractional inhibitory concentration (FIC) index, which is the sum of FICs for each agent. The FIC of each agent is calculated as the MIC of the agent in combination, divided by the MIC of the agent alone. Agent–agent interactions are considered synergic if the FIC index is <1.0, additive if the FIC is equal to 1.0 and less-than-additive if the FIC index is >1.0.

Results

The proportions of *P. aeruginosa* and *K. pneumoniae* clinical isolates from Chungshan Hospital resistant to the four commonly used antibiotics were examined, and the resistance rates of the two pathogens to these antibiotics were in the range 12.2–36.8%. Gentamicin was the antibiotic with the highest resistance rate in this study. The MIC values of garlic oil and four diallyl sulphides for *P. aeruginosa* and *K. pneumoniae* are presented in Table I and followed the order DAS > DADS > DAT > garlic oil = DATS ($P < 0.05$). For each test organism, there was no significant difference in the MIC value of test agent between antibiotic-susceptible and antibiotic-resistant strains ($P > 0.05$).

The bactericidal effects of garlic oil determined by *in vitro* time–kill curves are shown in the Figure. After 6 h incubation, the bactericidal effects of garlic oil against both organisms increased significantly with increasing garlic oil concentrations from 0.5 × MIC to 4 × MIC. Garlic oil at 4 × MIC could effectively reduce the original inoculum to <2 $\log_{10}$ in *P. aeruginosa* and *K. pneumoniae* within 6 and 8 h, respectively.

The interactions of DAT or DATS with the four antibiotics against both antibiotic-susceptible and -resistant *P. aeruginosa* and *K. pneumoniae*, determined according

Table I. MICs (mg/L) of garlic oil and four diallyl sulphides (DAS, DADS, DAT and DATS) against antibiotic-susceptible and -resistant *P. aeruginosa* and *K. pneumoniae* (data are expressed as mean ± S.D.)

	Garlic oil	DAS	DADS	DAT	DATS
P. aeruginosa					
susceptible	16 ± 2	80 ± 12	64 ± 8	32 ± 4	12 ± 2
CZ resistant	16 ± 4	84 ± 16	64 ± 4	32 ± 8	12 ± 2
GE resistant	16 ± 8	88 ± 12	64 ± 8	36 ± 4	12 ± 4
IM resistant	20 ± 2	80 ± 16	72 ± 8	36 ± 8	16 ± 2
ME resistant	16 ± 4	84 ± 12	64 ± 8	32 ± 8	16 ± 4
K. pneumoniae					
susceptible	24 ± 8	96 ± 12	72 ± 8	40 ± 8	20 ± 4
CZ resistant	28 ± 4	96 ± 16	72 ± 4	48 ± 4	24 ± 2
GE resistant	24 ± 4	104 ± 8	80 ± 8	48 ± 8	20 ± 8
IM resistant	24 ± 8	96 ± 8	80 ± 12	40 ± 4	24 ± 4
ME resistant	28 ± 8	104 ± 16	72 ± 8	40 ± 4	24 ± 8

CZ, ceftazidime; GE, gentamicin; IM, imipenem; ME, meropenem.

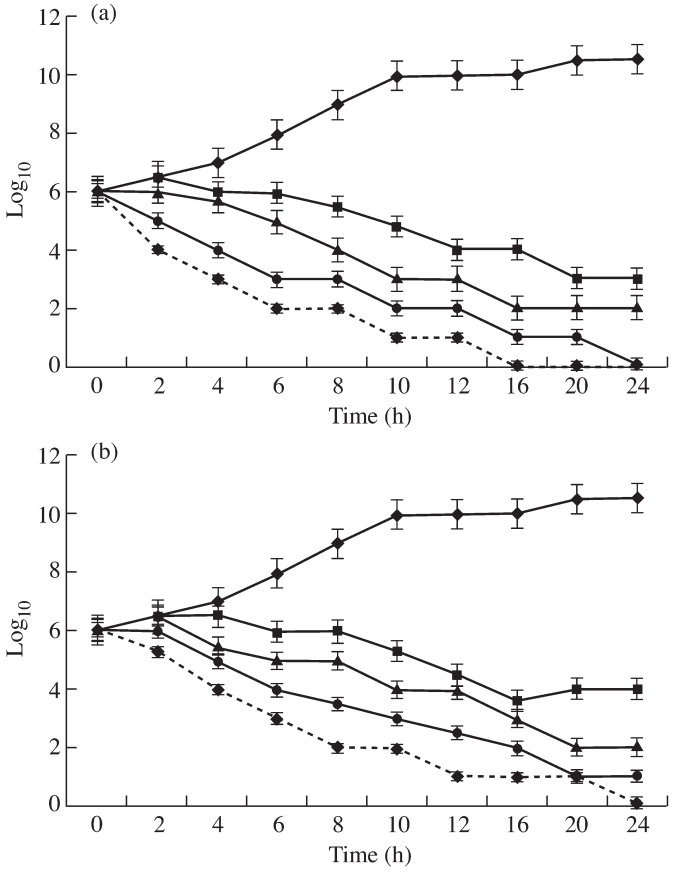

Figure. *In vitro* time–kill of garlic oil at various concentrations against *P. aeruginosa* (a) and *K. pneumoniae* (b) within 24 h. –◆–, control; ■, 0.5 × MIC; ▲, 1 × MIC; ●, 2 × MIC; - -◆- -, 4 × MIC. Data are expressed as mean ± S.D. (n = 12).

to the FIC index, are shown in Tables II and III. Two combinations of DAT with antibiotics were less-than-additive because their FIC indices were >1 (Table II). They were DAT plus ceftazidime against ceftazidime-resistant *K. pneumoniae*, and DAT plus gentamicin against gentamicin-resistant *K. pneumoniae*. The other interactions of DAT or DATS with the four antibiotics against both *P. aeruginosa* and *K. pneumoniae* were classed as synergic or additive because the FIC indices were <1 (Tables II and III).

Discussion

The antimicrobial activity of garlic oil against *H. pylori* and *Aspergillus* species has been reported.[5,6] The results of our present study show the antimicrobial activity of garlic oil against two other medically important pathogens. Since about 50% of garlic oil consisted of these four diallyl sulphides[9] and these sulphides also possessed antibacterial activities (Table I), the observed antibacterial activity of garlic oil is partly explained by them. It is known that the multiply antibiotic-resistant bacteria tested can cause severe nosocomial infections, which increase morbidity and mortality in hospitalized patients.[3,4] Garlic oil with the lower MICs (Table I) and effective bactericidal activity (Figure) may be considered as a functional food in human nutrition for prevention or treatment of nosocomial infections.

According to NCCLS standards, a breakpoint for antibiotic susceptibility is 8 mg/L. The MIC values of the four antibiotics tested for these antibiotic-resistant strains were >256 mg/L (data not shown). In practice, the MIC values

for DAS and DADS may be too high to allow their clinical use. Although the MIC values of DAT and DATS still exceed 8 mg/L, these two agents were more effective in inhibiting antibiotic-resistant *P. aeruginosa* and *K. pneumoniae* when compared with the antibiotics tested. Furthermore, it should be pointed out that loss of garlic oil and sulphides by volatilization might occur during experimental procedure, although these materials have been treated

Table II. Interaction of DAT with antibiotics, determined according to the FIC index, against antibiotic-susceptible and -resistant *P. aeruginosa* and *K. pneumoniae*

	P. aeruginosa		*K. pneumoniae*	
	susceptible	resistant	susceptible	resistant
DAT	0.5	0.75	0.5	0.75
CZ	0.25	0.25	0.5	0.375
FIC index[a]	0.75	1.0[b]	1.0[b]	1.125[c]
DAT	0.25	0.625	0.25	0.75
GE	0.5	0.25	0.75	0.5
FIC index	0.75	0.875	1.0[§]	1.25[c]
DAT	0.25	0.5	0.25	0.5
IM	0.5	0.375	0.5	0.5
FIC index	0.75	0.875	0.75	1.0[b]
DAT	0.25	0.75	0.625	0.625
ME	0.125	0.125	0.125	0.25
FIC index	0.625	0.875	0.75	0.875

CZ, ceftazidime; GE, gentamicin; IM, imipenem; ME, meropenem.
[a]The interaction of DAT with antibiotics was evaluated by the chequerboard method recommended by the NCCLS for each agent.
[b]The interaction was additive.
[c]The interaction was less than additive.

Table III. Interaction of DATS with antibiotics, determined according to the FIC index, against antibiotic-susceptible and -resistant *P. aeruginosa* and *K. pneumoniae*

	P. aeruginosa		*K. pneumoniae*	
	susceptible	resistant	susceptible	resistant
DATS	0.25	0.5	0.375	0.5
CZ	0.5	0.375	0.5	0.5
FIC index[a]	0.75	0.875	0.875	1.0[b]
DATS	0.5	0.5	0.75	0.75
GE	0.25	0.375	0.125	0.25
FIC index	0.75	0.875	0.875	1.0[b]
DATS	0.5	0.5	0.25	0.625
IM	0.125	0.25	0.5	0.25
FIC index	0.625	0.75	0.75	0.875
DATS	0.125	0.25	0.5	0.75
ME	0.5	0.5	0.25	0.125
FIC index	0.625	0.75	0.75	0.875

CZ, ceftazidime; GE, gentamicin; IM, imipenem; ME, meropenem.
[a]The interaction of DATS with antibiotics was evaluated by the chequerboard method recommended by the NCCLS for each agent.
[b]This interaction was additive.

with caution. Consequently, the MIC values obtained might be overestimated. On the other hand, these sulphide agents are naturally occurring components of garlic oil[9] and certain vegetables, such as garlic and onion.[13] The dietary nature of these vegetables may suggest the possible safety of these agents at these concentrations; however, further *in vivo* studies are needed to evaluate the metabolism, cytotoxicity, bactericidal efficiency and side effects of both.

In the studies by Chen *et al.*[11] and Naganawa *et al.*,[16] the anti-*K. pneumoniae* and anti-*Candida albicans* activities of DADS were significantly greater than DAS. O'Gara *et al.*[5] indicated a relationship between lower MICs and number of sulphur atoms/molecule for diallyl sulphides against *H. pylori*. Our present study found a similar relationship in inhibition of *P. aeruginosa*. These results agreed with previous studies,[5,11,16] and suggested that the number of sulphur atoms/molecule and/or disulphide bonds in these diallyl sulphides was an important factor in determining their antimicrobial activities.

In our present study, the inhibitory effects of DAS and DADS upon *K. pneumoniae* were close to those previously reported in a study[11] that also suggested that inhibition of arylamine *N*-acetyltransferase activity might be responsible. As the latter enzyme is also present in *P. aeruginosa*,[17,18] the same explanation may apply to the inhibition of growth reported here for this species. It is known that all four antibiotics could interfere with bacterial cell wall synthesis, increase bacterial membrane permeability and/or inhibit bacterial protein synthesis at the 30S subunit of ribosomes.[19–21] Therefore, the different modes of action of sulphide agents from antibiotics may be an important factor in the enhanced bactericidal efficacy observed when used in combination (Tables II and III). Since the combinations of diallyl polysulphides with antibiotics could inhibit both antibiotic-susceptible and antibiotic-resistant pathogenic bacteria, and most combinations were synergic or additive, the application of DAT or DATS combined with these antibiotics may be practical and beneficial in inhibiting both pathogens. Also, the required dosage of these antibiotics used in combination may be less than when used alone, which may further reduce the occurrence of side effects caused by these antimicrobials. Several studies have proved that DAS and DADS could be recovered in circulation via oral supply, and showed medical benefits.[22,23] The iv administration of the sulphides would be more suitable because antibiotics could be administered simultaneously.

In conclusion, garlic oil and its two components, DAT and DATS, possessed *in vitro* antibacterial activity against multiply antibiotic-resistant *P. aeruginosa* and *K. pneumoniae*. Both additive and synergic effects were observed in the combinations of ceftazidime, gentamicin, imipenem and meropenem with these two sulphide agents; therefore, garlic oil and both sulphides may have the potential to prevent or treat nosocomial infections caused by *P. aeruginosa* and *K. pneumoniae*.

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