ANTIMICROBIAL AGENTS AND RESISTANCE

In-vitro antimicrobial activity of four diallyl sulphides occurring naturally in garlic and Chinese leek oils

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The in-vitro antimicrobial activity of garlic oil, Chinese leek oil and four diallyl sulphides occurring naturally in these oils against *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), three *Candida* spp. and three *Aspergillus* spp. (total of 276 clinical isolates) was studied. The magnitude of activity of the four diallyl sulphides followed the order diallyl tetrasulphide > diallyl trisulphide > diallyl disulphide > diallyl monosulphide. These results suggest that disulphide bonds are an important factor in determining the antimicrobial capabilities of these sulphides. The concentration of four diallyl sulphides in garlic and Chinese leek oils was in the range 41.7–52.7% of total sulphides. Garlic oil, with a higher concentration of four diallyl sulphides, showed greater antimicrobial activity than Chinese leek oil. Diallyl disulphide, diallyl trisulphide, diallyl tetrasulphide and the oils rich in these sulphides may have a role in the prevention or treatment of infections.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), *Candida* spp. and *Aspergillus* spp. are the most commonly identified bacterial and fungal species responsible for severe nosocomial infections in Taiwan [1–3]. Such infections not only require expensive antibiotic treatments but also increase morbidity and mortality in hospitalised patients. To control these infections, there is a need for other agents with greater antimicrobial activity and less toxicity. The antimicrobial activity and other medical benefits of garlic oil have been widely recognised [4–6]. These benefits have been attributed to the presence of sulphides in garlic oil [5, 6]. Because they are easy to obtain or prepare as well as having good stability, the medical properties of the garlic constituents diallyl monosulphide and diallyl disulphide have been the focus of many studies [7–10]. A chemical analysis of garlic oil showed that 54.5% of the total sulphides comprised diallyl monosulphide, diallyl disulphide, diallyl trisulphide and diallyl tetrasulphide [11]. Although diallyl trisulphide and diallyl tetrasulphide accounted for 26.6% of the total sulphides in garlic oil, little attention has been paid to the medical benefits of these two

components. The inhibitory effect of diallyl disulphide, but not diallyl monosulphide, against *C. albicans* has been reported previously [12]; however, information regarding the activity of these two agents against *Aspergillus* spp. and MRSA is limited. The antimicrobial activity of garlic extract against *S. aureus* has been observed [13]; however, whether garlic oil can inhibit MRSA and fungal pathogens remains unknown. Also, the compounds in garlic oil responsible for any inhibitory effect require further elucidation.

Like garlic, Chinese leek is a member of the *Allium* family and a vegetable commonly used in oriental society. Apart from garlic and onion, little attention has been paid to the potential medical benefits of *Allium* plants. Recently, the antioxidant and antifungal activities of aqueous extracts from Chinese leek and other *Allium* plants were studied in this laboratory [14, 15]. The present study extended these observations by examining the antimicrobial activities of four diallyl sulphides occurring naturally in garlic and Chinese leek oils with a view to the development of new antibiotic agents or new functional foods.

Materials and methods

Sample preparation

Garlic bulbs (*Allium sativum* L.) and Chinese leeks (*A. odoratum* L.) were purchased directly from farms. The

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method of Ravid and Putievsky [16] was used to prepare essential oil. Fresh plant materials were steam-distilled for 3 h in a 100-L direct steam pilot plant apparatus. The oil recovered (2.2 ~ 4.3 g of oil/kg of garlic bulb; 1.1 ~ 3.5 g of oil/kg of Chinese leek) was stored at -80°C until used.

Standard preparation

Diallyl monosulphide (purity 97%) and crude diallyl disulphide (purity 80%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Diallyl disulphide was further purified by fractional distillation and its final purity was $>98\%$ when examined by high-performance liquid chromatography (HPLC). Diallyl trisulphide and diallyl tetrasulphide were obtained by fractional distillation from crude diallyl disulphide. The identification of diallyl trisulphide and diallyl tetrasulphide was confirmed by $^1\text{H-NMR}$ spectroscopy (CDCl_3 , 300 MHz) as described by Sparnins *et al.* [17]. Standards were stored at -80°C until used.

Analysis of four diallyl sulphides in garlic and Chinese leek oil

One mg of essential oil prepared from each plant was redissolved in 10 ml of acetonitrile immediately before compositional analysis by the method of Lawson *et al.* [11]. A 2- μl sample was injected into a Hitachi C18 HPLC with a Supelco LC-18, 250 mm $\times$ 4.6 mm $\times$ 5 μm column and acetonitrile:water:tetrahydrofuran (70:27:3) at a flow rate of 1 ml/min as eluent, and the absorbance of fractions was read at 240 nm. Standards were used to identify and quantify these diallyl sulphides in oils prepared from garlic and Chinese leek.

Strains and medium

S. aureus, MRSA, three *Candida* spp. (*C. albicans*, *C. krusei*, *C. glabrata*) and three *Aspergillus* spp. (*A. niger*, *A. flavus*, *A. fumigatus*) were isolated from infected patients in Chungshan Hospital (Taichung, Taiwan). The total numbers of clinical isolates of *S. aureus*, MRSA and fungi were 40, 60 and 176, respectively. All isolates were identified by conventional methods [18] and routinely maintained on nutrient agar or Sabouraud dextrose agar (Difco, Detroit, MI, USA) at 25°C until used.

Antibacterial tests

Diallyl monosulphide, diallyl disulphide, diallyl trisulphide, diallyl tetrasulphide standards and the two essential oils were tested against *S. aureus* and MRSA. Methicillin, penicillin, cefotaxime and tetracycline were purchased from Sigma and used as comparitors. Microdilution MICs were determined with strains grown in cation-adjusted Mueller-Hinton broth according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines [19]. The concentra-

tions of agents were 128–0.125 mg/L. All incubations were at 35°C . Clavulanic acid at a concentration of 2 mg/L in the medium was used to verify the production of β -lactamase in these clinical isolates.

Antifungal tests

The four diallyl sulphides and two essential oils were diluted 10-fold in polyethylene glycol because of their poor solubility. All agents were further diluted 1 in 5 in RPMI 1640 medium. A broth macro-dilution method was performed as described by the NCCLS [20] with agent concentrations of 128–0.125 mg/L. Agent-free and fungi-free controls were included. Turbidity was measured spectrophotometrically at 530 nm after incubation for 48 h at 35°C in RPMI 1640 medium containing 0.165 M morpholinepropanesulphonic acid (MOPS) (pH 7.0). The MIC was defined as the concentration that produced 80% reduction in turbidity, compared with that of agent-free controls. Amphotericin B (Sigma) was used for comparison in antifungal tests. Isolates were classified as susceptible if the MIC was ≤ 8 mg/L resistant if the MIC was > 64 mg/L.

Statistical analysis

MICs were expressed as the mean and SD of five experiments. Data were treated by analysis of variance (ANOVA) which was calculated with SAS [21]. Differences between means were determined by the least significance difference test with significance defined at $p \leq 0.05$.

Results

The concentrations of diallyl monosulphide, diallyl disulphide, diallyl trisulphide and diallyl tetrasulphide in garlic and Chinese leek oils is presented in Table 1. The concentrations of the four diallyl sulphides in garlic oil were higher than in Chinese leek oil ($p < 0.05$). The MIC values of four antibiotics, garlic oil, Chinese leek oil and the four diallyl sulphides against the 40 *S.*

Table 1. Content* of four diallyl sulphides in garlic oils and Chinese leek oils

Sulphides	Concentration ($\mu\text{g/g}$) in	
	Garlic oils	Chinese leek oils
DAS	112 (7)	104 (11)
DADS	1183 (42)	943 (45)
DAT	751 (24)	494 (37)
DATS	368 (19)	341 (21)
Sum [†]	2414 (227)	1882 (187)
Total sulphides	4581 (383)	4508 (432)
*Percent of sulphides	52.7	41.7

Data are expressed as mean (SD) ($n = 5$).

DAS, diallyl monosulphide; DADS, diallyl disulphide; DAT, diallyl trisulphide; DATS, diallyl tetrasulphide.

*Limit of detection is 5 $\mu\text{g/g}$.

[†]Sum = DAS + DADS + DAT + DATS.

*% = sum of four diallyl sulphides $\times$ 100/total sulphides.

aureus and 60 MRSA isolates are presented in Table 2. All agents tested inhibited the growth of non-MRSA *S. aureus* with the four antibiotics showing greater activity than the two essential oils and four diallyl sulphides. However, the antibiotic MIC values against MRSA were > 64 mg/L; diallyl disulphide, diallyl trisulphide and diallyl tetrasulphide showing greater inhibitory effects than these antibiotics. The MIC values for the two essential oils and four diallyl sulphides against six fungal pathogens are presented in Table 3. The MIC values of amphotericin B against the 176 clinical fungal isolates were in the range 0.25–4 mg/L (data not shown), indicating no resistance. The two essential oils, diallyl monosulphide and diallyl disulphide showed poorer antifungal activities than amphotericin B; however, diallyl trisulphide and diallyl tetrasulphide showed similar inhibitory effects.

Discussion

Garlic has been reported to have inhibitory activity against *S. aureus* [13]. The results of the present study extend the known antimicrobial activity of garlic oil to MRSA and six medically important fungi. Furthermore, the essential oil prepared from another member of the *Allium* family, Chinese leek, also possessed similar antimicrobial capabilities. The MIC values of garlic and Chinese leek oils against MRSA and six fungal pathogens were < 64 mg/L (Tables 2 and Table 3);

Table 2. MIC of four antibiotics, garlic oil, Chinese leek oil and four diallyl sulphides against 40 wild-type *S. aureus* and 60 MRSA

Agents	MIC (mg/L) against	
	<i>S. aureus</i>	MRSA
Methicillin	0.5 (0.25)	> 64.0
Penicillin	0.5 (0.125)	> 64.0
Cefotaxime	1.0 (0.25)	> 64.0
Tetracycline	1.0 (0.25)	> 64.0
Garlic oil	24.0 (4.0)	32.0 (8.0)
Chinese leek oil	36.0 (6.0)	48.0 (8.0)
Diallyl monosulphide	20.0 (4.0)	32.0 (8.0)
Diallyl disulphide	4.0 (1.0)	12.0 (2.0)
Diallyl trisulphide	2.0 (1.0)	8.0 (2.0)
Diallyl tetrasulphide	0.5 (0.125)	2.0 (0.5)

Data were expressed as mean (SD) (n = 5).

therefore, these two oils could be considered as potent functional foods or potential therapeutic agents for clinical MRSA and fungal infections. Further study is necessary to examine the concentrations reached in the circulation and recovery in urine after these two agents are taken orally.

Lawson *et al.* [11] reported that the sum of diallyl monosulphide, disulphide, trisulphide and tetrasulphide in garlic oil was 54.5% of total sulphides. The present study used a similar method to quantify the four diallyl sulphides and found that the sum of these sulphides in garlic oil was 52.7%, close to the figure of Lawson *et al.* [11]. The analysis of four diallyl sulphides was extended to Chinese leek oil and it was found that both oils contained high concentrations of three of these four diallyl sulphides. The concentration of diallyl monosulphide in these oils was quite low (*c.* 2.0%) and the antimicrobial activity of this agent was not as marked (Tables 2 and Table 3). Thus the contribution of this agent to the overall antimicrobial activity of these oils appears small. The concentration of the other three diallyl sulphides (diallyl disulphide + diallyl trisulphide + diallyl tetrasulphide) in garlic oil was significantly higher than in Chinese leek oil. This may explain why garlic oil showed greater antimicrobial activity than Chinese leek oil.

S. aureus can produce a β -lactamase, and this enzyme inactivates penicillin by hydrolysing the β -lactam ring of the antibiotic [22, 23]. In the present study, β -lactamase was produced by all clinical MRSA isolates. Furthermore, resistance to methicillin confers resistance to all penicillinase-resistant penicillins and cephalosporins; this resistance is due to the presence of the *mec* gene that encodes penicillin-binding proteins (PBPs) [24, 25]. So far, altered forms of PBPs such as PBP 1a and 2b have been implicated in the development of penicillin and cephalosporin resistance [24, 26]. Based on MICs, the six test agents were less active against 40 *S. aureus* isolates than four antibiotics; however, they showed greater inhibitory effects against MRSA than these antibiotics. Although the mechanism of action of these agents has not been investigated in gram-positive bacteria, it is unlikely that they are just affecting β -lactamase.

Table 3. MIC of garlic oil, Chinese leek oil and four diallyl sulphides against three *Candida* and three *Aspergillus* species

Species (number of isolates)	MIC (mg/L) of agent					
	Garlic oil	Chinese leek oil	DAS	DADS	DAT	DATS
<i>C. albicans</i> (39)	16.0 (2.0)	24.0 (2.0)	32.0 (4.0)	4.0 (1.0)	1.0 (0.25)	0.5 (0.125)
<i>C. krusei</i> (27)	24.0 (4.0)	48.0 (8.0)	72.0 (8.0)	12.0 (2.0)	8.0 (1.0)	4.0 (0.5)
<i>C. glabrata</i> (25)	32.0 (4.0)	40.0 (8.0)	54.0 (8.0)	8.0 (2.0)	4.0 (1.0)	2.0 (0.5)
<i>A. niger</i> (31)	20.0 (2.0)	32.0 (4.0)	40.0 (4.0)	8.0 (2.0)	2.0 (0.5)	1.0 (0.25)
<i>A. flavus</i> (26)	40.0 (8.0)	64.0 (8.0)	64.0 (8.0)	12.0 (4.0)	4.0 (2.0)	2.0 (1.0)
<i>A. fumigatus</i> (28)	32.0 (4.0)	56.0 (8.0)	54.0 (4.0)	12.0 (2.0)	8.0 (2.0)	4.0 (2.0)

DAS, diallyl monosulphide; DADS, diallyl disulphide; DAT, diallyl trisulphide; DATS, diallyl tetrasulphide. Data were expressed as mean (SD) (n = 5).

Previously, the inhibitory effect of diallyl monosulphide and diallyl disulphide against *Klebsiella pneumoniae*, an opportunist pathogen causing nosocomial infection, was observed [27]. In this case, the effect was due to inhibition of arylamine N-acetyltransferase activity in this organism with diallyl disulphide showing greater activity than diallyl monosulphide. In the study by Naganawa *et al.* [12], the anti-*C. albicans* activity of diallyl disulphide was also significantly greater than diallyl monosulphide. These authors indicated that the disulphide bond of diallyl disulphide was important for its antifungal activity. In the present study, both diallyl monosulphide and diallyl disulphide showed activity against MRSA and fungi, however, once again diallyl disulphide was more active than diallyl monosulphide. The antifungal effect of diallyl trisulphide against *Cryptococcus neoformans* has been described previously [28]. In the present study this compound as well as diallyl tetrasulphide was active against MRSA, *Candida* spp. and *Aspergillus* spp. (Tables 2 and Table 3), with the order of activity being diallyl tetrasulphide > trisulphide > disulphide. The number of disulphide bonds in the four diallyl sulphides is 0, 1, 2 and 3, respectively and it appears that the antimicrobial activity of these compounds is related to the number of bonds; the more disulphide bonds, the greater the antimicrobial activity.

The MICs of diallyl disulphide, diallyl trisulphide and diallyl tetrasulphide against MRSA, *Candida* spp. and *Aspergillus* spp. were all ≤ 12 mg/L and diallyl tetrasulphide was active at concentrations similar to conventional antibiotics. These agents occur naturally in foods such as garlic and Chinese leek and, therefore, the concentrations these agents achieve *in vivo* appear to be safe. However, further *in vivo* study is necessary to evaluate the clinical application of these three agents for infections.

In conclusion, diallyl disulphide, diallyl trisulphide, diallyl tetrasulphide and essential oils rich in these three sulphides possess potent antimicrobial activities, suggesting that they may be useful in the prevention or treatment of a variety of infections.

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