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Garlic extract and two diallyl sulphides inhibit methicillin-resistant *Staphylococcus aureus* infection in BALB/cA mice

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***Objectives*: The inhibitory effect of garlic extract, diallyl sulphide and diallyl disulphide against methicillin-resistant *Staphylococcus aureus* (MRSA) infection in BALB/cA mice was studied. The influence of these agents upon the levels of fibronectin, interleukin-6 and lipid oxidation in MRSA-infected mice was examined.**

***Methods*: Garlic extract at 100% and 50%; diallyl sulphide (DAS) at 10% and 5%; diallyl disulphide (DADS) at 1% and 0.5% were used in this study. Sixteen clinical MRSA isolates obtained from infected patients were used in this study (n = 16). Mice were infected by injecting 200 μL MRSA-PBS solution, which contained 10^7 cfu, via the tail vein. At 16 h post-infection (p.i.), garlic extract, DAS or DADS at 200 μL was administrated orally. At 24 h p.i., mice were killed and blood, liver, kidney and spleen of each mouse were collected. Plasma and the filtrate from each organ and serial dilutions were used to determine colony count. Plasma fibronectin level was determined by rabbit anti-rat fibronectin antibody and quantified by ELISA. Interleukin-6 levels were determined by commercial kit. Lipid oxidation was determined by measuring malondialdehyde levels.**

***Results*: The oral administration of these agents significantly decreased the viability of MRSA, in plasma, liver, kidney and spleen ($P < 0.05$). MRSA infection significantly increased fibronectin and interleukin-6 levels in plasma of MRSA-infected mice ($P < 0.05$); however, the oral administration of garlic extract and two diallyl sulphides significantly reduced both fibronectin and interleukin-6 levels ($P < 0.05$). MRSA infection also significantly enhanced lipid oxidation in plasma and three organs ($P < 0.05$). The treatments of garlic extract and two diallyl sulphides significantly decreased the malondialdehyde level and showed antioxidant protection ($P < 0.05$).**

***Conclusions*: These data strongly supported the conclusion that garlic extract, diallyl sulphide and diallyl disulphide possessed multiple protective functions against MRSA infection, in which diallyl sulphide and diallyl disulphide could be considered as novel therapeutic agents for the treatment of MRSA infection.**

Keywords: survival rate, fibronectin, interleukin-6, lipid oxidation

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common nosocomial pathogen in Taiwan and other countries.[1,2] Healthcare-associated MRSA strains are resistant to many antibiotics,[3,4] and MRSA infection markedly increases the morbidity and mortality in hospitalized patients.[5,6] In order to control this infection, there is a need to develop novel agents with greater inhibitory activity against MRSA.

The *in vitro* anti-MRSA activity of garlic extract and its two diallyl sulphides (diallyl sulphide, DAS; diallyl disulphide, DADS) has been studied previously in our laboratory.[7] Furthermore, our earlier study noted that the intake of fresh garlic extract by humans resulted in the presence of antimicrobial compounds in plasma, which further exhibited a marked inhibitory zone against MRSA.[8] Therefore, we designed an animal study to evaluate the *in vivo* inhibitory effects of garlic extract, DAS and DADS against MRSA infection.

In addition, bacterial infection often results in increased interleukin-6 (IL-6) secretion, and elevated oxidative stress and/or altered fibronectin levels in infected animals or humans.[9–13] Therefore, the influence of potential anti-MRSA agents upon the levels of fibronectin, IL-6 and lipid oxidation in MRSA-infected BALB/cA mice was also examined.

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Table 1. Survival rate of mice following oral administration (200 μL) of agent at various concentrations

Agent	Concentration of agent (%)	Survival rate (%)
Garlic extract	100	100
	50	100
DAS	100	0
	90	0
	75	0
	50	20
	25	50
	10	100
DADS	100	0
	90	0
	75	0
	50	5
	25	25
	10	50
	5	80
	1	100

Mineral oil was used to dilute DAS or DADS for various concentrations of preparations. Twenty mice were used for each agent at each concentration.

Materials and methods

Garlic extract and diallyl sulphides preparation

Peeled fresh garlic (100 g) was chopped and homogenized in 100 mL sterile distilled water in a Waring blender. After filtration through Whatman No. 1 filter paper, the filtrate was further sterilized by passing through a 22 μm pore-size filter. The filtrate was collected in a sterile vial and stored at 4°C until used. Diallyl sulphide (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%, as determined by HPLC.

Animals

Eight- to nine-week-old male BALB/cA mice (National Laboratory Animal Center, Science Council, Taipei City, Taiwan) were used in this study. Mice were housed on a 12 h light, 12 h dark schedule, and fed with rat and mouse standard diet no.1120 and water *ad libitum*. Use of the mice was reviewed and approved by the Chungshan Medical University Animal Care Committee. Various concentrations of garlic extract, DAS and DADS were tested to deduce sub-lethal levels in mice, and the survival rate was measured. Twenty mice were used for each agent at each concentration.

Bacterial strains

Sixteen clinical MRSA isolates were obtained from infected patients in Chungshan Medical University Hospital (Taichung City, Taiwan). 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 using vancomycin, methicillin, penicillin, cefotaxime and tetracycline were determined by disc diffusion. The antibiotic discs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Discs were placed on the surface of Mueller–Hinton agar plates supplemented with 2% NaCl and seeded with MRSA. Inhibition zones were measured after 24 h incubation at 35°C. Interpretation of resistance was based on the National Committee for Clinical Laboratory Standards (NCCLS) criteria.[14] The 16 MRSA isolates were susceptible to vancomycin and resistant to four other test antibiotics. All cultures were routinely maintained on Mueller–Hinton agar plates at 25°C until used. Overnight MRSA cultures in broth were diluted with PBS and adjusted to an optical density at 600 nm of 0.3 (about 10^{10} cfu/mL).[15] Before infection, MRSA were then diluted with PBS to 10^8 cfu/mL, which gave approximately 10^7 cfu per mouse in a volume of 200 μL.

Experimental design

Mice at 22–26 g were used. After overnight fasting, mice were infected by injecting 200 μL MRSA-PBS solution, which contained 10^7 cfu, via the tail vein. At 16 h post-infection (p.i.), garlic extract, DAS or DADS at 200 μL was administered orally, in which mineral oil was used as solvent for DAS and DADS. Vancomycin at 1% (200 μL of a 10 mg/L aqueous solution) was given orally as a comparator. At 24 h p.i. (8 h after administration of agents), mice were killed with carbon dioxide. Blood, liver, kidney and spleen from each mouse were collected. Plasma was separated from erythrocytes immediately after blood collection. Of each tissue, 200 mg was homogenized with 2 mL PBS (pH 7.2) in a motor-driven Teflon glass homogenizer (Glas-Col Co., CA, USA). The filtrate was used for analysis.

Culture

Plasma and the filtrate from each organ and serial dilutions at 100 μL were cultured on Mueller–Hinton agar plates supplemented with 2% NaCl. After incubation for 24 h at 35°C, colonies were counted and calculated as $\log_{10}$ cfu/mL or $\log_{10}$ cfu/g. The limit of detection was 200 cfu/mL.

Table 2. The organ weight (g) of mice with or without MRSA infection and oral administration of 100% garlic extract (GE), 10% DAS or 1% DADS

	Non-infected	MRSA infected	GE 100%	DAS 10%	DADS 1%
Liver	0.732 ± 0.023	0.723 ± 0.028	0.741 ± 0.025	0.735 ± 0.031	0.728 ± 0.026
Kidney	0.133 ± 0.015	0.174 ± 0.021*	0.165 ± 0.018*	0.170 ± 0.022*	0.168 ± 0.019*
Spleen	0.092 ± 0.011	0.124 ± 0.017*	0.128 ± 0.020*	0.131 ± 0.015*	0.125 ± 0.021*

Data are expressed as mean ± S.D.
*Value is significantly greater than that from non-infected group at $P < 0.05$.

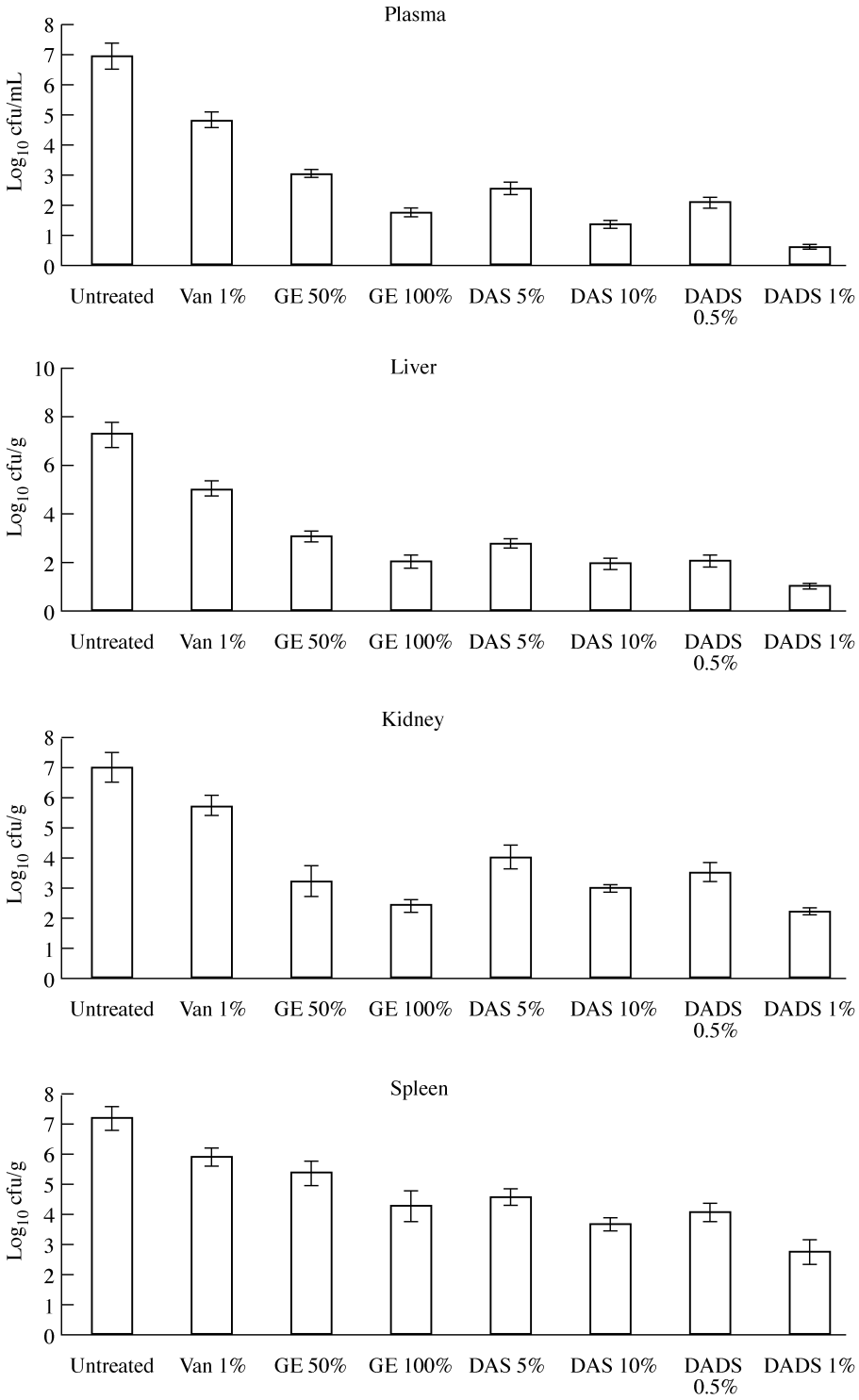

Figure 1. MRSA levels ($\log_{10}$ cfu/mL or $\log_{10}$ cfu/g) in plasma, liver, kidney and spleen from mice with infection without treatment (untreated) or treated with vancomycin (Van) at 1%, garlic extract (GE) at 50% and 100%, DAS at 5% and 10%, and DADS at 0.5% and 1%. Data are mean ± S.D. ($n = 16$).

Fibronectin and interleukin-6 assay

Plasma fibronectin level was determined by rabbit anti-rat fibronectin antibody and quantified by ELISA.[16] Plasma level of IL-6 was measured by ELISA using the Cytoscreen Immunoassay Kit (BioSource International Camarillo, Camarillo, CA, USA) according to the manufacturer's instructions.

Lipid oxidation determination

The concentration of malondialdehyde (MDA, nmol/mL) in plasma and the filtrate from each organ was determined by an HPLC method.[17] In brief, 0.2 mL plasma or filtrate was mixed with 0.8 mL PBS. Then, 0.5 mL trichloroacetic acid (30%) was added. After vortexing and standing in ice for 2 h, samples were centrifuged at 1500**g** for 15 min. Then 1 mL

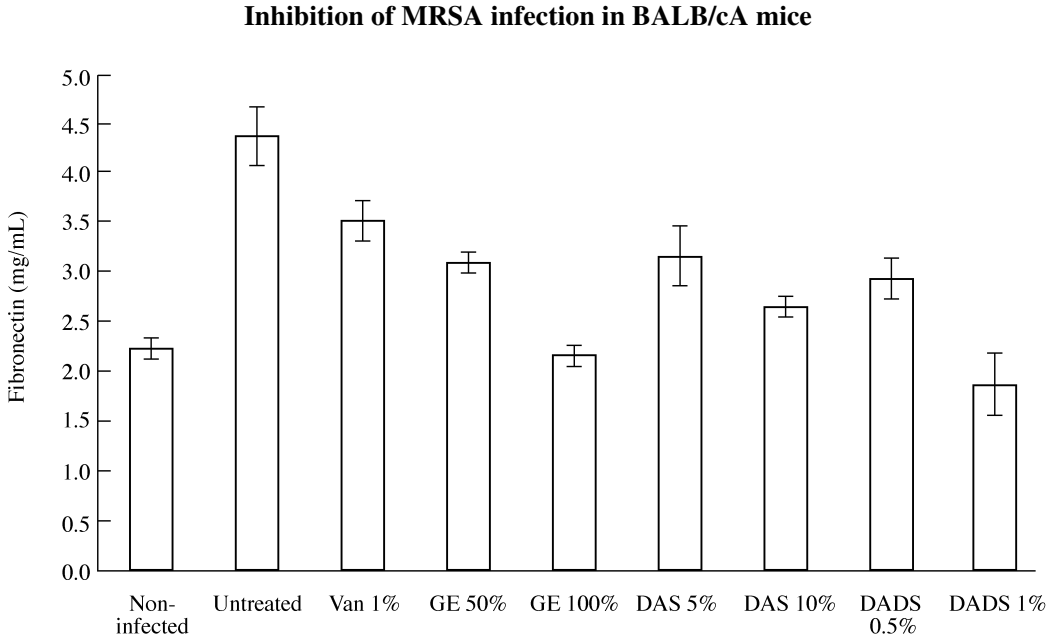

Figure 2. Fibronectin levels (mg/mL) in plasma from mice without infection (non-infected), and mice with MRSA infection without treatment (untreated) or treated with vancomycin (Van) at 1%, garlic extract (GE) at 50% and 100%, DAS at 5% and 10%, and DADS at 0.5% and 1%. Data are mean ± S.D. ($n = 16$).

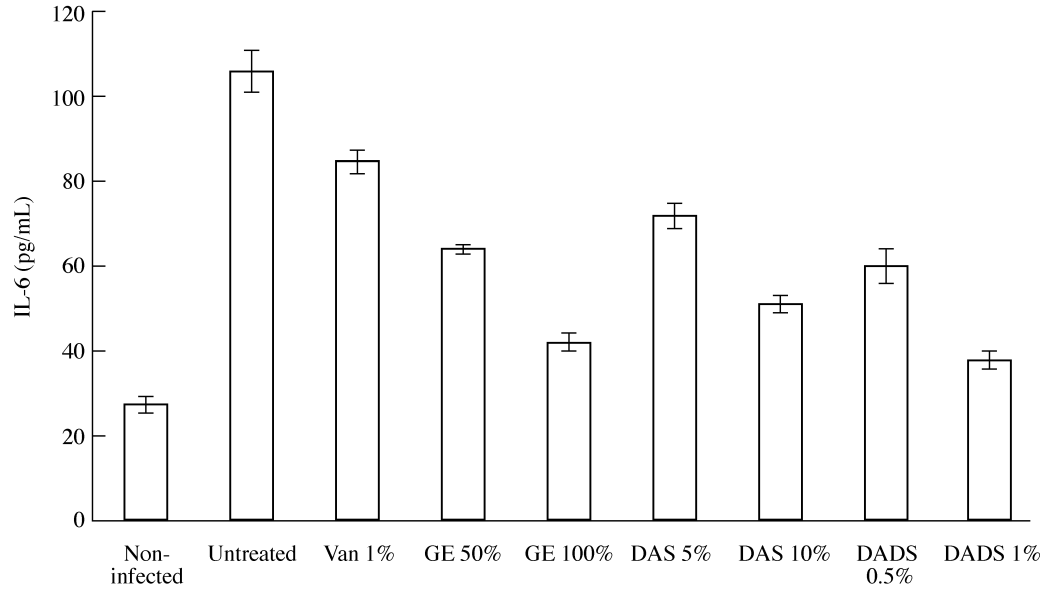

Figure 3. Interleukin-6 (IL-6) levels (pg/mL) in plasma from mice without infection (non-infected), and mice with MRSA infection without treatment (untreated) or treated with vancomycin (Van) at 1%, garlic extract (GE) at 50% and 100%, DAS at 5% and 10%, and DADS at 0.5% and 1%. Data are mean ± S.D. ($n = 16$).

supernatant was mixed with 0.25 mL thiobarbituric acid (TBA, 1%) and the mixture was kept in a boiling water bath for 15 min. The concentration of MDA–TBA complex was assayed using HPLC equipped with a reversed phase Shodex KC-812 column with a UV–Vis detector at 532 nm.

Statistical analysis

Sixteen clinical MRSA isolates obtained from infected patients were used in this study. Each isolate was used for one run experiment including all the above analyses. In every experiment, two mice were used for each agent at each concentration. Data were expressed as mean ± S.D. of 16 experiments (n = 16). Data were treated by analysis of variance (ANOVA) and computed using the SAS General Linear Model procedure.[18] Difference among means was determined by the Least Significance Difference Test with significance defined at $P \leq 0.05$.

Results

Three agents at various concentrations were evaluated to determine sub-lethal levels, and the survival rate is presented in Table 1. Garlic extract at 100%, DAS at 10%, and DADS at 1% resulted in 100% survival rate; therefore, garlic extract at 100% and 50%, DAS at 10% and 5%, and DADS at 1% and 0.5% were used for anti-infection and related experiments. Effects of MRSA infection and oral treatment with garlic extract, DAS or DADS on organ weights are shown in Table 2. MRSA infection significantly elevated kidney and spleen

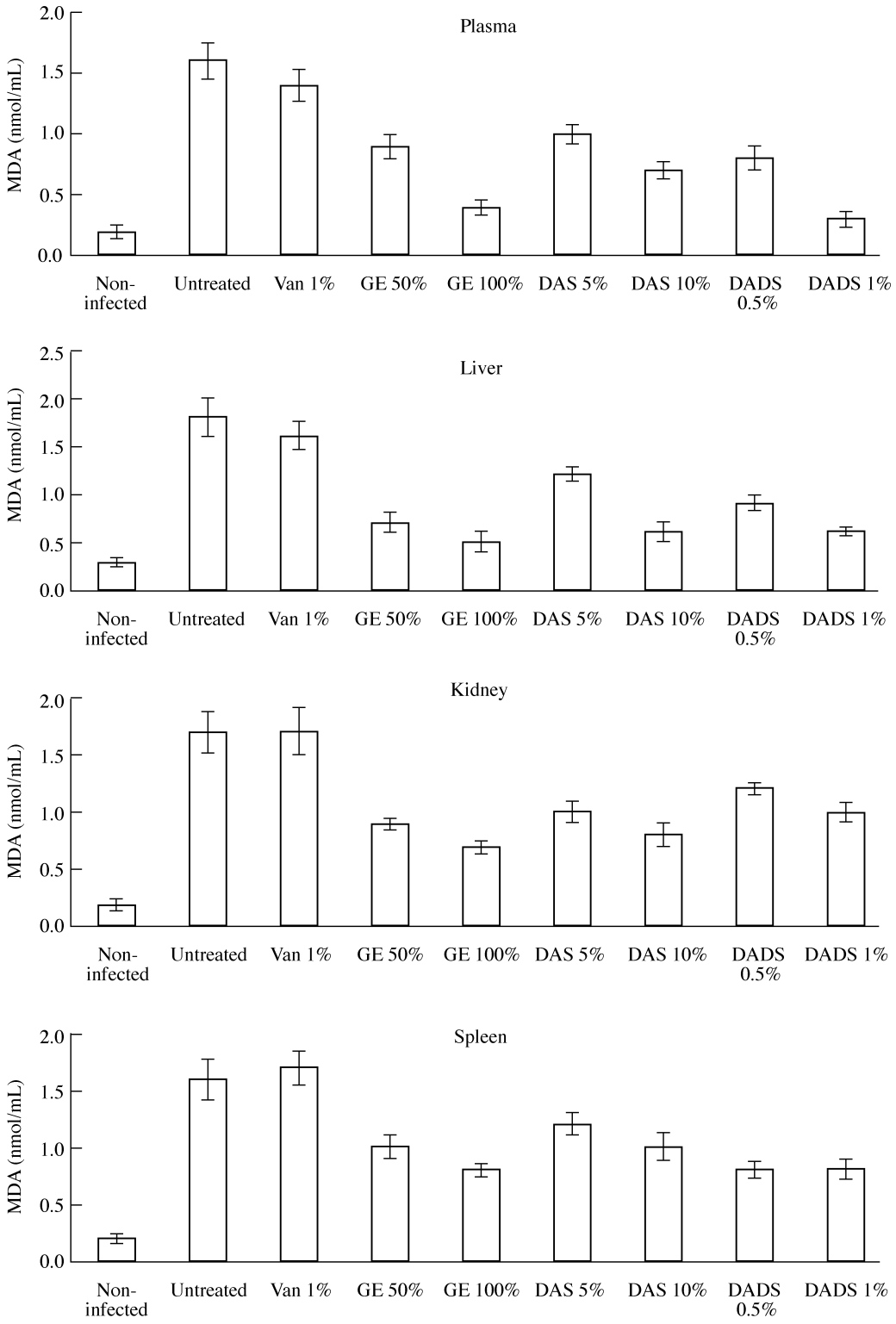

Figure 4. MDA levels (nmol/mL) in plasma, liver, kidney and spleen from mice without infection (non-infected), and mice with MRSA infection without treatment (untreated) or treated with vancomycin (Van) at 1%, garlic extract (GE) at 50% and 100%, DAS at 5% and 10%, and DADS at 0.5% and 1%. Data are mean ± S.D. ($n = 16$).

weights ($P < 0.05$), which were not affected by treatment with the three test agents ($P > 0.05$). The anti-MRSA effect of garlic extract, DAS and DADS on plasma, liver, kidney and spleen is shown in Figure 1. These three agents showed dose-dependent inhibitory effects against MRSA growth in plasma and three organs ($P < 0.05$). The influence of these agents on fibronectin and interleukin-6 is presented in Figures 2 and 3, respectively. MRSA infection significantly increased fibronectin and interleukin-6 levels in plasma

($P < 0.05$); however, the oral administration of garlic extract and two diallyl sulphides significantly reduced both fibronectin and interleukin-6 levels in plasma from infected mice ($P < 0.05$). The antioxidative effect of the three agents on plasma and three organs is shown in Figure 4. MRSA infection significantly enhanced lipid oxidation, determined by MDA level, in plasma and the three organs ($P < 0.05$), which were alleviated significantly by the treatment with garlic extract and two diallyl sulphides ($P < 0.05$). In this study, vancomycin did not exhibit suppressive effects on fibronectin, interleukin-6 or lipid oxidation levels ($P > 0.05$).

Discussion

Our present animal study has demonstrated that garlic extract, DAS or DADS effectively inhibited the growth of and killed MRSA located in plasma and three organs in a dose-dependent manner. Therefore, garlic extract, DAS or DADS are potential agents for *in vivo* MRSA infection therapy. DAS and DADS are compounds naturally formed in *Allium* foods such as garlic, Chinese leek and onion. In our present study, one single oral administration of 10% DAS or 1% DADS was equal to 21.5 and 3.6 μg, respectively, and was a safe and effective agent for MRSA infection therapy in mice with 22–26 g bodyweight. Therefore these two agents at these concentrations could be considered as novel antibiotics. Garlic extract might act as a nutritional supplement for infection prevention or therapy. Further study with other animal models such as New Zealand white rabbit is necessary to confirm the therapeutic effects of these agents against MRSA infection. In addition, the oral administration of DAS or DADS at high concentrations resulted in low survival rates in non-infected mice. This suggests that these agents may have toxicity toward the gastrointestinal system. Therefore, human toxicity and side effects of these agents need to be examined.

Fibronectin is an extracellular matrix protein and responsible for the adherence and internalization of pathogens to host cells.[9,19] Several studies have reported that MRSA infection elevated fibronectin-binding protein production, which enhanced the invasion of MRSA into host cells.[19–22] We have also found that MRSA infection markedly increases plasma fibronectin level, but garlic extract, DAS and DADS effectively suppressed fibronectin production to a greater extent than vancomycin. This suppression may reduce the fibronectin available to react with fibronectin-binding proteins, which consequently decreases the adherence and/or internalization of MRSA into host cells. Therefore, the suppressive effect from garlic extract, DAS and DADS on fibronectin production could effectively protect host cells against pathogen invasion.

It has been reported that MRSA infection resulted in the increased production of several cytokines such as interleukin-6 and these elevated cytokines consequently enhanced the pathogenic development not only in MRSA-associated diseases such as glomerulonephritis and nephritis[10,11,23] but also in other autoimmune/inflammatory diseases such as rheumatoid arthritis.[24–26] The results of our present study confirm that MRSA infection increased the IL-6 level in mice. Furthermore, our present study found that garlic extract, DAS and DADS markedly reduced the IL-6 level in these MRSA-infected mice, unlike vancomycin. This finding suggests that these agents are able to decrease immune complex formation and alleviate infection-induced inflammation reactions. Since these agents could markedly suppress IL-6 production, their use might be beneficial for patients with infection-related inflammatory diseases.

Oxidative stress resulting from bacterial infection such as *Streptococcus pneumoniae* meningitis has been reported.[13,27] Our present study is the first report regarding MRSA-induced oxidative damage. This infection-induced oxidation could further worsen host self-defence systems. In addition, the combined effects of infection, IL-6-related inflammation and elevated oxidation could enhance the development of inflammatory diseases and increase therapy difficulty. Several studies have indicated that garlic extract, DAS and DADS possessed both enzymic and non-enzymic antioxidant activities such as scavenging superoxide ions and enhancing glutathione peroxidase activity.[28–30] Thus, it was highly likely that these agents contributed to the observed oxidation alleviation in this present study via their antioxidant protection. This finding suggests that, based on their antioxidative capability, these agents could provide benefit in anti-infective therapy.

In conclusion, garlic extract, DAS and DADS could effectively inhibit MRSA infection, suppress infection-induced elevation of fibronectin and interleukin-6, and decrease MRSA-induced oxidative damage in MRSA-infected mice. These data strongly suggest that these agents possess multiple protective functions against MRSA infection.

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